JOURNAL OF BACTERIOLOGY, Mar. 1997, p. 2053–2059 Vol. 179, No. 6
0021-9193/97/$04.00+0

A Regulatory Gene (*ccaR*) Required for Cephamycin and Clavulanic Acid Production in *Streptomyces clavuligerus*: Amplification Results in Overproduction of Both β-Lactam Compounds

FRANCISCO J. PÉREZ-LLARENA,[1] PALOMA LIRAS,[1,2] ANTONIO RODRÍGUEZ-GARCÍA,[1] AND JUAN F. MARTÍN[1,2]*

Area of Microbiology, Faculty of Biology, University of León, 24071 León,[1] and Institute of Biotechnology INBIOTEC, 24006 León,[2] Spain

Received 28 October 1996/Accepted 16 January 1997

A regulatory gene (*ccaR*), located within the cephamycin gene cluster of *Streptomyces clavuligerus*, is linked to a gene (*blp*) encoding a protein similar to a β-lactamase-inhibitory protein. Expression of *ccaR* is required for cephamycin and clavulanic acid biosynthesis in *S. clavuligerus*. The *ccaR*-encoded protein resembles the ActII-ORF4, RedD, AfsR, and DnrI regulatory proteins of other *Streptomyces* species, all of which share several motifs. Disruption of *ccaR* by targeted double recombination resulted in the loss of the ability to synthesize cephamycin and clavulanic acid. Complementation of the disrupted mutant with *ccaR* restored production of both secondary metabolites. *ccaR* was expressed as a monocistronic transcript at 24 and 48 h in *S. clavuligerus* cultures (preceding the phase of antibiotic accumulation), but no transcript hybridization signals were observed at 72 or 96 h. This expression pattern is consistent with those of regulatory proteins required for antibiotic biosynthesis. Amplification of *ccaR* in *S. clavuligerus* resulted in a two- to threefold increase in the production of cephamycin and clavulanic acid.

Actinomycetes are well known producers of a variety of antibiotics and other secondary metabolites. *Streptomyces clavuligerus* produces the β-lactam antibiotic cephamycin C and the β-lactamase inhibitor clavulanic acid (4, 36). Control of the biosynthesis of antibiotics has been known to occur for many years (29), but the molecular mechanisms by which control is exerted are only slowly being unraveled (3, 31). Understanding the molecular mechanisms that control biosynthesis of pharmacologically active secondary metabolites will be of great value in the pharmaceutical industry.

A hierarchy of regulatory genes controls antibiotic production and sporulation (18). Individual antibiotic biosynthetic pathways are controlled by pathway-specific regulatory genes that are usually linked to the biosynthetic gene cluster (for reviews, see references 5 and 30). Gene *act*II-ORF4 encoding a transcriptional activator of actinorhodin biosynthesis was described for *Streptomyces coelicolor* (13, 16). Similarly, *redD*-ORF1 (32, 41), *afsR* (19), and *dnrI* (14, 27, 40) encode related proteins that regulate the biosynthesis of other pigments or antibiotics.

The cephamycin biosynthetic pathway has been studied in detail in *Nocardia lactamdurans* and *S. clavuligerus*. In *N. lactamdurans*, nine different genes encode enzymes that catalyze the entire biosynthetic pathway (except for the conversion of α-aminoadipic semialdehyde into α-aminoadipate) (6–8, 10, 11). Most of these genes occur also in *S. clavuligerus* (22, 23, 28, 45), although the arrangement is different from that observed in *N. lactamdurans*. The 3′-hydroxymethylcephem carbamoyltransferase of *S. clavuligerus* has been shown recently to be encoded by ORF9 (gene named *cmcH*) (11) (Fig. 1), and the methoxylase genes *cmcI* and *cmcJ* correspond to ORF7 and ORF8, respectively (35). No regulatory genes have been reported so far in the cephamycin biosynthetic clusters, although the *N. lactamdurans* cluster has been shown to contain three additional genes, *bla*, *pbp*, and *cmcT* (9), which encode, respectively, a β-lactamase which does not play an essential role in cephamycin biosynthesis (25), a penicillin-binding protein (PBP4), and a transmembrane protein. It was, therefore, of utmost interest to characterize pathway-specific regulatory genes that might be associated with the cephamycin gene cluster.

We report here that in the *S. clavuligerus* DNA region located between *lat* and *cmcH* there are three open reading frames, ORF10, ORF11, and ORF12; ORF10 and ORF12 encode, respectively, an *act*II-ORF4-like regulatory protein and a β-lactamase-inhibitory protein (BLIP)-like protein similar to the BLIP already described for *S. clavuligerus* (12).

MATERIALS AND METHODS

Strains and culture conditions. *S. clavuligerus* ATCC 27064 was used to isolate DNA and also as host for gene amplification and gene disruption studies. Spores of *S. clavuligerus* were maintained in 20% glycerol at −20°C. For cephamycin and clavulanic acid production in submerged cultures, *S. clavuligerus* was grown in GSPG medium containing the following (all concentrations are in grams per liter): glycerol (15), sucrose (20), proline (2.5), glutamic acid (1.5), NaCl (5), K_2HPO_4 (2), $CaCl_2$ (0.4), $MnCl_2 \cdot 4H_2O$ (0.1), $FeCl_3 \cdot 6H_2O$ (0.1), $ZnCl_2$ (0.05), $MgSO_4 \cdot 7H_2O$ (1), and distilled water (pH 7.0). This medium supports production of both cephamycin and clavulanic acid (36). Production of cephamycin and clavulanic acid by the different strains was confirmed in Trypticase soy broth (TSB) (Difco).

S. coelicolor JF1 *act177* (*act*II-ORF4) *red42 argA1 guaA1*, provided by F. Malpartida (13), was used for complementation studies with *ccaR*.

***S. clavuligerus* gene library and DNA sequencing.** A gene library of total DNA of *S. clavuligerus* was constructed in phage lambda GEM12. Recombinant phages were hybridized with DNA probes internal to the cephamycin biosynthetic genes labelled by nick translation with [^{32}P]dCTP. DNA manipulations were carried out according to standard procedures (38).

A 3.7-kb *Bam*HI-*Eco*RI DNA fragment subcloned from lambda GEM12-A10 was end filled with DNA polymerase (Klenow fragment) and subcloned in pBluescript KS+, giving rise to pULJP37. A nested set of deletion clones was obtained

* Corresponding author. Mailing address: Area of Microbiology, Faculty of Biology, University of León, 24071 León, Spain. Phone: 34 87 291505. Fax: 34 87 291506. E-mail: degjmm@unileon.es.

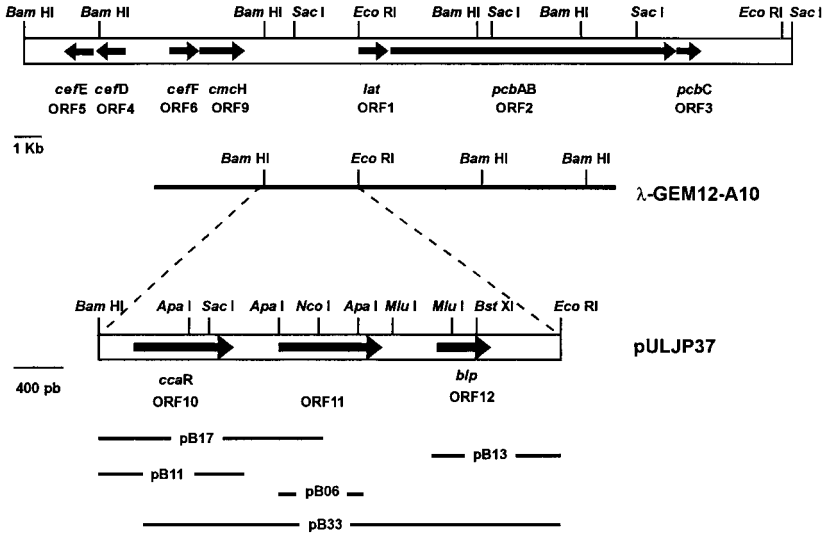

FIG. 1. Physical map of the DNA region of *S. clavuligerus* encoding the cephamycin gene cluster (upper box with arrows). The 16-kb DNA fragment cloned in λGEM12-A10 is indicated by a solid bar. The sequenced 3.7-kb *Bam*HI-*Eco*RI fragment (subcloned in pULJP37) containing ORF10, ORF11, and ORF12 is enlarged. DNA fragments in different plasmids of the pB series are indicated by thin lines at the bottom of the figure.

by the erase-a-base procedure (Promega). Both strands were sequenced with the AutoRead system (Pharmacia).

Southern hybridization. Total DNA was digested with *Bam*HI and *Mlu*I, and the fragments were resolved in 0.7% agarose gels (in duplicate). The DNA fragments were blotted and hybridized with probes internal to the *ccaR* gene or to the *aph*II gene. Probes were labelled with digoxigenin by random primer extension (Boehringer Mannheim Biochemicals).

Transformation of *Streptomyces lividans* and *S. clavuligerus*. Protoplasts of *S. lividans* and *S. clavuligerus* were obtained and transformed with plasmid DNA as described previously (15) except that protoplasts were subjected to a heat shock (10 min at 42°C) before transformation. Protoplasts were regenerated in R2YEG (R2YE with 10 g of glycerol per liter) (17). Transformants were selected by resistance to kanamycin (50 μg/ml) and/or thiostrepton (50 μg/ml), depending on the plasmids used.

Insertional inactivation of *S. clavuligerus ccaR* by gene replacement. Inactivation of the endogenous *ccaR* was performed with plasmid pB11B, which carries the 5′ and 3′ moieties of the *ccaR* gene separated by the aminoglycoside phosphotransferase (*aph*II) gene of transposon Tn*5* in a pIJ702 (pIJ101-derived) replicon, by using the strategy described by Paradkar and Jensen (33), which is based on the segregational instability of this replicon when allowed to sporulate under nonselective conditions. pB11B was constructed by subcloning in the *Pst*I site of pIJ702 a cassette (with blunt ends) consisting of the 5′ and 3′ moieties of *ccaR* separated by the 1.4-kb *aph*II gene of transposon Tn*5* subcloned in the *Apa*I site of *ccaR*. The *ccaR* gene was originally isolated from pB11 (Fig. 1) as a 1.2-kb *Bam*HI-*Xho*I fragment.

pB11B was transformed first into *S. lividans* and then into *S. clavuligerus*. The resulting Thio^s Km^r clones were identified in the spore progeny by replica plating. These putative recombinants (that have lost the plasmid) were confirmed by hybridization with probes internal to the *aph*II and the *ccaR* genes as indicated in Results.

Amplification of the *ccaR* gene and complementation of disrupted mutants. A *Bam*HI-*Nco*I DNA fragment from pULJP37 carrying *ccaR* was subcloned into the polylinker of pIJ2921 (20), giving rise to pB17 (Fig. 1). The fragment was rescued again with *Bgl*II ends from this construction and subcloned into the *Bgl*II site of pULVK99 (a positive-selection vector derived from pIJ699 [21] by replacing the DNA region between the two inverted terminators of pIJ699 [containing the *oriP15A* and the *neo* and *vph* genes] with an *aph*II gene and the pBR322 *ori* region [24]). This construction (named pB17B) was introduced into *S. lividans*, and transformants were selected in the presence of thiostrepton (50 μg/ml). pB17B was isolated from *S. lividans* and used to transform *S. clavuligerus* ATCC 27064 (wild type) or a *ccaR*-disrupted mutant of the same strain to study complementation.

RNA isolation and transcript analysis. RNA of *S. clavuligerus* ATCC 27064 was isolated from cultures in TSB medium grown at 28°C in 500-ml triple-baffled flasks (containing 100 ml of medium) on a rotary shaker at 220 rpm. Samples were taken at 24, 48, 72, and 96 h, and the RNA was extracted as described by Hopwood et al. (17) except that the cell pellet was frozen in liquid nitrogen and broken with alumina in a liquid nitrogen-refrigerated mortar.

Extracted RNAs (30 μg) were resolved in a denaturing formaldehyde (1.5%) agarose gel with the RNA molecular weight marker set of Boehringer Mannheim as standards. RNAs were then blotted onto nylon filters and hybridized overnight with [^{32}P]dCTP-labelled probes internal to the three cloned genes in buffer containing 50% formamide, 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× Denhardt's solution (38), and 0.1% sodium dodecyl sulfate (SDS). The filters were washed with 2× SSC containing 0.1% SDS at increasing temperatures up to 65°C for 20 min and were scanned in an Instant Imager (Packard) electronic autoradiography unit.

Cephamycin and clavulanic acid assays. Cephamycin and clavulanic acid levels were routinely determined by bioassay as described previously (36), and these assays were confirmed by high-pressure liquid chromatography (6).

Nucleotide sequence accession number. The nucleotide sequence obtained in this study has been assigned EMBL data bank accession number Z81324.

RESULTS

Cloning of the central region of the *S. clavuligerus* cephamycin gene cluster. The poorly known central region of the cephamycin C gene cluster upstream of the *lat* gene (42) was cloned from an *S. clavuligerus* DNA gene library in the lambda GEM12 vector by using as probe a 4.0-kb *Eco*RI-*Hin*dIII DNA fragment that contains the complete *lat* gene and part of the *pcbAB* gene (45). One of the recombinant phages, lambda GEM12-A10, which contained 16 kb of the cephamycin C cluster (*cmc*) and which extended about 8 kb upstream of the *lat* gene to the previously characterized *cmcH* gene (11), was mapped with several restriction endonucleases to confirm that it contained the expected *cefF-cmcH* region (Fig. 1). The *Bam*HI-*Eco*RI fragment was isolated from lambda GEM12-A10, end filled with Klenow DNA polymerase, and subcloned in both orientations into pBluescript KS+, forming plasmids pULJP37a and pULJP37b (Fig. 1). The entire 3.7-kb fragment was sequenced in both strands.

Three open reading frames are located between *cmcH* and *lat*. As shown in Fig. 2, three long open reading frames (ORF10, ORF11, and ORF12) were identified by using the GenePlot program of DNAstar (Madison, Wis.) with the codon usage of species of *Streptomyces* (44).

(i) ORF10. The first of the three open reading frames contained 768 nucleotides (nt) and started with a GTG codon at position 311 of the sequenced fragment, which was preceded immediately upstream by a hypothetical ribosome-binding site, GGAA, with relatively good complementarity to the 3′ ends of

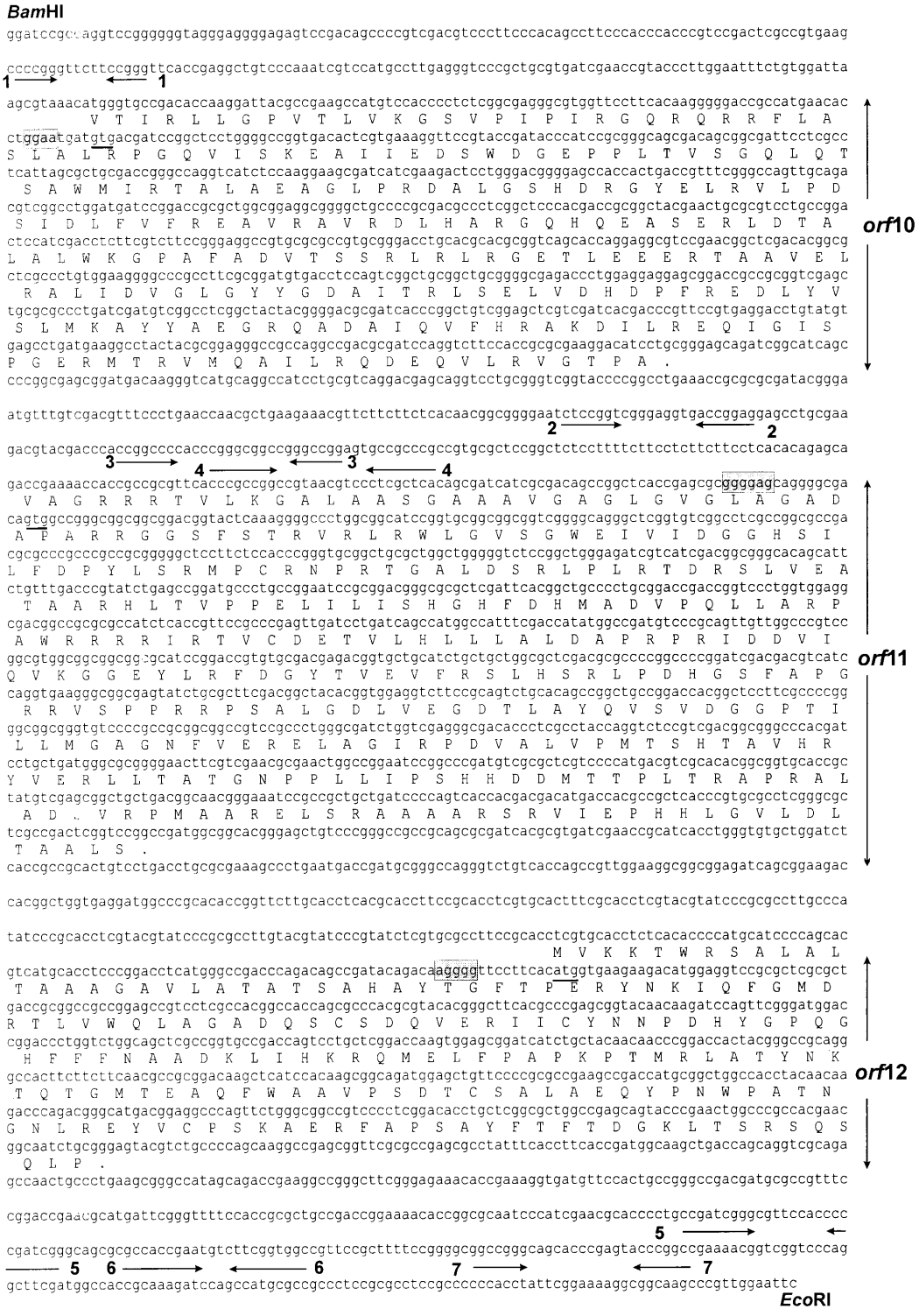

FIG. 2. Nucleotide sequence of the 3.7-kb *Bam*HI-*Eco*RI DNA fragment containing ORF10, ORF11, and ORF12. Putative ribosome-binding sites are in shaded boxes. The translation initiation codons are underlined. Inverted repeats are numbered 1 to 7 and are underlined by convergent arrows.

the 16S rRNA of several gram-positive bacteria, i.e., CCUCC UUUCU (which defines a Shine-Dalgarno 5′-AGAAAGG AGG sequence) (1). ORF10 encoded a deduced protein of 256 amino acids with a relative mass of 28,257 Da and a calculated isoelectric point of 6.52. A comparison of the protein encoded by ORF10 with other proteins in the SwissProt data bank with the FASTA program indicated that the encoded protein has significant homology with four different regulatory proteins of antibiotic clusters in different *Streptomyces* species. It showed 28.1% identical amino acids with the product of *redD*-ORF1 of

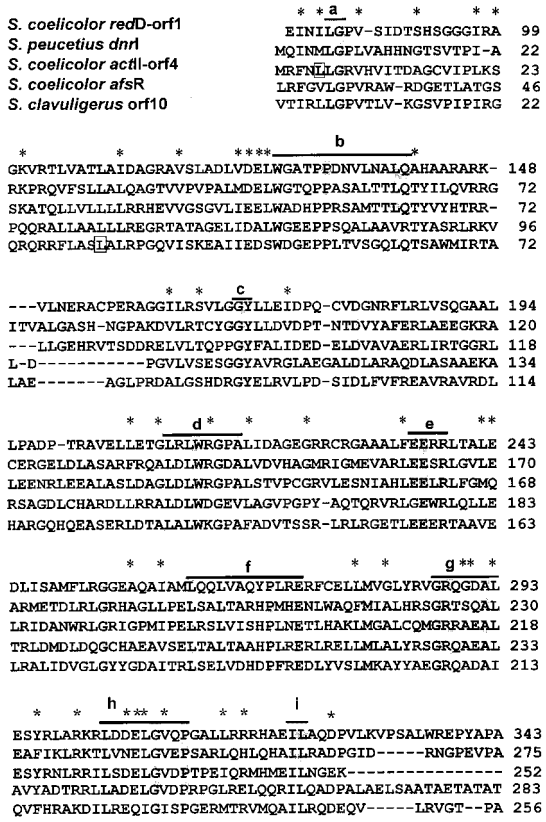

FIG. 3. Alignment of the deduced amino acid sequence of the product of *S. clavuligerus* ORF10 with those of the proteins encoded by *S. coelicolor redD*-ORF1, *S. peucetius dnrI*, and *S. coelicolor act*II-ORF4 and *afsR*. The identical amino acids are shaded, and functionally conserved amino acids are indicated by an asterisk. The TTA-encoded leucine is boxed. Conserved motifs a to i are overlined with a bar.

Streptomyces coelicolor, 26.9% identity with DnrI of *Streptomyces peucetius*, 25.9% identity with ActII-ORF4 of *S. coelicolor*, and 28.5% identity with AfsR (in a 253-amino-acid region out of the 993 residues of this protein). The nucleotide sequence of ORF10 had a high G+C content (69.1%), and its codon usage was in agreement with the characteristically biased codon preference of *Streptomyces* (44).

Multiple alignments of the proteins encoded by *redD*-ORF1, *dnrI*, *afsR*, and *act*II-ORF4 with the ORF10 product showed a series of nine motifs (Fig. 3) corresponding to identical or functionally conserved amino acids in these proteins. This alignment supported the choice of the first in-frame GTG triplet of the nucleotide sequence shown in Fig. 2 as the translation start codon since this gives a protein with an amino-terminal region similar to those of the proteins encoded by *dnrR1* of *S. peucetius* and *act*II-ORF4 of *S. coelicolor.*

A leucine (from a rare TTA codon) was found in the protein encoded by ORF10 that was also found in the ActII-ORF4 protein (13) (see Discussion). The protein encoded by ORF10 was shown to be involved in the control of cephamycin and clavulanic acid biosynthesis (see below) and has been named CcaR.

(ii) ORF11. The second open reading frame of this fragment contained 1,041 nt starting at a GTG at position 1403 (Fig. 2) which is preceded by ribosome-binding site sequence GGGAG. ORF11 encodes a putative 338-amino-acid protein with a deduced M_r of 36,552. A comparison of this protein with the protein databases did not reveal significant homologies with other proteins.

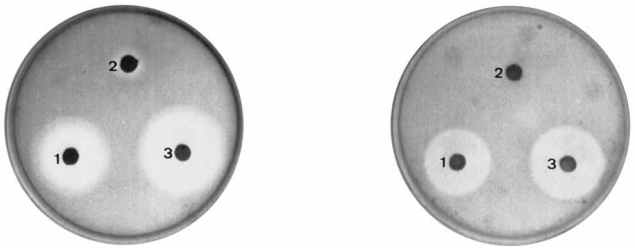

FIG. 4. Production of cephamycin (left petri dish) and clavulanic acid (right petri dish) in solid medium by the parental strain (1), the disrupted mutant (2), and a complemented transformant of the disrupted mutant (3). Note the lack of production in the disrupted mutant and the higher production in the complemented clone (3).

(iii) ORF12. A third open reading frame of 546 nt started at the ATG in position 2766 and was preceded by putative ribosome-binding site sequence AAGGG. It encodes a deduced protein of 182 amino acids with a molecular mass of 20,287 Da and an isoelectric point of 8.7 (7.78 for the mature protein after removal of the leader peptide) which contains an N-terminal 28-amino-acid sequence that appears to correspond to a leader peptide.

A search analysis of the SwissProt data bank with the FASTA program revealed significant homology (29.2% identical amino acids and 27.6% additional functionally conserved residues) with the extracellular BLIP produced by the same strain (12). The protein encoded by ORF12 was therefore named Blp for BLIP-like protein. The similarity in size between Blp and BLIP (both are small) and the conserved domains throughout both proteins suggest that the corresponding gene (*blp*) may have originated by a duplication of the first reported gene encoding BLIP.

Several inverted repeats (Fig. 2) were found in the upstream region of ORF10, in the intergenic regions between ORF10 and ORF11, and downstream of ORF12 (but not between ORF11 and ORF12). These inverted repeats may act as transcriptional terminators (see transcriptional studies below).

The CcaR protein is required for production of cephamycin C and clavulanic acid. In vivo disruption of the *ccaR* gene was of interest to establish the role of CcaR, an ActII-ORF4-like protein. Disruption of *ccaR* was achieved by targeted replacement of an internal fragment of this gene by the *aph*II gene by double recombination with the pB11B construction. This plasmid (8.35 kb) is a pIJ702 derivative containing a cassette of the 5′ and 3′ moieties of the *ccaR* gene separated by the *aph*II gene of transposon Tn*5*.

Disruption of the *ccaR* gene was confirmed by hybridizing total DNA of the wild type and the disrupted mutant with the following two probes: probe A, a 1.4-kb *Bam*HI fragment that contains the *aph*II gene and probe B, a 0.4-kb *Sac*I-*Xba*I fragment internal to *ccaR*. The wild type contains a *ccaR*-hybridizing *Bam*HI-*Mlu*I band of 2.3 kb and did not hybridize with the *aph*II probe, whereas the disrupted mutant shows a single band of 3.7 kb that hybridized with both probes A and B, indicating that *aph*II has been inserted into the endogenous *ccaR* gene by double crossover, causing the disruption. The disrupted clone did not produce either cephamycin C or clavulanic acid. These results were confirmed in solid and liquid TSB and MEY (maltose [10 g/liter], yeast extract [4 g/liter], $CaCl_2$ [0.01 g/liter]) (17) media in four different experiments (Fig. 4). The disrupted mutant showed normal growth kinetics.

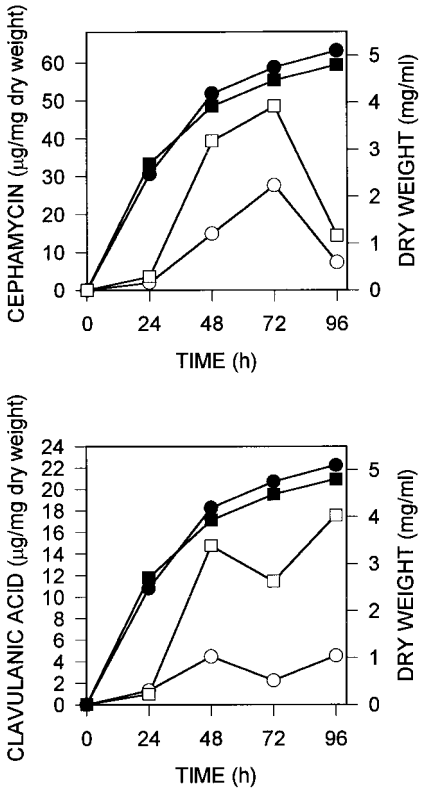

FIG. 5. Growth (●, ■) and production of cephamycin C (○, □ [upper panel]) and clavulanic acid (○, □ [lower panel]) in GSPG medium of a control culture of *S. clavuligerus* (pULVK99) (○, ●) and *S. clavuligerus* (pB17B) containing *ccaR* on a multicopy plasmid (□, ■).

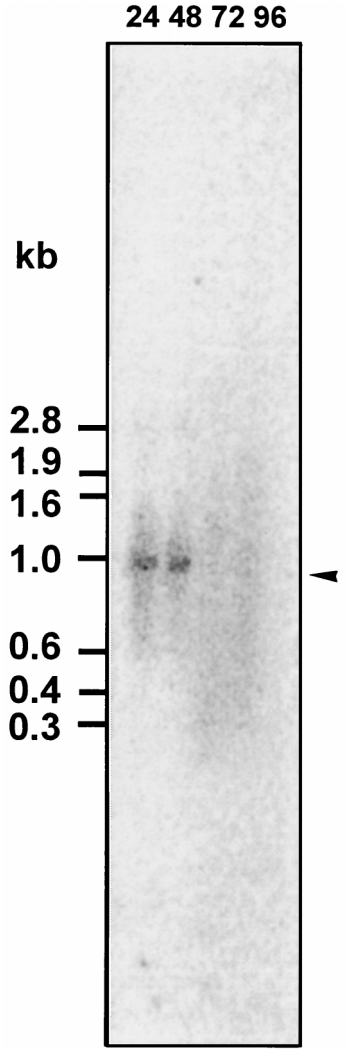

FIG. 6. Transcriptional analysis of the *ccaR* gene in *S. clavuligerus* ATCC 27064 by Northern hybridization with a 0.4-kb *Sac*I-*Xba*I probe internal to *ccaR*. Lanes 24, 48, 72, and 96 show the results for RNA extracted from cultures at 24, 48, 72, and 96 h, respectively, of incubation. Size markers are on the left. The 0.9-kb transcript is indicated by an arrowhead.

Complementation by *ccaR* restores production of cephamycin C and clavulanic acid. Complementation of the disrupted mutant with the *ccaR* gene was performed to elucidate whether the CcaR protein was functionally active in *trans* when amplified on a multicopy plasmid. As shown in Fig. 4, transformants carrying *ccaR* in a pULVK99 vector restored production of both cephamycin and clavulanic acid. The same results were obtained in solid and liquid cultures. Complementation resulted in higher cephamycin and clavulanic acid levels than those produced in the nondisrupted parental strain.

Amplification of the *ccaR* gene results in a two- to threefold increase in cephamycin and clavulanic acid. If the CcaR protein acts as an activator of cephamycin and clavulanic acid genes, its availability might be limiting for production of one or both of these metabolites. Results of amplification with the pULVK99 vector (more than 50 copies per cell for *S. lividans* since this plasmid contains the origin of replication of pIJ101 [17], although the copy number might be smaller for *S. clavuligerus*) showed that the specific yield of cephamycin increased about twofold at 48 and 72 h of cultivation and that that of clavulanic acid increased about threefold at the same times (Fig. 5).

***ccaR* does not complement an *S. coelicolor act*II-ORF4 mutant.** To study the specificities of induction in different strains, *S. coelicolor* JF1, an *act*II-ORF4 mutant (13), was transformed with pB17B used previously to complement the *ccaR*-disrupted mutant. Transformants were selected on the basis of resistance to thiostrepton and were tested for actinorhodin production in solid R5 medium. None of the transformants tested showed actinorhodin formation, indicating that *ccaR* is unable to complement the *act*II-ORF4 mutation or that it is not expressed in *S. coelicolor.*

Transcriptional analysis of the *ccaR*-ORF11-*blp* region. Total RNA of *S. clavuligerus* was obtained from cultures grown in TSB medium at 28°C under strongly aerobic conditions (220 rpm in triple-baffled flasks) as indicated in Materials and Methods, since these conditions are favorable for antibiotic production. Hybridizations with the different probes showed that *ccaR* is transcribed as a monocistronic transcript of 0.9 kb during the growth phase (24 and 48 h) but not at 72 and 96 h (Fig. 6); thus, transcription precedes cephamycin and clavulanic acid production (maximal antibiotic accumulation occurs at 72 h for cephamycin and at 96 h for clavulanic acid).

The *blp* gene was efficiently transcribed as a separate monocistronic messenger of 1.2 kb also at 24 and 48 h of incubation (data not shown). Under these conditions, ORF11 was not transcribed. No hybridization signal at any time during growth was obtained by hybridization with an *Apa*I probe corresponding to the first half (proximal to the 5′ end) of ORF11 (data not shown).

DISCUSSION

Individual antibiotic biosynthetic pathways are controlled by specific regulatory proteins that are frequently encoded by genes linked to the biosynthetic gene clusters. *S. coelicolor act*II-ORF4 (13) and *redD*-ORF1 (32) and *Streptomyces hygroscopicus brpA* (2) were reported to be transcriptional activators of the actinorhodin, undecylprodigiosin, and bialaphos genes, respectively. In the daunorubicin cluster of *S. peucetius*, regulatory locus *dnrR1* (40) includes two genes, *dnrI* and *dnrJ*. The product encoded by *dnrI* was homologous to the products of *act*II-ORF4, *redD*-ORF1, and *afsR* and corresponds to a transcriptional activator (27). Transcription of *dnrI* in *S. peucetius* is, in turn, controlled by another regulatory protein of the daunorubicin cluster, DnrN (14). The mechanism of action of the regulatory proteins encoded by *act*II-ORF4-like genes is not fully understood. No helix-turn-helix DNA-binding motifs are found in these proteins, which indicates that they may represent a novel family of DNA-binding regulatory proteins (5). It is likely that they require additional proteins to activate the biosynthetic structural promoters.

The protein encoded by *ccaR* is another example of a regulatory protein controlling antibiotic biosynthesis. Disruption of *ccaR* resulted in the inability to produce cephamycin C and clavulanic acid, suggesting that the *ccaR* product is an activator. A similar observation was made in the patent literature by Walter et al. (42). These authors showed that empirically derived nonproducing mutants of *S. clavuligerus* were complemented by a DNA fragment of about 7 kb that restored cephamycin and clavulanic acid production to the mutants. Although the mutants described were not known to be regulatory mutants, the patent claims that the 7.0-kb DNA fragment contains a regulatory gene that would correspond to the *ccaR* gene reported in this article. Our results support that claim.

In *S. coelicolor* a TTA codon has been shown to be involved in translational control of expression of actinorhodin biosynthesis genes (13). In *S. clavuligerus*, the leucine encoded by the TTA codon is located in position 32 of the CcaR protein, whereas in the protein encoded by *act*II-ORF4 the leucine corresponds to the fifth residue from the amino-terminal end of the protein. Leucine TTA codons are recognized by a rare tRNA encoded by the *bldA* gene in *S. coelicolor* (26). *bldA* mutants are unable to express TTA-containing genes. Phenotypically similar *bldA* mutants have also been found in *Streptomyces griseus* and may be widespread in actinomycetes (5). The presence of this rare TTA codon in the *ccaR* gene of *S. clavuligerus* suggests that it is a target for translational control by the corresponding *bldA*-encoded tRNA. Amplification of the *ccaR* gene on a multicopy plasmid resulted in a marked increase in the specific yields of cephamycin and clavulanic acid, indicating that the intracellular level of the CcaR protein is limiting for expression of both β-lactam compounds. Similarly, overexpression of *act*II-ORF4 results in an increase of actinorhodin production (16). The stimulatory effect of the CcaR protein on cephamycin and clavulanic acid biosynthesis is less dramatic than that of ActII-ORF4 on actinorhodin biosynthesis, which may suggest the presence of other bottlenecks in β-lactam antibiotic biosynthesis in *S. clavuligerus* that do not occur in actinorhodin biosynthesis.

It is interesting that the same regulatory gene controls the biosynthesis of cephamycin and clavulanic acid. Since the genes involved in the biosynthesis of these compounds are linked, forming a supercluster that extends for about 35 kb in the genome of *S. clavuligerus* (43), it seems that biosynthesis of cephamycin and the β-lactamase inhibitor clavulanic acid is closely coordinated for survival of the producing strain. Indeed, we reported previously the isolation of mutants impaired in the biosynthesis of both cephamycin and clavulanic acid (37).

ccaR failed to complement an *act*II-ORF4 mutant of *S. coelicolor*. It has been reported that *dnrI* can complement mutations in *act*II-ORF4 and that *act*II-ORF4 can stimulate daunorubicin production in *S. peucetius* (40), but *redD* and *act*II-ORF4 do not show cross-complementation (5). It seems, therefore, that only very similar biosynthetic genes (e.g., those of the actinorhodin and daunorubicin clusters) may be cross-regulated by activators of the *act*II-ORF4 family. It will be of interest to investigate if *ccaR* acts on β-lactam biosynthetic genes in other cephamycin and clavulanic acid producers.

The linkage of *ccaR* to *blp* is intriguing. BLIP has been shown to be a β-lactamase inhibitor that interacts with β-lactamases (39). We have found recently that *S. clavuligerus* contains a β-lactamase that is sensitive to BLIP and clavulanic acid (two β-lactamase inhibitors produced by the same strain) (34). The β-lactamase of *S. clavuligerus* possesses penicillin-binding activity and poor β-lactam hydrolytic ability, suggesting that its role may be that of a β-lactam-recognizing protein different from more evolved β-lactamases of pathogenic bacteria.

In liquid cultures both the *ccaR* and *blp* genes were expressed as monocistronic transcripts during transition from the growth phase to the production phase, but no hybridization signals were observed after 48 h of incubation; however, we cannot exclude the possibility of degradation of the mRNAs in the production phase. High-resolution transcript analysis is required to confirm the hypothesis that *ccaR* is transcribed only in the growth phase prior to antibiotic biosynthesis. Similarly, transcription of *redD* (41) and *act*II-ORF4 (16) occurs only during transition to the stationary phase in *S. coelicolor.*

ACKNOWLEDGMENTS

This work was supported by grants of the CICYT (BIO96-0827 and PETRI 93-0081). F. J. Pérez-Llarena received a fellowship from the Education Council of the Basque Government (Spain). A. Rodríguez was supported by a fellowship of the PFPI Program, Ministry of Education and Science (Madrid).

We thank F. Malpartida for providing *S. coelicolor* JF1 and A. L. Demain for reading the manuscript.

REFERENCES

1. **Amador, E., J. M. Castro, and J. F. Martín.** Structure and organization of the *rrn*D operon of *Brevibacterium lactofermentum*. Transcriptional analysis of the 16S rRNA gene. Submitted for publication.
2. **Anzai, H., T. Murakami, S. Imai, A. Satoh, K. Nagaoka, and C. J. Thompson.** 1987. Transcriptional regulation of bialaphos biosynthesis in *Streptomyces hygroscopicus*. J. Bacteriol. **169:**3482–3488.
3. **Bibb, M.** 1996. The regulation of antibiotic production in *Streptomyces coelicolor* A3(2). Microbiology **142:**1335–1344.
4. **Brown, A. G., D. Butterworth, M. Cole, G. Hanscomb, J. D. Hood, C. Reading, and G. N. Robinson.** 1976. Naturally occurring β-lactamase inhibitors with antibacterial activity. J. Antibiot. **29:**668–669.
5. **Chater, K. F., and M. J. Bibb.** Regulation of bacterial antibiotic production. *In* H. Kleinkauf and H. von Döhren (ed.), Products of secondary metabolism, in press. VCH, Weinheim, Germany.
6. **Coque, J. J. R., F. J. Enguita, J. F. Martín, and P. Liras.** 1995. A two-protein component 7α-cephem-methoxylase encoded by two genes of the cephamycin C cluster converts cephalosporin C to 7-methoxycephalosporin C. J. Bacteriol. **177:**2230–2235.
7. **Coque, J. J. R., P. Liras, L. Laiz, and J. F. Martín.** 1991. A gene encoding lysine 6-aminotransferase, which forms the β-lactam precursor α-aminoadipic acid, is located in the cluster of cephamycin biosynthetic genes in *Nocardia lactamdurans*. J. Bacteriol. **173:**6258–6264.
8. **Coque, J. J. R., J. F. Martín, and P. Liras.** 1993. Characterization and expression in *Streptomyces lividans* of *cef*D and *cef*E genes from *Nocardia lactamdurans*: the organization of the cephamycin gene cluster differs from that in *Streptomyces clavuligerus*. Mol. Gen. Genet. **236:**453–458.
9. **Coque, J. J. R., P. Liras, and J. F. Martín.** 1993. Genes for a β-lactamase, a penicillin-binding protein and a transmembrane protein are clustered with the cephamycin biosynthetic genes in *Nocardia lactamdurans*. EMBO J. **12:**631–639.

10. **Coque, J. J. R., J. F. Martín, J. G. Calzada, and P. Liras.** 1991. The cephamycin biosynthetic genes *pcb*AB, encoding a large multidomain peptide synthetase, and *pcb*C of *Nocardia lactamdurans* are clustered together in an organization different from the same genes in *Acremonium chrysogenum* and *Penicillium chrysogenum*. Mol. Microbiol. **5:**1125–1133.
11. **Coque, J. J. R., F. J. Pérez-Llarena, F. J. Enguita, J. L. de la Fuente, J. F. Martín, and P. Liras.** 1995. Characterization of the *cmc*H genes of *Nocardia lactamdurans* and *Streptomyces clavuligerus* encoding a functional 3′-hydroxymethylcephem *O*-carbamoyltransferase from cephamycin biosynthesis. Gene **162:**21–27.
12. **Doran, J. L., B. K. Leskiw, S. Aippersbach, and S. E. Jensen.** 1990. Isolation and characterization of a β-lactamase-inhibitory protein from *Streptomyces clavuligerus* and cloning and analysis of the corresponding gene. J. Bacteriol. **172:**4909–4918.
13. **Fernández-Moreno, M. A., J. L. Caballero, D. A. Hopwood, and F. Malpartida.** 1991. The *act* cluster contains regulatory and antibiotic export genes, direct targets for translational control by the *bld*A transfer RNA gene of *Streptomyces coelicolor*. Cell **66:**769–780.
14. **Furuya, K., and C. R. Hutchinson.** 1996. The DnrN protein of *Streptomyces peucetius*, a pseudo-response regulator, is a DNA-binding protein involved in the regulation of daunorubicin biosynthesis. J. Bacteriol. **178:**6310–6318.
15. **García-Domínguez, M., J. F. Martín, B. Mahro, A. L. Demain, and P. Liras.** 1987. Efficient plasmid transformation of the β-lactam producer *Streptomyces clavuligerus*. Appl. Environ. Microbiol. **53:**1376–1381.
16. **Gramajo, H. C., E. Takano, and M. J. Bibb.** 1993. Stationary-phase production of the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2) is transcriptionally regulated. Mol. Microbiol. **7:**837–845.
17. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Shrempf.** 1985. Genetic manipulation of *Streptomyces*: a laboratory manual. The John Innes Foundation, Norwich, United Kingdom.
18. **Horinouchi, S., and T. Beppu.** 1992. Autoregulatory factors and communication in actinomycetes. Annu. Rev. Microbiol. **46:**377–398.
19. **Horinouchi, S., M. Kito, M. Nishiyama, K. Furuya, S.-K. Hong, K. Miyake, and T. Beppu.** 1990. Primary structure of AfsR, a global regulatory protein for secondary metabolite formation in *Streptomyces coelicolor* A3(2). Gene **95:**49–56.
20. **Janssen, G. R., and M. J. Bibb.** 1993. Derivatives of pUC18 that have *Bgl*II sites flanking a modified multiple cloning site and that retain the ability to identify recombinant clones by visual screening of *Escherichia coli* colonies. Gene **124:**133–134.
21. **Kieser, T., and R. E. Melton.** 1988. Plasmid pIJ699, a multicopy positive selection vector for *Streptomyces*. Gene **65:**83–91.
22. **Kovacevic, S., and J. R. Miller.** 1991. Cloning and sequencing of the β-lactam hydroxylase gene (*cefF*) from *Streptomyces clavuligerus*: gene duplication may have led to separate hydroxylase and expandase activities in the actinomycetes. J. Bacteriol. **173:**398–400.
23. **Kovacevic, S., B. J. Weigel, M. G. Tobin, T. D. Ingolia, and J. R. Miller.** 1989. Cloning, characterization, and expression in *Escherichia coli* of the *Streptomyces clavuligerus* gene encoding deacetoxycephalosporin C synthetase. J. Bacteriol. **171:**754–760.
24. **Kumar, C. V., J. J. R. Coque, and J. F. Martín.** 1994. Efficient transformation of the cephamycin C producer *Nocardia lactamdurans* and development of shuttle and promoter-probe cloning vectors. Appl. Environ. Microbiol. **60:**4086–4093.
25. **Kumar, V., J. L. de la Fuente, A. L. Leitão, P. Liras, and J. F. Martín.** 1996. Effect of amplification or targeted disruption of the β-lactamase gene of *Nocardia lactamdurans* on cephamycin biosynthesis. Appl. Microbiol. Biotechnol. **45:**621–628.
26. **Leskiw, B. K., E. J. Lawlor, J. M. Fernández-Ábalos, and K. F. Chater.** 1991. TTA codons in some genes prevent their expression in a class of developmental antibiotic-negative *Streptomyces* mutants. Proc. Natl. Acad. Sci. USA **88:**2461–2465.
27. **Madduri, K., and C. R. Hutchinson.** 1995. Functional characterization and transcriptional analysis of the $dnrR_1$ locus, which controls daunorubicin biosynthesis in *Streptomyces peucetius*. J. Bacteriol. **177:**1208–1215.
28. **Madduri, K., C. Stuttard, and L. C. Vining.** 1991. Cloning and location of a gene governing lysine ε-aminotransferase, an enzyme initiating β-lactam biosynthesis in *Streptomyces* spp. J. Bacteriol. **173:**985–988.
29. **Martín, J. F., and A. L. Demain.** 1980. Control of antibiotic synthesis. Microbiol. Rev. **44:**230–251.
30. **Martín, J. F., and P. Liras.** 1989. Organization and expression of genes involved in the biosynthesis of antibiotics and other secondary metabolites. Annu. Rev. Microbiol. **43:**173–206.
31. **Martín, J. F., A. T. Marcos, A. Martín, J. A. Asturias, and P. Liras.** 1994. Phosphate control of antibiotic biosynthesis at the transcriptional level, p. 140–147. *In* A. Torriani-Gorini, E. Yagil, and S. Silver (ed.), Phosphate in microorganisms: cellular and molecular biology. ASM Press, Washington, D.C.
32. **Narva, K. E., and J. S. Feitelson.** 1990. Nucleotide sequence and transcriptional analysis of the *redD* locus of *Streptomyces coelicolor* A(3)2. J. Bacteriol. **172:**326–333.
33. **Paradkar, A. S., and S. E. Jensen.** 1995. Functional analysis of the gene encoding the clavaminate synthase 2 isoenzyme involved in clavulanic acid biosynthesis in *Streptomyces clavuligerus*. J. Bacteriol. **177:**1307–1314.
34. **Pérez-Llarena, F. J., J. F. Martín, M. Galleni, J. J. R. Coque, J. L. de la Fuente, J.-M. Frère, and P. Liras.** The *bla* gene of the cephamycin cluster of *Streptomyces clavuligerus* encodes a class A β-lactamase of low enzymatic activity. Submitted for publication.
35. **Pérez-Llarena, F. J., P. Liras, and J. F. Martín.** Unpublished results.
36. **Romero, J., P. Liras, and J. F. Martín.** 1984. Dissociation of cephamycin and clavulanic acid biosynthesis in *Streptomyces clavuligerus*. Appl. Microbiol. Biotechnol. **20:**318–325.
37. **Romero, J., P. Liras, and J. F. Martín.** 1988. Isolation and biochemical characterization of *Streptomyces clavuligerus* mutants in the biosynthesis of clavulanic acid and cephamycin C. Appl. Microbiol. Biotechnol. **27:**510–516.
38. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
39. **Strynadka, N. C. J., S. E. Jensen, K. Johns, H. Blanchard, M. Page, A. Matagne, J.-M. Frère, and M. N. G. James.** 1994. Structural and kinetic characterization of a β-lactamase-inhibitor protein. Nature **368:**657–660.
40. **Stutzman-Engwall, K. J., S. L. Otten, and C. R. Hutchinson.** 1992. Regulation of secondary metabolism in *Streptomyces* spp. and overproduction of daunorubicin in *Streptomyces peucetius*. J. Bacteriol. **174:**144–154.
41. **Takano, E., H. C. Gramajo, E. Strauch, N. Andres, J. White, and M. J. Bibb.** 1992. Transcriptional regulation of the *red*D transcriptional activator gene accounts for growth phase-dependent production of the antibiotic undecylprodigiosin in *Streptomyces coelicolor* A3(2). Mol. Microbiol. **6:**2797–2804.
42. **Walter, N., B. Barton, and A. Earl.** January 1994. Novel compounds. International patent application (PCT) WO 94/18326.
43. **Ward, J. M., and J. E. Hodgson.** 1993. The biosynthetic genes for clavulanic acid and cephamycin production occur as a "super-cluster" in three *Streptomyces*. FEMS Microbiol. Lett. **110:**239–242.
44. **Wright, F., and M. J. Bibb.** 1992. Codon usage in the G+C-rich *Streptomyces* genome. Gene **113:**55–65.
45. **Yu, H., E. Serpe, J. Romero, J. J. R. Coque, K. Maeda, M. Oelgeschläger, G. Hintermann, P. Liras, J. F. Martín, A. L. Demain, and J. Piret.** 1994. Possible involvement of the lysine ε-aminotransferase gene (*lat*) in the expression of the genes encoding ACV synthetase (*pcb*AB) and isopenicillin N synthase (*pcb*C) in *Streptomyces clavuligerus*. Microbiology **140:**3367–3377.