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Investigation of the *Streptomyces clavuligerus* Cephamycin C Gene Cluster and Its Regulation by the CcaR Protein

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As part of a search for transcriptional regulatory genes, sequence analysis of several previously unsequenced gaps in the cephamycin biosynthetic cluster has revealed the presence in *Streptomyces clavuligerus* of seven genes not previously described. These include genes encoding an apparent penicillin binding protein and a transport or efflux protein, as well as the CmcI and CmcJ proteins, which catalyze late reactions in the cephamycin biosynthetic pathway. In addition, we discovered a gene, designated *pcd*, which displays significant homology to genes encoding semialdehyde dehydrogenases and may represent the gene encoding the long-sought-after dehydrogenase involved in the conversion of lysine to α-aminoadipate. Finally, two genes, *sclU* and *rhsA*, with no obvious function in cephamycin biosynthesis may define the end of the cluster. The previously described CcaR protein displays homology to a number of *Streptomyces* pathway-specific transcriptional activators. The *ccaR* gene was shown to be essential for the biosynthesis of cephamycin, clavulanic acid, and non-clavulanic acid clavams. Complementation of a deletion mutant lacking *ccaR* and the adjacent *orf11* and *blp* genes showed that only *ccaR* was essential for the biosynthesis of cephamycin, clavulanic acid, and clavams and that mutations in *orf11* or *blp* had no discernible effects. The lack of cephamycin production in *ccaR* mutants was directly attributable to the absence of biosynthetic enzymes responsible for the early and middle steps of the cephamycin biosynthetic pathway. Complementation of the *ccaR* deletion mutant resulted in the return of these biosynthetic enzymes and the restoration of cephamycin production.

Streptomyces species are well known for their possession of gene clusters which orchestrate antibiotic biosynthesis. These clusters consist of resistance, transport, and regulatory genes physically linked and coordinately regulated with genes encoding biosynthetic enzymes (11). *Streptomyces clavuligerus* produces a number of β-lactam compounds, including cephamycin C, clavulanic acid, and several structurally related clavams which differ from clavulanic acid in the stereochemistry of the clavam nucleus and nature of the substituent groups. The genes responsible for cephamycin biosynthesis in *S. clavuligerus* are clustered and may be flanked by the genes encoding the Bla (43) and PcbR (40) resistance proteins. The genes encoding three of the earliest enzymes in the biosynthetic pathway, lysine ε-aminotransferase (LAT), δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS), and isopenicillin N synthase (IPNS), designated *lat*, *pcbAB*, and *pcbC* (42), are ordered sequentially in the gene cluster, and their transcriptional organization has been determined (45, 46). The *lat* promoter is thought to direct the synthesis of a polycistronic transcript of ~14 kb responsible for *lat*, *pcbAB*, and *pcbC* expression. As well, a promoter located within the 3′ end of the *pcbAB* coding region was shown to be responsible for the production of a monocistronic *pcbC* transcript.

The LAT protein catalyzes the first of a two-step reaction converting lysine to α-aminoadipate (29), while the second step has only recently been characterized. The product of LAT activity, 1-piperideine-6-carboxylate, requires the activity of a piperideine-6-carboxylate dehydrogenase enzyme to be converted into α-aminoadipate (14), and yet no candidate genes have been discovered in any bacterial cephamycin clusters studied to date (42). The ACVS enzyme catalyzes the condensation of the three precursor amino acids, valine, cysteine, and α-aminoadipate, into δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV), which in turn undergoes an oxidative cyclization by the IPNS enzyme (42).

The *cefD* and *cefE* genes, located adjacent to one another about 10 kb upstream of the *lat pcbAB pcbC* operon, encode the enzymes isopenicillin N epimerase (IPNE) and desacetoxycephalosporin C synthase (DAOCS), which function in the middle part of the pathway (42). IPNE converts isopenicillin N to penicillin N, and DAOCS then catalyzes a further conversion to desacetoxycephalosporin C (42). The *cefD* and *cefE* genes are also organized into an operon which gives rise to a polycistronic transcript together with other, as yet uncharacterized genes (31). Genes encoding enzymes which function later in the cephamycin pathway (*cefF* [30]) and (*cmcH* [13]) have also been located within the cephamycin cluster.

The genes responsible for clavulanic acid biosynthesis are located directly adjacent to the cephamycin biosynthetic cluster in *S. clavuligerus* (55), and a large portion of the cluster has been sequenced (25). It is unclear whether the *cas1* gene, encoding one of a pair of isozymes of clavaminate synthase (36), is part of a third group of biosynthetic genes responsible for the biosynthesis of non-clavulanic acid clavam compounds or is just the result of an apparent gene duplication event. Sequence analysis, both upstream and downstream of *cas1*, failed to demonstrate any additional homologs to genes within the cephamycin-clavulanic acid supercluster (56), and *cas1* is apparently not linked to the supercluster since it is separated from *cas2* by more than 40 kb (37).

Recently, Walters and coworkers (54) described the sequence analysis of a complementing fragment of DNA which restored clavulanic acid and cephamycin C production to non-producing mutants. The gene was designated *dclX* (for decreased clavulanic acid) and was believed to encode a tran-

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scriptional activator because of its similarity to a number of pathway-specific transcriptional activators from various *Streptomyces* spp. The existence of a species-specific transcriptional activator affecting cephamycin production would be consistent with previous results which showed that the *lat* promoter displayed very strong activity in *S. clavuligerus* but 155-fold-weaker activity in *S. lividans* (45). Pérez-Llarena et al. (44) subsequently provided conclusive evidence that this gene was essential for the biosynthesis of both cephamycin and clavulanic acid and renamed it *ccaR*, for cephamycin and clavulanic acid regulator. In addition to *ccaR*, biosynthesis of clavulanic acid is also specifically regulated by *claR*, encoding a LysR-type transcriptional activator, located within the clavulanic acid gene cluster (39). A functional *claR* is essential for expression of the biosynthetic late genes required for the conversion of the intermediate clavaminic acid to clavulanic acid.

When the deduced amino acid sequence of CcaR was aligned with the homologous transcriptional activators (RedD, ActIIORF4 (open reading frame 4), AfsR, and DnrI), many regions of similarity were found to be present throughout the primary sequence. The *ccaR* gene also contains a rare leucine-encoding TTA codon often found in the N-terminal end of many of these activators (11) and demonstrated to be involved in the hierarchy of regulation for antibiotic biosynthesis and sporulation in *S. coelicolor* (33). Since the *ccaR* designation more accurately describes the involvement of this gene in the regulation of both cephamycin and clavulanic acid biosynthesis, we have chosen to adopt this terminology for subsequent studies. The sequence analysis of two genes (designated *orf11* and *blp*) located downstream of *ccaR* has also been described (44). The *orf11* gene did not display any significant similarity to known sequences, while *orf12* was renamed *blp*, for β-lactamase-inhibitory protein (BLIP)-like protein because of its similarity to the *bli* gene previously described for *S. clavuligerus* (16). This terminology will also be used in the present study.

Insertional inactivation of *ccaR* has been shown to eliminate cephamycin and clavulanic acid biosynthesis in *S. clavuligerus* (44). We now show that biosynthesis of the clavams is also eliminated, and in the case of cephamycin biosynthesis, the *ccaR* mutation results in a failure to produce biosynthetic pathway enzymes.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. *S. clavuligerus* was maintained on MYM agar (51) or ISP Medium 3 (Difco, Detroit, Mich.). Plasmid-containing cultures were supplemented with thiostrepton (5 μg/ml; Sigma Chemical Corp., St. Louis, Mo.), apramycin (25 μg/ml; Provel Inc., Scarborough, Ontario, Canada) or hygromycin (200 μg/ml; Boehringer Mannheim Biochemicals, Laval, Quebec, Canada) as appropriate. *S. clavuligerus* strains were stored as spore stocks in 20% glycerol at −70°C.

Cultures for measurement of cephamycin production and/or production of cell extracts for Western analysis were prepared by inoculating spores of *S. clavuligerus* into Trypticase soy broth (Becton Dickinson and Company, Cockeysville, Md.) supplemented with 1% (wt/vol) soluble starch (TSBS) and incubating them on a rotary shaker for 30 to 36 h. Production cultures were prepared by subculturing seed cultures into fresh TSBS and incubating them for 48 to 72 h. All cultures of *S. clavuligerus* were incubated at 28°C and 250 or 280 rpm.

Production cultures for measurement of clavulanic acid or clavams were prepared as described above except that seed cultures were washed twice with sterile water before being subcultured into fresh TSBS, starch-asparagine (SA) medium (1), or soy medium (48) and incubated for 72 h.

Escherichia coli cultures were maintained on 2YT agar and grown in 2YT or Terrific Broth (49) at 37°C. Plasmid containing cultures were supplemented with ampicillin (100 μg/ml; Sigma), tetracycline (12.5 μg/ml; Sigma), apramycin (100 μg/ml), or hygromycin (50 μg/ml) as appropriate.

Transformation of *S. clavuligerus*. Protoplast formation, transformation, and selection of transformants were carried out as described by Hopwood et al. (26) and Bailey and Winstanley (6).

Recombinant DNA techniques. Restriction enzyme digestion was carried out according to the manufacturers' recommendations. Purification of DNA fragments from agarose gel blocks was done with the GlassMax DNA isolation system (GIBCO BRL, Burlington, Ontario, Canada). Ligation reactions, generation of blunt ends on DNA fragments by using Klenow or T4 DNA polymerase, plasmid isolation, PCR, dephosphorylation of DNA with alkaline phosphatase, random primer labeling, and *E. coli* transformations were all done as described by Sambrook et al. (49). Plasmid cloning vectors, oligonucleotide primers, and plasmid constructs used for sequence analysis, gene disruption and replacement, complementation, or high-level expression studies are listed in Tables 1 and 2. More detailed explanations of plasmid constructions or intermediate plasmids are available upon request.

Sequence analysis of *S. clavuligerus* cephamycin cluster. DNA fragments from the cephamycin cluster-containing cosmids, pOW309 and pLAFR/2-49, were subcloned into pBluescript KS(+), pBluescript SK(+), or pTZ18R, and exonuclease III-mediated deletions were generated with an Erase-a-Base kit (Promega Corp., Madison, Wis.). The resulting deletion clones were sequenced on both strands via ABI PRISM dye terminator cycle sequencing by the University of Alberta Department of Biological Sciences DNA synthesis lab, using a combination of universal and custom primers. No regions of the cluster which had already been sequenced by other groups were intentionally resequenced. Duplicated sequence was generated only when it was necessary to cross junctions of known and unknown regions or where sequences were published after completion of this sequencing project.

High-level production of CcaR and LAT in *E. coli*. To obtain large amounts of CcaR and LAT for preparation of polyclonal antibodies, each protein was overproduced in *E. coli*. Large amounts of purified IPNS were available from a previous study (18). Overproduction of both LAT and CcaR was achieved by fusion of the coding regions of each gene in frame with the gene encoding the maltose binding protein (MBP) in the vector pMAL-c2. These fusion clones were created by using PCR to introduce artificial *Eco*RI and *Bam*HI sites at the 3′ and 5′ ends, respectively, of each gene. For *ccaR*, the primers Dyl12 and Dyl13 and Vent DNA polymerase (New England Biolabs, Mississauga, Ontario, Canada) were used. For *lat*, the primers Dyl9 and Dyl10 and *Taq* DNA polymerase (Boehringer Mannheim) were used.

The amplified PCR fragments were inserted into pBluescript KS(+) and then subjected to sequence analysis. The *lat* clone contained a number of *Taq*-induced mutations; therefore, the entire ORF was replaced with wild-type DNA, leaving only a small amount of PCR-generated upstream sequence containing the desired *Bam*HI site. Amplification of the *ccaR* clone occurred without the introduction of any unintended mutations. The *ccaR* and *lat* genes were then excised as *Bam*HI/*Hin*dIII fragments and inserted into pMAL-c2 to create pMAL-CcaR and pMAL-LAT.

Cultures of *E. coli* HB101 carrying pMAL-CcaR and pMAL-LAT were grown in 2YT at 37°C to an optical density at 600 nm of about 0.5, induced by the addition of isopropyl-β-D-thiogalactopyranoside (0.5 mM, final concentration), and then incubated at 30°C for 4 h. The MBP fusion proteins were purified from induced cultures as directed by the manufacturer.

Preparation of polyclonal antibodies to CcaR, LAT, and IPNS. Purified MBP fusion proteins and purified IPNS protein, approximately 150 μg of each, were run on separate sodium dodecyl sulfate (SDS)–7.5 or 10% polyacrylamide gels (9), stained for 30 min with 0.1% (wt/vol) Coomassie blue, and then destained in water for 10 min. Protein bands were excised from the gels and homogenized in phosphate-buffered saline (49), and the gel slurries were used to immunize rabbits by standard procedures (23).

Creation of *ccaR* and *cob* mutants. (i) *ccaR*::*apr* mutant construct. A 3.7-kb *Eco*RI/*Bam*HI fragment carrying the *ccaR*, *orf11*, and *blp* (*cob*) group of genes was inserted into pTZ18R. The blunt-ended apramycin resistance (*apr*) cassette (see below) was inserted at the unique *Eco*ICRI site within the *ccaR* gene.

(ii) Δ*ccaR*::*tsr* mutant construct. The thiostrepton resistance (*tsr*) marker from pIJ486 was inserted as a 1.1-kb *Bcl*I fragment into pSL1180. A 2.8-kb *Nco*I/*Bam*HI fragment of *S. clavuligerus* DNA containing the *cefF-cmcH* pair of genes was inserted upstream of *tsr*. A 2.3-kb *Nru*I/*Eco*RI fragment containing the *orf11-blp* pair of genes was inserted downstream of *tsr*. The resulting construct carried a contiguous stretch of *S. clavuligerus* DNA extending from *cefF* to *blp* except that the 1.4-kb *Bam*HI/*Nru*I fragment containing *ccaR* had been replaced with *tsr*.

(iii) Δ*cob*::*tsr* mutant construct. By using the same strategy, a 2.8-kb *Nco*I/*Bam*HI fragment containing the *cefF-cmcH* pair of genes was inserted upstream of *tsr* and a 1.7-kb *Eco*RI/*Apa*I fragment containing the *lat* gene was inserted downstream of *tsr*, all within pSL1180. The resulting construct carried a contiguous stretch of *S. clavuligerus* DNA extending from *cefF* to *lat* except that the 3.7-kb *Bam*HI/*Eco*RI fragment containing the *cob* group of genes had been replaced with *tsr*.

All of the targeting vectors described above were prepared as parallel sets of constructs in which the antibiotic resistance marker was inserted in both orientations relative to the gene(s) being disrupted or deleted. In all cases where fragments with incompatible ends were to be ligated, the fragments were first made blunt by using Klenow or T4 DNA polymerase. The various disruption and deletion constructs are shown in Fig. 1. All of the targeting vectors described above were finally converted into *E. coli-Streptomyces* shuttle vectors by fusion with the *Streptomyces* replicon, pJOE829, at the unique *Hin*dIII site. The resulting gene targeting vectors were then introduced into *S. clavuligerus* by transformation. Disruption or deletion mutants defective in *ccaR* or the *cob* group of

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant features	Source or reference[a]
Bacterial strains		
S. clavuligerus		
NRRL 3585	Wild type	NRRL
Δ*cob*::*tsr*A/B	*ccaR orf11 blp* deletion mutant, Tsr^r	This study
Δ*ccaR*::*tsr*A/B	*ccaR* deletion mutant, Tsr^r	This study
ccaR::*apr*A/B	*ccaR* disruption mutant, Apr^r	This study
E. coli HB101	General cloning host strain	48
Plasmid vectors		
Cloning vectors		
pSL1180	*E. coli* general cloning vector, Amp^r	Pharmacia
pBluescript KS/SK	*E. coli* general cloning vector, Amp^r	Stratagene
pTZ18R	*E. coli* general cloning vector, Amp^r	U.S. Biochemicals
pUC119	*E. coli* general cloning vector, Amp^r	48
pJOE829	*Streptomyces* pIJ101 replicon, Hyg^r	J. Altenbuchner
pSET152	*E. coli* replicon, *Streptomyces* ØC31 attachment site, Apr^r	NRRL
pIJ486	*Streptomyces* pIJ101 replicon, Tsr^r	D. Hopwood
pMAL-c2	*E. coli* MBP fusion expression vector, Amp^r	New England Biolabs
Cephamycin cluster-containing cosmids		
pOW309	Cosmid containing some of the cephamycin cluster, Apr^r	P. Skatrud
pLAFR/2-49	Cosmid containing some of the cephamycin cluster, Tet^r	27
Gene-targeting vectors		
pDA517	*apr* cassette cloned into pBluescript KS	This study
pDA551/552	Shuttle vector carrying *ccaR*::*apr* construct	This study
pDA557/558	Shuttle vector carrying Δ*ccaR*::*tsr* construct	This study
pDA559/560	Shuttle vector carrying Δ*cob*::*tsr* construct	This study
E. coli high-level-expression constructs		
pMAL-CcaR	*ccaR* ORF inserted into pMAL-c2	This study
pMAL-LAT	*lat* ORF inserted into pMAL-c2	This study
Complementation constructs		
pDA1000	*tsr* marker inserted into pSET152	This study
pDA1001	Wild-type *cob* fragment inserted into pSET152	This study
pDA1002	*ccaR* mutant *cob* fragment inserted into pSET152	This study
pDA1003	*orf11* mutant *cob* fragment inserted into pSET152	This study
pDA1004	*blp* mutant *cob* fragment inserted into pSET152	This study
pDA1006	Wild-type *ccaR* fragment inserted into pSET152	This study
pDA1007	Mutant (*Eco*47III) *ccaR* fragment inserted into pSET152	This study
pDA1008	Mutant (*Eco*ICRI) *ccaR* fragment inserted into pSET152	This study

[a] NRRL, National Regional Research Laboratory. Some plasmids were generously provided by J. Altenbuchner, University of Stuttgart, Germany; D. Hopwood, John Innes Institute, Norwich, United Kingdom; and P. Skatrud, Eli Lilly, Indianapolis, Ind.

genes resulting from gene conversion by homologous recombination were isolated as described by Aidoo et al. (2). Disruption mutants defective in *ccaR* displayed an Apr^r hygromycin-sensitive (Hyg^s) phenotype, while deletion mutants defective in *ccaR* or the *cob* group of genes displayed a Tsr^r Hyg^s phenotype.

Creation of complementation constructs. (i) The *apr* cassette. The *apr* marker from pOW309 was introduced into pBluescript KS(+), and then the translational stop fragment adapter oligonucleotide Dyl19 was inserted immediately upstream of *apr* to give pDA517. The *apr* marker together with adapter oligonucleotide (*apr* cassette) could then be released from pDA517 as a unit by *Hin*dIII/*Sma*I digestion, made blunt by treatment with Klenow DNA polymerase, and inserted into any cloned gene where a nonsense mutation was desired. Once the *apr* cassette had been introduced into a gene with selection for Apr^r, the *apr* marker could be removed as a *Pst*I fragment and the remaining plasmid could be self-ligated to leave only the 37-bp translational stop fragment (TSF) which contains stop codons in all three reading frames.

(ii) Mutation of the *ccaR*, *orf11*, or *blp* gene. A plasmid construct carrying the *cob* group of genes was digested individually with each of the following enzymes which recognizes a unique site: *Eco*47III (near the 5′ end of *ccaR*), *Eco*ICRI (near the 3′ end of *ccaR*), *Nco*I (near the 5′ end of *orf11*), and *Not*I (near the 5′ end of *blp*). The blunt-ended *apr* cassette was inserted into these sites, which were also made blunt ended if necessary. After confirmation that the desired orientation of the *apr* cassette had been obtained, the *apr* marker was removed by *Pst*I digestion and religation, leaving behind the 37-bp TSF.

TABLE 2. Oligonucleotide primers used in this study

Oligonucleotide primer	Sequence[a]	Function
Dyl9	GCCGGATCCATGGGCGAAGCAGCACGCCACCCC	5′ *lat* primer
Dyl10	GCCGAATTCGCGTCAGACGCTCTCGGCGACCGG	3′ *lat* primer
Dyl12	GCCGAATTCGCGGTTTCAGGCCGGGGTACGAC	3′ *ccaR* primer
Dyl13	GCCGGATCCATGAACACCTGGAATGATGTGACG	5′ *ccaR* primer
Dyl19	CTAGACTAGTCTAGCATG	Creation of stop codons

[a] Restriction enzyme recognition sites or the positions of stop codons within each oligonucleotide primer are underlined.

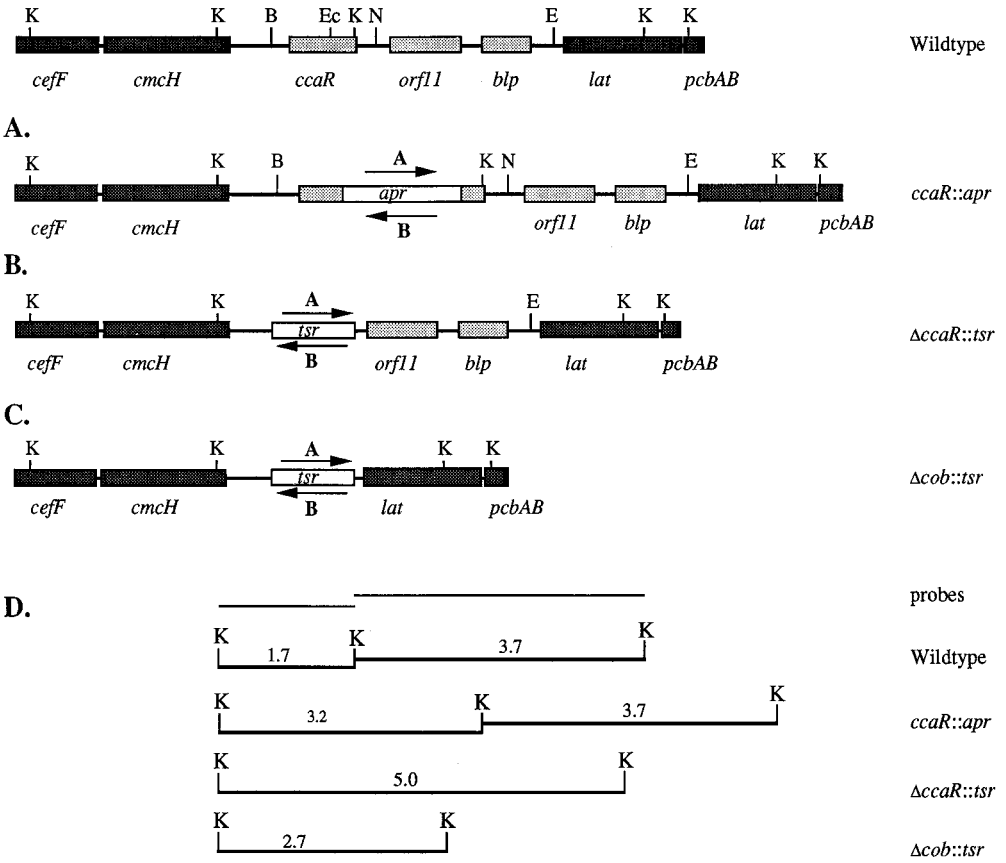

FIG. 1. Strategy for disruption or deletion of the *ccaR* gene or deletion of the *cob* group of genes by gene replacement. The lightly shaded boxes represent the target gene(s), the darker boxes represent other ORFs within the cephamycin cluster, and the clear box represents the antibiotic resistance marker. The antibiotic resistance markers were introduced in both orientations (A and B). (A) Insertion of the *apr* cassette into the *Eco*ICRI site of the *ccaR* gene; (B) deletion of the internal *Bam*HI/*Nru*I fragment containing the *ccaR* gene and replacement with the *tsr* marker; (C) deletion of the internal *Bam*HI/*Eco*RI fragment containing the *ccaR*, *orf11*, and *blp* genes and replacement with the *tsr* marker; (D) Southern hybridization pattern of *Kpn*I-digested genomic DNA from the wild type and *ccaR*::*apr*, Δ*ccaR*::*tsr*, and Δ*cob*::*tsr* mutants, using the 1.7- and 3.7-kb *Kpn*I fragments as hybridization probes. Abbreviations for restriction sites: B, *Bam*HI; E, *Eco*RI; Ec, *Eco*ICRI; K, *Kpn*I; N, *Nru*I.

(iii) pSET152 constructs. The 3.7-kb *Eco*RI/*Bam*HI fragment of wild-type *S. clavuligerus* DNA which carries the *cob* group of genes (pDA1001) and the corresponding fragments from the mutant constructs described above—*ccaR* mutant (TSF in *Eco*ICRI; pDA1002), *orf11* mutant (TSF in *Nco*I; pDA1003), and *blp* mutant (TSF in *Not*I; pDA1004)—were inserted into the integrating vector, pSET152 (see Fig. 5). As well, a 1.4-kb *Bam*HI/*Nru*I fragment of wild-type DNA which carries only *ccaR* (pDA1006) and the corresponding fragments from the mutant constructs described above—*ccaR* mutant (TSF in *Eco*47III near the 5′ end; pDA1007) and *ccaR* mutant (TSF in *Eco*ICRI near the 3′ end; pDA1008)—were inserted into pSET152. The *tsr* marker was also inserted into pSET152 to act as a negative control (pDA1000).

Southern hybridization analysis. Genomic DNA for Southern analysis was purified as described by Hopwood et al. (26). Confirmation of gene conversion by homologous recombination was obtained by Southern analysis of *Kpn*I-digested genomic DNA, using standard methods.

Heterologous probing of genomic DNA from a variety of β-lactam-producing and nonproducing actinomycetes was done with *Nco*I-digested genomic DNA separated by agarose gel electrophoresis, transferred to nylon membranes, and probed by standard techniques (49). Random primer-labeled probes were hybridized for 16 h followed by washing with 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7])–0.1% SDS for 20 min and 1× SSPE–0.1% SDS for 20 min, all at 55°C.

Antibiotic quantitation and cell extract preparation. Cultures of *S. clavuligerus* growing in SA, soy, or TSBS medium were sampled at 48 or 72 h. Culture filtrates from each sample were stored at −20°C and assayed within 1 week for cephamycin production (by bioassay [28]) and for clavulanic acid and clavam production (by high-pressure liquid chromatography [41]).

When cell extracts were to be analyzed, the cultures were harvested from TSBS cultures by centrifugation, washed with 0.85% (wt/vol) NaCl, and then resuspended in 1/5 original volume of TDE buffer (50 mM Tris-HCl [pH 7.0], 0.1 mM dithiothreitol, 0.01 mM EDTA). Washed cells were disrupted by sonication (two 20-s pulses; microprobe, power setting 2; Branson Sonifier 450; Branson Ultrasonic Corp., Danbury, Conn.) and then centrifuged for 5 min. Cell extracts were assayed for protein content (10) and stored at −20°C.

Western blot analysis. Five- or ten-microgram amounts of cell extract protein were separated on duplicate SDS–10% polyacrylamide gels. The gels were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, Mass.), using a Bio-Rad Transblot apparatus (Richmond, Calif.). The membranes were blocked in 5% (wt/vol) skim milk in Tris-buffered saline containing 0.1% (vol/vol) Tween 20 (TBST) for 16 h at 4°C. Proteins were detected by using the ECL (enhanced chemiluminescence) Western system reagents and protocols (Amersham, Chicago, Ill.). The primary antibodies were used at the following dilutions: CcaR, 1:5,000; LAT, 1:7,500; IPNS, 1:10,000; and DAOCS, 1:10,000. DAOCS antibodies were generously provided by C. Reeves, Panlabs Inc. Goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate (Amersham) was used as the secondary antibody.

Nucleotide sequence accession number. The sequence information obtained for gaps B, C, and D has been deposited under GenBank accession no. AF073895, AF073896, and AF073897, respectively; sequence information for flanking region A has been deposited under accession no. AF073894. Each deposited sequence includes six nucleotides of sequence overlapping with the previously published sequence.

RESULTS

Sequence analysis of gaps in the cephamycin biosynthetic cluster. Many of the structural genes encoding biosynthetic enzymes from the early and middle parts of the cephamycin C pathway of *S. clavuligerus* have been sequenced; however, several unsequenced gaps in the gene cluster remain. A rough

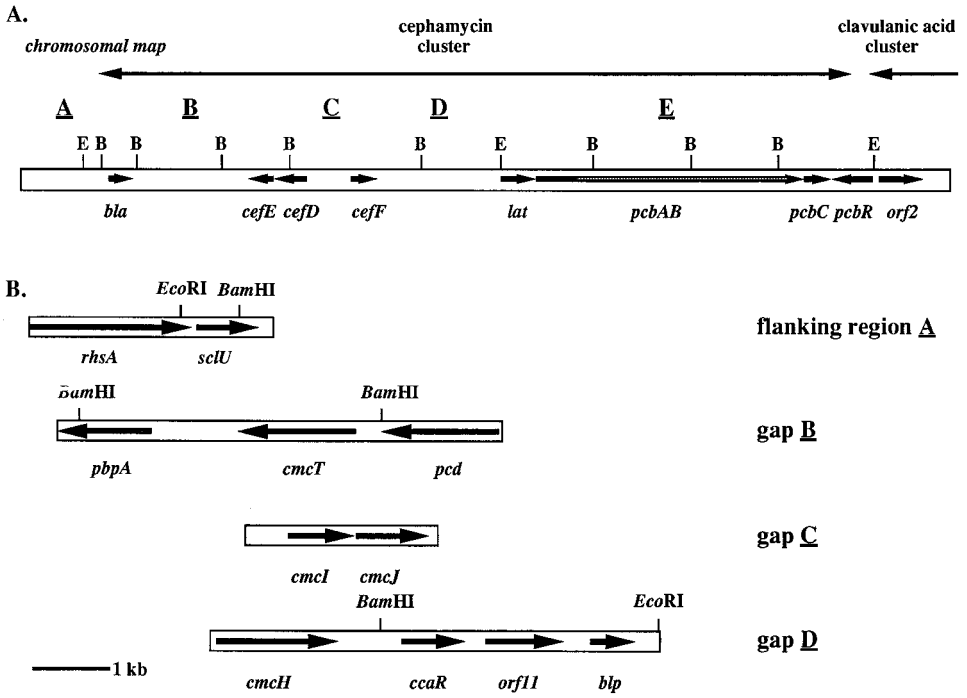

FIG. 2. Physical map of the cephamycin biosynthetic gene cluster in *S. clavuligerus*. (A) The chromosomal restriction map was created by compiling the restriction maps created in other studies (35, 50, 54). E and B, *Eco*RI and *Bam*HI restriction sites; solid arrows, genes previously sequenced; shaded bar, region of the *pcbAB* gene which has not been sequenced. Gaps within or regions flanking the known sequence of the cephamycin cluster are noted with underlined letters. (B) Diagrammatic representation of the genes present within the gaps or region flanking the cephamycin biosynthetic gene cluster as determined by sequence analysis. Arrows indicate the direction of transcription for each gene, positions of the *Eco*RI and *Bam*HI restriction sites within each fragment are shown.

restriction map of the cluster (Fig. 2A), indicating locations of the *Bam*HI and *Eco*RI restriction sites and positions of the genes for which sequence information was available at the onset of this study, was created by compilation of the restriction maps generated in other studies (35, 50, 54). Four of these unsequenced gaps (B through E) exist between regions of known sequence, while the other (flanking region A) lies adjacent to the previously sequenced *bla* gene and may represent a region beyond the end of the cephamycin cluster. The sizes of gaps B, C, and D were estimated to be 5.9, 2.3, and 6.0 kb, respectively. The gap E region represents about 8 kb from the middle of the *pcbAB* gene. In an attempt to identify a gene encoding a transcriptional activator which could be responsible for species-specific activation of the *lat* promoter (45), the remaining gaps of the cephamycin biosynthetic cluster were sequenced. Flanking region A was also sequenced to determine whether the genes present in this region appeared to be involved in cephamycin biosynthesis or whether *bla* represented the end of the cephamycin cluster.

Cosmid pOW309 (47) (generously provided by P. Skatrud, Eli Lilly Ltd.) was isolated from an *S. clavuligerus* DNA library constructed in the vector pKC462a when screened with a *pcbAB*-specific probe. Cosmid pLAFR/2-49 was isolated from an *S. clavuligerus* pLAFR3 cosmid library when screened with a *cefE*-specific probe (27). Cosmid pOW309 was mapped by using *Eco*RI and *Bam*HI, and the insert contained within this cosmid provided the template to complete the sequence of gaps located within the cephamycin cluster.

The *Bam*HI fragments were subcloned from pOW309 into pBluescript KS(+) or SK(+) vector and used to generate exonuclease III deletion subclones for sequencing. The *Eco*RI/*Bam*HI fragment was sequenced to obtain information on flanking region A. To obtain more DNA sequence information from this flanking region, digests of cosmid pLAFR/2-49 were hybridized with the *Eco*RI/*Bam*HI fragment, and a *Kpn*I/*Bam*HI fragment which provided a contiguous stretch of DNA extending downstream from the end of the pOW309 cosmid DNA was identified.

A combination of exonuclease III deletion subclones, custom primers, and additional deletion subclones created by using unique restriction enzymes was used to sequence the cluster fragments. Sequence across the junctions between subclones was confirmed by using custom primers or by sequencing overlapping subclones. All templates were sequenced on both strands by using cycle sequencing methodologies.

The sequence of the *pcbAB* gene from *S. clavuligerus* has been only partially determined at the 5′ (53, 57) and 3′ (17) ends, but since the unsequenced internal region of the gene (gap E) was judged to be unlikely to contain regulatory genes, it was left unsequenced. Regions of the cephamycin cluster for which published sequence information was available at the outset of this project were not sequenced again except to provide overlap with newly sequenced regions. Shortly after this project had been initiated, unpublished nucleotide sequence information for *ccaR* (*dclX* [54]) was generously provided by B. Barton, SmithKline Beecham, and so this region was not resequenced. Despite the fact that *ccaR* clearly resembled genes encoding transcriptional regulators, we persisted with the sequence analysis of unsequenced regions to ensure that all regulatory genes had been identified.

After the sequences of gaps B, C, and D and flanking region A had been completed, the sequences of the *cob*, group of genes (nucleotide accession no. Z81324) were reported by Pérez-Llarena et al. (44), and so these regions of sequence within gap D were inadvertently duplicated. All duplicated sequence information was checked for agreement with the

TABLE 3. Differences between newly determined sequence and published sequence

Sequence location in gap D[a]	Gene	Sequence comparison	
		Published[b]	This work
2278–2280	*ccaR* upstream	cc	ccc
2356–2358	*ccaR* upstream	cc	ccc
2450–2451	*ccaR* upstream	a	aa
3502–3503	*orf11* upstream	c	cc
3513	*orf11* upstream	gg	g
3643–3645	*orf11* upstream	ccg	cgc
3729–3730	*orf11* upstream	gg	gc
5754–5756	*blp* downstream	cc	ccc
5761–5764	*blp* downstream	ggg	gggg
5790–5791	*blp* downstream	g	gg
5803–5804	*blp* downstream	c	cc

[a] Nucleotide 7 represents the first base of new sequence adjacent to *cefF* published sequence (30).
[b] Sequence determined by Pérez-Llarena et al. (44) for wild-type *S. clavuligerus* ATCC 27064.

corresponding published sequence. Differences noted between the newly determined sequence and the published sequences are summarized in Table 3. It is not clear whether these discrepancies are due to allelic differences or sequencing errors, but careful reexamination of our raw sequence data and those of Walters et al. (54) failed to show any regions of ambiguity (7). Therefore, these changes may represent genuine differences between the two separate wild-type strains (NRRL 3585 and ATCC 27064) despite their recent common origin. Interestingly, all of these differences are located in the intergenic regions around the *cob* group of genes. Sequence information for a very small segment of the *cmcH* gene from *S. clavuligerus* was published previously by Coque et al. (13) but was not deposited in the databases, and so the entire region was resequenced.

A diagrammatic representation of the genes present within the gaps of the cephamycin cluster was generated from the compiled sequence containing both previously published and newly sequenced regions (Fig. 2B). The total amount of compiled sequence spanning from the *rhsA* gene to the 5′ end of the *pcbAB* gene was 26,035 nucleotides; when the unsequenced region of *pcbAB* and the sequences of the *pcbC* and *pcbR* genes were included, it was estimated that the entire cephamycin cluster would be approximately 38 kb. Nucleotide sequence accession numbers for previously sequenced genes used in this study are as follows: *bla*, Z54190 (43); *cefD*, M32324 (31); *cefE*, M24140 (31); *cefF*, M63809 (30); *lat* and the 5′ end of *pcbAB*, M64834 (53, 57). Each new gene was given a designation based on homology of its putative protein to known proteins from other species (see below). The organization of the genes was determined by using a combination of the CODONPREFERENCE program (15) from the Genetics Computer Group Wisconsin Package, which identifies ORFs on the basis of the characteristic third-position G+C bias typical of *Streptomyces*, as well as DNA Strider 1.2 and BLASTX and BLASTP searches of the databases at the National Center for Biotechnology Information (4, 5).

Analysis of ORFs in the newly sequenced regions. As a result of the sequence analysis and database homology searches of gaps B and C and flanking region A, we identified seven ORFs which have not been described previously for *S. clavuligerus*.

The PbpA protein shows weak homology to a number of low-molecular-weight penicillin binding proteins (PBPs). Although the *Nocardia lactamdurans* cephamycin gene cluster (12) also contains a *pbp* gene, that sequence was not identified as homologous in the database search. The *Nocardia* Pbp shows greater homology to D-Ala-D-Ala carboxypeptidases and β-lactamases. The PbpA protein in *S. clavuligerus* may function as an autoresistance mechanism with a role similar to that already demonstrated for PcbR, another PBP-type protein involved in resistance of *S. clavuligerus* to β-lactam antibiotics (40).

The CmcT protein shows homology to efflux pump proteins and therefore may function as a transporter for export of cephamycin from the cell.

The PCD protein shows strong homology to semialdehyde dehydrogenases. The location of *pcd* within the cephamycin cluster suggests that the PCD protein may function as a semialdehyde dehydrogenase in cephamycin biosynthesis. This dehydrogenase activity may be required to convert 1-piperideine-6-carboxylate to α-aminoadipate, the second of two steps required for the conversion of lysine into α-aminoadipate.

The CmcI and CmcJ proteins show significant homology only to the corresponding proteins from *N. lactamdurans* and are presumed to represent the cephalosporin-7-α-hydroxylase and methyltransferase activities responsible for the conversion of *O*-carbamoyl-desacetylcephalosporin C (OCDAC) to cephamycin C via 7-α-hydroxy-OCDAC (21).

Sequence information from flanking region A indicated the presence of two ORFs. The SclU (for *S. clavuligerus* unknown) protein, encoded by the first of these genes, does not shown significant homology to any known proteins, and therefore no presumed function can be ascribed. The sequence of the second ORF is apparently incomplete, but when analyzed by gapped BLASTP databases searches, a number of *rhs* genes from *E. coli* were identified as similar. The function of the putative Rhs proteins from *E. coli* is not completely understood, but the proteins contain a core ORF resembling a cell surface ligand binding protein and also contain a peptide motif that is repeated 28 times (24). The hybrid genes are also known to be recombination hot spots, for which they are named.

Neither the *sclU* nor the *rhsA* gene of *S. clavuligerus* has any apparent relationship to cephamycin biosynthesis, but since the function of each is unknown, neither gene can be eliminated as part of the cluster without further investigation.

Presence of homologous sequences in other β-lactam-producing actinomycetes. Genomic DNA preparations from a number of actinomycetes were subjected to Southern analysis using moderate-stringency conditions to look for evidence of cross-hybridization with various fragments of *S. clavuligerus* DNA from the cephamycin gene cluster. The *ccaR*, *orf11*, *blp*, and *pcd* genes were of particular interest because they had no apparent counterparts in the cephamycin gene cluster from *N. lactamdurans*. The *ccaR* probe was the same fragment as that generated by PCR for use in high-level expression, the *orf11* probe comprised two consecutive *Sma*I fragments extending from 130 bp downstream of the start codon to 80 bp upstream of the stop codon, and the *blp* probe was a *Not*I/*Pvu*I fragment extending from 40 bp downstream of the start codon to 170 bp downstream of the stop codon. The *pcd* probe was a *Kpn*I/*Bam*HI fragment extending from 48 bp downstream of the start codon to 27 bp downstream of the stop codon. The β-lactam-producing actinomycetes examined for evidence of hybridization were *S. clavuligerus*, *S. cattleya*, *S. griseus*, *S. jumonjinensis*, *S. lipmanii*, and *N. lactamdurans*. A variety of non-β-lactam-producing actinomycetes were also included: *S. antibioticus*, *S. fradiae*, *S. griseofuscus*, *S. lividans*, and *S. venezuelae*. Both the *ccaR* probe and the *pcd* probe hybridized to all of the β-lactam producers tested except *N. lactamdurans* (Table 4) and did not hybridize to any of the non-β-lactam producers.

TABLE 4. Presence of cross-hybridizing DNA in other β-lactam-producing actinomycetes

Actinomycete[a]	β-Lactam antibiotic(s)	Extent of hybridization[b]				
		lat	*ccaR*	*orf11*	*blp*	*pcd*
S. clavuligerus	Cephamycin C, clavams, clavulanic acid	+++	+++	+++	+++	+++
S. cattleya	Cephamycin C	++	+	−	−	++
S. griseus	Cephamycins A and B	++	+	−	−	++
S. jumonjinesis	Cephamycin C, clavulanic acid	+	+	+	−	+
S. lipmanii	7-Methoxycephalosporin C, clavaminic acid	++	+	++	−	+++
N. lactamdurans	Cephamycin C	++	−	−	−	−

[a] A combination of β-lactam-producing actinomycetes (*S. clavuligerus* NRRL 3585, *S. cattleya* NRRL 3841, *S. griseus* NRRL 3851, *S. jumonjinensis* NRRL 5741, *S. lipmanii* NRRL 3584, and *N. lactamdurans* NRRL 3802) was used.

[b] Qualitative score relative to the hybridization to the *S. clavuligerus* DNA, indicated as strong hybridization (+++), moderate hybridization (++), weak or very weak hybridization (+), and no hybridization (−).

The *orf11* probe hybridized only to *S. clavuligerus*, *S. jumonjinensis*, and *S. lipmanii* among the β-lactam producers and to none of the nonproducers, while the *blp* probe hybridized only to *S. clavuligerus* DNA. A *lat* probe (*Nco*I/*Bam*HI fragment extending from the *lat* start codon to 166 bp downstream of the *lat* stop codon), acting as a positive control for β-lactam producers, showed hybridization with all β-lactam-producing species, including *N. lactamdurans*, and was absent from all nonproducers.

The presence of *ccaR* homologs in all β-lactam producers except *N. lactamdurans* suggests that a common mechanism for the regulation of β-lactam biosynthesis exists in the β-lactam-producing *Streptomyces* spp. and that it proceeds through a CcaR-like regulator. The fact that *N. lactamdurans* is excluded from this group suggests that it must rely on a different regulator.

Disruption or deletion of the *ccaR* gene eliminates antibiotic production. The presence of a pathway-specific regulatory gene, *ccaR* (also named *dclX*), controlling antibiotic production in *S. clavuligerus* has recently been reported by two separate groups (44, 54). We have also investigated this regulatory gene in order to better understand its mechanism of regulation and the role, if any, of the two uncharacterized ORFs (*orf11* and *blp*, downstream of *ccaR*) in the regulation of β-lactam metabolite production in *S. clavuligerus*.

To investigate the roles of *ccaR*, *orf11*, and *blp* in antibiotic production in *S. clavuligerus*, we prepared a series of targeting vectors and used them to create mutants with disruptions or deletions in these genes. Plasmids pDA559 and pDA560 carry a stretch of *S. clavuligerus* DNA from the cephamycin gene cluster in which a 3.7-kb *Bam*HI/*Eco*RI fragment containing the *cob* group of genes has been replaced with the *tsr* marker in both orientations. The plasmid constructs were introduced into protoplasts of wild-type *S. clavuligerus* by transformation, and then transformants were allowed to sporulate in the absence of antibiotic selection to promote the loss of free plasmid. Mutants in which the *cob* genes had been deleted due to homologous recombination between the plasmid construct and the corresponding region of the chromosome (Δ*cob*::*tsr*) were detected initially by their Tsr^r Hyg^s phenotype and then confirmed by Southern analysis. Genomic DNA from wild-type *S. clavuligerus*, when digested with *Kpn*I, gave (i) a 1.7-kb fragment containing most of *ccaR* and part of *cmcH* and (ii) a 3.7-kb fragment containing *orf11*, *blp*, and part of *lat* (Fig. 1D). When these DNA fragments were labeled and used as probes, the *Kpn*I digests of genomic DNA from presumptive Δ*cob*::*tsr* mutants showed a single 2.7-kb hybridizing *Kpn*I fragment, consistent with the replacement of the *cob* genes with a *tsr* marker.

Four independently created Δ*cob*::*tsr* mutants (two mutants for each orientation of *tsr*) were grown on TSBS for 48 and 72 h, and then culture supernatants were assayed for cephamycin production by bioassay. All of the Δ*cob*::*tsr* mutants showed no detectable production of cephamycin, whereas the wild-type culture gave large zones of inhibition. The cephamycin-negative phenotype occurred regardless of the orientation of the *tsr* marker. Previous studies (3) had indicated that transcription can proceed through the *tsr* marker when it is oriented in the same direction as transcription. Therefore, the antibiotic-negative phenotype was presumably due to the loss of the *cob* genes and not to a polar effect on downstream genes.

In a related series of experiments, disruption mutants were prepared in which only the *ccaR* gene was affected. Using plasmids pDA551 and pDA552, we created disruption mutants (*ccaR*::*apr*) in which the wild-type *ccaR* gene was replaced with a mutant copy disrupted by insertion of the *apr* cassette into the unique *Eco*ICRI site. Similarly, using plasmids pDA557 and pDA558, we created deletion mutants (Δ*ccaR*::*tsr*) in which the wild-type *ccaR* gene was replaced with a *tsr* marker. As described earlier, each of these mutants was isolated based on its antibiotic resistance phenotype and then confirmed by Southern hybridization. The *ccaR*::*apr* mutants gave 3.2- and 3.7-kb hybridizing *Kpn*I fragments, while the Δ*ccaR*::*tsr* mutants gave a single 5.0-kb *Kpn*I fragment. Both of these results were consistent with the insertion of the *apr* cassette and deletion of the *ccaR* gene, respectively.

When these various *ccaR* and *cob* mutants were subsequently tested for cephamycin, clavulanic acid, and clavam production, all were found to be negative for all of these metabolites regardless of the orientation of the antibiotic resistance marker.

Complementation with *ccaR* restores β-lactam metabolite production. Since complete loss of β-lactam metabolite production was seen in all Δ*ccaR*::*tsr* and *ccaR*::*apr* mutants as well as in Δ*cob*::*tsr* mutants, any possible effects of mutations in *orf11* or *blp* on metabolite production were obscured by the overriding effect of *ccaR*. To test the function of each gene independently, we prepared three separate pSET152-based plasmid constructs carrying the *cob* group of genes in which one of the three genes was mutated by insertion of the TSF carrying stop codons in all three reading frames. Plasmid pSET152 integrates site specifically into the *Streptomyces* chromosome, using the *attP* site and integrase present on the plasmid (8), and therefore is present at single copy.

The Δ*cob*::*tsr* deletion mutant was transformed with the various pSET152 plasmid constructs containing wild-type or mutant *ccaR*, *orf11*, or *blp* genes (Fig. 3). Genomic DNA from the transformants was analyzed to confirm that the complementing plasmid had integrated into the chromosome; then transformants were grown in TSBS, soy, and SA media, and culture supernatants were analyzed for cephamycin, clavulanic acid,

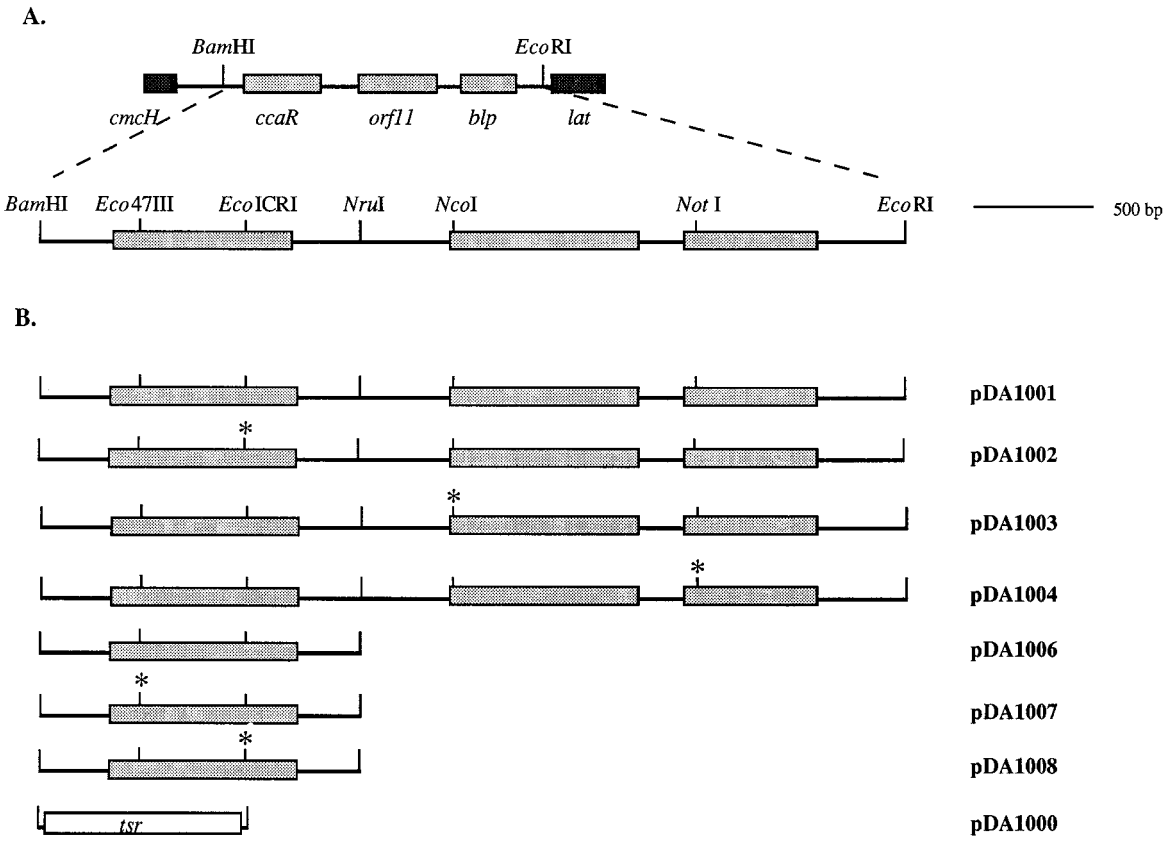

FIG. 3. The *cob* group of genes and pSET152 complementation constructs created with these genes. (A) Restriction map of the region of DNA containing the *cob* genes and unique restriction sites present within this region; (B) diagram of wild-type and mutant constructs created in pSET152 for the complementation experiments using the Δ*ccaR*::*tsr* or Δ*cob*::*tsr* deletion mutant strains. Asterisks represent locations of stop codon-containing TSFs.

and clavam metabolites. The pDA1001 construct carrying the three *cob* genes in wild-type form was capable of complementing Δ*cob*::*tsr* to restore production of cephamycin, clavulanic acid, and clavams. Mutation to either *orf11* or *blp* (pDA1003 or pDA1004, respectively) had no discernible effect on production of any of the metabolites, providing that an intact *ccaR* gene was present. Metabolite production was also restored to Δ*ccaR*::*tsr* mutants when a functional *ccaR* gene (pDA1006) alone was added. Since the mutations were designed to interrupt translation without affecting transcription, the effects observed could be unambiguously attributed to the mutated genes.

CcaR is essential for synthesis of the cephamycin C biosynthetic enzymes. Further studies into the CcaR-dependent regulation of β-lactam metabolite production concentrated on cephamycin biosynthesis since the biochemistry and genetics of this pathway are quite well understood (42), whereas the clavulanic acid and clavam biosynthetic pathways are only beginning to be elucidated (19, 25, 36, 41). Western analyses of cell extracts prepared from *ccaR* mutants were carried out to determine if expression of any of the genes encoding biosynthetic enzymes was affected in these mutants. Equivalent amounts of cell extract protein prepared from the various mutants were separated by SDS-PAGE, blotted onto PVDF membranes, and then probed with antisera specific for CcaR, LAT, IPNS, and DAOCS. As shown in Fig. 4, all four of these proteins were absent in all of the mutant strains, in contrast to the parental wild-type *S. clavuligerus* strain. The *ccaR* mutants also do not produce the ACVS protein, as determined by inspection of SDS-polyacrylamide gels, nor do they have detectable activities for the ACVS, IPNS, IPNE, and DAOCS biosynthetic enzymes (data not shown).

Complementation by *ccaR* restores production of cephamycin C biosynthetic enzymes. Introduction of plasmid pDA1006 into either the Δ*cob*::*tsr* or the Δ*ccaR*::*tsr* mutants resulted in integration of the *ccaR* gene in *trans* and at single copy into the chromosome, and this was sufficient to restore antibiotic production. Figure 5 shows that complementation resulted in restoration of the production not only of CcaR but also of three biosynthetic enzymes required for cephamycin production. Cephamycin production was, however, restored only to 89% of wild-type levels. Insertion of the TSF into the *Eco*47III or the *Eco*ICRI site of the *ccaR* gene used in complementation studies (Fig. 3) would be expected to create truncated CcaR proteins, 38 and 191 amino acids in length, respectively, instead of forming a full-length CcaR protein of 256 amino acids. Since both of these constructs (pDA1007 and pDA1008, respectively) were unable to complement a *ccaR* mutant, it was concluded that full-length CcaR gene products were needed before any complementation could occur. Western analysis did not show any evidence of these truncated forms of CcaR (Fig. 5, lanes 7 and 8), and therefore instability of the truncated products may prevent complementation.

DISCUSSION

Nucleotide sequence information has been obtained for several previously unsequenced regions of the cephamycin C biosynthetic gene cluster of *S. clavuligerus*; with this new informa-

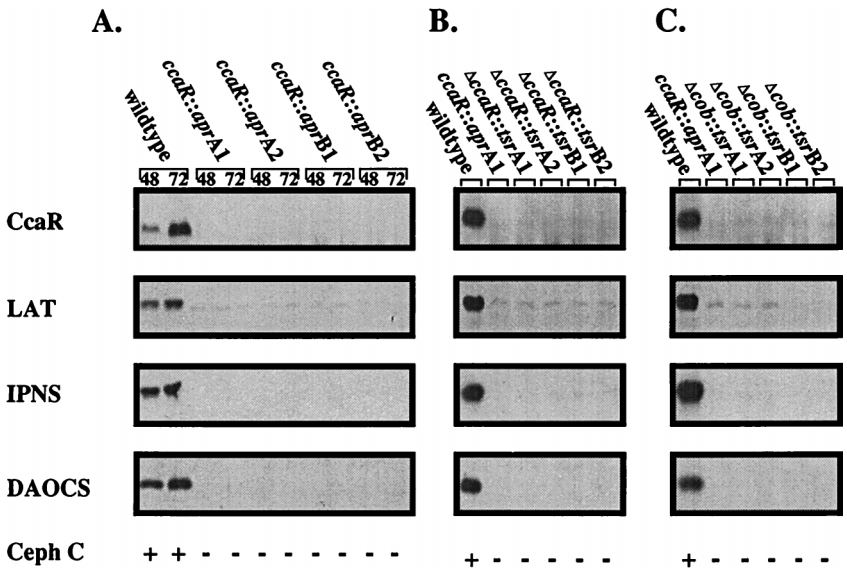

FIG. 4. Presence of the CcaR protein and cephamycin C biosynthetic enzymes in *ccaR* mutant strains. (A) Wild-type and *ccaR*::*apr* insertion strains; (B) wild-type and Δ*ccaR*::*tsr* deletion strains; (C) wild-type and Δ*cob*::*tsr* deletion strains. Ten micrograms of cell extract protein from each strain, harvested after 72 h of growth unless otherwise noted, was separated by SDS-PAGE (10% gel) and transferred to PVDF membranes. The resulting Western blots were developed with polyclonal antibodies specific for the CcaR, LAT, IPNS, and DAOCS proteins. Each strain's ability to produce cephamycin (Ceph) C was determined by bioassay, and the results were scored as + (production) and − (lack of production).

tion, the sequence of the cluster is complete except for an internal region of the *pcbAB* gene. Some uncertainty also remains as to whether *sclU* and/or *rhsA* represent one end of the cluster, but database searches do not suggest any identifiable roles for their putative proteins in cephamycin biosynthesis. New genes identified as a result of these sequence analyses appear to encode proteins responsible for resistance, transport, biosynthesis, and regulation functions in cephamycin biosynthesis. The *pbpA* gene likely acts as a resistance gene which along with *bla* (43) and *pcbR* (40) serves to protect the cell from the potentially lethal effects of the metabolites that it produces. Resistance genes also flank the cephamycin cluster in *N. lactamdurans* (12), but in *S. clavuligerus* the gene organization differs because *pcbR* has no counterpart in *N. lactamdurans*.

The *cmcT* gene could also be considered a resistance mechanism since genes encoding efflux proteins can confer resistance when cloned at high copy number, but its primary function more likely involves transporting cephamycin from the cell. The *cmcT* gene along with *pcd* and possibly *pbpA* may be transcribed as a polycistronic message together with the *cefD* and *cefE* genes, from a promoter located upstream of *cefD*. Kovacevic et al. (31) reported a *cefD*/*cefE* hybridizing mRNA of approximately 10 kb, which would be large enough to included all five of the genes within one transcript. Both the *bla* gene downstream of *pbpA* and the *cmcI* gene upstream of *cefD* are oriented in the opposite direction to the group of five genes and so cannot be part of the multicistronic transcript.

The *pcd* gene represents one of the more interesting genes present in the newly described sequences. The production of α-aminoadipate for cephamycin biosynthesis is a two-step process. The first step is catalyzed by LAT, which removes the ε amino group from lysine to create the intermediate 1-piperideine-6-carboxylate (cyclized form of aminoadipic semialdehyde). The second step, converting 1-piperideine-6-carboxylate to α-aminoadipate, is catalyzed by a PCD which has recently been purified from *S. clavuligerus* and characterized (14). However, a number of gene clusters from β-lactam-producing species have now been partially or fully sequenced and no candidate dehydrogenase-type genes have been identified (42), which led to speculation that the reaction might be catalyzed by a primary metabolic enzyme. *pcd* may represent that missing gene, a speculation consistent with the location of *pcd* within the cephamycin cluster and with the estimated size of the purified enzyme, 52.6 kDa, which corresponds well with the deduced molecular mass of the *pcd* gene product, 54.0 kDa. However, it seems unusual that since LAT and PCD are proposed to catalyze sequential steps in the pathway, their corresponding genes would not be more closely grouped, especially in view of the multicistronic transcriptional relationship that

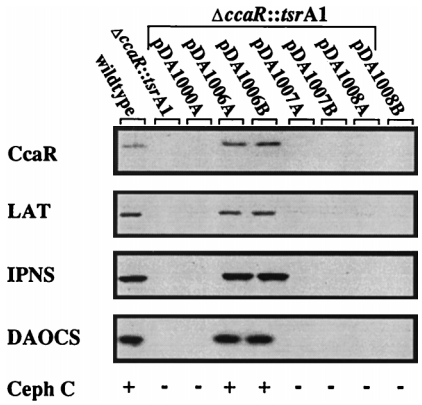

FIG. 5. Complementation of the Δ*ccaR*::*tsr*A1 mutant with pSET152 constructs. Western blot analysis and bioassay for production of cephamycin C were used to assess complementation. The designations A and B represent two independent clones generated during the same transformation. Five micrograms of cell extract protein from each strain, harvested after 48 h of growth, was separated by SDS-PAGE (10% gel) and transferred to PVDF membranes. The resulting Western blots were developed with polyclonal antibodies specific for the CcaR, LAT, IPNS, and DAOCS proteins. Each strain's ability to produce cephamycin (Ceph) C was determined by bioassay, and the results were scored as + (production) and − (lack of production).

exists between *lat* and the *pcbAB* and *pcbC* genes, encoding enzymes for the next two steps in the biosynthetic pathway. Instead, *pcd* is grouped together with genes encoding enzymes from the middle part of the pathway and is likely cotranscribed with them as a polycistronic message. A second area of concern is that no *pcd* gene has been identified in *N. lactamdurans* cephamycin cluster (consistent with the lack of cross-hybridization between *pcd* and *N. lactamdurans* genomic DNA), but PCD activity has been detected in *N. lactamdurans* (14). The fact that *N. lactamdurans* can form α-aminoadipate despite the absence of the *pcd* gene implies that another route is available. If that alternative route also exists in *S. clavuligerus*, then it follows that *pcd* may not actually be involved in α-aminoadipate formation but rather have some other unrecognized function which has no equivalent in *N. lactamdurans*. However, *pcd* was shown by Southern hybridization to have an equivalent in all other β-lactam species examined, and so whatever its role, it seems closely associated with the production of cephamycin.

cmcI and *cmcJ* are other newly identified biosynthetic genes from *S. clavuligerus* which act together with *lat*, *pcbAB*, *pcbC*, *cefD*, *cefE*, *cefF*, and *cmcH* (and possibly *pcd*) to make cephamycin. The *cmcI* and *cmcJ* genes were expected to be located within the unsequenced gaps in the *S. clavuligerus* cephamycin cluster, since similar genes had previously been located in *N. lactamdurans*. However, their organization within the *S. clavuligerus* cephamycin biosynthetic cluster differs markedly from that of *N. lactamdurans*. *cmcI* and *cmcJ* along with *cefF* and *cmcH* appear to have moved as a late gene cassette from their location between *cefD* and *ccaR* in *S. clavuligerus* to reside between *pcbC* and *bla* in *N. lactamdurans*. This variable organization could suggest that the different cephamycin-producing species have acquired component parts of the cephamycin biosynthetic gene cluster in separate stages rather than obtaining the entire cluster in a single horizontal gene transfer event. Alternatively, the different organization of the cephamycin gene cluster in *S. clavuligerus* may be related to constraints imposed by the presence of the adjacent clavulanic acid biosynthetic gene cluster, which is apparently coregulated with cephamycin production.

Like *pcd*, the *ccaR orf11 blp* group of genes is also absent from the *N. lactamdurans* cluster. No roles for the *orf11* and *blp* genes in β-lactam biosynthesis were evident from homology searches. However, *orf11* cross-hybridizing DNA was found in *S. jumonjinensis* and *S. lipmanii*, which produce clavulanic acid and clavulanic acid precursors like clavaminic acid, respectively, in addition to cephamycin (7, 20), but was absent in the other β-lactam producers. *blp*, which shows some sequence similarity to *bli*, the gene encoding the β-lactamase inhibitor protein from *S. clavuligerus*, hybridized only with genomic DNA from *S. clavuligerus*, and then only to itself and not to the *bli* gene.

Since this sequencing project was originally undertaken to search for potential transcriptional activators, the *ccaR* gene became the focus of subsequent studies. Involvement of CcaR in transcriptional regulation of the cephamycin biosynthetic pathway was predicted from its homology to other pathway-specific transcriptional activators, and an essential role in the production of both cephamycin and clavulanic acid has been demonstrated (44). We have confirmed this essential role for the CcaR protein in cephamycin and clavulanic acid production and have also demonstrated that CcaR is required for clavam production.

The biosynthetic gene clusters responsible for daunorubicin, actinorhodin, and undecylprodigiosin production in other *Streptomyces* spp. are known to be regulated by proteins homologous to CcaR (DnrI [52], ActII-ORF4 [22], and RedD [38], respectively). Mutants lacking the DnrI or RedD transcriptional activators displayed an antibiotic-nonproducing phenotype and lacked the transcripts responsible for the biosynthetic enzymes (34, 38). Disruption of the *ccaR* gene by insertion of the *apr* cassette or deletion of the *ccaR* gene, either by itself or together with *orf11* and *blp*, resulted in loss of biosynthetic ability for cephamycin, clavulanic acid, and the clavams. This makes the CcaR protein unique in that it simultaneously regulates the activity of three distinct biosynthetic pathways. Typically, multiple pathways within a single organism are each regulated by a pathway-specific transcriptional regulator, as in the case of *S. coelicolor*, where RedD and ActII-ORF4 regulate undecylprodigiosin biosynthesis and actinorhodin biosynthesis, respectively (22, 38).

Mutant strains defective in *ccaR*, *orf11*, and *blp* used in these studies demonstrated that only *ccaR* has a clear cut role in the regulation of β-lactam metabolite production. Plasmid constructs used to give rise to the mutants were designed to ensure that polar mutations could be ruled out, but this precaution may have been unnecessary since a previous study demonstrated that *ccaR* and *blp* were transcribed as monocistronic messages and no transcript was detected for *orf11* (44). Complementation of the Δ*cob*::*tsr* strain with the wild-type *cob* fragment rescued production of the metabolites. When complemented with *cob* constructs carrying mutations in any one of the three genes, only the *ccaR* mutated construct was unable to complement production of the metabolites. This eliminates an essential role for *orf11* or *blp* in the production of any of the metabolites. The lack of *orf11* involvement in metabolite production was further confirmed by demonstrating metabolite production in *orf11* gene disruption mutants resulting from replacement of the wild-type gene with a mutant version with the *apr* cassette inserted into the *Nco*I site of *orf11* (data not shown).

Despite this inability to demonstrate a role for *orf11* in production of any of the three classes of metabolites examined, it is still tempting to suggest that it may play a role in either clavulanic acid or clavam biosynthesis since *orf11* cross-hybridizing DNA was found in *S. jumonjinensis* and *S. lipmanii* but was absent in the other β-lactam producers. Perhaps *orf11* functions under environmental conditions which are difficult to reproduce in the laboratory.

The presence of *ccaR* cross-hybridizing DNA in *Streptomyces* β-lactam antibiotic-producing species but not in *N. lactamdurans* suggests that β-lactam production in *Streptomyces* spp. proceeds through a method of transcriptional regulation which is conserved throughout the genus but not in *N. lactamdurans*. As an alternative, transcriptional regulation of cephamycin production in *N. lactamdurans* has been suggested to involve the *orf12* gene product (12) or the *bla* gene product (32) rather than *ccaR*.

Since pathway-specific activators similar to *ccaR*, such as *dnrI*, *actII*-ORF4, and *redD*, typically control antibiotic biosynthesis by regulating the transcription of biosynthetic genes, *ccaR* mutants were characterized for the synthesis of selected enzymes required for cephamycin biosynthesis. As expected, mutants were unable to make the CcaR protein, but they were also shown to be unable to make the LAT, ACVS, IPNS, and DAOCS proteins, thus explaining the defect in cephamycin production. Therefore, the CcaR protein was essential to cephamycin production by activating production of at least these pathway enzymes. Presumably similar situations occur in the clavulanic acid and clavam biosynthetic pathways to prevent their biosyntheses.

The ability to complement a *ccaR* disruption mutant was demonstrated previously when the gene was cloned at a high

copy number (44). The present study shows that a *ccaR* deletion mutant can be complemented by a single copy of the *ccaR* gene delivered by the integrating vector pSET152 and that successful complementation rescues cephamycin biosynthesis by restoring the production of the cephamycin biosynthetic enzymes. The levels of cephamycin production were lower in complemented mutants than in the wild type, but this may be due to positional effects since pSET152 constructs integrate at the *attB* site. Alternatively, some upstream regulating elements needed for optimum expression of *ccaR* may have been unknowingly omitted from the complementing fragment used. The placement of stop codons within the *ccaR* gene created mutant alleles that were unable to complement, showing that *ccaR* does not contain alternate start codons capable of creating truncated but still functional versions of the CcaR protein.

In most instances, transcriptional activators like CcaR regulate a number of biosynthetic genes present within the cluster (11). Since LAT, ACVS, and IPNS arise from a tricistronic operon, CcaR could potentially regulate cephamycin production simply by controlling expression from the *lat* promoter. However, *pcbC* can also be transcribed as a monocistronic message from an independent promoter within the operon (46), yet no IPNS protein is seen in *ccaR* mutants. Similarly, DAOCS, an enzyme from the middle of the cephamycin pathway, arises from a separate polycistronic message which includes *cefD*, *cefE* (31), and possibly *pcd*, *cmcT*, and *pbpA*. The fact that DAOCS is also not produced in *ccaR* mutants indicates that CcaR must regulate expression from a number of separate promoters.

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