Functional Analysis of the Gene Encoding the Clavamate Synthase 2 Isoenzyme Involved in Clavulanic Acid Biosynthesis in *Streptomyces clavuligerus*

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A *Streptomyces clavuligerus* mutant disrupted in *cas2*, encoding the clavamate synthase (CAS2) isoenzyme, was constructed by a gene replacement procedure. The resulting *cas2* mutant showed no clavulanic acid production when grown in starch-asparagine medium. However, in soy medium, the *cas2* mutant did produce clavulanic acid, although in amounts less than those produced by wild-type cultures. This medium-dependent leaky phenotype correlated well with the presence of the *cas1* transcript, encoding the CAS1 isoenzyme, in cultures grown in soy medium and with its absence from those grown in starch-asparagine medium. This suggested that CAS1 and CAS2 both contribute to clavulanic acid production but that their production is regulated differently. Under nutritional conditions in which *cas1* expression is blocked, *cas2* becomes essential for clavulanic acid production. Northern (RNA) analysis revealed that while *cas1* is transcribed as a 1.4-kb monocistronic transcript only, *cas2* is transcribed both as a 1.2-kb monocistronic transcript and as part of a 5.3-kb polycistronic transcript. High-resolution S1 nuclease analysis located the transcription start point of the monocistronic *cas2* transcript at a C residue 103 nucleotides upstream from the *cas2* start codon.

Streptomyces clavuligerus is a gram-positive filamentous bacterium which produces two major groups of β -lactam compounds. The sulfur-containing β -lactam compounds have antibiotic properties and include penicillins, cephalosporins, and cephamycins. The oxygen-containing β -lactam compounds, commonly referred to as clavams, include clavulanic acid as well as a number of structurally related compounds. Clavulanic acid has potent β -lactamase inhibitory properties and is used clinically in combination with β -lactam antibiotics to combat infections caused by β -lactamase-producing microorganisms. The other clavams have a bicyclic ring structure similar to that of clavulanic acid but with an opposite stereochemical conformation and different side groups attached. These clavams include 2-hydroxymethylclavam, 2-formyloxymethylclavam, and clavam 2-carboxylate (9), which have antifungal activities, and alanylclavam (29), which has antifungal as well as antibacterial properties (Fig. 1A).

The branched biosynthetic pathway leading to penicillins, cephalosporins, and cephamycins has now been well characterized; many pathway enzymes have been purified, and corresponding genes have been cloned and sequenced (22). The biosyntheses of clavulanic acid and other clavam metabolites, while much less well understood, are nonetheless clearly separate from that of penicillins and cephamycins. The feeding of radiolabelled substrates has shown that the biosynthesis of clavulanic acid begins with the condensation of arginine and a three-carbon glycolytic intermediate and is followed by a series of reactions to form proclavaminic acid (Fig. 1B) (12, 13, 35, 37). Proclavaminic acid is then converted to clavaminic acid and finally to clavulanic acid. The latter conversion is thought to involve several steps, including a stereochemical conformational change in the bicyclic ring structure, but these steps have

not yet been fully characterized (6, 36). Recent experiments with labelled substrates have shown that proclavaminic acid can be incorporated into both clavulanic acid and the other clavam metabolites (19, 20). The biosyntheses of all clavam metabolites, including clavulanic acid, are therefore thought to follow a common pathway, at least up to proclavaminic acid and perhaps up to clavaminic acid.

Information regarding the enzymology and genetics of clavulanic acid biosynthesis is just beginning to emerge. Clavamate synthase (CAS), which catalyzes the conversion of proclavaminic acid to clavaminic acid, is the only well-characterized enzyme in the pathway. The activity was first demonstrated by Elson et al. (14), but Marsh et al. (24) reported recently that CAS exists as two isoenzymes, CAS1 and CAS2, encoded by two separate but very similar genes, *cas1* and *cas2* (referred to as *cas1* and *cas2* in this paper). The *cas2* gene has since been located within a cluster of genes encoding clavulanic acid biosynthetic enzymes, located adjacent to the cephamycin gene cluster (4, 39). A mutation in the gene *cla*, which is located 5.7 kb downstream from the gene encoding the cephamycin biosynthetic enzyme isopenicillin N synthase (*pcbC*), resulted in impaired clavulanic acid production (4). The deduced gene product of *cla* shows a high level of homology to amidinohydrolases and may be involved in the removal of the guanidino group from the intermediate 3-hydroxy-5-guanidino-2-(2-oxoazetidin-1-yl)pentanoic acid to form proclavaminic acid (12). Sequence analysis of the region flanking *cla* revealed that the gene immediately downstream of *cla* is *cas2* (4). However, the location of *cas1* in relation to this cluster remains unknown. Analysis of cosmid clones indicates that *cas1* and *cas2* are separated by at least 28 kb in the genome (24). Moreover, the rationale for the presence of two CAS isoenzymes and the role played by each in clavulanic acid biosynthesis are elusive.

In this paper we report the construction by gene replacement of a mutant disrupted in *cas2*. The characteristics of this mutant with respect to clavulanic acid production under two different nutritional conditions, along with transcriptional

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FIG. 1. (A) General structure of clavam metabolites other than clavulanic acid. (B) Pathway for biosynthesis of clavulanic acid.

analysis of *cas1* and *cas2* under these conditions, are also described.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *S. clavuligerus* NRRL 3585 was provided by the Northern Regional Research Laboratory, Peoria, Ill. *Streptomyces lividans* TK24 was provided by D. A. Hopwood (John Innes Institute, Norwich, United Kingdom). The indicator organism, *Escherichia coli* ESS, was obtained from A. L. Demain (Department of Biology, Massachusetts Institute of Technology, Cambridge), while *Staphylococcus aureus* N2 was obtained from the Department of Microbiology, University of Alberta, Edmonton, Canada.

The *E. coli* cloning vectors pUC119, pUC118, and pUC120 were provided by J. Vieira (Waksman Institute of Microbiology, Rutgers University, Piscataway, N.J.) (38). *Streptomyces* vector pIJ486 was obtained from D. A. Hopwood, and pIJ4083 was provided M. J. Bibb (John Innes Institute). pOW309, a plasmid based on the cosmid pKC462a (30) and containing a portion of the cephamycin biosynthetic gene cluster of *S. clavuligerus*, was generously provided by P. Skatrud (Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Ind.). The vector pCAT, a pUC119 derivative containing a chloramphenicol resistance gene (*cat*) inserted within the ampicillin resistance gene (*amp*), has been described earlier (11). *E. coli* strains were maintained as described earlier (32). *S. clavuligerus* was maintained on either MYM agar (34) or ISP medium no. 3 (Difco, Detroit, Mich.), while *S. lividans* was propagated on either MYM agar or R5 regeneration medium (without sucrose) (17). In the case of plasmid-containing cultures, either thiostrepton (50 µg/ml for *S. lividans*) and 10 µg/ml for *S. clavuligerus*) or apramycin (25 µg/ml for both species) was included.

Spores of *S. clavuligerus* were inoculated into seed medium which consisted of Trypticase soy broth supplemented with 1% starch, and the cultures were grown at 28°C on a rotary shaker (220 rpm) for 48 h. Mycelia from the seed cultures were washed twice with sterile water and used to inoculate starch-asparagine (SA) medium or soy medium at 1.5% (vol/vol) for clavulanic acid production. These cultures were grown under the same conditions as the seed cultures. SA medium (4) had the following composition (per liter): soluble starch, 10 g; L-asparagine, 2 g; 3-(*N*-morpholino)propanesulfonic acid (MOPS), 21 g; MgSO₄ · 7H₂O, 0.6 g; K₂HPO₄, 4.4 g; and trace element solution (FeSO₄ · 7H₂O, 100 mg; MnCl₂ · 4H₂O, 100 mg; ZnSO₄ · 7H₂O, 100 mg; and CaCl₂ · 3H₂O, 130 mg [per 100 ml of water]), 1 ml. The medium was adjusted to pH 6.8 with NaOH. Soy medium (31) consisted of the following (per liter): soybean flour, 15 g; soluble starch, 4.7 g; KH₂PO₄, 0.1 g; and FeSO₄ · 7H₂O, 0.2 g. The medium was adjusted to pH 6.8 with NaOH.

DNA isolation, recombinant DNA, and Southern blot analysis. Plasmid and genomic DNAs from *Streptomyces* spp. were isolated and purified as described by Hopwood et al. (17). *S. lividans* protoplasts were prepared, transformed, and regenerated as described earlier (17), while protoplasts of *S. clavuligerus* were prepared and transformed as described by Bailey and Winstanley (5) and heat shocked for 5 min at 45°C just before addition of the plasmid DNA. Restriction enzyme digestion and analysis, ligations, and transformation of *E. coli* were done as described by Sambrook et al. (32).

Southern blot analysis was done by standard procedures (32), using probe DNA which was radioactively labelled with [α -³²P]dATP by a nick translation procedure (17) and exposed to nylon blots under hybridization conditions (5× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA, pH 7.7], 0.5% sodium dodecyl sulfate [SDS], 5× Denhardt's solution) at 65°C overnight. After hybridization, membranes were washed at a high stringency (twice with 2× SSPE plus 0.1% SDS for 10 min at room temperature, once with 1× SSPE plus 0.1% SDS for 15 min at 65°C, and finally once with 0.1× SSPE plus 0.1% SDS for 10 min at 65°C) and exposed to X-ray film.

Generation of *NcoI* sites at the ends of the apramycin resistance gene cassette. A 1.45-kb *EcoRI*-*PstI* fragment carrying the apramycin resistance gene (*apr* cassette), which is expressed in both *Streptomyces* spp. and *E. coli*, was excised from pOW309, and *NcoI* restriction sites were engineered at both ends of this fragment in several steps. Briefly, the *EcoRI*-*PstI* fragment was made blunt by filling in with Klenow enzyme and inserted into the *SmaI* site of pUC120. This placed a *NcoI* site from pUC120 near the *EcoRI* site at one end of the *apr* cassette. A second *NcoI* site was generated at the other end of the cassette by the insertion of a *BamHI*-*NcoI* synthetic linker at the *BamHI* site. Finally, the unique *EcoRI* site from the multiple cloning site, present between the *apr* cassette and the *NcoI* site, was eliminated by *EcoRI* digestion followed by filling in and self-ligation, to facilitate further subcloning.

Insertional inactivation of *S. clavuligerus cas2* by gene replacement. The 12-kb *EcoRI* fragment located downstream from *pcbC* (21) contains four *NcoI* sites, one of which is located within *cas2*, 89 bp from the translation start codon. This 12-kb *EcoRI* fragment, which was previously subcloned from the cosmid K6L2 (4) into pUC119, was inserted into the pCAT vector to form pCATL2. The plasmid pCATL2 was then subjected to partial digestion with *NcoI* so as to obtain singly cut plasmid as the major digestion product. The DNA fragments obtained upon partial *NcoI* digestion were ligated with the *NcoI*-flanked 1.45-kb *apr* cassette, and the ligation mixture was used to transform *E. coli* with selection for apramycin and chloramphenicol resistance. Four types of plasmid DNA were identified in the transformants, each consisting of the entire pCATL2 with the *apr* cassette inserted into one of the four *NcoI* sites present within the 12-kb *EcoRI* insert. One such plasmid, pL2AP5, containing the *apr* cassette inserted at the *NcoI* site within *cas2* (and thus disrupting *cas2*) was used further in this study. An 8-kb *BglII* subfragment from pL2AP5 encompassing the disrupted *cas2* gene together with 2.5 kb of upstream and 4 kb of downstream sequences was inserted into the *BamHI* site of the *Streptomyces* vector pIJ486 to form pIJ486AP5. pIJ486AP5 was transformed first into *S. lividans* and from there into *S. clavuligerus*. The resulting *S. clavuligerus* Tsr^r Apr^r transformants were allowed to sporulate under nonselective conditions, and from the spore progeny Tsr^s Apr^r clones were identified by replica plating. Genomic DNA was isolated from these clones, digested with restriction enzymes, and subjected to Southern blot analysis with either a 0.8-kb *KpnI* fragment from the *cas2* region (see Fig. 2) or the 1.45-kb *apr* cassette as a probe to confirm the insertion of *apr* cassette within the *cas2* locus

in the chromosome. The 0.8-kb *KpnI* fragment, which consists of 339 bp from the 5' end of *cas2* and 483 bp of upstream sequence (extending into *cla*), was excised from pC18, a pUC119 derivative containing the fragment.

RNA isolation and Northern (RNA) blot analysis. *S. clavuligerus* cultures were grown in SA medium or soy medium, and total RNA was isolated from 48-h cultures by a method described earlier (40, 41). RNA samples (40 µg) were denatured with deionized glyoxal and dimethyl sulfoxide and size fractionated on 1.25% agarose gels by electrophoresis with a 10 mM Na₂HPO₄-NaH₂PO₄ (pH 7.0) recirculating buffer system at 4 V/cm. Phage λ DNA digested with *PstI* or *HindIII* and treated in a similar way served as size markers. After electrophoresis, agarose gels were blotted onto nylon membranes (Hybond; Amersham Canada, Oakville, Ontario, Canada). The membranes were then subjected to hybridization with radioactively labelled probes, washed, and exposed to X-ray film by using procedures similar to those described for Southern blot analysis. The probes were either double-stranded DNA fragments labelled by nick translation (17) or oligonucleotides labelled at the 5' end with T4 polynucleotide kinase (32). Probe A was an 18-mer oligonucleotide (5' ACTATCGGAGAGGCCA TG) with sequences complementary to the coding strand corresponding to nucleotides (nt) -1 to +17 relative to the *cas2* start codon and located upstream from the *apr* insertion site. Probe B was a 241-bp *NcoI-KpnI* fragment located immediately downstream from the *apr* insertion site. Probe C was a 531-bp *NruI* fragment internal to the open reading frame located immediately downstream of *cas2* (*orf-6*) (21).

The probes used to detect the *cas1* transcript were RMO1, a 36-mer oligonucleotide (5' GCGCGCAGCTCGGGGCGGTACGCGGTGCAGTCCACT) corresponding to nt +9 to +44, and AP11, a 27-mer oligonucleotide (5' CAGATG GCCGTCCTCGCTGCCCTCGGC) corresponding to nt +163 to +189 (both relative to the *cas1* translational start codon) (24).

High-resolution S1 nuclease mapping of the 5' end of the *cas2* transcript. High-resolution S1 nuclease mapping was carried out as described earlier (27). A 568-bp DNA probe fragment extending from nt -482 to +17 relative to the *cas2* start codon and containing 69 bp of nonhomologous sequences was generated for S1 nuclease mapping by PCR with probe A (described above) and the M13 reverse sequencing primer as primers and pC18 as the template. The PCR amplification reaction mixtures consisted of 70 mM Tris-HCl (pH 8.8), 2 mM MgCl₂ · 6H₂O, 0.1% (wt/vol) Triton X-100, 2.5 mM deoxynucleoside triphosphates, 1 U of *Taq* DNA polymerase, 20 pmol of each primer, and 10 ng of template DNA in a final volume of 100 µl. Thirty cycles, each consisting of 92°C for 30 s, 50°C for 30 s, and 72°C for 3 min, were performed. The amplified PCR product was electrophoresed and purified by using a GeneClean kit (Bio 101, La Jolla, Calif.) before further use. The amplified probe fragment was 5' end labelled with γ-³²P by using T4 polynucleotide kinase and standard procedures (32), extracted with phenol-chloroform, and precipitated with ethanol. The radioactivity incorporated in the probe DNA was measured by Cerenkov counting, and the labelled probe fragment was stored as a precipitate in ethanol at a final concentration of 50,000 cpm/µl.

The RNA samples used for S1 nuclease mapping were the same as those used for Northern blot analysis. Samples (40 µg) were allowed to anneal to the 5'-end-labelled probe fragment (50,000 Cerenkov cpm) in S1 formamide buffer (17) for 16 h at 50°C. S1 nuclease digestion was carried out in S1 digestion buffer (17) at 37°C. The reaction was stopped by the addition of ammonium acetate, and the mixture was extracted with phenol-chloroform and ethanol precipitated. The S1 nuclease-protected probe fragments were then visualized by electrophoresing samples on a 6% denaturing polyacrylamide gel alongside a ³⁵S-labelled sequencing ladder generated by using single stranded pC18 as a template and probe A as a primer (the same oligonucleotide used to generate the probe for S1 nuclease mapping).

HPLC assay of clavulanic acid. The high-pressure liquid chromatography (HPLC) assay has been described previously (15). Analysis was performed with a model M-45 pump, model 712 WISP automated sample injector, and model 480 variable-wavelength UV detector, all from Millipore Waters (Mississauga, Ontario, Canada). Samples (100 µl) were mixed with imidazole reagent (25 µl) and incubated at room temperature for 15 min to form imidazole-derivatized clavulanic acid (8). Derivatized samples (50 µl) were analyzed on a reverse-phase column (µBondapak-C18) with an isocratic buffer system consisting of 0.1 M KH₂PO₄-6% methanol, pH 3.2 (adjusted with H₃PO₄). Under these conditions, authentic clavulanic acid had a retention time of 6.5 min.

Bioassays of β-lactam antibiotics, clavulanic acid, and clavam metabolite. Bioassays were conducted by the agar plate diffusion method with appropriate indicator organisms seeded in soft nutrient agar. Total antibiotics were determined with *E. coli* ESS as the indicator organism (1), clavulanic acid was determined with *S. aureus* N2 as the indicator organism (4), and the antimetabolite activity of the clavam metabolite alanylclavam was determined with *Bacillus* sp. strain ATCC 27860 as the indicator organism (29).

RESULTS

Construction of an *S. clavuligerus cas2* mutant. In order to determine the role of the *cas2* gene in clavulanic acid biosynthesis, an *S. clavuligerus* mutant disrupted in *cas2* was constructed by a gene replacement procedure (see Materials and

TABLE 1. Clavulanic acid production in the wild-type strain and the *cas2* mutant

Strain	Specific clavulanic acid production (µg ml ⁻¹ OD unit ⁻¹) ^a in:	
	SA medium	Soy medium
Wild type	11.8	13.5
<i>cas2</i> mutant	ND ^b	8.7

^a Cultures were grown for 4 days, and the clavulanic acid content was determined quantitatively by integrating the peak areas obtained following HPLC analysis. Growth was measured by determining the optical density (OD) at 600 nm.

^b ND, not detected.

Methods). *S. clavuligerus* protoplasts were transformed with the recombinant plasmid pIJ486AP5, which confers thiostrepton resistance and also carries *cas2* disrupted by an apramycin resistance gene. Growth of the *S. clavuligerus* transformants on a medium without antibiotic selection followed by a single round of sporulation resulted in the loss of the Apr^r Tsr^r phenotype at an estimated frequency exceeding 98% of the CFU obtained. However, approximately 25% of the CFU showed an Apr^r Tsr^s phenotype. This was presumed to be due to replacement of the wild-type *cas2* gene in the *S. clavuligerus* chromosome with the *apr*-disrupted *cas2* from pIJ486AP5 as a result of homologous recombination involving a double crossover followed by loss of the plasmid sequences.

The gene replacement in one of the Apr^r Tsr^s integrant strains, AP5, was confirmed by Southern blot analysis. Genomic DNA preparations from the wild-type strain and from strain AP5 were singly digested with *Bgl*II and *Kpn*I and probed with a 0.8-kb *Kpn*I fragment from the *cas2* region (Fig. 2A)⁴ that spanned the *Nco*I site at which the *apr* cassette had been inserted. In *Bgl*II digests, the 6.6-kb hybridizing fragment present in the wild type was replaced by an 8.0-kb fragment in strain AP5, while in *Kpn*I digests, the 0.8-kb fragment in the wild type was replaced by 2- and 0.24-kb fragments in strain AP5 (Fig. 2B). These sizes of *Bgl*II and *Kpn*I fragments in strain AP5 are consistent with the integration of the 1.45-kb *apr* cassette into the chromosomal *cas2* gene. When the same blots were probed with the 1.45-kb *apr* cassette, no hybridization to wild-type DNA was seen and hybridization to strain AP5 DNA was consistent with integration occurring only within *cas2*, as expected (data not shown). The results from these digests also indicated that the *apr* gene within AP5 was oriented in the direction opposite to that of *cas2*.

Characterization of the *S. clavuligerus cas2* mutant. Strain AP5 (referred to as the *cas2* mutant from now on) produced approximately the same amounts of penicillin and cephamycin antibiotics as did the wild-type strain in both SA and soy media (data not shown). When supernatants from SA medium cultures were analyzed for clavulanic acid production by bioassay (data not shown) or quantitated by comparing peak areas obtained following HPLC analysis (Table 1), the wild-type strain showed production of clavulanic acid, but the *cas2* mutant did not. In contrast, when supernatants from soy medium cultures were analyzed, both the wild-type strain and the *cas2* mutant produced clavulanic acid; the amount produced by the *cas2* mutant was approximately 40% less than that produced by the wild type (Table 1). Although the data shown were obtained after 4 days of growth, similar results were obtained when samples were taken at different times during growth. The failure of the *cas2* mutant to produce clavulanic acid in SA medium despite the presence of an intact *cas1* gene suggested that

A

B

FIG. 2. (A) Model of integration of the *apr*-disrupted *cas2* gene into the *S. clavuligerus* wild-type chromosome at the *cas2* locus (not drawn to scale). The solid box represents the *apr* cassette, and the cross-hatched box represents the *cas2* gene. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; K, *Kpn*I; N, *Nco*I. (B) Southern blot analysis of genomic DNA from *S. clavuligerus* AP5 and the wild-type strain (wt) with a *cas2*-specific probe. Genomic DNA samples were digested with *Bgl*II or *Kpn*I and probed with the 0.8-kb *Kpn*I fragment from the *cla-cas2* region.

expression of the two *cas* genes might be regulated differently with respect to nutritional conditions.

Cultures were further characterized by bioassay for their abilities to produce one of the other clavam metabolites, alanylclavam, which has an antimetabolite activity that can be antagonized by methionine (29). When supernatants from SA medium-grown cultures were analyzed, neither the wild-type strain nor the *cas2* mutant produced any appreciable amounts of antimetabolite activity. However, when soy medium-grown cultures were analyzed, both wild-type and *cas2* supernatants showed antimetabolite activity, which was overcome by the addition of methionine (Fig. 3). This effect of growth medium composition on the production of alanylclavam was similar to that seen for the *cas1*-mediated production of clavulanic acid (in the *cas2* mutant) and suggests that some of the enzyme activities specific for the clavam branch of the pathway may have the same type of nutritional regulation as does *cas1*.

Northern analysis of the *cas1* gene. Total RNA was extracted from cultures grown in soy and SA media and was examined by Northern analysis with the *cas1*-specific oligonucleotide RMO1 as a probe. RNAs from both wild-type and *cas2* mutant cultures grown in soy medium hybridized to the *cas1* probe, showing an approximately 1.4-kb transcript, while the same amount of RNA from either culture grown in SA medium failed to hybridize (Fig. 4). *S. lividans* RNA, which

FIG. 3. Bioassay of alanylclavam in *S. clavuligerus* AP5 and wild-type (wt) cultures. Cultures were grown in either SA medium or soy medium for 4 days, and supernatants were bioassayed in the presence (A) or absence (B) of methionine at 200 µg/ml.

also failed to hybridize to the *cas1* probe, was included as a negative control. A second oligonucleotide probe, AP11, consisting of sequences from a different region of *cas1* gave identical results, indicating that the observed hybridization was *cas1* specific (data not shown). The size of the observed *cas1* transcript suggested it to be monocistronic in nature. Thus, the presence of the *cas1* transcript in cultures grown in soy medium, but not in SA medium, correlated well with the ability of the *cas2* mutant to produce clavulanic acid in soy medium but not in SA medium and suggested that *cas1*, when it was being expressed, could functionally replace *cas2* in clavulanic acid biosynthesis.

Northern analysis of the *cas2* gene. To study *cas2* at the transcriptional level, total RNA isolated from wild-type and *cas2* mutant cultures was examined by Northern analysis with three different probes (see Materials and Methods) (Fig. 5). Probes A and B, which are internal to *cas2* and located upstream and downstream of the *apr* insertion site, respectively, both hybridized to RNA extracted from the wild-type cultures, showing two major transcripts of approximately 5.3 and 1.2 kb (Fig. 5A and B). The sizes of these transcripts suggested that *cas2* is transcribed independently, resulting in the shorter 1.2-kb monocistronic transcript, as well as being cotranscribed along with other adjacent genes to form the longer 5.3-kb polycistronic transcript. A minor transcript of approximately 2.4 kb was also observed, but this transcript was not clearly

seen in all RNA preparations (26). Probe C, which is internal to the uncharacterized open reading frame located immediately downstream of *cas2* (*orf-6*) (2, 21), showed hybridization to a single transcript of approximately 1.3 kb (Fig. 5C). This suggests that *orf-6* is not cotranscribed with *cas2*, and therefore the 5.3-kb multicistronic *cas2* transcript must include upstream open reading frames. In support of this, a stable RNA stem-loop structure with a free energy of $-41.8 \text{ kcal mol}^{-1}$ (ca. -175 kJ mol^{-1}) which could act as a transcriptional terminator has been found between *cas2* and *orf-6* (3). RNA isolated from wild-type cultures grown in soy medium hybridized with probe A, showing the same two *cas2* transcripts seen in samples from SA medium. This indicates that, unlike *cas1*, *cas2* is expressed in both SA and soy media.

RNA isolated from the *cas2* mutant also hybridized with probe A but showed only a single main 5-kb transcript, and almost no evidence of the smaller 1.2-kb transcript was seen (Fig. 5A). The 5-kb transcript is about 0.3 kb smaller than the polycistronic *cas2* transcript seen in the wild type and is also present in much smaller amounts. Since the *cas2* mutant contains the *apr* cassette inserted at the *NcoI* site, the shortened polycistronic transcript may result from termination within the *apr* cassette. A second transcript representing the truncated form of the wild-type 1.2-kb transcript would then also be expected in RNA isolated from the *cas2* mutant, but this was not observed. Probe B did not show any hybridization to RNA from the *cas2* mutant, indicating that transcription of *cas2* sequences downstream from the *apr* cassette was not occurring (Fig. 5B). Probe C hybridized to RNA from the *cas2* mutant, showing a single transcript of the same size as seen in the wild type (Fig. 5C). This suggested that while the insertion of the *apr* cassette within *cas2* did have a polar effect on transcription of downstream sequences within *cas2*, it did not affect *orf-6* transcription. Therefore, transcription of *orf-6* must be separate from that of *cas2*.

Promoter activity in the DNA upstream from *cas2*. The promoter activity of a DNA fragment from upstream of *cas2* was tested to ensure that the 1.2-kb transcript was due to transcription initiation taking place immediately upstream from *cas2* and was not a result of degradation or processing of the larger, 5.3-kb transcript. A 0.8-kb *KpnI* fragment, which contains 339 bp from the 5' end of *cas2* and 483 bp of upstream sequences (extending into *cla*), was isolated from pC18 as an *EcoRI-XbaI* fragment and inserted into the promoter probe plasmid pIJ4083 (10). *S. lividans* transformants containing the resulting plasmid, pIJ4083C18, turned yellow in less than 5 min when sprayed with a 0.5 M catechol solution, indicating that a promoter had been inserted in front of the *xyIE* reporter gene in pIJ4083. The presence of a promoter within the 0.8-kb fragment suggested that the 1.2-kb *cas2* transcript is a primary transcript arising from its own promoter.

High-resolution S1 nuclease mapping of the *cas2* transcript. The transcription of *cas2* was further characterized by determining the 5' end of the 1.2-kb mRNA transcript by high-resolution S1 nuclease mapping. Total RNA extracted from 48-h cultures grown in SA medium was hybridized with a PCR-generated probe to identify any transcription start points within the protecting probe fragment. The 568-bp probe contained 69 bp of nonhomologous sequences at the 5' end distal to the *cas2* sequences and was labelled at both 5' ends. The nonhomologous sequences on one end of the probe would be removed by S1 nuclease treatment, making it possible to distinguish between the reannealed probe fragment and the fully protected probe.

As shown (Fig. 6), two major protected fragments were observed. One fragment corresponded to a transcription start

FIG. 4. Northern analysis of *cas1*. RNA samples isolated from *S. clavuligerus* *cas2* mutant and wild-type (wt) cultures grown in either SA medium or soy medium were subjected to hybridization with a 36-mer *cas1*-specific probe.

FIG. 5. Northern analysis of *cas2*. RNA samples isolated from *S. clavuligerus* *cas2* mutant and wild-type (*wt*) cultures grown in SA medium were subjected to hybridization with, as probes, an 18-mer oligonucleotide based in the 5' end of *cas2* (A), a 200-bp *NcoI-KpnI* *cas2* fragment located immediately downstream from the *apr* insertion site (B), and a 531-bp *NruI* fragment located internal to *orf-6* (C). The upper panel illustrates the region from which the three probes were generated and the arrangements of the transcripts in the wild-type strain and the *cas2* mutant. The solid box represents the *apr* cassette, the cross-hatched box represents the *cas2* gene, the open boxes represent adjacent genes flanking *cas2*, and dashed lines with arrows indicate transcripts.

point located at a C residue 103 nt upstream from the *cas2* start codon. The other fragment corresponded to the fully protected probe, which would be the result of a larger transcript originating from upstream of the probe fragment. *S. lividans* RNA, which was included as a negative control, failed to show any protected fragments. The RNA from the wild-type *S. clavuligerus* grown in soy medium also showed the same protected fragments (data not shown). When sequences centered around -10 and -35 regions upstream from the transcription start point were examined for promoter-like sequences, no significant similarity to *Streptomyces* consensus sequences was found, although a limited homology to other *Streptomyces* promoter sequences was noticed (33).

When RNA from the *cas2* mutant was analyzed, only the fully protected fragment corresponding to the long transcript was seen, and in amounts smaller than those seen in the wild type (data not shown). No protected fragment corresponding to a shorter *cas2* transcript was observed. Both of these observations are consistent with the results from Northern analysis.

DISCUSSION

As a step towards understanding the roles of the two CAS isoenzymes in clavulanic acid biosynthesis, we have con-

structed and characterized an *S. clavuligerus* mutant blocked specifically in CAS2. The mutant was constructed by disrupting *cas2* sequences by insertion of an *apr* gene, in vitro, and then replacing the wild-type chromosomal *cas2* with the disrupted copy, in vivo, by using the principle of homologous recombination. The resulting mutation resulted in a clavulanic acid-nonproducing phenotype in SA medium.

Characterization of the *cas2* mutant suggested that both CAS1 and CAS2 can contribute to clavulanic acid formation. However, since the *cas2* mutant showed a clavulanic acid-nonproducing phenotype in SA medium, it is clear that CAS2 is indispensable for clavulanic acid formation under some nutritional conditions. The mutant was also characterized in soy medium, since Salowe et al. (31) used this medium to prepare *S. clavuligerus* extracts and demonstrate the presence of the two CAS isoenzymes. In soy medium, the *cas2* mutant was able to produce clavulanic acid, and this medium-dependent leaky phenotype of the *cas2* mutant correlated well with the presence of the *cas1* transcript in cultures grown in soy medium and its absence in those grown in SA medium. Since CAS1 could complement the *cas2* defect in the mutant, it can compensate for the missing CAS2 in clavulanic acid biosynthesis.

Promoter probe studies and transcriptional analysis revealed that *cas2* is transcribed both as a 1.2-kb monocistronic tran-

FIG. 6. High-resolution S1 nuclease mapping of *cas2* transcripts. (A) Schematic representation of the PCR-derived DNA probe. The cross-hatched box represents *cas2* sequences, and the open box represents sequences upstream from the *cas2* start codon. The solid lines further upstream from nt -482 indicate nonhomologous *E. coli* plasmid sequences. (B) The 5'-end-labelled probe fragment was hybridized with RNA samples, and the resulting hybrids were treated with S1 nuclease and analyzed by electrophoresis. Hybridization signals due to probe-probe annealing (O) and corresponding to protection of the full-length probe fragment minus the 3' nonhomologous *E. coli* plasmid sequences (●) are indicated. *tsp*, hybridization signal due to protection by the shorter transcript.

script from a promoter which lies within the carboxy-terminal region of the upstream *cla* gene and as part of a 5.3-kb polycistronic transcript. The size of the longer transcript suggests that it could accommodate *cas2*, *cla*, and two open reading frames further upstream (*orf-2* and *orf-3*) (2, 21). A similar transcriptional arrangement is seen in the adjacent cephamycin gene cluster, in which the *pcbC* gene is transcribed as part of a polycistronic transcript together with *lat* and *pcbAB* but also as a monocistronic transcript (28). The promoter for the monocistronic *pcbC* transcript lies within the upstream *pcbAB* sequence. Perhaps the presence of separate promoters for some genes in an operon ensures that in addition to coordinate regulation with other genes in the operon, such genes can also be regulated independently. The *cas2* promoter sequence does not show any significant homology to the typical *Streptomyces* consensus sequence, which is in agreement with the observed promoter heterogeneity among streptomycetes (33).

Transcriptional analysis also revealed that in *cas2* mutants, insertion of the *apr* cassette caused premature termination of the longer *cas2* transcript. The hybridization signal corresponding to this truncated transcript was poorly resolved, which could be due to heterogeneous 3' ends resulting from transcript instability. The stem-loop structure normally present at the 3' end of *cas2* would not be present on the truncated 5-kb *cas2* transcript in the mutant, and such stem-loop structures are known to stabilize mRNA against degradation (7). The failure to detect the shorter truncated transcript could be due to antisense transcripts arising from within the *apr* cassette. Promoter probe studies have shown that the *apr* cassette contains a promoter for the *apr* gene and does not contain a terminator (26). Transcripts arising from the *apr* cassette would be antisense with respect to the *cas2* gene, and if such antisense transcripts span the 5' end of the shorter *cas2* transcript, they could form hybrids with this region and block not only translation but also transcription (16, 18, 25). The presence of the longer 5-kb transcript in the *cas2* mutant suggests that such antisense transcripts, if present, have less effect on the formation of the longer transcript. Moreover, the *cla* gene, which is encoded on this longer transcript immediately upstream from *cas2*, appears to be functional, since the *cas2* mutant was able to cosynthesize clavulanic acid when grown in a mixed culture with the *cla* mutant (26).

Thus, *cas1* and *cas2* represent redundant genes encoding two proteins which can be used for clavulanic acid biosynthesis, but there are significant differences in their transcriptional organization and regulation. *cas2* is clustered with other genes involved in clavulanic acid biosynthesis and is transcribed as both mono- and multicistronic transcripts, in both SA and soy media. *cas1* is transcribed as a monocistronic transcript only, and then in cultures grown in soy medium but not in those grown in SA medium.

The presence in this organism of two *cas* genes with similar functions but significant differences in transcriptional and regulatory characteristics is intriguing. As suggested earlier (24), it is possible that the conversion of proclavamate to clavamate is a rate-limiting step, which would explain the need for gene duplication. The observation that the *cas2* mutant, which produces only CAS1, makes less clavulanic acid in soy medium than does the wild type, which produces both CAS1 and CAS2, supports this idea. Analogous results which suggest that the reaction catalyzed by lysine ϵ -aminotransferase (encoded by *lat*) may be the rate-limiting reaction for cephamycin biosynthesis in *S. clavuligerus* have been reported recently (23). Insertion of an extra copy of *lat* into the chromosome led to a twofold increase in cephamycin production. If, as has been postulated, the biosynthetic pathways for clavulanic acid and

other clavams share reactions up to proclavaminic acid or clavaminic acid (19, 20), the two CAS isoenzymes may simply function to provide enough precursors to be channeled into the two pathways. Since alanylclavam was detected only in soy medium-grown cultures, in which both *cas1* and *cas2* are expressed, and not in SA medium, in which only *cas2* is expressed, this implies either that *cas1* is specifically required for clavam production and *cas2* cannot substitute or, more probably, that some as yet unidentified activity(ies) specific for the clavam branch of the pathway has the same type of nutritional regulation as does CAS1.

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