The Paralogous Pairs of Genes Involved in Clavulanic Acid and Clavam Metabolite Biosynthesis Are Differently Regulated in *Streptomyces clavuligerus*

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Carboxyethylarginine synthase, encoded by the paralogous *ceaS1* and *ceaS2* genes, catalyzes the first reaction in the shared biosynthetic pathway leading to clavulanic acid and the other clavam metabolites in *Streptomyces clavuligerus*. The nutritional regulation of *ceaS1* and *ceaS2* expression was analyzed by reverse transcriptase PCR and by the use of the enhanced green fluorescent protein-encoding gene (*egfp*) as a reporter. *ceaS1* was transcribed in complex soy medium only, whereas *ceaS2* was transcribed in both soy and defined starch-asparagine (SA) media. The transcriptional start points of the two genes were also mapped to a C residue 98 bp upstream of *ceaS1* and a G residue 51 bp upstream of the *ceaS2* start codon by S1 nuclease protection and primer extension analyses. Furthermore, transcriptional mapping of the genes encoding the beta-lactam synthetase (*bls1*) and proclavamate amidinohydrolase (*pah1*) isoenzymes from the paralogue gene cluster indicated that a single polycistronic transcript of ~4.9 kb includes *ceaS1*, *bls1*, and *pah1*. The expression of *ceaS1* and *ceaS2* in a mutant strain defective in the regulatory protein CcaR was also examined. *ceaS1* transcription was not affected in the *ccaR* mutant, whereas that of *ceaS2* was greatly reduced compared to the wild-type strain. Overall, our results suggest that different mechanisms are involved in regulating the expression of *ceaS1* and *ceaS2*, and presumably also of other paralogous genes that encode proteins involved in the early stages of clavulanic acid and clavam metabolite biosynthesis.

Members of the genus *Streptomyces* exhibit a complex life cycle that involves a hierarchy of regulatory genes controlling and coordinating antibiotic production and sporulation (14, 20). *Streptomyces clavuligerus* produces a number of β -lactam compounds, including cephamycin C, clavulanic acid, and at least four other known clavam metabolites (8, 12). Clavulanic acid and the other clavams differ from cephamycin C in that their bicyclic nucleus contains an oxygen atom instead of the sulfur atom found in the more conventional cephamycin-type antibiotics (6). Clavulanic acid is a clinically important inhibitor of β -lactamases, whereas the other clavam metabolites produced by *S. clavuligerus* show weak antibacterial and antifungal activities (35). The four clavam metabolites produced by *S. clavuligerus* are commonly referred to as the 5S clavams due to their stereochemistry, which differs from the 5R stereochemistry of clavulanic acid. The β -lactamase-inhibitory activity of clavulanic acid has been associated with this 5R stereochemistry (6). Since clavulanic acid is produced industrially by fermentation using *S. clavuligerus*, the regulation of clavulanic acid and 5S clavam biosynthesis is a point of great interest.

The biosynthetic pathway leading to clavulanic acid and the 5S clavams is partially shared, at least to the level of clavaminic acid (Fig. 1B) (10). However, in *S. clavuligerus*, the genes involved in the biosynthesis of clavulanic acid and the 5S clavams reside in three distinct gene clusters that are not physically linked (K. Tahlan, H. U. Park, and S. E. Jensen, unpublished data). The clavulanic acid gene cluster is situated

immediately downstream of the cephamycin gene cluster, and together they form a larger gene cluster often referred to as the β -lactam supercluster (1, 13, 44). This clavulanic acid gene cluster contains genes encoding enzymes involved in the early shared stages of the clavulanic acid and 5S clavam pathway (the early genes), as well as genes encoding proteins involved in the later stages of clavulanic acid biosynthesis only (the late genes) (15, 17, 21, 24). The clavam gene cluster encompasses the gene encoding clavamate synthase 1 (*cas1*), a paralogue of the *cas2* gene from the clavulanic acid gene cluster and one of the genes involved in the biosynthesis of both clavulanic acid and the 5S clavams. In addition to *cas1*, the clavam cluster includes other genes involved exclusively in the biosynthesis of the 5S clavams and some genes of unknown function (26). The paralogue gene cluster contains paralogues of additional genes found in the clavulanic acid gene cluster that encode enzymes involved in the early stages of both clavulanic acid and 5S clavam biosynthesis (18, 42).

The biosynthesis of both clavulanic acid and the 5S clavams initiates with the condensation of glyceraldehyde-3-phosphate and L-arginine to give N^2 -(2-carboxyethyl)arginine, catalyzed by the enzyme carboxyethylarginine synthase (CEAS) (Fig. 1B) (19). There are two copies of the gene encoding CEAS present in *S. clavuligerus*; *ceaS2* is located in the clavulanic acid gene cluster, and *ceaS1* is found in the paralogue gene cluster (Fig. 1A) (15, 33, 42). The next reaction in the pathway is catalyzed by the enzyme β -lactam synthetase (BLS) (3, 23), which is also encoded by two separate genes (*bls1* and *bls2*) (15, 42). BLS activity closes the β -lactam ring to give deoxyguanidino proclavaminic acid (3, 23), which is then converted to guanidino proclavaminic acid by clavamate synthase (CAS) (5). Two genes encode CAS in *S. clavuligerus* (22), *cas1* from

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FIG. 1. Early steps of clavulanic acid and 5S clavam metabolite biosynthesis in *S. clavuligerus*. (A) Genes involved in the early steps of clavulanic acid and 5S clavam biosynthesis from the clavam, clavulanic acid, and paralogue gene clusters. Genes flanking *cas1* in the clavam cluster are shown only to provide context; they do not encode early enzymes. (B) Diagrammatic representation of the early steps of clavulanic acid and 5S clavam metabolite biosynthesis showing the enzymes and genes involved. The 5S clavams are shown as a family of metabolites; R represents the site of side chain modification giving rise to the different members.

the clavam gene cluster and *cas2* from the clavulanic gene cluster (22, 26). Guanidino proclavaminic acid is hydrolyzed to give proclavaminic acid by the action of proclavamate amidinohydrolase (PAH) (46), which is encoded by *pah1* and *pah2* (1, 15, 18, 46). Next, in a series of two sequential reactions, CAS converts proclavaminic acid to clavaminic acid (4, 36). Clavaminic acid is thought to be the branch point of the pathway leading to clavulanic acid and the 5S clavams (10). The

only other step known in the pathway beyond clavaminic acid is the reduction of clavuldehyde to clavulanic acid by the action of the enzyme clavulanic acid dehydrogenase (27), which is encoded by *cad*, a late gene from the clavulanic acid gene cluster (15). It is still not known how clavaminic acid is converted to clavuldehyde, nor have any of the steps specifically leading from clavaminic acid to the 5S clavams been elucidated (16).

TABLE 1. Bacterial strains and plasmids used in this study

Strain, plasmid, or cosmid	Description ^a	Reference or source
<i>Bacillus</i> sp. strain ATCC 27860	Indicator strain for alanyl clavam bioassay	34
<i>K. pneumoniae</i> ATCC 15380	Indicator strain for clavulanic acid bioassay	35
<i>S. clavuligerus</i> NRRL3585	Wild type	National Regional Research Laboratory (NRRL)
<i>S. clavuligerus</i> $\Delta ccaR::tsrA$	<i>ccaR</i> deletion mutant; <i>ccaR</i> replaced by <i>tsr</i> ; Tsr ^r	2
<i>S. clavuligerus</i> C1G	Contains the <i>egfp</i> reporter gene fused to the <i>ceaS1</i> promoter; integrated at the ϕ C31 <i>attB</i> site in the chromosome	This study
<i>S. clavuligerus</i> C2G	Contains the <i>egfp</i> reporter gene fused to the <i>ceaS2</i> promoter; integrated at the ϕ C31 <i>attB</i> site in the chromosome	This study
Plasmids and cosmids		
K6L2	pLAFR3-based cosmid clone spanning the clavulanic acid gene cluster	1
p2.8-18	pUC18 containing the 5' end of <i>ceaS1</i> and upstream region	42
p5.7	pUC118 containing <i>blsI</i> , <i>pahI</i> , and flanking sequences	42
pBB5.3A	pUC119 containing the 5.3-kb BamHI/BglII fragment encompassing <i>pcbR</i> and <i>ceaS2</i> from pTZ40; Amp ^r	Jensen et al., unpublished
pCR2.1TOPO	<i>E. coli</i> phagemid cloning vector for rapid cloning of PCR fragments; Amp ^r Kan ^r	Invitrogen
pIJ8660	Promoterless EGFP reporter plasmid; free living in <i>E. coli</i> and integrates into the <i>Streptomyces</i> chromosome; Apr ^r	41
pIJ8660- <i>ceaS2</i>	EGFP reporter construct; pIJ8660 containing the 707-bp BamHI/KpnI fragment from pTOPO- <i>ceaS2</i> -8 encompassing the <i>ceaS2</i> promoter region fused to the <i>egfp</i> gene	This study
pSET152	<i>E. coli</i> cloning vector; integrates into the <i>Streptomyces</i> chromosome; Apr ^r	NRRL
pSET- <i>ceaS1</i>	EGFP reporter construct; pSET152 containing the 2.37-kb BamHI/EcoRI fragment from pTO6- <i>ceaS1</i> encompassing the <i>ceaS1</i> promoter fused to the <i>egfp</i> gene	This study
pTO6	pIJ8660 with <i>aac(3)IV</i> replaced by <i>aadA</i> ; Spc ^r	28
pTO6- <i>ceaS1</i>	EGFP reporter construct; pTO6 containing the 777-bp BamHI/KpnI fragment from pTOPO- <i>ceaS1</i> -4	This study
pTOPO- <i>ceaS2</i> -8	pCR2.1TOPO containing a 721-bp PCR fragment encompassing the <i>ceaS2</i> promoter region	This study
pTOPO- <i>ceaS1</i> -4	pCR2.1TOPO containing a 784-bp PCR fragment encompassing the <i>ceaS1</i> promoter region	This study
pTZ40	pTZ18R containing an ~26-kb BamHI/PstI fragment from cosmid K6L2 spanning the clavulanic acid gene cluster; Amp ^r	Jensen et al., unpublished

^a Amp^r, ampicillin resistance; Apr^r, apramycin resistance; Kan^r, kanamycin resistance; Spc^r, spectinomycin resistance; Tsr^r, thiostrepton resistance.

The transcriptional activators CcaR and ClaR are known to regulate the expression of the clavulanic acid biosynthetic genes (16). The *ccaR* gene lies within the cephamycin biosynthetic gene cluster, and CcaR is the pathway-specific transcriptional regulator for cephamycin biosynthesis, as well as controlling expression of the *claR* gene from the clavulanic acid gene cluster (2, 31, 32). ClaR is the pathway-specific transcriptional regulator for clavulanic acid biosynthetic genes, but it affects only expression of the late genes for clavulanic acid biosynthesis (29). The early genes responsible for the steps shared with the 5S clavams are not regulated by ClaR (29). In this manner, CcaR coordinates the biosynthesis of cephamycin C, a β -lactam antibiotic, with clavulanic acid, a β -lactamase inhibitor (16).

The *cas1* and *cas2* paralogues can functionally replace each other and are known to be nutritionally regulated. The *cas1* paralogue is expressed only when *S. clavuligerus* is grown on complex soy medium, whereas the *cas2* paralogue is expressed during growth on both complex soy and defined SA media (30). Thus, a *cas1* mutant can still produce clavulanic acid in both soy and SA media, because *cas2* is expressed in both media. In contrast, a *cas2* mutant produces clavulanic acid only when grown on soy medium, because *cas1* is not expressed in SA medium (30). Similar phenotypes were also observed when

mutants defective in each of the *ceaS*, *bls*, and *pah* paralogues were prepared and tested (15, 18, 42).

Since *ceaS1* and *ceaS2* encode proteins catalyzing the first reaction in the shared clavulanic acid and 5S clavam biosynthetic pathways, and since they are both located at the boundaries of their respective gene clusters (Fig. 1A), they seemed to be likely candidates for points of regulation. With this in mind, the nutritional regulation of *ceaS1* and *ceaS2* was examined at the transcriptional level to determine if they are regulated in a manner similar to *cas1* and *cas2*. Previous studies have suggested that ClaR does not regulate *ceaS2* transcription (29), but the effect of CcaR on *ceaS1* and *ceaS2* transcription is still not known. These effects were also examined and are discussed in the present study. Lastly, the transcriptional start points (TSPs) of *ceaS1*, *bls1*, *pah1*, and *ceaS2* were also mapped.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. The bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* cultures were grown as described earlier (38), and cultures containing plasmids were supplemented with ampicillin (100 μ g/ml), apramycin (50 μ g/ml), chloramphenicol (25 μ g/ml), kanamycin (50 μ g/ml), or spectinomycin (100 μ g/ml), as appropriate. *S. clavuligerus* cultures were maintained on either MYM (40) or ISP 4 medium, as described previously (42), and cultures containing plasmids were supplemented with apramycin (25 μ g/ml) or thiostrepton (5 μ g/ml). *S. clavuligerus*

TABLE 2. Oligonucleotide primers used in this study

Primer name	Sequence (5'→3') ^a	Description ^b
CAN 122	GGCCACCGCGACCTGCTGC	Reverse primer <i>hrdB</i> RT-PCR
CAN 123	CGGCCAAGCGCACCCTACC	Forward primer <i>hrdB</i> RT-PCR
ceaS1-S1-For	TCATGAATTCGGGTGGACGGAAGGGGACGG	Forward primer for <i>ceaS1</i> S1 probe
ceaS1-PR-EX	ATGGCTTTCGCGGTCTGTGT	Reverse primer for <i>ceaS1</i> S1 probe and primer extension
ceaS2-S1-For	TGGATCCGTCGCGAATCCAGGGAAGCCGAGC	Forward primer for <i>ceaS2</i> S1 probe
ceaS2-PR-EX	GGGCGGTGCGATACACGGG	Reverse primer for <i>ceaS2</i> S1 probe and primer extension
bls1-S1-For	TCGGATTAATACCTCGCTGCTCGCCGCCCTCAC	Forward primer for <i>bls1</i> S1 probe
bls1-S1-Rev	GGTCGGGGCCGGGCATGGTGAA	Reverse primer for <i>bls1</i> S1 probe
pah1-S1-For	CCAGATTAATGCGGCGCGGACGGTGCAG	Forward primer for <i>pah1</i> S1 and Northern probe
pah1-S1-Rev	CGGGGAGACGGCGGTGGACA	Reverse primer for <i>pah1</i> S1 and Northern probe
pah1-UP-Rev	CGCGGCTGCCCCCTCCCTC	Reverse primer for <i>pah1</i> S1 probe
ceaS1-RT-For	GCGCAGTCCGAGTCGTAC	Forward primer <i>ceaS1</i> RT-PCR
ceaS1-RT-Rev	TTGGCGGTGTAGGTGGTGAC	Reverse primer <i>ceaS1</i> RT-PCR
ceaS2-RT-For	AGGCCGCGTCGATTCTCTTC	Forward primer <i>ceaS2</i> RT-PCR
ceaS2-RT-Rev	CGGCGGGTTGGGGACGGT	Reverse primer <i>ceaS2</i> RT-PCR
KTA- <i>ceaS1</i> -For	GCGGGATCCGGGCGGTTCAGCACGGT	Forward primer for cloning <i>ceaS1</i> promoter
KTA- <i>ceaS1</i> -Rev	CCGGGTACCAAGGTCGCGAAGCACG	Reverse primer for cloning <i>ceaS1</i> promoter
KTA- <i>ceaS2</i> -For	AACCCAGGATCCGAGCCCCACCGTCACG	Forward primer for cloning <i>ceaS2</i> promoter
KTA- <i>ceaS2</i> -Rev	CGGCCGGGTACCCCAACACCTTCCCCACAC	Reverse primer for cloning <i>ceaS2</i> promoter

^a Nonhomologous sequences incorporated into oligonucleotide primers are underlined, and engineered restriction sites are shown in boldface letters.

^b Details of the function of each oligonucleotide primer are provided in Materials and Methods.

erus cultures for the isolation of chromosomal DNA were grown in trypticase soy broth supplemented with 1% starch (TSBS), and cultures for the isolation of exconjugants were grown in AS-1 medium supplemented with 10 mM MgCl₂, as described earlier (42). To prepare RNA from *S. clavuligerus*, spores were pregerminated for 4 h at 28°C in 2YT medium (38) and then used to inoculate SA or soy culture medium (30). RNA was isolated after 96 and 120 h of growth in soy medium and after 72 and 96 h of growth in SA medium. All *Streptomyces* liquid cultures were grown at 28°C on a rotary shaker at 250 rpm.

DNA isolation, manipulation, and Southern analysis. Routine manipulation of plasmid DNA isolated from *E. coli* cultures, including labeling of double-stranded DNA probes with [α -³²P]dCTP by nick translation and end labeling using [γ -³²P]dATP, was performed using standard procedures (38). DNA sequencing was carried out using the DYEnamic ET Terminator Cycle Sequencing kit (Amersham Pharmacia, Baie d'Urfe, Quebec, Canada) by the Molecular Biology Service Unit, University of Alberta. Manual sequencing of DNA was performed using the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing kit (U.S. Biochemical) according to the manufacturer's directions. DNA fragments fractionated by agarose and polyacrylamide gel electrophoresis (PAGE) were isolated using the QIAquick Gel Extraction kit (Qiagen Inc.) and the crush-and-soak method (38), respectively. PCRs were carried out using the Expand high-fidelity PCR system (Roche) according to the manufacturer's instructions. Plasmid DNA was introduced into *S. clavuligerus* by intergeneric conjugation as described previously (42). Southern analysis of *S. clavuligerus* DNA was also carried out as described elsewhere (38), using the following wash conditions. After overnight incubation with labeled probes, the nylon membranes were washed twice for 15 min each time with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) (38) at room temperature, once for 20 min with 1.0× SSC–0.1% SDS, and once for 20 min with 0.1× SSC–0.1% SDS. All incubations and washes were carried out at 65°C unless otherwise indicated.

RNA isolation, RT-PCR, S1 nuclease mapping, primer extension, and Northern blot analysis. RNAs from wild-type *S. clavuligerus* and *S. clavuligerus* Δ ccrR::tsrA were isolated using the modified Kirby procedure (20). Reverse transcriptase (RT)-PCR analysis of RNA was carried out using *C. therm* polymerase (Roche) for reverse transcription in two-step RT-PCR. All RT reactions were carried out at 62°C for 30 min according to the manufacturer's instructions with the following changes. The reactions were set up in a final volume of 10 μ l using 0.5 μ g of total RNA per reaction and 15.8 U of RNAGuard RNase Inhibitor (Amersham). The reverse primers ceaS1-RT-Rev, ceaS2-RT-Rev, and CAN 122 (Table 2) were used to synthesize cDNA corresponding to the *ceaS1*, *ceaS2*, and *hrdB* transcripts, respectively. The PCRs were performed using 5 μ l of the RT product from the reaction described above in a final volume of 50 μ l, employing the Expand high-fidelity PCR system with buffer 2. The primer pairs ceaS1-RT-For plus ceaS1-RT-Rev and ceaS2-RT-For plus ceaS2-RT-Rev were used for PCR amplification of the *ceaS1* and the *ceaS2* RT products, respectively.

Dimethyl sulfoxide (DMSO; 5% [vol/vol] final concentration) was used in these reactions with the following program: 94°C for 2 min, followed by 25 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min. The primers CAN 123 and CAN 122 were used to amplify the *hrdB* RT product by PCR using 10% (vol/vol) DMSO and the following program: 94°C for 2 min, followed by 25 cycles of 94°C for 1 min, 66°C for 1 min, and 72°C for 1 min. The RT-PCR products were analyzed by fractionation on 1.5% agarose Tris-borate-EDTA gels. The identities of the RT-PCR products were also verified by restriction analysis, followed by PAGE and by sequencing of both DNA strands (data not shown).

High-resolution S1 nuclease mapping was carried out using the sodium trichloroacetate method (20). All double-stranded DNA probes were prepared by PCR using custom primers. DMSO (5% [vol/vol]) was included in the reactions, with the following program: 94°C for 2 min, followed by 10 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s, and finally 15 cycles of 94°C for 45 s, 65°C for 45 s, and 72°C for 45 s. The primers ceaS1-S1-For and ceaS1-PR-EX, along with p2.8-18 as a template, were used to prepare the probe to map the *ceaS1* TSP. The primers ceaS2-S1-For and ceaS2-PR-EX, along with the template plasmid pBB5.3A, were used to prepare the probe for mapping the TSP of *ceaS2*. The primers bls1-S1-For and bls1-S1-Rev, and pah1-S1-For and pah1-UP-Rev, along with p5.7 as a template, were used to prepare probes for S1 protection analysis of *bls1* and *pah1*, respectively.

Primer extension analysis was performed using *C. therm* polymerase for reverse transcription in two-step RT-PCR (Roche) according to the manufacturer's instructions with the following changes. Twenty-microliter reaction mixtures were set up using 5 pmol of the end-labeled reverse primers ceaS1-PR-EX and ceaS2-PR-EX (Table 2) and 40 U of RNaseOUT Recombinant RNase Inhibitor (Invitrogen), with the following program: extension at 60°C for 60 min and termination at 80°C for 10 min.

DNA sequencing ladders were prepared for size estimation using the reverse primers and the template plasmids used in the preparation of the S1 probes or in the primer extension reactions. Samples were separated on 6% denaturing polyacrylamide sequencing gels for analysis as described earlier (38).

Northern blot analysis was carried out using established techniques (30) with 40 μ g of RNA isolated from wild-type *S. clavuligerus* grown on soy medium for 96 and 120 h. Molecular Weight Marker III (Roche) was run along with the RNA samples for size estimation. The primers pah1-S1-For and pah1-S1-Rev were used to generate a 288-bp probe by PCR using p5.7 as a template. This probe was used for both S1 nuclease protection analysis (data not shown) and Northern hybridization. Probe annealing and subsequent washes were carried out under the same high-stringency conditions used in Southern blot analysis.

Preparation of enhanced green fluorescent protein (EGFP) reporter constructs. A 781-bp DNA fragment spanning the *ceaS1* promoter region was amplified by PCR using p2.8-18 as a template and the primer pair KTA-*ceaS1*-For and KTA-*ceaS1*-Rev. Similarly, a 721-bp DNA fragment encompassing the *ceaS2* promoter region was amplified by PCR using pBB5.3A as the template and

the primers KTA-*ceaS2*-For and KTA-*ceaS2*-Rev. The PCR products were treated with *Taq* DNA polymerase before ligation to pCR2.1TOPO (Invitrogen) according to the manufacturer's instructions. This gave pTOPO-*ceaS1*-4 and pTOPO-*ceaS2*-8, which contain the *ceaS1* and *ceaS2* promoter regions in pCR2.1TOPO, respectively. The double-stranded DNA sequence of the inserts was obtained using universal primers to ensure that no mutations were introduced.

The *ceaS2* promoter region from pTOPO-*ceaS2*-8 was isolated as a BamHI/KpnI fragment and ligated into the corresponding sites of pIJ8660 to give pIJ8660-*ceaS2*. pIJ8660-*ceaS2* contains the *ceaS2* promoter region fused to a promoterless *egfp* gene for use as a reporter of expression driven by the *ceaS2* promoter.

For unexplained reasons, we were unable to subclone the *ceaS1* promoter region into pIJ8660 and therefore used an alternative approach. The *ceaS1* promoter region from pTOPO-*ceaS1*-4 was isolated as a BamHI/KpnI fragment and ligated into the corresponding sites of pTO6 to give pTO6-*ceaS1*. The entire cassette encompassing the *ceaS1* promoter region fused to the *egfp* gene from pTO6-*ceaS1* was isolated as a 2.37-kb BamHI/EcoRI fragment and introduced into the corresponding sites of pSET152 to give pSET-*ceaS1*, which served as the *ceaS1* reporter construct.

The plasmids pSET-*ceaS1* and pIJ8660-*ceaS2* were introduced into wild-type *S. clavuligerus* by conjugation. Strains that had the plasmids integrated at the ϕ C31 *attB* site in the chromosome were isolated based on apramycin resistance and were verified by Southern hybridization (data not shown).

Confocal microscopy. *S. clavuligerus* cultures harboring *egfp* reporter constructs were grown in TSBS for 36 h. Five-hundred-microliter amounts of the TSBS cultures were used to inoculate 25 ml of either SA or soy medium. After 72 h of growth, 1-ml amounts of the cultures were harvested and washed once in acetonitrile and then twice in sterile distilled water. The washed mycelia were mounted in 40% (vol/vol) glycerol before observations were made under the microscope.

Confocal microscopy was carried out using a Leica DM IRB inverted microscope. An argon laser (50 to 52% attenuation) provided excitation at 488 nm. Fluorescence due to EGFP excitation was detected between 505 and 520 nm, and corresponding differential interference contrast images were also obtained.

Western analysis. Five-milliliter amounts of *S. clavuligerus* cultures grown in soy and SA media were harvested and resuspended in 1 ml of lysing buffer (100 mM HEPES [pH 7.2], 0.5 mg of lysozyme/ml, 2× Complete EDTA free protease inhibitor cocktail [Roche]). The suspensions were incubated at 37°C for 30 min, the cell membranes were broken by ultrasonic disruption, and then the cell debris was removed by centrifugation. Samples of cell extracts (CFEs) containing 50 μ g of total protein were separated by SDS-PAGE (12% gels) as described earlier (38). The proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore) using a Bio-Rad Transblot apparatus. The BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit) (Roche) was used to detect proteins in accordance with the manufacturer's instructions. The primary antibody used to detect EGFP was the commercially available BD Living Colors A.v. Peptide Antibody (BD Biosciences) and was used at 1/400 dilution.

HPLC analyses of culture filtrates, bioassays, and growth determination. High-performance liquid chromatographic (HPLC) analysis of culture supernatants after imidazole derivatization was performed under previously described conditions (30). Bioassays were used to detect clavulanic acid and alanyl clavam production using *Klebsiella pneumoniae* ATCC 15380 and *Bacillus* sp. strain ATCC 27860, respectively, as the indicator organisms (18, 30). Growth of *S. clavuligerus* in fermentation medium was estimated by measuring the optical density of broken mycelia at 495 nm as described earlier (7).

RESULTS

Nutritional regulation of *ceaS1* and *ceaS2*. In previous studies, when *ceaS1* and *ceaS2* mutant strains were prepared individually, both mutant strains still retained some ability to produce both clavulanic acid and the 5S clavams, depending on the fermentation medium used (15, 33, 42). The *ceaS1* mutant produced both clavulanic acid and the 5S clavams in soy medium but only clavulanic acid in SA medium. In contrast, the *ceaS2* mutant produced small amounts of clavulanic acid and the 5S clavam metabolites in soy medium only, while no clavulanic acid or 5S clavam production was observed in cultures grown in SA medium (15, 42). Based on the observed pheno-

FIG. 2. Assessment of *ceaS1* and *ceaS2* transcript levels by RT-PCR. RNA samples isolated from various strains of *S. clavuligerus* were analyzed by RT-PCR using primers specific for *ceaS1*, *ceaS2*, and *hrdB*. (A) Analysis of RNAs from *S. clavuligerus* wild-type (WT) cultures grown on SA medium for 72 and 96 h and from cultures grown on soy medium for 96 and 120 h. (B) Analysis of RNAs from *S. clavuligerus* wild-type cultures grown on soy medium for 96 and 120 h and *S. clavuligerus* $\Delta ccaR::tsrA$ mutant cultures grown on soy medium for 96 and 120 h.

types, *ceaS1* was postulated to be expressed in soy medium only, whereas *ceaS2* was expressed in both soy and SA media. To verify this hypothesis, we examined the effects of growth in soy and SA media on both *ceaS1* and *ceaS2* expression at the transcriptional level.

RT-PCR was used to detect *ceaS1* and *ceaS2* transcripts, using RNA isolated from wild-type *S. clavuligerus* grown on SA medium for 72 and 96 h and on soy medium for 96 and 120 h. When RNA isolated from SA cultures was subjected to analysis, *ceaS1* transcripts were not detected, whereas the same samples showed the presence of *ceaS2* transcripts (Fig. 2A). In similar analyses of RNA isolated from soy-grown cultures, both *ceaS1* and *ceaS2* transcripts were detected by RT-PCR (Fig. 2A). The expression of *hrdB*, which encodes a constitutively expressed sigma factor in *Streptomyces*, was monitored as a control, and *hrdB* transcripts were detected at similar levels in all samples tested (Fig. 2). HPLC analysis of culture supernatants showed the expected levels of clavulanic acid and 5S clavams in cultures grown in both media used for RNA isolation and analysis (data not shown).

The promoter regions of *ceaS1* and *ceaS2* (−501 to +215 and −551 to +95 bp relative to the putative start codons of *ceaS1* and *ceaS2*, respectively) were subcloned in front of a promoterless *egfp* gene to give plasmids pSET-*ceaS1* and pIJ8660-*ceaS2*, respectively. Fluorescence arising due to EGFP expression was used as a reporter to monitor transcription driven from the promoters. These promoter constructs were introduced into wild-type *S. clavuligerus*, where they integrated into the chromosome via the ϕ C31 *attB* site. The integration of the reporter plasmids into the *S. clavuligerus* chromosome was confirmed by Southern hybridization using DNA probes specific for both *egfp* and the respective promoters (data not shown).

The *S. clavuligerus* reporter strains C1G and C2G (Table 1) were grown on soy and SA media for 72 h, after which mycelia were harvested and analyzed by confocal microscopy to exam-

FIG. 3. Use of EGFP as a reporter to detect *ceaS1* and *ceaS2* promoter activities. (A) Mycelia from *S. clavuligerus* EGFP reporter strains grown on SA and soy media for 72 h were analyzed by confocal microscopy. Both differential interference contrast (odd-numbered) and fluorescence (even-numbered) images were obtained and are shown side by side. Images 1 and 2, *ceaS1* reporter grown on SA medium; images 3 and 4, *ceaS2* reporter grown on SA medium; images 5 and 6, *ceaS1* reporter grown on soy medium; images 7 and 8, *ceaS2* reporter grown on soy medium. (B) Detection of EGFP in cell extracts (CFEs) from *S. clavuligerus* EGFP reporter strains grown on soy and SA media for 72 h. Cell extracts from samples subjected to microscopic analysis were also analyzed by Western blotting using commercially available antibodies raised against EGFP. C1G, lanes containing CFEs from the *ceaS1* reporter strain; C2G, lanes containing CFEs from the *ceaS2* reporter strain. The different culture media used to grow the reporter strains are indicated.

ine fluorescence arising due to EGFP expression and excitation. Fluorescence was observed in all samples except in the C1G strain grown on SA medium (Fig. 3A). Cell extracts (CFEs) were also prepared from the same samples that were subjected to confocal microscopy, and the presence of EGFP in the CFEs was analyzed by Western blotting. A 27-kDa band corresponding to EGFP was observed in all of the samples except for the lane containing CFE from the C1G strain grown on SA medium (Fig. 3B).

Bioassays indicated that all of the strains used in microscopic and Western analyses produced the expected levels of both clavulanic acid and alanyl clavam, and growth assays indicated that all the strains grew at similar rates (data not shown).

Transcriptional mapping of *ceaS1* and *ceaS2* promoters. S1 nuclease protection and primer extension analyses were used to map the TSPs of both *ceaS1* and *ceaS2*. RNA for the analysis was isolated from wild-type *S. clavuligerus* grown on soy medium (for 96 and 120 h). S1 protection analysis, using a probe extending from −261 to +26 bp relative to the putative *ceaS1* start codon, indicated that the *ceaS1* TSP was located 98 to 99

bp upstream of the *ceaS1* start codon (Fig. 4A). Primer extension analysis was also conducted using the reverse primer *ceaS1*-PR-EX (Fig. 4B), and the *ceaS1* TSP was mapped to a C residue located 98 bp upstream of the *ceaS1* ATG start codon (Fig. 4C).

A probe extending from −204 to +22 bp relative to the *ceaS2* start codon was used in an S1 protection assay to map the *ceaS2* TSP, which was located 51 to 52 bp upstream of the *ceaS2* start codon (Fig. 5A). Primer extension analysis using the reverse primer *ceaS2*-PR-EX (Fig. 5B) confirmed that the *ceaS2* transcript originated from a G residue located 51 bp upstream of the *ceaS2* ATG start codon (Fig. 5C).

Mapping of the *bls1* and *pah1* transcripts. Since *bls2* and *pah2* are transcribed as part of a larger polycistronic transcript that also includes *ceaS2* and *cas2* (30), the regions upstream of *bls1* and *pah1* were examined using S1 nuclease protection assays to determine if they were also expressed as part of a polycistronic message. The *bls1* transcript was mapped using RNA isolated from wild-type *S. clavuligerus* grown on soy medium for 96 h, together with a probe extending from −137 to

FIG. 4. Mapping of the *ceaS1* TSP. (A and B) Sequencing ladders (lanes G, A, T, and C) were prepared using the reverse primer *ceaS1*-PR-EX, along with p2.8-18 as a template. (A) A 297-bp probe (−261 to +26 bp relative to the putative *ceaS1* start codon plus 10 bp of nonhomologous sequence) was used in the S1 protection assay. Lanes 1 and 2, RNA from wild-type *S. clavuligerus* grown on soy medium for 96 and 120 h and subjected to analysis; lane P+S1, control lane with unprotected probe digested with S1 nuclease; lane P, undigested-probe control. (B) Primer extension analysis using the *ceaS1*-specific reverse primer, *ceaS1*-PR-EX. Lane 1, RNA isolated from wild-type *S. clavuligerus* grown on soy medium for 96 h and subjected to primer extension analysis. The most probable TSPs are marked by asterisks. (C) DNA sequence of the *ceaS1* promoter region. The open arrow represents *ceaS1*, with the arrowhead indicating the orientation of the gene. The bent arrow indicates the *ceaS1* TSP, and the respective −10 and −35 promoter regions are also shown.

+16 bp relative to the proposed *bls1* ATG start codon. Only full-length protection of the probe was observed, indicating that there was no individual TSP located in the 23-bp intergenic region between *ceaS1* and *bls1* (Fig. 6A).

The intergenic region between *bls1* and *pah1* was examined by S1 nuclease protection assays using a probe extending from −257 to +21 bp relative to the proposed *pah1* ATG codon, but once again, only full-length protection of the probe was observed (data not shown). Because the DNA sequence ladder was unclear in the region of the full-length protected probe, a second 116-bp probe (−257 to −151 bp upstream of the proposed *pah1* start codon) was also used in an S1 protection

assay. Again only full-length protection of the probe was observed (Fig. 6B), indicating that there was no promoter immediately upstream of *pah1*. Since the probes used for the S1 nuclease protection studies did not cover the entire 317-bp intergenic region between *bls1* and *pah1*, it was still possible that a TSP might be found further upstream of *pah1*. Northern analysis of RNA isolated from wild-type *S. clavuligerus* grown on soy medium for 96 and 120 h was conducted to investigate this possibility. The 288-bp probe prepared for the first *pah1* S1 protection assay was used as the *pah1* Northern probe. Only a single large band of ~4.9 kb hybridized to the probe (Fig. 7), indicating that *pah1* does not have an individual promoter and

FIG. 5. Mapping of the *ceaS2* TSP. (A and B) DNA sequencing ladders (lanes G, A, T, and C) were prepared for size estimation using the reverse primer *ceaS2*-PR-EX and the template plasmid pBB5.3A. The most probable TSPs are marked by asterisks. (A) S1 nuclease protection analysis using a 236-bp probe (−204 to +22 bp relative to the *ceaS2* start codon plus 10 bp of nonhomologous sequence) used to map the *ceaS2* TSP. Lanes 1 and 2, RNA from wild-type *S. clavuligerus* grown on soy medium for 96 and 120 h and subjected to analysis; lane P+S1, control lane with unprotected probe digested with S1 nuclease; lane P, undigested-probe control. (B) Primer extension analysis using the *ceaS2*-specific reverse primer, *ceaS2*-PR-EX. Lane 1, RNA from wild-type *S. clavuligerus* subjected to primer extension analysis. (C) DNA sequence of the *ceaS2* promoter region. The open arrow represents *ceaS2*, with the arrowhead indicating the orientation of the gene. The bent arrow indicates the most probable *ceaS2* TSP, and possible heptameric repeats, which are recognized by SARPs, are shown in solid boxes. The −10 and −35 promoter regions are also indicated.

that it is transcribed as part of a tricistronic operon together with *bls1* and *ceaS1*.

Effects of CcaR on *ceaS1* and *ceaS2* transcription. RNAs isolated from wild-type and Δ *ccaR::tsrA* strains of *S. clavuligerus* grown on soy medium for 96 and 120 h were analyzed by RT-PCR to monitor the effect of CcaR status on *ceaS1* and *ceaS2* expression. The transcription of *ceaS1* was comparable in both the wild-type and the Δ *ccaR::tsrA* strains (Fig. 2B). When *ceaS2* transcription was examined in the same RNA samples, almost no transcripts were detectable in the Δ *ccaR::tsrA* mutant compared to the wild-type strain

(Fig. 2B). The expression of *hrdB* was also monitored as a control and was found to be constant in all of the samples (Fig. 2B).

The levels of clavulanic acid and 5S clavam production in the wild-type and the Δ *ccaR::tsrA* mutant cultures used to isolate RNA were also determined by HPLC analysis of culture supernatants. The wild-type strain produced the expected levels of clavulanic acid and the 5S clavams after 96 and 120 h of growth on soy medium. Under the same conditions, the Δ *ccaR::tsrA* strain did not produce any detectable levels of clavulanic acid, whereas normal 5S clavam production was observed (data not shown).

FIG. 6. Mapping of the polycistronic transcript including *bls1* and *pah1* using S1 nuclease protection analysis. (A and B) DNA sequencing ladders (lanes G, A, T, and C) were prepared for size estimation using the reverse primers and template plasmids used for probe preparation. Lane S, RNA isolated from wild-type *S. clavuligerus* grown on soy medium for 96 h and subjected to analysis; lane P+S1, control lane with unprotected probe digested with S1 nuclease; lane P, undigested-probe control. Bands observed due to probe-probe reannealing and full-length protection of the probes, minus the 3' nonhomologous 10-nucleotide sequences, are indicated. Multiple bands due to degradation of the nonhomologous sequences were also observed above the full-length protected probe fragments. (A) A 163-bp probe (–137 to +16 bp relative to the proposed *bls1* start codon plus 10 bp of nonhomologous sequence) was used to map the *bls1* transcript. (B) A 116-bp probe (–257 to –151 bp upstream of the proposed *pah1* start codon plus 10 bp of nonhomologous sequence) was used to map the *pah1* transcript. (C) Diagrammatic representation of the *ceaS1*, *bls1*, and *pah1* polycistronic transcript. The large arrows represent the genes, with the arrowheads indicating the direction of transcription. The line represents the rest of the *S. clavuligerus* chromosome. The upper arrow represents the mRNA transcript, and bars 1 and 2 represent the S1 probes used to map the *bls1* and *pah1* transcripts, respectively (the diagram is not to scale).

DISCUSSION

Previous studies have shown that paralogous pairs of genes encode the enzymes involved in the early stages of the shared biosynthetic pathway leading to clavulanic acid and the 5S clavams (15, 18, 22, 42). In order to understand to what extent each of these sets of genes contributes to the production of clavulanic acid and the 5S clavams, the transcriptional regulation of the genes was examined.

The *ceaS1* transcript originated from a single TSP located at a C residue 98 bp upstream from the start codon, but the *ceaS1* promoter region showed little similarity to known *Streptomyces* promoters (Fig. 4C), a reflection of the large diversity found in *Streptomyces* promoter sequences (39). When the *bls1* and the *pah1* transcripts were analyzed by S1 nuclease protection assays, no individual TSP was detected for either of these genes and only full-length protection of the probes was observed. This suggested that neither *bls1* nor *pah1* has its own dedicated

promoter and that the upstream *ceaS1* promoter drives the transcription of these genes.

The large intergenic region of 317 bp that separates *bls1* and *pah1* was not fully covered by the probes used for S1 nuclease protection studies. Therefore, Northern analysis was used to ensure that any *pah1* transcript originating from a promoter located further upstream was not missed. Since *pah1* and *pah2* show 72% end-to-end identity at the nucleotide level (18), the probe used in Northern analysis was carefully chosen to be specific for *pah1*. The only hybridization seen was to a band ~4.9 kb in size, again indicating that *pah1* was transcribed as part of a large polycistronic transcript (Fig. 7). This 4.9-kb transcript is postulated to include *ceaS1*, *bls1*, and *pah1*, as the predicted length of a transcript extending from the *ceaS1* TSP to the stop codon of *pah1* would be ~4.7 kb. The next gene downstream of *pah1* is *oat1*, which is oriented in the direction opposite to *pah1* transcription, and therefore its presence on

FIG. 7. Northern analysis of wild-type *S. clavuligerus* RNA using a *pah1*-specific probe. Lane 1, molecular size marker; lanes 2 and 3, RNA from wild-type *S. clavuligerus* grown on soy medium for 96 and 120 h, respectively.

the 4.9-kb polycistronic transcript can be ruled out. In addition, computational analysis of the 126-bp intergenic region between *pah1* and *oat1* predicted the presence of considerable secondary structure, consisting of multiple stem-loops with a cumulative G of -101.4 , which could function as a transcriptional terminator (data not shown).

The transcriptional arrangement of *ceaS1*, *bls1*, and *pah1* is similar to that of their paralogous counterparts from the clavulanic acid gene cluster (Fig. 1A). The *ceaS2*, *bls2*, *pah2*, and *cas2* genes from the clavulanic acid gene cluster are transcribed as a 5.3-kb polycistronic transcript. In addition, *cas2* is also transcribed as a 1.2-kb monocistronic transcript (30). The most significant difference between the 5.3-kb transcript arising from the clavulanic acid gene cluster and the 4.9-kb transcript arising from the paralogue gene cluster is the absence of the *cas1* coding sequence in the paralogue gene cluster. *cas1* is located elsewhere on the *S. clavuligerus* chromosome and is expressed as a 1.4-kb monocistronic transcript (30).

The transcript comprising *ceaS2* was also mapped and was also found to arise from a single TSP located 51 bp upstream of the *ceaS2* start codon. As was the case for *ceaS1*, the proposed *ceaS2* promoter region did not show any significant

similarity to any known *Streptomyces* promoters. Since S1 nuclease and primer extension analyses were used to identify all of the TSPs described in this study, it should be noted that both of the analysis methods employed are predictive of the TSP, provided the mRNA is not processed.

The nutritional regulation of *ceaS1* and *ceaS2* transcription was examined using RT-PCR, which demonstrated that *ceaS1* was transcribed in soy medium only and not in SA medium. Under the same conditions and using the same RNA preparations, *ceaS2* was transcribed in both soy and SA media at comparable levels. The *ceaS1* and *ceaS2* promoter regions were also subcloned in front of a promoterless *egfp* gene, and EGFP expression was used as a reporter to monitor transcription driven by the respective promoters. Confocal microscopy was used to detect fluorescence due to EGFP expression and excitation, and the results confirmed that *ceaS1* was expressed in soy medium only and not in SA medium, whereas *ceaS2* was expressed in both media tested. The results obtained from confocal microscopy were confirmed by Western analysis, which indicated that the fluorescence observed in the samples was due to true EGFP expression. Since *ceaS1*, *bls1*, and *pah1* are expressed only as a 4.9-kb polycistronic message, it can be inferred that *bls1* and *pah1* will show the same general trend of nutritional regulation as *ceaS1*. In combination, our results indicate that *ceaS1*, *bls1*, and *pah1* are expressed in soy medium but not in SA medium, whereas *ceaS2*, *bls2*, *pah2*, and *cas2* are expressed in both soy and SA media. This explains the clavulanic acid- and 5S clavam-producing phenotypes observed when mutants defective in these genes were prepared and tested in previous studies (15, 18, 42).

The *ccaR* gene from the cephamycin gene cluster encodes a pathway-specific transcriptional regulator that coordinates the production of both cephamycin C and clavulanic acid (2, 31). Its effect on clavulanic acid biosynthesis is exerted, at least in part, through activation of the expression of a second pathway-specific transcriptional regulator, ClaR, from the clavulanic acid gene cluster. Mutants defective in CcaR do not produce any cephamycin C, and clavulanic acid production is also knocked out (2, 31), as the transcription of *claR* is reduced to near zero in these mutants (32). ClaR positively regulates genes involved exclusively in the biosynthesis of clavulanic acid (29). Previous studies have shown that *ceaS2* expression is not under the control of *claR* (29), but the detailed effects of CcaR on *ceaS1* and *ceaS2* expression are not known.

Our results indicate that *ceaS1* transcription is unaffected in the *ccaR* mutant compared to the wild-type strain, whereas the transcription of *ceaS2* is almost eliminated in the *ccaR* mutant. Therefore, CcaR controls the production of clavulanic acid through at least two routes, an indirect route mediated by ClaR and a second route that may involve CcaR directly regulating *ceaS2* promoter activity.

CcaR belongs to a family of transcriptional regulators called the *Streptomyces* antibiotic regulatory proteins (SARPs), which bind to specific heptameric repeats and promote transcription (45). Imperfect heptameric repeats can be identified in the region upstream of *ceaS2* (Fig. 5C), consistent with the idea that CcaR may bind directly to the *ceaS2* promoter region to regulate transcription. Such a notion, however, has not yet been demonstrated experimentally, and it is also possible that CcaR exerts its effect indirectly through additional proteins

(20a, 38a). Since *claR* is not expressed in the *ccaR* mutant, it can also be inferred that *claR* has no effect on *ceaS1* transcription, which was unaffected in the *ccaR* mutant. Similarly, due to the polycistronic nature of the transcript including *ceaS1*, *bls1*, and *pah1*, it follows that neither *bls1* nor *pah1* is affected by CcaR or ClaR.

The reason why *S. clavuligerus* possesses two sets of genes encoding enzymes involved in the early stages of clavulanic acid and 5S clavam biosynthesis is still unclear. One suggestion is that this could be a strategy to increase precursor and metabolite flux through the shared part of the pathway by increasing the gene dosage. This is consistent with the observation that both sets of the paralogous genes are expressed in complex soy medium, where precursor availability and growth would support greater metabolite production levels than are possible on defined SA medium, where only the *ceaS2-oat2* set of paralogues is expressed. In addition, the increased production of these secondary metabolites may be of greater advantage in complex medium to ward off competition, especially that posed by faster-growing organisms. In defined media, such as SA, the expression of only one set of paralogous genes may suffice to provide an adequate supply of precursors, given the lower levels of clavulanic acid and 5S clavam production.

Another explanation put forth is that the two sets of paralogous genes may belong to two separate pathways, with one leading to clavulanic acid and the other to the 5S clavams. Since the two pathways proceed through common early steps, a sharing of biosynthetic intermediates results, but the two pathways may be regulated differently. This is consistent with our observation that, although clavulanic acid production was knocked out in the *ccaR* mutant, the 5S clavams were still produced during growth on soy medium. The *ccaR* mutant shows *ceaS1* transcription equivalent to that seen in the wild-type strain and still produces wild-type levels of 5S clavams, whereas *ceaS2* transcription is almost absent and clavulanic acid production is lost. This suggests that *ceaS1*, *bls1*, and *pah1* may be more closely associated with the production of 5S clavams via a CcaR-independent pathway, whereas *ceaS2* and its related paralogues are associated with clavulanic acid production and are regulated by CcaR. This is an attractive hypothesis from the point of view that the producer organisms would be best served by coordinating production of a β -lactam antibiotic (cephamycin C) with production of a β -lactamase inhibitor (clavulanic acid) through the action of a common regulator (CcaR). In contrast, no apparent advantage would be gained by coordinating production of the 5S clavams with production of cephamycin C. Further investigation is required before any firm conclusions can be drawn. In previous studies (2), it was reported that production of both 5S clavam and clavulanic acid was lost in a *ccaR* mutant, whereas in our hands, mutation of *ccaR* had no effect on 5S clavam production. This inconsistency may be attributed to differences in the methodologies and growth media used for culture propagation in the two studies or to the extensive variability that has been observed in 5S clavam production profiles within this species (43).

The functional holoenzyme forms of CEAS2 (9), BLS2 (25), and PAH2 (11) have all been characterized structurally and shown to be oligomers. Since these proteins were overexpressed and purified from *E. coli*, only homo-oligomers were

observed. It is reasonable to expect that when the corresponding homo-oligomeric forms of CEAS1, BLS1, and PAH1 are expressed and purified, they may have somewhat different activities or kinetic properties, just as was seen for the CAS1 and CAS2 monomers (37). These differences in activities may be important under the specific nutritional conditions in which each of these isoenzymes is expressed. It is also possible that within *S. clavuligerus* the two isozymic forms of each protein can form hetero-oligomers, which could provide another mechanism to modulate enzyme activity based on nutritional and precursor availability.

Studies are under way to investigate the interactions among the different isoenzymes to gain greater understanding of the roles of the paralogous genes involved in clavulanic acid and 5S clavam biosynthesis.

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