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Carboxyethylarginine Synthase Genes Show Complex Cross-Regulation in *Streptomyces clavuligerus*

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Carboxyethylarginine synthase is the first dedicated enzyme of clavam biosynthesis in *Streptomyces clavuligerus* and is present in two isoforms encoded by two separate genes. When grown on a liquid soy medium, strains with *ceaS1* deleted showed only a mild reduction of clavam biosynthesis, while disruption of *ceaS2* abolished all clavam biosynthesis. Creation of an in-frame *ceaS2* deletion mutant to avoid polarity did not restore clavam production, nor did creation of a site-directed mutant altered only in a single amino acid residue important for activity. Reverse transcriptase PCR analyses of these mutants indicated that the failure to produce clavam metabolites could be traced to reduced or abolished transcription of *ceaS1* in the *ceaS2* mutants, despite the location of *ceaS1* on a replicon completely separate from that of *ceaS2*. Western analyses further showed that the CeaS1 protein (as well as the CeaS2 protein) was absent from the *ceaS2* mutants. Complementation experiments were able to restore clavam production partially, but only by virtue of restoring CeaS2 production. CeaS1 was still absent from the complemented strains. While this dependence of CeaS1 production on the expression of *ceaS2* from its native chromosomal location was seen in all of the *ceaS2* mutants, the effect was limited to growth in liquid medium. When the same mutants were grown on solid soy medium, clavam production was restored and CeaS1 was produced, albeit at low levels compared to the wild type.

Clavulanic acid is a powerful β-lactamase inhibitor used in conjunction with conventional β-lactam antibiotics to combat diseases caused by antibiotic-resistant pathogens. It is produced industrially in fermentations of *Streptomyces clavuligerus*. Due to its clinical value (1), the biochemical and genetic aspects of clavulanic acid biosynthesis in *S. clavuligerus* have been subjected to intensive research in recent years. It is now known that the biosynthetic pathway to clavulanic acid is partly shared with the pathway for structurally related 5*S* clavam metabolites that are also produced by *S. clavuligerus*, up to the level of clavaminic acid (2). The pathway branches at this point and goes on to form either clavulanic acid or 5*S* clavams (Fig. 1A). The enzymes involved in the early shared steps of the pathway have been well characterized (3), although the later stages remain to be clarified.

From their first discovery, it was known that there are two isozymes of clavaminic acid synthase (CAS), one of the early pathway enzymes, in *S. clavuligerus* (4, 5). The two isozymes are encoded by two *cas* genes, *cas1* and *cas2*, and disruption of either copy causes only partial loss of clavulanic acid production, while disruption of both genes causes a complete loss. *cas2* lies near one end of the chromosome within the clavulanic acid gene cluster, which includes all of the genes associated with the production of clavulanic acid (Fig. 1B), whereas *cas1* is located several megabases away in the clavam gene cluster. The gene *pah2*, encoding proclavaminic acid synthase (PAH), another of the early enzymes, lies adjacent to *cas2* in the clavulanic acid gene cluster. When disruption of *pah2* caused only partial loss of clavulanic acid production (6), this suggested that there must also be two *pah* genes. The *bls2* gene, which encodes β-lactam synthetase, follows this same paradigm, in that disruption of *bls2* caused only partial loss of clavulanic acid production, leading to prediction of a second *bls* gene, but the *ceaS2* gene stood apart. *ceaS2* encodes carboxyethylarginine synthase (CEAS), the first enzyme of the pathway. Initial studies showed that disruption of *ceaS2* with an apramycin resistance (*apr*) gene to generate a *ceaS2*::*apr* mutant caused complete loss of production of all clavam metabolites, suggesting that there might be only one *ceaS* gene. However, trace amounts of clavulanic acid (2 to 5% of wild-type levels) were produced by *ceaS2*::*apr* mutants on rare occasions (6). An early report by Perez Redondo et al. (7) also indicated that *ceaS2* mutants could produce clavulanic acid under certain growth conditions, but we could not reproduce this effect when using our *ceaS2* mutants. In all of our recent studies, *ceaS2* mutants were completely blocked in production of all clavam metabolites. This led us to conclude that initial findings of clavam production by *ceaS2* mutants were uncertain and perhaps attributable to limitations of early bioassay and high-performance liquid chromatography (HPLC) techniques. However, the eventual discovery of the paralogue gene cluster located on a giant linear plasmid (8–10) proved that there are second copies of all early genes, including a second *ceaS* gene. The second copies of these early genes were designated *ceaS1*, *bls1*, and *pah1*, to correlate with the previously named *cas1* gene and to distinguish them from the *ceaS2*, *bls2*, *pah2*, and *cas2* genes of the clavulanic acid gene cluster. The genes of the clavam and paralogue clusters are associated with the production of the 5*S* clavam metabolites, while the clavulanic acid cluster is involved with the production of clavulanic acid. The two processes are regulated independently of one another (11–16), although the shared early steps in the pathway mean that there is some cross talk between clavulanic acid and

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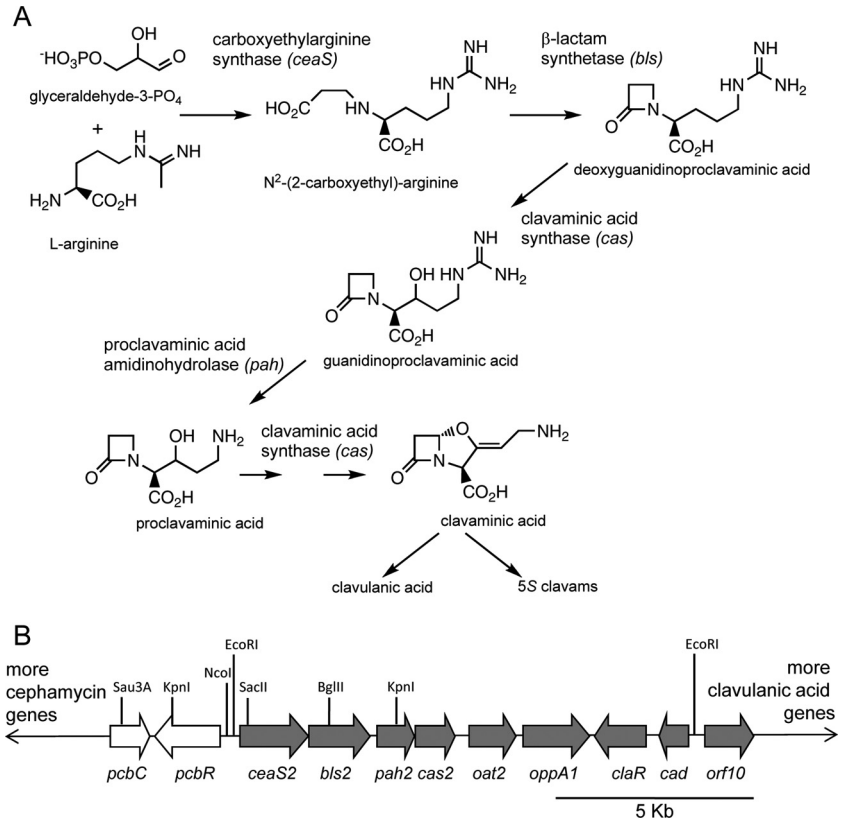

FIG 1 Production of clavam metabolites. (A) The biosynthetic pathway to clavaminic acid, the branch point intermediate leading to production of clavulanic acid and 5*S* clavams. Gene designations are shown in italics. (B) Part of the cephamycin-clavulanic acid gene cluster. Cephamycin-specific genes are shown in white, and clavulanic acid-specific genes are shown in gray. Locations of restriction endonuclease cleavage sites relevant to descriptions in the text are shown.

5*S* clavam biosynthesis. The *ceaS1* gene is highly similar to *ceaS2* (66% identity and 80% similarity at the amino acid level), but deletion of the *ceaS1* gene caused only a minor decrease in clavulanic acid production (10), which left open the question of why *ceaS2* mutants typically do not produce any clavam metabolites.

Investigation of the blocked phenotype of the *ceas2*::*apr* mutant was complicated by the fact that *ceaS2* is the lead gene of an operon encompassing all of the early genes of the clavulanic acid cluster (Fig. 1B) (17). To prevent polarity from influencing expression of downstream genes, a second *ceaS2* mutant was created in which the disrupting *apr* gene was replaced by a simple frameshift (FS) (6). However, the phenotype of the resulting *ceaS2*-FS mutant was the same as that of the *ceaS2*::*apr* mutant. In this study, we investigated further the basis for the severe effect of *ceaS2* disruption on clavam metabolite production in *S. clavuligerus*.

MATERIALS AND METHODS

Bacterial strains, plasmid and cosmid vectors, and culture conditions. Bacterial strains and the plasmid and cosmid vectors used in this study are listed in Table 1 (18–20). *S. clavuligerus* strains were maintained on International Streptomyces Project-4 (ISP-4) agar plates as described previously (21) and stored in 20% glycerol at −80°C as spore stocks. *Escherichia coli* strains were grown and maintained on Lennox broth (LB) medium at 37°C. Plasmid-containing strains were selected with appropriate antibiotics as described previously (10). Fermentation studies were performed by inoculating Trypticase soy broth plus 1% soluble starch (TSBS) with *S. clavuligerus* spore stocks. After 40 h of incubation at 28°C on a rotary shaker at 280 rpm, these seed cultures were used to inoculate 2% (vol/vol) soy (5) or starch asparagine (SA) medium (17), and incubation was continued for 72 to 96 h, when metabolite production was maximal in wild-type cultures. All production cultures were staged using 100 ml of medium in 500-ml flasks, since production of 5*S* clavam metabolites was found to be particularly sensitive to medium-to-flask volume ratios.

Metabolite production on solid medium was tested using plates containing 20 ml of soy medium solidified with agar (2%, wt/vol) and spread with ~10^8 spores. Sterile cellophane disks (22) were placed on the inoculated plates, and another 10^8 spores was spread on each cellophane disk. This double inoculation technique was necessitated by the insoluble nature of the soy medium nutrients. Inoculated plates were incubated at 28°C for 9 days.

DNA manipulation, PCR, and sequencing. Standard procedures were used to manipulate plasmid DNA purified from *E. coli* (23). PCRs were performed using the Expand high-fidelity PCR system (Roche) according to the manufacturer's protocol. Oligonucleotide primers are listed in Table S1 of the supplemental material and were purchased from Integrated DNA Technologies (IDT). DNA sequence analysis was carried out by the Molecular Biology Service Unit, University of Alberta. The BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for performing sequence alignments and similarity searches.

Construction of Δ*ceaS2*::*apr*, Δ*ceaS2* in-frame (IF), and E57W *ceaS2* mutants. Δ*ceaS2*::*apr* mutants were prepared by using the Redirect PCR-targeted mutagenesis procedure (24) as described in previous studies (10). Primers ceaS2-Redirect For and ceaS2-Redirect Rev were used to amplify the *apr* cassette to give a PCR product with sequences on each end targeting it to the *ceaS2* gene. The PCR product was transformed into *E. coli* BW25115/pIJ790 carrying cosmid 12B8, wherein the *ceaS2* gene was de-

TABLE 1 Bacterial strains, plasmids, and cosmids used in this study

Strain, plasmid, or cosmid	Description	Reference or source
E. coli strains		
BL21(DE3)	Host for T7 promoter-driven protein expression	Stratagene
BW25115/pIJ790	Host for Redirect PCR targeting system, produces λ Red proteins from temperature-sensitive plasmid pIJ790	24
DH5α	General cloning host	Gibco BRL
DH5α(BT340)	Produces FLP recombinase from temperature-sensitive plasmid BT340	24
ET12567/pUZ8002	Methylation-deficient host, expresses transfer functions from pUZ8002	22
S. clavuligerus strains		
NRRL 3585	Wild-type strain producing cephamycin C, clavulanic acid, and 5*S* clavams	Northern Regional Research Laboratory, Peoria, IL
ceaS2::*apr*	*ceaS2* insertional disruption mutant	6
ceaS2-FS	*ceaS2* frameshift mutant	10
Δ*ceaS1*::*apr*	Deletion mutant with *ceaS1* replaced by *apr* cassette	10
Δ*ceaS1*::*apr*/ *ceaS2*-FS	*ceaS1*/*ceaS2* double mutant	10
Δ*ceaS2*::*apr*	Deletion mutant with *ceaS2* replaced by *apr* cassette	This study
Δ*ceaS2*-IF	*ceaS2* in-frame deletion mutant	This study
Δ*ceaS2*-c5	*ceaS2* in-frame deletion mutant complemented with pSET152-c5	This study
Δ*ceaS2*-c11.6	*ceaS2* in-frame deletion mutant complemented with pSET152-c11.6	This study
E57W*ceaS2*	*ceaS2* site-directed mutant with codon 57 changed from glutamate to tryptophan	This study
E57W*ceaS2*-c5	*ceaS2* E57W mutant complemented with pSET152-c5	This study
E57W*ceaS2*-c11.6	*ceaS2* E57W mutant complemented with pSET152-c11.6	This study
Plasmids		
pBB5.3A	pUC119 carrying 5-kb DNA insert with *pcbR* and *ceaS2*	16
pET-9c	Protein expression vector	Novagen
pET-24b	Protein expression vector	Novagen
pET-9c-*ceaS1*	pET-9c derivative carrying *ceaS1*	This study
pET-24a-*ceaS2*	pET-24a derivative carrying *ceaS2*	C. Schofield, Oxford University
pIJ773	Plasmid carrying template for Redirect *apr* cassette	24
pSET152	*E. coli-Streptomyces* shuttle plasmid, integrates in *Streptomyces*	Northern Regional Research Laboratory
pSET152-ceaS1	pSET152 derivative carrying promoter region of *ceaS1* coupled to promoterless *egfp* gene	16
pSET152-c5	pSET152 derivative carrying 5-kb insert with *ceaS2* and upstream regions	This study
pSET152-c11.6	pSET152 derivative carrying 11.6-kb insert extending from mid-*pcbC* through *cad*	This study
pUC119	General cloning vector	18
pUWL-KS	*E. coli-Streptomyces* shuttle plasmid	19
pUWL-oriT	Derivative of pUWL-KS carrying *oriT*	20
pUWL-Δ*ceaS2*::apr	Derivative of pUWL-KS carrying 5.5-kb insert encompassing Δ*ceaS2*::*apr* mutation	This study
Cosmids		
pWE15	General purpose cosmid vector	Promega
12B8	pWE15-derived cosmid carrying *ceaS2* and downstream region	8
12B8-AP	12B8 derivative with *ceaS2* replaced by *apr* gene cassette	This study

leted and replaced by the *apr* cassette to generate mutant cosmid 12B8-AP. Cosmid 12B8-AP was then conjugated into wild-type *S. clavuligerus*, and Δ*ceaS2*::*apr* mutant strains were selected based on the presence of *apr* and confirmed by Southern analysis.

Δ*ceaS2*-IF mutants were prepared by cloning a 5.5-kb KpnI DNA fragment carrying the *apr* cassette and regions flanking the deleted *ceaS2* gene from cosmid 12B8-AP into pUWL-KS to create pUWL-Δ*ceaS2*::*apr*. pUWL-Δ*ceaS2*::*apr* was then transformed into *E. coli* DH5α(BT340), and transformants were grown at 42°C to induce FLP recombinase and excise the disruption cassette, leaving in its place an 81-bp scar sequence. The resulting plasmid, pUWL-Δ*ceaS2*-IF, was transformed into Δ*ceaS2*::*apr* protoplasts, where homologous recombination generated the Δ*ceaS2*-IF mutants.

E57W*ceaS2* mutants were created by PCR using plasmid pBB5.3A, which carries an ~5-kb DNA fragment extending from a Sau3A site inside *pcbC* to a BglII site inside *bls2* (Fig. 1B) (16), as the template, along with primers Mutate ceaS2-For and Mutate ceaS2-Rev to amplify a 580-bp fragment from the 5′ end and upstream of *ceaS2*. The amplified fragment encompassed naturally occurring NcoI and SacII sites and introduced a GA-to-TG mutation to change the glutamate 57 residue of CeaS2 to tryptophan. The fragment, digested with NcoI and SacII, was used to replace the corresponding wild-type fragment in pBB5.3A, and then the E57W mutant version of the entire 5-kb DNA fragment was subcloned into pUWL-oriT and conjugated into the *ceaS2*::*apr* mutant. Thiostrepton-resistant exconjugants were sporulated in the presence of thiostrepton and then again in its absence. Resulting spores were replica plated to identify thiostrepton-sensitive, apramycin-sensitive E57W*ceaS2* mutants. The identity of the mutants was verified by sequencing PCR products amplified from genomic DNA using primers ceaS2-RT-for and ceaS2-RT-rev.

Several independent isolates of each *ceaS2* mutant type were examined. All isolates sporulated and showed growth characteristics on solid

and liquid media that were indistinguishable from wild-type strains. Metabolite production patterns were consistent for all mutants, so a representative strain of each type was chosen for further studies.

Complementation of the Δ*ceaS2*-IF mutation in *S. clavuligerus*. The ~5-kb insert of *S. clavuligerus* DNA that encompasses *ceaS2* was released from pBB5.3A as a BamHI-XbaI fragment and cloned into pSET152 to create pSET152-c5. Similarly, an ~11.6-kb EcoRI fragment that extends from upstream of *ceaS2* to beyond the end of *cad* (Fig. 1B) was cloned into pSET152 to generate pSET152-c11.6. The plasmids were introduced into Δ*ceaS2*-IF and E57W*ceaS2* mutants by conjugation, and complemented strains were selected based on the presence of *apr*.

Overproduction of CeaS1 and CeaS2. Plasmid pET-24a-ceaS2 was generously provided by C. Schofield (Oxford University). Plasmid pET-9c-ceaS1 was prepared by amplifying *ceaS1* by PCR using primers ceas1-exp-fwd and ceaS1-exp-rev. The amplified fragment was subcloned into pTOPO2.1, sequenced to verify fidelity, and then transferred to pET9c. The two expression plasmids were transformed into *E. coli* BL21(DE3), and when freshly grown cultures reached an optical density at 600 nm of approximately 0.6, isopropyl-β-D-thiogalactoside (IPTG) was added to 0.5 mM to induce protein production, and cultures were incubated at 37°C for a further 4 h. Bacterial cells were harvested, resuspended in 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 0.1% Triton X-100, and lysozyme at 0.15 mg/ml, disrupted by sonication, and centrifuged for 10 min at 14,000 × *g* to sediment insoluble material.

RNA isolation and RT-PCR. Total RNAs were isolated from *S. clavuligerus* strains grown in soy medium for 48 h by using the modified Kirby procedure (22). Preliminary studies indicated that *ceaS1* and *ceaS2* transcript levels in wild-type cultures were greatest at this time point. Reverse transcriptase PCR (RT-PCR) analysis of RNA was carried out using SuperScript III (SSIII) reverse transcriptase (Invitrogen) to generate cDNA. RT reaction mixtures were set up according to the manufacturer's protocol but with the following changes: 1 μg of total RNA, 5% dimethyl sulfoxide (DMSO), and 0.5 μl of SSIII (200 U/μl) were included in each 10-μl reaction mixture, and reaction mixtures were incubated at 55°C for 1 h. Each 50-μl PCR mixture contained 10 μl of RT product, 5% DMSO, and 25 pmol of forward and reverse primers. PCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 28 cycles of denaturation at 94°C for 45 s, annealing at 62°C for 45 s, and elongation at 72°C for 45 s. Negative controls (no RT) were carried out with each RNA preparation to confirm the absence of contaminating chromosomal DNA.

Western blot analysis. Cell extracts (CFEs) were prepared from 5-ml aliquots of 48-h *S. clavuligerus* cultures grown in soy or SA medium, after supplementing with Complete protease inhibitor (Roche) and chilling to 4°C. Cultures were harvested by centrifugation, resuspended in 1 ml of lysis buffer (50 mM Tris-HCl [pH 7.4] containing Complete protease inhibitor), and disrupted by sonication. Cell debris was removed by centrifugation, and clarified cell lysates were stored at −80°C. Protein concentrations were determined using a Coomassie dye binding protein assay (Bio-Rad) with bovine gamma globulin as the standard.

CFEs were prepared from cultures grown on solid medium by scraping cell material from cellophane disks on the surface of soy agar plate cultures into lysis buffer, 1 ml per plate, and disrupting by sonication as described above.

Samples of total CFE protein were separated using 8% SDS-PAGE. Resolved proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Amersham) in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol) and using a mini-Trans-Blot apparatus (Bio-Rad). The enhanced chemiluminescent Western blotting system (ECL-Plus; Pierce) was used to determine protein production according to the manufacturer's instructions. As a cautionary note, CeaS proteins are very sensitive to proteolysis, and so blocking and hybridization solutions containing skim milk were prepared in small quantities immediately before use. Primary anti-CeaS2 antibodies were prepared by standard procedures (25) as described previously (26). Both primary antibody and horseradish peroxidase-linked anti-rabbit IgG secondary antibody (from donkey; Amersham) were used at a dilution of 1/15,000.

EGFP expression and confocal microscopy. pSET-ceaS1 (16), which carries the promoter region of *ceaS1* coupled to a promoterless *egfp* gene, was conjugated into wild-type and Δ*ceaS2*-IF mutant strains of *S. clavuligerus* with selection for *apr*. Confocal microscopy was carried out using a Leica DM IRB inverted microscope to detect enhanced green fluorescent protein (EGFP) production under conditions described previously (16), except samples were harvested after 48 h of growth in soy medium. An argon laser with 50 to 52% attenuation was used to provide excitation at 488 nm, and EGFP excitation was detected between 500 nm and 520 nm.

HPLC analyses. HPLC was used to measure clavulanic acid and 5*S* clavam metabolites in *S. clavuligerus* cultures grown in soy or SA medium. Supernatants from 72- and 96-h cultures were derivatized with imidazole and analyzed using a Phenomenex Bondclone C_{18} column (100 by 800 mm, 10 μm; Phenomenex, Torrance, CA) under previously described conditions (17).

Clavam metabolites produced during growth on solid soy medium were assessed by cutting 4-cm by 4-cm squares of agar from plate cultures after the cell material had been harvested for production of CFE. Agar squares were frozen at −80°C, thawed slowly, and pressed to release fluid. The resulting exudates (25-μl samples) were derivatized with imidazole and analyzed by HPLC as described above for culture supernatants.

RESULTS

Production of Δ*ceaS2*::*apr* and Δ*ceaS2*-IF mutants. Disruption of the *ceaS2* gene in *S. clavuligerus* gave *ceaS2*::*apr* mutants that routinely produced no clavam metabolites, and *ceaS2* frameshift mutants showed the same nonproducer phenotype (6, 10). Although the *ceaS2*-FS mutants were unmarked, translational termination of the out-of-frame 3′ end of the gene would occur 334 bp upstream of the normal stop codon, and so transcription of downstream genes could still be affected. Furthermore, both the *ceaS2*::*apr* and *ceaS2*-FS mutants could potentially produce truncated or C-terminally mistranslated CeaS2 proteins. Since the CeaS protein is known to exist as a tetramer (a dimer of dimers) (27), the defective Ceas2 subunits might interact with active CeaS1 subunits to affect activity.

To address these possibilities, two more *ceaS2* mutants were produced using PCR-targeted mutagenesis (24). In the mutant Δ*ceaS2*::*apr*, the entire *ceaS2* gene was replaced by an *apr* gene cassette, and in mutant Δ*ceaS2*-IF, the *apr* cassette was removed from Δ*ceaS2*::*apr* by FLP recombinase to leave an 81-bp unmarked, in-frame scar in place of the *ceaS2* gene. Both mutants were verified by Southern analysis and then analyzed along with wild-type *S. clavuligerus* and earlier versions of *ceaS2* mutants for the ability to produce clavulanic acid and 5*S* clavams. HPLC analysis of culture supernatants showed that all of the *ceaS2* mutant types had similar phenotypes, and the newly created Δ*ceaS2*::*apr* and Δ*ceaS2*-IF mutants, like the earlier versions, produced no detectable clavulanic acid or 5*S* clavams when grown in soy medium, whereas the wild-type strain produced all of the expected clavam metabolites (Fig. 2).

Effect of Δ*ceaS2*-IF mutation on *ceaS1* and *bls2* transcription. RT-PCR was carried out to investigate if the Δ*ceaS2*-IF mutant showed altered transcription of either *ceaS1* or downstream genes compared to the wild type. Total RNAs were isolated from *S. clavuligerus* wild type, Δ*ceaS2*-IF, and Δ*ceaS1*::*apr* mutants grown for 48 h in soy medium. Reverse primers (see Table S1 in the supplemental material) were used to generate cDNA, and specific primer pairs (see Table S1) were then used to determine if the genes of interest were transcribed in the mutant strains. When

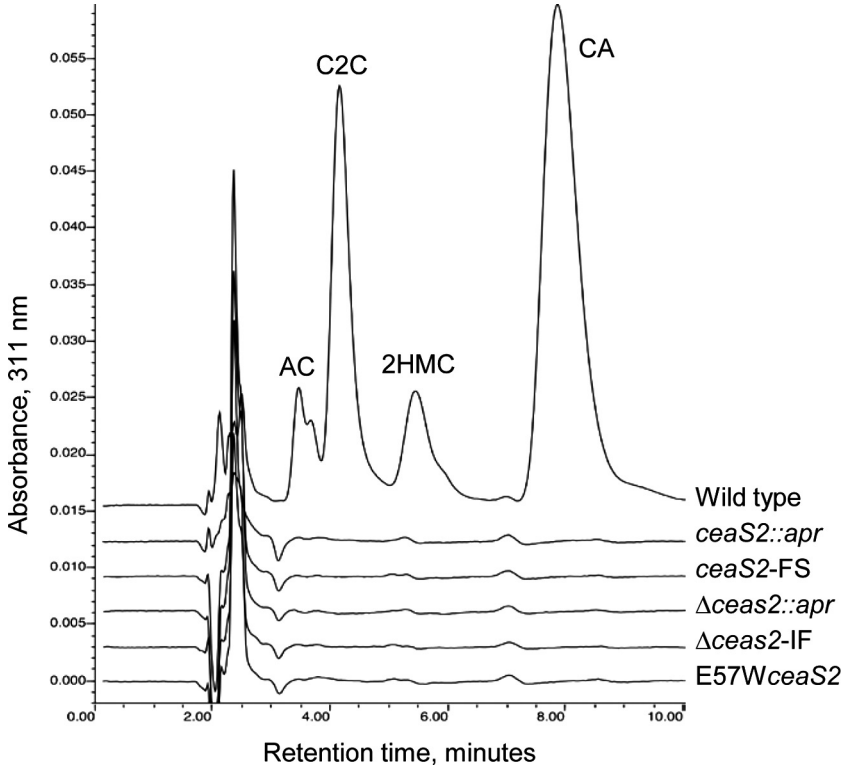

FIG 2 Production of clavam metabolites by *S. clavuligerus* wild-type and *ceaS2* mutant cultures. Culture supernatants from 96-h soy cultures were derivatized with imidazole to detect clavam metabolites and analyzed by HPLC. AC, alanylclavam; C2C, clavam-2-carboxylate; 2HMC, 2-hydroxymethylclavam; CA, clavulanic acid.

RNA from wild-type *S. clavuligerus* was subjected to RT-PCR, transcripts of *ceaS1*, *ceaS2*, *bls1*, and *bls2* were all observed (Fig. 3). When RNA from the Δ*ceaS1*::*apr* mutant was analyzed, only *ceaS2* and *bls2* transcripts were observed, consistent with previous studies showing that *ceaS-bls* gene pairs are cotranscribed in polycistronic transcripts (17). However, when RNA from the Δ*ceaS2*-IF mutant was analyzed, no *ceaS2* transcripts were seen, as expected, but *bls1* or *bls2* transcripts were also not detected, and only a faint band from *ceaS1* was observed. This indicated that transcription of *ceaS1* and *bls1* was severely impaired or absent in the Δ*ceaS2*-IF mutant, and also that transcription of *bls2* was blocked despite the supposedly nonpolar character of the mutation. All RNA samples showed similar transcript levels for *hrdB*, which served as a control for RNA quality and quantity (Fig. 3).

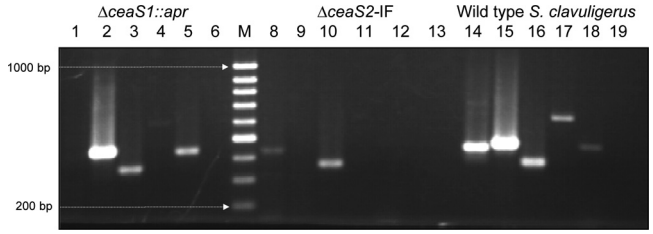

FIG 3 Assessment of transcript levels by RT-PCR. Total RNA was isolated from wild-type *S. clavuligerus*, Δ*ceaS2*-IF, and Δ*ceaS1*::*apr* mutants grown on liquid soy medium for 48 h. PCRs were carried out for 28 cycles, and transcripts examined were the following: *ceaS1* (lanes 1, 8, and 14), *ceaS2* (lanes 2, 9, and 15), *hrdB* (lanes 3, 10, and 16), *bls1* (lanes 4, 11, and 17), and *bls2* (lanes 5, 12, and 18). Lane M, GeneRuler 100-bp DNA ladder. Lanes 6, 13, and 19 represent negative controls for each RNA sample used, in which no reverse transcription was carried out.

Promoter activity analysis with *egfp*. To confirm that *ceaS1* transcription was affected in a Δ*ceaS2*-IF mutant, promoter activity of *ceaS1* was examined using EGFP as a reporter. pSET-ceaS1 carrying the promoter region of *ceaS1* coupled to *egfp* was conjugated into wild-type and Δ*ceaS2*-IF mutant strains of *S. clavuligerus*. The resulting reporter strains were grown in soy medium for 48 h, and samples were analyzed by confocal microscopy. Fluorescence was observed for wild-type *S. clavuligerus* carrying pSET-ceaS1 but not for the Δ*ceaS2*-IF strain carrying the same reporter construct (Fig. 4).

Production of CeaS1 and CeaS2 in *ceaS2* mutants. To gain further evidence that mutation of *ceaS2* affects production of CeaS1, Western analyses were carried out using CFEs from the *ceaS2* mutant strains. Recombinant CeaS1 and CeaS2 proteins were overproduced in *E. coli* and recovered from inclusion bodies for use as protein markers. Although only anti-CeaS2 antibody was available, Western analysis showed that the antibodies reacted with both CeaS1 and CeaS2 (Fig. 5). In addition, despite the similar molecular masses of CeaS1 and CeaS2 (59 and 61 kDA, respectively), the two proteins could be resolved from each other by prolonged SDS-PAGE (Fig. 5).

Previous studies showed that expression of *ceaS1* and *ceas2* is nutritionally regulated such that both *ceaS1* and *ceaS2* are expressed in soy medium but only *ceaS2* is expressed in SA medium (16). Western analyses confirmed these observations by showing that CeaS2 protein was present in CFEs from wild-type *S. clavu-*

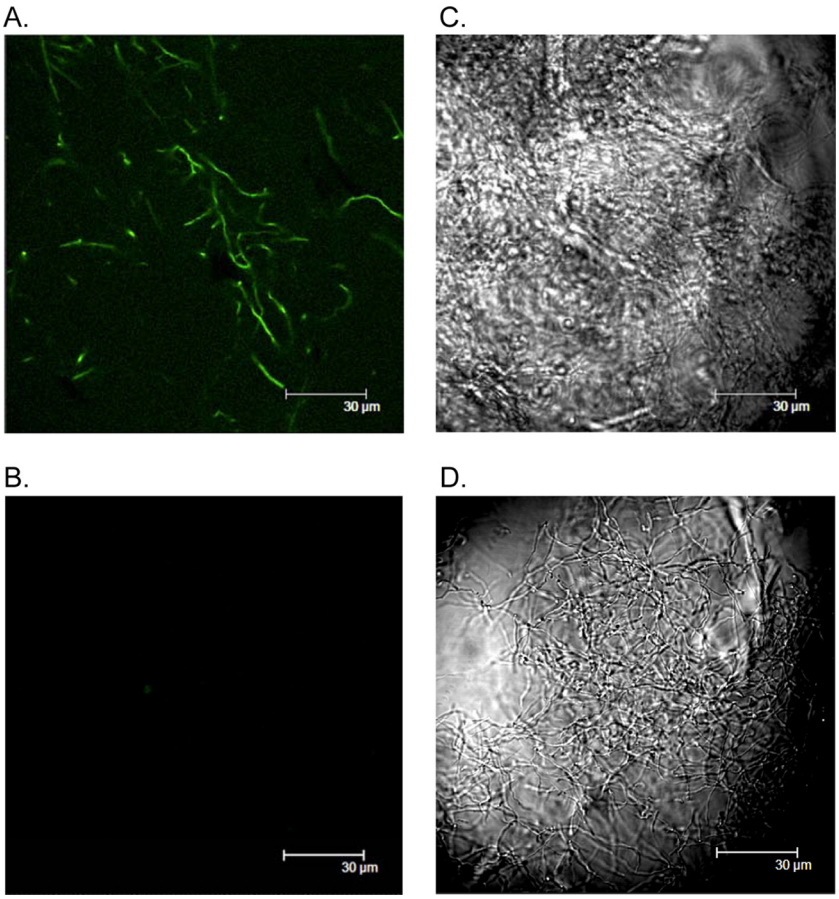

FIG 4 Detection of *ceaS1* promoter activity in wild-type *S. clavuligerus* and Δ*ceaS2*-IF mutant cultures by using EGFP as a reporter. (A) Mycelia of wild-type *S. clavuligerus* carrying the pSET-p-ceaS1 reporter construct. (B) Mycelia of Δ*ceaS2*-IF carrying pSET-ceaS1. Both reporter strains were grown in liquid soy medium for 48 h and then analyzed by confocal microscopy. Fluorescence images are shown on the left. Differential interference contrast (DIC) images are shown on the right.

ligerus grown on either soy or SA medium, while CeaS1 protein was almost undetectable in CFEs from SA-grown cultures (Fig. 6). Cross-reacting bands of unknown identity were observed between CeaS2 and CeaS1 and beneath Ceas1. These bands appeared to be degradation products of CeaS2, even though protease inhibitors were routinely included when preparing CFEs to prevent protein degradation.

CFEs of wild-type *S. clavuligerus* and *ceaS2*::*apr*, *ceaS2*-FS, Δ*ceaS2*::*apr*, Δ*ceaS2*-IF, Δ*ceaS1*::*apr*, and Δ*ceaS1*::*apr ceaS2*-FS mutants were prepared from cultures grown in soy medium and subjected to Western analysis. Protein bands corresponding to CeaS1 and CeaS2 were seen in the CFE of wild-type *S. clavuligerus*, and protein bands due to CeaS2 were absent from all of the *ceaS2* mutants (Fig. 7). However, protein bands representing CeaS1 were also absent from these mutants, indicating that neither CeaS1 nor CeaS2 protein was produced in the *ceaS2* mutants. In contrast, CeaS2 was observed in CFE from the Δ*ceaS1*::*apr* mutant. As expected, neither Ceas1 nor CeaS2 was seen in the *ceaS1*/*ceaS2* double mutants. The cross-reacting bands seen in the wild type CFE are missing from the various *ceaS2* mutants and from the *ceaS1 ceaS2* double mutant, which supports the contention that they are degradation products that arise from CeaS2.

Creation of an E57W*ceaS2* site-directed mutant. All four types of *ceaS2* mutants produced neither clavam metabolites nor CeaS1 protein. However, residual polarity affecting expression of downstream genes in the *ceaS2* operon was still evident, even in the Δ*ceaS2*-IF mutant. Furthermore, it was unclear whether the *ceaS2* mutants failed to produce CeaS1 because of the physical disturbance at the *ceaS2* locus caused by the mutations or if active CeaS2 protein was required for *ceaS1* expression. To gain further insights, a fifth type of *ceaS2* mutant was created in which the *ceaS2* gene was modified by a site-directed mutation of 2 bp, with GA changed to TG, such that the glutamate codon at amino acid 57 became a tryptophan codon. Structural analyses of CeaS2 have suggested that the glutamate residue at position 57 (E57) is intimately involved in the catalytic function of the protein (27), and so the E57W mutant CeaS2 protein was expected to be defective in CeaS2 activity but otherwise unchanged from the wild type. A cloned copy of the E57W mutant version of *ceaS2* was conjugated into a *ceaS2*::*apr* mutant, whereupon the *apr*-disrupted *ceaS2* was replaced by E57W*ceaS2*. Upon cultivation in soy medium, culture filtrates of the E57W mutants, like all of the previous *ceas2* mutants, showed a complete inability to produce clavam metabolites (Fig. 8A).

Complementation of *S. clavuligerus* Δ*ceaS2*-IF and E57W*ceas2* mutants with *ceaS2*. Complementation experiments to restore

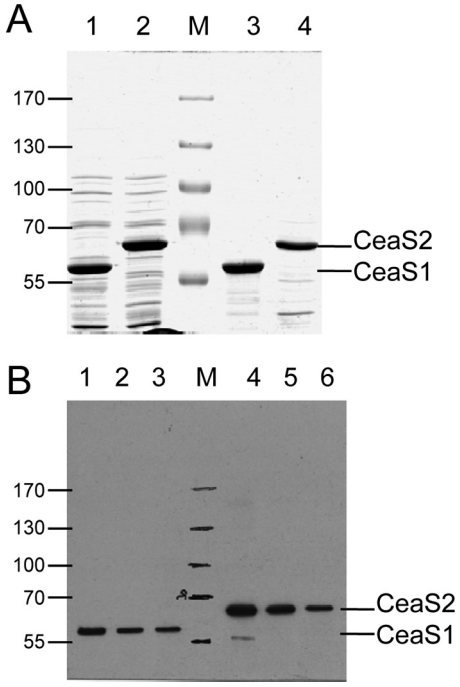

FIG 5 Western analysis of recombinant CeaS1 and CeaS2 proteins. Broken cell suspensions of *E. coli* expressing *ceaS1* and *ceaS2* were separated into soluble and insoluble fractions by centrifugation and then analyzed by electrophoresis on 8% SDS-PAGE gels. (A) Coomassie-stained gel, Lanes 1 and 3, soluble (8.5 μg of protein) and insoluble (2.5 μg of protein) CeaS1; lanes 2 and 4, soluble (8.5 μg of protein) and insoluble (2.5 μg of protein) CeaS2. (B) Western analysis of insoluble CeaS1 (lanes 1 to 3, 50, 25, and 12.5 ng of protein, respectively) and CeaS2 (lanes 4 to 6, 50, 25, and 12.5 ng of protein, respectively) samples used in panel A.

clavam production to the Δ*ceaS2*-IF and E57W*ceas2* mutant strains were carried out using two different complementation constructs. Both constructs were based on the integrative vector, pSET152, and both employed the native *ceaS2* promoter. In the first instance, an ~5-kb Sau3A-BglII DNA fragment was used, which encompassed the *ceaS2* gene extending upstream into *pcbC* and downstream into *bls2* (Fig. 1B). This construct, pSET152-c5, was introduced into the Δ*ceaS2*-IF and E57W*ceaS2* mutant strains by conjugation, and exconjugants were cultured in soy medium. Culture filtrates were analyzed by HPLC and showed small

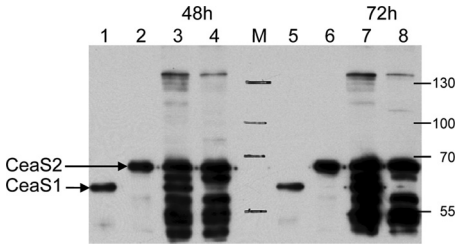

FIG 6 Western analysis of CeaS proteins from cell extracts of *S. clavuligerus* grown on soy or SA medium. Samples were separated by electrophoresis on 8% SDS-PAGE gels and transferred to PVDF membranes for analysis. Lanes 1 and 5, CeaS1 standard (25 ng of protein); lanes 2 and 6, Ceas2 standard (25 ng of protein); lanes 3 and 7, soy medium-grown *S. clavuligerus* CFE (200 ng of protein); lanes 4 and 8, SA medium-grown *S. clavuligerus* CFE (100 ng of protein); M, molecular weight marker proteins.

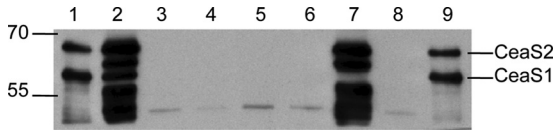

FIG 7 Western analysis of CeaS proteins from the wild type and mutant strains of *S. clavuligerus*. Cell extracts were isolated from soy medium-grown cultures of wild-type *S. clavuligerus* (lane 2), *ceaS2*::*apr* (lane 3), *ceaS2*-FS (lane 4), Δ*ceaS2*::*apr* (lane 5), Δ*ceaS2* (lane 6), Δ*ceaS1*::*apr* (lane7), and Δ*ceaS1*::*apr*/*ceaS2*-FS (lane 8). Lanes 1 and 9 contained 25 ng each of CeaS1 and CeaS2 standard proteins. Cell extract proteins (200-ng amounts) were separated by electrophoresis on 8% SDS-PAGE gels and transferred to PVDF membranes for analysis.

amounts of clavam metabolite production in the complemented Δ*ceaS2*-IF strain and somewhat greater complementation in the E57W*ceaS2* strain, but production was still well below wild-type levels (Fig. 8A). A second complementation construct, pSET152-c11.6, was prepared in which an ~11.6-kb EcoRI DNA fragment, extending from 183 bp upstream of *ceaS2* to beyond the end of *cad* (Fig. 1B) and including all of the genes in the *ceaS2* operon was inserted in pSET152. When this larger complementation construct was inserted into the Δ*ceaS2*-IF and E57W*ceaS2* mutants, clavulanic acid and 5*S* clavam production were again partially restored, but only to levels similar to those seen with the 5-kb complementing construct (Fig. 8A).

When CFEs of the complemented strains Δ*ceaS2*-c5, Δ*ceaS2*-c11.6, E57W*ceaS2*-c5, and E57W*ceaS2*-c11.6 were examined by Western analysis, protein bands corresponding to CeaS2 were observed in all of them. The amounts of CeaS2 produced varied, and the level of Ceas2 protein did not necessarily reflect the level of clavam metabolites produced. However, and more significantly, the CeaS1 protein band was still absent in all of these complemented strains (Fig. 8B).

Clavam metabolite production by *ceaS2* mutants on solid medium. None of the *ceaS2* mutants produced clavam metabolites, and none expressed *ceaS1* even when they were complemented with an intact *ceaS2* gene. Since no conditions were encountered under which *ceaS1* was expressed and clavam metabolites produced in the absence of CeaS2, the question of whether CeaS1 is functionally equivalent to CeaS2 remained unanswered. Therefore, we looked for cultural conditions that would allow the *ceaS2* mutant strains to express *ceaS1* and produce clavam metabolites. We knew from previous studies that expression of *ceaS1* and *ceaS2* is subject to different nutritional regulation and that production of 5*S* clavam metabolites is very sensitive to changes in growth conditions, such as the culture medium-to-flask volume ratio and shaker speed (unpublished observations). Despite testing various growth conditions and media (including the GSPG medium originally reported by Perez-Redondo et al. as supporting clavulanic acid production by *ceaS2* mutants [7]), no conditions supporting clavam metabolite production in liquid medium by *ceaS2* mutants were identified. Surprisingly, however, if the *ceaS2* mutants were grown in solid culture on the surface of soy medium-based agar plates, all of the mutants regained at least some ability to produce clavam metabolites (Fig. 9A). Furthermore, when CFEs were prepared from cell material growing on cellophane disks on the surface of the plates, Western analyses showed that CeaS1 was now clearly present (Fig. 9B), although at low levels compared to the wild type. This indicated that at least

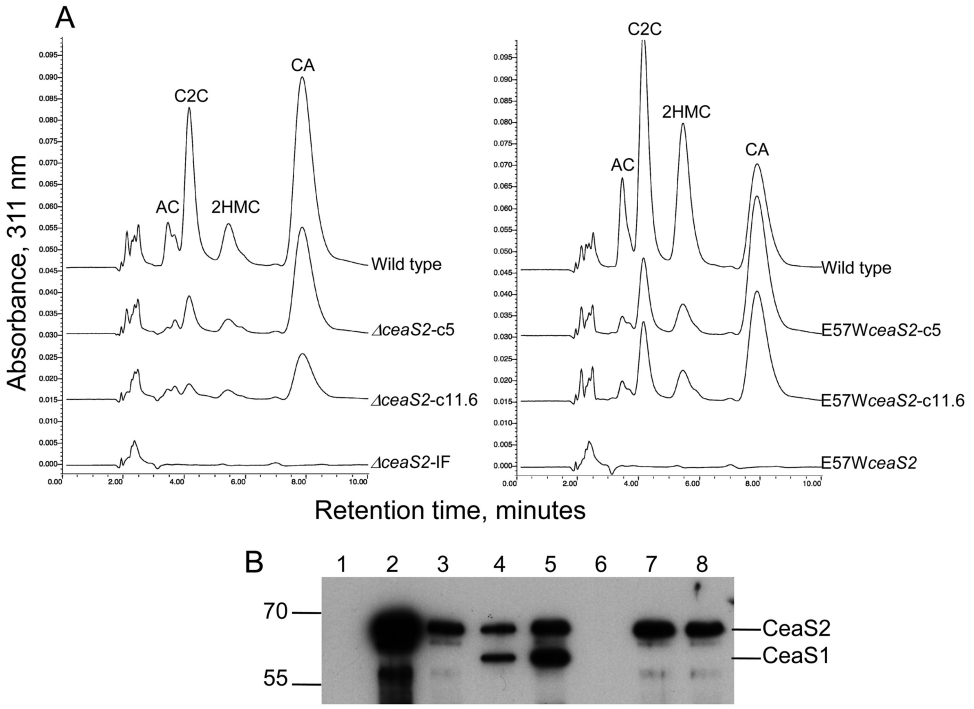

FIG 8 Analysis of clavam metabolite and CeaS1 and CeaS2 production in complemented *ceaS2* mutants. (A) Culture supernatants from 96-h soy cultures were derivatized with imidazole to detect clavam metabolites and then analyzed by HPLC. AC, alanylclavam; C2C, clavam-2-carboxylate; 2HMC, 2-hydroxymethylclavam; CA, clavulanic acid. (B) Cell extra proteins (200-ng amounts) from wild-type, Δ*ceaS2* and E57W*ceaS2* mutant and complemented mutant cultures were separated by electrophoresis on 8% SDS-PAGE gels and transferred to PVDF membranes for analysis. Lane 1, Δ*ceaS2*; lane 2, Δ*ceaS2*-c5; lane 3, Δ*ceaS2*-c11.6; lane 4, 25-ng amounts of CeaS1 and CeaS2 standard proteins; lane 5, wild type; lane 6, E57W*ceaS2*; lane 7, E57W*ceaS2*c5; lane 8, E57W*ceaS2*-c11.6.

some transcription of *ceaS1* takes place in the absence of CeaS2, providing that cells are grown on solid medium.

DISCUSSION

S. clavuligerus ceaS2::*apr* and *ceaS2*-FS mutants typically showed a complete inability to produce clavam metabolites, which was unexpected given that they contain intact *ceaS1* genes that should be able to compensate for the loss of CeaS2. Since *ceaS2* is transcribed together with *bls2*, *pah2*, and *cas2*, a Δ*ceaS2*-IF mutant was constructed to ensure that polar effects on downstream genes were not contributing to this defect (17). Like the other *ceaS2* mutants (6, 10), the Δ*ceaS2*-IF mutant produced no clavam metabolites, but RT-PCR analyses indicated that transcription of the downstream *bls2* gene was still blocked despite the mutation being unmarked and in frame.

Although polarity persisted in Δ*ceaS2*-IF mutants, this alone could not explain why clavam production was so severely affected in *ceaS2* mutants. Even if disruption of *ceaS2* silenced the entire *ceaS2* operon, the redundant *ceaS1* operon was still present and so a reduced level, rather than a total loss of clavam production, would be expected. As an alternative, we considered whether CeaS1 might not be functionally equivalent to CeaS2. However, RT-PCR, eGFP, and Western analyses all clearly showed that expression of *ceaS1* was severely depressed or abolished in *ceaS2* mutants, making the failure of these mutants to produce CeaS1 a more likely reason for the defect in clavam production, rather than any differences in activities between the two proteins.

Interestingly, when the E57W site-directed mutant version of *ceaS2* was generated to produce full-length but enzymatically inactive CeaS2 protein, clavam metabolite and CeaS1 production were still completely blocked, suggesting that active Ceas2 protein must be required for *ceaS1* expression. The inability to detect the E57W mutant form of CeaS2 by Western analysis further suggested either that the mutation in the protein rendered it so unstable that all remnants of it escaped detection by Western analysis or that active CeaS2 protein is also required for efficient *ceaS2* expression.

Despite their locations on separate replicons within the cell (*ceaS2* resides on the chromosome, while *ceaS1* is located on a giant linear plasmid), cross-regulation must tie the expression of *ceaS1* to *ceaS2*. In this regard, complementation studies showed that CeaS1 production was not restored in the complemented *ceaS2* mutant strains, even though both CeaS2 and some clavam metabolite production were observed. The failure of complementation to restore expression of *ceaS1* suggested that there might be some type of a positional effect, such that the location from which the *ceaS2* gene is expressed, as well as the resulting gene product itself, are critical in order to support efficient *ceaS1* expression. But whether this might involve a topological effect related to supercoiling, or possibly involve sense or antisense noncoding RNA, or the involvement of small effector molecules, is not clear at present. Alternatively, if mutation of *ceaS2* at its native location on the chromosome somehow negatively impacts expression of genes further downstream from *cad* in the clavulanic acid gene cluster, this could account for some of the complementation results.

Expression of developmental genes associated with fruiting body formation in *Myxococcus xanthus* requires activator proteins that bind to DNA elements similar to enhancer sequences from

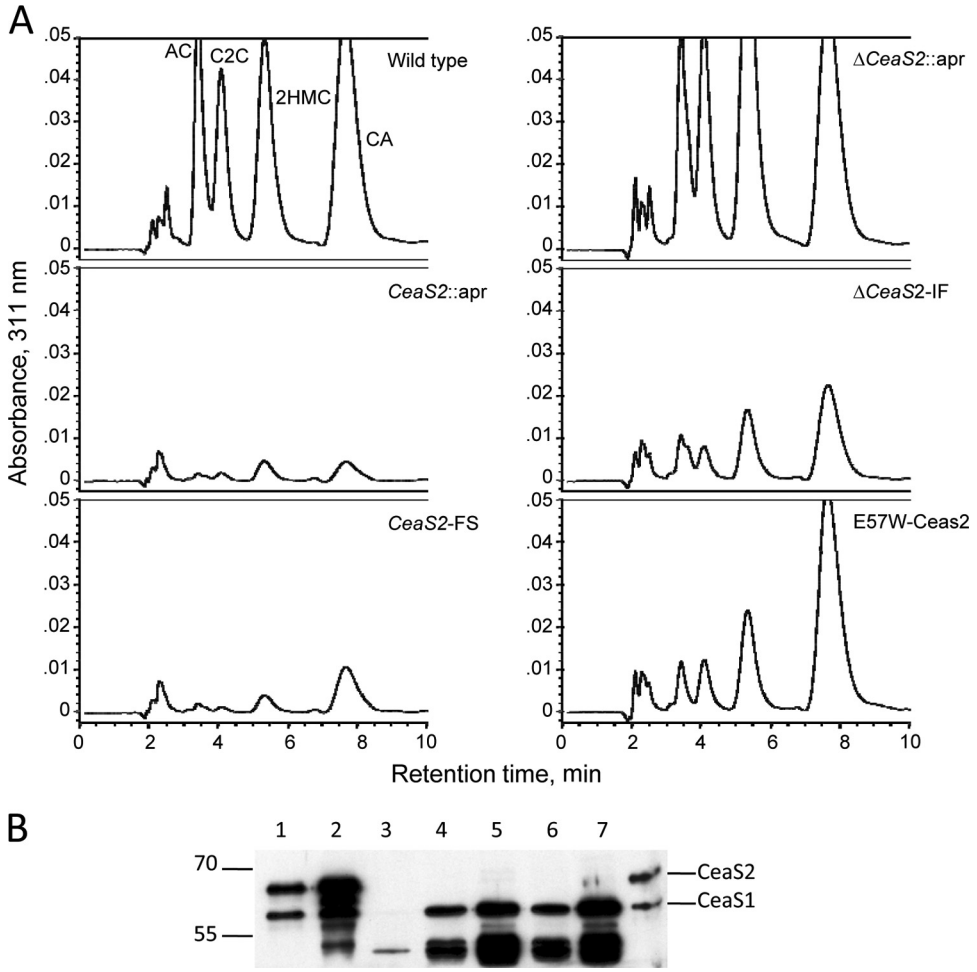

FIG 9 Analysis of clavam metabolite and CeaS1 and CeaS2 production in wild-type and mutant strains of *S. clavuligerus* grown on solid soy medium. (A) Freeze-and-squeeze exudates from 9-day-old solid soy medium-grown cultures were derivatized with imidazole to detect clavam metabolites and then analyzed by HPLC. AC, alanylclavam; C2C, clavam-2-carboxylate; 2HMC, 2-hydroxymethylclavam; CA, clavulanic acid. (B) Cell extract proteins (200-ng amounts) from wild-type and various *ceaS2* mutant cultures harvested after 9 days growth on solid soy medium were separated by electrophoresis on 8% SDS-PAGE gels and transferred to PVDF membranes for analysis. Lanes 1 and 8, CeaS1 and CeaS2 standards (25 ng of each protein); lane 2, wild-type *S. clavuligerus*; lane 3, *ceaS2*::*apr*; lane 4, *ceaS2*-FS; lane 3, Δ*ceaS2*::*apr*; lane 6, Δ*ceaS2*; lane 7, E57W*ceaS2*.

eukaryotes to activate transcription from their promoters (28, 29). While analysis of genome sequence information has not indicated the presence of such enhancer binding-type activator proteins in *Streptomyces* spp., perhaps active CeaS2 plays such a role, but how these effects would be transmitted to *ceaS1*, residing as it does on a separate replicon within the cell, remains unclear.

In previous studies, Kyung et al. showed that expression of genes encoding both enzymes and transcriptional regulators involved in antibiotic biosynthesis were spatially and temporally regulated in *S. clavuligerus* (30). Prokaryotes are typified by a lack of subcellular compartments, but if the enzymes of clavulanic acid and 5*S* clavam biosynthesis associate into multienzyme complexes as they are produced and reside in separate cellular locations, as has been described for bacillaene production in *Bacillus subtilis* (31), this may provide an opportunity for higher levels of regulation and organization more similar to those seen in eukaryotes.

Finally, the ability to obtain clavam metabolite production when *ceaS2* mutants are grown on solid medium demonstrated that Ceas1 protein is functional in its own right and can support clavam metabolite production. While the specific production of Ceas1 in *ceaS2* mutants was low compared to the production levels for CeaS1 and CeaS2 in the wild type on solid medium, *ceaS1* was nonetheless expressed and clavams were produced in the absence of any CeaS2 protein. This also implied that there must be two separate pathways to achieve *ceaS1* expression, a CeaS2-dependent pathway that is functional during growth in both liquid and solid medium and a Ceas2-independent pathway seen only during growth on solid medium. This Ceas2-independent pathway may respond to signals reflecting the physical environment, such as osmolarity, concentrations of volatile metabolites, or oxygen tension, in the same way that such signals may affect other processes, such as sporulation of *Streptomyces* spp. on solid versus liquid media. Previous studies have shown that an atypical two-component regulatory system specifically controls the production of the 5*S* clavam metabolites, and so growth conditions may influence *ceaS1* expression through interaction with this system (12).

Given the complexity of the process, a model that attempts to incorporate all of these possible explanations to account for the

cross-regulation of *ceaS1* expression by CeaS2 would be overly speculative at this stage and must await clarification through further studies. Since sequencing of the *S. clavuligerus* genome sequence has recently been completed (9, 32), microarray-type analyses comparing transcription patterns of a Δ*ceaS2*-IF mutant grown in liquid and solid medium may help to identify additional genes that are involved and provide greater insights into this complex regulatory system.

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We have no conflicts of interest to declare.

REFERENCES

1. **Demain AL.** 2000. Small bugs, big business: the economic power of the microbe. Biotechnol. Adv. **18**:499–514.
2. **Egan LA, Busby RW, IwataReuyl D, Townsend CA.** 1997. Probable role of clavaminic acid as the terminal intermediate in the common pathway to clavulanic acid and the antipodal clavam metabolites. J. Am. Chem. Soc. **119**:2348–2355.
3. **Jensen SE, Paradkar AS.** 1999. Biosynthesis and molecular genetics of clavulanic acid. Antonie Van Leeuwenhoek **75**:125–133.
4. **Marsh EN, Chang MD, Townsend CA.** 1992. Two isozymes of clavaminate synthase central to clavulanic acid formation: cloning and sequencing of both genes from *Streptomyces clavuligerus*. Biochemistry **31**:12648–12657.
5. **Salowe SP, Marsh EN, Townsend CA.** 1990. Purification and characterization of clavaminate synthase from *Streptomyces clavuligerus*: an unusual oxidative enzyme in natural product biosynthesis. Biochemistry **29**:6499–6508.
6. **Jensen SE, Elder KJ, Aidoo KA, Paradkar AS.** 2000. Enzymes catalyzing the early steps of clavulanic acid biosynthesis are encoded by two sets of paralogous genes in *Streptomyces clavuligerus*. Antimicrob. Agents Chemother. **44**:720–726.
7. **Perez-Redondo R, Rodriguez-Garcia A, Martin JF, Liras P.** 1999. Deletion of the *pyc* gene blocks clavulanic acid biosynthesis except in glycerol-containing medium: evidence for two different genes in formation of the C3 unit. J. Bacteriol. **181**:6922–6928.
8. **Jensen SE, Wong A, Griffin A, Barton B.** 2004. *Streptomyces clavuligerus* has a second copy of the proclavaminate amidinohydrolase gene. Antimicrob. Agents Chemother. **48**:514–520.
9. **Medema MH, Trefzer A, Kovalchuk A, van den Berg M, Muller U, Heijne W, Wu L, Alam MT, Ronning CM, Nierman WC, Bovenberg RA, Breitling R, Takano E.** 2010. The sequence of a 1.8-Mb bacterial linear plasmid reveals a rich evolutionary reservoir of secondary metabolic pathways. Genome Biol. Evol. **2**:212–224.
10. **Tahlan K, Park HU, Wong A, Beatty PH, Jensen SE.** 2004. Two sets of paralogous genes encode the enzymes involved in the early stages of clavulanic acid and clavam metabolite biosynthesis in *Streptomyces clavuligerus*. Antimicrob. Agents Chemother. **48**:930–939.
11. **Alexander DC, Jensen SE.** 1998. Investigation of the *Streptomyces clavuligerus* cephamycin C gene cluster and its regulation by the CcaR protein. J. Bacteriol. **180**:4068–4079.
12. **Kwong T, Zelyas NJ, Cai H, Tahlan K, Wong A, Jensen SE.** 2012. 5*S* clavam biosynthesis is controlled by an atypical two-component regulatory system in *Streptomyces clavuligerus*. Antimicrob. Agents Chemother. **56**:4845–4855.
13. **Paradkar AS, Aidoo KA, Jensen SE.** 1998. A pathway-specific transcriptional activator regulates late steps of clavulanic acid biosynthesis in *Streptomyces clavuligerus*. Mol. Microbiol. **27**:831–843.
14. **Perez-Llarena FJ, Liras P, Rodriguez-Garcia A, Martin JF.** 1997. A regulatory gene (*ccaR*) required for cephamycin and clavulanic acid production in *Streptomyces clavuligerus*: amplification results in overproduction of both β-lactam compounds. J. Bacteriol. **179**:2053–2059.
15. **Perez-Redondo R, Rodriguez-Garcia A, Martin JF, Liras P.** 1998. The *claR* gene of *Streptomyces clavuligerus*, encoding a LysR-type regulatory protein controlling clavulanic acid biosynthesis, is linked to the clavulanate-9-aldehyde reductase (*car*) gene. Gene **211**:311–321.
16. **Tahlan K, Anders C, Jensen SE.** 2004. The paralogous pairs of genes involved in clavulanic acid and clavam metabolite biosynthesis are differently regulated in *Streptomyces clavuligerus*. J. Bacteriol. **186**:6286–6297.
17. **Paradkar AS, Jensen SE.** 1995. Functional analysis of the gene encoding the clavaminate synthase 2 isoenzyme involved in clavulanic acid biosynthesis in *Streptomyces clavuligerus*. J. Bacteriol. **177**:1307–1314.
18. **Vieira J, Messing J.** 1987. Production of single stranded plasmid DNA. Methods Enzymol. **153**:3–11.
19. **Wehmeier UF.** 1995. New multifunctional *Escherichia coli-Streptomyces* shuttle vectors allowing blue-white screening on XGal plates. Gene **165**:149–150.
20. **Zelyas N, Tahlan K, Jensen SE.** 2009. Use of the native *flp* gene to generate in-frame unmarked mutations in *Streptomyces* spp. Gene **443**:48–54.
21. **Jensen SE, Paradkar AS, Mosher RH, Anders C, Beatty PH, Brumlik MJ, Griffin A, Barton B.** 2004. Five additional genes are involved in clavulanic acid biosynthesis in *Streptomyces clavuligerus*. Antimicrob. Agents Chemother. **48**:192–202.
22. **Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA.** 2000. Practical *Streptomyces* genetics. John Innes Foundation, Norwich, England.
23. **Sambrook J, Fritsch EF, Maniatis T.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
24. **Gust B, Challis GL, Fowler K, Kieser T, Chater KF.** 2003. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc. Natl. Acad. Sci. U. S. A. **100**:1541–1546.
25. **Harlow E, Lane D.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
26. **Paradkar AS, Aidoo KA, Wong A, Jensen SE.** 1996. Molecular analysis of a β-lactam resistance gene encoded within the cephamycin gene cluster of *Streptomyces clavuligerus*. J. Bacteriol. **178**:6266–6274.
27. **Caines MEC, Elkins JM, Hewitson KS, Schofield CJ.** 2004. Crystal structure and mechanistic implications of N_2-(2-carboxyethyl)arginine synthase, the first enzyme in the clavulanic acid biosynthesis pathway. J. Biol. Chem. **279**:5685–5692.
28. **Buck M, Gallegos MT, Studholme DJ, Guo Y, Gralla JD.** 2000. The bacterial enhancer-dependent σ^{54} (σ^{N}) transcription factor. J. Bacteriol. **182**:4129–4136.
29. **Jelsbak L, Givskov M, Kaiser D.** 2005. Enhancer-binding proteins with a forkhead-associated domain and the σ^{54} regulon in *Myxococcus xanthus* fruiting body development. Proc. Natl. Acad. Sci. U. S. A. **102**:3010–3015.
30. **Kyung YS, Hu W, Sherman DS.** 2001. Analysis of temporal and spatial expression of the CcaR regulatory element in the cephamycin C biosynthetic pathway using green fluorescent protein. Mol. Microbiol. **40**:530–541.
31. **Straight PD, Fischbach MA, Walsh CT, Rudner DZ, Kolter R.** 2007. A singular enzymatic megacomplex from *Bacillus subtilis*. Proc. Natl. Acad. Sci. U. S. A. **104**:305–310.
32. **Song JY, Jeong H, Yu DS, Fischbach MA, Park HS, Kim JJ, Seo JS, Jensen SE, Oh TK, Lee KJ, Kim JF.** 2010. Draft genome sequence of *Streptomyces clavuligerus* NRRL 3585, a producer of diverse secondary metabolites. J. Bacteriol. **192**:6317–6318.