

Alanylclavam Biosynthetic Genes Are Clustered Together with One Group of Clavulanic Acid Biosynthetic Genes in *Streptomyces clavuligerus*^{∇§}

Nathan J. Zelyas, Hui Cai, Thomas Kwong, and Susan E. Jensen*

Department of Biological Sciences, University of Alberta, Edmonton, Alberta T6G 2E9, Canada

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***Streptomyces clavuligerus* produces at least five different clavam metabolites, including clavulanic acid and the methionine antimetabolite, alanylclavam. In vitro transposon mutagenesis was used to analyze a 13-kb region upstream of the known paralogue gene cluster. The paralogue cluster includes one group of clavulanic acid biosynthetic genes in *S. clavuligerus*. Twelve open reading frames (ORFs) were found in this area, and mutants were generated in each using either in vitro transposon or PCR-targeted mutagenesis. Mutants with defects in any of the genes *orfA*, *orfB*, *orfC*, or *orfD* were unable to produce alanylclavam but could produce all of the other clavams, including clavulanic acid. *orfA* encodes a predicted hydroxymethyltransferase, *orfB* encodes a YjgF/YER057c/UK114-family regulatory protein, *orfC* encodes an aminotransferase, and *orfD* encodes a dehydratase. All of these types of proteins are normally involved in amino acid metabolism. Mutants in *orfC* or *orfD* also accumulated a novel clavam metabolite instead of alanylclavam, and a complemented *orfC* mutant was able to produce trace amounts of alanylclavam while still producing the novel clavam. Mass spectrometric analyses, together with consideration of the enzymes involved in its production, led to tentative identification of the novel clavam as 8-OH-alanylclavam, an intermediate in the proposed alanylclavam biosynthetic pathway.**

The biochemistry and genetics behind the production of β -lactam antibiotics by microbes have long been an area of active research due to the clinical importance of these compounds. *Streptomyces clavuligerus* is a bacterium capable of producing a number of β -lactam metabolites, including, most notably, the β -lactamase inhibitor clavulanic acid (9). Although clavulanic acid has only weak antimicrobial activity, it inhibits serine β -lactamase enzymes, which are responsible for much of the penicillin resistance that is seen in clinical settings. When used in combination with conventional β -lactam antibiotics, clavulanic acid provides a powerful tool to combat infections by antibiotic-resistant pathogens. Understanding the complete biosynthetic pathway of clavulanic acid could lead to the design of new strains of *S. clavuligerus* able to produce higher levels or more potent variants of clavulanic acid.

In addition to clavulanic acid, *S. clavuligerus* also produces the following other β -lactam metabolites that are structurally similar to clavulanic acid: clavam-2-carboxylate, 2-formyloxymethylclavam, 2-hydroxymethylclavam, and alanylclavam (10, 42) (Fig. 1). All of these β -lactam compounds, including clavulanic acid, are called clavams by virtue of containing a β -lactam ring fused to a five-membered oxazolidine ring. While this bicyclic nucleus in clavulanic acid has 5*R* stereochemistry, the other clavams mentioned above have a 5*S* configuration and are thus called the 5*S* clavams. The β -lactamase inhibitory

activity displayed by clavulanic acid is associated with its 5*R* stereochemistry (3), and therefore the 5*S* clavams exhibit antifungal or antibacterial activities but do not have β -lactamase inhibitory properties (10, 42).

Clavulanic acid and the 5*S* clavams share the same early steps in their biosynthesis. However, once clavaminic acid is synthesized, the pathway splits into two branches, one specific for clavulanic acid production and the other for 5*S* clavam production (13) (Fig. 1). Two reactions from the late stages of the pathway leading to clavulanic acid have been characterized (2, 21, 35), while only one intermediate leading specifically to the 5*S* clavams, 2-carboxymethylideneclavam, has been identified (51).

Three unlinked gene clusters containing genes involved in clavam biosynthesis have been identified in *S. clavuligerus* (52). These are the clavulanic acid, the clavam, and the paralogue gene clusters. The clavulanic acid gene cluster contains genes involved in the early steps required for the biosynthesis of clavaminic acid, as well as genes specific to the late steps of clavulanic acid production (17, 21, 23, 29, 31, 40). The clavam gene cluster carries primarily genes required for the late steps of 5*S* clavam biosynthesis but also includes *cas1* encoding clavaminic synthase, an early biosynthetic enzyme. *cas1* is a paralogue of the *cas2* gene from the clavulanic acid gene cluster (34, 53). Except for *cas1*, analogues for all of the other early biosynthetic genes of the clavulanic acid gene cluster are found in the paralogue gene cluster (22, 53). The paralogue gene cluster also contains genes involved specifically in the late steps of 5*S* clavam biosynthesis (22, 51, 53).

Five genes required specifically for the late steps of 5*S* clavam biosynthesis have been described. In the clavam gene cluster, *cvm1*, *cvm2*, and *cvm5* are necessary for the production

* Corresponding author. Mailing address: Department of Biological Sciences, University of Alberta, Edmonton, AB T6G 2E9, Canada. Phone: (780) 492-0830. Fax: (780) 492-9234. E-mail: susan.jensen@ualberta.ca.

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Strains of *S. clavuligerus* were grown on maltose-yeast extract-malt extract agar (50) or ISP4 (Difco) agar plates at 28°C. Liquid cultures used for determining antibiotic production levels were grown as previously described (21). Briefly, seed cultures were prepared by inoculating 10⁸ spores from glycerol stocks into 25-ml amounts of Trypticase soy broth supplemented with 1% (wt/vol) soluble starch. These cultures were incubated for 40 h and then inoculated at 2% (vol/vol) into soy flour medium (21). Culture supernatants were collected after 72 and 96 h for assessment of metabolite levels. All *S. clavuligerus* liquid cultures were grown at 28°C and shaken at 250 rpm. Mutant strains were grown at least three times to determine metabolite-producing capabilities. Antibiotic-resistant strains of *S. clavuligerus* were grown with Apr (25 µg/ml) or thiostrepton (2.5 to 10 µg/ml).

DNA manipulations. Plasmid and cosmid DNA isolation from *E. coli*, restriction endonuclease digestions, PCRs, Southern analyses, and transformations of *E. coli* were carried out using standard techniques (45).

Isolation of genomic DNA from *S. clavuligerus* was performed using a standard technique (25). Plasmid and cosmid DNA was introduced into *S. clavuligerus* through intergeneric conjugation from *E. coli* ET12567(pUZ8002) as previously described (25). Exconjugants were isolated on AS-1 plus 10 mM MgCl₂ medium (4) agar using nalidixic acid (25 µg/ml) to counterselect the donor *E. coli* strain, and an appropriate antibiotic was used to select for exconjugants.

Both Southern analysis and mycelial PCR were used to confirm mutations in *S. clavuligerus*. PCRs were typically carried out using mycelia from 40-h seed cultures that had been washed twice with 10.3% sucrose, suspended to their original volume in dimethyl sulfoxide, and then added directly to PCR mixtures to give a final dimethyl sulfoxide concentration of 5% (vol/vol). Oligonucleotide primers used in this study are listed in Table S2 in the supplemental material.

In vitro transposon mutagenesis. In vitro transposition reactions were carried out according to the manufacturer's specifications (Epicentre, Madison, WI). Briefly, Tn5062 (carrying Apr^r, *egfp*, and *oriT*) was liberated from pQM5062 (8) by restriction digestion and combined with cosmid 6G9, transposase, and reaction buffer. The reaction was incubated at 37°C for 2 h and then terminated by the addition of a stop solution and heating at 70°C for 10 min. Reaction mixtures were introduced into *E. coli* Transformax EC100, and transformants were selected on Apr-containing agar.

DNA sequence analysis. Cosmid 6G9 carries the paralogue gene cluster and its flanking regions. Within 6G9, an 11-kb EcoRI fragment comprises much of the region upstream of the paralogue cluster, and this was targeted for sequencing. Mutant forms of 6G9 carrying individual transposon insertions in the 11-kb EcoRI fragment were identified via restriction analysis. Thirty such mutant cosmids were used as templates, together with primers that bind to the upstream and downstream edges of Tn5062, to sequence outward from each insertion. This provided overlapping sequence information for most of the region upstream of the paralogue gene cluster. When required, sequence-specific primers were used to fill in gaps and to sequence the ends of the 11-kb EcoRI fragment.

A portion of the region immediately upstream of *ceaS1* was not contained on the 11-kb EcoRI fragment. Plasmid p2.8-18 carries a 2.8-kb EcoRI fragment comprising the 5' end of *ceaS1* and upstream DNA, and it abuts the 11-kb EcoRI fragment. It was used as a sequencing template to bridge the gap between the paralogue gene cluster and the 11-kb EcoRI fragment.

Sequence analysis was performed using the DYEnamic ET Terminator cycle sequencing system (Roche, Basel, Switzerland) by the Molecular Biology Service Unit, University of Alberta. The double-stranded sequence was obtained for all novel genomic DNA sequences. Open reading frames (ORFs) were predicted using the online program FramePlot 3.0beta (<http://watson.nih.gov/~jun/cgi-bin/frameplot-3.0b.pl>) (19), and similarity searches were performed using the BLAST programs made available by the National Institute for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (1). GeneTools version 2.0 (BioTools, Inc., Edmonton, Alberta, Canada) was used to produce DNA sequence assemblies, perform sequence alignments, and identify restriction endonuclease sites. Protein domains were located using the InterProScan Sequence Search (57) provided by the European Molecular Biology Laboratory-European Bioinformatics Institute (<http://www.ebi.ac.uk/InterProScan/>).

Generation of mutants. Mutant versions of cosmid 6G9 carrying transposon insertions in all of the ORFs in the upstream region of interest, except for *orfB*, were obtained by in vitro transposon mutagenesis. The mutant cosmids were conjugated into *S. clavuligerus*, and Apr^r kanamycin-sensitive exconjugants (indicating a double crossover had taken place) were isolated to give mutant strains of *S. clavuligerus* with insertions in each of the genes *orfA*, *orfC*, *orfD*, *orfI*, *orfJ*, *orfK*, and *orfL*. However, mutant strains with transposon insertions in *orfE*, *orfF*, *orfG*, and *orfH* were never successfully generated by this process, despite repeated efforts, and so mutants in these ORFs and in *orfB* were instead generated by employing a PCR-targeting protocol (16). A second *orfC* mutant (in addition

to the one generated by transposon mutagenesis) was also generated by PCR targeting. For mutants generated by PCR targeting, cosmid 14E10 rather than 6G9 was used as the template, because the genes targeted for mutagenesis are more centrally located in the insert of this cosmid than they are in cosmid 6G9. All mutants were confirmed using Southern analysis or mycelial PCR.

Construction of pSET-AT-*orfC*. The *tsr* gene from pDA504 was inserted as a 1.1-kb BglIII/BamHI fragment into the BamHI site of pSET152 to give pSET-AT. A 1.9-kb AgeI/EcoRI fragment containing *orfC* and its upstream region was isolated from cosmid 6G9 and inserted into pSET-AT after passage through pSL1180 to pick up compatible restriction sites. This construct, pSET-AT-*orfC*, was then conjugated into an *S. clavuligerus* *orfC*::Tn5062 mutant.

Bioassays. Clavulanic acid production was detected using a disc-diffusion bioassay with *Klebsiella pneumoniae* ATCC 29665 as the indicator organism (34). Alanylclavam and 2-hydroxymethylclavam were detected using a disc-diffusion bioassay with *Bacillus* sp. ATCC 27860 as the indicator organism (42).

HPLC and LC-MS analyses. Culture filtrates were analyzed for clavulanic acid and 5S clavams by high-performance liquid chromatography (HPLC) after imidazole derivatization using a Bondclone C₁₈ column (100 by 8.0 mm, 10 µm; Phenomenex, Torrance, CA) under previously described conditions (42). Culture filtrates containing novel metabolites were further analyzed using LC-mass spectrometry (MS) as previously described (21).

Analysis of the novel clavam. Five-milliliter amounts of culture supernatants from both wild-type and *orfD* mutants of *S. clavuligerus* grown in soy flour medium for 96 h were acidified by the addition of 20 µl of 5 N HCl and then centrifuged for 5 min at 13,000 × *g* to remove precipitated matter. Supernatants were passed through SepPak Classic C₁₈ cartridges (Waters Scientific, Milford, MA), and the eluant (unretained fraction) containing the clavam metabolites was immediately neutralized with NaOH. Samples (0.5 ml) of each neutralized eluant were reduced to dryness in a SpeedVac, dissolved in 100 µl of water, and derivatized by the addition of 25 µl of imidazole reagent (7). After 15 min at 21°C, 5-µl samples of each were analyzed by HPLC on an XTerra column (0.21 by 10 cm; Waters Scientific) operating under isocratic conditions in 0.1% acetic acid adjusted to pH 3.7 with NH₄OH (96%), methanol (4%) at 0.2 ml/min with detection at 311 nm and on-line analysis of effluents by electrospray MS using a ZMD-2 single quadrupole spectrometer (Waters Scientific).

Nucleotide sequence accession number. The nucleotide sequence of this region of the *S. clavuligerus* chromosome has been appended to the existing file in GenBank for the paralogue gene cluster under accession number AY426768.

RESULTS

In vitro transposon mutagenesis of *S. clavuligerus*. The paralogue gene cluster of *S. clavuligerus* includes genes involved in the early stages of biosynthesis of all clavam metabolites as well as genes specific for the production of 5S clavam metabolites. In order to analyze the region of the *S. clavuligerus* chromosome located upstream of the paralogue cluster, in vitro transposon mutagenesis of cosmid 6G9 was undertaken. The resulting mutant cosmids were introduced into *E. coli*, and thousands of clones carrying Tn5062 in 6G9 were obtained. Among these, 156 mutant clones were subjected to restriction analysis to determine the Tn5062 insertion point.

6G9 can be digested into the following six EcoRI fragments: an 8.2-kb fragment (pWE15) and 20-kb, 11-kb, 5.7-kb, 2.8-kb, and ~100-bp fragments derived from the genomic DNA insert. The relative positions of the 5.7- and 2.8-kb fragments were already known from prior sequencing, and Southern analysis revealed that the 11-kb fragment (Fig. 2) carries the region of interest lying upstream of the paralogue gene cluster (data not shown). Thirty of the 156 mutant 6G9 cosmids carried Tn5062 in the 11-kb upstream fragment, as determined by restriction analysis. The number of Tn5062 insertions in the different EcoRI fragments is indicated in Table 1. Only 134 cosmids are represented in the table because 22 of the mutant cosmids were not readily interpretable by analysis of EcoRI restriction digests. For the 134 mutant cosmids, frequencies of insertion correlate well with the size of each fragment.

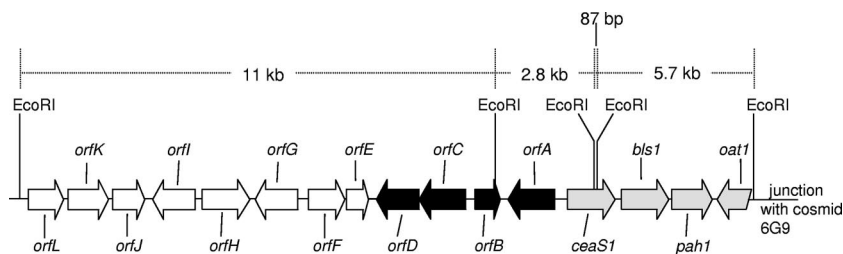


FIG. 2. The paralogue gene cluster. Gray arrows represent ORFs of the previously described paralogue gene cluster. Black arrows represent newly discovered ORFs specifically required for alanylclavam biosynthesis. White arrows indicate newly discovered ORFs not required for alanylclavam biosynthesis.

Sequencing and analysis of the region upstream of the paralogue gene cluster. The 30 6G9 cosmids carrying Tn5062 in the 11-kb EcoRI fragment were used as templates to sequence bidirectionally outward from each transposon insertion point. Gaps in the resulting assembled sequence were filled by sequencing from custom primers. The end of the 2.8-kb EcoRI segment that abuts the 11-kb EcoRI fragment was also sequenced to bridge the gap between the known paralogue gene cluster and the newly sequenced region. Taken together, this provided 13,003 bp of new sequence upstream of the paralogue gene cluster.

Examination of the sequence revealed 12 new ORFs (Fig. 2; Table 2). Of these, four encode products with similarities to proteins involved in amino acid metabolism. *orfA* encodes a putative serine hydroxymethyltransferase, an enzyme typically involved in the reversible conversion of serine to glycine (46). *orfA* is also notable in that it contains a TTA codon as the 126th codon of the 391-codon ORF. The product encoded by *orfC* strongly resembles aminotransferases, which catalyze the transfer of amino groups from amino acids to oxoacids, such that the ketone oxygen is replaced by an amino group (30). *orfD* encodes a protein similar to threonine dehydratases. Threonine dehydratases (also called threonine deaminases) catalyze the dehydration and deamination of threonine or serine to yield 2-ketobutyrate or pyruvate, respectively (15). Finally, the protein encoded by *orfJ* shows similarity to dihydrofolate reductases, which carry out the reduction of dihydrofolate to tetrahydrofolate (48). This reaction is an essential step in the production of glycine, purines, and deoxythymidine phosphate.

Two of the new ORFs are similar to amino acid transport proteins. Proteins encoded by *orfE* and *orfF* have similarity to

AzlD and AzlC, respectively. In *Bacillus subtilis*, AzlC and AzlD cause 4-azaleucine resistance when overproduced and are believed to be involved in branched-chain amino acid transport (5).

orfB, *orfI*, and *orfL* code for proteins which show similarities to regulatory proteins. OrfB, based on its amino acid sequence, belongs to the YjgF/YER057c/UK114 family of proteins. In eukaryotes, proteins in this family have been implicated in RNase activity, mitochondrial genome maintenance, and isoleucine biosynthesis (33, 26, 37). In prokaryotes, this family of proteins has been found to be involved in the repression of purine and isoleucine biosynthesis (14, 43). OrfI appears to be a LysR-type transcriptional regulator. LysR-type transcriptional regulators are found in many prokaryotic systems. In fact, ClaR, a protein that positively regulates clavulanic acid biosynthesis in *S. clavuligerus*, is itself a LysR-type protein (38, 41). OrfL shows similarity to the ArsR family of transcriptional repressors, proteins that regulate expression of arsenical resistance operons (12).

The three other ORFs in the region upstream of the paralogue gene cluster do not have any commonalities with the other ORFs or with each other. The protein encoded by *orfG* shows very limited similarity to protein kinases, the *orfH* protein product is similar to multidrug resistance transporters, and *orfK* codes for a protein with limited similarity to activators of Hsp90 ATPase.

Generation and analysis of mutants. To determine whether any of the newly discovered ORFs play a role in the biosynthesis of 5S clavams or other metabolites produced by *S. clavuligerus*, mutants with defects in each of the ORFs were obtained. Mutants in *orfA*, *orfC*, *orfD*, *orfI*, *orfJ*, *orfK*, and *orfL* were generated by conjugating cosmid 6G9 derivatives carrying Tn5062 in the ORF of interest into *S. clavuligerus*. Recombination between the mutant cosmids and the chromosome allowed gene conversion to occur, thus transferring the mutations to the chromosomal copy of the gene. However, even after repeated attempts, no *orfE*, *orfF*, *orfG*, or *orfH* mutations could be obtained via this route. Although this initially suggested that these ORFs may be essential for cellular functions, PCR-targeting mutagenesis was subsequently performed successfully to generate mutants (16). Since there were no mutant cosmids carrying a transposon insertion in *orfB*, the mutation of *orfB* was also carried out via PCR targeting. Thus, transposon-bearing mutants were generated in *orfA*, *orfC*, *orfD*, *orfI*, *orfJ*, *orfK*, and *orfL*, and mutants carrying an antibiotic resistance gene cassette replacing the ORF of interest were pre-

TABLE 1. Frequencies of transposon insertion into the different EcoRI fragments of cosmid 6G9

EcoRI fragments of cosmid 6G9 ^b	No. of cosmids with Tn5 insertion in a specific EcoRI fragment ^a	Frequency of Tn5 insertion (%)	Fragment size as a proportion of total cosmid size (%)
~20	68	50.7	41.9
11	30	22.4	23.1
8.2	16	12	17.2
5.7	14	10.4	11.9
2.8	6	4.5	5.9
87	0	0	0

^a Results are based on the 134 transposon-containing cosmids examined.

^b All values are kilobases except the last entry, which is shown as base pairs.

TABLE 2. Newly sequenced ORFs from the region upstream of the paralogue cluster of *S. clavuligerus*

ORF	Size (aa) ^a	Most similar protein, accession no., similarity/identity (%) ^b	Proposed function
<i>orfA</i>	390	Serine hydroxymethyltransferase from <i>Methanocaldococcus jannaschii</i> , AAB99615, 32/47 over 380 aa	Hydroxymethyltransferase
<i>orfB</i>	126	YjgF/YER057c/UK114 family protein from <i>Saccharopolyspora erythraea</i> , YP_001107461, 55/70 over 126 aa	Amino acid biosynthesis regulator
<i>orfC</i>	393	Aminotransferase from " <i>Solibacter usitatus</i> ," YP_824873, 37/53 over 344 aa	Aspartic/tyrosine/aromatic aminotransferase
<i>orfD</i>	351	Threonine dehydratase from an <i>Acidobacteria</i> bacterium, YP_591159, 39/56 over 314 aa	Threonine dehydratase
<i>orfE</i>	102	Hypothetical protein from <i>S. erythraea</i> , YP_001104250, 54/69 over 102 aa	AzID-type amino acid permease
<i>orfF</i>	253	Branched-chain amino acid permease from <i>Rhodococcus</i> sp., 53/66 over 204 aa	AzID-type amino acid permease
<i>orfG</i>	364	Hypothetical protein from <i>Streptomyces avermitilis</i> , NP_821993, 51/65 over 353 aa	Serine/threonine-type protein kinase
<i>orfH</i>	416	Transporter from <i>Streptomyces ambofaciens</i> , CAK50890, 45/60 over 397 aa	AraJ-type arabinose efflux permease
<i>orfI</i>	356	LysR-type transcriptional regulator from <i>S. erythraea</i> , YP_001107820, 48/62 over 294 aa	Transcriptional regulator
<i>orfJ</i>	201	Dihydrofolate reductase-type protein from <i>Frankia alni</i> , YP_714368, 84/91 over 189 aa	Bifunctional deaminase-reductase
<i>orfK</i>	277	Hypothetical protein from <i>Frankia</i> sp., YP-481895, 36/49 over 240 aa	Uncharacterized conserved protein
<i>orfL</i>	229	ArsR-type transcriptional regulator from <i>Rhodococcus</i> sp., YP_707049, 66/82 over 195 aa	Transcriptional regulator

^a aa, amino acids.

^b Percent similarities/identities were determined using the online BLASTp program.

pared in *orfB*, *orfE*, *orfF*, *orfG*, and *orfH*. All mutations were confirmed through either Southern analyses or mycelial PCR.

None of the mutants showed any morphological effects or dramatic changes in either cephamycin C or clavulanic acid production (data not shown). Most of the mutants also showed no changes in production of 5S clavams. However, mutants with defects in *orfA*, *orfB*, *orfC*, and *orfD* were specifically defective in the production of alanylclavam (Fig. 3). Interestingly, analysis of culture filtrates of *orfC* and *orfD* mutants

showed the presence of an additional clavam-type peak eluting just before the alanylclavam retention time on HPLC chromatograms, indicating that these mutants were producing a novel metabolite (Fig. 3).

Mass determination of the novel clavam. Underivatized clavam metabolites have poor retention on reverse-phase HPLC columns, and so to investigate the structure of this new metabolite, imidazole-derivatized clavams from wild-type and *orfD* culture supernatants were subjected to HPLC with on-

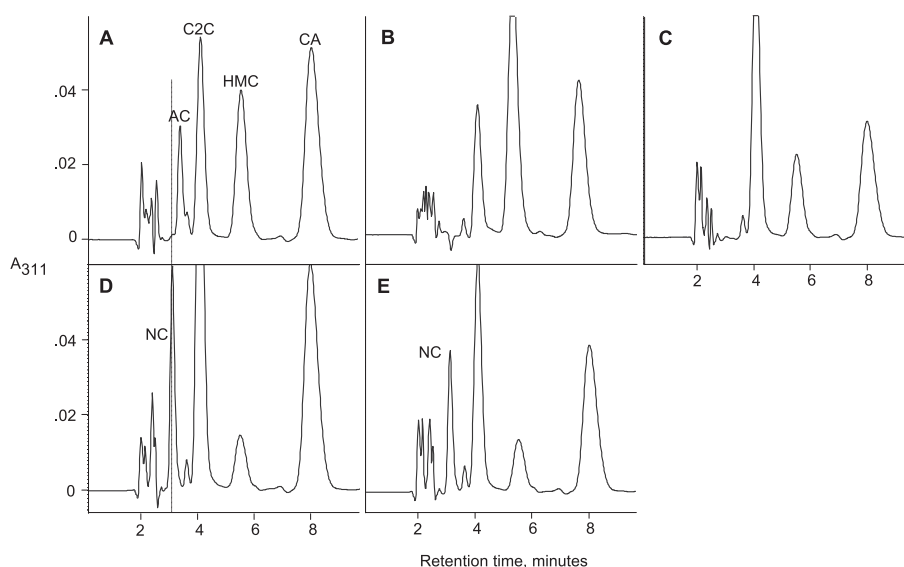


FIG. 3. HPLC analysis of clavam metabolites produced by wild-type and mutant strains of *S. clavuligerus*. Wild type (A), *orfA* mutant (B), *orfB* mutant (C), *orfC* mutant (D), and *orfD* mutant (E) are shown. AC, alanylclavam; C2C, clavam-2-carboxylate; HMC, 2-hydroxymethylclavam; CA, clavulanic acid; NC, novel clavam. The faint dotted line shows the expected location of the novel clavam in the HPLC profile from the wild-type strain.

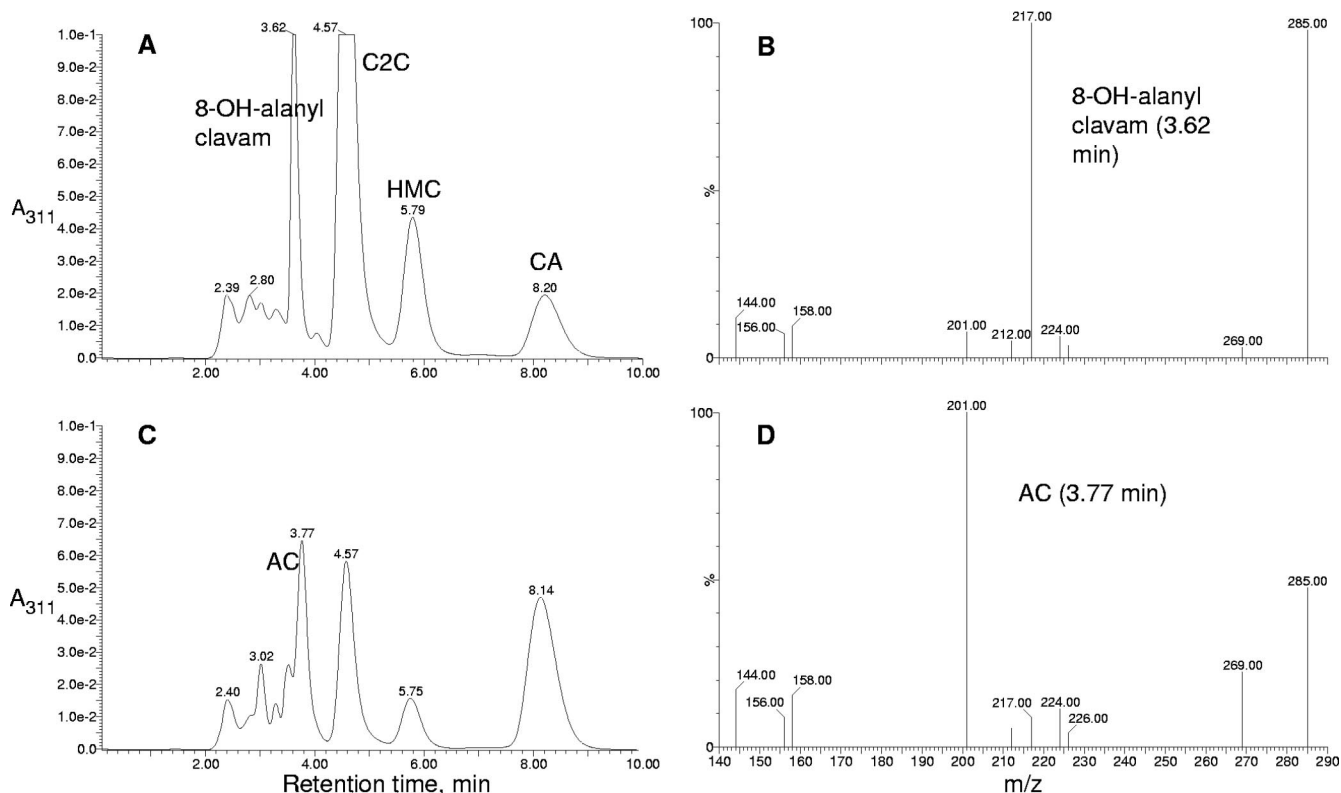


FIG. 4. MS analysis of the novel clavam accumulated by *orfC* and *orfD* mutants of *S. clavuligerus*. HPLC profile of *orfD* mutant of *S. clavuligerus* (A), mass spectrum of the novel clavam peak (retention time, 3.62 min) seen in panel A (B), HPLC profile of wild-type *S. clavuligerus* (C), and mass spectrum of the alanylclavam peak (retention time, 3.77 min) seen in panel C (D) are shown. Abbreviations for metabolite peaks are given in legend to Fig. 3.

line electrospray MS analysis (Fig. 4). Analysis of wild-type culture supernatants showed an alanylclavam peak that gave the expected prominent mass ions of 269 (intact imidazole derivative) and 201 (fragmented derivative), whereas the novel clavam from *orfD* mutant culture supernatants gave a peak with mass ions of 285 (intact imidazole derivative) and 217 (fragmented derivative). These masses are consistent with the novel clavam having a structure of 8-OH-alanylclavam. The HPLC profile of the wild-type culture supernatant shows a shoulder on the leading edge of the alanylclavam peak corresponding in retention time to that of 8-OH-alanylclavam. That shoulder peak also shows low levels of mass ions at 285 and 217, indicating that 8-OH-alanylclavam may normally be present in wild-type culture supernatants but at low levels compared to those of alanylclavam. In contrast, the 8-OH-alanylclavam peak from the *orfD* mutant culture supernatant showed no appreciable levels of alanylclavam (Fig. 4A).

Complementation of the *orfC* mutant. Mutants with defects in either *orfC* or *orfD* accumulate the same novel clavam, suggesting either that the OrfC and OrfD proteins are both needed for a single reaction in alanylclavam synthesis or that the genes are transcribed together in an operon and that transposon insertion in the upstream *orfC* has polar effects on the expression of *orfD*. This latter possibility seemed most likely, since the *orfC* stop codon overlaps the *orfD* start codon, and this in turn suggested that the loss of OrfD alone could be responsible for accumulation of the novel metabolite.

To test whether these two genes are transcribed in an operon, the *orfC* mutant was complemented. *orfC* was inserted into pSET-AT, which confers thiostrepton resistance and integrates into the chromosome at the Φ C31 phage attachment site (6). *orfC* mutant strains carrying pSET-AT-*orfC* were fermented, and culture filtrates were analyzed (Fig. 5). Chromatograms revealed that the complemented mutants still produced primarily the novel clavam (retention time, 3.0 min), but a small peak (retention time, 3.3 min) consistent with the presence of alanylclavam was also seen (Fig. 5C). This shoulder peak is not found in the uncomplemented *orfC* mutant (Fig. 5B).

Complementation of the *orfC* mutant with pSET-AT-*orfC* was able to restore only trace levels of alanylclavam production, supporting the hypothesis that the *orfC* mutation was polar on *orfD*. To obtain additional evidence, we attempted to produce an *orfC* in-frame deletion mutant, which would not be polar on *orfD*. As a first step, a Δ *orfC*::*apra* mutant was created by PCR-targeted mutagenesis. HPLC analysis of culture filtrates from this mutant showed the same chromatographic profile as was seen in the *orfC* transposon mutant, with accumulation of the novel clavam (data not shown). However, when the Δ *orfC*::*apra* mutant was subjected to *flp*-mediated recombination to remove the Apr^r cassette and replace it with an 81-bp in-frame scar (16), no in-frame mutants were recovered, despite two independent attempts.

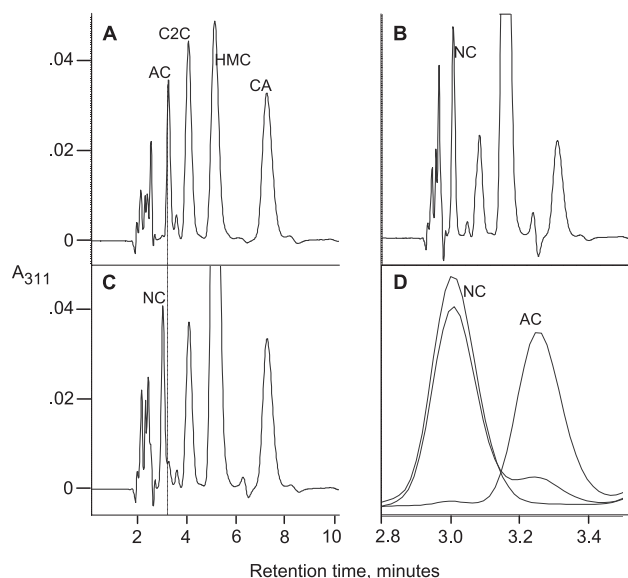


FIG. 5. HPLC analysis of clavam metabolites produced by the complemented *orfC* mutant of *S. clavuligerus*. Wild type (A), *orfC* mutant (B), complemented *orfC* mutant (C), and overlay and expansion of panels A to C in the region of retention times from 2.8 to 3.5 min (D) are shown. The faint dotted line indicates the presence of low levels of alanylclavam in the complemented *orfC* mutant. Abbreviations for metabolite peaks are given in legend to Fig. 3.

DISCUSSION

The known sequence of the paralogue gene cluster has been increased to 23,328 bp through the use of in vitro transposon mutagenesis, the first time this technology has been applied to *S. clavuligerus*. Within the 13 kb of new DNA sequence, 12 new ORFs were located. Using a combination of in vitro transposon mutagenesis and PCR-targeted mutagenesis, mutations were generated in each of the newfound ORFs.

For unknown reasons, Tn5062 could not be introduced into all of the genes on the chromosome. Mutant cosmids with Tn5062 inserted into *orfE*, *orfF*, *orfG*, and *orfH* were obtained, but transfer of these mutations into the *S. clavuligerus* chromosome was unsuccessful, despite repeated attempts. This suggested that the ORFs might be essential for *S. clavuligerus* survival, but PCR-targeted mutagenesis subsequently proved that not to be the case. The Tn5062 system is currently being used to mutate the entire *Streptomyces coelicolor* genome, and no indication of difficulties has been reported in that species (8, 18). Although the exact cause is not known, in vitro transposon mutagenesis using Tn5062 may not be ideal for saturation mutagenesis of *S. clavuligerus*, but it is still useful for DNA sequencing.

Of the 12 newly described ORFs, four were specifically required for alanylclavam biosynthesis: *orfA*, *orfB*, *orfC*, and *orfD*. Each of these ORFs encodes a protein with similarities to those involved in amino acid regulation or biosynthesis. OrfA shows similarity to serine hydroxymethyltransferases, enzymes that catalyze the reversible interconversion of glycine and 5,10-methylenetetrahydrofolate to serine and tetrahydrofolate. While OrfA contains three of the four amino acids involved in glycine binding, it lacks five of the six amino acids involved in

5,10-methylenetetrahydrofolate binding (46). This suggests that OrfA may catalyze a reaction between glycine and a substrate other than 5,10-methylenetetrahydrofolate.

OrfB belongs to the YjgF/YER057c/UK114 family of proteins (14, 26, 33, 37, 43). While these proteins have been implicated in many cellular functions in both eukaryotes and prokaryotes, involvement in regulation of isoleucine biosynthesis has been observed in a number of organisms. YjgF from *Salmonella enterica* serovar Typhimurium is necessary for isoleucine biosynthesis in that it is required for normal IlvB (an aminotransferase directly involved in isoleucine biosynthesis) activity (14, 47). As well, members of the YjgF/YER057c/UK114 family in *Haemophilus influenzae* and *E. coli* are capable of binding to α -keto acids, such as 2-ketobutyrate, the first intermediate of isoleucine biosynthesis (11, 39). This led to the formulation of a model in which YjgF binds to an α -keto acid, possibly an intermediate in the isoleucine biosynthetic pathway such as 2-ketobutyrate, that would, in its unbound form, repress IlvB activity (47). By binding to the α -keto acid, YjgF sequesters the metabolite and prevents it from inhibiting the aminotransferase, thus allowing isoleucine production to continue (47).

Examination of the OrfB amino acid sequence reveals that it contains all of the eight residues implicated in ligand binding (Y18, G32, Q33, F86, N90, P102, R104, and E118) as determined from the crystal structures of *E. coli* and human YjgF/YER057c/UK114 family proteins (32, 55). Although C107 in TdcF (an *E. coli* family member) has also been identified as an important residue for 2-ketobutyrate binding, it is not as conserved as the other eight active site residues and is not found in either the human YjgF/YER057c/UK114 family protein nor OrfB (11, 55). Therefore, OrfB is likely to be capable of binding to α -keto acids.

Analysis of the OrfC amino acid sequence shows that OrfC belongs to family I of the aminotransferases (20, 30). Family I contains aspartate, alanine, and aromatic amino acid aminotransferases (30), but OrfC contains only two of the six amino acids involved in substrate binding found in similar family I aminotransferases (24, 36, 44), suggesting that OrfC may catalyze aminotransferase reactions but use atypical substrates.

OrfD is similar to threonine and serine dehydratases. It contains all of the residues likely involved in substrate binding found in the *E. coli* biosynthetic threonine dehydratase (15), the *S. enterica* serovar Typhimurium threonine deaminase TdcB (49), and the rat liver serine dehydratase (56). For this reason, it is probable that OrfD catalyzes a reaction similar to that carried out by threonine and serine dehydratases, involving a substrate structurally analogous to threonine or serine.

From the apparent activities of OrfA, OrfB, OrfC, and OrfD, it is possible to envision a putative alanylclavam biosynthetic pathway. OrfA may use glycine as a substrate and fuse it to an as-yet-unobserved aldehyde-containing clavam intermediate in a reaction analogous to that catalyzed by serine hydroxymethyltransferase (Fig. 1). This would produce 8-OH-alanylclavam, which has the same mass as the novel clavam accumulated by *orfC* and *orfD* mutants. OrfD, by analogy to threonine deaminase, would then carry out the dehydration/deamination of 8-OH-alanylclavam to produce a clavam intermediate with a pyruvyl side chain attached at C2 (Fig. 1). Alanylclavam would finally be generated by transamination of

the keto group on the pyruvyl side chain through the action of OrfC (Fig. 1). In this putative pathway, the role of OrfB is regulatory in nature. If the pyruvylclavam intermediate (an α -keto acid) inhibits OrfC activity, analogous to the way α -keto acids inhibit aminotransferase activity in isoleucine biosynthesis, OrfB may function to sequester this intermediate, thereby allowing OrfC to remain active (Fig. 1). Although more detailed analyses will be required to confirm the identity of 8-OH-alanylclavam, the proposed structure is in agreement with the observed mass of the compound and is consistent with the proposed biosynthetic pathway.

The proposed pathway can also help to explain the observed phenotypes of the *orfC* and *orfD* mutants and of the complemented *orfC* mutant. In the *orfD* mutant, 8-OH-alanylclavam would be expected to accumulate, since its further conversion requires OrfD. Because *orfC* and *orfD* form an apparent operon, the polar effects of *orfC* disruption on *orfD* expression would also result in a buildup of 8-OH-alanylclavam in the *orfC* mutant. However, despite the operon arrangement, *orfD* may be expressed at low levels in the *orfC* mutants due to some level of transcription originating within the transposon or to the presence of a weak promoter located within *orfC* but after the point of Tn5062 insertion. In an *orfC* mutant, this low level of OrfD would generate correspondingly small amounts of the pyruvylclavam intermediate (not observed), but the main product would be 8-OH-alanylclavam. In the complemented *orfC* mutant, OrfD would remain at the same low level, and so even full restoration of OrfC would still result in 8-OH-alanylclavam as the main product, although low levels of alanylclavam would now also be produced. The fermentation profiles observed for these mutants and complemented strains are consistent with this proposed pathway and the transcriptional arrangement of the *orfC* and *orfD* genes. Interestingly, our unsuccessful attempts to generate an in-frame *orfC* mutant, which should have decoupled expression of *orfD* from that of *orfC*, suggests that a buildup of the predicted α -keto acid product of OrfD might be toxic to the cell.

Regulation of alanylclavam production is also of interest in view of the observation that *orfA* contains a TTA codon. This TTA codon represents only the second such codon found in any of the genes associated with production of β -lactam metabolites in *S. clavuligerus*. The only other TTA codon is found in *ccaR*, a gene that encodes a pathway-specific transcriptional regulator controlling cephamycin and clavulanic acid production. *bldA* is a rare leucyl tRNA for the translation of UUA codons in *Streptomyces* spp. (27). Genes containing TTA codons are typically strongly dependent on *bldA* for expression and are usually genes that are expressed during the later stages of growth, such as those involved in secondary metabolite production (28).

Examination of *S. clavuligerus bldA* mutants has revealed that they can produce all of the clavams except alanylclavam (unpublished results). *orfA* would make a logical target for regulation of alanylclavam biosynthesis by *bldA*, since it encodes the first dedicated enzyme of the proposed alanylclavam branch of the 5S clavam biosynthetic pathway, but additional studies will be required to confirm its involvement, since *ccaR* expression was found unexpectedly to proceed in a *bldA* mutant of *S. clavuligerus*, despite the presence of a TTA codon (54).

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