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5*S* Clavam Biosynthetic Genes Are Located in Both the Clavam and Paralog Gene Clusters in *Streptomyces clavuligerus*

Kapil Tahlan,[1,3] Cecilia Anders,[1] Annie Wong,[1] Roy H. Mosher,[1,4] Perrin H. Beatty,[1] Michael J. Brumlik,[1,5] Allison Griffin,[2] Claire Hughes,[2] John Griffin,[2] Barry Barton,[2] and Susan E. Jensen[1,*]
[1] Department of Biological Sciences, University of Alberta, Edmonton, Alberta T6G 2E9, Canada
[2] GlaxoSmithKline Pharmaceuticals, Worthing, West Sussex BN14 8QH, England
[3] Present Address: Department of Biochemistry and Biomedical Sciences, McMaster University, 1200 Main Street West, Hamilton, Ontario L8N 3Z5, Canada
[4] Present Address: Department of Biological Sciences, Wagner College, One Campus Road, Staten Island, NY 10301, USA
[5] Present address: University of Texas Health Sciences Center at San Antonio (UTHSCSA), MED/Med Oncology 7884, San Antonio, TX 78229-3900, USA
*Correspondence: susan.jensen@ualberta.ca
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SUMMARY

The *Streptomyces clavuligerus* clavam gene cluster was examined to identify genes specifically involved in 5*S* clavam biosynthesis. A reduction/loss of 5*S* clavam production was seen in *cvm2* and *cvm5* gene mutants, and a clavam metabolite not previously observed, 2-carboxymethylideneclavam, accumulated in the *cvm5* mutant. Disruption of additional genes from the region of the clavam cluster did not have any effect on 5*S* clavam production. Examination of the paralog gene cluster region for 5*S* clavam biosynthetic genes led to the identification of *cvm6P* and *cvm7P*, which encode a putative aminotransferase and a transcriptional regulator, respectively. Mutants defective in *cvm6P* and *cvm7P* were completely blocked in 5*S* clavam but not clavulanic acid production. The loss of 5*S* clavam production in *cvm7P* mutants suggests that this gene encodes a transcriptional regulator specific for 5*S* clavam metabolite biosynthesis.

INTRODUCTION

Streptomyces clavuligerus is well known as the species used for the industrial production of the β-lactam metabolite, clavulanic acid. Due to its ability to inhibit many β-lactamases, clavulanic acid is used clinically in combination with other β-lactam antibiotics to combat antibiotic resistant infections caused by β-lactamase-producing bacteria [1]. In addition to clavulanic acid, *S. clavuligerus* also produces penicillin and cephamycin antibiotics, as well as four more clavam metabolites, collectively referred to as the 5*S* clavams due to their 5*S* stereochemistry [2, 3] (Figure 1). In contrast, clavulanic acid has a 3*R*, 5*R* configuration [4].

The biosynthetic pathway leading to clavulanic acid and the 5*S* clavams is partially shared in *S. clavuligerus*, at least up to the level of clavaminic acid [5] (Figure 1). Although the early, shared part of this pathway has been well studied, little is known about the late steps beyond clavaminic acid that lead specifically to clavulanic acid or the 5*S* clavams. The last reaction in clavulanic acid biosynthesis involves the conversion of clavaldehyde into clavulanic acid [6], and recently, the enzyme *N*-glycyl-clavaminic acid synthetase, which converts clavaminic acid to *N*-glycyl-clavaminic acid [7], was identified; however, details of the intervening reactions remain unclear, and none of the reactions involved in the late steps of 5*S* clavam biosynthesis has as yet been elucidated.

The 3*R*, 5*R* stereochemistry of clavulanic acid is associated with its β-lactamase inhibitory activity, as both clavulanic acid and clavaldehyde share this stereochemistry and are inhibitory. In contrast, the 5*S* clavams show a variety of weak antifungal and antibacterial activities, but are not β-lactamase inhibitors [8]. In the industrial production of clavulanic acid, the 5*S* clavams represent unwanted contaminants that consume metabolic resources and complicate downstream processing. Therefore, elimination of 5*S* clavam production while retaining full clavulanic acid productivity is one reason to seek a full understanding of the interrelationship between clavulanic acid and 5*S* clavam metabolite biosynthesis. The observation that both clavaldehyde and clavulanic acid are bioactive is consistent with previous studies showing that variations in side chains at the C2 position are compatible with β-lactamase inhibition [4]. Since the 5*S* clavams differ from each other and from clavulanic acid in their C2 side chains, this raises the additional possibility that the enzymes for 5*S* clavam biosynthesis may provide a biological route to the production of clavulanic acid analogs with altered C2 side chains. However, a full understanding of the late stages of clavulanic acid and 5*S* clavam biosynthesis is first required.

The biosynthesis of several large classes of antibiotics, such as the polyketides and nonribosomal peptides,

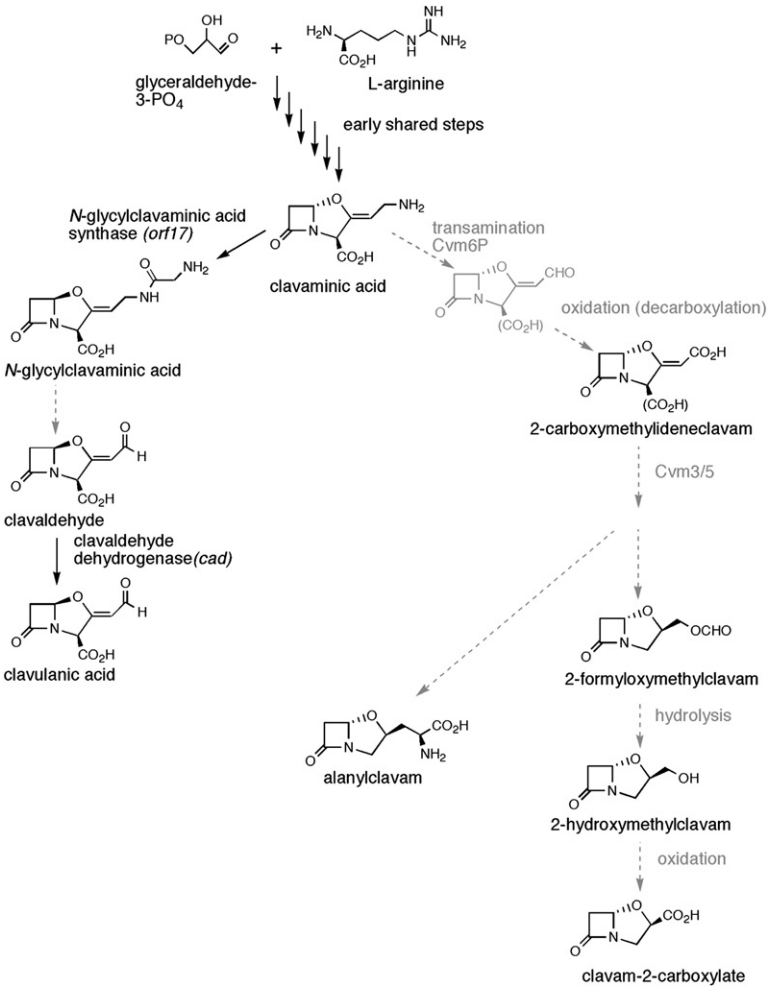

Figure 1. The Clavulanic Acid and 5*S* Clavam Biosynthetic Pathway in *S. clavuligerus*

Solid lines represent known steps and broken lines indicate multiple intermediates or unknown portions of the pathway. Structures and enzymes shown in gray are hypothetical.

follows such conserved patterns that the biosynthesis of newly discovered metabolites in these classes can be largely predicted from translation of gene sequences alone. In contrast, the β-lactams are less widespread in nature, and each subgroup follows a separate biosynthetic route. As a result, details of clavam metabolite biosynthesis cannot be inferred from known pathways. Furthermore, since the reactions giving rise to the final products are unknown, gene sequences have little predictive value, and genes involved in biosynthesis can only be identified by mutating each candidate individually.

The genes involved in the biosynthesis of clavulanic acid and the 5*S* clavams reside in at least three separate locations on the *S. clavuligerus* chromosome [9] (Figure 2A). The clavulanic acid gene cluster is located next to the cephamycin C gene cluster [10]. It comprises the *ceaS2*, *bls2*, *pah2*, *cas2*, and *oat2* genes, all involved in the biosynthesis of clavaminic acid and, therefore, contributing to the production of both clavulanic acid and the 5*S* clavams, along with additional genes involved in the formation of clavulanic acid only [11–15]. However, genes encoding enzymes for the early shared part of the pathway are each present in two copies on the *S. clavuligerus* chromosome [16–18] (Figure 2A). *cas1*, encoding a second form (paralog) of clavaminate synthase, does not reside together with *cas2* in the clavulanic acid gene cluster, but instead is located separately [17]. The region surrounding *cas1* is referred to as the clavam gene cluster, since mutants defective in genes from this region have lost 5*S* clavam but not clavulanic acid production [19]. The paralogs of the *ceaS2*, *bls2*, *pah2*, and *oat2* genes are not located in either the clavulanic acid or the clavam gene clusters. Instead, they form yet another gene cluster—the paralog cluster [18] (Figure 2A). In the present study, we examined the regions of the *S. clavuligerus* chromosome in the vicinity of the clavam and paralog gene clusters to identify additional genes that are involved in the biosynthesis and regulation of the 5*S* clavams.

RESULTS

Isolation and Analysis of Genes from the Clavam Gene Cluster

In a previous study, Mosher et al. [19] identified four genes (*cvm1*, *cvm2*, *cvm4*, and *cvm5*) and partially sequenced

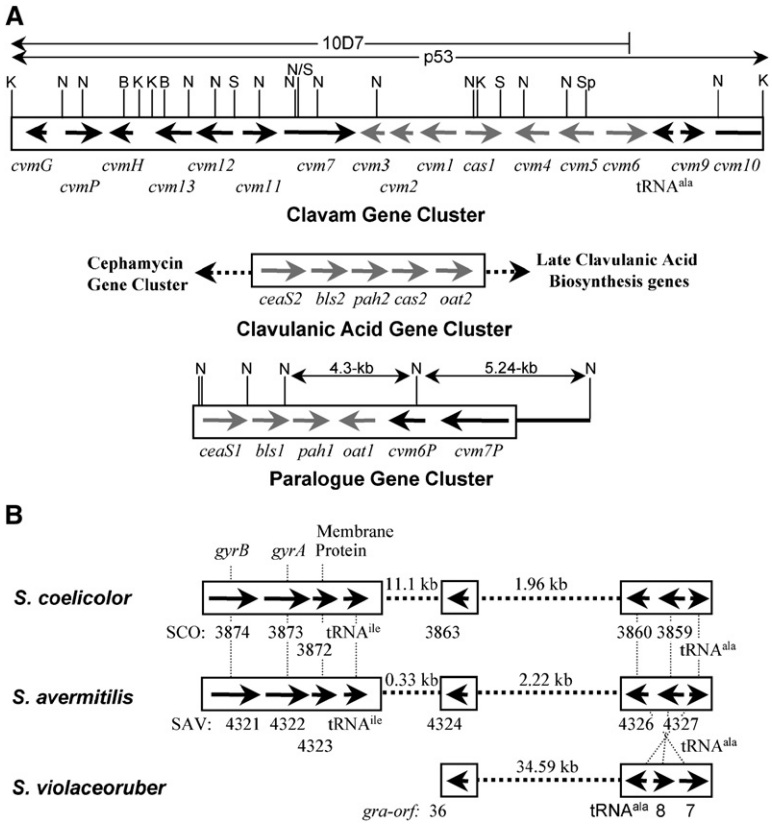

Figure 2. Genes Involved in the Biosynthesis of Clavam Metabolites in *S. clavuligerus*

(A) Diagrammatic representation of the three gene clusters involved in clavulanic acid and 5*S* clavam metabolite biosynthesis in *S. clavuligerus*. Gray arrows represent previously sequenced genes, whereas black arrows indicate genes identified in the present study, and arrowheads indicate the direction of transcription (not drawn to scale). The location of the cephamycin gene cluster and genes specific for clavulanic acid biosynthesis are also indicated. Restriction endonuclease sites used to subclone DNA fragments for sequencing are also shown. B, BamHI; K, KpnI; N; *Nco*I; S, SalI and Sp, *Sph*I.

(B) Diagrammatic representation of the region corresponding to the clavam gene cluster in *S. coelicolor*, *S. avermitilis*, and *S. violaceoruber* (not to scale). The designation and annotation of some homologous ORFs are shown. The broken line represents the region corresponding to the clavam gene cluster in *S. clavuligerus*, and the relative sizes (in kb) of these regions in *S. coelicolor*, *S. avermitilis*, and *S. violaceoruber* are indicated.

two open reading frames (ORFs; *cvm3* and *cvm6*) located in the vicinity of the *cas1* gene in *S. clavuligerus*. This group of genes was named the clavam cluster because *cvm1* and *cvm4-cvm5* mutants did not produce any 5*S* clavams, while clavulanic acid production was unaffected [19]. Due to the apparent involvement of *cvm1* and *cvm4-cvm5* in 5*S* clavam production, the region surrounding the known clavam gene cluster was investigated to search for additional genes involved in 5*S* clavam production.

The cosmid clones 10D7 [19] and p53 (Jensen et al., unpublished data), which contain DNA inserts from the regions upstream and downstream of the known clavam gene cluster (Figure 2A), were used to isolate smaller restriction fragments for DNA sequencing. DNA inserts within the cosmids were compared with chromosomal DNA by Southern analysis to confirm that no rearrangements had occurred during cosmid isolation. In total, nine new ORFs and a tRNA gene were identified, extending the known sequence of the *S. clavuligerus* clavam gene cluster to 23.3 kb (Figure 2A and Table 1). The remaining DNA sequence of the previously incomplete *cvm3* and *cvm6* genes was also obtained [19]. The predicted Cvm3 gene product contains a flavin reductase-like FMN-binding domain typical of reductases associated with mono-oxygenases involved in secondary metabolite biosynthesis [20, 21], although the highest degree of similarity was shown by a flavin reductase-like protein from *Novosphingobium aromaticivorans*. The sequence of *cvm6* revealed that it encodes a putative class-III aminotransferase similar to ornithine/acetylornithine aminotransferases (ArgD) that catalyze the transfer of an amino group from acetylornithine to α-ketoglutarate [22].

Seven of the newly sequenced ORFs lie downstream of *cvm3* at the left end of the clavam cluster and include two potential transcriptional regulators (Figure 2A and Table 1). The Cvm7 gene product has an unusual two-domain structure, but shows end-to-end similarity to the transcriptional regulator, PimR, from *S. natalensis* [23]. The N-terminal domain resembles transcriptional activators of the *Streptomyces* antibiotic regulatory protein (SARP) type, while the C-terminal domain contains a tetratricopeptide repeat sequence typically found in LuxR-type regulators. The Cvm12 gene product is predicted to contain a gluconate operon transcriptional repressor (GntR)-type winged helix-turn-helix domain. In addition, amino acids 145–389 show the presence of a class I and class II aminotransferase binding motif similar to transcriptional regulators of unknown function from the MocR subfamily of GntR regulators [24].

cvm11 (Figure 2A), lies between *cvm7* and *cvm12*, and encodes an integral membrane protein similar to the LysE and RhtB-type translocator family of proteins involved in lysine and threonine export [25]. The four ORFs downstream of *cvm12* include *cvm13*, encoding an asparaginase-like protein, *cvmH*, encoding a thioesterase-like protein, and *cvmP*, the predicted gene product of which

Table 1. Predicted Functions for Previously Unreported or Partially Sequenced ORFs from the Clavam and the Paralog Gene Clusters of *Streptomyces clavuligerus*

ORF	Gene Cluster Size (aa)[a]	Most Similar Protein, Source, Accession Number, Similarity/Identity[b]	Proposed Function[c]
cvm2[d]	Clavam, 151	Hypothetical protein, *Ralstonia eutropha*, AAZ64037, 60/47	Isomerase
cvm3[e]	Clavam, 188	Putative flavin reductase, *Novosphingobium aromaticivorans*, EAP38005, 65/49	Flavin Reductase
cvm4[d]	Clavam, 328	Putative homoserine acetyltransferase, *Burkholderia fungorum*, ZP_00284696, 56/41	Acetyltransferase
cvm5[d]	Clavam, 394	Putative oxidoreductases, *Novosphingobium aromaticivorans*, EAP37999, 52/35	Mono-oxygenase/Oxidoreductase
cvm6[e]	Clavam, 442	Putative aminotransferase from *Chloroflexus aurantiacus*, EAO57759, 57/42	Aminotransferase
cvm7	Clavam, 1114	Pimaricin regulator PimR, *Streptomyces natalensis*, CAE51066, 45/32	Transcriptional regulator
cvm9	Clavam, 182	Gra-orf8, *Streptomyces violaceoruber*, CAA09629, 96/94	Transcriptional regulator
cvm10	Clavam, NA[f]	Gra-orf7, *Streptomyces violaceoruber*, CAA09628, 96/89	Protein Kinase
cvm11	Clavam, 216	Putative LysE family translocator, *Nocardia farcinica*, Q5Z268, 63/52	Efflux Protein
cvm12	Clavam, 445	Transcriptional regulatory protein from *Bradyrhizobium japonicum*, Q89HX9, 63/49	Transcriptional regulator
cvm13	Clavam, 390	Putative asparaginase YbiK, *Shigella flexneri*, AAN42412, 53/40	Asparaginase
cvmH	Clavam, 305	LanU-like protein, *Streptomyces murayamaensis*, AAO65353, 60/51	Hydrolase
cvmP	Clavam, 687	Putative protein-arginine deiminase, *Gibberella zeae*, EAA72393, 53/39	Arginine deiminase
cvmG	Clavam, 92	Gra-orf36, *Streptomyces violaceoruber*, CAA09663, 39/32	Putative secreted protein
cvm6P	Paralog, 437	Cvm6, the clavam gene cluster of *Streptomyces clavuligerus*, 73/63	Aminotransferase
cvm7P	Paralog, 818	Pimaricin regulator PimR, *Streptomyces natalensis*, CAE51066, 31/23	Transcriptional regulator for 5*S* clavams

[a] aa, number of amino acids predicted in the encoded protein.
[b] Percent similarities/identities were determined using the online BLASTp program.
[c] Proposed functions are based on predicted motifs and on the functions of the most similar proteins present in GenBank.
[d] The DNA sequence of these ORFs has been reported previously [19] and are included because they were subjected to gene disruption analysis in this study.
[e] The partial DNA sequence of these ORFs has been reported previously [19].
[f] NA, not applicable as only the partial DNA sequence of the ORF is known.

contains a partial protein-arginine deiminase (PAD) motif. In mammals, PADs modify protein-bound arginine residues to citrulline, but their function in prokaryotes is yet to be demonstrated [26]. The most downstream ORF to be sequenced was *cvmG*, encoding a hypothetical protein of unknown function, but similar to *gra-orf36*, a gene located at one edge of the granaticin gene cluster [27].

On analysis of the genes beyond *cvm6* at the right end (Figure 2A) of the clavam cluster, an alanyl-tRNA gene ($tRNA^{ala}$), and two ORFs, *cvm9*, the gene product of which is virtually identical to a hypothetical protein encoded by *gra-orf8*, and *cvm10* (partial sequence only), resembling the putative serine-threonine protein kinase, *gra-orf7*, were discovered. These three genes show identical arrangement to the $tRNA^{ala}$, *gra-orf7* and *gra-orf8* genes found at the other edge, opposite to *gra-orf36*, in the granaticin biosynthetic gene cluster of *S. violaceoruber* [27] (Figure 2B).

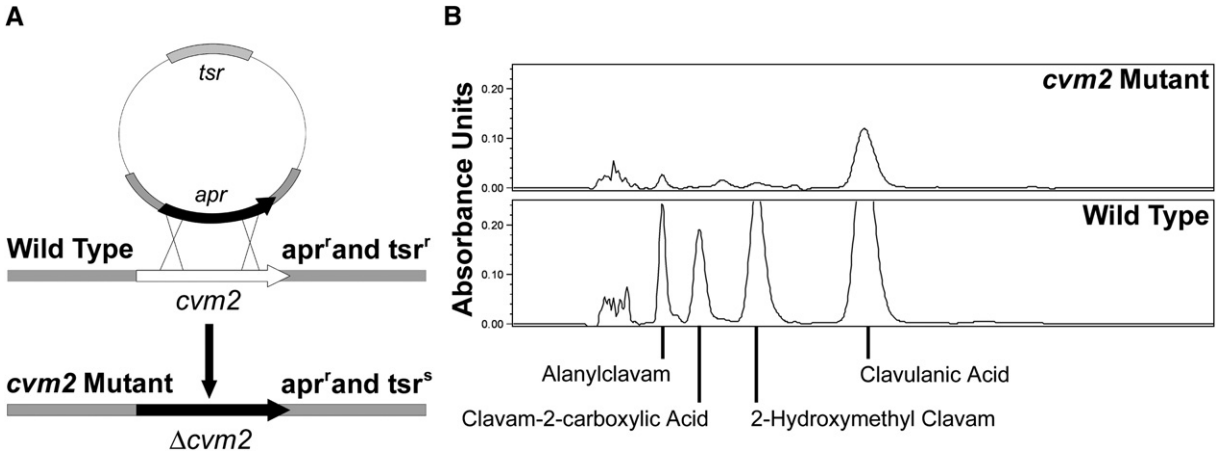

Figure 3. Preparation and Analysis of the *cvm2* Mutant

(A) Diagrammatic representation of the process leading to the isolation of the *cvm2* mutant using a specific gene-targeting plasmid. The gray bar represents the *S. clavuligerus* chromosome, white arrows represent *cvm2*, and black arrows represent the disruption cassette. The antibiotic resistance profiles of wild-type *S. clavuligerus* containing disruption vector and the *cvm2* mutant are also indicated. Terms apr^r, tsr^r, and tsr^s refer to apramycin- or thiostrepton-resistant or -sensitive phenotypes, respectively.

(B) HPLC analysis of the *cvm2* mutant. The identities of the various clavam metabolites produced by wild-type *S. clavuligerus* and the *cvm2* mutant are indicated.

Preparation of Mutants from the Clavam Gene Cluster

Activities can be inferred for some of the nine new ORFs identified in the region of the clavam gene cluster (Table 1), but actual functions for natural product biosynthetic genes can be quite different from what might be predicted based on primary sequence homology. Therefore, gene disruption mutants were prepared to determine if they are involved in clavulanic acid and/or 5*S* clavam metabolite biosynthesis. Although *cvm2* had been sequenced previously [19], it had not been subjected to mutational analysis. Therefore, a *cvm2* mutant was prepared by a modified version of the polymerase chain reaction (PCR) targeting procedure [28] (Figure 3A). Analysis of the *cvm2* mutant revealed that it was severely compromised in alanylclavam and 2-hydroxymethylclavam synthesis, and totally blocked in clavam-2-carboxylate production (Figure 3B).

Mosher et al. [19] reported the partial DNA sequence of *cvm3* and *cvm6*, but did not prepare mutants defective in these ORFs. They also prepared a mutant in which both *cvm4* and *cvm5* were deleted together, and found that this mutant did not produce any of the 5*S* clavams. Therefore, it was not clear which of *cvm4* or *cvm5* was responsible for the observed phenotype. To investigate further the roles of *cvm3*, *cvm4*, *cvm5*, and *cvm6* in 5*S* clavam metabolite biosynthesis, individual mutants were prepared in each of the ORFs by insertion of apramycin (*apr*) or neomycin (*neo*) resistance genes within their coding regions, oriented in the same direction of transcription. Analysis of soy culture supernatants from *cvm3*, *cvm4*, and *cvm6* mutant cultures indicated that they all still produced wild-type levels of clavulanic acid and 5*S* clavams (data not shown).

High-pressure liquid chromatography (HPLC) analysis of *cvm5* mutant cultures showed that production of the known 5*S* clavams was completely blocked in this mutant, but that a new clavam metabolite (retention time, 6.13 min) had accumulated (Figure 4A). Once recognized, the new clavam metabolite was also noted to be present at low levels in supernatants from the wild-type strain. Liquid chromatography-mass spectrometry (LC-MS) analysis of imidazole-derivatized soy culture supernatants confirmed the accumulation of this metabolite in the *cvm5* mutant cultures (retention time, 13.68; *m/z* = 238) (Figure 4A). Furthermore, LC-MS-MS analyses identified fragments with *m/z* values of 238, 170, 152, 126, 110, 98, and 84 (Figure 4B), which led to the predicted structure of the novel clavam, tentatively named 2-carboxymethylidene-clavam, as shown in Figure 4C, structures 1 and 2. The two possible structures for this metabolite could not be distinguished by this analysis, since the imidazole derivatization procedure used for HPLC analysis would remove a C3 COOH group, if present.

Sequence analysis of *cvm7* indicated that its predicted protein product would contain two distinct domains: an N-terminal SARP-like domain and a C-terminal LuxR-like domain. Two *cvm7* knockout mutants were prepared by the insertion of *apr* at 130 bp, in the SARP-like region, and at 2064 bp, in the LuxR-like region, of the predicted ORF. On fermentation analysis, it was found that both types of mutants produced clavulanic acid and 5*S* clavams at levels comparable to the wild-type strain, indicating that Cvm7 was not involved in regulating 5*S* clavam biosynthesis.

Mutants defective in *cvm9*, *cvm10*, *cvm11*, *cvm12*, *cvm13*, *cvmP*, and *cvmG* were also prepared by PCR targeting, or by the insertion of *apr* within their coding

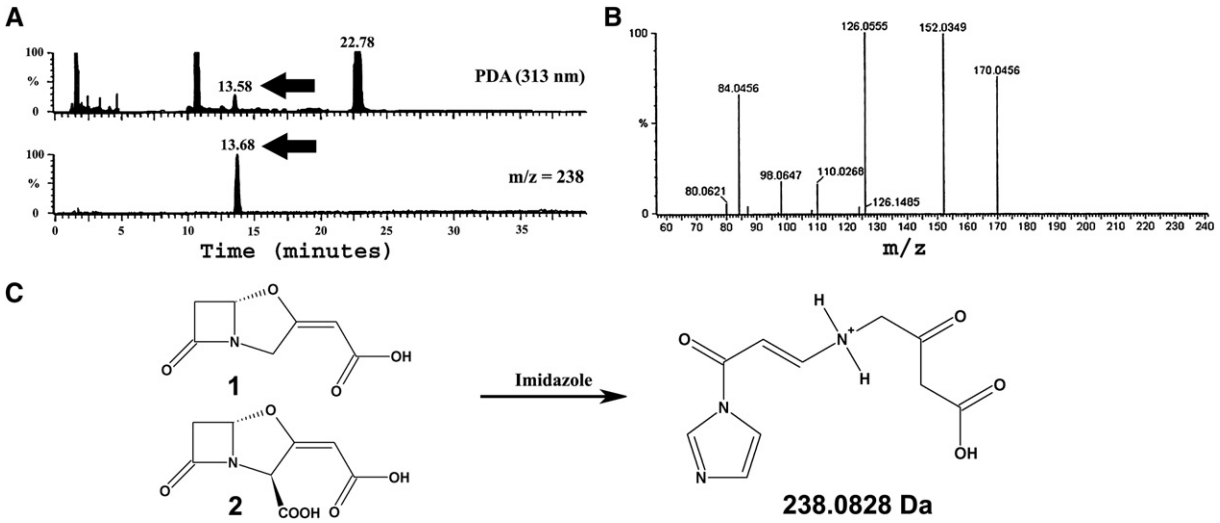

Figure 4. Identification of the Clavam Metabolite Accumulating in the *cvm5* Mutant

(A) LC-MS analysis of *cvm5* mutant culture supernatants after imidazole derivatization showing the UV absorbance (PDA) and scan for mass (*m/z* = 238).

(B) Tandem LC-MS-MS analysis of the peak corresponding to *m/z* = 238 showing its fragmentation pattern.

(C) Deduced structures of the possible compounds capable of giving the parent ion seen in (A) and corresponding to the fragmentation pattern seen in (B).

regions, but none of the mutants was affected in clavulanic acid or 5*S* clavam metabolite production. Mutation of *cvmH* was also attempted, but disruption constructs were unstable and did not lead to the isolation of *cvmH* mutants despite repeated attempts.

Isolation and Analysis of Genes from the Paralog Gene Cluster

Preparation and analysis of thirteen new mutants from the clavam gene cluster revealed that only the *cvm2* and *cvm5* disruption mutants had observable phenotypes in terms of 5*S* clavam production, in addition to the previously described *cvm1* mutant. This led us to conclude that any additional biosynthetic genes needed to form the four 5*S* clavam metabolites are likely to be located elsewhere on the chromosome.

To investigate the possibility that genes involved in the later stages of 5*S* clavam metabolite biosynthesis might reside in the vicinity of the paralog gene cluster, additional ORFs from the region downstream from *oat1* were analyzed. The corresponding region downstream from *oat2* in the clavulanic acid gene cluster comprises the rest of the clavulanic acid biosynthetic genes. DNA sequence was obtained from restriction fragments subcloned from the cosmid 14E10 and from a 4.3-kb *Nco*I genomic DNA fragment cloned previously by Jensen et al. [16], leading to the identification of two new ORFs, *cvm6P* and *cvm7P* (Figure 2A).

The putative Cvm6P gene product shares 73% end-to-end similarity with Cvm6 from the clavam gene cluster, and, like Cvm6, it resembles the ArgD protein (Table 1). *cvm7P*, located upstream of *cvm6P* (Figure 2A), encodes a putative bipartite transcriptional regulator. Once again, the predicted Cvm7P gene product is so-named because it shares 30% end-to-end similarity with Cvm7 from the clavam gene cluster, and, therefore, also resembles the pimaricin regulator, PimR [23] (Table 1).

Preparation of Mutants from the Paralog Gene Cluster

cvm6P mutants were prepared by the insertion of *neo* within the ORF, in the same orientation as the gene. Fermentation analysis showed that production of the 5*S* clavams was abolished in the *cvm6P* mutants, whereas clavulanic acid and cephamycin C production was unaffected (data not shown).

A *cvm7P* mutant strain was prepared using the PCR targeting system, as described earlier [28]. HPLC analysis of culture supernatants showed that the *cvm7P* mutant and the wild-type strains produced similar levels of clavulanic acid. Like the *cvm6P* mutants, bioassays and LC-MS indicated that the *cvm7P* mutants did not produce detectable levels of any of the 5*S* clavams (data not shown).

DISCUSSION

Genes involved in the biosynthesis of 5*S* clavam metabolites in *S. clavuligerus* have been located in the clavam gene cluster, but many steps in the proposed biosynthetic pathway have no corresponding genes in this cluster, and no transcriptional regulators specific for 5*S* clavam production are known. Therefore, the DNA sequence of the *S. clavuligerus* clavam gene cluster was extended, and mutants defective in genes from flanking regions were

prepared and analyzed to assess their involvement in 5*S* clavam biosynthesis. Examination of the *cvm2* mutant prepared in this study showed that the production of the 5*S* clavams was greatly reduced (alanylclavam and 2-hydroxymethylclavam) or completely eliminated (clavam-2-carboxylate). The predicted amino acid sequence of Cvm2 is similar to proteins possessing ketosteroid isomerase-like domains. Ketosteroid isomerases are involved in the intramolecular transfer of protons resulting in the isomerization of steroids [29], essential steps in steroid biosynthesis in mammals; however, in bacteria these enzymes are linked to the degradation of steroids [30]. The reason for involving an isomerase in 5*S* clavam biosynthesis is unknown, but it is clearly important, since mutation severely reduced the level of 5*S* clavams produced.

The predicted Cvm5 gene product resembles flavin-dependent mono-oxygenases [19] that utilize reduced flavin provided by a flavin reductase. The best matches for Cvm5 found by BLAST analysis were luciferase-like mono-oxygenases from *Sphingomonas* and *Novosphingobium* spp. Similarly, the predicted Cvm3 gene product resembles flavin reductases, and the most similar proteins by BLAST analysis are the flavin reductases from *Sphingomonas* and *Novosphingobium* spp., which partner with their corresponding mono-oxygenases. Therefore, Cvm3 presumably functions to provide reduced flavin to Cvm5.

Analysis of the *cvm5* mutant showed that it did not produce any of the known 5*S* clavams. Instead, there was accumulation of a novel clavam, 2-carboxymethylideneclavam. Since this novel clavam is also present in small amounts in the wild-type, it may be an intermediate in 5*S* clavam biosynthesis. Furthermore, since the *cvm5* mutant produces none of the known 5*S* clavam products, Cvm5 must function soon after clavaminic acid in the 5*S* arm of the branched pathway. The lack of a phenotype for the *cvm3* mutants is inconsistent with Cvm3 being required for Cvm5 activity. However, the flavin reductase merely provides reduced FAD or FMN to the mono-oxygenase, and so, perhaps, other flavin reductases may take its place, as has been shown for SnaC, which can functionally replace ActVB in the biosynthetic pathway leading to actinorhodin [21].

In considering reactions that could give rise to 2-carboxymethylideneclavam, an aminotransferase-type activity could convert the amino group at C2 of clavaminate to the corresponding aldehyde, which would then be oxidized to give the carboxylic acid seen in 2-carboxymethylideneclavam (Figure 1). Alternatively, 2-carboxymethylideneclavam may be a shunt product arising from an attack on the proposed reactive aldehyde by a nonspecific oxygenase/dehydrogenase. However, assuming that 2-carboxymethylideneclavam is a true pathway intermediate, the Cvm3/Cvm5 couple, alone or in combination with other gene products, must then further convert 2-carboxymethylideneclavam into 2-formyloxymethyl clavam and subsequent products of the 5*S* pathway, as proposed by Egan et al. [5]. Although the putative Cvm6 gene product resembles aminotransferases, we were unable to establish a role for Cvm6 in clavam biosynthesis, as *cvm6* mutants showed no phenotype.

We also prepared and analyzed a mutant defective in the previously reported *cvm4* gene, as Mosher et al. [19] showed that a *cvm4-cvm5* double mutant did not produce any of the 5*S* clavams. Examination of the new *cvm4* mutant revealed that it was not affected in either clavulanic acid or 5*S* clavam metabolite biosynthesis, indicating that the defect in 5*S* clavam production seen earlier in the *cvm4-cvm5* double mutant [19] was due solely to the disruption of *cvm5*. The predicted Cvm4 gene product resembles homoserine *O*-acetyltransferases [19], which transfer acetate from acetyl-CoA to homoserine [31] during methionine biosynthesis in some bacteria. Examination of the *S. coelicolor* and *S. avermitilis* genomes suggests that these streptomycetes do not use homoserine *O*-acetyltransferases, but instead channel cysteine and serine into the methionine biosynthetic pathway via cystathionine γ-lyase and cystathionine β-synthase, respectively. In keeping with this, mutagenesis of *cvm4* did not lead to methionine auxotrophy in *S. clavuligerus* (data not shown). *cvm4* also shows a lesser degree of similarity (46% over 156 aa) to *cefG*, encoding the acetyltransferase involved in formation of cephalosporin C in *Acremonium chrysogenum* [32]. However, no corresponding reaction is involved in the production of cephamycin C in *S. clavuligerus*, and the disruption of *cvm4* had no effect on cephamycin C production. Despite the lack of phenotype for *cvm4* mutants, the location of *cvm4* in the clavam gene cluster suggests that it may play a conditional role in 5*S* clavam biosynthesis.

Other ORFs identified in this study from the region surrounding the clavam gene cluster were also subjected to insertional inactivation. Results showed that none of the putative transcriptional regulators located in this region affect 5*S* clavam production. Therefore, it is predicted that these ORFs are not directly involved in 5*S* clavam biosynthesis.

The disruption of *cvmG*, *cvm9*, and *cvm10* from the clavam gene cluster (Figure 2A), which are very similar to *gra-orf36*, *gra-orf8*, and *gra-orf7*, respectively (Table 1), genes flanking either edge of the granaticin biosynthetic gene cluster of *S. violaceoruber* [27], did not affect 5*S* clavam production. Examination of the *S. coelicolor* [33] and *S. avermitilis* [34] genome sequences showed that *gra-orf36*, *gra-orf8*, and *gra-orf7* homologs are also found in these organisms, suggesting that they are not true granaticin biosynthetic genes but merely flank the cluster in *S. violaceoruber* (Figures 2A and 2B). The location of these genes on the chromosomes of *S. coelicolor* and *S. avermitilis* can give insights into the presumptive locations of the clavam and granaticin gene clusters in the genomes of *S. clavuligerus* and *S. violaceoruber*. In both *S. coelicolor* and *S. avermitilis*, the *gra-orf36* and *gra-orf8* homologs are located within the conserved core of the chromosome near the origin of replication and separated from each other by only about 2 kb. In contrast, in *S. violaceoruber* and *S. clavuligerus*, they are separated by the intervening granaticin (34.59 kb) or clavam

(21.05 kb) gene clusters, respectively (Figures 2A and 2B). The discrepancies between these species in this region of the genome suggests that *S. violaceoruber* and *S. clavuligerus* might have acquired their granaticin and clavam gene clusters by horizontal gene transfer, by incorporating them into a hot spot for the insertion of exogenous DNA found at this chromosomal locus. All four of these *Streptomyces* species contain one or more tRNA genes in this region (Figures 2A and 2B), genes often associated with insertions and rearrangements of genetic material in bacteria [35]. Comparative analysis of clavam biosynthetic gene clusters from other producer species should help shed light on this hypothesis.

Since further examination of the clavam gene cluster did not uncover any genes involved in regulation or in the specific late steps of 5*S* clavam production, we explored the possibility that additional genes involved in the biosynthesis of these metabolites might reside in the paralog gene cluster. The sequence of the known paralog gene cluster was extended, leading to the isolation of *cvm6P* and *cvm7P* (Figure 2A). These genes are similar to their counterparts, *cvm6* and *cvm7*, genes from the clavam gene cluster. Cvm6P, like Cvm6, resembles pyridoxal-5′-phosphate (PLP)-dependent ArgDs (Table 1). The crystal structure of human ArgD ($ArgD^{HS}$) has been solved, showing that the active site comprises R180, E235, and R413 residues, with PLP covalently bound to K292 [36]. The predicted amino acid sequences of Cvm6 and Cvm6P contain sites for the covalent attachment of PLP (K280 and K267, respectively), but lack two of the three above-mentioned active-site residues, with only E184 (= $E235^{HS}$) being conserved. In comparison, the predicted *S. clavuligerus* ArgD contains the K246 residue for PLP attachment and the active site residues R135, E189, and R363 [37]. Therefore, it is possible that Cvm6 and/or Cvm6P function as aminotransferases, but with a substrate other than ornithine/acetylornithine. While disruption of *cvm6* had no effect on 5*S* clavam biosynthesis, production was completely abolished in the *cvm6P* mutant, indicating that the two gene products have different activities in *S. clavuligerus*. If 5*S* clavam biosynthesis proceeds by deamination of clavaminic acid, Cvm6P may represent the aminotransferase responsible for this reaction.

During arginine biosynthesis in *Streptomyces* spp., ArgD catalyzes the transamination of *N*-acetylglutamyl-γ-semialdehyde to give *N*-acetylornithine, which is then converted to ornithine by the action of OAT (ArgJ), thereby recycling the acetyl group [22]. Within the paralog cluster, *cvm6P* (similar to *argD*) resides next to *oat1* (Figure 2A), which has been proposed to encode an unusual OAT-like protein (similar to ArgJ) [38, 39]. Therefore, the putative transamination activity encoded by *cvm6P*, which is essential for 5*S* clavam biosynthesis, may also be coupled to an acetylation activity encoded by *oat1*, although neither OAT1 nor -2 activity is essential for clavam biosynthesis [18].

The *cvm7P* gene, like *cvm7*, encodes a putative transcriptional regulator similar to the pimaricin regulator from *S. natalensis* [23] (Table 1). While mutation of *cvm7* had no discernable phenotype, disruption of *cvm7P* resulted in the loss of all of the 5*S* clavams, with no effect on clavulanic acid. Therefore, Cvm7P apparently functions as a pathway-specific regulator for 5*S* clavam biosynthesis, analogous to ClaR in clavulanic acid biosynthesis [40], making it the first 5*S* clavam-specific transcriptional regulator to be identified. The Cvm7P regulon is currently under investigation to determine whether it extends beyond the paralog cluster to include clavam and clavulanic acid cluster genes, and to determine if Cvm7P regulates the production of the 5*S* clavams directly or indirectly. Since pathway-specific transcriptional regulators involved in antibiotic biosynthesis are normally located within their respective biosynthetic gene clusters, studies are also being carried out to isolate and characterize additional ORFs from the region of the paralog gene cluster to determine if they are involved in the biosynthesis of the 5*S* clavams.

With the discovery of the *cvm6P* and *cvm7P* genes, it becomes apparent that the paralog gene cluster not only contains paralogs to genes from the clavulanic acid gene cluster (*ceaS1*, *bls1*, *pah1*, and *oat1*), but also to genes from the clavam gene cluster (Figure 2A). However, while the paralogous *ceaS1-2*, *bls1-2*, *pah1-2*, and *oat1-2* genes can functionally replace each other in clavulanic acid and 5*S* clavam metabolite biosynthesis [18], the same is not true for the *cvm6-cvm6P* and the *cvm7-cvm7P* genes. Only *cvm6P* and *cvm7P* are functional relative to their counterparts from the clavam gene cluster. Perhaps the proximity of *cvm6P* and *cvm7P* to the "early" biosynthetic genes from the paralog gene cluster is important for coregulation and/or coordination of enzymatic activities. Alternatively, since *cvm6*- and *cvm7*-type genes are represented twice, the redundant genes from the clavam gene cluster may have lost their functionality over time, and only the genes specifically required for biosynthesis remained intact.

SIGNIFICANCE

The *Streptomyces clavuligerus* clavam and paralog gene clusters both contain genes involved in 5*S* clavam metabolite biosynthesis. Genes identified from the clavam gene cluster included a two-component flavin-dependent mono-oxygenase complex—the first example of such a system in β-lactam metabolite biosynthesis. Disruption of the mono-oxygenase-encoding gene, *cvm5*, led to the accumulation of the novel clavam, 2-carboxymethylideneclavam, proposed to function as an intermediate in 5*S* clavam biosynthesis. In addition, 2-carboxymethylideneclavam is the first metabolite to be identified in the 5*S* clavam biosynthetic pathway beyond clavaminic acid. Further analysis revealed that additional genes involved in the biosynthesis of the 5*S* clavams are located in the paralog, rather than the clavam gene cluster. A transcriptional regulator was identified that functions as a pathway-specific regulator for 5*S* clavam metabolite

Table 2. Oligonucleotide Primers Used in this Study

Name	Oligonucleotide Sequence (5′–3′)	Function[a]
CAN 60	GGTCTGGTGCAGTGGAGCC	Forward primer for cloning *cvm9*
CAN 65	TCCGCGTCCGATCGGGTCG	Reverse primer for cloning *cvm9*
CVM2-FWD	CCAGCACAGCCACCCGGAATCTCTGGAGGTCGTT TCATTCCGGGGATCCGTCGACC	Forward primer for *cvm2* REDIRECT mutagenesis
CVM2-REV	CCGGAGGCCGGGTCACAGCCACGGGTAACGCGC GGTGTCTGTAGGCTGGAGCTGCTTC	Reverse primer for *cvm2* REDIRECT mutagenesis
CVM12-FWD	CGGGAAGCGGTCCACCGGACAGGGGAGGTACGG GAGATGATTCCGGGGATCCGTCGACC	Forward primer for *cvm12* REDIRECT mutagenesis
CVM12-REV	GCCCGCCCGGCGCGCTCAGTCCAGGGCCAGGTC CTCCGGTGTAGGCTGGAGCTGCTTC	Reverse primer for *cvm12* REDIRECT mutagenesis
CVM13-FWD	CGGAGCGTCTCTCACCCCCGTCCCGAGGAGTGC CCGATGATTCCGGGGATCCGTCGACC	Forward primer for *cvm13* REDIRECT mutagenesis
CVM13-REV	GTCAGGCCCGCAGGCAGCCACCGGTCGGCGGGC GGATCATGTAGGCTGGAGCTGCTTC	Reverse primer for *cvm13* REDIRECT mutagenesis
CVM7P-FOR	GGCGCCGTCAGCCACGCAGAGAAGATCGGATAC GCAGTGATTCCGGGGATCCGTCG	Forward primer for *cvm7P* REDIRECT mutagenesis
CVM7P-REV	GCGCCGCCCGCCGCCGAGGACCCCGGGGCCCGG GACTCATGTAGGCTGGAGCTGCT	Reverse primer for *cvm7P* REDIRECT mutagenesis
GRA-FWD	GCACCCGCCGGACGGACGCCCTCGGCACATGCC CCGGTGATTCCGGGGATCCGTCGACC	Forward primer for *cvmG* REDIRECT mutagenesis
GRA-REV	GCCGGCCGGAGAGCTGGGCTCCGGTGCTGCGGG GCCTCATGTAGGCTGGAGCTGCTTC	Reverse primer for *cvmG* REDIRECT mutagenesis
HYD-FWD	TCCCGACCGCTCCACTCCCCAGGAGTCCGCCCGAT GGTG ATTCCGGGGATCCGTCGACC	Forward primer for *cvmH* REDIRECT mutagenesis
HYD-REV	GCCCCCGGGCCCGACCCGGGTCAGCGCTGACCC GGGTCATGTAGGCTGGAGCTGCTTC	Reverse primer for *cvmH* REDIRECT mutagenesis
PAD-FWD	GTCCGGCCCACTCCCGCGTGCGAAAAGTCGCCC ACTGTGATTCCGGGGATCCGTCGACC	Forward primer for *cvmP* REDIRECT mutagenesis
PAD-REV	CGGGACCCCGCGTTCACGCTGCCGCCCTGACCG GTGTTCATGTAGGCTGGAGCTGCTTC	Reverse primer for *cvmP* REDIRECT mutagenesis

[a] The function of each oligonucleotide primer is described in detail under Experimental Procedures.

biosynthesis—the first such regulator of 5*S* clavam biosynthesis to be identified. A better understanding of the biochemical pathway leading to the 5*S* clavam metabolites and, in particular, the enzymatic activities responsible for forming the C2 side chains of the 5*S* clavams may provide routes for the biological modification of the clavulanic acid C2 side chain. In addition, identification of the full complement of biosynthetic and regulatory genes needed for production of the 5*S* clavams, and elucidation of their interactions with clavulanic acid production, could provide further insight into the evolution and regulation of related antibiotic biosynthetic pathways. Such an understanding could impact rational strain improvement and combinatorial biosynthesis methodologies, whereby multiple antibiotic gene clusters could be introduced into the same organism and manipulated to produce useful compounds.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions

S. clavuligerus NRRL3585 and *S. lividans* TK24 [41] were used in this study. Wild-type and mutant *S. clavuligerus* strains were maintained on either MYM [42] or ISP-4 (Difco) medium. AS-1 medium [43] supplemented with 10 mM $MgCl_2$ was used in the isolation of *S. clavuligerus* exconjugants. Trypticase soy broth supplemented with 1% soluble starch (TSBS) was used to grow *S. clavuligerus* cultures for seeding and plasmid/chromosomal DNA isolation. Soy fermentation medium, composed largely of soy flour and starch, was used to assay antibiotic production [12]. *S. lividans* was maintained on either R5 or MYM medium, and grown in TSB liquid culture to isolate plasmid DNA [41]. *Streptomyces* plasmid-bearing cultures were supplemented with apramycin (50 μg ml^{-1} for *S. lividans* and 25 μg ml^{-1} for *S. clavuligerus*), neomycin (60 μg ml^{-1} for *S. lividans* and 50 μg ml^{-1} for *S. clavuligerus*), or thiostrepton (50 μg ml^{-1} for *S. lividans* and 5 μg ml^{-1} for *S. clavuligerus*). All *Streptomyces* spp. cultures were propagated at 28°C on a rotary shaker at 250 rpm.

Escherichia coli DH5α (GIBCO BRL) and XL1-Blue (Stratagene) were used as host strains for cloning, and were grown on Luria broth (LB)

and maintained on LB agar medium [44]. *E. coli* ER1447 provided by Dr. R. Losick (Harvard University, Cambridge, MA) was used to isolate nonmethylated plasmid DNA for transformation into *Streptomyces* spp. *E. coli* ET12567 (pUZ8002) was used for intergeneric conjugation of plasmid/cosmid DNA into *Streptomyces* spp [41].

DNA Manipulation and Southern Hybridization

Standard procedures, including transformation, restriction analysis, blunting of fragments, subcloning, and labeling with [α-^{32}P]dCTP by nick translation, were used for manipulating plasmid DNA isolated from *E. coli* [44]. Southern hybridization and washing of membranes were carried out at 65°C, as previously described [44]. Plasmid and chromosomal DNA was isolated from *Streptomyces* spp. by previously described procedures [41]. Preparation and transformation of *S. lividans* [41] and *S. clavuligerus* [45] protoplasts were performed as described previously. Unmethylated plasmid DNA isolated from *E. coli* ER1447 was passaged through *S. lividans* before transformation into *S. clavuligerus*. Introduction of cosmid DNA into *S. clavuligerus* by conjugation was carried out as described previously [18].

PCRs using oligonucleotide primers listed in Table 2 were carried out using the Expand high-fidelity PCR system according to the manufacturer's instructions (Roche).

DNA Sequence Analysis

The cosmids p53, from a pFD666-based [46] library containing *S. clavuligerus* chromosomal DNA (Jensen et al. unpublished data), and 10D7 [19] were used to isolate DNA fragments to extend the sequence of the clavam cluster beyond the previously sequenced *cvm3* and *cvm6* genes. The region of the *S. clavuligerus* chromosome downstream of *oat1* [18] from the paralog gene cluster was also sequenced. The plasmids pO4H4 and pO4H5 [16] were used to obtain the sequence of the 3′-end of *cvm6P*. A 5.24 kb *Nco*I fragment from the cosmid 14E10 [16] was sequenced using universal and custom primers to yield the 5′-end of *cvm6P* as well as *cvm7P*. Some sequence information was also obtained directly using cosmid 14E10 as template to complete the double-stranded DNA sequence of *cvm6P* and *cvm7P*.

DNA sequencing was carried out by the Molecular Biology Service Unit, University of Alberta (AB, Canada). ORFs were predicted based on codon preference with the online program Frame Plot 2.3.2 (http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl) and similarity searches were performed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Preparation of Mutants

Mutants with defects in genes from the region surrounding the clavam cluster were prepared either by insertion of antibiotic resistance cassettes into appropriate restriction endonuclease sites within cloned copies of the genes, or, more recently, by PCR-targeted mutagenesis [28]. Genes mutated by the former procedures include *cvm3* (*apr* inserted into an *Nco*I site 140 bp from the start codon), *cvm4* (*neo* inserted into a *Bcl*I site 284 bp from the start codon), *cvm5* (*apr* inserted into a *Bsa*BI site 772 bp from the start codon), *cvm6* (*apr* inserted into a *Bst*EII site 158 bp from the start codon), *cvm7*-5′ end (*apr* inserted into a *Cla*I site 130 bp from the start codon), *cvm7*-3′ end (*apr* inserted into an *Nco*I site 2064 bp from the start codon), *cvm9* (*apr* inserted in the *Pfl*MI site 234 bp from the start codon), *cvm10* (*apr* inserted in the *Xcm*I site 239 bp from the start codon), and *cvm11* (*apr* inserted in the *Nco*I site 344 bp from the start codon). In each case, once the subcloned gene had been disrupted within an *E. coli*-based plasmid, the disrupted gene was either transferred to the *Streptomyces*-based plasmid, pIJ486, or the entire plasmid construct was fused to pIJ486 to create a shuttle vector. The disruption constructs were then transformed into *S. clavuligerus*, where the wild-type gene was replaced by the disrupted allele, as described previously [11].

The *cvmP*, *cvm2*, *cvm12*, *cvm13*, and *cvmG* mutants were prepared, and creation of a *cvmH* mutant was attempted, with the cosmid p53 and the previously described PCR-targeting method [28]. Due to structural instability of cosmid p53 and its derivatives, fragments carrying the disrupted genes were subcloned into pUWL-KS [47] for conjugation into *S. clavuligerus* to generate mutants.

Mutants defective in *cvm6P* were prepared using an approximately 1 kb EcoRI/PstI fragment encoding *neo*, isolated from pFDNeo-S [46]. This fragment was inserted into a subcloned copy of *cvm6P*, 708 bp from the start codon, in place of a native *Eco*NI fragment. The *neo*-disrupted *cvm6P* was transferred into the shuttle vector pUWL-KS, which was then transformed into *S. clavuligerus*.

S. clavuligerus cvm7P mutants were prepared using PCR targeting in cosmid 14E10 [28]. All of the mutants prepared in this study were verified by Southern analysis and/or PCR.

HPLC, MS, Bioassays, and Growth Assays

Supernatants or solid media extracts from *S. clavuligerus* cultures grown on soy medium were analyzed by HPLC to measure clavulanic acid and 5*S* clavam production using previously described conditions [48]. When novel peaks were observed, culture supernatants were also analyzed by LC-MS using conditions described previously [12]. Soft ESI-MS analysis to obtain fragmentation data for unknown identification was performed as described previously [7].

Bioassays were also used to monitor clavulanic acid, alanylclavam, and cephamycin C production under previously described conditions [12].

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Accession Numbers

The nucleotide sequence information for the clavam and the paralog gene clusters has been appended to existing files deposited in GenBank under accession numbers AF124928, AF124929, and AY426768, respectively.

