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Five Additional Genes Are Involved in Clavulanic Acid Biosynthesis in *Streptomyces clavuligerus*

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An approximately 12.5-kbp region of DNA sequence from beyond the end of the previously described clavulanic acid gene cluster was analyzed and found to encode nine possible open reading frames (ORFs). Involvement of these ORFs in clavulanic acid biosynthesis was assessed by creating mutants with defects in each of the ORFs. *orf12* and *orf14* had been previously reported to be involved in clavulanic acid biosynthesis. Now five additional ORFs are shown to play a role, since their mutation results in a significant decrease or total absence of clavulanic acid production. Most of these newly described ORFs encode proteins with little similarity to others in the databases, and so their roles in clavulanic acid biosynthesis are unclear. Mutation of two of the ORFs, *orf15* and *orf16*, results in the accumulation of a new metabolite, *N*-acetylglycylclavaminic acid, in place of clavulanic acid. *orf18* and *orf19* encode apparent penicillin binding proteins, and while mutations in these genes have minimal effects on clavulanic acid production, their normal roles as cell wall biosynthetic enzymes and as targets for β-lactam antibiotics, together with their clustered location, suggest that they are part of the clavulanic acid gene cluster.

Among prokaryotes, the *Streptomyces* spp. are unusual for both their morphological and biochemical versatility. When these filamentous soil bacteria grow on solid substrates, they exhibit a complex life cycle which progresses from spores through vegetative and aerial mycelia and back to spores. Often, coincident with these morphological changes, the organisms produce a diverse array of exocellular enzymes and secondary metabolites.

Streptomyces clavuligerus is notable in this regard for its ability to produce β-lactam-type compounds. In particular, it is the species used industrially for the production of clavulanic acid, a multi-billion-dollar/annum product useful for its β-lactamase inhibitory activity. While the biosynthesis of clavulanic acid has been the subject of intense investigation in recent years, the details of its production are still not completely worked out.

Previously Jensen et al. characterized an approximately 15-kb stretch of chromosomal DNA located adjacent to the cephamycin gene cluster in *S. clavuligerus* and demonstrated that the open reading frames (ORFs) were involved in clavulanic acid production (12). Genes located in this region include *ceaS* (encoding carboxyethylarginine synthase) (14), *bls* (β-lactam synthetase) (3, 18), *pah* (proclavaminate amidinohydrolase) (2, 11, 34), *cas2* (clavaminate synthase) (4, 17, 26, 27), and *cad* (clavulanic acid dehydrogenase) (21), all enzymes for recognized steps in the biosynthetic pathway. In addition, *oat*, a gene encoding a protein with ornithine acetyltransferase activity, has been identified (13), but its role in clavulanic acid is not yet understood, and *claR*, a gene encoding a transcriptional regulator that controls the late steps in clavulanic acid biosynthesis, is also located in this region (22, 24). Two additional genes, *orf7* and *orf10*, encode an apparent peptide transport protein and a cytochrome P450 type protein, respectively (11, 12), as judged by BLAST analysis. While both have been shown to be essential for clavulanic acid biosynthesis, their precise roles are unclear.

Genes within this region account for all of the early steps and for the final step in clavulanic acid biosynthesis (Fig. 1). However, the late step(s) of the pathway from clavaminic acid to clavaldehyde remains unaccounted for. While it has been suggested that the cytochrome P450 protein encoded by *orf10* might carry out this conversion (16), no demonstration of activity in this regard has followed. Furthermore, it is not clear that a single protein could accomplish both the inversion of stereochemistry of the clavam nucleus and the side chain modifications at C2 that this step involves. Since other candidate genes encoding enzymes capable of bringing about these changes were not obvious within this 15-kb region, several groups, including our own, looked beyond the end of the known clavulanic acid biosynthetic gene cluster for additional genes involved in biosynthesis of this important metabolite.

Recently, Li et al. (16) provided evidence for two additional genes, *orf11* and *orf12*, extending the clavulanic acid gene cluster; they encode an apparent ferredoxin and a protein showing some similarity to β-lactamases, respectively. While the ferredoxin is presumed to work in concert with the cytochrome P450 encoded by *orf10*, the role of the β-lactamase-like protein is again unclear, although gene disruption showed it to be essential for clavulanic acid biosynthesis. Similarly, Mellado et al. (19) provided additional sequence information giving evi-

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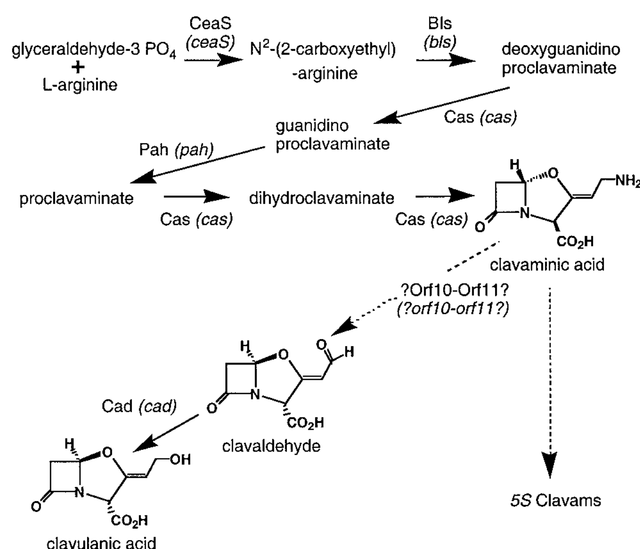


FIG. 1. The biosynthetic pathway leading to clavulanic acid and the *5S* clavams.

dence for the presence of *orf13* to *orf19* in this region. Through gene disruption they demonstrated an involvement of *orf14* in clavulanic acid biosynthesis, but the rest of the ORFs remain uncharacterized. While it is not immediately apparent from BLAST similarity searches how any of the ORFs would be involved in the clavaminate to clavaldehyde conversion, it is also not clear what type of ORFs would be expected for this conversion. Furthermore, the close spacing of the ORFs does suggest operon arrangements indicating that the genes might be involved in related processes. Here we describe a mutational analysis of *orf11* to *orf19* and provide evidence to suggest that all of these ORFs, with the possible exception of *orf18* and *orf19*, are part of the clavulanic acid biosynthetic gene cluster.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* strains were maintained on Luria broth (28) agar plates and grown in Luria broth liquid medium at 37°C. Plasmid-bearing cultures were supplemented with ampicillin (100 μg/ml) or apramycin (25 μg/ml) as appropriate.

S. clavuligerus wild-type and mutant strains were maintained on either MYM agar (29) or ISP #4 medium (Difco) at 28°C, except for *orf18*::*apr* mutants of the *S. clavuligerus* p*gyl-orf18* strain, which were maintained on ISP #4 supplemented with 1% glycerol. For assessment of metabolite production, cultures were grown as previously described (20). Briefly, glycerol spore stocks of *S. clavuligerus* were used to inoculate Trypticase soy broth–1% soluble starch seed medium. Seed cultures (40 to 48 h) were used to inoculate both starch asparagine (SA) and soy flour (Soy) media to 2% (vol/vol), and samples were removed at 72 and 96 h for analysis. All cultures were incubated at 28°C and 250 rpm. Representative mutants were grown in duplicate cultures, and all experiments were repeated at least twice in two separate laboratories. Plasmid-carrying cultures were supplemented with apramycin (25 μg/ml), thiostrepton (5 μg/ml), or neomycin (50 μg/ml) as appropriate.

DNA manipulations. Plasmid and genomic DNA preparations from *S. clavuligerus* were isolated using standard techniques (15). Plasmid DNA was typically introduced into *Streptomyces* spp. by transformation of protoplasts. Protoplasts were prepared and used for transformation procedures as described previously (23).

Where indicated, plasmids were also introduced into *S. clavuligerus* spores through the use of the conjugation procedure described by Kieser et al. (15), with *E. coli* ET12567 (pUZ8002) as the donor strain. Exconjugants were isolated on AS-1 (5) (with appropriate antibiotic selection) rather than on soy-mannitol medium.

Plasmid DNA isolation from *E. coli* cultures, restriction endonuclease digestions, ligations, PCRs, Southern analyses, and transformations of *E. coli* were all performed using standard techniques (28).

DNA sequence analysis. Cosmid clone K6L2, which carries an approximately 30-kb fragment of *S. clavuligerus* chromosomal DNA in the vector pLAFR3 (2), was digested with *Pst*I and *Eco*RI to generate a unique DNA fragment of 11.2 kb. This fragment, now known to extend from the beginning of *orf10* to near the 5′ end of *orf18* in the clavulanic acid gene cluster, was subcloned into plasmid pTZ18R to yield plasmid pCEC001. Restriction analysis of K6L2 indicated that a 2.8-kb *Pst*I-*Bam*HI fragment is located adjacent to the 11.2-kb *Pst*I-*Eco*RI fragment. This 2.8-kb *Pst*I-*Bam*HI fragment, now known to contain the 5′ end of *orf18* and all of *orf19*, was subcloned from cosmid K6L2 into pUC119 to yield plasmid p667-3PB. The DNA sequence for part of the pCEC001 insert was already known from previous studies (12). The DNA sequence for the remaining part, and for the entire insert of p667-3PB, was determined essentially as described previously (12). Subclones generated by restriction endonuclease digestion as well as ordered sets of deletion subclones were sequenced from universal primers. Sequence-specific oligonucleotide primers were used for regions which were not obtained from analyses using universal primers. All sequence information was verified on both strands, and the arrangement of restriction fragments was confirmed by obtaining sequence information for cross-fragment junctions. Sequence analyses were performed using a DYEnamic ET Terminator cycle sequencing kit (Amersham Pharmacia, Baie d'Urfe, Quebec, Canada) by the Molecular Biology Service Unit, University of Alberta.

Gene disruption of *orf11* to *orf19*. The region of the *S. clavuligerus* chromosome encompassing *orf10* to *orf19* is represented diagrammatically in Table 2, and restriction sites of relevance to the gene disruption studies are indicated. In preparing the disruption constructs described below, whenever DNA fragments with incompatible ends were ligated they were first made blunt by digestion with either the Klenow fragment of DNA polymerase I or with T4 DNA polymerase.

Disruption of *orf11*. A 2.2-kb *Sph*I fragment of *S. clavuligerus* DNA which extends from near the 3′ end of *orf10* to within *orf13* was subcloned into pBluescript II SK(+).

The resulting plasmid was linearized by digestion with *Bgl*II at the unique site centrally located in *orf11* and ligated to a 1.45-kb *Nco*I fragment carrying the apramycin resistance gene (*apr*^{r}) from pUC120apr (23). The disrupted *orf11* gene (*apr*^{r} in the same orientation as *orf11*) was then excised as a *Bam*HI-*Hin*dIII fragment and inserted into the *Streptomyces* vector pIJ486 to give pLOG221. pLOG221 was transformed through *S. lividans* and into wild-type *S. clavuligerus*. Apramycin- and thiostrepton-resistant transformants were subjected to two rounds of sporulation on solid ISP #4 medium with no antibiotic supplementation and then replica plated onto antibiotic-supplemented medium. The presence of putative gene replacement mutants (apramycin resistant and thiostrepton sensitive) was confirmed by Southern analysis.

Disruption of *orf12*. A 1.8-kb *Nru*I-*Sph*I fragment of *S. clavuligerus* DNA which extends from just downstream of *orf11* to within *orf13* was subcloned into pBluescript II SK(+). The resulting plasmid was linearized at the *Bst*EII site located within *orf12* and ligated to the *apr*^{r} cassette. The disrupted *orf12* gene (*apr*^{r} in the orientation opposite to *orf12*) was transferred to pIJ486 as a *Bam*HI-*Hin*dIII fragment to yield pLOG240. pLOG240 was transformed through *S. lividans* and into wild-type *S. clavuligerus*, after which putative gene replacement mutants were selected as described above and then confirmed by Southern analysis.

Disruption of *orf13*. A 2.9-kb *Bst*EII fragment of *S. clavuligerus* DNA carrying part of *orf12*, all of *orf13* and *orf14*, and the 3′ end of *orf15* was subcloned into pBluescript II SK(+). The resulting plasmid, pCEC028, was linearized at the unique *Nru*I site within *orf13* and ligated to the *apr*^{r} cassette. pCEC028 was linearized with *Hin*dIII and ligated to *Hin*dIII-digested pIJ486 to give a shuttle plasmid, pCEC047 (*apr*^{r} in the orientation opposite to *orf13*). pCEC047 and the transformation protocol described for *orf11* were used to generate mutants of *S. clavuligerus*.

Disruption of *orf14*. pCEC028 was linearized by digestion at the unique *Bal*I site located within *orf14* and ligated to the *apr*^{r} cassette. The resulting plasmid was fused to pIJ486 at the *Hin*dIII site to give a shuttle plasmid, pCEC046 (an *apr*^{r} fragment in the same orientation as *orf14*), and pCEC046 and the transformation protocol described for *orf11* were used to generate mutants of *S. clavuligerus*.

Disruption of *orf15*. A 4-kb *Nru*I fragment of *S. clavuligerus* DNA carrying part of *orf13*, all of *orf14* and *orf15*, and part of *orf16* was subcloned into pBluescript II SK(+) to generate pCEC004. pCEC004 was digested with *Sac*I and *Xba*I and religated to delete the *Bst*XI site from the multiple cloning site. The resulting plasmid was linearized at the remaining *Bst*XI site within *orf15*, ligated to the *apr*^{r}

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	Relevant features[a]	Source or reference
Strains		
S. clavuligerus		
NRRL 3585	Wild type; cephamycin and clavulanic acid producer	NRRL
E. coli		
XLI-blue	General cloning host	Stratagene
ET12567(pUZ8002)	Methylation deficient; transfer functions from pUZ8002	15
Plasmids		
Cloning vectors		
pBluescript II KS(+)	*E. coli* general cloning vector, Amp^r	Stratagene
pSL1180	*E. coli* general cloning vector, Amp^r	Pharmacia
pTZ18R	*E. coli* general cloning vector, Amp^r	US Biochemicals
pUC119	*E. coli* general cloning vector, Amp^r	32
pSET152	*E. coli* replicon, *Streptomyces* φC31 attachment site, apt^r	NRRL
pCR 2.1-TOPO	*E. coli* vector for cloning PCR products, Amp^r	Invitrogen
pJV326	*par*-defective, *oriT*-containing, *Streptomyces-E. coli* shuttle	9
pIJ486	*Streptomyces* pIJ101 replicon, Tsr^r	33
pMT3226	pSET152 derivative, *xylE* controlled by *gyl*P_1P_2 promoters	10, 31
Antibiotic resistance cassettes		
pUC120apr	Apr^r cassette in pUC120	23
pSKNeo	Neo^r cassette in pBluescript II KS(+)	20
Intermediate constructs		
K6L2	*E. coli* cosmid carrying *S. clavuligerus* genomic DNA	2
p667-3PB	middle of *orf18* to end of *orf19* in pUC119	This study
pCEC001	Start of *orf10* to middle of *orf18* in pTZ18R	This study
pCEC004	Middle of *orf13* to middle of *orf16* in pBluescript II SK(+)	This study
pCEC028	Middle of *orf12* to end of *orf15* in pBluescript II SK(+)	This study
pCEC062	End of *orf17* to middle of *orf18* in pBluescript II KS(+)	This study
Gene disruption constructs		
pLOG221	*orf11*::*apr* disruption construct	This study
pLOG 240	*orf12*::*apr* disruption construct	This study
pCEC046	*orf14*::*apr* disruption construct	This study
pCEC047	*orf13*::*apr* disruption construct	This study
pCEC063	*orf15*::*apr* disruption construct	This study
pCEC068	*orf16*::*apr* disruption construct	This study
pCEC076	*orf17*::*apr* disruption construct	This study
pCEC085	*orf18*::*neo* disruption construct	This study
pCEC086	*orf15*-Δ*fs* replacement construct	This study
pCEC089	*orf19*::*apr* disruption construct	This study
pCEC179	Δ*orf16* replacement construct	This study
Gene insertion construct		
pMT8.34	p*gyl*::*orf18* construct in pSET152	This study

[a] Abbreviations: Amp, ampicillin; Apr, apramycin; Neo, neomycin; Tsr, thiostrepton.

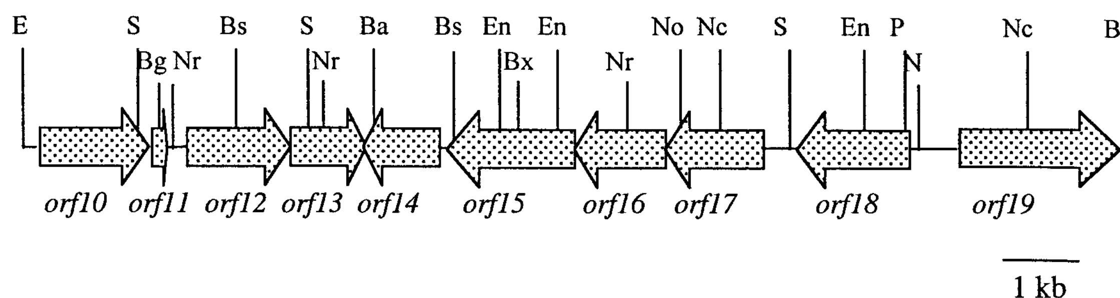
cassette, and then fused to pIJ486 at the *Hin*dIII site. The resulting shuttle plasmid, pCEC063 (*apr*^{r} in the orientation opposite to *orf15*), and the transformation protocol described for *orf11* were used to generate mutants of *S. clavuligerus*.

A second version of the *orf15* mutant was prepared in which the *apr*^{r} cassette was removed and replaced by a deletion frameshift mutation. To prepare these *orf15*-Δ*fs* mutants, pCEC004 was linearized by digestion with *Eco*NI, which removes a 0.75-kb fragment from within *orf15*. The 0.75-kb *Eco*NI fragment encompasses the *Bst*XI site that is the site of the disruption in the original *orf15*::*apr* mutant. The linearized plasmid was made blunt and then religated, generating a deletion frameshift mutation in *orf15*. The resulting plasmid was fused to pIJ486 at the *Sac*I site to give the shuttle plasmid, pCEC086. pCEC086 was transformed through *S. lividans* and into the *orf15*::*apr* mutant. Since the desired *orf15*-Δ*fs* mutants lack a selectable marker, presumptive mutants were chosen on the basis of their loss of apramycin resistance after sporulation on nonselective medium; the mutations were confirmed by Southern analysis.

Disruption of *orf16*. A 2.7-kb *Bst*XI-*Nco*I fragment of *S. clavuligerus* DNA extending from the middle of *orf15* to the middle of *orf17* was subcloned into pSL1180. The resulting plasmid was linearized by digestion at the unique *Nru*I site within *orf16*, ligated to the *apr*^{r} cassette, and then fused to pIJ486 at the *Hin*dIII site. The resulting shuttle plasmid, pCEC068 (*apr*^{r} in the same orientation as *orf16*), and the transformation protocol described for *orf11* were used to generate mutants of *S. clavuligerus*.

A second version of the *orf16* mutant was prepared in which the *apr*^{r} cassette was removed and replaced by a deletion spanning most of *orf16*. To prepare these Δ*orf16* mutants, two 1-kb fragments from the upstream and downstream flanks of *orf16* were amplified by the PCR. The upstream fragment, PCR I, included DNA sequence encoding the first 14 amino acid residues of *orf16* and was generated using primers 5′-CCCCGACCCCGAGGCCGTGG and 5′-*ATCGAT*CGGCAGCGCGTCCGTCAC (italicized residues represent a *Cla*I digestion site introduced for cloning). The downstream fragment, PCR II, included a DNA sequence encoding the last 13 amino acid residues and the stop codon of *orf16* and was generated using primers 5′-GCGCGGCGTACGTGCACATTG and 5′-*ATCGAT*GGCACCACCGACACCGATCAGA (italicized residues represent a *Cla*I digestion site introduced for cloning). Both PCR I and PCR II fragments were cloned into pCR2.1-TOPO through the use of a TOPO TA cloning kit (Invitrogen) and sequenced to confirm fidelity. The two fragments were then assembled, and the resulting 2-kb PCR I-PCR II fragment was transferred into the partition-defective *E. coli-Streptomyces* shuttle vector, pJV326, to yield pCEC179. pCEC179 was then transformed into *E. coli* ET12567 (pUZ8002) and from there introduced into the *S. clavuligerus orf16*::*apr* mutant by conjugation. Thiostrepton-resistant exconjugants were subjected to two rounds of sporulation on ISP #4 medium with no antibiotic supplementation, and presumptive mutants were chosen on the basis of their loss of both apramycin and thiostrepton resistance after sporulation on nonselective medium. The identity of the mutants was confirmed by Southern analysis.

TABLE 2. Characteristics and arrangement of the newly described ORFs from the clavulanic acid gene cluster[a]



ORF	Highest similarity[b] (GenBank accession no.); % identity	Start-stop coordinates[c] (bp)	Length[c] (bp)	Gap before next ORF (bp)	Direction
orf11	*S. griseolus* ferredoxin (P18325); 46% over 64 aa[d]	Incomplete–128	128	233	→
orf12	*S. cattleya* β-lactamase (CAD18987); 43% over 251 aa	362–1,738	1,377	−4	→
orf13	*Shigella flexneri* amino acid metabolite efflux pump (NP707443); 42% over 289 aa	1,735–2,757	1,023	−49	→
orf14	*Deinococcus radiodurans* acetyltransferase[e] (NP285343); 31% over 352 aa	2,709–3,728	1,020	93	←
orf15	*S. coelicolor* oligopeptide binding lipoprotein (NP629613); 39% over 555 aa	3,822–5,510	1,689	−4	←
orf16	*Bradyrhizobium japonicum* hypothetical protein (NP771774); 26% over 228 aa	5,507–6,712	1,206	−4	←
orf17	*Rhizobium* sp. carboxylase[e] (NP444046); 28% over 419 aa	6,709–8,022	1,314	272	←
orf18	*S. coelicolor* PBP (NP628195); 76% over 495 aa	8,295–9,779	1,485	606	←
orf19	*S. avermitilis* PBP (NP826635); 74% over 710 aa	10,386–12,539	2,154	56+	→

[a] The top panel represents the ORFs found in the inserts from pCEC001 and p667-3PB. B, *Bam*HI; Ba, *Bal*I; Bg, *Bgl*II; Bs, *Bst*EII; Bx, *Bst*XI; E, *Eco*RI; En, *Eco*NI; N, *Nae*I; Nc, *Nco*I; No, *Not*I; Nr, *Nru*I; P, *Pst*I; S, *Sph*I. Only sites mentioned in the text are shown.
[b] Protein with highest level of similarity to ORF-encoded protein.
[c] Includes stop codon.
[d] aa, amino acids.
[e] Protein was annotated as a hypothetical protein, but conserved domains were detected, suggesting that the protein was an acetyltransferase *(orf14)* or carboxylase *(orf17)*.

Disruption of *orf17*. An approximately 3-kb *Not*I-*Pst*I fragment of *S. clavuligerus* DNA including most of *orf17* and *orf18* was subcloned into pBluescript II KS(+) to give pCEC062. pCEC062 was subjected to partial digestion with *Nco*I to obtain a singly cut plasmid as the major digestion product. DNA fragments obtained upon partial *Nco*I digestion were then ligated with the *apr*[r] cassette to give plasmids in which *apr*[r] had inserted into one of the three *Nco*I sites present on the plasmid. Screening by restriction analysis identified one plasmid that contained *apr*[r] inserted at the *Nco*I site within *orf17* (in the orientation opposite to *orf17*), and that plasmid was fused to pIJ486 at the *Hin*dIII site. The resulting shuttle plasmid, pCEC076, and the transformation protocol described for *orf11* were used to generate mutants of *S. clavuligerus*.

Disruption of *orf18*. pCEC062 was linearized by digestion at the unique *Eco*NI site within *orf18* and ligated to the neomycin resistance gene cassette (*neo*[r]) isolated from pSKNeo (20) as a 1-kb *Acc*I fragment. The resulting plasmid was fused to pIJ486 at the *Sst*I site to give the shuttle plasmid, pCEC085 (*neo*[r] in the orientation opposite to *orf18*). pCEC085 was transformed through *S. lividans* and into *S. clavuligerus* as described for *orf11*. Despite repeated attempts, no *orf18* mutants were isolated.

For an alternative host strain in which to attempt the disruption of *orf18*, an additional copy of *orf18* under the transcriptional control of the glycerol-inducible promoter was introduced into the *S. clavuligerus* chromosome to create a strain diploid with respect to *orf18*. To accomplish this, the pSET152-based vector pMT3226 (10, 31) was digested with *Bam*HI and *Xba*I to release the *xylE* gene and a 1.6-kb *Sph*I-*Nae*I fragment of *S. clavuligerus* DNA extending from 39 bp upstream to 109 bp downstream of *orf18* was inserted in its place. The resulting plasmid, pMT8.34, carries the *orf18* gene positioned in the correct orientation just downstream from the *gyl*P_1P_2 promoters. pMT8.34 was then introduced into wild-type *S. clavuligerus* by transformation, and apramycin-resistant transformants were analyzed by PCR to confirm that the plasmid had integrated at the ΦC31 attachment site rather than by recombination with the native *orf18* gene. The resulting diploid strain was designated the p*gyl*::*orf18* strain.

pCEC085 was then transformed into the p*gyl*::*orf18* strain with selection for neomycin resistance, and putative mutants were isolated from transformants after two rounds of sporulation on nonselective ISP #4 medium supplemented with 1% glycerol. Southern analysis was carried out on these putative disruptants to determine whether the *neo*[r] cassette had disrupted the native copy of *orf18* or the ectopic p*gyl*::*orf18*.

Disruption of *orf19*. A 1.6-kb DNA fragment internal to *orf19* was amplified by PCR using oligonucleotides 5′-GCATA*GGATCC*GGATACGGCTCGTGGTC GTCCAGATACTGG (italicized residues represent a *Bam*HI digestion site introduced for cloning) and 5′-GCATC*GAATTC*GAGGGCCGCGTAGATCGT CGCCATCTGGATG (italicized residues represent a *Eco*RI digestion site introduced for cloning) as primers. The PCR product was cloned into pSL1180 and then linearized by digestion at the unique *Nco*I site located midway through the *orf19* fragment and ligated to the *apr*[r] cassette. The resulting plasmid was fused to pIJ486 at the *Eco*RI site, and the resulting shuttle plasmid, pCEC089 (*apr*[r] in the same orientation as *orf19*), and the transformation protocol described for *orf11* were used to generate mutants of *S. clavuligerus*.

HPLC and mass spectrometric analyses. Culture filtrates were routinely analyzed for the presence of clavulanic acid and the other clavam metabolites by high-pressure liquid chromatography (HPLC) after imidazole derivatization, as described previously (20). In those cases in which new clavam metabolites were detected, the imidazole-derivatized culture supernatants were reanalyzed by an alternative HPLC procedure employing a volatile buffer system; effluents were then analyzed by electrospray ionization mass spectrometry. Derivatized samples (5 μl) were analyzed on an XTerra column (Waters Scientific, Milford, Mass.) (0.21 by 10 cm) at a flow rate of 0.25 ml/min. The mobile phase consisted of solvent A (10 mM ammonium bicarbonate, pH 10) and solvent B (acetonitrile) used in a binary gradient system as follows: 100% solvent A for 5 min, linear

TABLE 3. Differences between newly determined sequence and published sequence

orf	Location in new sequence[a] (nt)	Comparison of sequences from indicated sources	
		Published[b]	This work[b]
16	6374–6382	GGCCCCGGG *A G P*	GGCCTCGGG *A E P*
16	6590–6598	GAGAGCCGG *L A P*	GAGATCCGG *L D P*
17	6853–6861	GCGCTTAAG *R K L*	GCGCTGGAG *R Q L*
17	6865–6873	CTTGACGCC *D V G*	CTTGTCGCC *K D G*
19	10521–10538	TAC CGC AGA AGG CCG TCG V R R R P S	TAC GCG GAG AAG GCG TCG V A E K A S

[a] New DNA sequence deposited in Genbank under accession number AY258009.
[b] Published sequence refers to the work of Mellado et al. (19). Changes to the DNA or amino acid sequences are shown underlined. Amino acid sequences shown in italics are from the reverse complement of the DNA sequence shown.

gradient to 85% solvent A over 10 min, 85% solvent A for 5 min, linear gradient to 100% solvent A over 1 min, and 100% solvent A for 9 min. Eluant was monitored at 311 nm to detect the imidazole derivatives, and mass spectra were acquired using a ZMD-2 single-quadrupole instrument (Waters Scientific).

Bioassays. In an indirect bioassay for β-lactamase inhibitors, clavulanic acid was detected using *Klebsiella pneumoniae* ATCC 29665 as the indicator organism growing on Trypticase soy agar (Becton Dickinson) containing penicillin G at 6 μg/ml. Alanylclavam was detected by a bioassay using *Bacillus* sp. ATCC 27860 as the indicator organism growing on a defined agar medium (25). Cephamycin C was detected in culture filtrates by a bioassay using the indicator organism *E. coli* ESS (1) growing on Trypticase soy agar.

Nucleotide sequence accession number. The nucleotide sequence of this region of the *S. clavuligerus* chromosome has been deposited in GenBank (accession number AY258009).

RESULTS

DNA sequence information from the clavulanic acid gene cluster. Previously, we reported the DNA sequence for an approximately 15-kb region of the *S. clavuligerus* chromosome (GenBank accession number AF205427) encoding a series of structural genes involved in clavulanic acid production. While these genes accounted for many of the expected activities, it was not clear that all of the genetic information needed to form clavulanic acid was present in this region. In view of the possibility that additional genes might be required for the production of clavulanic acid, we investigated the DNA sequence of the region beyond this already reported area. Beginning immediately adjacent to the sequence information reported previously (12), we have determined an additional 12,595 bp of DNA sequence ending at a *Bam*HI site. The previously deposited sequence information terminated partway through what is now recognized as *orf11.* Examination of the new DNA sequence information indicates the presence of the rest of *orf11* as well as of eight additional complete open reading frames, *orf12* to *orf19* (carrying on with the numbering established for the previous sequence information) (Table 2). Two of these ORFs (*orf11* and *orf12*) have been reported previously by Li et al. (16). Recently, Mellado et al. (19) also reported additional DNA sequence information extending from *orf10* through *orf18* and into *orf19*.

The sequence information presented in this study covers largely the same region as that reported by Mellado et al. but extends further to give the complete sequence for *orf19*. With the benefit of their published sequence information for comparison, we were able to identify several areas where our sequence data differed. These differences are noted in Table 3. Careful reexamination of our data in these regions did not show any ambiguous or unclear bases, and so these may represent differences between isolates or they may represent sequencing errors. None of these sequence discrepancies affected the reading frame of the putatively encoded proteins, but they did result in a number of amino acid changes. Analysis of the sequenced region by database searching allowed us to recognize the same similarities between the proteins encoded by *orf11* to *orf19* and other sequenced proteins reported by Mellado et al. (Table 2) (19). However, we did choose different start codon possibilities for several of the ORFs in the region, resulting in different sizes for the encoded proteins (Table 2). Because of the presence of a more convincing ribosome binding site, we designated the ATG codon at nucleotide (nt) 362 the start of *orf12*, making the ORF 1,377 bp in length (including the stop codon), 24 bp shorter than that suggested by Mellado et al. The predicted ORF12 protein shows only limited similarity to other proteins in the databases, and no useful similarities which could help to clarify the start codon were noted in the extreme 5′ end of the ORF. Similarly, we chose a different start site for *orf16* (nt 6712), making the gene 1,206 bp in length, 24 bp longer than that suggested by Mellado et al., and providing a 4-bp overlap and presumed translational coupling with *orf17*. This is consistent with the Frame analysis of the region (http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl) (Fig. 2), which shows an abrupt transition from *orf16* to *orf17* indicative of translational coupling with no intergenic region. We also chose a different start site for *orf17* (nt 8022), making the gene 1,314 bp in length, 276 bp shorter than that suggested by Mellado et al., and introducing an intergenic region in place of the proposed 4-bp overlap. Once again this change is consistent with the Frame analysis of the region, which shows clear evidence of an intergenic region between the two genes (Fig. 2). In addition, examination of the amino acids that would be encoded by this otherwise intergenic region shows a predominance of arginine and alanine residues quite unlike that seen with the rest of the ORF17 protein (data not shown). While the ultimate determination of start codons for the various ORFs will require biochemical analyses, the existence of these

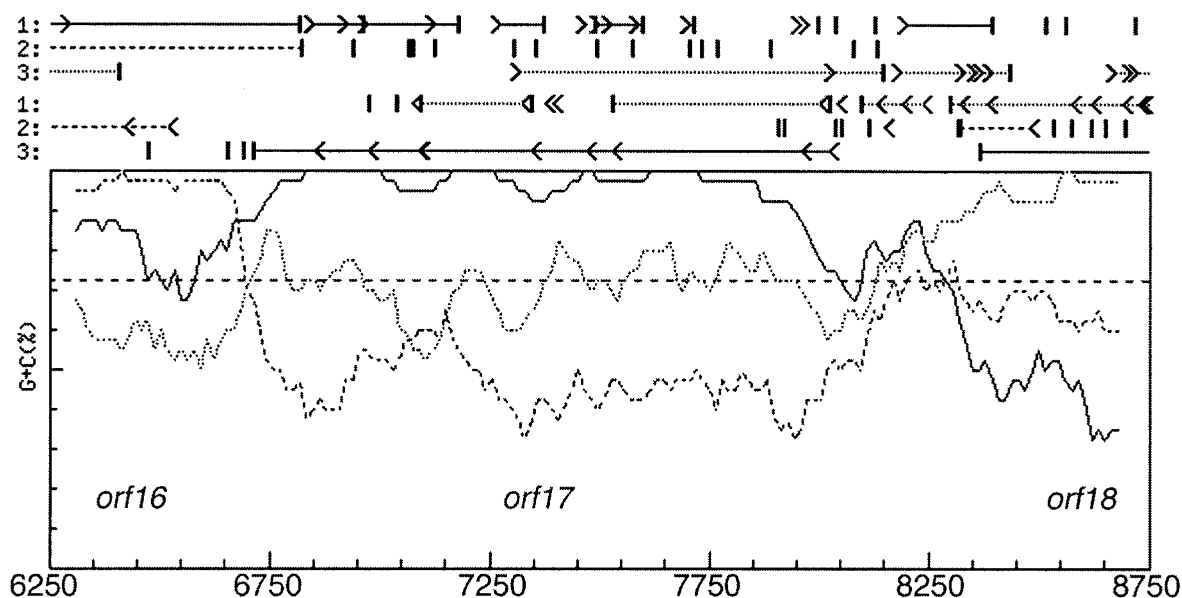


FIG. 2. Frame analysis of the *orf16-orf18* region. nt 6250 to 8750 of the deposited sequence were subjected to Frame analysis. Frame analysis examines percentages of G+C content of DNA as a function of codon position and is useful for the prediction of protein coding regions in organisms with genomic DNA with high levels of G+C content (see http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl). Genes within organisms with genomic DNA with high levels of G+C content exhibit biased codon usage, resulting in extremely high levels of G+C distribution at the third-letter position of each codon. Lines marked with arrowheads and bars above the main graph indicate the presence of potential start and stop codons in each of the six possible reading frames.

various possible alternatives should be considered if expression studies are planned in the meantime.

In addition to the sequence information, Li et al. also presented evidence based on gene disruption mutation to indicate that *orf12* is essential for clavulanic acid production (16). Similarly, Mellado et al. presented evidence based on mutant studies suggesting that *orf14* is important but not essential for clavulanic acid production (19). Furthermore, they reported an unpublished result from Lorenzana and Liras indicating that *orf15* was essential for clavulanic acid production. No information was presented regarding the involvement of the remaining ORFs in clavulanic acid production except to note that the close spacing of the ORFs suggested an operon arrangement consistent with their involvement in clavulanic acid biosynthesis.

Targeted disruption of *orf11* to *orf19*. To assess the involvement of these newly described ORFs in clavulanic acid production, each was mutated using established procedures. In each case, the isolated gene was disrupted by insertion of an antibiotic resistance gene into the open reading frame; the disrupted gene was then introduced into the wild-type strain and allowed to replace the resident wild-type copy of the gene by homologous recombination. Presumptive mutants were initially isolated on the basis of their antibiotic resistance phenotypes and then confirmed by Southern analysis. Disruption mutants were prepared successfully for all of the ORFs except *orf18*. In each case, the size of the fragment(s) observed upon Southern analysis of the disruption mutant was consistent with the insertion of the antibiotic resistance marker at the intended site.

Clavulanic acid production by disruption mutants. Each disruption mutant was then characterized to determine the effect of the mutation on clavulanic acid production. Mutants were cultivated on both Soy and SA media, and samples were withdrawn after 72 and 96 h of incubation and assessed for metabolite production by bioassay and HPLC analysis. All mutant cultures were compared to similarly grown wild-type *S. clavuligerus* reference cultures. Effects of the mutations on clavulanic acid production are shown in Table 4.

TABLE 4. Effect of gene disruption mutations on clavulanic acid production

Mutant gene (similarity[a])	Reduction in clavulanic acid production[b] (% relative to wild type) in indicated medium	
	Soy	SA
orf11 (ferredoxin)	70–80	25–65
orf12 (β-lactamase)	100	100
orf13 (efflux pump)	75–95	75–95
orf14 (acetyltransferase)	99.5	100
orf15 (peptide binding protein)	100	100
orf16 (hypothetical protein)	100	100
orf17 (carboxylase)	100	100
orf18 (PBP)	Not done	Not done
orf19 (PBP)	0	0

[a] Protein with highest level of similarity to that encoded by indicated ORF.

[b] Representative mutants were cultured in duplicate and growth experiments were repeated on two or more occasions in two different locations. Typical clavulanic acid production levels for the wild type were 225 μg/ml in Soy medium and 20 μg/ml in SA medium. The limit of detection for clavulanic acid was about 1 μg/ml in Soy medium grown cultures.

Disruption of *orf11* resulted in a decreased ability of the mutant strains to produce clavulanic acid. When grown on SA medium, clavulanic acid levels were reduced by 25 to 65%; when grown on Soy medium, mutant strains showed a 70 to 80% reduction in clavulanic acid production. Mutants disrupted in *orf13* were severely compromised but not completely defective in their ability to produce clavulanic acid on both Soy and SA media. Levels of clavulanic acid were reduced by 75 to 95% relative to those of the wild-type control strain. Mutants disrupted in *orf14* were even more severely affected such that clavulanic acid production was at the limits of detection. However, both bioassays and HPLC analyses indicated the presence of trace amounts of clavulanic acid. We estimate that production was at about 0.5% of wild-type levels (about 0.8 to 1.0 μg/ml). In contrast, mutants with a defect in *orf19* showed no demonstrable effects on either growth or clavulanic acid production. Finally, mutants with defects in *orf12*, *orf15*, *orf16*, or *orf17* were unable to produce detectable clavulanic acid (detection limit, about 1 μg/ml) when grown on either growth medium at either of the time points tested.

Production of a new clavam metabolite by *orf15* and *orf16* mutants. *S. clavuligerus* has been shown to produce at least four other clavam metabolites, clavam-2-carboxylate (C2C), 2-hydroxymethylclavam (2HMC), 2-formyloxymethylclavam, and alanylclavam, in addition to clavulanic acid when grown on Soy medium (20). These other clavam metabolites have no β-lactamase inhibitory activity and differ from clavulanic acid in their stereochemistry and side chain modifications at C-2 and C-3. Clavulanic acid displays *3R,5R* stereochemistry in contrast to the *5S* stereochemistry of the other clavam metabolites. These other clavam metabolites will hereinafter be referred to as *5S* clavams. When imidazole-derivatized samples of Soy grown culture supernatants were examined by HPLC, the various disruption mutants were all found to produce at least some levels of the *5S* clavam metabolites. Production levels of the *5S* clavam metabolites are typically more variable than those of clavulanic acid even in the wild-type strain, making small changes in production difficult to follow (30). However, obvious changes in the levels of production of the *5S* clavam metabolites were apparent for *orf11*::*apr* mutants, which showed a 50 to 90% decrease in the production of C-2-C and 2-HMC (as detected by HPLC) and only traces of alanylclavam (as detected by bioassays). Similarly, *orf13*::*apr* mutants showed a 90 to 100% decrease in production of these *5S* clavams. The remaining mutants showed variable amounts of the *5S* clavams, but for all of the mutant types, at least some of the *5S* clavams were present even in those strains in which no clavulanic acid production could be detected.

Although neither *orf15*::*apr* nor *orf16*::*apr* mutant cultures produced any clavulanic acid, both of them showed evidence of the presence of a new metabolite when examined by HPLC. This peak was only seen in culture supernatants derivatized with imidazole, suggesting that the compound had a clavam structure. Furthermore, the UV spectrum of the new peak showed an absorption maximum at 312 nm consistent with the presence of a clavam structure (data not shown). Previously, Elson et al. (8) had investigated an uncharacterized non-clavulanic-acid-producing mutant of *S. clavuligerus* (designated *dcl8*) and found that it accumulated a new clavam compound, *N*-acetylglycylclavaminic acid (NAG-clavam). To gain additional information about the new metabolite accumulated by the *orf15*::*apr* and *orf16*::*apr* mutant cultures, culture filtrates were reanalyzed by HPLC using a volatile solvent system so that effluents could be subjected to electrospray ionization mass spectrometric analyses. Under these conditions, the new imidazole-derivatized metabolite had a retention time of 18.6 min and gave a spectrum with a parent ion of mass 322.08 and a major fragment of mass 254.09 (Fig. 3). The total mass, together with the mass of the major fragment which resulted from removal of the imidazole group, was consistent with the new peak representing NAG-clavam. When authentic NAG-clavam was subjected to HPLC and mass spectrometric analysis, it showed results with respect to retention time, UV absorption spectrum, mass, and fragmentation pattern identical to those of the unknown peak in the *orf15*::*apr* and *16*::*apr* mutants.

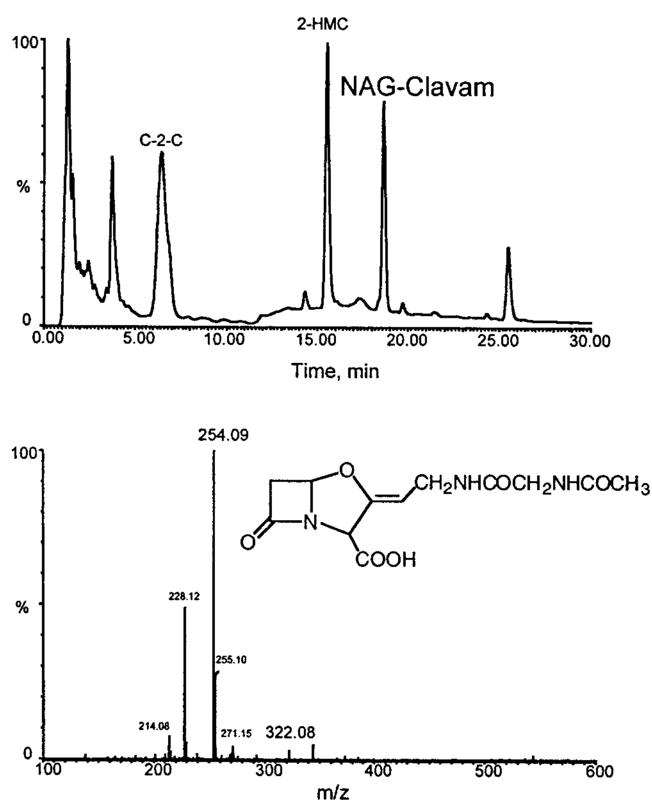


FIG. 3. HPLC and mass spectrometric analyses of culture filtrates from an *orf15*::*apr* mutant. (A) HPLC elution profile with detection at 311 nm. 2-Hydroxymethyl clavam (2-HMC) and clavam 2-carboxylate (C-2-C) are two of the *5S* clavams produced by *S. clavuligerus*; NAG-clavam is the new *5S* clavam found in *orf15* and *orf16* mutants. (B) Mass spectrum of the NAG-clavam peak at 18.6 min.

Although NAG-clavam was observed in the *orf15*::*apr* mutant, it is the *orf14* gene located just downstream which encodes a protein showing similarity to acetyltransferases. In view of the orientation and close spacing of the ORFs (Table 2), it seemed possible that the production of NAG-clavam might also be due to a polar effect of the *orf15* disruption on *orf14*. To explore this further, we constructed a new *orf15* mutant in which the apramycin disruption cassette was removed and replaced with a simple frameshift deletion muta-

tion less likely to affect the expression of a downstream gene. When this new *orf15*-Δ*fs* mutant was examined it showed the same non-clavulanic-acid-producer phenotype and accumulation of NAG-clavam as the original *orf15*::*apr* mutant, suggesting that the accumulation of this new metabolite is truly a result of mutation of *orf15*. By the same argument, the ability of the *orf16* mutation to cause accumulation of NAG-clavam might also be a reflection of a polar effect of the *orf16* mutation on the downstream *orf15* gene. To eliminate this possibility, a second version of the *orf16* mutant was also constructed in which the apr^r cassette was removed and replaced by an in-frame deletion of the entire *orf16* gene. Once again, the phenotype of the new Δ*orf16* mutant was unchanged compared with that of the original mutant, suggesting that mutations in both *orf15* and *orf16* have the effect of causing the mutant strains to accumulate NAG-clavam.

Mutation of the *orf18* gene. The deduced amino acid sequences encoded by *orf18* and *orf19* show high similarity to those of penicillin binding proteins (PBPs) from *S. coelicolor* and other species. While the disruption of *orf19* gave rise to mutants on the first attempt, however, we were unable to disrupt *orf18* in either the wild-type background or in any of a variety of non-antibiotic-producer mutants. On the assumption that *orf18* might be an essential gene, the integrating plasmid, pSET152, was used to introduce a second copy of *orf18* into the chromosome. This second copy of *orf18* was first engineered to replace most of the native upstream sequences with a glycerol-regulated promoter from *S. coelicolor* (10, 31). Following insertion of the glycerol-regulated copy of *orf18*, the resulting p*gyl*-*orf18* strain was subjected to the same gene replacement strategy that was unsuccessful in the wild-type strain. Presumptive gene replacement mutants were selected on the basis of their antibiotic resistance phenotypes, and Southern analysis confirmed that disruption of *orf18* had taken place in each case. Of the five mutants analyzed, four had the disruption in the extra copy of *orf18* inserted ectopically at the *att* site via pSET152 (p*gyl*-*orf18*::*neo*) and one was mutant in the native chromosomal copy of *orf18* (*nat*-*orf18*::*neo*).

To test whether *orf18* was essential in *S. clavuligerus*, the *nat*-*orf18*::*neo* mutant was grown on medium in the absence of added glycerol to suppress expression of the glycerol-regulated *orf18* gene, which still remained intact. When cultivated on either Soy or SA liquid medium with no added glycerol, no difference was seen between the extent of growth of the *nat*-*orf18*::*neo* mutant and that of either the wild type or a control strain in which pSET152 with no insert was integrated at the *att* site. While these results suggested that *orf18* is not essential, a second control strain transformed with pSET152 carrying an *xylE* reporter gene under the control of the same glycerol-regulated promoter (p*gyl*-*xylE*) showed low levels of catechol 2,3-dioxygenase (C23O) activity when grown on both Soy and SA liquid media even without added glycerol. Similarly, cultures of the p*gyl*-*xylE* strain grown on Soy medium agar plates without added glycerol still showed low levels of C23O activity. However, when the p*gyl*-*xylE* strain was grown on SA agar plates, C23O activity was absent. Under these same conditions, growth of the *nat*-*orf18*::*neo* mutant was noticeably inhibited and the resulting colonies were of various sizes (Fig. 4E). Addition of glycerol to the SA solid medium stimulated growth of the *nat*-*orf18*::*neo* mutant but not back to the level of that of wild type (Fig. 4F).

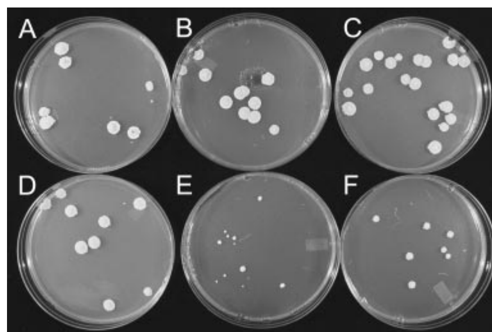


FIG. 4. Response of *orf18* mutants and control strains to growth on media with and without added glycerol. (A) *S. clavuligerus* wild type growing on SA–1% glycerol. (B) *S. clavuligerus* p*gyl*-*orf18*::*neo* mutant growing on SA–1% glycerol. (C) *S. clavuligerus* p*gyl*-*orf18* strain growing on SA–1% glycerol. (D) *S. clavuligerus* p*gyl*-*xylE* strain growing on SA–1% glycerol. (E) *S. clavuligerus nat*-*orf18*::*neo* strain growing on SA with no added glycerol. (F) *S. clavuligerus nat*-*orf18*::*neo* strain growing on SA–1% glycerol.

All of these strains produced clavulanic acid; any changes seen in the levels of metabolite production were difficult to attribute to the *orf18* mutation, because glycerol affects clavulanic acid production even in the wild type.

DISCUSSION

The clavulanic acid gene cluster has been extended by the addition of new DNA sequence information to give a total size of 25,344 bp (from the start codon of *ceaS* to the *Bam*HI site down stream from *orf19*) for the cluster. While many of the ORFs added as a result of this study do not appear to encode enzymes directly involved in the biosynthesis of clavulanic acid, i.e., transport proteins, β-lactamase-type proteins, and PBPs, evidence is presented from gene disruption studies to indicate that all (with the possible exception of *orf18* and *orf19*) are involved in some aspect of clavulanic acid biosynthesis. From previous studies, *orf10*, encoding a putative cytochrome P450, was known to be essential for clavulanic acid biosynthesis. *orf11* lies immediately downstream and in the same orientation as *orf10*, and its high degree of similarity to ferredoxins has led to the suggestion that it works in concert with ORF10 as a electron transport partner. In this regard, our observation that disruption of *orf11* causes a decrease but not a loss of clavulanic acid production and that disruption of *orf10* causes a complete loss of clavulanic acid production suggests that ORF11 cannot be the only possible electron transport partner for ORF10.

The ORF10-ORF11 pair of proteins has been suggested to be a likely candidate for the enzymes needed to carry out the oxidative deamination and enantiomerization involved in the conversion of clavaminic into clavulanic acid. However, it is of interest that disruption of genes encoding proteins (such as Cad and ClaR) that function in the late stages of the clavulanic acid biosynthetic pathway (after the branching point from clavaminate to clavulanic acid) do not have major effects on the amount of the *5S* clavam metabolites formed (12). In contrast,

disruption of both *orf10* and *orf11* does cause marked decreases in the ability of the mutant strains to form the *5S* clavam metabolites as well as similar or even more dramatic effects on production of clavulanic acid. If the sole function of the ORF10-ORF11 pair was to catalyze the conversion of clavaminic acid to clavaldehyde, then it is not clear why mutation of these proteins should decrease production of the *5S* clavam metabolites. If anything, mutation of ORF10-ORF11 might be expected to channel more of the clavaminic acid in the direction of the *5S* clavam metabolites, resulting in increased production levels. Alternatively, de la Fuente et al. (7) recently observed that mutants disrupted in *orf10* showed elevated levels of production of the unrelated antibiotic holomycin, suggesting a complex interregulation between these two antibiotic gene clusters and making interpretation of mutant phenotypes more complex.

orf12 apparently encodes a protein with similarities to β-lactamases, although only some of the conserved motifs which characterize these proteins are present. Our studies agree with those of Li et al. (16) showing that disruption of *orf12* causes a complete loss of clavulanic acid production ability. Similarly, *orf13*, which encodes an export pump-type protein, also appears to be important, although not completely essential, for production of both clavulanic acid and the *5S* clavams. Mutants with disruptions in *orf13* produce only about 5% of the wild-type levels of clavulanic acid and the *5S* clavams. Since the ORF13 protein resembles other transport-type proteins, it may represent an export system involved in the excretion of all clavam metabolites.

In our hands, disruption of *orf14* produced mutants with a phenotype that differs from those described by Mellado et al. (19). In that study, disruption of *orf14*, which encodes an apparent acetyltransferase activity, caused only a partial loss of production (about 33% of wild-type levels), whereas our mutants were almost completely (>99%) blocked in the production of clavulanic acid. This discrepancy cannot easily be explained by differences in disruption methodology, since Mellado et al. replaced an internal *Sal*I fragment of the *orf14* gene with the resistance gene, resulting in a disruption starting about 250 bp into the ORF, whereas our disruption was closer to the 3′ end of the ORF. If anything, our mutant should have been less severely affected.

Disruption of both *orf15* and *orf16* caused the complete loss of clavulanic acid production but the appearance of a new metabolite, NAG-clavam. The putative ORF15 protein is highly similar (about 50% identity) to the ORF7 protein of the clavulanic acid cluster. There is a growing body of evidence to suggest that genes encoding enzymes from early in the biosynthetic pathway to clavulanic acid are duplicated in *S. clavuligerus* (12). However, *orf7* and *orf15* cannot be considered paralogues in the same way that other genes such as *cas1* and *cas2* are because the putative proteins that they encode are not functionally equivalent. Mutants with defects in either *orf7* or *orf15* are unable to produce clavulanic acid; therefore, neither gene product can compensate for a mutation in the other. Furthermore, the *orf7* mutant does not make NAG-clavam whereas the *orf15* mutant does.

The *orf16* mutant shows limited similarity to other proteins in the databases and then only to hypothetical proteins. Nonetheless, in similarity to the results seen with the *orf15* mutant, clavulanic acid production was lost and NAG-clavam was produced in *orf16* mutants. Because of the close spacing of the *orf14*, *orf15*, and *orf16* genes, additional care was taken to prepare gene disruption mutants, with simple deletion or frame shift mutations replacing the antibiotic resistance gene insertions to minimize the possibility of polar effects. No evidence of polarity was seen, and so we conclude that accumulation of NAG-clavam is a genuine feature of mutation of either *orf15* or *orf16*. Accumulation of this metabolite has been seen previously in non-clavulanic-acid-producer mutants of *S. clavuligerus*. Elson et al. (8) reported that a non-clavulanic-acid-producer mutant, *dcl8*, accumulated novel acylated derivatives of clavaminic acid (including NAG-clavam) in amounts equivalent to that seen with the clavulanic acid produced by the wild-type parent as well as minor amounts of *N*-acetylclavaminic acid and trace amounts of *N*-glycylclavaminic acid. Our *orf15* mutant produced NAG-clavam but in amounts equivalent to only about 10% of the clavulanic acid amount produced by the wild type. On initial analysis, we did not see evidence of the other two acylated metabolites, but on closer scrutiny, we noted a small peak at retention time 17.5 min in *orf15*::*apr* (Fig. 3) and *orf16:apr* culture filtrates which eluted at about the same position as the clavaminic acid peak in the wild type. Upon mass spectrometric analysis, this peak was concluded to consist of a mixture of two compounds, clavaminic acid (fragmented mass, 155) and *N*-glycylclavaminic acid (fragmented mass, 212). In *orf16*::*apr* mutants, the *N*-glycylclavaminic peak was even more prominent (data not shown). No evidence of *N*-acetylclavaminic acid production was noted in the *orf15*::*apr* and *orf16:apr* culture filtrates, although the chromatographic conditions may not have been suitable for its detection.

Elson et al. suggested that the *dcl8* mutant was likely blocked in the conversion of clavaminic acid to clavulanic acid, and the resultant accumulation of clavaminic acid in the mutants led to the formation of various acylated derivatives of clavaminic acid. This would imply that NAG-clavam is a shunt metabolite rather than a biosynthetic intermediate. However, in view of similarity that the product of *orf14* shows to acetyltransferases, if ORF14 functions immediately before ORF15 and ORF16 in the pathway to form NAG-clavam as a product then mutation of *orf15* and *orf16* could result in accumulation of NAG-clavam.

ORF18 and ORF19 show strong similarities to PBPs. While *orf19* was disrupted with no apparent effect on viability or metabolite production, mutation of *orf18* was more complex. Attempts to disrupt the gene in the wild type or in any of a variety of non-antibiotic-producing mutant backgrounds were unsuccessful. When a second copy of the *orf18* gene was introduced on an integrating plasmid, however, disruptions could be obtained in either copy, but not in both copies, of the *orf18* gene. The introduced second copy of *orf18* was designed to be regulatable (under the control of a glycerol-regulated promoter) to demonstrate unequivocally that the gene was essential. However, the promoter was found to be leaky in *S. clavuligerus* such that promoter activity could be detected in most growth media even in the absence of added glycerol. The tightest regulation by the glycerol-regulated promoter was observed on solid SA medium, on which growth of the *nat-orf18*::*neo* mutant was severely inhibited but not totally

abolished. These results suggest that *orf18* is essential but that even low levels of expression are sufficient to support growth and that the glycerol-regulated promoter does not give tight enough control to see an absolute response.

Since *orf18* and *orf19* encode PBPs, proteins normally associated with primary metabolism in other species, and since disruption of these genes had no marked effects on metabolite production, this could be taken as evidence that *orf18* and *orf19* are not part of the cephamycin C-clavulanic acid gene supercluster. However, since β-lactam metabolites such as clavulanic acid and cephamycin C specifically target PBPs and related enzymes, it seems unlikely that genes encoding two such enzymes should be located at the boundary of the cephamycin C-clavulanic acid gene supercluster just by chance. If *orf18* and *orf19* truly encoded primary metabolic enzymes, then they might be expected to be distributed on the *S. clavuligerus* chromosome in a way similar to that seen with the corresponding genes from *S. coelicolor*, since a considerable degree of synteny has been noted in actinomycete genomes that have been analyzed to date (6). The *S. coelicolor* genome contains 13 genes which are annotated as potential PBPs, and these are quite widely distributed throughout the chromosome (http://www.sanger.ac.uk/Projects/S_coelicolor/), extending from 0.879 Mb to 5.773 Mb. In particular, the two PBPs from *S. coelicolor* which show the greatest similarity (72 to 76% identity at the amino acid level) to *orf18* and *orf19* are located at 2.831 Mb (similar to *orf18*) and 4.409 Mb (similar to *orf19*) on the *S. coelicolor* chromosome. Therefore, we conclude that *orf18* and *orf19* are likely to be part of the cephamycin-clavulanic acid supercluster and that additional genes will have to be analyzed before it can be concluded that the end of the cluster has been reached.

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