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Two Sets of Paralogous Genes Encode the Enzymes Involved in the Early Stages of Clavulanic Acid and Clavam Metabolite Biosynthesis in *Streptomyces clavuligerus*

Kapil Tahlan,* Hyeon Ung Park,† Annie Wong, Perrin H. Beatty, and Susan E. Jensen

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

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Recently, a second copy of a gene encoding proclavaminate amidinohydrolase (*pah1*), an enzyme involved in the early stages of clavulanic acid and clavam metabolite biosynthesis in *Streptomyces clavuligerus*, was identified and isolated. Using Southern analysis, we have now isolated second copies of the genes encoding the carboxyethylarginine synthase (*ceaS*) and β-lactam synthetase (*bls*) enzymes. These new paralogues are given the gene designations *ceaS1* and *bls1* and are located immediately upstream of *pah1* on the chromosome. Furthermore, sequence analysis of the region downstream of *pah1* revealed a second copy of a gene encoding ornithine acetyltransferase (*oat1*), thus indicating the presence of a cluster of paralogue genes. *ceaS1*, *bls1*, and *oat1* display 73, 60, and 63% identities, respectively, at the nucleotide level to the original *ceaS2*, *bls2*, and *oat2* genes from the clavulanic acid gene cluster. Single mutants defective in *ceaS1*, *bls1*, or *oat1* were prepared and characterized and were found to be affected to variable degrees in their ability to produce clavulanic acid and clavam metabolites. Double mutants defective in both copies of the genes were also prepared and tested. The *ceaS1*/*ceaS2* and the *bls1*/*bls2* mutant strains were completely blocked in clavulanic acid and clavam metabolite biosynthesis. On the other hand, *oat1*/*oat2* double mutants still produced some clavulanic acid and clavam metabolites. This may be attributed to the presence of the *argJ* gene in *S. clavuligerus*, which encodes yet another ornithine acetyltransferase enzyme that may be able to compensate for the lack of OAT1 and -2 in the double mutants.

The genus *Streptomyces* comprises gram-positive filamentous soil-dwelling organisms, which are renowned for their ability to produce a wide variety of chemically distinct antibiotics and secondary metabolites (25). When grown in soy medium, *Streptomyces clavuligerus* produces a variety of β-lactam compounds, including cephamycin C, penicillin N, clavulanic acid, and at least four other clavams (9, 17). *S. clavuligerus* is used commercially for the production of the clinically important β-lactamase inhibitor clavulanic acid on an industrial scale (21). Although clavulanic acid and the other clavams are structurally related to each other, only clavulanic acid is inhibitory to β-lactamases (3). The β-lactamase inhibitory activity of clavulanic acid has been attributed to its *3R*, *5R* stereochemistry, which differs from the *5S* stereochemistry of all of the other known clavams (hereafter referred to as *5S* clavams) produced by *S. clavuligerus* (21).

Although separate pathways produce cephamycin C and clavulanic acid, the two gene clusters involved in their biosynthesis are found grouped on the *S. clavuligerus* chromosome, forming a supercluster (1, 19, 45). Clavulanic acid and the *5S* clavams arise by the condensation of L-arginine and glyceraldehyde-3-phosphate (24). This reaction is catalyzed by the enzyme carboxyethylarginine synthase and leads to the formation of N^2-(2-carboxyethyl)arginine, the first dedicated intermediate in the biosynthesis of clavulanic acid and the *5S* clavams (24) (Fig. 1.). Carboxyethylarginine then undergoes intramolecular ring closure to form the β-lactam ring-containing intermediate, deoxyguanidinoproclavaminate. This ring formation is mediated by the action of the enzyme β-lactam synthetase (β-LS) (2, 28) encoded by *bls*, the second gene in the clavulanic acid gene cluster (18, 19, 20). Subsequently, deoxyguanidinoproclavaminate is hydroxylated in the first of three reactions catalyzed by the enzyme clavaminate synthase (CAS) to form guanidinoproclavaminate (6). Guanidinoproclavaminate is then converted to proclavaminate by the removal of the guanidino group in a reaction catalyzed by the enzyme proclavaminate amidinohydrolase (PAH) (15). Next, CAS mediates the formation of the bicyclic nucleus of clavulanic acid and the *5S* clavams in a two-step reaction involving oxidative cyclization, followed by desaturation to form clavaminate (5, 40). Clavaminate is thought to be the branch point between the biosynthetic pathways leading to clavulanic acid and the *5S* clavams (14). The pathway beyond clavaminate is not well characterized, and the only other known intermediate between clavaminate and clavulanic acid is clavaldehyde (33). Clavaldehyde has the same stereochemistry as clavulanic acid, shows β-lactamase inhibitory activity, and is reduced to clavulanic acid by the action of the enzyme clavulanic acid dehydrogenase (CAD) (33). However, the mechanism by which clavaminate undergoes stereochemical inversion and side chain modification to form clavaldehyde is unknown. Similarly, the reactions leading from clavaminate to the *5S* clavams are not known (3).

In addition to genes encoding enzymes with clearcut roles in

* Corresponding author. Mailing address: Department of Biological Sciences, University of Alberta, Edmonton, Alberta T6G 2E9, Canada. Phone: (780) 49-4434. Fax: (780) 492-9234. E-mail: ktahlan@ualberta.ca.

† Present address: National Cancer Institute, NIH, Bethesda, MD 20892.

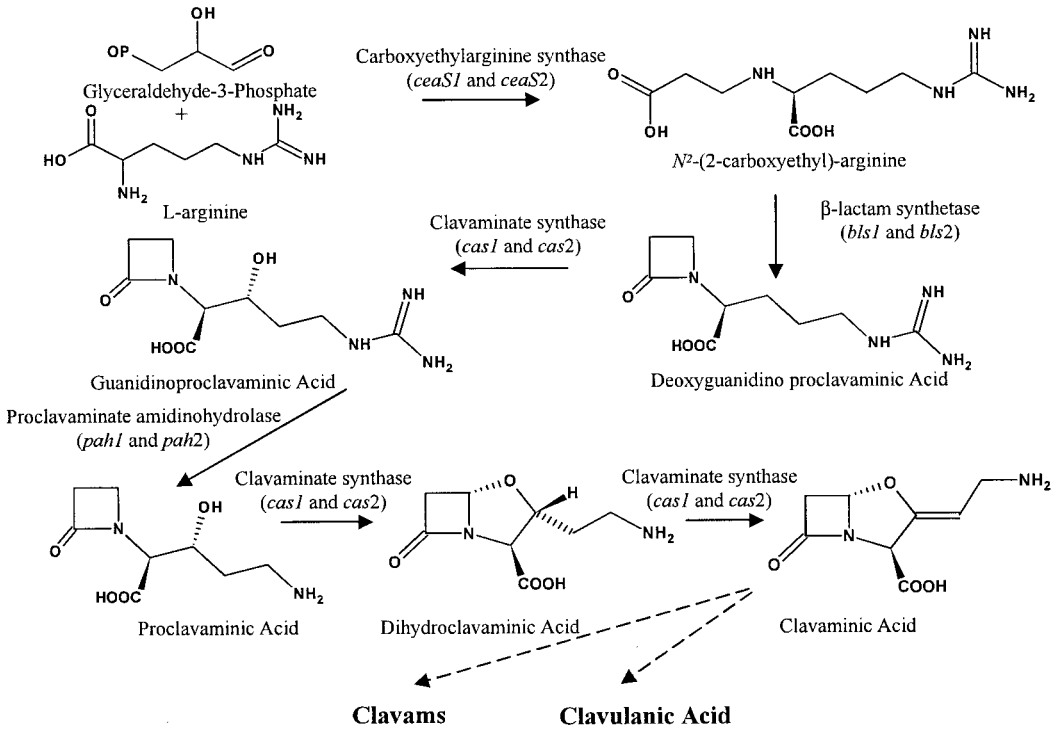

FIG. 1. Early steps of clavulanic acid and *5S* clavam metabolite biosynthesis.

clavulanic acid biosynthesis, a gene (*orf6*) apparently encoding an ornithine acetyltransferase (OAT) is also present within the clavulanic acid gene cluster. Although its function in clavulanic acid and clavam metabolite biosynthesis is unclear, mutation of *orf6* has been shown to decrease metabolite production (20). Recently, *orf6* was shown to encode a protein with OAT activity (23). OATs are normally involved in arginine biosynthesis (12), and since arginine is a precursor of clavulanic acid and the *5S* clavams, perhaps *orf6*, hereafter called *oat*, functions to increase the flux of arginine into the pathway.

S. clavuligerus possesses two CAS isozymes, encoded by two separate paralogous genes, *cas1* and *cas2* (4, 27). *cas2* is located within the clavulanic acid gene cluster (1, 19, 45), whereas *cas1* is located elsewhere on the chromosome, surrounded by genes that are involved in *5S* clavam but not clavulanic acid biosynthesis (27, 32). The two *cas* paralogues are regulated differently, and transcriptional studies have shown that *cas2* is transcribed in both complex soy and defined starch-asparagine (SA) medium, whereas *cas1* is transcribed exclusively in soy medium (34). The continued production of some clavulanic acid and *5S* clavams on soy medium even when *cas2* was disrupted by insertional inactivation indicated that *cas1* could partially complement the *cas2* mutation (34). Very similar phenotypes were observed when individual mutants defective in each of *ceaS*, *bls*, *pah*, and *oat* from the clavulanic acid gene cluster were prepared and analyzed, suggesting that paralogues may also exist for these genes (20). This was recently shown to be true for *pah*, since a paralogue was isolated and characterized (22a). The *pah* paralogues were designated as *pah1* and *pah2*, with *pah2* located adjacent to *cas2* in the clavulanic acid gene cluster. Preliminary studies indicated that *pah1*, on the other hand, is not located in the vicinity of *cas1* (22a).

In the present study we report the isolation and characterization of paralogues for the *ceaS*, *bls*, and *oat* genes, all of which are located in the region of the *S. clavuligerus* chromosome flanking *pah1*. Mutants defective in each of the paralogous genes, and double mutants defective in both genes, were prepared by targeted gene disruption and tested for their abilities to produce clavulanic acid and *5S* clavam metabolites.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. All bacterial strains and plasmids used in this study are described in Table 1. Cultures of *Escherichia coli* were grown in liquid culture in Luria broth (LB) and maintained on LB agar medium at 37°C (42). Plasmid-containing cultures were supplemented with ampicillin (100 μg/ml), apramycin (50 μg/ml), chloramphenicol (25 μg/ml), or kanamycin (50 μg/ml). *S. clavuligerus* was maintained either on MYM (43) or ISP4 medium agar plates (Difco, Detroit, Mich.) at 28°C. Plasmid-bearing *Streptomyces* cultures were supplemented with apramycin (25 μg/ml), kanamycin (50 μg/ml), or thiostrepton (5 μg/ml for *S. clavuligerus* and 50 μg/ml for *S. lividans*). Cultures for the isolation of chromosomal DNA were grown in Trypticase soy broth supplemented with 1% starch. Cultures for analysis of clavulanic acid and *5S* clavam metabolite production were grown on both SA medium and on soy medium as described previously (34). All *Streptomyces* liquid cultures were grown at 28°C on a rotary shaker at 250 rpm.

DNA isolation, manipulation, and Southern analysis. Plasmid DNA isolation from *Escherichia coli* cultures, restriction endonuclease digestion, ligation, generation of blunt-ended fragments, and *E. coli* transformation were carried out by standard procedures (42). In all subsequent procedures, when DNA fragments with incompatible ends were to be ligated, they were first made blunt by treatment with the Klenow fragment of DNA polymerase I. The QIAquick gel extraction kit (Qiagen, Inc.) was used for the isolation of DNA fragments separated by agarose gel electrophoresis. Plasmid and genomic DNA isolation from *Streptomyces* spp. and preparation and transformation of *Streptomyces lividans* protoplasts were conducted as described earlier (25). Preparation and transfor-

TABLE 1. Bacterial strains and plasmids used in this study

Strain, plasmid, or cosmid	Description[a]	Source reference
Strains		
Bacillus sp. strain ATCC 27860	Indicator strain for alanyl clavam bioassay	36
E. coli ESS	Indicator strain for cephamycin C bioassay	A. L. Demain, Drew University, Madison, N.J.
E. coli BW25113/pIJ790	Recombination host for Redirect PCR targeting system	16
K. pneumoniae ATCC 15380	Indicator strain for clavulanic acid bioassay	37
S. clavuligerus NRRL3585	Wild type	Northern Regional Research Laboratory, Peoria, Ill.
S. clavuligerus 4B and 4B-C	*ceaS2* disruption mutant (*ceaS2*::*apr*)	20
S. clavuligerus O2FS	*ceaS2* frameshift mutant	This study
S. clavuligerus orf3::*apr*	*bls* disruption mutant	20
S. lividans TK24	Plasmid-less cloning host (SLP2$^-$ SLP3$^-$), *str-6*	D. A. Hopwood, John Innes Institute, Norwich, United Kingdom
Plasmids and cosmids		
6G9	pWE15-derived cosmid carrying DNA fragment encoding *ceaS1*, *bls1*, *pah1*, and *oat1* and flanking sequences	22a
14E10	pWE15-derived cosmid carrying DNA fragment encoding *ceaS1*, *bls1*, *pah1*, and *oat1* and flanking sequences	22a
p2.8-18	pUC18 containing 2.8-kb *Eco*RI fragment carrying part of *ceaS1*	This study
p5K-6	pUC118 containing 4.3-kb *Nco*I fragment carrying *pah1*, *oat1*, and upstream sequences but with an internal *Kpn*I fragment deleted to remove *pah1*	This study
p5.7	pUC118 containing 5.7-kb *Eco*RI fragment carrying the 3′ end of *ceaS1*, all of *bls1*, *pah1*, and *oat1*, and flanking sequences	This study
p5.7-T	p5.7 containing *tsr* from pTSR#8 inserted at *Fse*I site within *bls1*	This study
p5.7-TH	*E. coli-Streptomyces* shuttle vector formed by fusing p5.7T to pJOE829	This study
pApOrf6	Disruption construct carrying *oat2* disrupted with *apr*	20
pCAD2-3	pUC120 carrying *ceaS2* and *bls2*	20
pCAD2-3(L1-5)	pCAD2-3 but with a frameshift mutation in *ceaS2*	This study
pCAD2-3(L1-5)486	*E. coli-Streptomyces* shuttle vector: pCAD2-3(L1-5) fused to pIJ486	This study
pFDNeo-S	pUC18 carrying *neo* from Tn*5*	13
pIJ486	*Streptomyces* plasmid vector, Tsr^r	D. A. Hopwood
pIJ773	Template plasmid for PCR targeting; *acc(3)IV* plus *oriT*	16
pJOE829	*Streptomyces* plasmid vector; Hyg^r	J. Altenbucher, University of Stuttgart, Stuttgart, Germany
pNEO5K-6A	p5K-6 containing *neo* from pFDNeo-S inserted at *Rsr*II site in *oat1*	This study
pTSR#8	pUC118 containing *tsr* gene from pIJ702	1
pUC18	*E. coli* cloning vector; Amp^r	Stratagene
pUC118	*E. coli* phagemid; Amp^r	44
pUC120Apr	pUC120 containing the *apr* gene flanked by *Nco*I sites	34

[a] Hyg^r, hygromycin resistance; Amp^r, ampicillin resistance.

mation of *S. clavuligerus* protoplasts was as described by Paradkar and Jensen (34). Studies using the PCR were performed with the Expand High-Fidelity PCR system according to the manufacturer's instructions (Roche). Conjugative transfer of DNA from *E. coli* into *S. clavuligerus* was carried out as described for *S. coelicolor* (25), except that AS-1 medium supplemented with 10 mM $MgCl_2$ was used for the isolation of exconjugants (7). Southern analysis of *S. clavuligerus* DNA fragments and the labeling of double-stranded DNA probes with [α-^{32}P]dCTP by nick translation was conducted as described by Sambrook et al. (42).

Isolation and DNA sequence of *ceaS1*, *bls1*, and *oat1*. The *ceaS1* and the *bls1* genes were located on *Eco*RI fragments subcloned from the cosmids 14E10 and 6G9, respectively. The 2.85-kb *Eco*RI insert from plasmid p2.8-18 carries the 5′ end of *ceaS1*; the rest of *ceaS1* was found on the 5.7-kb *Eco*RI insert of p5.7, which also encodes *bls1*. DNA sequence of the *ceaS1* and *bls1* regions of plasmids p2.8-18 and p5.7 was obtained by a combination of subcloning and analysis with both universal and sequence-specific primers. Similarly, the DNA sequence of *oat1* was obtained by analysis of appropriate subclones isolated from the 4.3-kb *Nco*I fragment previously found to encode *pah1* (22a).

All DNA sequence information was confirmed on both strands, and sequence information was obtained to cross all junctions of subclones in order to ensure that no small fragments were lost during subcloning. Sequencing reactions were carried out by using the DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia, Baie d'Urfe, Quebec, Canada) by the Molecular Biology Service Unit, University of Alberta.

DNA sequence analysis. The nucleotide sequence data obtained were compiled and analyzed by using GeneTools 1.0 (BioTools, Inc.). Prediction of open reading frames (ORFs) based on codon preference was done with the online program FramePlot 2.3.2 (http://watson.nih.go.jp/~jun/cgi-bin/frameplot.p1). Similarity and homology searches were performed by using the online basic local alignment search tool (BLAST) program at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). The PROSITE online

program at the ExPASy home page was used to search for specific peptide motifs (http://ca.expasy.org/prosite/).

Creation of targeted gene replacement mutants. The plasmid pCAD2-3 was used to prepare *ceaS2* frameshift mutants (*ceaS2*-Fs). pCAD2-3 was linearized by digestion at the unique *Not*I site located 674 bp from the proposed start codon of *ceaS2.* The linearized plasmid was made blunt by treatment with the Klenow fragment of DNA polymerase I and then recircularized to give pCAD2-3(L1-5) with a 4-bp insertion resulting in a +1 frameshift mutation in *ceaS2*. The *Streptomyces* vector pIJ486 was fused to pCAD2-3(L1-5) at the *Hin*dIII site to give the *E. coli-Streptomyces* shuttle vector pCAD2-3(L1-5)486, which was passed through *S. lividans* TK24 and then transformed into the *S. clavuligerus ceaS2*::*apr* mutant, 4B. *ceaS2*::*apr-4B* has a disruption in the *ceaS2* gene resulting from insertion of an apramycin resistance gene cassette (*apr*) (20). Apramycin- and thiostrepton-resistant (Apr^r and Tsr^r, respectively) transformants were allowed to sporulate twice on nonselective medium to isolate Apr^s and Tsr^s mutants. Southern analysis was then used to confirm the replacement of the original *ceaS2*::*apr* by *ceaS2*-Fs. An 855-bp *Eco*RI-*Not*I fragment which contained the 5′ region of *ceaS2* and some upstream sequence was used as the *ceaS2*-specific probe.

ceaS1 mutants were prepared by using the Redirect PCR targeting system described by Gust et al. (16). The Redirect PCR targeting materials were supplied by Plant Bioscience, Ltd., Norwich, United Kingdom. The primers KTA14 (5′-CCATCCCGCGCCCGTCCGTGCGAAGGAGATCTCCATGATTCCGG GGATCCGTCGACC) and KTA15 (5′-CGGGGCCGGGCATGGTGAACT CGTCCTCCACGGTGGTCATGTAGGCTGGAGCTGCTT) were used to amplify the disruption cassette from the template plasmid pIJ773. The disruption cassette comprised the *acc3(IV)* gene, conferring Apr^r, and an RK2 origin of transfer (*oriT*) flanked by DNA sequence homologous to regions immediately upstream and downstream of *ceaS1*. The cosmid 14E10 was used to prepare the *ceaS1* disruption construct in *E. coli* BW25113/pIJ780 (16), and the entire gene was deleted from the cosmid and replaced by the disruption cassette (Δ*ceaS1*::*apr*) to produce the mutant cosmid 14E10-AP. 14E10-AP was then introduced into wild-type *S. clavuligerus* by conjugation, and exconjugants were selected based on Apr^r and kanamycin sensitivity (Kan^s) on AS-1 medium supplemented with 10 mM $MgCl_2$ (7). These isolates were allowed to sporulate under nonselective conditions to isolate unigenomic Apr^r Kan^s spores.

ceaS1/*ceaS2* double mutants were prepared as described for the *ceaS1* mutants, except that the mutant cosmid 14E10-AP was conjugated into the *ceaS2*-Fs mutant strain, O2FS, instead of the wild type. Disruption of the wild-type copy of *ceaS1*, in the *ceaS1* mutants and in the *ceaS1*/*ceaS2* double mutants, was confirmed by Southern hybridization. A 777-bp *Eco*RI-*Nru*I fragment internal to *ceaS1* was used as the *ceaS1*-specific probe.

bls1 mutants were prepared by isolating the thiostrepton resistance gene cassette (*tsr*) from pTSR#8 as an *Eco*RI/*Hin*dIII fragment and inserting it into the *Fse*I site of p5.7, located within *bls1*, 507 bp from the proposed start codon, to give p5.7-T (*tsr* in the opposite orientation to *bls1*). The construct was converted to an *E. coli-Streptomyces* shuttle vector (p5.7-TH) by fusing p5.7-T to pJOE829 at their *Hin*dIII sites. p5.7-TH was passed through *S. lividans* and into wild-type *S. clavuligerus* to generate gene replacement mutants, as described previously (34). Replacement of the wild-type copy of *bls1* by the *tsr*-disrupted copy was confirmed by Southern hybridization. A 1,862-bp *Nco*I fragment, including *bls1* and 407 bp of upstream sequence, was used as the *bls1*-specific probe. In addition to the isolation of *bls1* mutant strains, cured wild-type strains were also isolated as controls. These control strains were derived from primary transformants that subsequently lost the targeting vector without undergoing gene replacement versus the same primary transformants that underwent gene replacement to produce the mutants.

The *bls1*/*bls2* double mutants were prepared by transformation of the *bls2* mutant strain (originally called *bls*::*apr* [20]) with the *bls1* disruption construct, p5.7-TH. Apr^r Tsr^r transformants were selected, and the *bls1*/*bls2* double mutation was confirmed by Southern analysis.

oat1 was disrupted by linearization of p5K-6 at the *Rsr*II site located in the middle of *oat1* and ligation to a *Pst*I/*Eco*RI fragment from pFDNeo-S carrying the neomycin resistance cassette (*neo*). The resulting plasmid, pNEO5K-6A (*neo* in the same orientation as *oat1*), was digested with *Bam*HI and fused to pIJ486 digested with *Bgl*II to yield an *E. coli-Streptomyces* shuttle disruption construct. The disruption construct was then passed through *S. lividans* and into wild-type *S. clavuligerus* to generate gene replacement mutants, essentially as described by Paradkar and Jensen (34). Southern analysis was used to confirm the *oat1* disruption. A 319-bp *Sal*I fragment internal to *oat1* was used as the *oat1*-specific probe. In addition to the *oat1* mutants, cured wild-type strains were also isolated as controls as was described for the *bls1* mutants.

oat1/*oat2* double mutants were generated by transforming protoplasts of the *oat1*::*neo* mutant strain prepared in the present study with the *oat2*::*apr* disruption construct pApOrf6, prepared earlier (20). Southern analysis was then used to verify the replacement of the genomic wild-type copies of *oat1* and *oat2* with the plasmid encoded *neo* and *apr* disrupted copies, respectively. A 531-bp *Nru*I fragment internal to *oat2* was used as the *oat2*-specific probe.

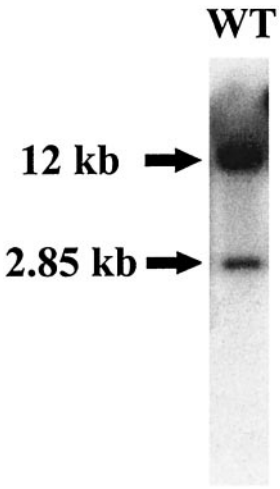

FIG. 2. Southern analysis of genomic DNA from wild-type *S. clavuligerus* after *Eco*RI digestion. The fractionated and blotted genomic DNA was probed with a *ceaS2*-specific probe.

HPLC analyses of culture filtrates. High-performance liquid chromatographic (HPLC) analysis of culture supernatants after imidazole derivatization was performed as described earlier (34).

Bioassays and growth assays. The production of clavulanic acid was also detected by bioassays with *Klebsiella pneumoniae* ATCC 15380 as the indicator organism as described previously (Jensen et al., unpublished). Cephamycin C was detected in culture supernatants by bioassay against the indicator organism *E. coli* ESS (22). The production of alanylclavam was also assayed with *Bacillus* sp. ATCC 27860 as the indicator organism (34).

The extent of growth of *S. clavuligerus* in fermentation medium was determined by using an indirect assay for deoxyribose (10).

Nucleotide sequence accession number. The nucleotide sequence encompassing *ceaS1*, *bls1*, and *oat1* from *S. clavuligerus* has been deposited in GenBank under the accession number AY426768.

RESULTS

Location of paralogues for *ceaS*, *bls*, and *oat*. Recently, it was shown that there is a second copy of the gene encoding PAH in *S. clavuligerus* (22a). Together with the previously discovered *cas1* and *cas2* paralogues (41), the *pah* genes provide the second example of a pair of paralogous genes encoding enzymes involved in the early stages of the shared clavulanic acid-*5S* clavam biosynthetic pathway (Fig. 1). Furthermore, when *ceaS*, *bls*, *pah2*, *cas2*, and *oat* mutant strains were examined for clavulanic acid production (20, 32), their phenotypes could best be explained if all of these genes, not just *pah2* and *cas2*, also have paralogues.

To investigate the possibility of a second *ceaS* gene in *S. clavuligerus*, chromosomal DNA from wild-type *S. clavuligerus* was digested with *Eco*RI and analyzed by Southern hybridization with a *ceaS*-specific probe. The 855-bp *Eco*RI-*Not*I fragment from pCAD2-3, which contained the 5′ region of *ceaS* and some upstream coding sequence, was used as the *ceaS* specific probe. A DNA fragment of 12 kb hybridized strongly with the probe, while a 2.85-kb fragment gave a weaker hybridization signal (Fig. 2). From previous studies it was known

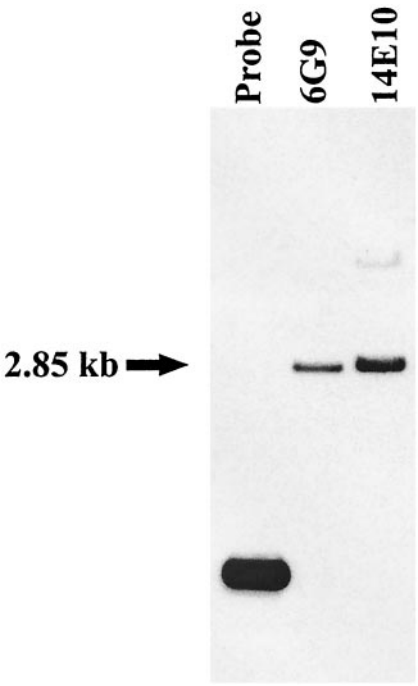

FIG. 3. Southern analysis of cosmids 6G9 and 14E10 with a *ceaS2*-specific probe. Lane 1, *ceaS2* probe (control); lanes 2 and 3, *Eco*RI-digested 6G9 and 14E10, respectively.

that the *ceaS* gene in the clavulanic acid gene cluster is carried on a 12-kb *Eco*RI fragment (20). The 2.85-kb *Eco*RI fragment was therefore postulated to encode a second copy of *ceaS*, the putative *ceaS* paralogue.

Previous DNA sequence analyses had not located any putative paralogues in the regions flanking *cas1* (32), and *pah1* is apparently not linked to *cas1*. However, it seemed possible that other paralogues, if they exist, might be clustered with *pah1*. Cosmids 14E10 and 6G9, previously shown to carry *pah1* and flanking regions of the chromosome, were digested with *Eco*RI and subjected to Southern analysis with the *ceaS*-specific probe. A 2.85-kb hybridizing fragment was observed in both of the cosmids (Fig. 3), and the fragment was subcloned for further study. Sequence analysis indicated the presence of an incomplete ORF showing similarity to the 5′ end of *ceaS*, located at one end of this fragment. The same *Eco*RI digests of the cosmids 14E10 and 6G9 were also screened with a probe specific for the gene encoding β-lactam synthetase (*bls*), to search for a *bls* paralogue in the same region (data not shown).

TABLE 2. Three newly sequenced ORFs found adjacent to *pah1*, extending the *S. clavuligerus* paralogue gene cluster

ORF	Size (bp/aa)[a]	% G+C content	Molecular mass of encoded protein (Da)	Similarity to known proteins from *S. clavuligerus*[b]	% Identity[b]
ceaS1	1,668/555	70.1	59,078	Carboxyethylarginine synthase	66
bls1	1,584/527	75.6	55,042	β-LS	49
oat1	1,176/391	76.8	39,747	OAT	47

[a] aa, amino acids.
[b] Similarities at the amino acid level were determined by searching the database by using the BLASTp online program.

The 582-bp *Bgl*II-*Nco*I fragment from pCAD2-3 was used as the *bls*-specific probe. A 5.7-kb fragment seen in digests of the cosmid 6G9 but not in 14E10, hybridized to the probe, and was therefore subcloned and partially sequenced. At one end of this fragment the 3′ end of the *ceaS*-like ORF was found. The complete ORF was 1,668 bp in size, and the predicted amino acid sequence of this ORF showed 66% identity (77% similarity) to CeaS. Downstream of the *ceaS*-like ORF, separated by an intergenic region of 23 bp, a 1,584-bp ORF was found that displayed 49% identity (59% similarity) to β-LS at the amino acid level (Table 2). The previously described *pah1* gene was found to be located immediately downstream and in the same orientation as the *bls*-like ORF, separated by a gap of 314 bp (Fig. 4). These newly found ORFs were designated *ceaS1* and *bls1*, respectively, due to their linkage to *pah1*.

The *pah1* gene was initially found on a 4.3-kb *Nco*I fragment that also contained flanking sequences from the *S. clavuligerus* chromosome (22a). When the region downstream of *pah1* was sequenced, an 1,176-bp ORF was found separated by an intergenic gap of 124 bp. The predicted amino acid sequence of this ORF showed 47% identity (58% similarity) to OAT from the clavulanic acid gene cluster. This ORF was therefore called *oat1* to distinguish it from *oat2*, which lies adjacent to *cas2* in the clavulanic acid gene cluster. Like OAT2, the predicted OAT1 protein also contains an ArgJ family domain, and *oat1* was predicted to be transcribed in the orientation opposite to *pah1* transcription (Fig. 4). *S. clavuligerus* also possesses another known OAT encoded by *argJ*, which is part of the arginine biosynthetic gene cluster (38) and the predicted OAT1 showed 29% identity to ArgJ.

The gene cluster comprising *ceaS1*, *bls1*, *pah1*, and *oat1* is hereafter referred to as the paralogue gene cluster (Fig. 4) to distinguish it from the clavulanic acid gene cluster which com-

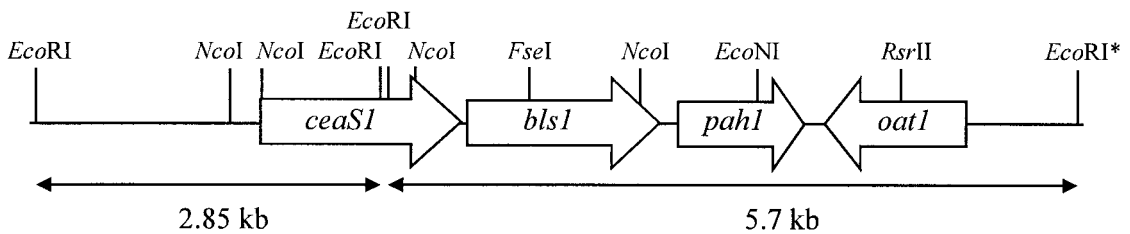

FIG. 4. Diagram of the genes encoded by the paralogue gene cluster thought to be involved in the early stages of clavulanic acid and *5S* clavam metabolite biosynthesis. Only restriction sites referred to in the text are shown. *Eco*RI* denotes an *Eco*RI site arising from the multiple cloning site of the cosmid vector, pWE15. (The diagram is not to scale.)

prises *ceaS2*, *bls2*, *pah2*, *cas2*, and *oat2* in addition to other genes involved in clavulanic acid and *5S* clavam metabolite synthesis.

Generation of a *ceaS2* frameshift mutant. In order to study the involvement of *ceaS1* in clavulanic acid and *5S* clavam metabolite biosynthesis, *ceaS1* single and *ceaS1*/*ceaS2* double mutants were prepared. However, as a first step, a new *ceaS2* single-mutant strain was constructed in which the *apr* resistance cassette of the original *ceaS2*::*apr* mutant (20) was replaced by a simple frameshift mutation. This enabled the Apr^r gene cassette to be used in the preparation of the *ceaS1* disruption mutant. The new *ceaS2* mutant was generated by the introduction of a plasmid construct, pCAD2-3(L1-5)486 carrying a frameshifted mutant copy of *ceaS2* (*ceaS2*-Fs), into the previously prepared *ceaS2*::*apr* mutant strain (20), and by screening for double-crossover events with a loss of Apr^r.

To confirm the replacement of the *apr* disrupted copy of *ceaS2* by *ceaS2*-Fs, genomic DNA from the new *ceaS2* mutant and wild-type strains was analyzed by Southern hybridization after digestion with *Eco*RI and *Nru*I. When the *Eco*RI-*Not*I fragment from pCAD2-3 (includes both the *ceaS2* and *bls2* genes) was used as a *ceaS2*-specific probe, a 2.0-kb fragment hybridized to the probe in the wild-type and the new *ceaS2*-Fs mutant samples (Fig. 5). In contrast, the same probe hybridized to a 3.5-kb fragment in samples from the parental *ceaS2*::*apr* mutants (Fig. 5). When the same blot was stripped and reprobed with *apr* from pUC120Apr as a probe, no hybridizing bands were seen in lanes containing DNA from the wild type and the *ceaS2*-Fs mutants, whereas a 3.5-kb fragment hybridized to the probe in lanes containing DNA from the *ceaS2*::*apr* mutant (Fig. 5). These results were consistent with the replacement of *ceaS2*::*apr* by *ceaS2*-Fs on the *S. clavuligerus* chromosome.

Three *ceaS2*-Fs mutants were fermented in SA and soy media in single shake flask cultures, along with wild-type and parental *ceaS2*::*apr* strains. Supernatants from 72- and 96-h cultures were analyzed by HPLC and bioassays for clavulanic acid and *5S* clavam metabolite production. HPLC analysis showed that the *ceaS2*-Fs mutants produced clavulanic acid at levels of up to 13% compared to the wild-type strain in soy medium. Various amounts of clavam metabolites were also detected in these culture supernatants, but production of all of the detectable clavam metabolites was depressed. As predicted earlier, both the *ceaS2*::*apr* and *ceaS2*-Fs mutants were completely blocked in clavulanic acid and clavam metabolite biosynthesis in SA medium (20).

Generation of a *ceaS1* mutant. The *ceaS1* mutant was created by using the recently described Redirect PCR targeting system (16). The PCR primers used were designed as such that *ceaS1* was completely deleted and replaced by the *acc(3)IV* + *oriT* cassette (Δ*ceaS1*::*apr*).

Five mutants were isolated and confirmed by Southern analysis (Table 3) and then characterized by fermentation in SA and soy media. On HPLC analysis of SA and soy culture supernatants, a reduction in clavulanic acid production compared to the wild-type strain was observed (Table 4). *5S* clavam metabolite biosynthesis also varied in soy medium, but no specific pattern was identified. The *ceaS1* disruption did not have any effect on alanylclavam or cephamycin production, as indicated by bioassays.

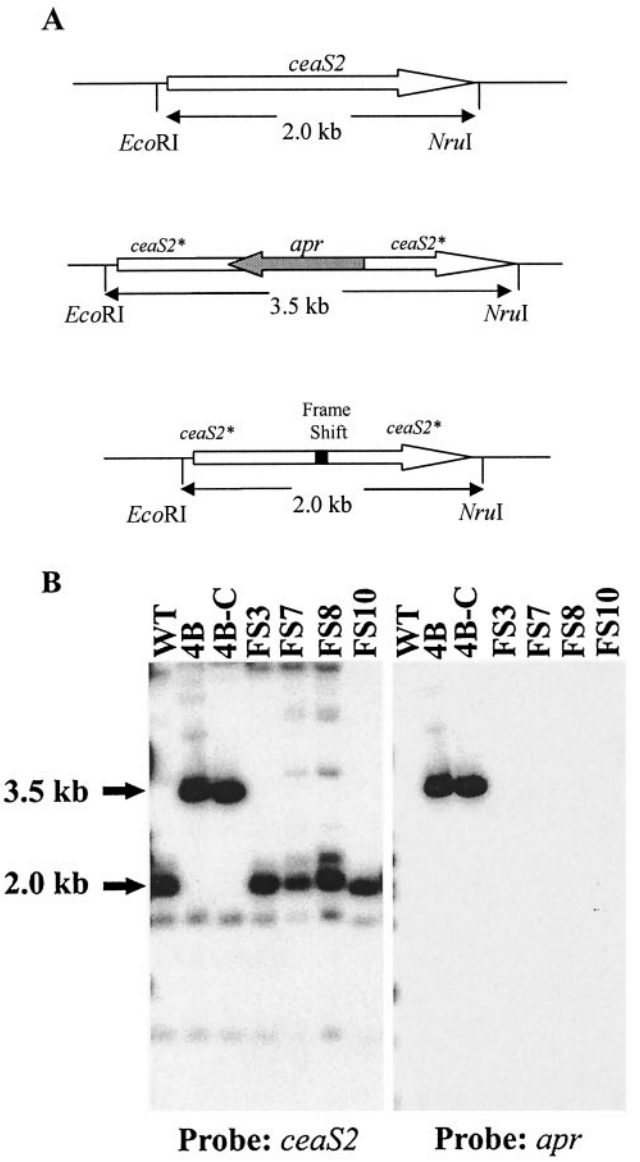

FIG. 5. Southern analysis of the *ceaS2*-Fs mutant. (A) Diagram of the *ceaS2* region of the *S. clavuligerus* chromosome in the wild type, the *ceaS2*::*apr* mutant, and the *ceaS2*-Fs mutant. The gray arrow represents the *apr* disruption cassette, and the open arrow represents *ceaS2* with the direction of transcription represented by the direction of the arrowheads. The solid bar represents the frame shift mutation, and the fine lines represent the rest of the *S. clavuligerus* chromosome. (B) Southern analysis of *Eco*RI- and *Nru*I-digested genomic DNA from *S. clavuligerus* wild-type and *ceaS2* mutant strains. DNA from the wild type, from *ceaS2*::*apr* mutants 4B and 4B-C, and from *ceaS2*-Fs mutants FS3, FS7, FS8, and FS10 was probed with a *ceaS2*-specific probe and an *apr*-specific probe.

Generation of an *S. clavuligerus ceaS1* and *ceaS2* double mutant. Since both the *ceaS1* and the *ceaS2* single mutants produced some clavulanic acid and *5S* clavams, a *ceaS1*/*ceaS2* double-mutant strain was prepared to verify that the two genes are indeed true paralogues. The *ceaS1*/*ceaS2* double mutant was prepared by conjugating the mutant cosmid 14E10-AP containing Δ*ceaS1*::*apr* into the *ceaS2*-Fs mutant strain. Three parental *ceaS2*-Fs mutants were used to isolate six *ceaS1*/*ceaS2*

TABLE 3. Hybridization profiles from Southern analyses of wild-type and mutant strains

Mutant	Restriction endonuclease(s)	Probe[a]	Hybridizing fragment (kb)	
			Parent[b]	Mutant
ceaS2-Fs	*Eco*RI/*Nru*I	*ceaS2*	3.5	2.0
		apr	3.5	None
Δ*ceaS1*::*apr*	*Nco*I	*ceaS1*	1.2[c]	None[c]
		apr	None	3.4
Δ*ceaS1*::*apr*/*ceaS2*-Fs	*Nco*I	*ceaS1*	1.2[c]	None[c]
		apr	None	3.4
bls1::*tsr*	*Nco*I	*bls1*	1.9[c]	3.0[c]
		tsr	None	3.0
bls1::*tsr*/*bls2*::*apr*	*Nco*I	*bls1*	1.9[c]	3.0[c]
		tsr	None	3.0
oat1::*neo*	*Bgl*II	*oat1*	4.3	2.4 and 2.9
		neo	None	2.4 and 2.9
oat1::*neo*/*oat2*::*apr*	*Bgl*II	*oat2*	6.5	7.95
		neo	None	7.95

[a] Gene-specific probes are described in Materials and Methods. An approximately 1.5-kb fragment from pUC120Apr was used as the apramycin-specific probe. An approximately 1-kb *Eco*RI-*Hin*dIII fragment from pTSR#8 was used as the thiostrepton-specific probe. An approximately 1-kb *Bam*HI-*Hin*dIII fragment from pFDNeo-S was used as the neomycin-specific probe.

[b] *ceaS2*::*apr* was the parental strain for the *ceaS2*-Fs mutant, *ceaS2*-Fs was the parental strain for the Δ*ceaS1*::*apr*/*ceaS2*-Fs double mutant, *bls2*::*apr* was the parental strain for the *bls1*::*tsr*/*bls2*::*apr* double mutant, and *oat1*::*apr* was the parental strain for the *oat1*::*neo*/*oat2*::*apr* double mutant. For all other mutants, the parental strain was the wild type.

[c] Faint cross-hybridizing bands were observed which can be attributed to the presence of the respective paralogues.

mutants, and Southern analysis confirmed that gene disruption had taken place in each case (Table 3).

The six isolated *ceaS1*/*ceaS2* double mutants were analyzed for clavulanic acid, *5S* clavam, and cephamycin production after 72 and 96 h of growth in SA and soy medium. No clavulanic acid or *5S* clavam production was detected in either SA or soy culture supernatants by HPLC or bioassays. Bioassays also indicated that the *ceaS1*/*ceaS2* mutants were unaffected in cephamycin biosynthesis.

TABLE 4. Clavulanic acid production by single and double mutants of ORFs located in the paralogue gene cluster

Wild type or mutant	Clavulanic acid produced[a] (% relative to wild type)	
	SA medium	Soy medium
Wild type	100	100
ceaS2-Fs	0	5–13
Δ*ceaS1*::*apr*	17–63	2–63
ceaS2-Fs/Δ*ceaS1*::*apr*	0	0
bls1::*tsr*	103–162	107–173
bls1::*tsr*/*bls2*::*apr*	0	0
oat1::*neo*	30–154	57–145
oat1::*neo*/*oat2*::*apr*	12–62	31–67

[a] The amount of clavulanic acid produced after 96 h of growth was measured by HPLC analysis.

Generation of a *bls1* mutant. The *bls1* mutant was prepared by insertion of a Tsr^r gene cassette into the *bls1* gene in the opposite orientation to *bls1*. Nine mutants were isolated, verified by Southern analysis (Table 3), and analyzed for their ability to produce clavulanic acid and clavam metabolites. On HPLC analysis of SA and soy culture supernatants, the mutants appeared to be little affected in clavulanic acid or *5S* clavam metabolite biosynthesis. In SA medium the *bls1* mutants produced between 102 to 312% and 103 to 162% of the wild-type levels of clavulanic acid after 72 and 96 h of growth, respectively. Similar results were obtained when soy culture supernatants were analyzed. After 72 and 96 h of growth in soy medium, the mutants produced between 50 to 247% and 107 to 173% of the wild-type levels clavulanic acid. Once again, the level of *5S* clavams varied from mutant to mutant, with some *bls1* mutants accumulating elevated levels of *5S* clavam metabolites in soy culture, whereas others produced much reduced levels. At 96 h clavam-2-carboxylate and 2-hydroxymethyl clavam production varied from 10 to 301% and 5 to 355%, respectively, compared to cured wild-type controls. Cephamycin production was again unaffected in the *bls1* mutant strains.

Generation of an *S. clavuligerus bls1* and *bls2* double mutant. When *bls1* and *bls2* were knocked out individually, the mutants still retained the ability to produce clavulanic acid (20). To establish unequivocally the involvement of both *bls1* and *bls2* in clavulanic acid biosynthesis, a *bls1*/*bls2* double-mutant strain was prepared and tested for its ability to produce clavulanic acid and clavam metabolites. On analysis of culture supernatants from *bls1*/*bls2* double mutants grown in soy and SA media, no clavulanic acid or *5S* clavam production was detected by HPLC or bioassays. Although clavulanic acid and clavam metabolite biosynthesis was completely abolished in these mutants, they still produced wild-type levels of cephamycin.

Generation of an *oat1* mutant. The involvement of *oat1*, which lies immediately downstream of *pah1* (Fig. 4), in clavulanic acid and clavam metabolite biosynthesis was investigated by preparing a mutant with *neo* disrupting the *oat1* gene. The *oat1* mutation was verified by Southern analysis (Table 3), and its effect was surveyed by fermenting different *oat1* mutants in soy and SA medium along with wild-type cured strains. After 72 h of growth, clavulanic acid production was between 28 to 86% and 14 to 87% in SA and soy medium, respectively, compared to cured wild-type strains. After 96 h of growth, even more variation in clavulanic acid production was observed in both media compared to the cured wild-type controls. In SA medium clavulanic acid production varied from 30 to 154% and in soy medium from 57 to 145% compared to the wild-type strain. No specific trend in *5S* clavam production was observed, and cephamycin production was unaffected in the *oat1* mutants.

Generation of an *oat1* and *oat2* double mutant. Previously, when supernatants from *oat2* mutant cultures were analyzed, the mutants still produced clavulanic acid in soy medium, although at levels only 40% of that produced by the wild-type strain (20). To determine whether the ability of the *oat2* mutant to produce clavulanic acid could be attributed to the presence of *oat1*, we prepared *oat1*/*oat2* double-mutant strains of *S. clavuligerus* and studied their effect on clavulanic acid and *5S* clavam biosynthesis. This was done by introducing the previously prepared *oat2*::*apr* disruption (20) into the *S. clavulig-*

erus oat1::*neo* mutant strain prepared in the present study. Seven mutants were isolated, verified by Southern analysis (Table 3), and characterized by fermentation. After 72 h of growth, HPLC analyses revealed that clavulanic acid production by the double mutant was between 3 to 9% and 24 to 56% of wild-type levels, in SA and soy medium, respectively. A similar decrease in clavulanic acid production was also observed in the culture supernatants analyzed after 96 h of growth (Table 4). Again, there was a high degree of variation in the levels of *5S* clavam metabolites produced, and no specific trend was observed. Cephamycin production was unaffected in the *oat1*/*oat2* mutants.

DISCUSSION

This study extends the work of Jensen et al. (20), who found that *S. clavuligerus ceaS*, *bls*, *pah2*, *cas2*, and *oat* mutants, when prepared individually, still retained some ability to produce clavulanic acid and *5S* clavam metabolites in complex soy medium but not in defined SA medium. In the case of CAS and PAH, it is known that there are two copies of the genes encoding each of these enzymes present in *S. clavuligerus* (22a, 27). Based on similarities in the observed phenotypes of the *ceaS*, *bls*, *pah2*, *oat*, and *cas2* mutants, it was proposed that paralogues may also exist for *ceaS*, *bls*, and *oat* (20).

In the present study we report the isolation and characterization of three additional paralogues of genes encoding proteins involved in the early stages of clavulanic acid and *5S* clavam biosynthesis. The genes *ceaS1*, *bls1*, and *oat1* were found grouped together, along with the previously reported *pah1* (22a), in a cluster designated the paralogue gene cluster. The paralogue cluster is distinct from the clavulanic acid gene cluster that encodes *ceaS2*, *bls2*, and *oat2* and other genes involved in clavulanic acid and clavam metabolite biosynthesis (20, 26, 29). *ceaS1*, *bls1*, and *oat1* are 73, 60, and 63% identical to *ceaS2*, *bls2*, and *oat2*, respectively, while the predicted proteins encoded by *ceaS1*, *bls1*, and *oat1* are 66, 49, and 47% identical to those encoded by *ceaS2*, *bls2*, and *oat2*, respectively. From the paralogue gene cluster, *pah1* shows the highest level of identity to its paralogous counterpart *pah2*, which was 71% at the amino acid level (22a).

OAT catalyzes the formation of ornithine by transferring an acetyl group from *N*-acetylornithine to glutamate, a key step in the biosynthesis of arginine (12). Recently, *orf6* from the clavulanic acid gene cluster, referred to as *oat2* in the present study, was shown to display OAT activity (23). In addition, *S. clavuligerus* contains another OAT, ArgJ, that is encoded by the arginine biosynthetic gene cluster (39), making the predicted OAT1 the third OAT so far known to be present in *S. clavuligerus*. Although *argJ* is presumably required for arginine biosynthesis, *oat2* and *oat1* are associated with genes involved in the biosynthesis of clavulanic acid and the *5S* clavams, which utilize arginine as a precursor. To test whether *oat1* was involved in the biosynthesis of clavulanic acid or the clavams, an *oat1* mutant was prepared, but no marked decrease in clavulanic acid or *5S* clavam production by the *oat1* mutant strain was observed. Since the *oat1* mutant still has wild-type copies of *oat2* and *argJ*, it is possible that OAT2 and ArgJ can compensate for the *oat1* mutation. Alternatively, OAT1 activity may not be essential for clavulanic acid or *5S* clavam metabolite biosynthesis. To investigate the former hypothesis, we prepared an *oat1*/*oat2* double-mutant strain and saw that the levels of clavulanic acid and *5S* clavams produced by the double mutant dropped in both SA and soy media compared to the wild-type strain, although some production still remained (Table 4). If *oat1* and *oat2* indeed encode OATs involved in providing arginine for clavulanic acid and clavam biosynthesis, then the residual production of these metabolites by the *oat1*/*oat2* double mutant may be attributed to the presence of wild-type ArgJ in this mutant. Although a role for *oat1* and *oat2* in providing increased precursor availability for metabolite synthesis seems most plausible, their role in some unidentified step in clavulanic acid or *5S* clavam biosynthesis cannot be ruled out.

All of the known or putative OATs sequenced to date contain the autoproteolytic cleavage motif KGXGMXXPX-(M/L)AT(M/L)L, with cleavage taking place between the alanine and threonine residues (11). OAT2 is expressed as a 42-kDa peptide that undergoes posttranslational autoproteolytic cleavage to form a small 19-kDa subunit and a large 25-kDa subunit, which oligomerize to form an 84-kDa heterotetramer (23). The cleavage occurs between alanine 180 and threonine 181 residues of the motif ^{169}KGVGMLEPDMATLL^{183} (23). A similar motif, ^{168}KGAGMLAPGLATTLL^{181}, is also found in *S. clavuligerus* ArgJ, but posttranslational cleavage has yet to be demonstrated. The predicted amino acid sequence of OAT1 in this region is ^{167}KGPGTGPAEQDDRSTL^{182}, which deviates from the consensus sequence, and it is also missing the adjacent alanine and threonine residues where cleavage is thought to take place. Therefore, more work is required to confirm whether *oat1* actually encodes an active OAT or if it could have some other, yet-unknown function in clavulanic acid or clavam biosynthesis.

The *ceaS1* and the *bls1* genes described here were isolated by screening *S. clavuligerus* chromosomal DNA, using sequences from *ceaS2* and *bls2* as probes, respectively. When *ceaS2* was first sequenced, it showed striking similarity to genes encoding acetohydroxyacid synthases (AHAS) based on observed homologies and conservation of five of the eight amino acids forming the active center of AHAS (35). Subsequently, CeaS2 was shown to catalyze the thiamine pyrophosphate (TPP)-dependent condensation of glyceraldehyde-3-phosphate and L-arginine to form carboxyethylarginine, the first reaction in the clavulanic acid-clavam biosynthetic pathways (24). These five amino acids, associated with the active centers of AHAS enzymes, which are found in CeaS2 (^{69}E,^{132}Q,^{472}G,^{499}N, and ^{503}G), are also conserved in CeaS1 (^{45}E,^{112}Q,^{446}G,^{472}N, and ^{476}G). Since CeaS2 utilizes TPP, it also contains the TPP binding motif (^{446}IGAQMARPDQPTFLIAGDGG^{465}), and a similar TPP binding motif (^{428}MAAQIARPGEPVFLIAGDGG^{447}) is also present in the predicted CeaS1.

β-Lactam synthetase, as the name suggests, is responsible for the formation of the β-lactam ring of clavulanic acid and the clavams (2, 28). It catalyzes the second reaction in the clavulanic acid and *5S* clavam biosynthetic pathway and requires ATP and Mg^{2+} (2, 28). Crystallographic studies on β-LS2 have identified certain amino acids that are involved in substrate binding and catalysis (30, 31), and these amino acids were also found to be conserved in β-LS1 (Fig. 6). Both β-LS2

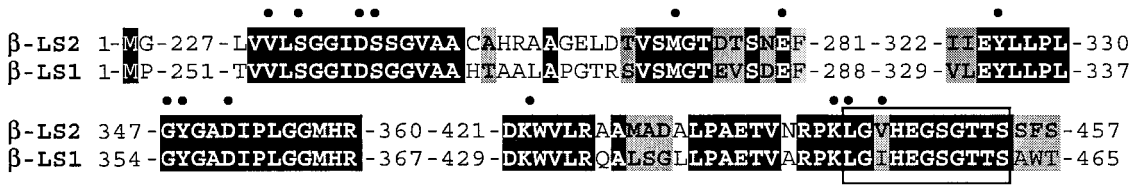

FIG. 6. Similarities between regions of β-LS1 and β-LS2. Heavy shading indicates conserved residues, and light shading indicates similar residues. Amino acids that have been shown to be involved in substrate binding, either directly or indirectly, are indicated by dots. The boxed residues represent a loop thought to form a part of the active site of β-LS2.

and β-LS1 show similarities to asparagine synthases (AS-B) from different organisms. Asparagine synthases belong to a family of enzymes called Ntn amidotransferases that have a conserved cysteine residue at their N terminus (8). This conserved cysteine residue is missing at the N terminus of both β-LS2 and β-LS1 (Fig. 6) and, in the case of β-LS2, the absence of this residue is consistent with its function in clavulanic acid and *5S* clavam metabolite biosynthesis rather than asparagine biosynthesis (2, 28). Therefore, based on homologies and the presence of conserved residues, it seems probable that β-LS1 may perform a function similar to β-LS2 in *S. clavuligerus*.

Due to the high levels of similarities between the predicted CeaS1 and β-LS1 proteins and the characterized CeaS2 and β-LS2, respectively, similar functions were envisioned for these pairs of proteins. To test whether *ceaS1* and *bls1* were involved in clavulanic acid or clavam biosynthesis, *ceaS1* and *bls1* mutants were prepared and tested individually. Since the *ceaS1* and the *bls1* mutants still contain functional wild-type copies of *ceaS2* and *bls1*, respectively, any clavulanic acid or clavams produced by these mutants can be attributed to the presence of these genes. Large variations in the levels of *5S* clavam production were observed in the *bls1* mutant. It is possible that the production of these metabolites may be extremely sensitive to minor variations in culture conditions. Despite our best efforts to produce replicate cultures, these variations persist and are observed in all of our fermentation studies. To confirm the hypothesis that *ceaS1* and *ceaS2* and *bls1* and *bls2* were indeed true paralogues, *ceaS1*/*ceaS2* and *bls1*/*bls2* double mutants were prepared and characterized. Both the *ceaS1*/*ceaS2* and the *bls1*/*bls2* double mutants were found to be completely blocked in clavulanic acid and clavam biosynthesis, lending further evidence to the paralogue hypothesis.

Results from the present study and from other work (22a) show that the genes encoding enzymes involved in the early stages of clavulanic acid and clavam metabolite biosynthesis, at least up to the level of clavaminic acid, are duplicated in *S. clavuligerus*. At present, the paralogue gene cluster contains paralogues of four genes from the clavulanic acid gene cluster in a similar but not an identical arrangement to their counterparts in the clavulanic acid gene cluster (20). Surprisingly, *cas1*, the paralogous counterpart of *cas2*, is found elsewhere on the *S. clavuligerus* chromosome and is surrounded by genes that are involved exclusively in *5S* clavam metabolite biosynthesis and not in the biosynthesis of clavulanic acid (32). In addition to the striking absence of the *cas1* paralogue, the relative orientation of *oat1* is opposite to that of *oat2* with respect to its neighboring genes. Therefore, the paralogue gene cluster does not appear to have arisen from a direct duplication of a portion of the clavulanic acid gene cluster; rather, a somewhat more complex evolution is indicated.

The reasons why *S. clavuligerus* has two sets of genes encoding enzymes involved in the early part pathway that is shared between clavulanic acid and the *5S* clavams is not clear. One explanation could be to provide a gene dosage effect and thereby increase the level of clavaminate production, which is a precursor of both clavulanic acid and the *5S* clavams. A second possibility is that the two sets of paralogues have arisen to serve separate, parallel biosynthetic pathways, which happen to share intermediates up to the level of clavaminate. To date there is no evidence for paralogues of other genes in the clavulanic acid gene cluster, based on mutational phenotypes and genetic studies (20), suggesting that these other genes are dedicated to the biosynthesis of either clavulanic acid or the *5S* clavams with no need for duplication and increased gene dosage (20). Therefore, in conclusion, the genes involved in clavulanic acid and clavam biosynthesis are now shown to be grouped into three distinct gene clusters in *S. clavuligerus*, the clavulanic acid gene cluster, the *cas1*-associated clavam gene cluster and the paralogue gene cluster, with no evidence of linkage between the clusters. Duplication of all of the genes encoding enzymes involved in the early shared steps of the pathway is evident, with paralogues to the original clavulanic acid gene cluster genes located in both the *cas1* clavam gene cluster and in the newly described paralogue gene cluster.

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