ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2004, p. 514–520 Vol. 48, No. 2
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.2.514–520.2004

Streptomyces clavuligerus Has a Second Copy of the Proclavaminate Amidinohydrolase Gene

Susan E. Jensen,[1]* Annie Wong,[1] Alison Griffin,[2] and Barry Barton[2]

Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9,[1] and Glaxo SmithKline Pharmaceuticals, Worthing, West Sussex BN14 8QH, England[2]

Received 29 April 2003/Returned for modification 30 August 2003/Accepted 19 October 2003

Past genetic studies have indicated that the genes encoding early enzymes of clavulanic acid biosynthesis may be duplicated in *Streptomyces clavuligerus*. We observed cross-hybridizing bands upon Southern analyses of proclavaminate amidinohydrolase (*pah*)-defective mutant strains of *S. clavuligerus* screened with a *pah*-specific probe. The DNA fragment responsible for this cross hybridization was cloned and sequenced and shown to encode a second copy of the *pah* gene. The new *pah* gene (*pah1*) was 1,056 bp in length, and its sequence was 72% identical to that of the original *pah* gene (*pah2*). Disruption mutants with defects in *pah1* showed no significant effects on production of clavulanic acid or any of the clavam metabolites with stereochemistries opposite that of clavulanic acid (*5S* clavams) produced by *S. clavuligerus* when they were grown on starch asparagine or soy medium. However, double mutants with defects in both *pah1* and *pah2* were defective in the production of both clavulanic acid and all of the *5S* clavam metabolites.

Streptomyces clavuligerus is a filamentous soil bacterium with an unusual facility for the production of β-lactam metabolites. In addition to cephamycin C, this organism produces clavulanic acid and at least four other clavam compounds with stereochemistries opposite that of clavulanic acid (hereafter referred to as *5S* clavams) (7). Clavulanic acid is an important industrial product because it irreversibly inactivates a wide range of β-lactamase enzymes. When used in combination with conventional β-lactam antibiotics, it restores their effectiveness against antibiotic-resistant microbes. As a result, the biosynthesis of clavulanic acid and the genes associated with its production have come under intense investigation in recent years.

The cluster of genes responsible for clavulanic acid biosynthesis has been found to lie immediately adjacent to the cephamycin C gene cluster on the chromosome of *S. clavuligerus*, and the two pathways are coregulated by a single pathway-specific transcriptional regulator, CcaR (2, 16). The full details of the clavulanic acid biosynthetic pathway remain to be elucidated, but the early enzymes in the pathway are now known (up to the level of clavaminate), and the genes responsible for their synthesis are grouped at one end of the clavulanic acid gene cluster (Fig. 1A). These include genes encoding carboxyethylarginine synthase (*ceaS*) (10), β-lactam synthetase (*bls*) (3, 13), proclavaminate amidinohydrolase (*pah*) (1, 5, 22), and clavaminate synthase (*cas2*) (12), as well as a gene encoding ornithine acetyltransferase (*oat*) (6, 9). The involvement of *oat* in clavulanic acid synthesis is unclear, but it is grouped with the rest of the early genes because it shows the same pattern of regulation of gene expression (6). The biosynthetic steps catalyzed by these activities are represented in Fig. 1B.

cas, the gene encoding clavaminate synthase, was the first gene of the cluster to be cloned and sequenced (12). Purification of the Cas enzyme as an initial step in the reverse genetic procedure used to clone the gene showed that there were actually two closely related forms of clavaminate synthase in the cells (18). Ultimately, two highly similar genes were identified as encoding the two forms of Cas. The two copies of the *cas* gene were 85.5% identical at the nucleotide level and encoded proteins showing 88% similarity, including 82% identical residues at the amino acid level. The two genes were named *cas1* and *cas2*, and it has since been shown that *cas2* is the gene located within the clavulanic acid cluster (1, 21). In contrast, *cas1* resides elsewhere on the chromosome and is apparently unlinked to *cas2* (12, 14). Examination of the genetic material flanking *cas1* has implicated it in the biosynthesis of the *5S* clavam metabolites produced by *S. clavuligerus*, since a mutation in *cvm1*, located immediately upstream of *cas1*, or a deletion which spanned parts of both *cvm4* and *cvm5*, located downstream of *cas1*, caused a complete loss of production of all of the *5S* clavam metabolites with no effect on clavulanic acid production (14).

Studies on the genes encoding early enzymes of the clavulanic acid biosynthetic pathway revealed that each of these genes could be disrupted by gene replacement procedures, yet the mutant strains could still synthesize clavulanic acid, albeit at somewhat reduced levels (6, 17). Several of these genes encode enzymes with unusual activities very different from the activities of any enzymes found to be part of primary metabolism, and so it seemed unlikely that the residual clavulanic acid synthesis seen in the disruption mutants could be due to endogenous activity from enzymes with broad specificities. Similarly, since the intermediates of clavulanic acid biosynthesis have unusual structures, these compounds are unlikely to be found to preexist in bacterial growth media. This led to the hypothesis that, as is the case for *cas*, there might be second copies, or paralogues, for each of the genes encoding the early enzymes of clavulanic acid biosynthesis in *S. clavuligerus* (6, 17). These paralogues would then enable clavulanic acid production to continue in the face of mutations in seemingly essential structural genes.

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

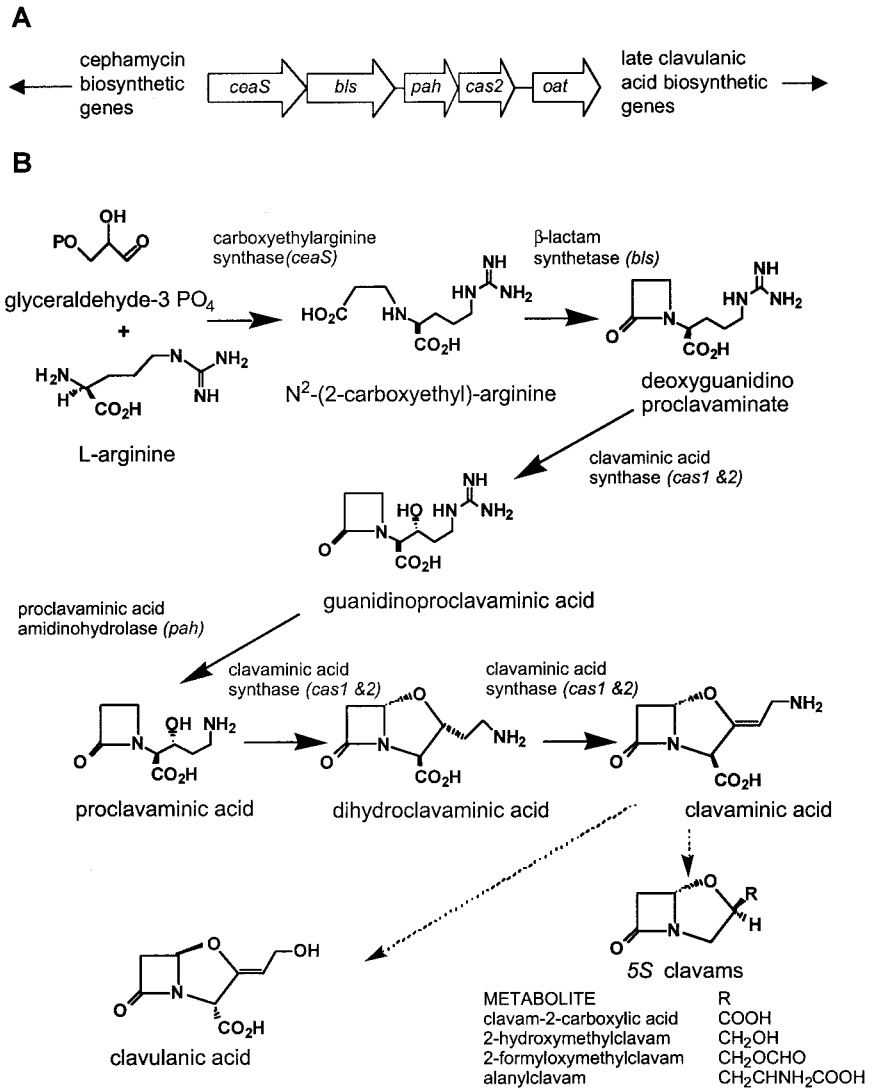

FIG. 1. Early steps in the biosynthesis of clavulanic acid and the *5S* clavams. (A) Diagrammatic representation of the first five genes (encoding enzymes involved in early steps of the pathway) from the clavulanic acid gene cluster; (B) enzyme activities involved in the early steps of clavulanic acid and *5S* clavam biosynthesis.

Proclavaminate amidinohydrolase (Pah) is the only enzyme from the early part of the pathway, other than Cas, which has been purified from *S. clavuligerus*. This purified Pah was partially sequenced, and the resulting data were compared with the amino acid sequence deduced from the *pah* gene sequence (4). Unlike the case for Cas, no evidence suggesting that there are two forms of Pah was found, and only a single copy of the *pah* gene was identified (1, 5). Subsequent analysis showed that the *pah* gene is located in the clavulanic acid cluster immediately adjacent to *cas2* (1), and no paralogue of *pah* was found in the corresponding location adjacent to *cas1* (14).

In view of these conflicting pieces of evidence regarding the likelihood that a paralogue for *pah* might exist, a search for a second copy of *pah* was undertaken. In this study we report on the isolation of a paralogue for the *pah* gene and examine its involvement in the production of clavulanic acid and the *5S* clavams in *S. clavuligerus*.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. *S. clavuligerus* NRRL 3585 was obtained from the Northern Regional Research Laboratory (Peoria, Ill.). *S. clavuligerus* was maintained on ISP Medium 4 agar plates (Difco, Detroit, Mich.). Cultures for the isolation of chromosomal DNA were grown on a 2:3 mixture of Trypticase soy broth and yeast extract malt extract (YEME) medium as described by Alexander et al. (2). Cultures for analysis of the production of clavulanic acid and *5S* clavam metabolites were grown both on starch asparagine (SA) medium and on soy medium as described previously (15). All liquid cultures were grown at 28°C on a rotary shaker at 250 rpm. Plasmid-containing cultures were supplemented with apramycin (50 μg/ml; Apralan; Provel, Division of Eli Lilly, Indianapolis, Ind.) or thiostrepton (5 μg/ml; Sigma, Oakville, Ontario, Canada), as appropriate.

Manipulation of DNA in *Escherichia coli* was done by using strain XL-1 Blue (Stratagene, La Jolla, Calif.). *E. coli* cultures were maintained on Luria-Bertani (LB) agar medium and grown in liquid culture in LB medium at 37°C (19). Plasmid-containing cultures were supplemented with ampicillin (100 μg/ml; Sigma) or apramycin (50 μg/ml), as appropriate.

The plasmids used in this study included pUC120 (20) and pUC120apr (15), a pUC120 derivative carrying an apramycin resistance cassette flanked by *Nco*I

sites. pDA501 is a shuttle vector prepared by fusing *Streptomyces* plasmid pIJ486 (11) to *E. coli* plasmid pTZ18R (Stratagene) by means of their *Eco*RI and *Bam*HI sites (this study). pDA501 is segregationally unstable in *S. clavuligerus* in the absence of antibiotic selection and so is useful for the introduction of gene disruption constructs. pWE15 is an *E. coli* cosmid cloning vector (Promega, Madison, Wis.). A library of *S. clavuligerus* genomic DNA fragments in the cosmid pWE15 was generously provided by W. Jin, Seoul National University.

DNA manipulations. Standard DNA manipulations, such as *E. coli* plasmid isolation, restriction endonuclease digestion, generation of blunt-ended fragments, ligation, ^{32}P labeling of DNA probes by nick translation, and *E. coli* transformation, were carried out as described by Sambrook et al. (19). Isolation of plasmid and genomic DNA from *Streptomyces* spp. was conducted as described by Kieser et al. (11). Southern analysis of *S. clavuligerus* DNA fragments for the detection of the *pah* paralogue was conducted at high stringency, as described by Sambrook et al. (19). The hybridization membranes (Hybond-N; Amersham Pharmacia) were washed twice for 10 min each time in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at room temperature and then once for 15 min in 1.0× SSC–0.1% SDS and twice for 10 min each time in 0.1× SSC–0.1% SDS, all at 65°C.

HPLC analyses of culture filtrates. Culture filtrates were derivatized with imidazole as described previously before analysis by high-pressure liquid chromatography (HPLC) (14).

Bioassays. Clavulanic acid was detected in an indirect bioassay for β-lactamase inhibitors by using *Klebsiella pneumoniae* ATCC 29665 as the indicator organism growing on Nutrient Agar (Difco) containing penicillin G at 6 μg/ml. Cephamycin C was detected in culture filtrates by bioassay with the indicator organism *E. coli* ESS (8).

Cloning and sequence analysis of the *pah1* gene. Total genomic DNA from *S. clavuligerus* was digested to completion with *Nco*I, and the resulting fragments were separated by electrophoresis on a 0.8% agarose gel. Regions of the gel corresponding to DNA fragments of 4 to 5 kb were excised with a sharp blade, and the DNA was recovered by using a QIAquick gel extraction kit (Qiagen, Mississauga, Ontario, Canada). The purified DNA fragments were inserted into pUC120 digested with *Nco*I, and the ligation mixture was transformed into *E. coli* XL-1 Blue. Ampicillin-resistant transformants were screened by colony hybridization with a 0.46-kb *Sal*I fragment from the original *pah* gene which was ^{32}P labeled by nick translation for use as a probe. Plasmid DNA was isolated from hybridizing transformants and was confirmed to carry a 4.3-kb *Nco*I fragment. The *pah1* gene within the 4.3-kb *Nco*I fragment was then sequenced by the Molecular Biology Service Unit, University of Alberta, by using the DYEnamic ET Terminator cycle sequencing kit (Amersham Pharmacia, Baie d'Urfe, Quebec, Canada). A 0.46-kb *Sal*I fragment from within the *pah1* gene was subcloned into pUC118R and sequenced by the use of universal primers. The remainder of the *pah1* gene sequence was obtained by using sequence-specific primers. Sequence information was obtained in full for both strands.

Insertional inactivation of *pah1* by gene disruption. The *pah1* gene was initially isolated as a 4.3-kb *Nco*I fragment of *S. clavuligerus* genomic DNA cloned into the *Nco*I site of pUC120. This construct was digested with *Kpn*I (one site within the cloned fragment and one site in the vector) and religated to reduce the size of the insert to 1.65 kb, thereby generating plasmid p4K-1, which still carried the complete *pah1* gene. The *pah1* gene within p4K-1 was disrupted by digestion at its centrally located *Eco*NI site and insertion of an apramycin resistance gene cassette from pUC120apr after both fragments had been made blunt by treatment with the Klenow fragment of DNA polymerase I. The *Kpn*I-*Nco*I insert carrying the disrupted *pah1* gene was then inserted into the *Eco*RI site of pDA501 after blunting of the ends of both the insert and the vector. Constructs with the *apr* gene in both orientations relative to that of *pah1* were obtained.

The disruption constructs were then passaged through *Streptomyces lividans* and into *S. clavuligerus* to generate gene replacement mutants, essentially as described by Paradkar and Jensen (15). For each mutant strain a corresponding cured wild-type control was also obtained. The cured control strain originated from the same primary transformant that gave rise to the mutant, but represents the case in which the disruption plasmid was simply lost from the cell rather than recombined with the chromosome.

Production of *pah1* and *pah2* double mutants. The method used for the production of mutants with disruptions in *pah2* has been described previously (1). Double mutants with disruptions in both *pah1* (*apr*) and *pah2* (*thio*) were produced by using the gene disruption construct originally constructed for the disruption of *pah2* (1) and introducing it into one of the newly created *pah1* mutants (mutant 5A).

Nucleotide sequence accession number. The DNA sequence of *pah1* has been deposited in GenBank under accession no. AY394923.

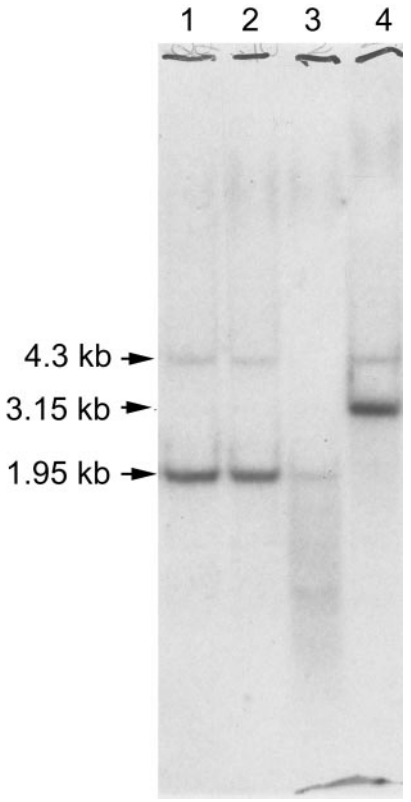

FIG. 2. Southern analysis of genomic DNA from *S. clavuligerus* wild-type and *pah* mutant strains. Genomic DNA was digested with *Nco*I, separated by electrophoresis on agarose, blotted onto a nylon membrane, and probed with a *pah*-specific probe. The nylon membrane was exposed to X-ray film for 10 days at −70°C. Lane 1, *S. clavuligerus* wild type; lanes 2 and 3, thiostrepton-resistant isolates selected as potential *pah* mutants but shown to carry a wild-type *pah* gene (not *pah* mutants); lane 4, thiostrepton-resistant true *pah* mutant.

RESULTS

Cloning and sequence analysis of *pah1*. Previous studies have shown that a mutant strain of *S. clavuligerus* in which the *pah* gene was knocked out by a gene replacement procedure was nonetheless still able to produce clavulanic acid (6). One possible interpretation of these results is that a second *pah* gene may be present and able to compensate for the mutated gene. In the case of *cas2*, in which a second copy of the gene is known to exist, the *cas1* gene is 85.5% identical to *cas2*, which is sufficient to give extensive cross-hybridization upon Southern analysis unless probes are carefully designed to target regions of dissimilarity between the two genes.

When data from the Southern analyses used in previous studies of *pah* disruption mutants were reexamined, autoradiograms that had been exposed for longer durations than normal showed evidence of additional weakly hybridizing bands (Fig. 2). In particular, genomic DNA from the wild-type strain of *S. clavuligerus* (lane 1) digested with *Nco*I showed a band of about 4.3 kb which hybridized weakly to the *pah*-specific probe, as well as the 1.95-kb strongly hybridizing band which corresponded to the known *pah* gene. The *pah* disruption mutant (lane 4) lacked the 1.95-kb strongly hybridizing band and showed a new 3.15-kb hybridizing band which corresponded to the *pah* gene

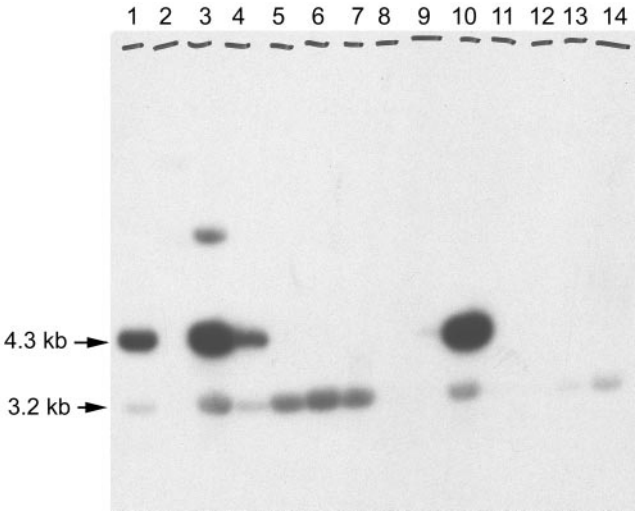

FIG. 3. Southern analysis of clones hybridizing to a *pah*-specific probe. *S. clavuligerus* genomic DNA was digested with *Nco*I, and fragments of 4 to 5 kb were cloned into pUC120. Transformants were analyzed by colony hybridization with a *pah*-specific probe, and plasmids from hybridizing colonies were subjected to Southern analysis after *Nco*I digestion. Lanes 1 and 3 to 14, *Nco*I-digested plasmids from hybridizing colonies; lane 2, *Pst*I digest of bacteriophage lambda DNA.

disrupted by introduction of a 1.2-kb thiostrepton resistance gene. However, the *pah* disruption mutant also showed the same 4.3-kb weakly hybridizing band seen in the wild type. Since the weakly hybridizing bands were the same size in both the wild-type and the *pah* mutant strains, this suggested that they were not just artifacts resulting from incomplete digestion of DNA fragments carrying the known *pah* gene. On the basis of the assumption that this 4.3-kb *Nco*I fragment might carry a second copy of the *pah* gene, a library of *Nco*I-digested genomic DNA fragments, biased to contain primarily fragments of 4 to 5 kb, was prepared in plasmid vector pUC120 and transformed into *E. coli*. About 800 transformants were patched onto nylon membranes and then analyzed by colony hybridization with a radiolabeled *pah*-specific probe. Colonies showing levels of hybridization above the background levels were selected for further analysis.

When plasmid DNA was isolated from 13 of these transformants, digested with *Nco*I, and subjected to Southern analysis, hybridization with the *pah*-specific probe was associated with the presence of the desired 4.3-kb *Nco*I insert fragment in four of the plasmids (Fig. 3). The band seen at 3.2 kb represents the pUC120 plasmid and shows some hybridization due to the presence of a small amount of contaminating plasmid in the *pah*-specific probe. Within the 4.3-kb *Nco*I fragment, the *pah*-hybridizing region was further localized to a 0.46-kb *Sal*I fragment. Subcloning and DNA sequence analysis of this 0.46-kb *Sal*I fragment indicated the presence of a gene with significant similarity to *pah*. Additional DNA sequence analysis with sequence-specific oligonucleotide primers was used to complete the determination of the DNA sequence of this *pah* paralogue. Since the clavaminate synthase-encoding gene located in the clavulanic acid gene cluster is named *cas2*, we propose that the original *pah* gene which lies adjacent to it should be called *pah2*. The newly isolated *pah* paralogue would then be named *pah1*. The newly identified *pah1* gene was 1,056 bp in length, whereas the known *pah2* gene is 942 bp. Sequence alignment indicated that it showed 72% end-to-end sequence identity with *pah2*. The corresponding protein predicted by the gene sequence showed 81% similarity, including 71% residues identical to the residues in the Pah2 protein (Fig. 4). The boldface underlined residues in Fig. 4 indicate the locations of two predicted manganese-binding sites that are typically found in proteins belonging to the arginase family, including Pah. The amino acid sequences of the two Pah proteins are identical across both of these sites.

When the 0.46-kb *Sal*I fragment, which is internal to the *pah1* gene, was used as a probe to screen cosmids from a pWE15 library of *S. clavuligerus* genomic DNA fragments, two recombinant cosmids which hybridized specifically with *pah1* were identified. Neither of these recombinant cosmids hybridized to a probe specific for the *cas1* gene, nor was there any overlap between the inserts of *cas1*- and *pah1*-bearing cosmids (data not shown). Therefore, there is no evidence of a physical linkage between the *cas1* gene and the *pah1* gene, at least within a range of about 40 kb (the average insert size of cosmids in this library).

Involvement of *pah1* in clavulanic acid and clavam metabolite biosynthesis. Although the *pah1* gene showed considerable similarity to *pah2*, no direct evidence was available to link it to the production of clavulanic acid or the *5S* clavams. In order to investigate the role of *pah1* in clavulanic acid and *5S* clavam metabolite biosynthesis, the gene was disrupted by a gene replacement technique similar to that used to disrupt *pah2*. The original disruption of *pah2* used a thiostrepton resistance gene as a disruption marker, and so an apramycin resistance gene (*apr*) was used for disruption of *pah1* to facilitate the eventual creation of a double mutant. *apr* was inserted in both orientations into an *Eco*NI site located centrally within *pah1* (Fig. 5). The disrupted forms of *pah1* carried on 3.1-kb

```
                10        20        30        40        50
Pah1     VSTAVSPRYAQPATFMRLRHRPDPIGHDVVVVGAPYDGGTSYRPGARFAPRAIRHES
         ... ::::::: ::::: : :.: :.:::::.:::::::::::::::::::.:::: ::
Pah2   MERIDSHVSPRYAQIPTFMRLPHDPQPRGYDVVVIGAPYDGGTSYRPGARFGPQAIRSES
          10        20        30        40        50        60

          60        70        80        90       100       110
Pah1   SLIHGVGIDRGPGVFDRIDVVDGGDIDLSPFSMDLAMDTATVALTRLLERNDAFLMLGGD
       .:::::::::::::.:: :. ::.:::.:.:.:.:..:.:::   :. ::. : ::::.:::
Pah2   GLIHGVGIDRGPGTFDLINCVDAGDINLTPFDMNIAIDTAQSHLSGLLKANAAFLMIGGD
          70        80        90       100       110       120

         120       130       140       150       160       170
Pah1   HSLSLAALRAVHARHGRVAVLHLDAHSDTNPPVYGGTYHHGTPFRWAIEEGLVDPERLVQ
       :::..:::::::  .:: .:.:::::::::  ::: ::::::::: .:.: :.::  .::
Pah2   HSLTVAALRAVAEQHGPLAVVHLDAHSDTNPAFYGGRYHHGTPFRHGIDEKLIDPAAMVQ
          130       140       150       160       170       180

         180       190       200       210       220       230
Pah1   VGIRGHNPRPDSLDYARGHGVSIVTAADFTRRSPRGIAEQIRRTVGGLPLYVSVDIDVVD
       .:::::::.:::::::::::::: .::: .: . .  : :. ::. ::  :.:::::::::::
Pah2   IGIRGHNPKPDSLDYARGHGVRVVTADEFGELGVGGTADLIREKVGQRPVYVSVDIDVVD
          190       200       210       220       230       240

         240       250       260       270       280       290
Pah1   PAYAPGTGTPAPGGLSSREVLTLLDVVGQLRPVGFDVVEVSPAYDPSGITSLLAAEIGAE
       ::.:::::::::::::: ::::::.::  ::.:.:::::::::::: :: .:::::.::.:::::
Pah2   PAFAPGTGTPAPGGLLSREVLALLRCVGDLKPVGFDVMEVSPLYDHGGITSILATEIGAE
          250       260       270       280       290       300

         300       310       320       330       340       350
Pah1   LLYQYARATTSPASAPVDSPLPPGAAADDAENAENAVDAVDAESAVDFAGQRWGZ
       ::::::::
Pah2   LLYQYARAHRTQL
          310
```

FIG. 4. Similarity between the proteins predicted to be encoded by *pah1* and *pah2*. Identical residues are indicated by colons, and similar residues are indicated by dots. The locations of two motifs associated with binding of manganese in arginase-type enzymes are shown underlined and in boldface.

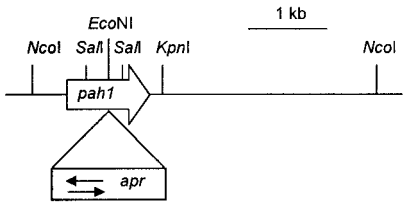

FIG. 5. Diagrammatic representation of the 4.3-kb *Nco*I fragment carrying the *pah1* gene. The open arrow indicates the size and direction of the *pah1* gene. The open box indicates the size of the *apr* gene, and the arrows indicate that it was inserted in both directions relative to the orientation of the *pah1* gene. Only the restriction sites mentioned in the text are shown.

*Nco*I-*Kpn*I fragments (originally 1.65 kb before insertion of the 1.45-kb apramycin cassette) were then transferred into *E. coli-Streptomyces* shuttle plasmid pDA501. The disruption constructs were passaged through *S. lividans* before transformation into *S. clavuligerus*. Thiostrepton-resistant, apramycin-resistant transformants were then subjected to two rounds of sporulation in the absence of selection. Thiostrepton-sensitive, apramycin-resistant colonies were subjected to Southern analysis to confirm their status as *pah1* mutants.

Southern transfers of *Nco*I-digested DNA fragments were hybridized with linearized plasmid pUC120apr carrying the apramycin marker cassette. Disruption of *pah1* in the mutants was evident because the 4.3-kb *Nco*I fragment seen in wild-type strains disappeared from the mutants and was replaced by a 5.75-kb hybridizing fragment corresponding to the 4.3-kb *Nco*I fragment plus the 1.45-kb apramycin resistance cassette (data not shown). No hybridization to the apramycin probe was seen by cured wild-type strains.

Single-flask cultures of eight of the *pah1* mutants, including strains carrying the *apr* gene in both the forward and the reverse orientations relative to the orientation of the *pah1* gene, were surveyed for clavulanic acid and *5S* clavam metabolite production (Table 1). The level of production volumetrically was compared to that of the cured wild-type controls, and the strains were grown in both soy and SA media. Mutants grown in SA medium showed similar or slightly increased levels of clavulanic acid production compared to the levels of production by wild-type control strains, whereas mutants grown in soy medium showed slightly decreased levels of production relative to the levels of production of the wild-type controls. Changes in the levels of production of the *5S* clavam metabolites were highly variable, but no definite pattern related to the mutation was observed. The extent of growth of the mutants (determined by measurement of the DNA content) was similar to that of the wild-type cultures on both media, and so when data were corrected for growth, no change in the production trends was observed (data not shown). Similarly, no differences in whether the disruption cassette was in the forward or the reverse orientation relative to the orientation of the *pah1* gene were noted. Cephamycin C production was also unaffected by the mutation in *pah1*, as judged by a bioassay (data not shown).

Creation of a *pah1* and *pah2* double mutant. Mutation of the *pah1* gene had little effect on the level of production of clavulanic acid or any of the *5S* clavams. This suggested that the *pah2* gene is able to fulfill the requirement for Pah enzyme activity in a *pah1* mutant. To test this theory, we created a *pah1* and *pah2* double mutant. Initial attempts to create this double mutant used the *pah1* disruption construct as described above, but the construct was transformed into protoplasts of a *pah2* mutant rather than into the wild type. For reasons which are not clear, we were unable to obtain double mutants by this approach, even though apramycin-resistant primary transformants were obtained. When these transformants were subjected to sporulation under nonselective conditions, only apramycin-sensitive cured isolates were obtained, even though many thousands of colonies were screened. In contrast, when the *pah2* disruption construct used to create the original *pah2* mutant (1) was introduced into one of the newly created *pah1* mutants (mutant 5A), primary transformants were obtained, giving rise to numerous double mutants upon sporulation in the absence of selection.

Confirmation by Southern analysis. Five double mutants were examined by Southern analysis to confirm the identities of the mutations. Genomic DNA preparations from each of the mutants and from the corresponding *pah1* parental strain were digested with *Apa*I and analyzed by Southern hybridiza-

TABLE 1. Production of clavulanic acid and the *5S* clavams by *pah1* mutants

Medium and mutant or wild-type strain[a]	Concn (μg/ml)[b]			
	Clavulanic acid	Alanyl-clavam	Clavam-2-carboxylic acid	2-Hydroxy-methylclavam
SA medium				
M-5A	13.0	0	0	0
M-5B	16.0	0	0	0
WT-5	5.6	0	0	0
M-6A[c]	10.7	0	0	0
M-6B[c]	13.1	0	0	0
WT-6	9.6	0	0	0
M-11A	18.6	0	0	0
M-11B	25.4	0	0	0
M-11E	22.9	0	0	0
M-11F	19.9	0	0	0
WT-11	15.2	0	0	0
Soy medium				
M-5A	145	42	79	49
M-5B	180	40	60	34
WT-5	441	0.5	66	121
M-6A[c]	151	0.1	1	0
M-6B[c]	155	0.4	3	0
WT-6	132	0.9	3	0
M-11A	113	45	114	65
M-11B	161	37	87	44
M-11E	188	19	37	20
M-11F	91	45	121	79
WT-11	175	24	75	45

[a] Mutant strains are designated with the letter M, followed by a number to indicate the primary transformant which gave rise to the mutant, and then the letters A, B, etc., indicate the particular mutant strain. Wild-type strains are plasmid-free (cured) isolates from each primary transformant that gave rise to mutants and are designated WT, followed by a number to indicate the primary transformant. The cultures were grown for 96 h in SA or soy medium before analysis by HPLC.

[b] The levels of production of the *5S* clavam metabolites are expressed in clavulanic acid equivalents. Peak areas from HPLC analyses were converted to concentrations by using a clavulanic acid standard with a known concentration.

[c] Mutants M-6A and M-6B both carried the apramycin cassette in the opposite orientation relative to that in the *pah1* gene. All other mutants carried the disruption cassette in the same orientation as that in the *pah1* gene.

tion with a radiolabeled 0.8-kb *Kpn*I fragment comprising part of *pah2* and part of *cas2* as a probe. The *pah1* parental strain gave a single strongly hybridizing band of 1.3 kb that corresponded to the wild-type *pah2* gene. In contrast, each of the presumed mutant strains gave a strongly hybridizing band of 2.5 kb, and the 1.3-kb band was absent, confirming that the *pah2* disruption had been introduced into the strains which already carried the *pah1* disruption. Hybridization with a thiostrepton resistance gene probe also confirmed the identities of the mutants.

Production of β-lactam metabolites by the *pah1* and *pah2* double mutants. When mutants defective for both copies of the *pah* genes were grown in soy medium and SA medium and their levels of production of β-lactam metabolites were compared to that for wild-type *S. clavuligerus*, the production of clavulanic acid (retention time, 9.0 min) and the *5S* clavam metabolites was found to be abolished, as determined by HPLC analyses (Fig. 6) and bioassays for clavulanic acid.

DISCUSSION

Past genetic studies with disruption mutants with defects in the early genes encoding clavulanic acid biosynthetic enzymes suggested the possibility that second functionally equivalent copies may exist for each of these genes. The existence of such a second copy was already known for *cas*, and the Cas enzyme was shown to be a mixture of two closely related isozymes. In contrast, Pah, another of the early enzymes, had been purified to homogeneity with no indication that it was a mixture of two isozymes. However, we had observed in past studies that digests of genomic DNA from *S. clavuligerus* showed evidence of a weakly hybridizing band when they were probed with a *pah*-specific probe, in addition to the expected strongly hybridizing band due to the known *pah* gene. When the DNA fragment representing this weakly hybridizing band was cloned and sequenced, the genetic material responsible for this hybridization was shown to be a second copy of the *pah* gene. This second copy of the *pah* gene, now called *pah1*, showed a high degree of similarity to the original *pah* gene, now called *pah2*, at both the nucleotide (72% identical) and amino acid (71% identical) sequence levels. Although this level of similarity is lower than that seen between the two forms of the *cas* gene, it is nonetheless high enough to suggest that the two Pah enzymes catalyze the same reaction.

Mutants with disruptions in *pah2* showed some modest reduction in the levels of production of clavulanic acid, and the disruptions had no effect on the levels of production of the *5S* clavam metabolites when the mutants were grown on a soy medium but resulted in the complete loss of metabolite production on SA medium (6). When mutants with defects in the *pah1* gene were created and analyzed for their levels of clavulanic acid and *5S* clavam metabolite production, they showed an even less clear-cut phenotype than the *pah2* mutants. Neither clavulanic acid nor *5S* clavam metabolite production was greatly affected by the *pah1* mutation, regardless of whether the cultures were grown on SA medium or soy medium. From this result we concluded that the *pah2* gene alone is able to produce sufficient Pah enzyme under the growth conditions used to support the full level of clavulanic acid and *5S* clavam biosynthesis seen in the wild-type strain. These results are

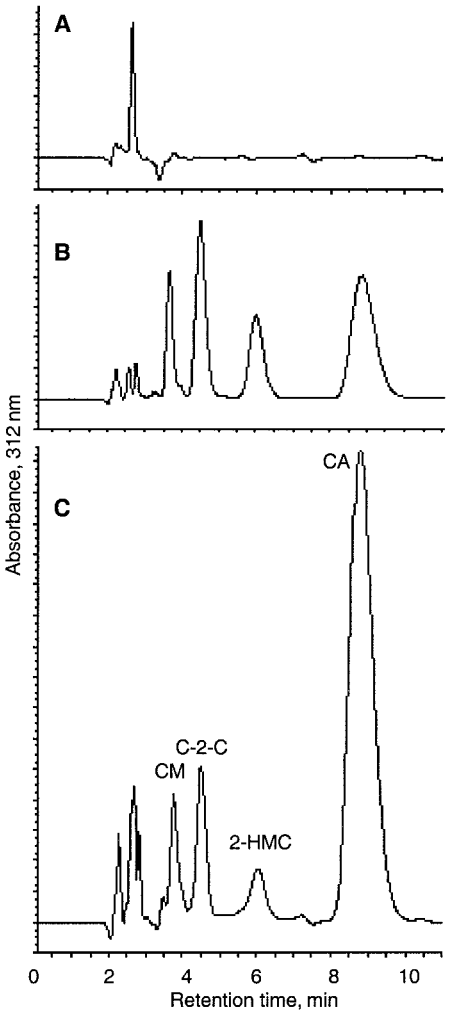

FIG. 6. Analysis of culture filtrates from the wild type, the *pah1* mutant, and the *pah1* and *pah2* double mutants of *S. clavuligerus* by HPLC. Culture filtrates from 96-h cultures grown on soy medium were analyzed by HPLC after derivatization with imidazole. The elution profiles for the *pah1* and *pah2* double mutant culture (A), the *pah1* mutant culture (mutant 5A) (B), and the wild type (C), with detection at 311 nm, are shown. CM, clavaminic acid; C-2-C, clavam-2-carboxylic acid; 2-HMC, 2-hydroxymethylclavam; CA, clavulanic acid.

different from those seen when disruption mutants with defects in *cas1* were prepared. In that case the levels of clavulanic acid production were little affected during growth on SA medium but were substantially decreased during growth on soy medium (14). For Pah, it appears that there is sufficient biosynthetic capability in the enzyme output from the *pah2* gene alone to provide the intermediates needed for metabolite biosynthesis under the growth conditions used. However, since the Cas enzyme catalyzes three separate reactions in clavulanic acid biosynthesis, a single copy of the gene may be insufficient to meet all of the metabolic needs for cultures growing on soy medium.

Since disruption of *pah1* had so little effect on the level of production of clavulanic acid or the other *5S* clavam metabolites, it called into question the assumption that *pah1* encoded an enzyme functionally equivalent to *pah2*. Creation of a *pah1* and *pah2* double mutant was undertaken to establish that *pah1*

was dispensable only under circumstances in which there was an intact copy of *pah2*. When these double mutants were prepared and analyzed, they were found to be defective in clavulanic acid and *5S* clavam metabolite production on both SA and soy media, thereby establishing the functional equivalence of Pah1 and Pah2.

The discovery of a paralogue for *pah*, taken together with the known existence of the pair of *cas* genes, lends strong support to the theory that all of the genes encoding early enzymes for clavulanic acid biosynthesis are duplicated in *S. clavuligerus*. The reason for this duplication is far less evident. A sizeable number of *Streptomyces* species are known to produce various mixtures of *5S* clavam metabolites, and a smaller number of species are known to produce clavulanic acid only (7). However, *S. clavuligerus* is the only species in this group of clavam producers that makes both clavulanic acid and *5S* clavam metabolites. We originally postulated that *S. clavuligerus* may accomplish the production of both of these types of clavam metabolites by having evolved or otherwise acquired two complete clusters of biosynthetic genes, one for clavulanic acid production and one for the production of the *5S* clavam metabolites. Since the early steps in the biosynthesis of both clavulanic acid and the *5S* clavams up to the level of clavaminic acid are common, the organism would therefore carry duplicate genes for these early steps in the pathway. Other species would have one cluster or the other but not both.

However, when the newly discovered *pah1* gene was used as a probe to screen a library of cosmids carrying fragments of chromosomal DNA from *S. clavuligerus*, two cosmids carrying the *pah1* gene were identified. Neither of these cosmids hybridized to a *cas1*-specific probe, and no evidence was obtained to indicate that these *pah1*-bearing cosmids could cross-react with cosmids carrying *cas1*. Therefore, although two gene clusters, one for clavulanic acid production and one for production of the *5S* clavam metabolites, were predicted, at present there is no evidence that *pah1* and *cas1* form a cluster. Studies are in progress to establish the organizational relationship between the clavulanic acid gene cluster, the *cas1* gene cluster associated with production of the *5S* clavam metabolites, and the newly described *pah1* gene.

ACKNOWLEDGMENTS

This study was supported by GlaxoSmithKline, the Natural Sciences and Engineering Research Council of Canada, and the Canadian Institutes of Health Research.

REFERENCES

1. **Aidoo, K. A., A. Wong, D. C. Alexander, R. A. Rittammer, and S. E. Jensen.** 1994. Cloning, sequencing and disruption of a gene from *Streptomyces clavuligerus* involved in clavulanic acid biosynthesis. Gene **147:**41–46.
2. **Alexander, D. C., and S. E. Jensen.** 1998. Investigation of the *Streptomyces clavuligerus* cephamycin C gene cluster and its regulation by the CcaR protein. J. Bacteriol. **180:**4068–4079.
3. **Bachmann, B. O., R. Li, and C. A. Townsend.** 1998. β-Lactam synthetase: a new biosynthetic enzyme. Proc. Natl. Acad. Sci. USA **95:**9082–9086.
4. **Elson, S. W., K. H. Baggaley, M. Davison, M. Fulston, N. H. Nicholson, G. D. Risbridger, and J. W. Tyler.** 1993. The identification of three new biosynthetic intermediates and one further biosynthetic enzyme in the clavulanic acid pathway. J. Chem. Soc. Chem. Commun. **1993:**1212–1214.
5. **Hodgson, J. E., A. P. Fosberry, N. S. Rawlinson, H. N. M. Ross, R. J. Neal, J. C. Arnell, A. J. Earl, and E. J. Lawlor.** 1995. Clavulanic acid biosynthesis in *Streptomyces clavuligerus*: gene cloning and characterization. Gene **166:** 49–55.
6. **Jensen, S. E., K. J. Elder, K. A. Aidoo, and A. S. Paradkar.** 2000. Enzymes catalyzing the early steps of clavulanic acid biosynthesis are encoded by two sets of paralogous genes in *Streptomyces clavuligerus.* Antimicrob. Agents Chemother. **44:**720–726.
7. **Jensen, S. E., and A. S. Paradkar.** 1999. Biosynthesis and molecular genetics of clavulanic acid. Antonie Leeuwenhoek **75:**125–133.
8. **Jensen, S. E., D. W. Westlake, and S. Wolfe.** 1982. Cyclization of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine to penicillins by cell-free extracts of *Streptomyces clavuligerus.* J. Antibiot. (Tokyo) **35:**483–490.
9. **Kershaw, N. J., H. J. McNaughton, K. S. Hewitson, H. Hernandez, J. Griffen, C. Hughes, P. Greaves, B. Barton, C. V. Robinson, and C. J. Schofield.** 2002. ORF6 from the clavulanic acid gene cluster of *Streptomyces clavuligerus* has ornithine acetyltransferase activity. Eur. J. Biochem. **269:**2052–2059.
10. **Khaleeli, N., R. F. Li, and C. A. Townsend.** 1999. Origin of the β-lactam carbons in clavulanic acid from an unusual thiamine pyrophosphate-mediated reaction. J. Am. Chem. Soc. **121:**9223–9224.
11. **Kieser, T., M. J. Bibb, M. J. Buttner, K. F. Chater, and D. A. Hopwood.** 2000. Practical *Streptomyces* genetics. John Innes Foundation, Norwich, England.
12. **Marsh, E. N., M. D. Chang, and C. A. Townsend.** 1992. Two isozymes of clavaminate synthase central to clavulanic acid formation: cloning and sequencing of both genes from *Streptomyces clavuligerus.* Biochemistry **31:** 12648–12657.
13. **McNaughton, H. J., J. E. Thirkettle, Z. H. Zhang, C. J. Schofield, S. E. Jensen, B. Barton, and P. Greaves.** 1998. β-Lactam synthetase: implications for β-lactamase evolution. Chem. Commun. **1998:**2325–2326.
14. **Mosher, R. H., A. S. Paradkar, C. Anders, B. Barton, and S. E. Jensen.** 1999. Genes specific for the biosynthesis of clavam metabolites antipodal to clavulanic acid are clustered with the gene for clavaminate synthase 1 in *Streptomyces clavuligerus.* Antimicrob. Agents Chemother. **43:**1215–1224.
15. **Paradkar, A. S., and S. E. Jensen.** 1995. Functional analysis of the gene encoding the clavaminate synthase 2 isoenzyme involved in clavulanic acid biosynthesis in *Streptomyces clavuligerus.* J. Bacteriol. **177:**1307–1314.
16. **Perez-Llarena, F. J., P. Liras, A. Rodriguez-Garcia, and J. F. Martin.** 1997. A regulatory gene (*ccaR*) required for cephamycin and clavulanic acid production in *Streptomyces clavuligerus*: amplification results in overproduction of both β-lactam compounds. J. Bacteriol. **179:**2053–2059.
17. **Perez-Redondo, R., A. Rodriguez-Garcia, J. F. Martin, and P. Liras.** 1999. Deletion of the *pyc* gene blocks clavulanic acid biosynthesis except in glycerol-containing medium: evidence for two different genes in formation of the C3 unit. J. Bacteriol. **181:**6922–6928.
18. **Salowe, S. P., E. N. Marsh, and C. A. Townsend.** 1990. Purification and characterization of clavaminate synthase from *Streptomyces clavuligerus*: an unusual oxidative enzyme in natural product biosynthesis. Biochemistry **29:** 6499–6508.
19. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
20. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153:**3–11.
21. **Ward, J. M., and J. E. Hodgson.** 1993. The biosynthetic genes for clavulanic acid and cephamycin production occur as a 'super-cluster' in three *Streptomyces.* FEMS Microbiol. Lett. **110:**239–242.
22. **Wu, T. K., R. W. Busby, T. A. Houston, D. B. McIlwaine, L. A. Egan, and C. A. Townsend.** 1995. Identification, cloning, sequencing, and overexpression of the gene encoding proclavaminate amidino hydrolase and characterization of protein function in clavulanic acid biosynthesis. J. Bacteriol. **177:** 3714–3720.