Possible involvement of the lysine ε-aminotransferase gene (lat) in the expression of the genes encoding ACV synthetase (pcbAB) and isopenicillin N synthase (pcbC) in Streptomyces clavuligerus

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Streptomyces clavuligerus produces the β-lactam antibiotics penicillin N, O-carbamoyldeacetylcephalosporin C and cephamycin C. We characterized a wild-type DNA region which restores antibiotic formation to a mutant strain named NP1, previously shown to exhibit depressed activities for two early enzymes of cephalosporin synthesis, δ-(L-ε-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS) and isopenicillin N synthase (IPNS). L-Lysine ε-aminotransferase (LAT) assays and α-AAA feeding experiments suggested that strain NP1 is a lat mutant. NP1 recovered LAT, ACVS and IPNS activities when transformed with the cloned region. DNA sequencing showed that this region encodes the entire LAT gene (lat), required for the conversion of L-lysine to the β-lactam precursor L-ε-aminoadipic acid (ε-AAA), as well as the upstream half of the ACVS gene (pcbAB). The activities of ACVS and IPNS appear to depend upon LAT expression. Gene fusions constructed to investigate promoter activities in the cloned region support a model of interdependence in the expression of the genes for LAT, ACVS and IPNS (pcbC).

Key words: Streptomyces clavuligerus, ACV synthetase (pcbAB), isopenicillin N synthase (pcbC), cephamycin synthesis, β-lactam antibiotics, lysine aminotransferase (LAT)

INTRODUCTION

Streptomyces clavuligerus is a prokaryotic producer of medically important β-lactam antibiotics. One pathway leads to the formation of three biologically active β-lactams, penicillin N, O-carbamoyldeacetylcephalosporin C and cephamycin C (Fig. 1). In an early step of the pathway, the amino acids L-ε-aminoadipic acid (ε-AAA), L-cysteine and L-valine are joined to form the tripeptide δ-(L-ε-aminoadipyl)-L-cysteinyl-D-valine (ACV) by the enzyme ACV synthetase (ACVS), encoded by the pcbAB gene. The tripeptide is cyclized by the action of the isopenicillin N synthase enzyme (IPNS; cyclase) encoded by the pcbC gene. α-AAA is produced from L-lysine by the sequential action of L-lysine ε-aminotransferase (LAT; encoded by lat) and 1-piperideine-6-carboxylate dehydrogenase (PCDH; Kern et al., 1980). Madduri et al. (1989, 1991) have shown that LAT activity occurs in β-lactam-producing actinomycetes but not in non-producing species, representing the first dedicated step of β-lactam biosynthesis in these organisms.

We reported previously the isolation and characterization of S. clavuligerus strain NP1, a cephamycin-deficient
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Demain, 1987; Piret

mutant derived from S. clavuligerus NRRL 3585 (Mahro & Demain, 1987; Piret et al., 1990). Assays showed that the activities of both ACVS and IPNS are significantly reduced in NP1. A wild-type cloned DNA region which restores antibiotic production and the two enzyme activities to a significant degree to strain NP1 was isolated (Piret et al., 1990). The complementing plasmid, pNBR1, carried a 7.2 kb insert originating from a region of the S. clavuligerus genome which lies near, but does not include, the pcbC gene. Furthermore, the cloned region was probably too small to encode the entire pcbAB gene, since in the closely related Nocardia lactamderans, this gene is 10.9 kb in length (Coque et al., 1991b). Thus it was hypothesized that the cloned sequences in pNBR1 might play a regulatory role in the β-lactam pathway. In further characterization of mutant NP1, we have found that it exhibits an even more complex phenotype than previously thought since at least three steps of the β-lactam pathway are affected. We have also analysed the insert in pNBR1 by DNA sequencing and probed it for promoter activities. Our results point to interdependence in the gene expression and enzymic activities of the first three steps of the S. clavuligerus β-lactam pathway.

METHODS

Bacterial strains, transformation and microbiological methods. Streptomyces clavuligerus NRRL 3585 (equivalent to ATCC 27064) was obtained from the US Department of Agriculture, Peoria, IL. S. clavuligerus NP1 is a cephalosporin-deficient mutant isolated following nitrosoguanidine treatment (Mahro & Demain, 1987). S. clavuligerus spore production and fermentation procedures were as described by Piret et al. (1990). Protoplasts were prepared and transformed according to Bailey & Winstanley (1986). Regeneration agar was based on R5 of these authors; dextrin was replaced by maltose and the trace elements were those used for the R2YE agar of Hopwood et al. (1985). After 48 h at 30 °C, transformation plates were overlaid with 2.5 ml soft agar composed of one-third TSA (Tryptone Soya Agar; Oxoid) containing 50 pg thiostrepton ml\(^{-1}\) and incubated at 37 °C. The diameters of zones of growth inhibition around the plugs or disks were measured and quantified using a calibration curve. To confirm the β-lactam nature of the inhibitory compound(s) produced, narrow spectrum penicillinase (Difco Bacto Penase Concentrate, 1 μl ml\(^{-1}\) or 10000 IU ml\(^{-1}\) final) or broad spectrum β-lactamase (Sigma P-4524 Penicillinase; 10 μg ml\(^{-1}\)) were added to the assay plates.

**Fig. 1. Pathway for cephalosporin biosynthesis in S. clavuligerus NRRL 3585.** The three extracellular antibiotic products are boxed.
The penicillinase destroys only penicillins; the \( \beta \)-lactamase destroys penicillins and cephalosporins.

**Enzyme assays.** ACVS and IPNS activities were measured according to Zhang et al. (1989b). LAT activity was assayed as described by Kern et al. (1980). As described in Results, transformants produced a range of antibiotic levels. The enzyme assay data are for a single transformant chosen arbitrarily for its ability to produce high levels of antibiotic.

**Deletion analysis of the cloned DNA.** Using a detailed restriction map of pNBR1, fragments were either deleted and the remaining plasmid re-ligated, or fragments were moved to pJ702 (Katz et al., 1983). Thus pNBR1.10, pNBR1.20 and pNBR1.30 were produced by complete digestion of pNBR1 with BglII, SstI or Asp718, respectively, and re-ligation. pNBR1.11 and pNBR1.21 were constructed by deletion of Asp718 fragments from pNBR1.10 and pNBR1.20, respectively. pNBR1.12 was obtained by digestion of pNBR1.10 with BglII followed by limited digestion with Bae31 (10 units; 37 °C, 45 min, 0.2 mg DNA ml\(^{-1}\)), filling-in using Klenow Polymerase (Boehringer Mannheim) and re-ligation. pNBR1.40 and pNBR1.41 (two orientations of the insert) were constructed by introducing the 2.8 kb BglII fragment from pNBR1 into pJ702. To clone individual Asp718 fragments, the 4.2 kb BglII–SphI fragment from pNBR1.10 was moved into the E. coli vector pUC19 (Messing, 1983), producing pNJR1. pNJR1 digested to completion with Asp718 was then ligated with pJ702 and the reaction used to transform Streptomyces lividans 66 (Lomovskaya et al., 1972). Constructions recovered in this host were then tested in S. clavuligerus. In this way, pNBR1.50 and pNBR1.60 were obtained. Plasmid DNA extracted from representative colonies and digested with restriction enzymes showed that the plasmids were of the expected sizes and structures. The transformation protocol used in this work (Bailey & Winstanley, 1986) resulted in transformants carrying plasmids at comparable copy number levels, easily visible by small-scale plasmid isolation methods, in contrast with some of our earlier results with a different transformation protocol where copy numbers varied markedly (discussed by Piret et al., 1990).

**DNA manipulations.** Plasmids were isolated from Streptomyces using the small- and large-scale alkaline lysis methods of Hopwood et al. (1985; procedure 2), except that for S. clavuligerus large-scale isolations, plasmids were extracted from four 25 ml cultures and pooled prior to CsCl gradient purification. Restriction enzyme digestions and ligations were carried out as recommended by the manufacturers (Boehringer Mannheim or New England Biolabs).

**DNA sequencing.** The insert from pNJR1 was excised by digestion with EcoRI and HindIII and introduced into pBluescript SK(+) and KS(+) to obtain both orientations of the insert. The 'Erase-A-Base' system (Promega) was used according to the manufacturer's instructions to generate a set of nested deletions of the cloned region. Both DNA strands were sequenced by the dideoxy chain termination method (Sanger et al., 1977) using Taq polymerase (TaqTrack Sequencing System, Promega) and 7-deaza-dGTP. The sequence data were analysed using the frame computer program of Bibb et al. (1984) as modified by Uchiyama & Weisblum (1985; gift of S. Jensen), the DNA Inspector Ile program (Textco) and DNA Strider version 1.2 (Marck, 1988).

**Gene fusions.** Restriction fragments from the pNJR1 insert were introduced into pUC19 or pSP72 (Promega), rescued as EcoRI–HindIII fragments and ligated into pJ1487 (Ward et al., 1986) digested with the same enzymes. The constructs were recovered in the commonly used intermediate host S. lividans, then tested in S. clavuligerus.

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**RESULTS**

Mutant NP1 appears to have a lat defect with pleiotropic effects on steps two and three of the cephamycin pathway

As described in the Introduction, the behaviour of strain NP1 (pNBR1) suggested that the cloned insert may encode a regulatory gene for the \( \beta \)-lactam pathway of S. clavuligerus. Another possibility was that pNBR1 carries the wild-type lat gene and that NP1 is defective in lat, the first step of the pathway. Assays of cell extracts prepared from NP1 and from the parent showed that LAT activity was severely reduced in strain NP1, representing about 4% of the parental activity. Cephalosporin production was reduced to 1% of parental activity. Feeding experiments were also carried out in which \( \alpha \)-AAA, the product of LAT and PCDH, was added to liquid fermentation cultures in complex medium. The presence of \( \alpha \)-AAA stimulated antibiotic production by NP1, increasing it from trace amounts to 17% of the parental level. This relatively low level may reflect poor uptake of the amino acid by the cells. The parent was not stimulated by \( \alpha \)-AAA addition under these conditions.

LAT activity was also assayed in extracts of NP1 (pNBR1). The activity was restored to 72% (mean of three experiments) of parental levels by the presence of the cloned sequences. The presence of pJ702 (the vector backbone of pNBR1) alone had no effect. Since LAT activity is essentially abolished in the mutant, whereas the ACVS and IPNS activities are depressed to 17% of wild-type levels, it appears that NP1 carries a lat mutation. The activities of isopenicillin N epimerase and desacetoxy-cephalosporin C synthase (expandase), two later steps in the pathway, were found to be normal in NP1, as reported.

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**Fig. 2.** Restriction and gene map of subclones derived from pNBR1 and restoration of antibiotic production in NP1 transformed with these plasmids. Left box, pchAB ORF; right box, lat ORF. A, Asp718; B, BamHI; Bg, BglII; P, PstI; S, SstI; *, endpoint of a BglIII deletion. Boxes below the restriction map represent subcloned fragments. Right, plasmid designations and antibiotic production by mutant strain NP1 transformed with each construction as determined by plug plate bioassay. +, antibiotic production > 10% of parent; +/-, < 10% of parent; −, none detectable.
Fig. 3. For legend see facing page.
\[ S. \text{clavuligerzts} \]
\[ p-lactam \]
\[ \text{lat} \]
\[ \text{affects} \]
\[ \text{pcbAB} \]
\[ \text{and} \]
\[ \text{pcbC} \]
\[ ~ \]
\[ CAGCGCGAGCGGCTTCAGC \]
\[ TGGAACGCGACC \]
\[ TGGC \]
\[ CTTCCC \]
\[ GGACCAGCGCCTCAACGACCTGGTCGAGGCGGCCGTGCGG \]
\[ CGGTCGCC \]
\[ ACCTGCCGTACCCGAGATTCGCTGACCGGTGCGGGGCTGCGG \]
\[ ACCTGGAAGGCGCCGGGTACCGACCGATCCGAGCCAGCGGTCGT \]
\[ CGGTCGTCTTC \]
\[ ACACAGCGGCTGACCTATCG \]
\[ GAGGTCGACGCGCGGGC \]
\[ CCGGTTCGCGCACTGG \]
\[ C \]
\[ CTCGGTCCGGGTCTCG \]
\[ GTCCGTTCGCAGCAGCTCGTCGGGATCTTTCTGGACAAGAGCGATCTC \]
\[ GGTGGTCGCCACGCTCGGC \]
\[ ATCTGGGAAGGCGGGCGCGGCCTATGTGCCGATCGACCCGGCCTATCCG \]
\[ GGAGCGGGTGCGGTTCGCGGTCGGTGACACCG \]
\[ TGCGG \]
\[ GGCATCGTCACCAACCGGCAC \]
\[ CACGCGGGGCGGCTGCGG \]
\[ GATCCTGGC \]
\[ CGCGGAGCACGC \]
\[ ATGTGAC \]
\[ TCGTCGAGATCGAGTCC \]
\[ GTGCTCGACGAGCAGGCCGCCGCC \]
\[ CACCGACGGCCTGCTGAGTGTG \]
\[ GCCCGAGCTGGCGCTGGGGGTACGGGATCTGGCCTATCTC \]
\[ ACCTACACCTCCGGCACCACCGGTGTGCCG \]
\[ G \]
\[ TGCCCAAGTACCACGACAGTGTCGTGAACAGCATCACGGACCTGTCGGAGCGG \]
\[ TACGACATGCGGCGGCCGG \]
\[ ACGGAGCG \]
\[ TCGCGCTC \]
\[ CGCCTCGTACGTC \]
\[ CGAGCCGCATCTGC \]
\[ A \]
\[ CGCTGATCGCGCTC \]
\[ ATCAACGGGCAGACGCTGGTCGTCGTCCCCGA \]
\[ GGTCCGGCTCGACCCGGACCGCTT \]
\[ CCCCGCGTACATCGAGGAGCACGGCGTCACG \]
\[ TACCTCAACGCGACGGGTTCGGTGCTGC \]
\[ CACTTCGATTG \]
\[ ACGCGGCCGGGCTGCGCCAGCTCCGC \]
\[ GCGGTTCTCC \]
\[ GC \]
\[ ATCGTCAACGAGTACGCCTTCACCGA \]
\[ CGGCGTTCGTCACCGCC \]
\[ GTGAAGAAGTTCGCGCCGGGCGTCACCGAGCGCGCG \]
\[ CCGCAGCATC \]
\[ CCGACCGGTGCGGAATGTG \]
\[ GTGGTATGTCCTCAGCCAG \]
\[ GATCTGAAGCGGTTGCCGGTCGGGGCGAT \]
\[ GGCGAGCTGTACATCGGC \]
\[ CTGC \]
\[ TGGCGCC \]
\[ CTATCTG \]
\[ CCGCGACGACCTG \]
\[ ACGGCCGAGCGGTTCCTCAATCCGTACGCCTCGGCTG \]
\[ GCGCGGATGCTGCCGTCCG \]
\[ GAGGTCGA \]
\[ ;TTCATGGG \]
\[ ATCGAGGCGCAGGCCACCGAGTATGCG \]
\[ TCCGCAAGTGCGTGGTCATCGCCCGTGA \]
\[ GCGGGC \]
\[ CGGCAGCGACCGCCATCTG \]
\[ GTCGGCTACTACCTGACGGAGCCC \]
\[ CCGGGGTGACC \]
\[ GGCGGAGCTGCTGTCCTTCCTGGAGCGGCG \]
\[ TCATCC \]
\[ ATCATGGTC \]
\[ CCCGGCGCGGATGGTCCCGC \]
\[ GAAGAGCATCCCGTCCTCGCCCGC \]
\[ QGRLR \]
\[ QRERLQRWNATDGDFPADQRLNDLVEAAVR \]
\[ RSPDREAVVFGTQRLTYREVDARANRFAHW \]
\[ LLGPGLGVRSQQLVGIFLDKSDLGVVATLG \]
\[ IWKAGAAYVPIDPAYPAERVRFAVGDTGLR \]
\[ GIVTNRHHHAGRLREILGAEHADVTTVIIES \]
\[ VLTDEQAADTDGLLSSVFKPELALALGVRDLAYL \]
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\[ CCGGGACACG \]
\[ CGGCGGCGCGGTGGGCTCCACCACGACC \]
\[ TGTCCA \]
\[ TGAGCTGCGC \]
\[ GATC \]
\[ 4029 \]

**Fig. 3.** DNA sequence and predicted protein products encoded by the pNJR1 (pNBR1.10) insert. The translational start sites of \text{lat} and \text{pcbAb} are labelled with arrowheads. Proposed ribosomal binding sites (RBS) are labelled and overlined. Sequences resembling the consensus (Strohl, 1992) for \text{E. coli} \text{E}^-\text{p}^-\text{like streptomycete promoters located upstream of} \text{lat} (\text{-10}) and in the 3' \text{region of} \text{lat} (\text{-35}) are underlined and labelled. The GC-rich inverted repeat located downstream from \text{lat} is underlined with dashed arrows. The Box A-like sequence is underlined and labelled. The Sa/I sites \#2 and \#3 used in the construction of transcriptional fusions are underlined and labelled.

Earlier (Piret et al., 1990). When pNBR1 is present in NP1, LAT activity and cephalosporin production increase to 72% and 67% of parental levels, respectively, whereas the ACVS and IPNS activities reach 39 and 35%.

**Deletion analysis of the complementing DNA region**

To confirm that pNBR1 carries \text{lat}, sequencing of the cloned insert was undertaken following generation of in vitro deletions and testing for complementation by specific restriction fragments (see Methods). NP1 transformants carrying each of 10 plasmid subclones were tested for cephalosporin formation in plate bioassays. Control strains were untransformed NP1 and NP1 (pJ702), both of which produced trace amounts of antibiotic as expected. Isolation and restriction analysis of the plasmids showed that they were structurally intact.

As drawn in Fig. 2, the right half of the pNBR1 insert, present in pNBR1.20 and pNBR1.10, is sufficient to
restore antibiotic production to strain NP1. Deletion of about 1.6 kb from the right side of the inserts in these plasmids, producing pNBR1.21 and pNBR1.11, abolishes the complementation. The data for pNBR1.12 suggest that sequences near the middle of the insert may be important also. We observed no obvious variation in plasmid copy number for any of the plasmids tested in NP1, as evidenced by visual observations of bands on gels. On this basis, copy number effects were apparently not a factor in the partial complementation by pNBR1.12.

The collection of transformants obtained with the three complementing plasmids produced a range of antibiotic levels. Among the 44 pNBR1.10 transformants obtained, 37 (84%) produced more antibiotic than the NP1 control strains, while seven produced less; among the 29 pNBR1.20 transformants, 21 (72%) produced more antibiotic than the controls. Re-transformation of NP1 with pNBR1.10 isolated from low-producing transformants resulted in mixtures of low- and high-producers once again, indicating that the plasmids were unaltered. It was possible that complementation of the NP1 phenotype might require recombination of the cloned region into the host chromosome. To investigate this, an experiment to cure NP1(pNBR1) of its plasmid was performed. NP1(pNBR1) was grown in the absence of selection for the plasmid marker (thiostrepton), spores were plated for individual colonies and screened for the loss of pNBR1 (by thiostrepton sensitivity and plasmid isolation). All of the nine thiostrepton-sensitive colonies (found among 128 colonies screened) exhibited the mutant phenotype, producing only trace levels of antibiotic. Re-transformation of several of the cured strains with pNBR1 restored antibiotic production. The data suggested either that the cloned sequences can complement NP1 in trans from the autonomous plasmid or, if complementation by the cloned sequences requires chromosomal integration (in cis), that the NP1 mutation(s) lies near one end of the cloned region where marker exchange would be unlikely.

**The complementing DNA region encodes the lat gene and the truncated pcbAB gene**

To characterize the complementing region, the sequence of the insert in pNBR1.10 was determined. Computer analysis of the data revealed the presence of one complete (1374 bp) and one truncated (2415 bp) open reading frame (ORF; Fig. 3), separated by 153 bp and transcribed in the same direction (right to left in Fig. 2). Both exhibit strong **Streptomyces** codon bias (Seno & Baltz, 1989) with a G+C content greater than 90 mol % in the third position of the triplets (Bibb et al., 1984). The putative translation start sites (both ATG) for the two ORFs (+88 and +1615 nt in Fig. 3) were selected on the basis of analyses using the DNA Inspector IIe program and identification of putative ribosome binding sites lying upstream of the two ORFs. These are located 8–14 nt upstream of the first ATG of the complete ORF (AGGAGTT) and 8–13 nt upstream from the start of the truncated ORF (GGAGAG); in good agreement with the locations of Shine–Dalgarno sequences for most characterized **Streptomyces** genes involved in antibiotic production (Seno & Baltz, 1989). The calculated molecular mass of the product of the upstream ORF was 49.83 kDa.

Extensive similarity (80.4% identity at the DNA level and 80.9% identity at the protein level, allowing for gaps) between the upstream ORF and the LAT (lat) gene from another actinomycete, **Nocardia lactamdurans** (Coque et al., 1991a; Martin, 1992), identified it as the **S. clavuligerus** lat gene. Comparison of the sequence of the downstream truncated ORF with the published sequence of the ACVS gene, pcbAB, of N. lactamdurans (76.2% similarity) and its protein product (68.5% identity) indicated that it encodes the 5' end portion of pcbAB. During the course of this work, Tobin et al. (1991) reported the sequence of a DNA fragment which encodes the S. clavuligerus lat gene as well as a 5' portion of the pcbAB gene. We compared our data with theirs and, as expected, found almost identical sequences in the region of overlap. However, a few differences should be noted. Within the lat ORF, the C residue at nt 455 (Fig. 3) was reported to be a G by Tobin et al. (1991), our data implying the presence of a proline rather than an arginine residue in the predicted protein sequence. At nt 1313 and 1392, our results show T and C residues rather than C and G, respectively, which would leave the polypeptide sequence unchanged. At nt 1441, we found a G rather than a C residue, thus alanine rather than proline. In the intergenic region between lat and pcbAB, we have data for a C residue (nt 1502) absent from the sequence of Tobin et al. (1991). We do not know whether these discrepancies are due to allelic differences or sequencing errors.

Sequence data for pcbAB genes available for several other
microbial sources have shown that they are very large genes (about 11 kb) which share three similar repeated structural motifs termed the A-, B- and C-domains first observed by Smith et al. (1990b) and predicted to be involved in the sequential activation and polymerization of the three amino acid components of the tripeptide ACV. The \textit{S. clavuligerus pcbAB} sequence data presented here extends 1535 bp further downstream of that reported by Tobin et al. (1991), representing about 20\% of the predicted total for the gene. Comparison of these data with the core region of the \textit{pcbAB} A-domain from \textit{N. lactamdurans} (Coque et al., 1991b) showed that the sequenced region comprises most of the A-domain. Tobin et al. (1991) reported partial amino acid sequence data for the \textit{S. clavuligerus pcbAB} A-domain core region, specifically N-terminal and C-terminal sequences of the domain. Comparison with our results revealed several differences. In the N-terminal region of the A-domain; we found that nt 2524–2526 encode leucine rather than proline residues, in both cases increasing the number of identities between the \textit{S. clavuligerus} sequence and the consensus sequences for the domain. We also found that nt 2524–2526 encode leucine rather than histidine and nt 2548–2551 encode serine, not arginine. Again, the reasons for these sequence differences could be allelic or technical.

**Table 1. Comparison of possible promoter regions internal to the lat ORF with consensus sequences for \textit{Streptomyces} and \textit{E. coli} vegetative promoters**

Matches with the \textit{Streptomyces} consensus are underlined. The sequences of the mapped lat, pcbC and cepD promoters are given for comparison.

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<tr>
<td>\textit{Streptomyces}</td>
<td>TTGAC (Pu)</td>
<td>17 ± 1</td>
<td>TAg (Pu) (Pu) T</td>
<td>Strohl (1992)</td>
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<tr>
<td>\textit{E. coli}</td>
<td>TTGAC(G)</td>
<td>17 ± 1</td>
<td>TATAAT</td>
<td>McClure (1985)</td>
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<tr>
<td>\textbf{lat}</td>
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<tr>
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<td>16</td>
<td>CGGGGT</td>
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</tr>
<tr>
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<td>TCGACA</td>
<td>17</td>
<td>AAGTAC</td>
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<tr>
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<td>CAGAAT</td>
<td>Kovacevic et al. (1990)</td>
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</table>

*As numbered in Fig. 3.

†The cefD promoter controls transcription of the co-transcribed \textit{S. clavuligerus} isopenicillin N epimerase and desacetoxycephalosporin C synthase genes, cefD and cefE.

Promoter activities in the lat–pcbAB region

The close physical juxtaposition of \textit{lat} and \textit{pcbAB} prompted us to ask whether the two genes are co-transcribed or expressed separately. We looked for promoter activities in the lat–pcbAB region by fusing fragments of the cloned DNA to a kanamycin resistance reporter gene in the multicopy vector pIJ487 (Ward et al., 1986), producing the pNH7 series of plasmids (Fig. 4). In gradient plate assays, these constructions were tested for their ability to confer kanamycin resistance on \textit{S. clavuligerus} grown on medium which supports antibiotic production (TSA) and thus expression of the cephalosporin genes. The absence of translational fusions between the cloned fragments and the reporter gene was verified in each case. \textit{S. clavuligerus} carrying pIJ487 without insert exhibited a background level of resistance to about 25 \(\mu\)g kanamycin ml\(^{-1}\) under these conditions. Interestingly, the pattern of kanamycin resistance in \textit{S. lividans}, which was used as an intermediate host in the construction of the fusions but which is not known to make \(\beta\)-lactam antibiotics, was similar to that observed in \textit{S. clavuligerus}. Petrich et al. (1992) made the same observation in their study of transcriptional fusions to the \textit{pcbC} (IPNS gene) promoter in these two hosts.

pNH7.52, constructed to test for promoter activity directed toward \textit{pcbAB} in the lat–pcbAB intergenic region (Fig. 4), conferred no net kanamycin resistance to the host suggesting the absence of promoter activity in this region. In contrast, pNH7.12 and pNH7.30, which carry overlapping inserts (\textit{Asp718} \#3–SalI \#9 fragment) from within the 3' region of the \textit{lat} ORF and directed toward \textit{pcbAB}, conferred significant resistance to the host (\(\geq\) 400 and 60 \(\mu\)g kanamycin ml\(^{-1}\), respectively). Examination of the DNA sequence data revealed that these inserts featured two sets of sequences located in the common \textit{Asp718} \#3–SalI \#9 fragment which resemble (8 matches in each pair of hexamers) the \textit{Streptomyces} promoter consensus region proposed for \textit{E. coli}-like Eσ\(^{30}\) promoters (Strohl, 1992; Table I and Fig. 3). It is noteworthy that the -10 bp region of one of these (located at nt 992–1014) is identical at four of six residues to the same region in the \textit{pcbC} promoter mapped by Petrich et al. (1992).

A highly GC-rich inverted repeat is located in the
**Fig. 5.** Potential stem-loop structure located in the lat-pcbAB intergenic region. The numbers (bp) indicate the positions of the ends of the structure in the sequence data. Calculated $\Delta G = -54.4$ kcal mol$^{-1}$ at 25°C (Tinoco et al., 1973).

lat-pcbAB intergenic region, commencing 4–6 bp downstream of the lat ORF stop codon (Fig. 3). It could form a stable hairpin structure with a stem of 15–17 bp and a loop of 3 bp (Fig. 5) having a calculated $\Delta G$ value of $-54.4$ kcal mol$^{-1}$ (Tinoco et al., 1973). This element, which resembles a transcription terminator (Platt, 1986), lies within the pNHY7.52 insert, downstream of the lat sequences found to have promoter activity in pNHY7.12 and pNHY7.30. A search of the 111 bp region between this structure and the pcbAB start codon revealed no sequences closely related to Streptomyces promoter consensus sequences; all candidates had a total of five or more mismatches in each pair of hexamers.

Evidence for antisense transcription was also found in these experiments. The pNHY7.10 insert, which includes the entire pNHY7.12 insert, imparts high kanamycin resistance ($\geq 400 \mu$g ml$^{-1}$). Since the Asp718 #2-SalI #9 fragment fails to elicit resistance (pNHY7.11), promoter activities may originate from both DNA strands in the SaI #9–Asp718 #3 region and result in overlapping transcription. The pNHY7.01 insert shares the SaI #10–Asp718 #3 fragment with pNHY7.30 and confers a similar resistance level (about $80 \mu$g ml$^{-1}$), again suggesting promoter activity in both directions.

**DISCUSSION**

The complex phenotype of the *S. clavuligerus* mutant NP1 includes very low levels of cephalosporin production and LAT activity, and depressed ACVS and IPNS activities. Antibiotic production is stimulated when NP1 is fed $\alpha$-AAA, the product of LAT (and PCDH). Upon transformation of the mutant with pNBR1, a multicyclic plasmid carrying the entire wild-type lat gene and a truncated 5′ region (about 20%) of the pcbAB gene, LAT activity and antibiotic production are increased to approximately 70% of wild type, while ACVS and IPNS activities are restored to a lesser extent. Plasmid deletions which eliminate the lat gene also abolish complementation. Thus our current interpretation is that NP1 is a lat mutant and that it is complemented by the cloned wild-type lat gene. The observation of diminished complementation when a small internal portion of the cloned pcbAB sequences is deleted from pNBR1 (pNBR1.12) is intriguing and awaits further investigation. One possibility is that NP1 also carries a mutation in pcbAB. Otherwise, possible reasons for the effect of the deletion on repair of NP1 could be specific, such as elimination of a binding site involved in the regulation of the pathway, or general, such as alterations in mRNA structure leading to decreased half-life of the message.

Whether complementation of NP1 occurs in trans from the autonomous plasmids, or in cis by reconstitution of the wild-type region in the chromosome by recombination, is not yet known. If complementation requires integration, the plasmid curing data suggest that the NP1 mutation lies near one end of the cloned region in the chromosome where the frequency of marker exchange with the cloned sequences would be relatively low, as would be expected for a mutation in lat. This can be confirmed in the future by sequencing the lat gene from NP1. If, on the other hand, NP1 complementation occurs by the lat gene in trans, at least two mechanisms are possible. $\alpha$-AAA, provided by the action of LAT (and endogenous PCDH), may be an inducer of pcbAB expression and ACV, the tripeptide product of the ACVS reaction, may in turn induce pcbC. Alternatively, protein–protein interactions between the LAT, PCDH, ACVS and IPNS proteins themselves could be necessary for proper functioning of the pathway.

The levels of antibiotic production restored to the NP1 transformants are consistently lower than those of the wild type. Whether complementation requires integration or not, the cloned region is probably expressed from vector promoter sequences. The DNA data reported here include just 87 bp upstream of the putative lat start codon and Petrich (1993) has recently mapped the 5′ end of the lat transcript at the −88 bp position. The promoter region for the tyrosinase gene (mel) in pJ702 lies immediately upstream of the cloned lat and in the correct orientation; however this promoter is apparently not expressed in *S. clavuligerus* (Bailey et al., 1984; our observations). It is therefore likely that transcription is mediated by the vector rep promoter mapped by Kendall & Cohen (1988). Alternatively, an AT-rich sequence (TAAAAAT) closely resembling the eu bacterial -10′ consensus for vegetative promoters (TATAAT; McClure, 1985) and the corresponding region in streptomycete promoters [TAg(Pu)(Pu)T; Strohl, 1992] which is located just 5 bp from the end of the cloned region in pNBR1, may be sufficient to stimulate transcription. This element, read through from rep or a fortuitous promoter is likely to be responsible for the expression of the cloned region. The absence of a complete native promoter may explain why the levels of antibiotic production restored to the NP1 transformants are lower than those of the wild type.

Several lines of evidence suggest interplay in the expression of the *S. clavuligerus* lat, pcbAB and pcbC genes. They are closely clustered (Smith et al., 1990a; Madduri et al., 1991; Martín, 1992; Tobin et al., 1991) and arranged in the same order as their product enzymes in the biosynthetic pathway. Physiological studies have shown that the formation and activities of ACVS and IPNS are controlled by the sources of nitrogen and phosphate provided (Zhang et al., 1989a, b). Such influences on LAT production are likely to contribute significantly to the overall expression of the cloned genes, particularly if their native promoters are not recognized by the vector system.
cluster may also be transcribed in its entirety, producing a large mRNA containing evidence for promoter sequences. However, their mRNA transcriptional fusions showed that the SacB-t (Steinmetz et al., 1985) are analogous structures from Bacillus subtilis. Situations were found for pcbC, the third gene in the cluster. Similarly, Garcia-Dominguez et al. (1991) reported that lat-pcbAB-pcbC region lacks a transcriptional start site, promoter activity and recognizable promoter sequences which we detected in gene fusions with fragments from upstream, but not downstream, of this structure. Although other roles, such as in mRNA stability, have been found for such structures, a transcription terminator would allow selective expression of lat only under certain physiological circumstances, while preventing expression of the downstream cephalosporin biosynthetic genes. Under conditions where antibiotic formation is desirable, the expression of distal genes may then be permitted via an anti-termination mechanism.

Malmberg et al. (1993) have reported some evidence for separate expression of lat and pcbA. Upon construction of an S. clavuligerus strain containing a chromosomal duplication of the wild-type lat gene, they found that while LAT activity was increased several-fold, ACV synthetase activity was not altered under their conditions. Li et al. (1991) have postulated an anti-termination mechanism in the regulation of the Streptomyces actuoso nshR gene, nshR. They proposed that the putative terminator is preceded by a 'Box A' sequence resembling conserved sequences implicated in transcription anti-termination in Escherichia coli. Fig. 6 shows a comparison of several Box A sequences with a region located upstream of the inverted repeat in the lat-pcbAB intergenic sequence (Fig. 3). The S. clavuligerus sequence most closely resembles (10 identities among 16 positions) two Box A regions in the E. coli bgl (β-glucosidase utilization) operon. Thus the possibility of regulation by transcription termination in the lat-pcbAB-pcbC region merits further consideration.

ACKNOWLEDGEMENTS

This research was supported by the National Science Foundation, Biochemie GmbH, Lederle Laboratories and Northeastern University. We thank Gerhard Weber and Ernst Leinser (Biochemie GmbH) for the exchange of hybridization probes and sharing of experimental results. We are also grateful to Venetka Agayn, Manoj Ratnakaye, Xinga Xiao and Trien Vihn Ho for their valuable assistance and to Susan Jensen for a Macintosh version of the FRAME program.

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Received 21 February 1994; revised 6 June 1994; accepted 14 June 1994.