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(54) Title: ISOLATION AND SEQUENCE OF THE ACETYL COA:DEACETYLCEPHALOSPORIN ACETYLTRANSFERASE GENE

(57) Abstract

The invention relates to a DNA sequence with a nucleotide sequence encoding a polypeptide in a host cell having Cephalosporin C synthetase (DAC acetyltransferase) activity which is the last enzyme in the synthesis of cephalosporins. Embodiments of the invention relate to methods and compositions for detecting the gene or its product, mutants and hybrids of the gene, replicative cloning and expression vectors, and processes for preparing recombinant DAC acetyltransferase polypeptide useful for host cell synthesis of, as well as cell-free synthesis of natural and synthetic cephalosporin antibiotics.

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ISOLATION AND SEQUENCE OF THE ACETYL COA:DEACETYLCEPHALOSPORIN ACETYLTRANSFERASE GENE

Field of the Invention

The invention relates generally to genetic engineering involving recombinant DNA technology, and particularly to DNA sequences for the final gene in cephalosporin antibiotic synthesis that encodes Acetyl CoA:Deacetylcephalosporin acetyltransferase useful in recombinant and cell-free synthesis of intermediates in cephalosporin synthesis, and in increased synthesis of Cephalosporin C antibiotics.

Background of the Invention

Beta lactam antibiotics including the penicillins and cephalosporins have had incalculable social impact in freeing mankind from the scourge of bacterial diseases. The emergence of antibiotic resistance in bacteria has spurred efforts to create, through chemical means and genetic engineering, new classes of antibiotics. Cephalosporins are particularly useful because they affect both gram negative and gram positive bacteria, are more resistant to beta-lactamases than penicillins, and do not elicit allergic responses in those allergic to penicillins. Intermediates in the synthesis of Cephalosporin C are very useful reagents in chemical synthesis of novel antibiotics, which, while retaining the penicillinase resistant beta-lactam ring characteristics of Cephalosporin C, have increased antibiotic potency.

Quite recently techniques have become available for genetically engineering in filamentous fungi that produce cephalosporins, but unfortunately, many of the approaches employed in other genetic systems are not easily amenable to use with filamentous fungi. Despite technical challenges, 3 of the 5 genes involved in the synthesis of Cephalosporin C have recently been cloned. The genes for Acetyl CoA:Deacetylcephalosporin acetyltransferase and epimerase have proven to be elusive, (in part because the enzymes they encode are unstable). It is the acetyltransferase gene which is a subject of the present disclosure.

Throughout the specification, the notation "(#)" is used to refer to the documents in the appended Citation section.

Cephalosporin producing organisms are shown in Table I below. It has only recently become possible to study filamentous fungi with recombinant genetic engineering techniques, and of the cephalosporin producing organisms listed in Table I only *Cephalosporium acremonium* and the bacteria *Streptomyces clavuligerus* have been extensively studied. *C. acremonium* was first isolated from near a sewage outflow on the coast of Sardinia in 1948, and has been highly evolved through mutational and selectional cloning for more than two decades as a commercial source of beta lactam antibiotics and cephalosporins.

TABLE I

Cephalosporin Antibiotic-Producing Organisms

Arachnomyces	*minimus*
Anixiopsis	*peruviana*
Cephalosporium	*acremonium*
	purpurascens
	polyalenrum
	curtipes
Emericellopsis	*terricola*
	minima
	synnematicola
	glabra
	mirabilis
	salmosynnemata
Paecilomyces	*carneus*
	persicinus
Spiroidium	*fuscum*
Streptomyces	*clavuligerus*
	hygroscopicus
	lipmanii

Chemical derivatization, modification, and substitution have been used for more than two decades to alter the properties of cephalosporins. Such efforts, while providing invaluable antibiotics, have resulted in complex, time consuming, low-yield, and costly chemical processes. Obtaining low-cost sources of intermediates and efficient biochemical methods is highly desirable and could result in considerable savings in production of antibiotics.

Attempts to purify enzymes and intermediates from cell extracts have not solved the major problem of cost, because the purification processes are still complex and time consuming and several of the enzymes are unstable. A source

of enzymes from a recombinant genetically engineered source would be highly desirable.

Molecular biology in *C. acremonium* is at an early stage compared with the extensive studies conducted with *Saccharomyces cerevisiae*. Several laboratories have independently developed transformation systems in filamentous fungi, and quite recently stable integrative transformation of *C. acremonium* was demonstrated, although at a low frequency (1-3). This has offered (for the first time) the possible opportunity to specifically and predictably alter antibiotic production in *C. acremonium*. However, approaches commonly employed in other genetic systems are not easily amenable for routine use with *C. acremonium*: namely, screening for complementation of mutant phenotypes, gene disruption techniques, or gene replacement techniques. In addition, the most useful industrial strains of *C. acremonium* transform even less efficiently than wild type ATCC 11550, (e.g., the strain used by Queener et al. 1985 (1), and Skatrud et al. 1987 (3)). Studies have been hampered by the lack of cloned genes, and particularly those studies attempting to delineate the regulatory interactions and rate limiting steps in cephalosporin synthesis, i.e., studies designed to cut the costs of antibiotic production by improving efficiency.

Biosynthesis of Cephalosporin C involves the sequential action of at least 5 genes, and their 6 enzymatic activities, as depicted in FIGURE 1. (Unlike bacteria, in *C. acremonium* the cef E and cef F genes are fused (cef E/F), giving a total of 5 instead of six genes.) The 6 enzymatic activities oxidatively condense L-alpha-aminoadipic acid, L-cysteine and L-valine precursors into Cephalosporin C through a series of intermediates. All of the intermediates with the exception of the first (delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine; LLD-ACV or simply ACV) contain a beta lactam ring. The final step in the synthesis of Cephalosporin C involves the addition of an acetyl group from acetyl CoA to the cephem ring. The reaction is catalyzed by acetyl CoA: Deacetyl cephalosporin acetyltransferase (also commonly known as 'Cephalosporin C synthetase', 'DAC acetyltransferase', 'acetyl CoA: cephalosporin acetyl-transferase' or 'cephalosporin acetyltransferase'). The gene encoding Cephalosporin C synthetase (cefG) has not previously been cloned.

In *C. acremonium*, three cephalosporin pathway genes (encoding 4 enzymatic activities) have been cloned and sequenced: namely, pcbAB (plasmid, cephalosporin biosynthesis AB), encoding ACV synthetase (4); 2) pcbC, encoding isopenicillin N synthetase (IPNS; 1,5); and, 3) cef E/F gene, encoding both deacetoxycephalosporin C synthetase (DAOCS, also commonly known as

'expandase') and deacetylcephalosporin C synthetase (DACS, also commonly known as 'hydroxylase'), respectively (6,7). Cloning in these references was accomplished using a portion of the deduced amino acid sequence of the enzyme to synthesize oligonucleotide probes which were used to screen cosmid libraries of *C. acremonium* DNA prepared in *E. coli*. The Cephalosporin C synthetase enzyme is relatively unstable, and cloning of cefG gene has not been disclosed previously.

Nucleotide sequence homologies are reported between cephalosporin genes coding for the same enzyme in different genus and species of filamentous fungi (8-12). In the case of IPNS, homology of about 74% exists at the nucleotide sequence level between the genes in *C. acremonium* and *Penicillium chrysogenum* (8). In comparing DACS/DAOCS encoded by cef E/F in *C. acremonium* with the bacterial cef E gene product of *S. clavuligerus*, it is reported that there is 57% identity at the amino acid level and 65% identity in the gene sequence (11).

The genes encoding the enzymatic activities involved in the Cephalosporin C biosynthetic pathway have been reported to be located on separate chromosomes in *C. acremonium* with IPNS on chromosome 6 and cef E/F on chromosome 2 (13).

Selection, mutagenesis and screening have been useful for producing *C. acremonium* variants useful for producing Cephalosporin C on an industrial scale. However, such methods are time consuming, costly, nonspecific, unpredictable, and the resultant variants are frequently unstable. It is reportedly routine for tens of thousands of mutagenized colonies to be screened and tested with none showing an improvement in antibiotic synthesis (14 at p. 483). Genetic engineering offers an attractive alternative to improve synthesis of cephalosporins (15). Regulation of cephalosporin gene expression is not well understood; thus, it is difficult at present to predict what types of genetic manipulations may lead to increased cephalosporin production.

Genetic transformation and selection for increased Cephalosporin C production has been reported by Skatrud et al. (14) and Ingolia et al. (16) by using plasmid vectors containing the cef E/F gene and the hygromycin drug resistance gene (as a selectable marker) to transform a commercial high-Cephalosporin C-producing strain of *C. acremonium* (394-4; derived from the wild type ATCC 11550). A resultant selected strain reportedly showed a 15% improvement in Cephalosporin C production in pilot scale (150 liter) fermentation (relative to untransformed controls).

Progress in the protein chemistry and enzymology of Cephalosporin C biosynthesis has been characterized as, (quoting from Martin, 15), ". . . very slow due, in part, to the intrinsic instability of the enzymes involved." An enzymatic

activity involved in acetylation of DAC to Cephalosporin C (DAC acetyl-transferase) was identified by Liersch et al. (17) and Fujisawa et al. (18) in mycelial extracts of mutants of *C. acremonium* producing both DAOC and DAC. Liersch et al. (17) partially purified the enzyme activity using ammonium sulfate fractionation and Sephadex G-200 molecular-sieve chromatography. Mutant *C. acremonium* strains have been reported which excreted DAC and in which no DAC acetyltransferase activity could be detected (17,18).

Rate limiting steps in synthetic pathways are important points where improving efficiency may significantly decrease cost. Two studies have recently attempted to identify points in Cephalosporin C synthesis where cellular enzyme activity may be rate limiting. In the first, gene dosage of pcbC (the IPNS gene) was reported increased (ie. in transformants) and the effect on cephalosporin synthesis studied (2)). The findings reported that IPNS enzyme may not be rate-limiting in this production strain. In a second study, quantitative measurements were made of the levels of the intermediates isopenicillin N, penicillin N, and DAC and these were compared with the levels of Cephalosporin C produced. The results reportedly suggested that DAOCS/DACS may be limiting in strain 394-4 (an industrial production strain of *C. acremonium* derived from ATCC 11550). In support of this notion, the transformation of this production strain (discussed above) with cef E/F resulted in increased production of Cephalosporin C.

Cellular extracts for synthesis of penicillins and cephalosporins is known in the art. Demain et al. (19) reported conversion of penicillin N, but not its precursors, to a cephalosporin, suggesting that the cell-free system lacked IPNS and epimerase but contained all the other necessary enzyme activities. Production of isopenicillin derivatives in a cell-free system using a *C. acremonium* extract was subsequently reported (20) suggesting that the extract contained IPNS, but not epimerase. Conversion of isopenicillin N to penicillin N using fresh (not frozen) extracts of *C. acremonium* containing IPNS and epimerase activity was reported, suggesting preservation of epimerase activity (21). Synthesis of unnatural cephalosporins from polypeptide precursors using *C. acremonium* extracts containing IPNS, epimerase, and expandase activities was reported (22).

The relative instability of the enzymes involved in Cephalosporin C biosynthesis makes it highly desirable to have a genetically-engineered source for all the enzymes in this pathway. This would provide a low-cost reproducible source of enzymes for cell-free synthesis of intermediates in cephalosporin biosynthesis. These intermediates are useful reagents in chemical synthesis of cephalosporin derivatives. Improving the rate and efficiency of Cephalosporin C

synthesis would result in significant cost savings. It would also be desirable to identify and clone the cefG gene, so that specific probes and screening methods could be used to identify variants with increased gene copy number or level of expression. The availability of the cefG gene would offer significant improvements over the present time consuming and costly methods of mutagenizing, selecting, and screening.

Summary of the Invention

Pursuant to the present invention DNA sequences of the entire cefG gene are provided for encoding acetyl CoA:cephalosporin acetyltransferase polypeptide, which is the last enzyme in Cephalosporin C synthesis in *C. acremonium*. These DNA sequences are useful in replicative cloning and shuttle vectors, and in expression vectors for transforming host cells. In another aspect the invention provides methods for constructing mutant DNA sequences related to the cefG gene by deletion and conservative substitution, and which encode polypeptides having modified enzyme activity. Other aspects provide assays including nucleic acid probes and immunoassays for measuring cefG gene and its translation products. Methods are provided using these assays to identify and select novel variants and strains of filamentous fungi having relatives of the cefG gene. Other aspects of the invention relate to novel methods and transformants with multiple copies of the cefG gene that synthesize remarkably greater amounts of Cephalosporin C than non-transformed Cephalosporin C producing strains.

Brief Description of the Drawings

FIGURE 1 is a schematic drawing which shows the biosynthetic pathway, enzymes, and genes reportedly involved in synthesis of cephalosporin antibiotics, as described in the Background section of the specification.

FIGURE 2 is a flow chart which shows schematically the steps involved in cloning and sequencing the gene encoding acetylCoA:deacetylcephalosporin acetyltransferase (cefG), as described in Examples I-VIII of the Detailed Description of the Invention.

FIGURE 3 shows by Northern blot hybridization that transformation of the mutant M40 strain (incapable of synthesizing acetyltransferase) with the cefG gene resulted in expression of acetyltransferase mRNA, as described in Example XI.

FIGURE 4 shows by antibiotic bioassay that cefG transformed mutant M40 strains express Cephalosporin C which is fully active in neutralizing the bacterial growth of *A. faecalis*, as described in Example XIII.

FIGURE 5 shows by high pressure liquid chromatography that the cefG transformant strain M40-T_3 produces Cephalosporin C antibiotic, as described in Example XIV.

FIGURE 6 confirms by high pressure liquid chromatography that the Cephalosporin C produced by the cefG transformant strain M40-T_3 is indistinguishable from purified Cephalosporin C standard, as described in Example XIV.

FIGURE 7 shows by high pressure liquid chromatography that a second transformant M40-T_5 strain also produces cephalosporin antibiotic, as described in Example XIV.

FIGURE 8 shows by high pressure liquid chromatography that the mutant M40 strain, from which the cefG transformants were derived, is incapable of synthesizing Cephalosporin C.

FIGURE 9 shows the genomic organization of the acetylCoA:Deacetyl-cephalosporin Acetyltransferase gene (cefG) as deduced following nucleotide sequencing of the entire gene.

FIGURE 10 shows similarities in the amino acid sequences in the N-terminal open reading frame between the amino acid sequence of Acetyl CoA:Deacetylcephalosporin Acetyltransferase (encoded by cefG; and termed AACACTCDNA in the FIGURE) and the amino acid sequence of Homoserine Acetyltransferase of *Ascobollus immersus* (MET2 & ASCIM).

FIGURE 11A shows the nucleotide sequence of bases 1-720 of cefG cDNA and FIGURE 11B shows the remaining nucleotide sequence (bases 721-1183) of cefG cDNA.

FIGURE 12A shows the nucleotide sequence of bases 1-720 of cefG genomic DNA, FIGURE 12B shows the nucleotide sequence of bases 721-1575, and FIGURE 12C shows base 1575-1807 of cefG genomic DNA.

Detailed Description of the Preferred Embodiment

The present invention provides an isolated cefG DNA sequence which encodes acetyl CoA:cephalosporin acetyltransferase, the last enzyme in the synthesis of *Cephalosporin C*. Since it has proven difficult to obtain an amino acid sequence for this relatively unstable enzyme, two different interrelated cloning approaches were employed successfully to identify and isolate the cefG gene from *C. acremonium* that encodes acetyltransferase. These approaches are depicted diagrammatically in FIGURE 2.

The first approach, labeled "Genomic Cloning" in FIGURE 2, involved investigating gene sequences upstream of the cef E/F gene. Clones were selected

from a *C. acremonium* genomic library (Examples I-IV) on the basis of their hybridization with oligonucleotide probes constructed to the 3' and 5' regions of the cef E/F gene. A clone with the correct restriction pattern was identified as containing cef E/F. DNA sequence analysis in the region upstream of cef E/F revealed a long open reading frame (FIGURE 9; ATG = initiation codon; TAA = stop codon; introns are indicated by boxes and the positions are numbered; SEQ ID No. 4; FIGURE 12). The protein coded for by this open reading frame show significant amino acid sequence similarity to the homoserine acetyltransferase from *Ascobollus immersus* (FIGURE 10; METZ & ASCIM is *Ascobollus immersus* amino acid sequence and AACACTDNA is the deduced amino acid sequence; colons indicate identical amino acids; FIGURE 10; SEQ ID No. 5). The sequence similarity with an acetyltransferase, and the linkage with cef E/F indicated to us that this open reading frame may code for the Cephalosporin C acetyltransferase. To test whether the gene encoded Cephalosporin C acetyltransferase, M40 mutants lacking Cephalosporin C acetyltransferase were transformed (Example XI). The transformants synthesized Cephalosporin C (Example XIII) which was biochemically indistinguishable from natural Cephalosporin C (Example XIV). DNA from the cefG gene was used to make restriction fragment probes that were used to screen a *C. acremonium* cDNA library (Examples V-VIII).

In the second approach, labeled "cDNA Cloning" in FIGURE 2, the same nucleotide coding sequence was identified (SEQ ID No. 3, FIGURE 11), and this approach also revealed the presence of two introns in the cefG gene. In this respect, the *C. acremonium* cefG gene is distinguished from any putative bacterial cefG gene.

To further verify the identification of the cefG gene, other experiments were conducted in which *Aspergillus niger* (a genus and species naturally lacking the genetic information for synthesizing acetyltransferase) was transformed with the cefG gene. These transformants now synthesized acetyltransferase activity. These findings and others (below) confirm that the cefG gene, encoding the last enzyme in the synthesis of Cephalosporins has finally been discovered.

In another aspect, the present invention provides recombinant vectors containing the cefG gene capable of transforming a host cell to produce a recombinant host cell. For production of a purified recombinant acetyl CoA:cephalosporin acetyltransferase polypeptide suitable host cells include *E. coli*, *Streptomycetes*, *Cephalosporium*, or *Saccharomyces*, while for production of

Cephalosporin C (or intermediates as detailed for example in FIGURE 1), suitable host cells include, e.g., *Cephalosporium* or *Streptomyces* cells.

C. acremonium is a well-known microorganism, and several suitable host cell strains from the American Type Culture Collection under accession numbers such as ATCC 20339 (*Cephalosporium* sp. strain F12), ATCC 14553 (*C. acremonium*), and ATCC 36255 (*Acremonium strictum*, of which M-0198 is a nonproducing mutant. Other strains of host cells known to those skilled in the art are of course also useful including many species of Streptomyces, for example, *S. lipmanii* (A16884, MM4550, MM13902), *S. clavuligerus*, *S. lactamdurans*, *S. griseus*, *S. hygroscopicus*, *S. madayamensis* (WS-3442-D), *S. chartreusis* (SF1623), *S. heteromorphus* and *S. panayensis* (C2081X); *S. cinnamonensis*, *S. fimbriatus*, *S. halstedii*, *S. rochei*, and *S. viridochromogenes; S. cattleya*; and *S. olivaceus*, *S. flavovirens*, *S. flavus*, *S. fulvoviridis*, *S. argenteolus* and *S. sioyaensis* (MM4550 and MM13902).

Genetic transformation of *Cephalosporium* or *Streptomyces* host cells with plasmid vectors containing the nucleic acids of the present invention (e.g., SEQ ID No. 3-4; FIGURE 11-12) is conveniently accomplished using for example the pIT_{221} expression plasmid system described by Chapman et al., U.S. Patent No. 4,762,786 (incorporated herein by reference). This vector and others which contain the DNA sequence of the invention operably linked (e.g., in the correct reading frame) to suitable control sequences, e.g., *Cephalosporium*-functional autonomous replication sequences, transcriptional and translational activating sequences, promoters and enhancers and integration sequences, are useful for producing transformed host cells which express the polypeptide gene products of the invention (e.g., SEQ ID No. 5; FIGURE 10). These transformed recombinant host cell systems are referred to herein as expression systems.

Useful shuttle vectors for transferring genetic material between *E. coli*, *Cephalosporium*, and many related *Streptomyces* species including related Nocardia are for example pETS702 and pPLS221 and other plasmid vectors described in Chapman et al., op.cit. These vectors and the plasmid and cosmid cloning vectors operative in *Escherichia coli* (e.g., pBR322, λgt10, λgt11, etc.; ref. 23) are all referred to herein as replicative cloning vectors. Methods for preparing these replicative cloning vectors are well known to those skilled in the art (23).

Embodiments of the invention are also useful for preparing vectors containing nucleotide sequences that are related to the nucleotide sequence of the cefG gene (i.e., SEQ ID No. 4; FIGURE 12) or its mRNA (i.e., SEQ ID No. 3;

FIGURE 11), e.g., by deletion or nucleotide substitution. The invention provides that recombinant host cells transformed with vectors containing these related DNA sequences produce enzymes with modified enzyme activity, e.g., decreased, increased, showing altered response to regulatory control or improved commercial properties. Recombinant host cells expressing decreased enzyme activity are useful for preparing intermediates while those expressing increased activity are useful for preparing Cephalosporin C, as well as the acetyl CoA:cephalosporin acetyltransferase enzyme. Recombinant host cells expressing enzymes with altered response to regulatory control, e.g., less sensitive to end-product feedback inhibition of enzyme activity, are useful in cell-free synthesis of antibiotics. Recombinant host cells expressing enzyme with improved commercial properties include enzymes, e.g., with greater stability and improved performance in cell-free synthesis of antibiotics.

Mutagenesis and selection for cefG are useful as targeted methods for creating and selecting desirable strains and variants producing intermediates in Cephalosporin biosynthesis. For example, mutations created in the cefG gene (e.g., SEQ ID No. 4, FIGURE 12) by site-directed mutagenesis are useful to create new strains of fungi, yeast and bacteria as expression systems for use in antibiotic synthesis. The nucleotide sequences of the disclosure thus provides one skilled in the art the necessary road map to introduce specific mutations (e.g., deletions, insertions, substitutions). The resultant mutagenized DNA sequences can then be used in replicative cloning vectors, shuttle vectors, or expression vectors (above). Host cells transformed with the vectors are useful as a source of enzyme having modified activity (above). Embodiments of the present invention thus anticipate production of mutations in the cefG gene, and in the related cefG genes in other strains of filamentous fungi (and bacteria) as well as selection methods for distinguishing among these mutants for desirable traits such as (but not limited to) increased production of intermediates in Cephalosporin C biosynthesis which may be useful for subsequent production of other chemically modified cephalosporin antibiotic derivatives.

The transformed host cells of the invention are a useful source for preparing cellular extracts for synthesis of antibiotics. For example, one illustrative method of preparing a cellular extract for synthesis of antibiotics is to lyse a protoplast pellet made from whole cells obtained from 40-70 hr. mycelia and treated, e.g., with Cytophagolytic enzyme L_1 preparation and Zymolyase-5000. After treatment with the enzymes the extract is clarified by centrifuging, chilled (but not frozen) at sub-zero temperatures, and re-centrifuged. Preferably, the

enzyme is used in substantially pure form, e.g., by chromatographic separation from the cell-free extract. Synthesis of Cephalosporin C and its intermediates may be accomplished using this cell-free extract for example as described by Demain et al., U.S. Patent No. 4,307,192 and A. Scheidegger et al., J. Antibiotics 37:522-531, 1984. Semi-purified enzymes such as this can be prepared from numerous microorganisms, e.g., *Cephalosporium acremonium* (such as strains ATCC 48272 and ATCC 36225), *Streptomyces clavuligerus* and *Streptomyces lipmanii*. The cephalosporin products and intermediates obtained from these enzymatic processes are useful intermediates in synthesis of known cephalosporin semi-synthetic antibiotics. For example, the oral antibiotic cephalexin can be prepared by deacylating the 3-carboxyphenylacetyl group and the adipoyl group of the product is then deacylated to the corresponding 3-substituted 7-β-amino-3 cepham nucleus. The latter is then reacylated to provide the desired 7-β-acylamino cephalosporin. Other known cephalosporin antibiotics are also obtained by well-known acylation methods, examples are provided in Baldwin E.P.O. 0,268,343, published 25.05.88.

In another aspect, the invention provides methods for modifying Cephalosporin C synthesis (e.g., increasing or decreasing synthesis) in Cephalosporin C producing organisms by transforming host cells of these organisms with vectors containing the cefG gene. Synthesis of Cephalosporin C is increased when extra copies of the cefG gene are introduced into host cells to form transformed host cells (e.g., Example XVI). Synthesis of Cephalosporin C may be decreased by introducing a mutant cefG gene into host cells and selecting transformed host cells with either decreased synthesis of Cephalosporin C; or, decreased levels of cefG mRNA; or, decreased cephalosporin acetyltransferase enzyme activity. For example, upstream regulatory regions (i.e., upstream from the ATG start codon in SEQ. ID. No. 4 and FIGURE 12A) are particularly attractive targets for site-directed mutagenesis to create the mutagenized cefG genes useful in this aspect, although other regions of the nucleotide sequence are also useful.

In another aspect the invention provides nucleic acid probes (labeled probes) for identifying the presence or the amount of the nucleotide sequence of the cefG gene and related nucleotide sequences (e.g., by deletion and/or nucleotide substitution) in cell extracts of organisms. Detection of the cefG gene may be accomplished, for example, using labeled probes prepared from genomic fragments, DNA fragments, cDNA fragments (e.g., SEQ ID No. 3; FIGURE 11), synthetic and natural nucleic acids and oligonucleotides that hybridize to the cefG gene of *Cephalosporium acremonium* (as provided in SEQ ID No. 4; FIGURE 12)

under stringent conditions. These probes are useful in identifying the cefG gene in cellular extracts of other organisms, e.g., using suitable enzyme-, fluorescent- and ^{32}P-radiolabeling methods and stringent hybridization conditions (23). For example, it is possible to identify related genes synthesizing Cephalosporin C synthetase in other strains of filamentous fungi (or bacteria) by using labeled oligonucleotide probes under stringent conditions for identification of positive clones in genomic and cDNA libraries. It is also possible to examine the restriction fragments of these clones using Northern and Southern blotting, respectively; and, it is routine for those skilled in the art to determine the nucleotide sequence of the related gene and to determine the relationships between nucleic acid sequences based on deletion and substitution, such as conservative substitution of a purine base for a purine base or a pyrimidine for a pyrimidine.

The labeled nucleic acid probes of the invention are useful in direct screening for new species and strains of filamentous fungi (and bacteria) having modified enzyme activity (as detailed above). For example, using the nucleic acid probes of the invention for Northern and Southern blotting one can rapidly screen cellular extracts containing DNA's or mRNA's prepared from hundreds of natural strains, mutagen treated cultures, or transformed recombinant host cells looking for increased or decreased expression of the cefG mRNA (e.g., by methods similar to those used in Examples II-VIII, below). In this way the invention provides screening and selection methods for identifying and isolating cephalosporin producing strains with novel properties.

Other aspects of the invention provide polypeptides of the enzyme encoded by the cefG gene. Peptide portions of the acetyltransferase enzyme (i.e., SEQ ID No. 5; FIGURE 10) are useful for constructing synthetic peptides and polypeptides identical to, homologous with, or related to certain portions of the Cephalosporin C synthetase polypeptide. For example, useful homologous synthetic peptides may be at least 40% identical to a sequence of at least five amino acids; related peptides may be related by substitution of one amino acid for another amino acid of like physical properties (ie. a polar amino acid for a polar amino acid; a negatively charged for a negatively charged; a polar for a polar; a hydrophobic for a hydrophobic; etc.). These amino acid substitutions can be engineered by changing a nucleotide base (or series of bases) or a deletion of nucleotide(s) in a nucleotide sequence of the cefG gene. The synthetic polypeptides are useful for evoking specific binding partners, such as but not limited to monoclonal and polyclonal antibodies. They are also useful for regulating the activity of other

enzymes in the Cephalosporin C biosynthesis pathway, such as through feedback inhibition of enzyme synthesis and/or specific negative and positive feedback regulatory interactions as they occur between the different enzymes in the Cephalosporin C biosynthetic pathway, e.g., mechanisms involved in coordinate control of cephalosporin biosynthesis.

Specific binding partners, as exemplified by antibody reagents developed to synthetic peptides (above) are useful for qualitative and quantitative immunoassays such as those outlined below. The antibody reagents are also useful for preparing substantially pure preparations of Cephalosporin C acetyltransferase (and portions thereof), as for example (but not limited to) by immunoaffinity chromatography. The antibody reagents are also useful for blocking the activity of the Cephalosporin C acetyltransferase, such as (but not limited to) during cell-free synthesis of intermediates in Cephalosporin C synthesis using cellular extracts. An aspect of the invention provides immunoassays for quantitatively and qualitatively assessing the amount and type acetyl CoA:cephalosporin acetyltransferase produced by filamentous fungi. Immunoassays for expression of acetyltransferase enzyme, such as using monoclonal and polyclonal antibody reagents produced against synthetic peptides, are useful for selecting among natural, mutant, and recombinant cells to find those which express altered levels (e.g., intracellular or extracellular) or types (i.e., mutant) or amounts of Cephalosporin C acetyltransferase. The assays are also useful for identifying the sources, methods, and procedures for obtaining fragments and portions of Cephalosporin C acetyltransferase, as well as for assessing recombinant production of the Cephalosporin C acetyltransferase polypeptide or for assessing amounts of the enzyme in cellular extracts as that may be useful in cell-free synthesis of cephalosporins. Examples of useful immunoassay formats which are well known to those skilled in the art include radioimmunoassay (RIA), enzyme-linked immunoassay (ELISA), fluorescence immunoassay (FIA), time resolved immunofluorescence assay, Western immunoblot assays, immunoelectrophoresis assays, and radial immunodiffusion assays. The RIA, ELISA, and FIA assays (for example) may be run in a homogeneous or heterogeneous assay format and in a stepwise or simultaneous assay mode, and bound can be separated from free ligand (or antibody) using solid-phase reagents such as for example bound to beads, dipsticks, microtiter plates, or tubes. The antibody reagents are also useful for purifying acetyl CoA:cephalosporin acetyltransferase, e.g., by affinity chromatography such as from cellular extracts or filamentous fungi, bacteria, or transformed recombinant host cells.

The ultimate possibility offered by the cloning of all five *C. acremonium* cephalosporin synthetic genes is that at some time in the future it may be possible to introduce them into cells from other species which may offer a significant improvements over filamentous fungi.

As used in this specification and appended claims, the following terms are intended to have the meanings set forth below:

"Isolated DNA sequence" means a substantially pure molecule of deoxyribonucleic acid having a defining sequence of nucleotides.

"Oligonucleotide sequence" means a molecule having a sequence of nucleotide bases in an indicated manner.

"Complementary" nucleotide sequence means an oligonucleotide sequence that hybridizes with DNA or RNA because it contains nucleotide bases capable of base-pairing with the bases in the DNA or RNA (e.g., a G pairing with a C or an A with a T or a U).

"Encode" refers to the genetic code by which triplets of nucleotides within a nucleotide sequence directs the selection of a particular amino acid for inclusion in a chain of amino acids.

"Amino acid sequence" is a proscribed sequence of amino acids in a chain.

"Polypeptide" means a chain of more than 30 amino acids having an amino acid sequence.

"Peptide" means a chain of 5 to 29 amino acids.

"Protein" means a polypeptide chain which has an activity, eg. enzymatic, or antigenic.

"Labeled probe" means an oligonucleotide having a label which permits its detection or quantitation (e.g., an enzyme-label, fluorescent-label, or radioactive label such as ^{32}P; Example III).

"Cephalosporin C synthetase" is used synonymously with DAC acetyltransferase, as they are commonly used in the art, to refer to the acetyl CoA:cephalosporin acetyltransferase.

"Deletion" means an oligonucleotide sequence which differs from the native oligonucleotide sequence by the absence of at least one nucleotide base.

"Homologous amino acid substitution" means that the replacement of one amino acid with another which has similar physical properties, e.g., an acidic amino acid by another acidic amino acid, or a basic with a basic, or polar with a polar, or a hydrophobic with a hydrophobic.

"Conservative nucleotide substitution" means the substitution of an nucleotide phosphate base with a corresponding complementary base, e.g. an A for a T; a T for an A; a G for C; or, a C for G.

"Hybridizing under stringent conditions" means binding (i.e., greater than 30% double-stranded) of one oligonucleotide sequence to another under conditions such as those included within the specification (e.g., Example VII), or alternatively, at page 387-389 of Maniatis et al. (23).

"Replicative cloning vector" means an oligonucleotide sequence capable of increasing in copy number when introduced into a cell.

"Expression vector" means an oligonucleotide sequence which contains at least regulatory elements, such as (but not limited to) a promoter, and a nucleotide sequence encoding Cephalosporin C acetyltransferase in such a manner that the introduction of the oligonucleotide sequence into a cell results in the expression of Cephalosporin C acetyltransferase.

"Expression" means transcription of an oligonucleotide sequence encoding Cephalosporin C acetyltransferase into complementary mRNA or translation of the mRNA to obtain production of the encoded Cephalosporin C acetyltransferase.

"To transform" means to introduce into a cell nucleic acids, such as an isolated DNA sequence or oligonucleotide sequence, for the purpose of altering the phenotype of the cell, such as (but not limited to) cases in which gene expression may be down-regulated, or alternatively, where expression of a gene may be turned on which was not previously present in the cell.

The foregoing and other aspects of the invention may be more fully understood in connection with the following representative examples, which are set forth for purposes of illustration and not by way of limitation of the inventive concepts.

Example I. C. acremonium Culture Conditions

Cephalosporin acremonium strains used in these procedures include a Cephalosporin C producing strain (Panlabs production strain PF14-1) and a mutant strain (M40) which does not produce Cephalosporin C. Analysis of M40 fermentations shows that the mutant strain produces the immediate Cephalosporin C precursor, deacetylcephalosporin. Strains were maintained on slants containing complete medium composed of sucrose, 20g; agar, 20g; peptone, 4g; yeast extract, 4g; $NaNO_3$, 3g; KH_2PO_4, 0.5g; K_2HPO_4, 0.5g; KCl, 0.5g; $MgSO_4.7H_2O$, 0.5g; $FeSO_4.7H_2O$, 0.01g; in 1 liter of distilled water, pH 6.6. After ten days of growth at 28°C and 65% relative humidity, 6 ml of sterilized water was added to the slants, and the culture growth was scraped from the agar

surface. The resulting suspension was transferred to a sterile screw-top tube containing glass beads. After macerating the culture growth by vortexing for a few minutes, 3.5 ml of the resulting suspension was used to inoculate liquid cultures. This suspension was also used to provide lyophiles of the cultures for storage at 4°C. The suspension of macerated culture growth was centrifuged, the pellet resuspended in 5% skim milk, and aliquots lyophilized in sterile ampoules.

A two-stage fermentation of the strains in shake flasks was used for the production of cephalosporins or for the production of mycelia as a source of DNA and RNA. The seed stage was initiated by adding the inoculum to 15 ml/250 ml flask of medium with the following composition: glucose, 5g; sucrose, 40g; corn starch, 30g; beet molasses, 50g; soybean meal, 65g; $CaSO_4.2H_2O$, 15.8g; ammonium acetate, 8g; $CaCO_3$ (pptd), 5g; $(NH_4)_2SO_4$, 7.5g; $MgSO_4.7H_2O$, 3.5g; KH_2PO_4, 1g; soybean oil, 0.15 ml/flask in 1 liter of distilled water at pH 6.2. Incubation was at 25°C and 65% relative humidity on a rotary shaker with a 70 mm diameter amplitude at 220 rpm. After 96 hours of incubation, the production stage was initiated by transferring 2 ml of vegetative seed to a fresh 15 ml/250 ml flask of the above-described media. Incubation was continued under the same conditions.

When mycelia were needed to generate protoplasts for transformation or as a source of DNA, the strains were grown in 100 ml/500 ml flask of complete media composed of: glycerol, 20g; peptone, 4g; yeast extract, 4g; KH_2PO_4, 0.5g; K_2HPO_4, 0.5g; KCl, 0.5g; $MgSO_4.7H_2O$, 1g; $NaNO_3$, 3g; $FeSO_4.7H_2O$, 0.01g in 1 liter of distilled water. Incubation was at 30°C on a rotary shaker at 200 rpm.

Example II. Isolation of Cephalosporin Genomic DNA

The vegetative mycelial growth from a 48-hour culture prepared in accordance with Example I was collected by filtration through cheesecloth. The collected mycelia were washed once with water, twice with 50mM Tris pH8, 100mM EDTA, 150mM NaCl, and again with water before they were frozen in liquid nitrogen and lyophilized overnight. The dried mycelia were ground with sand in a mortar and pestle and resuspended in 25 ml of 100mM LiCl, 50mM EDTA, 10mM Tris pH8, 4% SDS. After heating the suspension to 50-55°C in a 60°C water bath, the mixture was extracted first with 1M Tris (pH8) saturated phenol, followed by Tris-saturated phenol:chloroform (1:1,v:v) and then chloroform. RNA was precipitated from the aqueous phase by the addition of an equal volume of 6M LiCl cooled at -20°C for two to three hours. After centrifugation at 12000xg for 20 minutes at 4°C, the supernatant was made 66% (v/v) ethanol and cooled to -20°C for 15 minutes to precipitate the DNA. After

centrifugation as described above, the DNA pellet was washed with 70% ethanol, dried and resuspended in TE Buffer (10mM Tris-HCL, pH7.5, 1mM EDTA). The DNA concentration was estimated by comparison to known DNA standards when stained with ethidium bromide in agarose gel electrophoresis.

Example III. Construction of a Gene Library and Isolation of a DNA Fragment Containing the cef E/F Gene Encoding DAOCS/DACS

C. acremonium PF14-1 genomic DNA obtained by the procedure of Example II was partially digested with 100 µg Sau3AI for 5, 10, and 20 minutes (23) to achieve an average fragment size of 10 to 20 kb. Centrifugation of the digest on a 5 to 20% NaCl density gradient provided a further enriched 10 to 20 kb fraction which was ligated into the BamHI site of the lambda vector 2001. The ligation was mixed with a lambda DNA packaging system (Gigapack from Stratagene, La Jolla, CA) and the resulting plaque forming units (PFU) plated on agarose in NZCYM media (10 g NZ-amine, 5 g NaCl, 5 g yeast extract, 2 g $MgSO_4$-$7H_2O$, pH7.5) in five 157 mm petri dishes (approximately 10,000 PFU/plate). Plaques were transferred to nitrocellulose and hybridized with an oligonucleotide probe end-labeled with (γ^{32}P) ATP by T4 polynucleotide kinase. An oligonucleotide probe was constructed to complement the coding sequence of a DNA region at the 3'-end of the published sequence of the cef E/F gene (Samson et al., 1987 (12)). The probe was synthesized by cyanoethyl phosphoramidite chemistry (Pharmacia Gene Assembler instrumentation) and it is shown in SEQ ID No. 1. Those clones that hybridized with the SEQ ID No. 1 probe were isolated and their DNA prepared for restriction enzyme mapping. The SEQ ID No. 2 oligonucleotide probe constructed to be complementary with the 5'-end of the cef E/F gene, was used to orient the gene within the cloned genomic fragments by Southern analysis of the restriction digests of the lambda clones.

A 7.2 Kb fragment thus shown to contain the cef E/F gene was gel purified from a BamHI digest of the DNA from a selected lambda clone and ligated into the BamHI site of a pUC18 vector. Restriction mapping of the resulting subclone, 104.80.1, and Southern blot analysis with the 3' and 5' cef E/F oligonucleotides probes (SEQ ID No. 1 and No. 2) revealed the position and orientation of the gene within the subclone. This clone provided a source of DNA for construction of fungal transformation vectors and subclones for DNA sequencing.

Example IV. Subcloning of the cefG (acetyltransferase) gene into M13 Vectors for DNA Sequencing

A 5.0kb genomic fragment was isolated from a BamHI/SstI digest of the 104.80.1 plasmid by separation in and elution from a 0.7% low melting point agarose gel. The DNA fragment was ligated to M13mp18 and M13mp19 DNAs which had been previously digested with BamHI and SstI. The ligations were done in a final volume of 10 µl for one hour at room temperature. Dilutions of the ligation mixture were used to transfect competent MV1190 cells (Bio-Rad) using electroporation (Gene Pulser, Bio-Rad, Richmond, CA). Preparation of the competent MV1190 cells and electroporation conditions were both according to the manufacturer's recommendations. The transfection mixture was plated on yeast tryptone (YT) plates in 3 ml of YT soft agar (5.0 g yeast extract, 8.0 g tryptone, 2.5 g NaCl per liter, pH 7.2) containing 40 µg/ml X-gal, 0.1 M IPTG, and 100 µl of an overnight culture of MV1190 grown in YT broth. Following overnight incubation of the YT plates at 37°C, recombinant M13 phages were identified by the colorless plaque formation technique involving insertional inactivation of the plasmid vector β-galactosidase gene activity. The colorless plaques were picked from the YT plates as agar plugs using a pasteur pipet and transferred into 1.5 ml of YT broth containing 10 µl of an MV1190 overnight culture. The cultures were grown six hours at 37°C on a rotary shaker and the cells pelleted by centrifuged at 12000xg for five minutes at 4°C. The supernatant was decanted to a fresh tube to serve as phage stock.

Preparation of Single-Stranded Template

Single-stranded template DNA was prepared by inoculating 1 ml of TY broth with 5 µl of M13mp18 and M13mp19 vector stock and 10 µl of a MV1190 overnight culture, incubating six hours at 37°C on a rotary shaker, centrifuging at 12000 xg for five minutes at 4°C and decanting the supernatant to fresh tubes. Two-hundred µl of 20% polyethylene glycol/(PEG, MW3350), 2.5M NaCl were added to the supernatant and the mixture was incubated for 15 minutes at room temperature. The tubes were spun at 12000xg for five minutes at 4°C and the supernatant was drawn off and discarded. The pellet was resuspended in 100 µls of TE buffer after which 50 µl of a Tris-saturated phenol:chloroform (1:1,v:v) mix was added. The tubes were vortexed 15 seconds, left and room temperature for five minutes, vortexed another 15 seconds, and then centrifuged at 12000xg for five minutes at 4°C. The top 80 ul of the aqueous layer was removed and 3 µl of 3M sodium acetate and 200 µl of ethanol were added, and the mixture was then placed at -70°C for ten minutes. The single-stranded DNA was pelleted by

centrifugation, washed with 70% ethanol, dried and resuspended in 18 µl of TE buffer.

DNA Sequencing

Two 1 ml cultures were prepared per template and combined into the final volume of 18 µl. Sequencing primers were annealed to the single-stranded templates, and the sequencing reactions were performed using the USB Sequenase Version 2.0 DNA Sequencing Kit per the manufacturer's instructions (USB, Cleveland, Ohio). Synthetic oligonucleotide sequencing primers for the sequence extension reactions were synthesized on an automated DNA synthesizer (Pharmacia, Piscataway, New Jersey), according to the manufacturer's instructions.

Example V. Isolation of Cephalosporin Total RNA and mRNA

Cultures of *C. acremonium* PF14-1 were grown for 96 hours in 15 ml fermentation medium (fermentation conditions previously described), at 25°C on a rotary shaker at 220 rpm. Mycelia were collected by filtration through a Whatman #1 filter under vacuum and washed with approximately 50 ml water. The mycelia were immediately scraped from filter, resuspended in 5 ml of "breaking buffer" (50mM Tris-HCL pH 7.4, 150mM NaCl, 5mM EDTA pH 8.0, 5% SDS), frozen in liquid nitrogen and lyophilized. After overnight lyophilization, 5 ml of water containing 0.1% DEPC and 5 ml of 1M Tris (pH8) saturated phenol:chloroform:isoamyl alcohol (50:50:1) were added and the mixture was left to thaw at 37°C for 20 minutes with shaking. The mixture was centrifuged at 12000xg for ten minutes at 4°C, and the aqueous layer was removed and re-extracted first with 1M Tris (pH8) saturated phenol:chloroform:isoamyl alcohol (50:50:1), and second with 1M Tris (pH8) saturated phenol, and third with chloroform. An equal volume of 6M LiCl was combined with the final aqueous layer, and the solution was left at -20°C for a minimum of four hours. The total RNA was pelleted at 12000xg for 20 minutes at 4°C, the pellet dissolved in 0.3 ml TE buffer plus 0.03 ml of 3M sodium acetate, and 2.5 volumes of ethanol were added to reprecipitate the RNA. The final pellet was dissolved in 0.1 ml of TE buffer and the RNA concentration was determined spectrophotometrically using absorbance at 260 nm.

mRNA was isolated from total RNA using an mRNA Purification Kit per the manufacturer's instructions (Pharmacia LKB Biotechnology, Piscataway, New Jersey). The kit uses prepacked oligo(dT)-cellulose columns for affinity purification of polyadenylated RNA.

Example VI. Construction of a lambda gt10 cDNA Library

Synthesis of cDNA was accomplished with a cDNA Synthesis Kit, per the manufacturer's instructions (Pharmacia LKB Biotechnology, Piscataway, New Jersey) and poly(A+) RNA isolated from *C. acremonium* PF14-1 as described above. The cDNA synthesis kit created cDNAs with cohesive EcoRI ends which were ligated to EcoRI cut lambda gt10 arms (Bethesda Research Labs, Gaithersburg, MD). One µg of EcoRI cut lambda gt10 arms and the total cDNA synthesis product were ligated overnight at 15°C in a total volume of 10 µl. cDNA in four µl of the ligation mixture was packaged using an *in vitro* packaging extract purchased as "Gigapack Gold-tm" (Stratagene, La Jolla, CA), and the resultant mixture was plated on NZCYM plates (above) using *E. coli* strains C600 (permissive) and C600 hf1A150 (non-permissive), obtained (Bethesda Research Labs, Gaithersburg, MD), to determine the titer and frequency of recombinants. The permissive and non-permissive *E. coli* strains were grown overnight in NZCYM broth plus 100 µl of 20% maltose. After overnight growth the cells were pelleted by centrifugation and resuspended in one-half volume of 10mM $MgSO_4$ for storage at 4°C until use.

Example VII. Screening of the lambda gt10 cDNA Library

Approximately 14,000 plaque forming units (pfu's) were screened by standard methods (Maniatis et al. 27) using *E. coli* strain C600hf1A150 (above; Example VI). A 1.2kb HindIII fragment (which contains 430bp of the 5' end of the *C. acremonium* cefG gene) was isolated from a HindIII digest of the pUC18 vector subclone 104.80.1 (Example III, above) by separation in and elution from a 0.7% low melting point agarose gel, and was labeled with ^{32}P for use as a probe to screen the cDNA library. Labeling of the isolated fragment was accomplished by random-primer extension reaction with (^{32}P) dCTP and an Oligolabeling Kit, per the manufacturer's instructions (Pharmacia, Piscataway, New Jersey). Hybridization reactions were performed in the presence of 50% formamide, 5X SSC (0.15M NaCl, 0.015M sodium citrate pH7), 0.1% SDS, 5X Denhardt's (5g ficoll, 5g polyvinylpyrolidone, and 5g BSA for 500 ml of 50X stock) and 100 µg/ml calf thymus DNA, at 37°C overnight. Hybridization assays were performed at least in duplicate. Three plaques termed, Clones #1, #2, and #3 were identified having overlapping nucleotide sequence, a composite of which is provided in SEQ ID No. 3; FIGURE 11. Clones 1-3 cDNA hybridized strongly on duplicate filters with the isolated ^{32}P-labeled probe and these were rescreened under identical

hybridization conditions and continued to show strong hybridization to the isolated ^{32}P-labeled *C. acremonium* cefG probe.

Example VIII. Subcloning of the acetyltransferase cDNA clones into M13 Vectors

The phage in the supernatant of the three positive plaques (identified in Example VII) was transferred into 1 ml of SM (0.1M NaCl, 0.1M $MgSO_4$, 0.05M Tris-HCL pH7.5, and 5 ml of 2% gelatin (w/v)) using 50 µl of chloroform and the mixture was incubated at room temperature for one hour. Phage suspensions (500 µl; i.e., of the plaque supernatant) were mixed with 50 µl of an overnight culture of *E. coli* strain C600Hf1A150 (Example VI, above) and incubated for 20 minutes at 37°C to adsorb the phage to the host bacteria; and, eight ml of NCZYM broth was then added and the cultures were grown overnight at 37°C on a rotary shaker. Cell debris from the resulting lysed cultures were removed by centrifugation at 1500xg for ten minutes at 4°C, and the supernatant was transferred to a new tube. RNase A and DNase I were added to a final concentration of 1 µg/ml each and the mixture was placed at 37°C for 30 minutes. An equal volume of SM containing 20% PEG and 2M NaCl was added to precipitate the phage and the mixture was left on ice for one hour. The PEG-precipitated phage were pelleted by centrifugation at 12000xg for 20 minutes at 4°C, resuspended in 500 µl SM, and centrifuged again at 12000xg for 2 minutes at 4°C. The supernatant, now containing the phage, was transferred to a new tube and 5 µl of 10% SDS and 5 µl of 0.5M EDTA were added to remove the protein coat from the phage DNA. After the tube was incubated for 15 minutes at 68°C, the solution was extracted with an equal volume of Tris saturated phenol:chloroform. The aqueous layer was transferred to a new tube; an equal volume of isopropanol was added; and, the tube was placed at -70°C for 20 minutes. The phage DNA was pelleted, washed with 70% ethanol, dried under vacuum, and resuspended in TE buffer. The DNA was digested with EcoRI (New England BioLabs, Beverly, MA) for 2 hours at 37°C and the desired 1.2 kb fragment was purified by electrophoresis on and elution from a 0.7% low melting point agarose gel. The isolated fragment was ligated in an EcoRI cut M13mp18 vector (Bethesda Research Labs, Gaithersburg, MD; one hour at room temperature). Dilutions of the ligation mixture were transformed into competent cells from *E. coli* strain MV1190 (Bio-Rad, Richmond, CA) using electroporation (Gene Pulser Apparatus, Bio-Rad) and the *E. coli* were plated in soft TY agar containing 40 µg/ml X-gal and 0.1M IPTG poured over the surface TY agar plates. Fifteen individual recombinant M13mp18 plaques were identified by the colorless plaque formation technique and each was collected as an agar plug from the plate using pasteur

pipets. The agar plugs were each transferred to 1.5 ml of TY broth containing 10 µl of an MV1190 overnight culture. The resulting cultures were grown for six hours at 37°C on a rotary shaker, and the resulting phage lysates were transferred to fresh tubes after first removing the residual cell debris by centrifugation. Two such lysates were chosen for DNA sequence analysis.

Example IX. Construction of Fungal Transformation Vectors

The 7.2 kb isolated BamHI fragment was purified from a BamHI digest of the 104.80.1 plasmid by electrophoresis on and elution from 0.7% low melting temperature agarose gels, and the isolated fragment was ligated into the unique BamHI site of the *Aspergillus niger* (*A. niger*) transformation vector pJL21. Integration of the pJL21 vector by *A. niger* confers a benomyl resistant phenotype on the transformants. pJL21 transformation vector was constructed by the addition to a pUC18 *E. coli* plasmid backbone to a 4.3 kb EcoRI fragment containing a beta-tubulin gene isolated from a benomyl resistant *A. niger* mutant. pJL21 also contains a 550 bp lambda cos fragment which enables the vector to be used for cosmid formation when appropriate size inserts are included.

A *C. acremonium* transformation vector was constructed with a phleomycin resistant gene as a dominate selectable marker. This was accomplished first by isolating a 660 bp fragment, containing the phleomycin resistance gene (a phleomycin binding protein gene from *Streptoalloteichus hindustanus*) and also coupled to a yeast cytochrome C1 terminator from a BamHI/HindIII digest of plasmid pUT713 (CAYLA, Toulouse Cedex, France) by electrophoresis on and elution from agarose gels. The isolated fragment was ligated into the BamHI/HindIII sites of M13mp18 bacteriophage vector. Next, an 840 bp NcoI fragment, containing the promoter region of the *C. acremonium* isopenicillin N synthetase gene, was isolated (by electrophoresis on and elution from agarose gels) from an NcoI digest of a genomic clone containing the IPNS gene (Samson et al., 1985). The IPNS-promoter fragment isolated in this manner was ligated into the M13 Rf construct at the NcoI site which is at the ATG start codon of the phleomycin resistant gene. Orientation of the promoter to the phleomycin resistant gene was confirmed by complementation of the following oligonucleotide to the single-stranded M13 subclone: 5'-CTCGAAAATCAGAAGAGC-3'. The oligonucleotide was synthesized as the inverse complement of a portion of the *C. acremonium* IPNS-promoter sequence. The 1.5 kb BamHI/HindIII fragment containing the IPNS-promoted, phleomycin resistance gene was transferred to the BamHI/HindIII site of the pUC18 plasmid to make an *E. coli* shuttle vector. This vector was shown to efficiently transform phleomycin resistance into the M40 and

PF14-1 *C. acremonium* strains. The 5.0 kb fragment upstream of the DAOCS/DACS structural gene was isolated (by agarose gel electrophoresis and elution) from an SstI/BamHI digest of 104.80.1 and the isolated fragment was ligated into the SstI/BamHI site of the *C. acremonium* transformation vector. The resulting vector construct, termed pCLS5, was used to transform cells of the M40 strain of *C. acremonium* to determine if it was capable of complementing the missing functions in the mutant strain such that it would restore the ability of the strain to produce Cephalosporin C.

Example X. Isolation of Plasmid DNA

E. coli cultures containing the pCLS5 plasmid were grown in 500ml LB broth (20g/l of LB Broth Base (Gibco, Paisley, Scotland), with 100 µg/ml ampicillin on a rotary shaker at 220 rpm for 12-16 hours at 37°C. The cells were pelleted by centrifugation at 4000xg for ten minutes at 4°C. The cell pellet was resuspended in 18 ml of Glucose Buffer (50mM glucose, 25mM Tris pH8.0, 10mM EDTA) and 2ml of 40mg/ml lysozyme (Sigma, St. Louis, MO) in glucose buffer was added, mixed, and the mixture was incubated at room temperature for 15 minutes. Forty ml of a freshly prepared solution of 0.2N NaOH, 1% SDS was added, and the mixture swirled gently and placed on ice for ten minutes. Thirty ml of 5M potassium acetate pH4.8 were then added, mixed well, and the resultant mixture was placed on ice for an additional ten minutes. The cellular debris were pelleted by centrifugation at 4000 xg for ten minutes at 4°C and the resulting supernatant was filtered through a cheesecloth filter. Isopropanol (0.6 volumes) was added to the clarified supernatant to precipitate the plasmid DNA, and the precipitate was formed during incubation at room temperature for 20 minutes. The plasmid DNA was pelleted at 4000xg for 20 minutes at 4°C and then washed with 70% ethanol and dried briefly. The pellet was resuspended in 9 ml TE buffer, then 10 grams of CsCl and 0.387 ml of a 10mg/ml ethidium bromide solution were added. This solution was centrifuged at 313,100xg for 24 hours. The resulting plasmid band in the ethidium gradient was visualized with ultraviolet light, isolated, and then the ethidium bromide was removed using water saturated butanol for extraction. The CsCl in the plasmid preparation was then removed by dialysis against TE buffer, and finally the DNA was concentrated using PEG (MW8000). Concentration of DNA was determined spectrophotometrically using an absorbance reading at 260nm.

Example XI. Transformation of Non-Acetyltransferase expressing mutants with the Fungal Transformation Vector

Protoplasts from strains PF14-1 and M40 were created by growing the cells for 48 hours at 30°C in CCM broth. Transformation of the *C. acremonium* protoplasts was carried out as described by Queener et al., 1985, with modifications described by Skatrud et al., 1987. Transformation vectors are described in Example IX, above.

Northern Blotting

Twenty µg of total RNA, isolated as previously described in Example V, were loaded onto each lane of a 0.7% agarose-formaldehyde gel and the RNAs were separated by electrophoresis. RNA molecular weight markers (0.16-1.77 kb ladder; and, 0.24-9.5 kb ladder, supplied by Bethesda Research Labs) were also applied to the gel for size comparisons. The gel was blotted onto nitrocellulose (Schleicher and Schuell, Keene, NH), baked for one hour at 80°C in a vacuum oven, prehybridized in 50% formamide, 5X SSC, 0.1% SDS, 5X Denhardt's, and 100 µg/ml calf thymus DNA at 55°C for 3 hours, and hybridized in the same buffer overnight at 37°C. The filter was washed three times at room temperature with 2X SSC and then once with 1X SSC at room temperature, and then autoradiographed using Kodak X-Omat AR film with intensifying screens.

The results presented in FIGURE 3 show hybridization of the isolated cloned acetyltransferase DNA (prepared in Example IV, above) with mRNA (prepared in Example V, above) from the PF14-1 strain (expressing acetyltransferase), but not with m-RNA prepared from the M40 acetyltransferase mutant strain. The results also show that two M40 transformants with the acetyltransferase gene, termed M40-T_3 and M40-T_5, both expressed m-RNA hybridizing with the acetyl-transferase probe (FIGURE 3). FIGURE 3 shows a Northern blot of total RNA extracted from Cephalosporin C producing cultures of PF14-1 (production strain), M40 (acetyl transferase mutant), and two transformants of M40. The blot was probed with P-32 labeled cloned cephalosporin acetyltransferase gene. These transformation results showed successful transfer and expression (at the mRNA level) of the cefG gene. The acetyltransferase gene was also transferred into *Aspergillus niger*, i.e., which does not contain acetyltransferase activity. The resultant transformant also expressed cefG mRNA (Example XII, below).

Example XII. Transformation of *Aspergillus niger*

Spheroplasts were produced by first growing the conidia from *A. niger* strain NRRL 3 (Northern Regional Research Laboratory, U.S. Dept. of Agriculture, Peoria, IL) in Clutterbuck's complete medium (50 ml of 20X Clutterbuck's salts (120g Na2NO3, 10.4 g KCL, 10.4 g MgSO4-7H20, 30.4 g KH2PO4, 2.0 ml Vogel's Trace Elements (0.3M citric acid, 0.2M ZnSO4, 25mM Fe(NH4)2(SO4)2.6H20, 10mM CuSO4, 3mM MnSO4, 8mM boric acid, 2mM Na2Mo04-2H20), 5 g tryptone, 5 g yeast extract, 10 g glucose), for 24 hours at 35°C on a rotary shaker. Second, the mycelia were harvested by filtration on cheesecloth filters, transferred to fresh tubes, and resuspended in 50 ml of KMP (0.7M KCL, 0.8M mannitol, 0.02M KPO4 pH6.3), containing 250 mg of Novozym 234 (Novo BioLabs, Bagsvaerd, Denmark). Third, after an overnight incubation at 30°C on a rotary shaker at 80rpm, the spheroplasts were separated by filtration through cheesecloth/glasswool filters and pelleted by centrifugation at 350xg for 15 minutes. The pelleted spheroplasts were washed twice with 15 ml of KMP and resuspended in KMPC (KMP with 50mM CaC12) to a concentration of $5x10^7$ cells/ml.

For transformation of *A. niger* 200 µl of the spheroplast suspension was added to DNA (1-10 µg of M13 vector DNA in 6.2 µl of KMPC with 5 mg/ml heparin) and the mixture was incubated at room temperature for 10-20 minutes. Fifty µl of PPC (40%PEG (MW3500), 20mM KPO4 pH6.3, 50mM CaC12) was added, and the mixture was left on ice for 30 minutes. One ml of PPC was gently mixed into the transformation mixture, and the entire solution was added to 50 ml of molten (50°C) regeneration agar (CM plus 1.3M mannitol and 3% agar) containing 1 µg/ml benomyl (DuPont, Wilmington, DE). The transformation mixture was then distributed between 4 petri dishes and the dishes were incubated for 4 hours at 30°C or until onset of germ tube growth. The plates were then overlayed with 1% peptone in 1% agar containing 1 µg/ml benomyl. The amount of the overlay was equal to the amount of the regeneration agar. The plates were incubated at 35°C for 3-10 days and observed for generation of transformant colonies.

These results support the interpretation that the Aspergillus transformants, (and the previous M40-T_3 and M40-T_5 transformants; Example XI, above), represent a successful transfer and expression (at the mRNA level) of a novel genomic fragment closely linked to the cef E/F gene. To test whether the closely linked gene was cefG gene, i.e., encoding acetyltransferase, capable of enabling cephalosporin synthesis, cephalosporin bioassays, (Example XIII, below), and high

pressure liquid chromatography (HPLC), (Example XIV, below), were used to characterize the biosynthetic products of the transformants.

Example XIII. Antibiotic Assays

An agar diffusion bioassay was used to screen the culture filtrates of M40 transformants for the restoration of Cephalosporin C production. Twenty µl of culture filtrate was applied to 5mm discs on an agar plate (nutrient agar, DIFCO 0001) containing penicillinase/ (1 unit/ml) seeded with *Alcaligenes faecalis* ATCC 8750 which is susceptible to Cephalosporin C. After 15 hours incubation at 37°C a halo of inhibited growth of the indicator bacteria around the disk indicates the presence of Cephalosporin C in the filtrate (FIGURE 4). FIGURE 4 shows a Cephalosporin C Plate Assay where culture filtrates and a Cephalosporin C standard were spotted on filter disks and then overlayed with *Alcaligenes faecalis* in the presence and absence of penicillinase. An estimation of the concentration of cephalosporin was determined by comparing the ring diameter to a standard curve of the ring diameters resulting from known concentrations of a Cephalosporin C standards (0.5 µg/ml to 10 µg/ml). Culture filtrates of the M40 (blocked) mutant strain had no effect on *A. faecalis* growth (FIGURE 4, M40) indicating that they did not produce cephalosporin, whereas filtrates of the Cephalosporin C producing strain (PF14-1 or M40 strains transformed with the M13 vector pCLS5), in this case M40-T_3 and M40-T_5 (FIGURE 3), show a halo around the disc representing growth inhibition of the indicator *A. faecalis* strain. The controls in this experiment included CPC, which is Cephalosporin C, and agar containing penicillinase/ (+PENASE) or no penicillinase/ (-PENASE), as a control for possible production for non-cephalosporin beta-lactam antibiotics.

Example XIV. HPLC Assay of Cephalosporin C

High performance liquid chromatography (HPLC) was used to assay the Cephalosporin C in culture filtrates of the *C. acremonium* strains and from the acetyltransferase assay of *A. niger* transformants. The analysis was done on Waters system with 625 solvent delivery system, 490E variable wavelength detector set at 360 nm, 825 Maxima data system, and a Novo-C18 column as the stationary phase. The mobile phase (at a 1 ml/min. flow rate) consisted of a 15 minute, 5 to 25% linear gradient of methanol/0.02 M KH_2PO_4, pH 4.0 with 2 mM tetrabutylammonium bromide added to both the 5% and 25% solvents as an ion pairing agent. A known concentration of Cephalosporin C standard was used to determine the retention time for Cephalosporin C and quantitate the peak area. The results presented in FIGURE 5 shows cephalosporin synthesized by the M40-T_3 transformed strain; FIGURE 6 shows that the M40-T_3 product is indistinguishable

from a cephalosporin standard (CPC STD, FIGURE 6) when mixed and co-separated on HPLC; FIGURE 7 shows the cephalosporin produced by the M40-T_5 strain; and, FIGURE 8 shows that the control mutant M40 parent does not synthesize cephalosporin. FIGURE 5 shows an HPLC tracing showing restoration of Cephalosporin C production in the supernatant culture fluid from an M40 (cefG mutant) transformed with the cefG gene. The transformant was designated T3. FIGURE 6 shows an HPLC tracing of the supernatant culture fluid from transformant T3 spiked with Cephalosporin C. FIGURE 7 shows an HPLC tracing demonstrating restoration of Cephalosporin C production in supernatant culture fluid from an M40 (cefG mutant) transformed with the cefG gene. The transformant was designated T5. FIGURE 8 shows an HPLC tracing demonstrating the absence of Cephalosporin C production in supernatant culture fluid from a control M40 (cefG mutant). These combined results indicate that the novel genomic fragment closely linked to the cef E/F gene and present in the M13 pCLS5 vector enables synthesis of biologically active Cephalosporin C in M40 transformants. Since M40 is known to lack acetyltransferase activity, the results strongly support the notion that the genomic fragment closely linked to the cef E/F gene is the cefG gene encoding Acetyl COA:Deacetylcephalosporin acetyltransferase. To further confirm that the M40 transformants express acetyltransferase, the enzymatic activity was tested; see below, Example XV.

Example XV. Enzymatic Assay for Acetyl CoA:DAC Acetyltransferase

Cell-free extracts of each strain were made from cultures incubated for 4 days at 28°C by extracting 100mg of lyophilized vegetative growth with 500 µl of 0.01 M potassium phosphate buffer, pH 7.0, containing 1 mM 2-mercaptoethanol. The enzyme assay was performed by adding 20 µl of the cell-free extract to the reaction mixture containing 30 µl of 0.5 M potassium phosphate buffer (pH 7.5, 10 µl of 22.8 mM deacetylcephalosporin C, 10 µl of 7.8 acetyl CoA, 10 µl of acetyl-1-^{14}C-CoA (20 µCi/mmole), and 20 µl of 20 mM $MgSO_4$. After 20 minutes incubation at 30°C the reaction was stopped by the addition of 60 µl ethanol. After centrifugation, an aliquot was co-injected with Cephalosporin C standard into the HPLC and analyzed by the previous conditions described in Example XIV, above. The absorption peak corresponding to the retention time of the Cephalosporin C standard was collected in the column effluent by a fraction collector and the radioactivity in the fractions determined by scintillation counting.

Example XVI. Increased cefG Gene Copy Number Increases Cephalosporin C Synthesis

We have demonstrated that increasing the copy number of the cefG gene in recombinant *Cephalosporium* host cells increases *Cephalosporin C* antibiotic biosynthesis. The mutant strain M40 (or wild type (WT) *C. acremonium*; ATCC #11550) was transformed with a vector pCLS5 which contained the *Cephalosporium* acetyltransferase gene and the resulting transformants were analyzed for copy number and Cephalosporin C production. The copy number was determined by comparing intensities of hybridizing bands on a Southern blot which was probed with a cefG genomic DNA probe. The intensities of the bands were measured by a densitometer using a single copy control, i.e., the endogenous acetyltransferase gene. The results are presented in Table II. The T3 M40 transformant (M40-T3) showed 3-4 extra copies of the acetyltransferase gene and M40-T5 showed only 1 extra copy. A Northern blot was probed with the same cefG probe, and measured on a densitometer. The results agreed with the initial copy number determination, i.e., M40-T3 having 3-4 extra copies and M40-T5 having one extra copy. Cephalosporin C production was measured by HPLC (as above, Example XIV) and the transformant M40-T3 showed three times higher (.9mg/ml) Cephalosporin C production than T5 (0.3 mg/ml). Wild type *C. acremonium* synthesized Cephalosporin C (0.625 mg/ml) and transformants WT-T11 (4-5 extra gene copies), WT-T20 (1 extra copy), and WT-T22 (3 extra copies) all showed significantly increased Cephalosporin C synthesis in relation to the number of extra cefG gene copies.

TABLE II

Cephalosporin C Synthesis in Transformants Having Multiple Copies of the cefG Gene

Transformant	Extra Gene Copy No.	Cephalosporin C (mg/ml
M40-T3	3-4	0.9
M40-T5	1	0.3
Control (M40)	0	0
WT-T11	4-5	1.9
WT-T20	1	0.88
WT-T22	3	1.14
Control (WT)	0	0.625

Citations

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APPENDIX I
"Sequence Listing"

(1)GENERAL INFORMATION
(i) APPLICANT
Mathison,Lee,
Soliday,Charles,
Rambosek,John,
(ii)TITLE OF THE INVENTION
"Isolation and Sequence of the Acetyl CoA: De acetyl-cephalosporin C acetyl transferase gene"
(iii)NUMBER OF SEQUENCES
5
(iv)CORRESPONDENCE ADDRESS
(A)ADDRESSEE:
Christensen, O'Connor, Johnson and Kindness
(B)STREET:
2800 Pacific First Center, 1420 Fifth Avenue
(C)CITY:
Seattle
(D)STATE:
Washington
(E)COUNTRY:
USA
(F)ZIP:
98101-2347
(v)COMPUTER READABLE FORM
(A)MEDIUM TYPE:
Diskette-5.25 inch, 1.2Mb storage
(B)COMPUTER:
IBM PC/386 Compatible
(C)OPERATING SYSTEM:
MS-DOS 4.01
(D)SOFTWARE:
Word 5.5-t
(vi)CURRENT APPLICATION DATA
(A)APPLICATION NUMBER:
none
(B)FILING DATE:
new application
(C)CLASSIFICATION:
none
(vii)PRIOR APPLICATION DATA
(A)APPLICATION NUMBER:
none
(B)FILING DATE:
none
(viii)AGENT INFORMATION
(A)NAME:
Sundsmo,John,S.
(B)REGISTRATION NUMBER:
34,446
(C)REFERENCE/DOCKET NUMBER:
PANL-1-5380

(ix)TELECOMMUNICATION INFORMATION
(A)TELEPHONE:
1-206-682-8100
1-206-224-0727 (direct)
(B)TELEFAX:
1-206-224-0779
(C)TELEX:
4938023

(2)INFORMATION FOR SEQ ID NO:1:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH:
33 bp
(B)TYPE
nucleic acid
(C)STRANDEDNESS
single stranded
(D)TOPOLOGY
linear
(ii)MOLECULE TYPE
oligonucleotide probe
-oligonucleotide probe constructed to complement 3' sequences in the cefE/F gene -see Example VI

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
  3       9       15      21      27      33
  |       |       |       |       |       |
1 GAC AGG GGC AGC CGC GGG GAC AGC CGC CTC CGC 33
```

(2)INFORMATION FOR SEQ ID NO:2:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH:
33bp
(B)TYPE
nucleic acid
(C)STRANDEDNESS
single stranded
(D)TOPOLOGY
linear
(ii)MOLECULE TYPE
oligonucleotide probe

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
  3       9       15      21      27      33
  |       |       |       |       |       |
1 GGT GGT GAC GGC CTC GGC GAG CTC GGT GAG GAC 33
```

(2) INFORMATION SEQ. ID. NO.:3:

(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH:
1183 bp
(B)TYPE
nucleic acid
(C)STRANDEDNESS
double stranded
(D)TOPOLOGY
linear
(ii)MOLECULE TYPE
cDNA
(A)DESCRIPTION
-see Example VIII; Figure 11
(xi) SEQUENCE DESCRIPTION:
-see Figure 11; Example VIII
-see Example VIII; Figure 11
-start is at position 1; stop at 1117

SEQ ID NO: 3

```
ATGTCGCCTC AGATCGCCAA TCGCTTCGAG GCTTCGCTAG ATGCCCAAGA CATAGCCAGA   60
TACAGCGGAG TCTAGCGGTT AGCGAAGCTC CGAAGCGATC TACGGGTTCT GTATCGGTCT

ATATCGCTCT TCACACTGGA ATCTGGCGTC ATCCTTCGCG ATGTACCCGT GGCATACAAA  120
TATAGCGAGA AGTGTGACCT TAGACCGCAG TAGGAAGCGC TACATGGGCA CCGTATGTTT

TCGTGGGGTC GCATGAATGT CTCAAGGGAT AACTGCGTCA TCGTCTGCCA CACCTTGACG  180
AGCACCCCAG CGTACTTACA GAGTTCCCTA TTGACGCAGT AGCAGACGGT GTGGAACTGC

AGCAGCGCCC ATGTCACCTC GTGGTGGCCC ACACTGTTTG GCCAAGGCAG GGCTTTCGAT  240
TCGTCGCGGG TACAGTGGAG CACCACCGGG TGTGACAAAC CGGTTCCGTC CCGAAAGCTA

ACCTCTCGCT ACTTCATCAT CTGCCTAAAT TATCTCGGGA GCCCCTTTGG GAGTGCTGGA  300
TGGAGAGCGA TGAAGTAGTA GACGGATTTA ATAGAGCCCT CGGGGAAACC CTCACGACCT

CCATGTTCAC CCGATGCAGA AGGCCAGCGC CAGCGCCCGT ACGGGGCCAA GTTTCCTCGC  360
GGTACAAGTG GCCTACGTCT TCCGGTCGCG GGCATGCCCA TGCCCCGGTT CAAAGGAGCG

ACGACGATTC GAGATGATGT TCGTATTCAT CGCCAGGTGC TCGACAGGTT AGGCGTCAGG  420
TGCTGCTAAG CTCTACTACA AGCATAAGTA GCGGTCCACG AGCTGTCCAA TCCGCAGTCC

CAAATTGCTG CCGTAGTCGG CGCATCCATG GGTGGAATGC ACACTCTGGA ATGGGCCTTC  480
GTTTAACGAC GGCATCAGCC GCGTAGGTAC CCACCTTACG TGTGAGACCT TACCCGGAAG

TTTGGTCCCG AGTACGTGCG AAAGATTGTG CCCATCGCGA CATCATGCCG TCAGAGCGGC  540
AAACCAGGGC TCATGCACGC TTTCTAACAC GGGTAGCGCT GTAGTACGGC AGTCTCGCCG

TGGTGCGCAG CTTGGTTCGA GACACAGAGG CAGTGCATCT ATGATGACCC CAAGTACCTG  600
ACCACGCGTC GAACCAAGCT CTGTGTCTCC GTCACGTAGA TACTACTGGG GTTCATGGAC
```

GACGGGGAGT ACGACGTAGA CGACCAGCCT GTCCGGGGGC TCGAAACAGC GCGCAAGATT 660
CTGCCCCTCA TGCTGCATCT GCTGGTCGGA CAGGCCCCCG AGCTTTGTCG CGCGTTCTAA

GCGAATCTCA CGTACAAGAG CAAACCTGCG ATGGACGAGC GCTTCCATAT GGCTCCAGGA 720
CGCTTAGAGT GCATGTTCTC GTTTGGACGC TACCTGCTCG CGAAGGTATA CCGAGGTCCT

GTCCAAGCCG GCCGGAATAT CAGCAGCCAG GATGCGAAGA AGGAAATCAA CGGCACAGAC 780
CAGGTTCGGC CGGCCTTATA GTCGTCGGTC CTACGCTTCT TCCTTTAGTT GCCGTGTCTG

AGCGGCAACA GCCACCGTGC TGGCCAGCCC ATTGAAGCCG TATCTTCCTA TCTCCGGTAC 840
TCGCCGTTGT CGGTGGCACG ACCGGTCGGG TAACTTCGGC ATAGAAGGAT AGAGGCCATG

CAGGCCCAGA AGTTTGCCGC GAGCTTCGAC GCCAACTGCT ACATCGCCAT GACACTCAAG 900
GTCCGGGTCT TCAAACGGCG CTCGAAGCTG CGGTTGACGA TGTAGCGGTA CTGTGAGTTC

TTCGACACCC ACGACATCAG CAGAGGCCGG GCAGGATCAA TCCCGGAGGC TCTGGCAATG 960
AAGCTGTGGG TGCTGTAGTC GTCTCCGGCC CGTCCTAGTT AGGGCCTCCG AGACCGTTAC

ATTACACAAC CAGCGTTGAT CATTTGCGCC AGGTCAGACG GTCTGTACTC GTTTGACGAG 1020
TAATGTGTTG GTCGCAACTA GTAAACGCGG TCCAGTCTGC CAGACATGAG CAAACTGCTC

CACGTTGAGA TGGGGCGCAG TATCCCAAAC AGTCGTCTTT GCGTGGTGGA CACGAATGAG 1080
GTGCAACTCT ACCCCGCGTC ATAGGGTTTG TCAGCAGAAA CGCACCACCT GTGCTTACTC

GGTCATGACT TCTTTGTAAT GGAAGCGGAC AAGGTTTAAT GATGCCGTCA GAGGATTCCT 1140
CCAGTACTGA AGAAACATTA CCTTCGCCTG TTCCAAATTA CTACGGCAGT CTCCTAAGGA

CGATCAGTCA TTAATGTGAG GCTATGGAGG TGTCAGAAAA AAA 1183
GCTAGTCAGT AATTACACTC CGATACCTCC ACAGTCTTTTT TT

(2)INFORMATION FOR SEQ ID NO:4:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH:
1807bp
(B)TYPE
nucleic acid
(C)STRANDEDNESS
double stranded (only one strand shown)
(D)TOPOLOGY
linear
(ii)MOLECULE TYPE
genomic DNA
(A)DESCRIPTION
-see Example VII; Figure 12
(xi) SEQUENCE DESCRIPTION: ACTCEPH
-see Example VII; Figure 9; Figure 12
-ATG start codon is at position 230-232;
stop is at 1489-1494.

SEQUENCE NO. 4

TGCGGACGGG CCGCCGCCGT CGATGCCGGC CAAGGCTTGT CGTGCATGAT AGATGCTGCC 60

GTCGGCCCAA GTGGCCCGTC TAAAGCCGGA CCCCTTTCCC CCGAGTCTCT CCCCGATCCC 120

GCACGGGGCC GTCACTTTCG CTGCCCTCGC TCCTTGTCAT AACCTACCTA TATTCTCATC 180

CCGGCAAATG CTGCGGGATA GCCTCACCTA CAGCCACACG TCGCCCACCA TGTCGCCTCA 240

GATCGCCAAT CGCTTCGAGG CTTCGCTAGA TGCCCAAGAC ATAGCCAGAA TATVGCTCTT 300

CACACTGGAA TCTGGCGTCA TCCTTCGCGA TGTACCCGTG GCATACAAAT CGTGGGGTCG 360

CATGAATGTC TCAAGGGATA ACTGCGTCAT CGTCTGCCAC ACCTTGACGA GCAGCGCCCA 420

TGTCACCTCG TGGTGGCCCA CACTGTTTGG CCAAGGCAGG GCTTTCGATA CCTCTCGCTA 480

CTTCATCATC TGCCTAAATT ATCTCGGGAG CCCCTTTGGG AGTGCTGGAC CATGTTCACC 540

GGACCCCGAT GCAGAAGGCC AGCGCCCGTA CGGGGCCAAG TTTCCTCGCA CGACGATTCG 600

AGATGATGTT CGGTAGGTAA GCGCACCGAT CCAGCTTGTC TCAATATCGA GTGGTCAGGA 660

CAATCCAGGC TAAGCTTTCC GTGTCCAAAA GTATTCATCG CCAGGTGCTC GACAGGTTAG 720

GCGTCAGGCA AATTGCTGCC GTAGTCGGCG CATCCATGGG TGGAATGCAC ACTCTGGAAT 780

GGGCCTTCTT TGGTCCCGAG TACGTGCGAA AGATTGTGCC CATCGCGACA TCATGSSGTC 840

AGAGCGGCTG GTGCGCAGCT TGGTTCGAGA CACAGAGGCA GTGCATCTAT GATGACCCCA 900

AGTACCTGGA CGGGGAGTAC GACGTAGACG ACCAGCCTGT CCGGGGGCTC GAAACAGCGC 960

GCAAGATTGC GAATCTCACG TACAAGAGCA AAGCTGCGAT GGACGAGCGC TTCCATATGG 1020

CTCCAGGAGT CCAAGCCGGT GAGTTTATAG ATGCCTTGCC GTCGGTCGAT GCTCAGAGCT 1080

AATCAGACCG AACCCGCTGC TAGGCCGGAA TATCAGCAGC CAGGATGCGA AGAAGGAAAT 1140

CAACGGCACA GACAGCGGCA ACAGCCACCG TGCTGGCCAG CCCATTGAAG CCGTATCTTC 1200

CTATCTCCGG TACCAGGCCC AGAAGTTTGC CGCGAGCTTC GACGCCAACT GCTACATCGC 1260

CATGACACTC AAGTTCGACA CCCACGACAT CAGCAGAGGC CGGGCAGGAT CAATCCCGGA 1320

GGCTCTGGCA ATGATTACAC AACCAGCGTT GATCATTTGC GCCAGGTCAG ACGGTCTGTA 1380

CTCGTTTGAC GAGCACGTTG AGATGGGGCG CAGTATCCCA AACAGTCGTC TTTGCGTGGT 1440

GGACACGAAT GAGGGTCATG ACTTCTTTGT AATGGAAGCG GACAAGGTTA ATGATGCCGT 1500

CAGAGGATTC CTCGATCAGT CATTAATGTG AGGCTATGGA GGTGTCAGCC TGCCGGTGCG 1560

CGTACTTGCC AGGGTGATCG ATGTACTCTC AGATAGTCTC CATGTGAGTA TGGATTTCGC 1620

TGTTTCCGCT CGGATATAGG CACTCTCAGG CCATCTCGCA GTAGGTATCA GAACAGCAGC 1680

TGAGGCCTTC TCGTAAAGTA GGTTGTGTCA ATAGATTCAT AAAGCGTCAA ATAAAGCCCA 1740

AAGTCGCAGT AGACTCATCG CATCGCAAGT CTCAGAGGGT CGACTCGGCA GATTCGAGGC 1800

ATTGTAG 1807

(2)INFORMATION FOR SEQ ID NO:5
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH:
372 amino acids
(B)TYPE: amino acid
(D)TOPOLOGY: linear
(ii)MOLECULE TYPE: polypeptide
(A)DESCRIPTION
-amino acid sequence encoded by SEQ.ID.NO. 3 or 4
(xi)SEQUENCE DESCRIPTION:
-see Figure 10

SEQ ID NO: 5
Predicted Amino Acid Sequence

Met	Ser	Pro	Gln	Ile	Ala	Asn	Arg	Phe	Glu	Ala	Ser	Leu	Asp	Ala	
1				5					10						15
Gln	Asp	Ile	Ala	Arg	Ile	Ser	Leu	Phe	Thr	Leu	Glu	Ser	Gly	Val	
				20					25						30
Ile	Leu	Arg	Asp	Val	Pro	Val	Ala	Tyr	Lys	Ser	Trp	Gly	Arg	Met	
				35					40						45
Asn	Val	Ser	Arg	Asp	Asn	Cys	Val	Ile	Val	Cys	His	Thr	Leu	Thr	
				50					55						60
Ser	Ser	Ala	His	Val	Thr	Ser	Trp	Trp	Pro	Thr	Leu	Phe	Gly	Gln	
				65					70						75
Gly	Arg	Ala	Phe	Asp	Thr	Ser	Arg	Tyr	Phe	Ile	Ile	Cys	Leu	Asn	
				80					85						90
Tyr	Leu	Gly	Ser	Pro	Phe	Gly	Ser	Ala	Gly	Pro	Cys	Ser	Pro	Asp	
				95					100						105
Pro	Asp	Ala	Glu	Gly	Gln	Arg	Pro	Tyr	Gly	Ala	Lys	Phe	Pro	Arg	
				110					115						120

```
Thr Thr Ile Arg Asp Asp Val Arg Ile His Arg Gln Val Leu Asp
                125                 130                 135

Arg Leu Gly Val Arg Gln Ile Ala Ala Val Val Gly Ala Ser Met
                140                 145                 150

Gly Gly Met His Thr Leu Glu Trp Ala Phe Phe Gly Pro Glu Tyr
                155                 160                 165


Val Arg Lys Ile Val Pro Ile Ala Thr Ser Cys Arg Gln Ser Gly
                170                 175                 180

Trp Cys Ala Ala Trp Phe Glu Thr Gln Arg Gln Cys Ile Tyr Asp
                185                 190                 195

Asp Pro Lys Tyr Leu Asp Gly Glu Tyr Asp Val Asp Asp Gln Pro
                200                 205                 210

Val Arg Gly Leu Glu Thr Ala Arg Lys Ile Ala Asn Leu Thr Tyr
                215                 220                 225

Lys Ser Lys Pro Ala Met Asp Glu Arg Phe His Met Ala Pro Gly
                230                 235                 240

Val Gln Ala Gly Arg Asn Ile Ser Ser Gln Asp Ala Lys Lys Glu
                245                 250                 255

Ile Asn Gly Thr Asp Ser Gly Asn Ser His Arg Ala Gly Gln Pro
                260                 265                 270

Ile Glu Ala Val Ser Ser Tyr Leu Arg Tyr Gln Ala Gln Lys Phe
                275                 280                 285

Ala Ala Ser Phe Asp Ala Asn Cys Tyr Ile Ala Met Thr Leu Lys
                290                 295                 300

Phe Asp Thr His Asp Ile Ser Arg Gly Arg Ala Gly Ser Ile Pro
                305                 310                 315

Glu Ala Leu Ala Met Ile Thr Gln Pro Ala Leu Ile Ile Cys Ala
                320                 325                 330

Arg Ser Asp Gly Leu Tyr Ser Phe Asp Glu His Val Glu Met Gly
                335                 340                 345

Arg Ser Ile Pro Asn Ser Arg Leu Cys Val Val Asp Thr Asn Glu
                350                 355                 360

Gly His Asp Phe Phe Val Met Glu Ala Asp Lys Val
                365                 370
```

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. An isolated DNA nucleotide sequence encoding acetyl CoA:Deacetylcephalosporin acetyltransferase.

2. The DNA nucleotide sequence of Claim 1 encoding *Cephalosporium* acetyl CoA:deacetylcephalosporin acetyltransferase.

3. The DNA nucleotide sequence of Claim 1 that encodes *Cephalosporium acremonium* acetyl CoA:cephalosporin acetyltransferase.

4. The DNA nucleotide sequence of SEQ. ID. NO. 3 or 4.

5. A vector comprising the DNA nucleotide sequence of Claim 1.

6. A vector of Claim 5 wherein the vector is selected from the group of: replicative cloning vectors, shuttle vectors, and expression vectors.

7. A recombinant host cell transformed with an expression vector of Claim 5.

8. A recombinant host cell of Claim 7 wherein the host cell is a member of the genus *Cephalosporium*.

9. A recombinant host cell transformed with the expression vector of Claim 6.

10. A recombinant polypeptide synthesized by a recombinant host cell of Claim 7.

11. A synthetic peptide homologous or related to a portion of SEQ. ID. NO. 5.

12. An isolated nucleotide sequence capable of hybridizing under stringent conditions with a nucleotide sequence of Claim 1.

13. An isolated nucleotide sequence capable of hybridizing under stringent conditions with SEQ. ID. NO. 3 or SEQ. ID. NO. 4.

14. An assay for determining the presence or amount of mRNA complementary to SEQ. ID. NO. 3 in a cellular extract comprising the steps of:

a. contacting the cellular extract with a labeled probe comprising at least 9 nucleotide bases of SEQ. ID. NO. 3;

b. determining the amount of the labeled probe bound to the mRNA as an indication of the presence or amount of mRNA complementary to SEQ. ID. NO. 3 in the cellular extract.

15. The assay of Claim 14 wherein variants of a parent Cephalosporin C producing organism with modified expression of acetylCoA: deacetylcephalosporin acetyltransferase are identified comprising the steps of:

a. contacting the cellular extract with a labeled probe comprising at least a portion of SEQ. ID. NO. 3 or 4;

b. determining the amount of the labeled probe bound to the mRNA as an indication of the presence or amount of mRNA complementary to SEQ. ID. NO. 3 or 4 in the cellular extract; and

c. comparing the amount of binding of the labeled probe determined in step b. with the amount of binding under similar conditions in a control cellular extract from the parent Cephalosporin C producing organism with known expression of AcetylCoA: Deacetylcephalosporin acetyltransferase, thereby permitting identification of organisms having modified expression of Cephalosporin C and that are variants of the parent organism.

16. A method for modifying Cephalosporin C synthesis in a Cephalosporin C producing organism, comprising the steps of:

transforming host cells of the organism with an expression vector of Claim 5;

selecting transformed host cells which have modified synthesis of Cephalosporin C as compared to the unmodified organism.

17. A method for constructing recombinant host cells having blocked Cephalosporin C synthesis, comprising the steps of:

transforming the host cells with a mutagenized SEQ. ID. NO. 3;

selecting for host cells with decreased Cephalosporin C synthesis.

18. A method for constructing transformed recombinant host cells having increased synthesis of Cephalosporin C, comprising the steps of:

(a) introducing an expression vector of Claim 5 into host cells to create a transformed recombinant host cell;

(b) selecting host cells that have increased synthesis of Cephalosporin C.

19. The method of Claim 18, wherein the host cells are selected from members of the genus *Cephalosporium*.

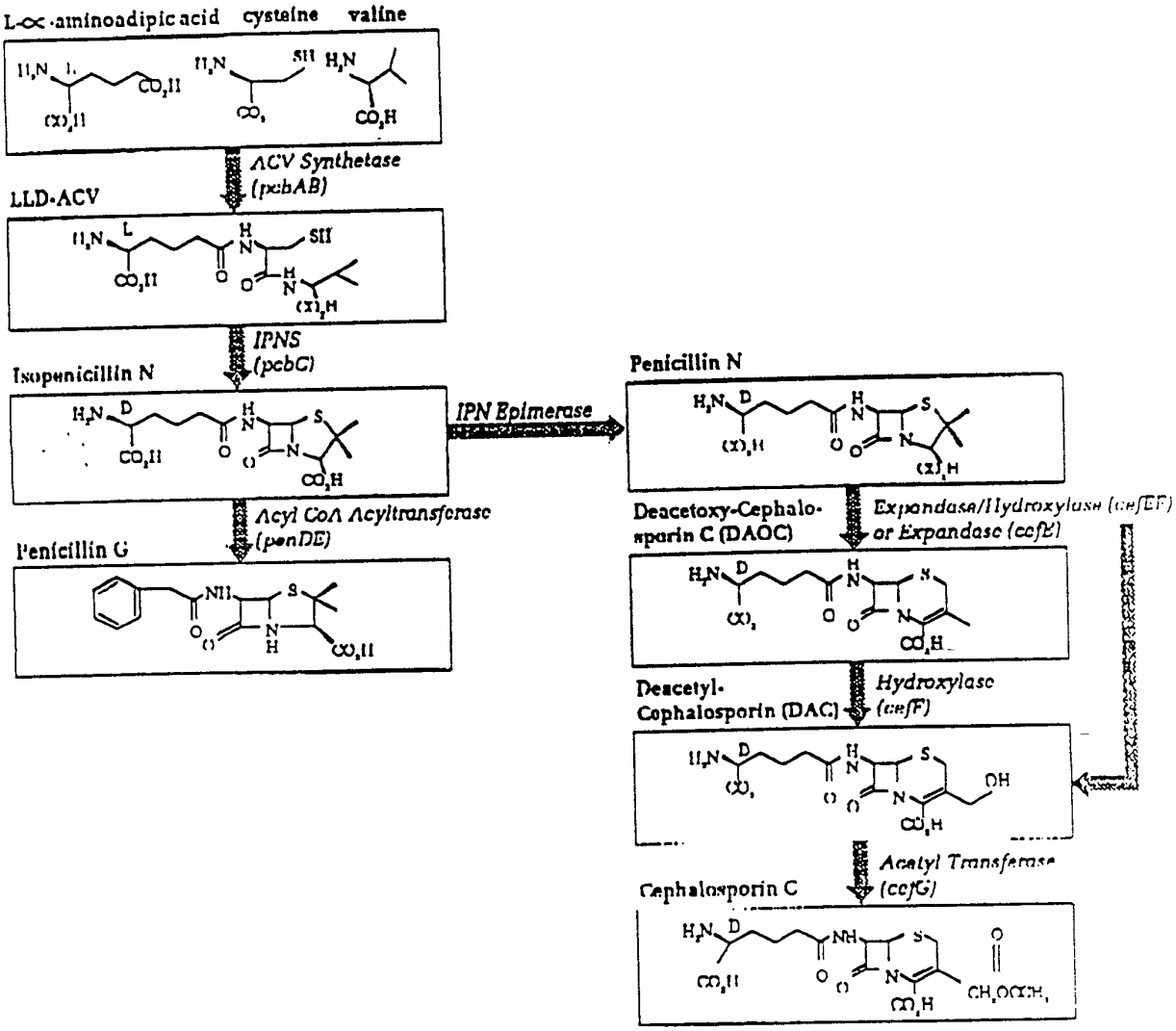
L-α-aminoadipic acid
cysteine
valine
ACV Synthetase
(pcbAB)
LLD-ACV
IPNS
(pcbC)
Isopenicillin N
IPN Epimerase
Penicillin N
Acyl CoA Acyltransferase
(penDE)
Penicillin G
Deacetoxy-Cephalosporin C (DAOC)
Expandase/Hydroxylase (cefEF)
or Expandase (cefE)
Deacetyl-Cephalosporin (DAC)
Hydroxylase
(cefF)
Acetyl Transferase
(cefG)
Cephalosporin C

FIGURE 2

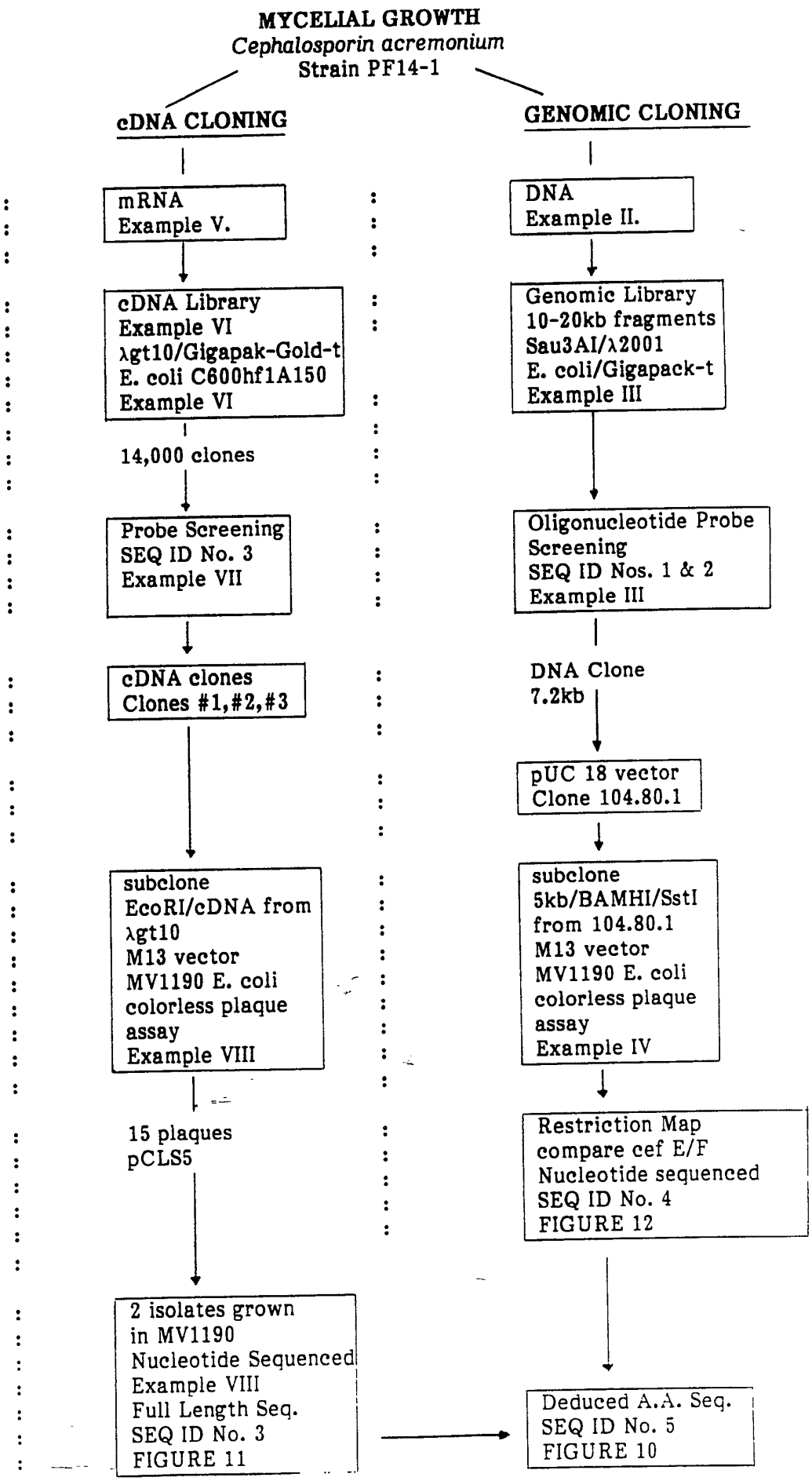
MYCELIAL GROWTH
Cephalosporin acremonium
Strain PF14-1
cDNA CLONING
GENOMIC CLONING
mRNA
Example V.
DNA
Example II.
cDNA Library
Example VI
λgt10/Gigapak-Gold-t
E. coli C600hf1A150
Example VI
Genomic Library
10-20kb fragments
Sau3AI/λ2001
E. coli/Gigapack-t
Example III
14,000 clones
Probe Screening
SEQ ID No. 3
Example VII
Oligonucleotide Probe
Screening
SEQ ID Nos. 1 & 2
Example III
cDNA clones
Clones #1,#2,#3
DNA Clone
7.2kb
pUC 18 vector
Clone 104.80.1
subclone
EcoRI/cDNA from
λgt10
M13 vector
MV1190 E. coli
colorless plaque
assay
Example VIII
subclone
5kb/BAMHI/SstI
from 104.80.1
M13 vector
MV1190 E. coli
colorless plaque
assay
Example IV
15 plaques
pCLS5
Restriction Map
compare cef E/F
Nucleotide sequenced
SEQ ID No. 4
FIGURE 12
2 isolates grown
in MV1190
Nucleotide Sequenced
Example VIII
Full Length Seq.
SEQ ID No. 3
FIGURE 11
Deduced A.A. Seq.
SEQ ID No. 5
FIGURE 10

Figure 3

96 HR FERMENTATIONS

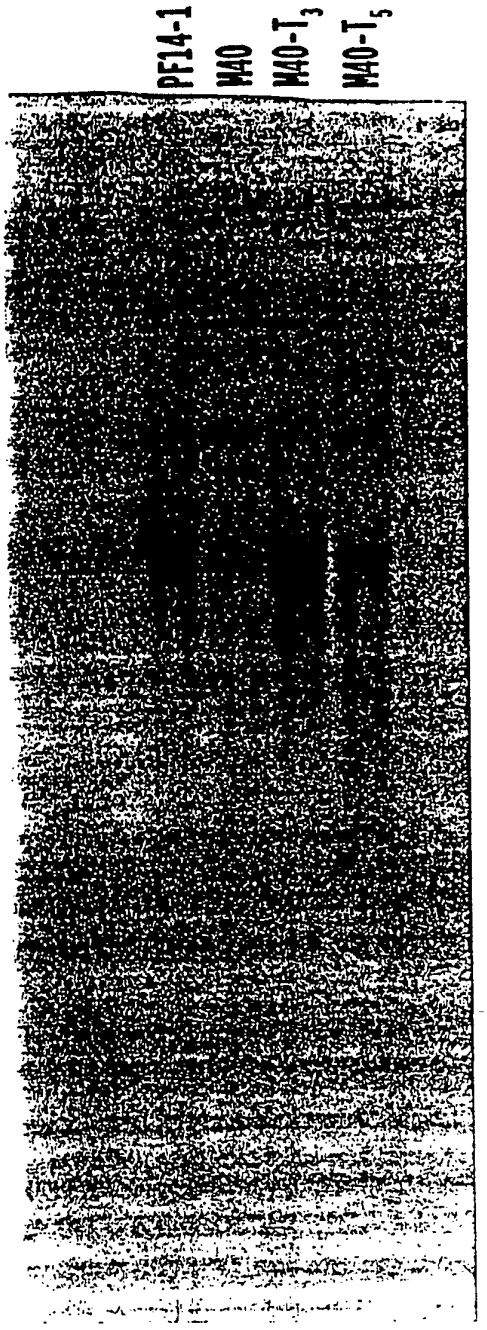

Fig. 4

BIO-ASSAY WITH ALCALIGENES FAECALIS USING 96 HR FERMENTATION BROTHS

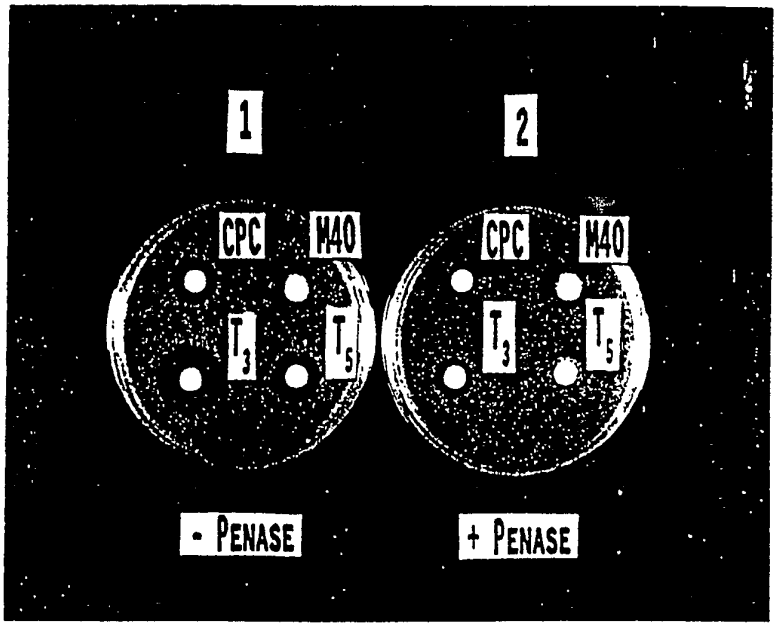

Figure 5

MAO-T_3

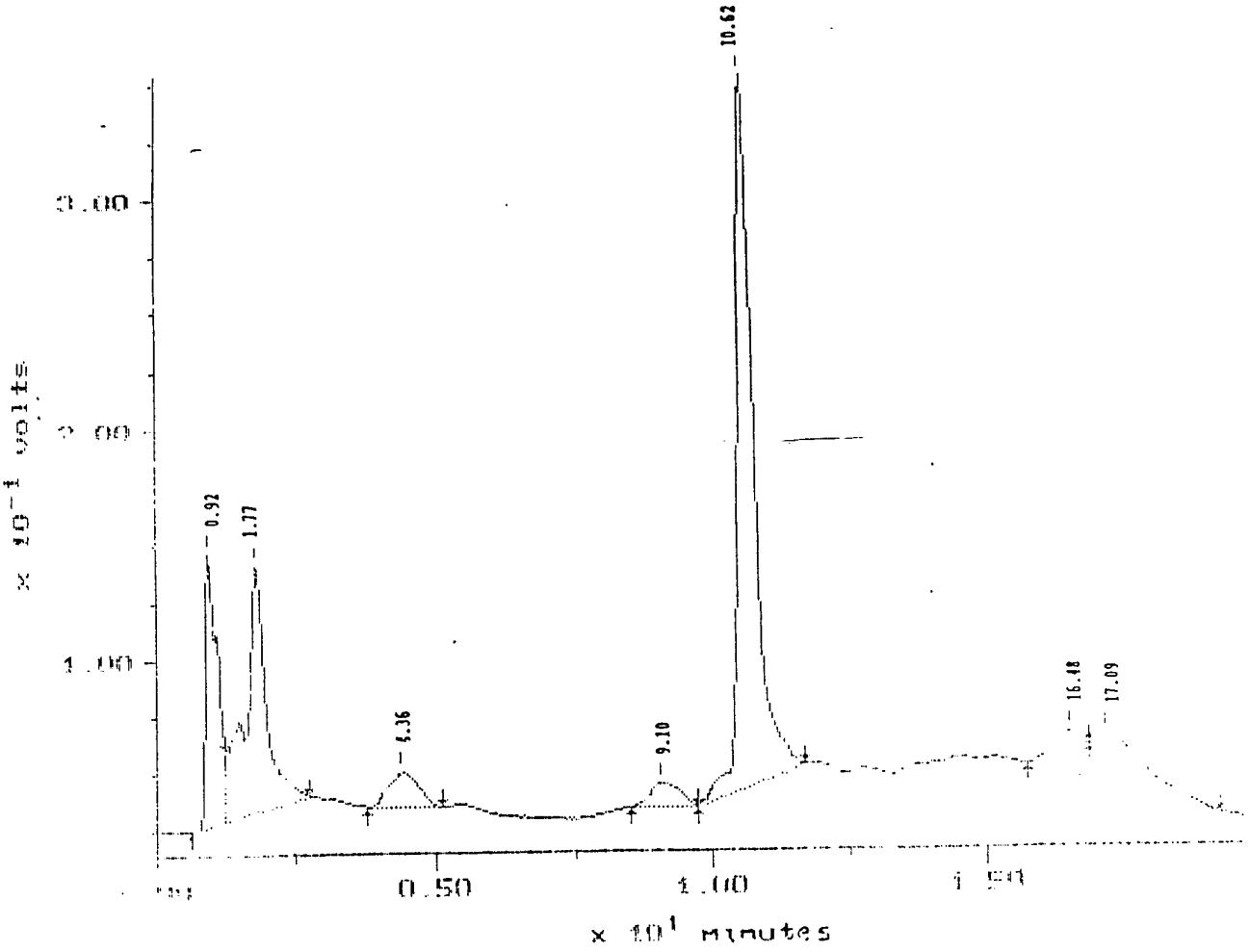

Figure 6

M40-T_3 + CPC STD

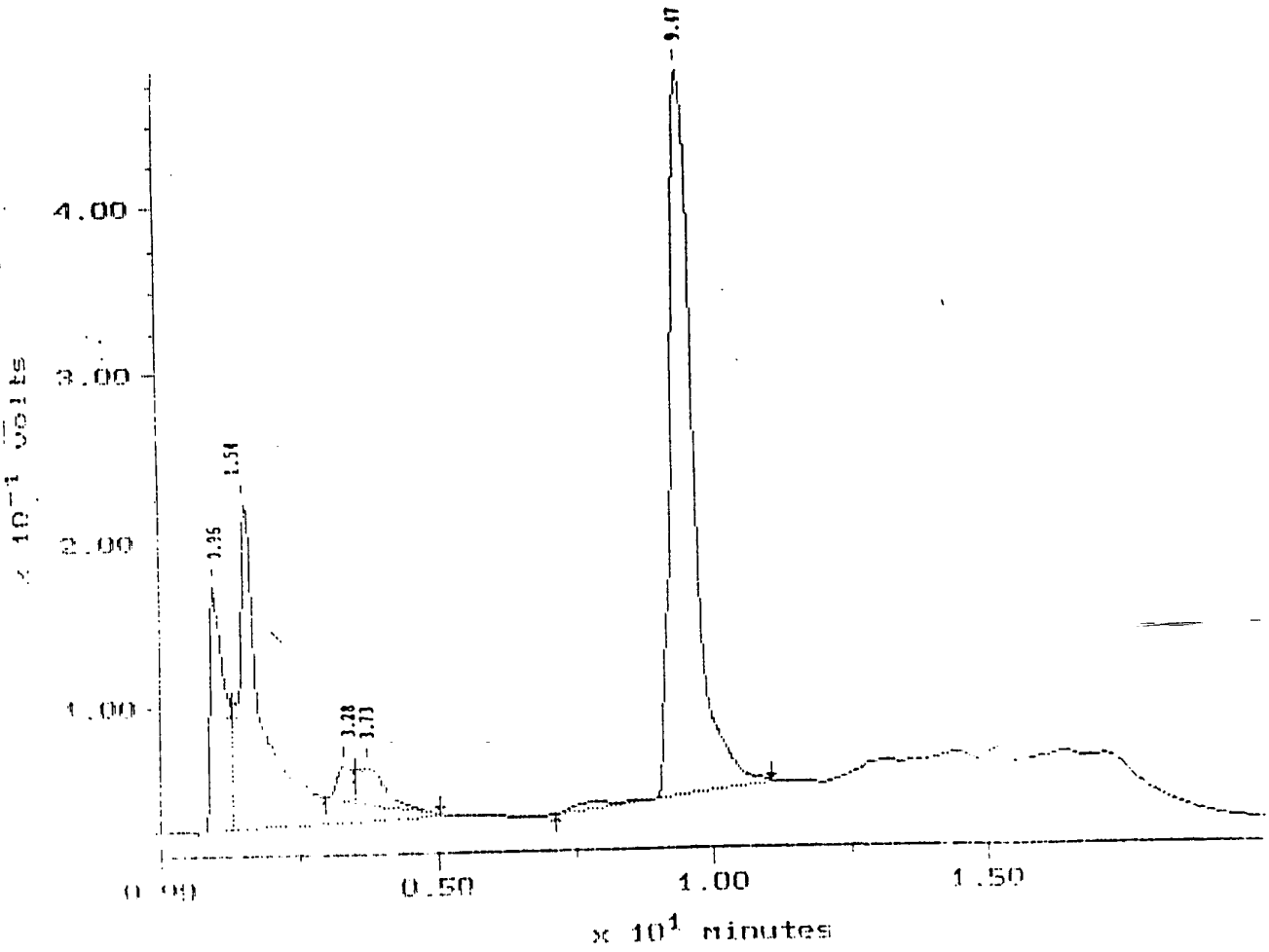

Figure 7

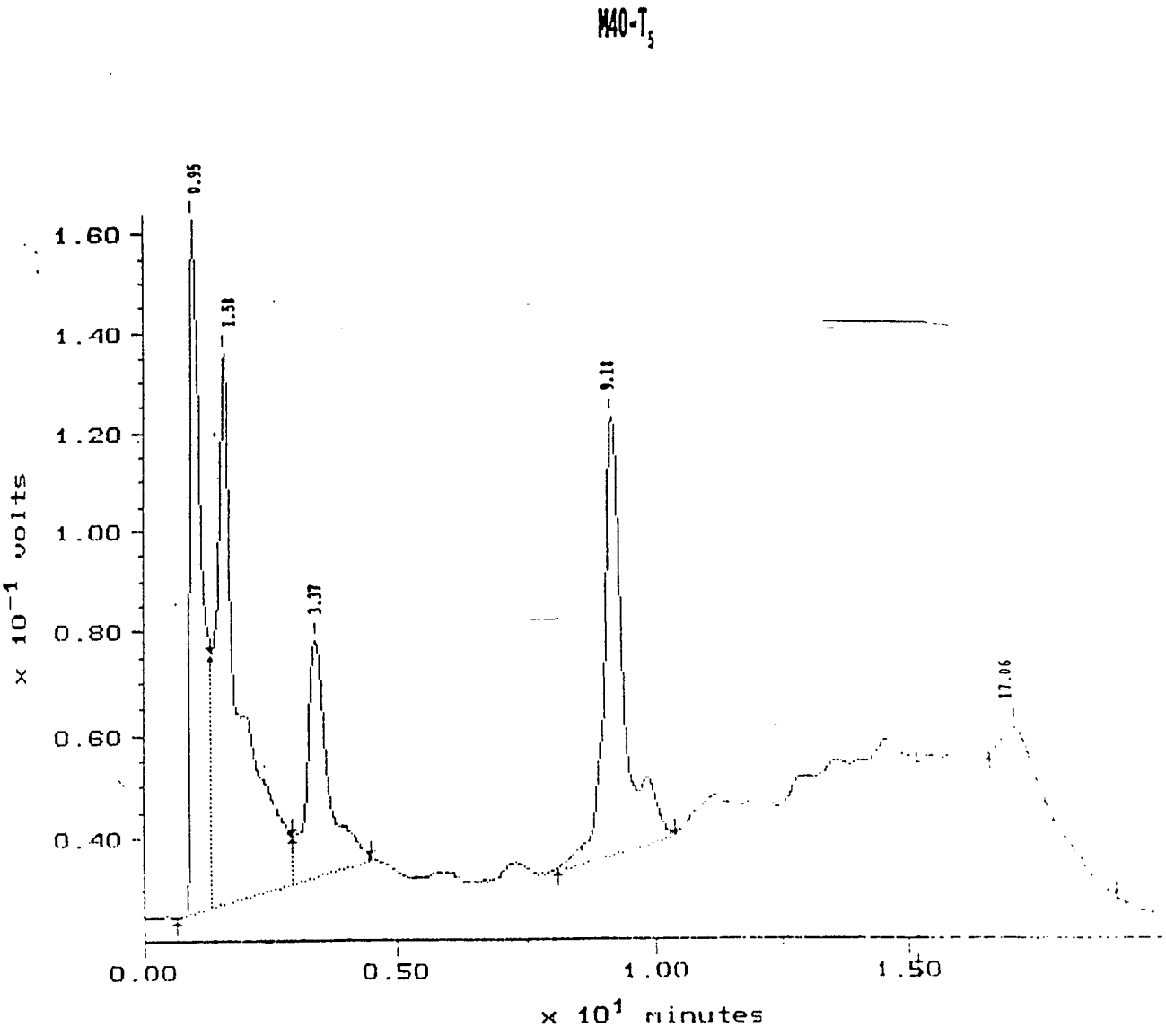
MAO-T5
0.95
1.58
3.37
9.18
17.06
1.60
1.40
1.20
1.00
0.80
0.60
0.40
x 10⁻¹ volts
0.00
0.50
1.00
1.50
x 10¹ minutes

Figure 8

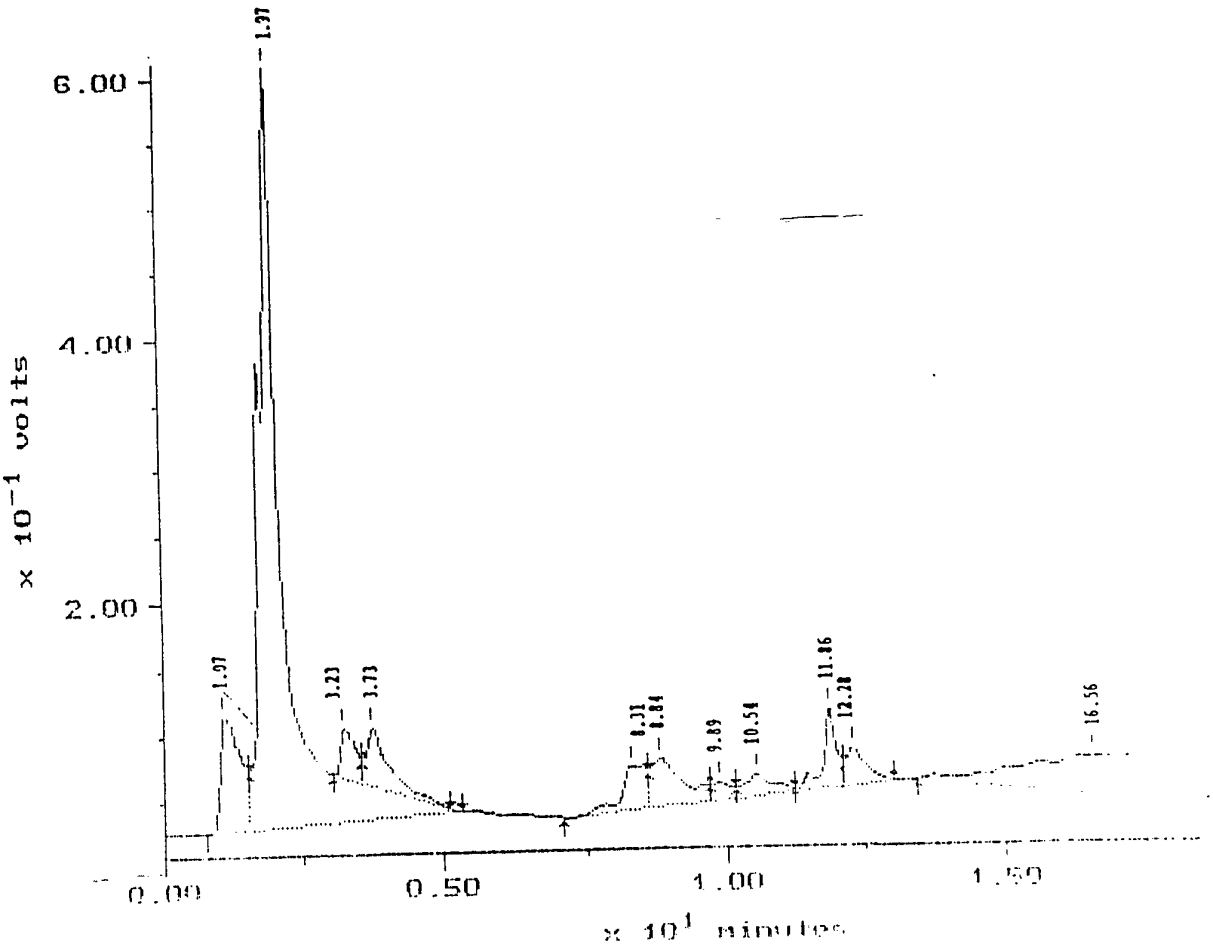
6.00
4.00
2.00
x 10⁻¹ volts
0.00
0.50
1.00
1.50
x 10¹ minutes
1.97
3.23
3.73
8.31
8.84
9.89
10.54
11.86
12.28
16.56

Figure 9

C. ACREMONIUM ACETYLTRANSFERASE

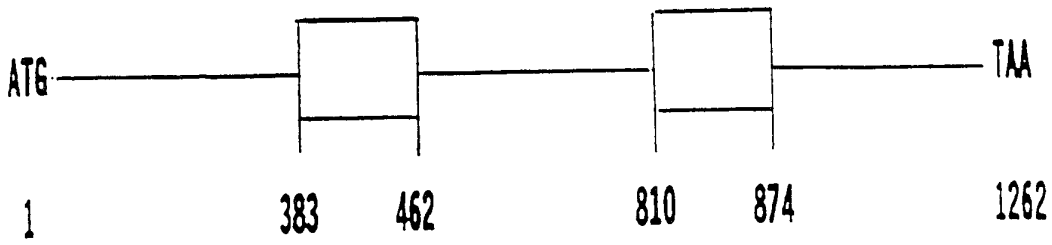

Figure 10

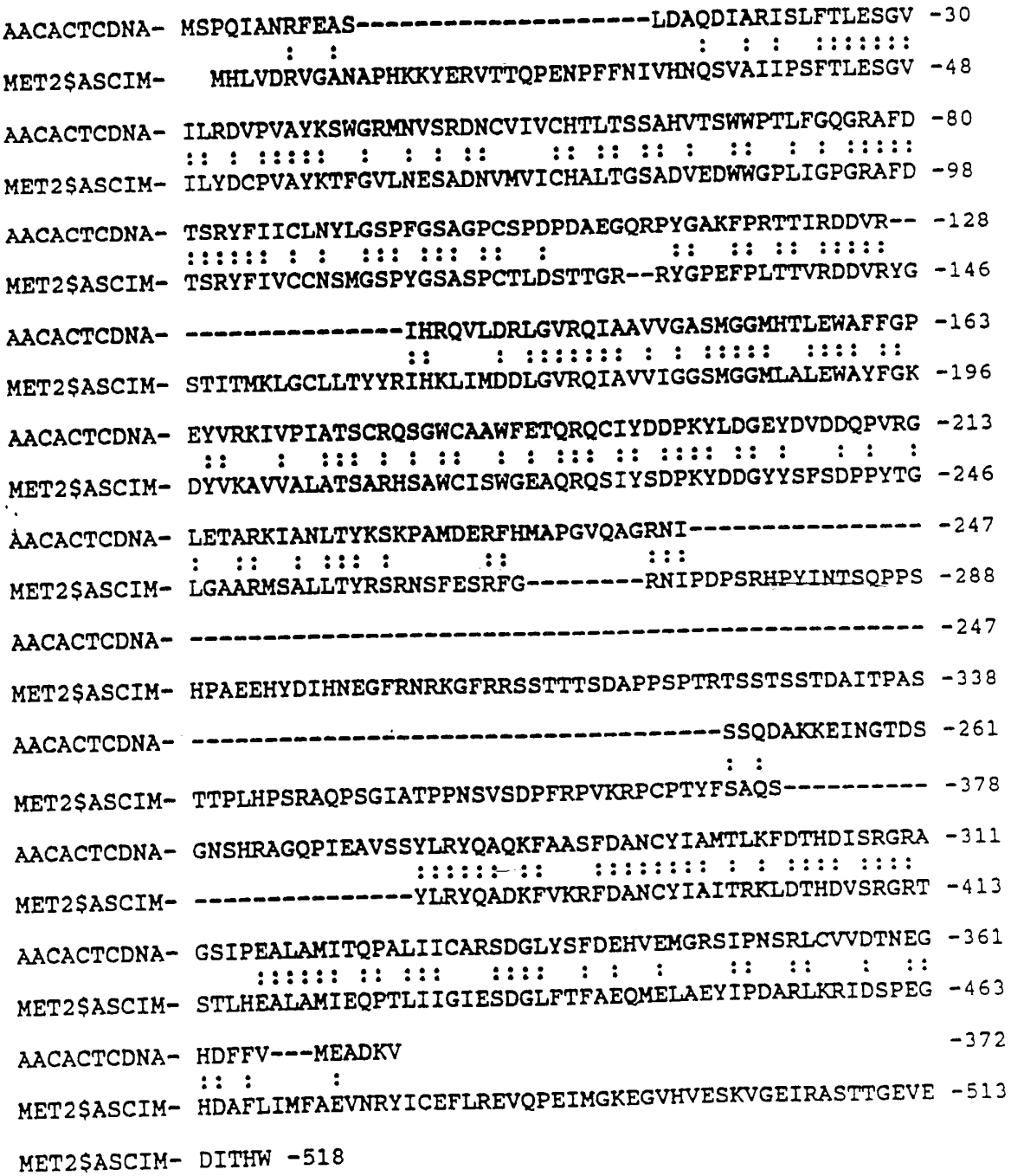
AACACTCDNA- MSPQIANRFEAS----------LDAQDIARISLFTLESGV -30
MET2$ASCIM- MHLVDRVGANAPHKKYERVTTQPENPFFNIVHNQSVAIIPSFTLESGV -48
AACACTCDNA- ILRDVPVAYKSWGRMNVSRDNCVIVCHTLTSSAHVTSWWPTLFGQGRAFD -80
MET2$ASCIM- ILYDCPVAYKTFGVLNESADNVMVICHALTGSADVEDWWGPLIGPGRAFD -98
AACACTCDNA- TSRYFIICLNYLGSPFGSAGPCSPDPDAEGQRPYGAKFPRTTIRDDVR-- -128
MET2$ASCIM- TSRYFIVCCNSMGSPYGSASPCTLDSTTGR--RYGPEFPLTTVRDDVRYG -146
AACACTCDNA- ----------IHRQVLDRLGVRQIAAVVGASMGGMHTLEWAFFGP -163
MET2$ASCIM- STITMKLGCLLTYYRIHKLIMDDLGVRQIAVVIGGSMGGMLALEWAYFGK -196
AACACTCDNA- EYVRKIVPIATSCRQSGWCAAWFETQRQCIYDDPKYLDGEYDVDDQPVRG -213
MET2$ASCIM- DYVKAVVALATSARHSAWCISWGEAQRQSIYSDPKYDDGYYSFSDPPYTG -246
AACACTCDNA- LETARKIANLTYKSKPAMDERFHMAPGVQAGRNI---------- -247
MET2$ASCIM- LGAARMSALLTYRSRNSFESRFG--------RNIPDPSRHPYINTSQPPS -288
AACACTCDNA- ---------- -247
MET2$ASCIM- HPAEEHYDIHNEGFRNRKGFRRSSTTTSDAPPSPTRTSSTSSTDAITPAS -338
AACACTCDNA- ----------SSQDAKKEINGTDS -261
MET2$ASCIM- TTPLHPSRAQPSGIATPPNSVSDPFRPVKRPCPTYFSAQS---------- -378
AACACTCDNA- GNSHRAGQPIEAVSSYLRYQAQKFAASFDANCYIAMTLKFDTHDISRGRA -311
MET2$ASCIM- ----------YLRYQADKFVKRFDANCYIAITRKLDTHDVSRGRT -413
AACACTCDNA- GSIPEALAMITQPALIICARSDGLYSFDEHVEMGRSIPNSRLCVVDTNEG -361
MET2$ASCIM- STLHEALAMIEQPTLIIGIESDGLFTFAEQMELAEYIPDARLKRIDSPEG -463
AACACTCDNA- HDFFV---MEADKV -372
MET2$ASCIM- HDAFLIMFAEVNRYICEFLREVQPEIMGKEGVHVESKVGEIRASTTGEVE -513
MET2$ASCIM- DITHW -518

FIGURE 11A

```
          3       9       15      21      27      33      39      45
          |       |       |       |       |       |       |       |
    1 ATG TCG CCT CAG ATC GCC AAT CGC TTC GAG GCT TCG CTA GAT GCC
      TAC AGC GGA GTC TAG CGG TTA GCG AAG CTC CGA AGC GAT CTA CGG

   46 CAA GAC ATA GCC AGA ATA TCG CTC TTC ACA CTG GAA TCT GGC GTC
      GTT CTG TAT CGG TCT TAT AGC GAG AAG TGT GAC CTT AGA CCG CAG

   91 ATC CTT CGC GAT GTA CCC GTG GCA TAC AAA TCG TGG GGT CGC ATG
      TAG GAA GCG CTA CAT GGG CAC CGT ATG TTT AGC ACC CCA GCG TAC

  136 AAT GTC TCA AGG GAT AAC TGC GTC ATC GTC TGC CAC ACC TTG ACG
      TTA CAG AGT TCC CTA TTG ACG CAG TAG CAG ACG GTG TGG AAC TGC

  181 AGC AGC GCC CAT GTC ACC TCG TGG TGG CCC ACA CTG TTT GGC CAA
      TCG TCG CGG GTA CAG TGG AGC ACC ACC GGG TGT GAC AAA CCG GTT

  226 GGC AGG GCT TTC GAT ACC TCT CGC TAC TTC ATC ATC TGC CTA AAT
      CCG TCC CGA AAG CTA TGG AGA GCG ATG AAG TAG TAG ACG GAT TTA

  271 TAT CTC GGG AGC CCC TTT GGG AGT GCT GGA CCA TGT TCA CCG GAC
      ATA GAG CCC TCG GGG AAA CCC TCA CGA CCT GGT ACA AGT GGC CTG

  316 CCC GAT GCA GAA GGC CAG CGC CCG TAC GGG GCC AAG TTT CCT CGC
      GGG CTA CGT CTT CCG GTC GCG GGC ATG CCC CGG TTC AAA GGA GCG

  361 ACG ACG ATT CGA GAT GAT GTT CGT ATT CAT CGC CAG GTG CTC GAC
      TGC TGC TAA GCT CTA CTA CAA GCA TAA GTA GCG GTC CAC GAG CTG

  406 AGG TTA GGC GTC AGG CAA ATT GCT GCC GTA GTC GGC GCA TCC ATG
      TCC AAT CCG CAG TCC GTT TAA CGA CGG CAT CAG CCG CGT AGG TAC

  451 GGT GGA ATG CAC ACT CTG GAA TGG GCC TTC TTT GGT CCC GAG TAC
      CCA CCT TAC GTG TGA GAC CTT ACC CGG AAG AAA CCA GGG CTC ATG

  496 GTG CGA AAG ATT GTG CCC ATC GCG ACA TCA TGC CGT CAG AGC GGC
      CAC GCT TTC TAA CAC GGG TAG CGC TGT AGT ACG GCA GTC TCG CCG

  541 TGG TGC GCA GCT TGG TTC GAG ACA CAG AGG CAG TGC ATC TAT GAT
      ACC ACG CGT CGA ACC AAG CTC TGT GTC TCC GTC ACG TAG ATA CTA

  586 GAC CCC AAG TAC CTG GAC GGG GAG TAC GAC GTA GAC GAC CAG CCT
      CTG GGG TTC ATG GAC CTG CCC CTC ATG CTG CAT CTG CTG GTC GGA

  631 GTC CGG GGG CTC GAA ACA GCG CGC AAG ATT GCG AAT CTC ACG TAC
      CAG GCC CCC GAG CTT TGT CGC GCG TTC TAA CGC TTA GAG TGC ATG

  676 AAG AGC AAA CCT GCG ATG GAC GAG CGC TTC CAT ATG GCT CCA GGA
      TTC TCG TTT GGA CGC TAC CTG CTC GCG AAG GTA TAC CGA GGT CCT
```

Figure 11 B

```
 721 GTC CAA GCC GGC CGG AAT ATC AGC AGC CAG GAT GCG AAG AAG GAA
     CAG GTT CGG CCG GCC TTA TAG TCG TCG GTC CTA CGC TTC TTC CTT

 766 ATC AAC GGC ACA GAC AGC GGC AAC AGC CAC CGT GCT GGC CAG CCC
     TAG TTG CCG TGT CTG TCG CCG TTG TCG GTG GCA CGA CCG GTC GGG

 811 ATT GAA GCC GTA TCT TCC TAT CTC CGG TAC CAG GCC CAG AAG TTT
     TAA CTT CGG CAT AGA AGG ATA GAG GCC ATG GTC CGG GTC TTC AAA

 856 GCC GCG AGC TTC GAC GCC AAC TGC TAC ATC GCC ATG ACA CTC AAG
     CGG CGC TCG AAG CTG CGG TTG ACG ATG TAG CGG TAC TGT GAG TTC

 901 TTC GAC ACC CAC GAC ATC AGC AGA GGC CGG GCA GGA TCA ATC CCG
     AAG CTG TGG GTG CTG TAG TCG TCT CCG GCC CGT CCT AGT TAG GGC

 946 GAG GCT CTG GCA ATG ATT ACA CAA CCA GCG TTG ATC ATT TGC GCC
     CTC CGA GAC CGT TAC TAA TGT GTT GGT CGC AAC TAG TAA ACG CGG

 991 AGG TCA GAC GGT CTG TAC TCG TTT GAC GAG CAC GTT GAG ATG GGG
     TCC AGT CTG CCA GAC ATG AGC AAA CTG CTC GTG CAA CTC TAC CCC

1036 CGC AGT ATC CCA AAC AGT CGT CTT TGC GTG GTG GAC ACG AAT GAG
     GCG TCA TAG GGT TTG TCA GCA GAA ACG CAC CAC CTG TGC TTA CTC

1081 GGT CAT GAC TTC TTT GTA ATG GAA GCG GAC AAG GTT TAA TGA TGC
     CCA GTA CTG AAG AAA CAT TAC CTT CGC CTG TTC CAA ATT ACT ACG

1126 CGT CAG AGG ATT CCT CGA TCA GTC ATT AAT GTG AGG CTA TGG AGG
     GCA GTC TCC TAA GGA GCT AGT CAG TAA TTA CAC TCC GAT ACC TCC

1171 TGT CAG AAA AAA A
     ACA GTC TTT TTT T.

Total number of bases is: 1183.
DNA sequence composition:    278 A;   328 C;   330 G;   247 T;

Sequence name: NCACTCDNA.
```

Figure 12A

```
      3         9        15        21        27        33        39        45
      |         |         |         |         |         |         |         |
  1 TGC GGA CGG GCC GCC GCC GTC GAT GCC GGC CAA GGC TTG TCG TGC
    ACG CCT GCC CGG CGG CGG CAG CTA CGG CCG GTT CCG AAC AGC ACG

 46 ATG ATA GAT GCT GCC GTC GGC CCA AGT GGC CCG TCT AAA GCC GGA
    TAC TAT CTA CGA CGG CAG CCG GGT TCA CCG GGC AGA TTT CGG CCT

 91 CCC CTT TCC CCC GAG TCT CTC CCC GAT CCC GCA CGG GGC CGT CAC
    GGG GAA AGG GGG CTC AGA GAG GGG CTA GGG CGT GCC CCG GCA GTG

136 TTT CGC TGC CCT CGC TCC TTG TCA TAA CCT ACC TAT ATT CTC ATC
    AAA GCG ACG GGA GCG AGG AAC AGT ATT GGA TGG ATA TAA GAG TAG

181 CCG GCA AAT GCT GCG GGA TAG CCT CAC CTA CAG CCA CAC GTC GCC
    GGC CGT TTA CGA CGC CCT ATC GGA GTG GAT GTC GGT GTG CAG CGG

226 CAC CAT GTC GCC TCA GAT CGC CAA TCG CTT CGA GGC TTC GCT AGA
    GTG GTA CAG CGG AGT CTA GCG GTT AGC GAA GCT CCG AAG CGA TCT

271 TGC CCA AGA CAT AGC CAG AAT ATV GCT CTT CAC ACT GGA ATC TGG
    ACG GGT TCT GTA TCG GTC TTA TAB CGA GAA GTG TGA CCT TAG ACC

316 CGT CAT CCT TCG CGA TGT ACC CGT GGC ATA CAA ATC GTG GGG TCG
    GCA GTA GGA AGC GCT ACA TGG GCA CCG TAT GTT TAG CAC CCC AGC

361 CAT GAA TGT CTC AAG GGA TAA CTG CGT CAT CGT CTG CCA CAC CTT
    GTA CTT ACA GAG TTC CCT ATT GAC GCA GTA GCA GAC GGT GTG GAA

406 GAC GAG CAG CGC CCA TGT CAC CTC GTG GTG GCC CAC ACT GTT TGG
    CTG CTC GTC GCG GGT ACA GTG GAG CAC CAC CGG GTG TGA CAA ACC

451 CCA AGG CAG GGC TTT CGA TAC CTC TCG CTA CTT CAT CAT CTG CCT
    GGT TCC GTC CCG AAA GCT ATG GAG AGC GAT GAA GTA GTA GAC GGA

496 AAA TTA TCT CGG GAG CCC CTT TGG GAG TGC TGG ACC ATG TTC ACC
    TTT AAT AGA GCC CTC GGG GAA ACC CTC ACG ACC TGG TAC AAG TGG

541 GGA CCC CGA TGC AGA AGG CCA GCG CCC GTA CGG GGC CAA GTT TCC
    CCT GGG GCT ACG TCT TCC GGT CGC GGG CAT GCC CCG GTT CAA AGG

586 TCG CAC GAC GAT TCG AGA TGA TGT TCG GTA GGT AAG CGC ACC GAT
    AGC GTG CTG CTA AGC TCT ACT ACA AGC CAT CCA TTC GCG TGG CTA

631 CCA GCT TGT CTC AAT ATC GAG TGG TCA GGA CAA TCC AGG CTA AGC
    GGT CGA ACA GAG TTA TAG CTC ACC AGT CCT GTT AGG TCC GAT TCG

676 TTT CCG TGT CCA AAA GTA TTC ATC GCC AGG TGC TCG ACA GGT TAG
    AAA GGC ACA GGT TTT CAT AAG TAG CGG TCC ACG AGC TGT CCA ATC
```

Figure 12B

```
 721 GCG TCA GGC AAA TTG CTG CCG TAG TCG GCG CAT CCA TGG GTG GAA
     CGC AGT CCG TTT AAC GAC GGC ATC AGC CGC GTA GGT ACC CAC CTT

 766 TGC ACA CTC TGG AAT GGG CCT TCT TTG GTC CCG AGT ACG TGC GAA
     ACG TGT GAG ACC TTA CCC GGA AGA AAC CAG GGC TCA TGC ACG CTT

 811 AGA TTG TGC CCA TCG CGA CAT CAT GSS GTC AGA GCG GCT GGT GCG
     TCT AAC ACG GGT AGC GCT GTA GTA CSS CAG TCT CGC CGA CCA CGC

 856 CAG CTT GGT TCG AGA CAC AGA GGC AGT GCA TCT ATG ATG ACC CCA
     GTC GAA CCA AGC TCT GTG TCT CCG TCA CGT AGA TAC TAC TGG GGT

 901 AGT ACC TGG ACG GGG AGT ACG ACG TAG ACG ACC AGC CTG TCC GGG
     TCA TGG ACC TGC CCC TCA TGC TGC ATC TGC TGG TCG GAC AGG CCC

 946 GGC TCG AAA CAG CGC GCA AGA TTG CGA ATC TCA CGT ACA AGA GCA
     CCG AGC TTT GTC GCG CGT TCT AAC GCT TAG AGT GCA TGT TCT CGT

 991 AAC CTG CGA TGG ACG AGC GCT TCC ATA TGG CTC CAG GAG TCC AAG
     TTG GAC GCT ACC TGC TCG CGA AGG TAT ACC GAG GTC CTC AGG TTC

1036 CCG GTG AGT TTA TAG ATG CCT TGC CGT CGG TCG ATG CTC AGA GCT
     GGC CAC TCA AAT ATC TAC GGA ACG GCA GCC AGC TAC GAG TCT CGA

1081 AAT CAG ACC GAA CCC GCT GCT AGG CCG GAA TAT CAG CAG CCA GGA
     TTA GTC TGG CTT GGG CGA CGA TCC GGC CTT ATA GTC GTC GGT CCT

1126 TGC GAA GAA GGA AAT CAA CGG CAC AGA CAG CGG CAA CAG CCA CCG
     ACG CTT CTT CCT TTA GTT GCC GTG TCT GTC GCC GTT GTC GGT GGC

1171 TGC TGG CCA GCC CAT TGA AGC CGT ATC TTC CTA TCT CCG GTA CCA
     ACG ACC GGT CGG GTA ACT TCG GCA TAG AAG GAT AGA GGC CAT GGT

1216 GGC CCA GAA GTT TGC CGC GAG CTT CGA CGC CAA CTG CTA CAT CGC
     CCG GGT CTT CAA ACG GCG CTC GAA GCT GCG GTT GAC GAT GTA GCG

1261 CAT GAC ACT CAA GTT CGA CAC CCA CGA CAT CAG CAG AGG CCG GGC
     GTA CTG TGA GTT CAA GCT GTG GGT GCT GTA GTC GTC TCC GGC CCG

1306 AGG ATC AAT CCC GGA GGC TCT GGC AAT GAT TAC ACA ACC AGC GTT
     TCC TAG TTA GGG CCT CCG AGA CCG TTA CTA ATG TGT TGG TCG CAA

1351 GAT CAT TTG CGC CAG GTC AGA CGG TCT GTA CTC GTT TGA CGA GCA
     CTA GTA AAC GCG GTC CAG TCT GCC AGA CAT GAG CAA ACT GCT CGT

1396 CGT TGA GAT GGG GCG CAG TAT CCC AAA CAG TCG TCT TTG CGT GGT
     GCA ACT CTA CCC CGC GTC ATA GGG TTT GTC AGC AGA AAC GCA CCA

1441 GGA CAC GAA TGA GGG TCA TGA CTT CTT TGT AAT GGA AGC GGA CAA
     CCT GTG CTT ACT CCC AGT ACT GAA GAA ACA TTA CCT TCG CCT GTT

1486 GGT TAA TGA TGC CGT CAG AGG ATT CCT CGA TCA GTC ATT AAT GTG
     CCA ATT ACT ACG GCA GTC TCC TAA GGA GCT AGT CAG TAA TTA CAC

1531 AGG CTA TGG AGG TGT CAG CCT GCC GGT GCG CGT ACT TGC CAG GGT
```

Figure 12C

```
     TCC GAT ACC TCC ACA GTC GGA CGG CCA CGC GCA TGA ACG GTC CCA

1576 GAT CGA TGT ACT CTC AGA TAG TCT CCA TGT GAG TAT GGA TTT CGC
     CTA GCT ACA TGA GAG TCT ATC AGA GGT ACA CTC ATA CCT AAA GCG

1621 TGT TTC CGC TCG GAT ATA GGC ACT CTC AGG CCA TCT CGC AGT AGG
     ACA AAG GCG AGC CTA TAT CCG TGA GAG TCC GGT AGA GCG TCA TCC

1666 TAT CAG AAC AGC AGC TGA GGC CTT CTC GTA AAG TAG GTT GTG TCA
     ATA GTC TTG TCG TCG ACT CCG GAA GAG CAT TTC ATC CAA CAC AGT

1711 ATA GAT TCA TAA AGC GTC AAA TAA AGC CCA AAG TCG CAG TAG ACT
     TAT CTA AGT ATT TCG CAG TTT ATT TCG GGT TTC AGC GTC ATC TGA

1756 CAT CGC ATC GCA AGT CTC AGA GGG TCG ACT CGG CAG ATT CGA GGC
     GTA GCG TAG CGT TCA GAG TCT CCC AGC TGA GCC GTC TAA GCT CCG

1801 ATT GTA G
     TAA CAT C

Total number of bases is: 1807.
DNA sequence composition:    405 A;   515 C;   493 G;   391 T;  3 OTHER;

Sequence name: NACTCEPH.
```

==========

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/02087

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :536/27; 435/6, 69.8, 172.1, 193, 252.3, 254, 320.1; 530/300
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, BIOSIS, MEDLINE, APS, search terms: cephalosporin, synthesis or produc?, gene or sequence
GENBANK, EMBL, PIR, SWISS-PROT, GENESEQ for Seq ID Nos. 3 and 4

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X Y	EP, A, 0,450,758 (Matsuda, A. et al.) 09 October 1991, entire document.	1-10, 12, 17 11, 13-16, 18, 19
P, X Y	EP, A, 0,437,378 (Maishman et al.) 17 July 1991, entire document.	1-10, 12-13, 16, 18-19 11, 14-15, 17
P, X Y	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Volume 182, No. 3, issued 14 February 1992, A. Matsuda et al., "Molecular Cloning of Acetyl Coenzyme A: Deacetylcephalosporin C O-Acetyltransferase cDNA from Acremonium Chrysogenium: Sequence and Expression of Catalytic Activity in Yeast", pages 995-1001, entire document.	1-7, 9-10, 12-13 8, 11, 14-19
P, Y	JOURNAL OF INDUSTRIAL MICROBIOLOGY, Volume 9, No. 2, issued February 1992, J.F. Martin, "Clusters of Genes for the Biosynthesis of Antibiotics: Regulatory Genes and Overproduction of Pharmaceuticals", pages 73-90, particularly 73-74 and 78-82.	1-19

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
17 JULY 1992

Date of mailing of the international search report
31 JUL 1992

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/02087

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	JOURNAL OF BACTERIOLOGY, volume 17, No. 7, issued April 1991, S. Gutierrez et al. "Characterization of the Cephalosporium acremonium pcbAB Gene Encoding α-Aminoadipyl-Cysteinyl-Valine Synthetase, a Large Multidomain Peptide Synthetase: Linkage to the pcbC Gene as a Cluster of Early Cephalosporin Biosynthetic Genes and Evidence of Multiple Functional Domains", pages 2354-2365, particularly pg 2363, final paragraph	1-19
Y	BIOTECHNOLOGY, Volume 7, No. 5, issued May 1989, P.L. Skatrud et al., "Use of Recombinant DNA to Improve Production of Cephalosporin C By Cephalosporium Acremonium", pages 477-485, entire document.	16, 18, 19
Y	BIOTECHNOLOGY, Volume 5, No. 11, issued November 1987, S.M. Samson et al., "Cloning and Expression of the Fungal Expandase/Hydroxylase Gene Involved in Cephalosporin Biosynthesis, pages 1207-1214, particularly pages 1207 and 1208.	1-19
Y	CHEMICAL ABSTRACTS, Volume 104, No. 2, issued January 1986, A. Scheidegger et al. "Investigation of Acetyl-CoA:Deacetyl-cephalosporin C O-Acetyltransferase of Cephalosporium Acremonium", see page 238, column 1, abstract no. 16857m, Journal of Biotechnology, No 3, pages 109-117.	10
Y	CURRENT GENETICS, Volume 12, issued September 1987, P.L. Skatrud et al. "Efficient Integrative Transformation of Cephalosporium acremonium, pages 337-348, entire document.	5-10, 16, 18-19
Y	US, A, 4,302,204 (Wahl et al.) 24 November 1981, entire document.	14, 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/02087

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C07K 15/00; C12N 1/15, 1/21, 9/10, 15/00, 15/54, 15,80; C12P 21/06; C12Q 1/68