

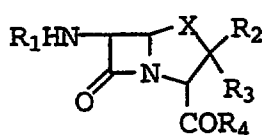
MUTANT PENICILLIN G ACYLASES**Field of the Invention**

The present invention relates to mutated genes
5 encoding Type II penicillin G acylases, to penicillin G
acylases encoded by these genes resulting in altered
properties, and to methods for the synthesis of β -lactam
antibiotics using these penicillin G acylases.

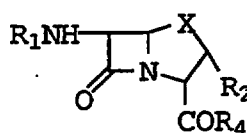
Background of the Invention

10 Today, semisynthetic β -lactam derivatives such as
ampicillin, amoxicillin, cephalexin, cefadroxil, and
cefprozil are, on an industrial scale, prepared by chemical
methods. The synthesis of these antibiotics catalyzed by
15 enzymes constitutes a clear example of an enzymatic
reaction of possible industrial importance. The enzymatic
approach has several advantages as compared to conventional
chemical methods: (1) avoidance of toxic reagents and
solvents; (2) enzyme specificity renders protection of
20 carboxyl groups in the antibiotic nucleus unnecessary; (3)
avoidance of side reactions, including racemization.

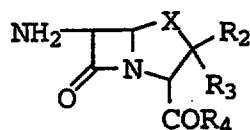
In this context, penicillin G acylase offers a great
advantage. Penicillin G acylase, also called penicillin G
amidase or benzylpenicillin amidohydrolase [EC. 3.5.1.11],
25 refers to a group of hydrolases from microorganisms,
especially bacteria, capable of hydrolyzing the 6 acyl
group of penicillins or the 7 acyl group of cephalosporins
having the general structures of I and II to their
corresponding free amine forms (6-APA and its derivatives,
30 III, and 7-ACA and its derivatives, IV).



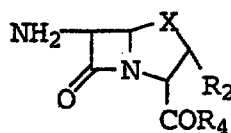
I



II



III



IV

wherein:

R1= phenylacetyl, phenoxyacetyl,
 5 hydroxyphenylglycine, phenylglycine and their derivatives,
 acetyl, adipyl and their derivatives

R2, R3= aliphatic or aromatic entities with or
 without one or more O, S, N atoms

R4= aliphatic or aromatic alcohols and their
 10 derivatives with or without one or more O, S, N atoms

The preferred acyl group is phenylacetyl, although
 other aromatic and aliphatic (hydrophobic, or
 charged/polar) acyl groups can also be hydrolyzed to
 varying degrees (generally less). The preference for
 15 different acyl groups are not necessarily true for the
 reverse reaction, namely the formation of amide bonds
 between the acyl groups and 6-APA and 7-ACA (III and IV).
 For instance, the chloroacetyl group can be put on to 7-ACA
 much faster than most aromatic acyl groups (patent
 20 JP08000284-A). For many currently marketed β -lactam
 antibiotics, the acyl groups are aromatic functions with
 varying degrees of hydrophobicity. The wild type
 penicillin G amidase can catalyze the semisyntheses (amide
 bond formation) of these antibiotics, but the reactions
 25 rarely go to completion under suitable or economical
 conditions for the production of these antibiotics.
 Improvements in the production yield and efficiency of
 these reactions are highly desired.

There are many reports in the literature of penicillin G acylases which contain altered amino acid residues exhibiting altered substrate specificity or changes in catalytic activity. Prieto et al. (I. Prieto et al., Appl. Microbiol. Biotechnol. 33 (1990) 553-559) replaced Met168 in penicillin G acylase from K. citrophila with Ala, Asp, Val, Asn, and Tyr resulting in modified kinetic parameters for penicillin G and penicillin V deacylation; the substitution of Asn with Lys375 or Tyr with His481 did not. Martin et al. (J. Martin & I. Prieto, Biochimica et Biophysica Acta 1037 (1990) 133-139) describe a mutant of penicillin G acylase with different substrate specificity and enhanced thermal stability when Met168 was changed to Ala. Wang Min et al. (Wang Min et al. Shiyuan Shengwu Xuebao 24 (1991), 1, 51-54) reported the replacement of Ser177 in E.coli penicillin G acylase with Gly, Thr, Leu, Arg, all of which changes resulted in inactive enzymes. Kyeong Sook et al. (Kyeong Sook et al. Journal of Bacteriology 174 (1992) 6270-6276) and Slade et al. (Slade et al. Eur.J. Biochem. 197 (1991) 75-80) have demonstrated Ser290 to be an essential amino acid residue of penicillin G acylase from E.coli. Substitution of Ser290 with Cys completely inactivated the enzyme. Niersbach et al. (Niersbach et al. Biotechnology Letters 17, 1, (1995) 19-24) replaced Gly359 with aspartic acid in penicillin G acylase from E.coli. The mutant enzyme lost the ability to hydrolyze penicillin G but exhibited the novel ability to hydrolyze phthalyl-L-leucine and phthalyl-glycyl-L-proline. An enhanced stability at alkaline pH was demonstrated by a site-directed mutant of penicillin G acylase from E.coli when Trp431 was changed to Arg (Gabriel del Rio et al. Biotechnology and Bioengineering 48 (1995) 141-148).

The inventors herein present mutant penicillin G acylases having altered enzymatic activities when compared with the wild type enzyme.

Summary of the Invention

In one aspect of the invention the DNA sequence of the Type II wild-type penicillin G acylase, preferably from prokaryotic organisms (the structure of the enzyme from E. coli is given in Figures 1A through 1D), is altered to encode mutant penicillin G acylases. Type II acylases all share a common molecular structure. Type II acylases are heterodimers composed of a small subunit (alpha; 16-26 kilodaltons (kDa)) and a large subunit (beta; 54-66 kDa). As used herein the term "penicillin G acylase" is intended to mean prokaryotic Type II acylase as well as its preenzyme and preproenzyme forms. The DNA sequence (SEQ.ID.NO.:1) and corresponding amino acid sequence (SEQ.ID.NO.:2) for the alpha subunit of the wild type penicillin G acylase from E. coli are shown in Figure 1A. The DNA sequence (SEQ.ID.NO.:3) and corresponding amino acid sequence (SEQ.ID.NO.:4) for the beta subunit of the wild type penicillin G acylase from E. coli are shown in Figures 1B through 1D. In accordance with the present invention, one or more selected amino acid residues are substituted with different amino acid residues from the group of natural amino acids. Of course, in the mutated DNA sequences of the invention corresponding changes in the DNA sequence are made in order to encode the desired amino acid(s) at the desired position(s). The structural changes were determined based on the X-ray crystallographic structure of the wild-type penicillin G acylase. The DNA and amino acid sequence changes for each substitution in accordance with the present invention are shown in Figure 2.

In accordance with the invention the following substitutions at one or more of the designated sites are provided:

1. On the alpha subunit:

DNA basepairs:A424-426 (MetA142 - Ala)

DNA basepairs:A436-438 (PheA146 - Ala)

2. On the beta subunit:

DNA basepairs: B70-72 (PheB24 - Ala, Leu, Val,
Pro, Tyr, Met, Ser, Cys, Gly, Asp, Lys, Arg,
Tyr, Thr, Ile, Glu, Gln, Asn or His).

5 DNA basepairs: B166-168 (ValB56 - Ser or Thr)

DNA basepairs: B529-531 (IleB177 - Phe)

In the nomenclature used above, "A" represents the alpha subunit, "B" represents the beta subunit; the numbered positions are conventional amino terminus to carboxy terminus for amino acid sequences, and 5' to 3' for DNA sequences; the amino acid preceding the amino acid position number represents the wild type amino acid and the amino acid following the amino acid position number represents the substitute amino acid, for example, "ValB56 - Ser or Thr" means that amino acid at position 56 in the wild type beta subunit is valine which is substituted with either serine or threonine to make a mutant acylase of the invention.

20 The altered acylases of the invention have altered enzymatic activities when compared with the corresponding wild-type penicillin G acylase.

The most preferred altered (mutant) penicillin G acylase has a single amino acid alteration (PheB24-Ala), and is capable of synthesizing β -lactam antibiotics with significantly higher yield and efficiency than the wild type enzyme.

30 In other aspects the present invention is also directed to vectors which comprise the altered nucleic acid sequences of the invention, and microorganism host cells transformed with said vectors. The invention also concerns processes for production of the altered acylases comprising culturing the host cells of the invention, preferably followed by isolation of the acylase.

35 In yet another aspect the invention provides methods to use the said mutant penicillin G acylase for the semisyntheses of β -lactam antibiotics (e.g., cefadroxil,

cefprozil, amoxicillin). Conditions, such as substrate concentrations, pH values and temperatures, are presented hereinafter. The yields and efficiencies of the semisynthetic reactions using the mutant penicillin G acylases are preferably improved when compared with the wild type enzyme.

Brief Description of the Drawings

Figure 1A: Nucleotide (DNA) sequence of alpha subunit of the *E. coli* wild-type penicillin G amidase gene and the corresponding amino acid sequence encoded by the nucleotide sequence.

Figure 1B: Nucleotide (DNA) sequence of beta subunit of the *E. coli* wild-type penicillin G amidase gene and the corresponding amino acid sequence encoded by the nucleotide sequence.

Figure 1C: continuation of Figure 1B.

Figure 1D: continuation of Figure 1C.

Figure 2: DNA and amino acid sequences of the relevant fragments of penicillin G acylase illustrating the exact sites of mutations in accordance with the invention. DNA fragment 1 is SEQ.ID.NO.:5, amino acid fragment 1 is SEQ.ID.NO.:6, DNA fragment 2 is SEQ.ID.NO.:7, amino acid fragment 2 is SEQ.ID.NO.:8, DNA fragment 3 is SEQ.ID.NO.:9, amino acid fragment 3 is SEQ.ID.NO.:10, DNA fragment 4 is SEQ.ID.NO.:11, amino acid fragment 4 is SEQ.ID.NO.:12, and DNA fragment 5 is SEQ.ID.NO.:13, amino acid fragment 5 is SEQ.ID.NO.:14.

Figure 3: Illustration of PBM vector referred to in Example 1 containing mutated DNA sequences of penicillin G acylases

Figure 4: Computer graphics image of the structure of the penicillin G acylase substrate binding site. For clarity, protein backbone trace is not shown. The isolated amino acids constituting the site are shown as stick structural diagrams. Atom types are coded by different shades of gray, i.e., polypeptide backbone carbons, white;

side chain carbons, light gray; nitrogens, dark gray; oxygens, black. The cleaved substrate, phenylacetic acid, is marked by an arrow at the center of the site. The phenylalanine side chain B24 is also labeled with an arrow.

5 As can be seen, the aromatic side chain ring of the B24 residue occupies an important position central to the site, in contact with the substrate, and shielding the substrate from solvent. The image was prepared from the X-ray crystallographic coordinates of the phenylacetic acid -

10 penicillin G acylase complex.

Detailed Description of the Invention

The penicillin G acylases which are the subject of this invention have altered substrate specificity and/or

15 altered specific activity when compared with the wild type enzyme. The enzymes of the invention preferably exhibit enhanced yield and/or efficiency when compared with the wild type enzyme. It is possible that routine experimentation may be required to determine optimal

20 conditions for use of the altered enzymes of the invention. The wild type enzyme utilized herein to prepare the altered enzymes of the invention is obtained from prokaryotes such as Escherichia coli, Kluyvera citrophila, Providencia rettgeri, Pseudomonas sp., Alcaligenes faecalis, Bacillus megaterium, Arthrobacter viscosus, and the like. The

25 acylase preferably has the following characteristics: (1) is isolated from the prokaryote E. coli (e.g., ATCC 11105) (2) is translated as a single peptide chain precursor (3) is processed after translation resulting in a

30 heterodimer with a small N-terminal domain (the alpha subunit) and a larger C-terminal domain (the beta subunit). The molecular weight of the preferred alpha subunit is about 24000 and the molecular weight of the preferred beta subunit is about 62000. The active form of the preferred

35 enzyme is typically found in the periplasmic of E. coli.

Current LC-MS data suggest that during post-translational processing in E. coli the alpha subunit is

truncated at the C-terminus by about 10 to 15 amino acids, most likely by 12 or 13 amino acids. Similarly, the same data indicate that during post-translational processing the alpha subunit is truncated at the N-terminus by 1 or 2 amino acids. Thus, the present invention includes mutant penicillin G acylases wherein the alpha subunit has been truncated at the N-terminus by 1 or 2 amino acids and/or wherein said alpha subunit has been truncated at the C-terminus by 10 to 15 amino acids, preferably by 12 or 13 amino acids.

The alteration of the substrate specificity of penicillin G acylases is achieved in such a way that the mutant enzymes are able to cleave or synthesize penicillin and cephalosporin derivatives possessing side chains other than phenylacetyl, which is the natural side chain of penicillin G. Examples of side chains which are presently not significantly affected by penicillin G acylases are acyl groups derived from the dicarboxylic acids succinic acid, glutaric acid adipic acid, aminoadipic acid, and the like.

The mutated enzymes of the invention may exhibit increased stereospecificity which can result in improved enantiomeric excess in conversion with racemic mixtures of chiral compounds. Such a property might make the acylases very useful for synthesis of enantiomerically pure semisynthetic antibiotics from racemic mixtures of phenyl acetyl side chains or activated derivatives of the phenylacetyl side chains (e.g., phenylglycine-amides or esters therefrom, p-hydroxyphenylglycine-amides or esters therefrom, and the like) containing a chiral alpha carbon due to the presence of an amino group (e.g., as in, for example, ampicillin, cefalexin, amoxicillin, cefadroxyl, cefaclor) or a hydroxyl group (as in, for example cefamandole).

The present invention also relates to the identification of penicillin G mutants derived from wild-type enzyme via recombinant DNA methodology known in the

art substituting one amino acid residue for a new residue. Mutants were analyzed for both hydrolytic and synthetic activity. Penicillin G acylase variants are preferred in which the transferase activity is improved with respect to the hydrolase activity. This makes the enzyme more useful in synthetic conversions. Mutants with improved performance in the enzymatic synthesis of antibiotics such as amoxicillin, cefadroxil, cefprozil, and cephalixin are preferred.

10 The introduction of a mutation in determined sites of a gene can be carried out by modification of a defined site of a DNA sequence using synthetic oligonucleotides. Mutants of penicillin G acylase in the present invention can be prepared by a process which comprises:

15 (1) introducing a mutation in specific sites of the gene encoding penicillin G acylase by standard polymerase chain reaction site-directed mutagenesis. Specific oligodeoxynucleotides for these mutations were synthesized by a commercial source. The oligonucleotides are
20 homologous to the sequence to be mutagenized except for an internal portion which determines the mutation.

(2) cloning the mutagenized gene in a cloning vector.

(3) transforming a host strain with the recombinant
25 vector.

(4) cultivating the host strain on a suitable culture medium.

(5) separating and immobilizing the mutant penicillin G acylase thus obtained.

30 (6) assaying the mutants with regard to hydrolytic and synthetic activity.

The mutagenesis of penicillin G acylase in accordance herein introduces new substrate specificity and/or altered enzymatic activity. To introduce point
35 mutations, a rational approach is taken, relying on protein crystallography, molecular modeling, molecular biology, and protein chemistry techniques. According to the present

invention, specific amino acid positions have been identified as important positions with regard to the catalytic properties of the enzyme. These residues include MetA142, PheA146, PheB24, ValB56, and IleB177. The
5 identification of these residues is based on X-ray crystallographic structure.

To compare the enzymes of the invention with the wild type enzyme, the mutant and the wild type penicillin G acylases are in the form of crude cell lysates or
10 immobilized solids, preferably the latter. The enzyme of the invention having mutation PheB24 - Ala demonstrates an improved synthesis activity for β -lactam antibiotics and is therefore preferred.

The present invention also includes an expression
15 vector comprising a nucleic acid sequence of the invention operably linked to a promoter sequence capable of directing its expression in a host cell. Preferred vectors are plasmids such as plasmid PBMPGA shown in Figure 3. Expression vectors useful in the present invention
20 typically contain an origin of replication, a promoter located in front (i.e., upstream of) the DNA sequence and followed by the DNA sequence coding for all or part of the mutant acylase. The DNA sequence coding for all or part of the mutant acylase is followed by transcription termination
25 sequences and the remaining vector. The expression vectors may also include other DNA sequences known in the art, for example, stability leader sequences which provide for stability of the expression product, secretory leader sequences which provide for secretion of the expression
30 product, sequences which allow expression of the structural gene to be modulated (e.g., by the presence or absence of nutrients or other inducers in the growth medium), marking sequences which are capable of providing phenotypic selection in transformed host cells, and sequences which
35 provide sites for cleavage by restriction endonucleases. The characteristics of the actual expression vector used must be compatible with the host cell which is to be

employed. For example, when cloning in an E. coli cell system, the expression vector should contain promoters isolated from the genome of E. coli cells (e.g., tac, lac, and trp). Suitable origins of replication in E. coli various hosts include, for example, a ColEI plasmid replication origin. Suitable promoters include, for example, the tac, lac, and trp and the neo-r gene promoter from E. coli. Suitable termination sequences include, for example, the penicillin G acylase, T7 phage gene 10, and the neo-r gene terminators from E. coli. It is also preferred that the expression vector include a sequence coding for a selectable marker. The selectable marker is preferably antibiotic resistance. As selectable markers, ampicillin resistance, and neomycin resistance can be conveniently employed. All of these materials are known in the art and are commercially available.

Suitable expression vectors containing the desired coding and control sequences may be constructed using standard recombinant DNA techniques known in the art, many of which are described in Sambrook et al. Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).

The present invention additionally concerns host cells containing an expression vector which comprises a DNA sequence coding for all or part of the mutant acylase. The host cells preferably contain an expression vector which comprises all or part of one of the DNA sequences having one or more mutations shown in Figure 2. Further preferred are host cells containing an expression vector comprising one or more regulatory DNA sequences capable of directing the replication and/or the expression of, and operatively linked to a DNA sequence coding for, all or part of mutant acylase. Suitable host cells include, for example, E. coli HB101 (ATCC 33694) available from Life Technologies, Inc., P.O. Box 6009, Gaithersburg, MD 20897; BL21 available from Novagen, Inc., 597 Science Drive, Madison, WI 53711; and the like.

Expression vectors may be introduced into host cells by various methods known in the art. For example, transfection of host cells with expression vectors can be carried out by the polyethylene glycol mediated protoplast transformation method. However, other methods for
5 introducing expression vectors into host cells, for example, electroporation, biolistic injection, or protoplast fusion, can also be employed.

Once an expression vector has been introduced into
10 an appropriate host cell, the host cell may be cultured under conditions permitting expression of the desired mutant acylase.

A host cell, E. coli BL21, containing plasmid pBMPGA ((pBMF1PGA)+) was deposited with the American Type Culture
15 Collection, Rockville, Maryland 20852, under the provisions of the Budapest Treaty on September 4, 1997 and has the designation ATCC 98537.

Host cells containing an expression vector which contains a DNA sequence coding for all or part of the
20 mutant acylase may be identified by one or more of the following five general approaches: (a) DNA-DNA hybridization; (b) the presence or absence of marker gene functions; (c) assessment of the level of transcription as measured by the production of penicillin G acylase mRNA
25 transcripts in the host cell; (d) detection of the gene product immunologically; and (e) enzyme assay (colorimetric detection, etc.).

The DNA sequences of expression vectors, plasmids or DNA molecules of the present invention may be determined by
30 various methods known in the art. For example, the dideoxy chain termination method as described in Sanger et al., Proc. Natl. Acad. Sci. USA 74, 5463-5467 (1977), or the Maxam-Gilbert method as described in Proc. Natl. Acad. Sci. USA 74, 560-564 (1977) may be employed.

35 It should, of course, be understood that not all expression vectors and DNA regulatory sequences will function equally well to express the DNA sequences of the

present invention. Neither will all host cells function equally well with the same expression system. However, one of ordinary skill in the art may make a selection among expression vectors, DNA regulatory sequences, and host
 5 cells using the guidance provided herein without undue experimentation and without departing from the scope of the present invention.

All amino acid residues identified herein are in the natural L-configuration. In keeping with standard
 10 polypeptide nomenclature, J. Biol. Chem. 243, 3557-3559 (1969), abbreviations for amino acid residues are as shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

15

<u>SYMBOL</u>		<u>AMINO ACID</u>
<u>1-Letter</u>	<u>3-Letter</u>	
Y	Tyr	L-tyrosine
G	Gly	L-glycine
F	Phe	L-phenylalanine
M	Met	L-methionine
A	Ala	L-alanine
S	Ser	L-serine
I	Ile	L-isoleucine
L	Leu	L-leucine
T	Thr	L-threonine
V	Val	L-valine
P	Pro	L-proline
K	Lys	L-lysine
H	His	L-histidine
Q	Gln	L-glutamine
E	Glu	L-glutamic acid
W	Trp	L-tryptophan
R	Arg	L-arginine
D	Asp	L-aspartic acid
N	Asn	L-asparagine
C	Cys	L-cysteine

All amino acid sequences are represented herein by formulas whose left to right orientation is in the conventional direction of amino-terminus to carboxy-terminus.

The polypeptides of the present invention may be obtained by synthetic means, i.e. chemical synthesis of the polypeptide from its component amino acids, by methods known to those of ordinary skill in the art. For example, the solid phase procedure described in Houghton et al., Proc. Natl. Acad. Sci. 82, 5131-5135 (1985) may be employed. It is preferred that the polypeptides be obtained by production in prokaryotic host cells expressing a DNA sequence coding for the mutant acylase, or by in vitro translation of the mRNA encoded by a DNA sequence coding for the mutant acylase. For example, the DNA sequence may be synthesized using PCR as described above and inserted into a suitable expression vector, which in turn may be used to transform a suitable host cell. The recombinant host cell may then be cultured to produce enzyme. Techniques for the production of polypeptides by these means are known in the art, and are described herein.

The polypeptides produced in this manner may then be isolated and purified to some degree using various protein purification techniques. For example, chromatographic procedures such as ion exchange chromatography, gel filtration chromatography and immunoaffinity chromatography may be employed.

The polypeptides of the present invention have been defined by means of determined DNA and deduced amino acid sequencing. Due to the degeneracy nature of the genetic code, which results from there being more than one codon for most of the amino acid residues and stop signals, other DNA sequences which encode the same amino acid sequence as depicted in Figure 1 may be used for the production of the polypeptides of the present invention. In addition, it will be understood that allelic variations of these DNA and

amino acid sequences naturally exist, or may be intentionally introduced using methods known in the art. These variations may be demonstrated by one or more amino acid differences in the overall sequence, or by deletions, 5 substitutions, insertions, inversions or additions of one or more amino acids in said sequence. Such amino acid substitutions may be made, for example, on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the 10 residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups or nonpolar head groups having similar hydrophilicity values include the 15 following: leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, tyrosine. Other contemplated variations include salts and esters of the aforementioned polypeptides, as well as precursors of the aforementioned polypeptides, for example, 20 precursors having N-terminal substituents such as methionine, N-formylmethionine used as leader sequences. All such variations are included within the scope of the present invention.

The invention also contemplates a process for 25 producing a mutant penicillin G acylase of the invention comprising culturing a host cell of the invention under conditions suitable for production of the mutant acylase of the invention. For bacterial host cells, typical culture conditions are liquid medium containing the appropriate 30 antibiotic and induction agent. Cultures are shaken or stirred at a temperature suitable for optimal production of enzyme, e.g., about 28°C to about 29°C. Typical appropriate antibiotics include kanamycin, chloroamphenicol, tetracyclin and the like. Typical 35 induction agents include IPTG, lactose and the like.

The present invention also includes a process for producing a semi-synthetic 6-acylated penicillanic acid, a

7-acylated cephalosporanic acid or a salt or ester thereof which comprises contacting a corresponding 6-amino β -lactam or 7-ACA or salt or ester thereof, respectively, and an acylating agent with a mutant acylase of the invention

5 under conditions suitable for acylation to occur. Typical acylating agents include esters or amides of the sidechains of amoxicillin, cefdroxil, cefprozil, etc.. Typical acylating agents include, but are not limited to, phenylglycine, parahydroxyphenylglycine, phenylacetic acid,

10 phenoxyacetic acid, their esters or amides. The preferred form of acylating agent is the ester of above mentioned acids. The alcohol portion of these esters including, but are not limited to, methanol and its longer-chain analogues and their stereoisomers, ethylene glycol and its longer-

15 chain analogues and their stereoisomers. Most preferred are ethylene glycol esters. Typical acylating conditions are in aqueous buffer at neutral pH or below, with constant stirring. A typical temperature is about 0°C to about 35°C. The mutant acylase for use in the above process can

20 be made in situ by the host cells or can be pre-made by the host cells. If cell-free mutant acylase is used, it can be in a crude cell lysate, can be partially purified, or can be purified to homogeneity. It is preferred that the mutant acylase is immobilized. Typical immobilization supports

25 for use herein include celite, dicalite, or UOP Beads.

The following examples are further illustrative of the present invention. These examples are not intended to limit the scope of the present invention, but provide further understanding of the invention.

30 In the following examples, some reagents, restriction enzymes, and other materials were obtained from commercial sources and used according to the indication by suppliers. Operations employed for the purification, characterization and the cloning of DNA are well known in

35 the art and can be adapted from published literature.

Example 1Site-specific Mutagenesis

At selected positions, amino acid mutations were generated using the PCR site-directed method described above. The oligonucleotides used for introducing the desired mutations were obtained commercially. In particular the oligonucleotides have the following sequences:

- (1) 5'CAGAGAAGCGGTTTGCCCGGGTGCCCCACAAATATC3'
- 10 (SEQ.ID.NO.:15)- A:142 MET - ALA
- (2) 5'CGCTAGTGCTATCAGAGGCGGGTTTGCCATGGTGCC3'
- (SEQ.ID.NO.:16)- A:146 PHE - ALA
- (3) 5'AGCCAGGCCCATAACCAGCCCTGCGGACCATTATACCATG3'
- (SEQ.ID.NO.:17)-B:24 PHE - ALA
- 15 (4) 5'AGCCAGGCCCATAACCAGCCCACTGCGGACCATTATACCATG3'
- (SEQ.ID.NO.:18)- B:24 PHE - VAL
- (5) 5'AGCCAGGCCCATAACCAGCCGAACTGCGGACCATTATACCATG3'
- (SEQ.ID.NO.:19)- B:24 PHE - LEU
- (6) 5'AGCCAGGCCCATAACCAGCCCACTGCGGACCATTATACCATG3'
- 20 (SEQ.ID.NO.:20)- B:24 PHE - GLY
- (7) 5'AGCCAGGCCCATAACCAGCCATCCTGCGGACCATTATACCATG3'
- (SEQ.ID.NO.:21)- B:24 PHE - MET
- (8) 5'AGCCAGGCCCATAACCAGCCACACTGCGGACCATTATACCATG3'
- (SEQ.ID.NO.:22)- B:24 PHE - CYS
- 25 (9) 5'AGCCAGGCCCATAACCAGCCAGACTGCGGACCATTATACCATG3'
- (SEQ.ID.NO.:23)- B:24 PHE - SER
- (10) 5'AGCCAGGCCCATAACCAGCCGGTCTGCGGACCATTATACCATG3'
- (SEQ.ID.NO.:24)- B:24 PHE - PRO
- (11) 5'AGCCAGGCCCATAACCAGCCCTGCTGCGGACCATTATACCATG3'
- 30 (SEQ.ID.NO.:25)- B:24 PHE - ASP
- (12) 5'AGCCAGGCCCATAACCAGCCGTGCTGCGGACCATTATACCATG3'
- (SEQ.ID.NO.:26)- B:24 PHE - HIST
- (13) 5'AGCCAGGCCCATAACCAGCCATACTGCGGACCATTATACCATG3'
- (SEQ.ID.NO.:27)- B:24 PHE - TYR
- 35 (14) 5'AGCCAGGCCCATAACCAGCCTTTCTGCGGACCATTATACCATG3'
- (SEQ.ID.NO.:28)- B:24 PHE - LYS

- (15) 5'AGCCAGGCCCATACCAGCCCTCTGCGGACCATTTACCATG3'
 (SEQ.ID.NO.:29)- B:24 PHE - ARG
 (16) 5' CACACCATTATGACCAAAAGACAGCCCAGGATAGGCAAAT3'
 (SEQ.ID.NO.:30)- B: 56 VAL - SER
 5 (17) 5'CACACCATTATGACCAAAAGTCAGCCCAGGATAGGCAAAT'3'
 (SEQ.ID.NO.:31)- B: 56 VAL -THRE
 (18) 5'GCGAAACAAGCACTGGACCTTCAAACCTGGTACTATGCTG3'
 (SEQ.ID.NO.:32)- B: 177ILE - PHE
 (19) 5'AGCCAGGCCCATACCAGCCAAATCTGCGGACCATTTACCATG3'
 10 (SEQ. ID NO.:33)- B: 24 PHE - ILE
 (20) 5'AGCCAGGCCCATACCAGCCAGTCTGCGGACCATTTACCATG3'
 (SEQ. ID. NO.:34)- B: 24 PHE - THR
 (21) 5'AGCCAGGCCCATACCAGCCTTGCTGCGGACCATTTACCATG3'
 (SEQ. ID. NO.:35)-B: 24 PHE - GLN
 15 (22) 5'AGCCAGGCCCATACCAGCCATTCTGCGGACCATTTACCATG3'
 (SEQ. ID. NO.:36)-B: 24 PHE - ASN
 (23) 5'AGCCAGGCCCATACCAGCCTTCCTGCGGACCATTTACCATG3'
 (SEQ. ID. NO.:37)-B 24 PHE - GLU
 (24) 5'AGCCAGGCCCATACCAGCCCCACTGCGGACCATTTACCATG3'

20 (SEQ. ID. NO.:38)-B 24 PHE - TYRP

(1) The gene for penicillin G acylase is inserted into the PBM plasmid (Figure 3) which is used as a template for the synthesis of the mutated gene.

25 (2) An oligonucleotide is designed which is complementary to the sequence to be mutagenized except for an internal portion which determines the mutation.

(3) Using standard PCR techniques, the synthetic oligonucleotide is annealed to the template and the template is amplified. The megaprimer product is purified
 30 to be used for a second round of PCR to generate the double-stranded mutant. The mutant DNA is then purified from a preparative agarose gel.

Example 2

Cloning and Expression of Mutant Penicillin G Acylases

The mutated penicillin G acylase gene is cloned into plasmid PBM which contains the tac promoter and is induced
5 by lactose or IPTG. The recombinant plasmids can be introduced into a host organism selected from the E.coli group. These microorganisms are then cultivated under appropriate conditions and colonies selected.

(1) Both the PBM plasmid and the DNA sequence encoding the
10 mutagenized enzyme are digested with the restriction enzymes HindIII and BamHI. The products are gel purified.

(2) The digested DNA sequences are ligated and an aliquot of the ligation reaction is used to transform competent E. coli cells. The transformants were subsequently selected
15 on LB plates containing kanamycin and lactose.

(3) To assay, individual colonies were chosen and grown overnight at 28°C in LB media containing lactose and kanamycin.

(4) To verify the mutations, a kit from Ambion Inc. was
20 utilized. This method is based on the fact that certain RNases can selectively cleave double stranded RNA at a position with a single mismatched base pair, indicating mutation has occurred.

25

Example 3

Culture of Microorganism

Transformed E. coli colonies are used to inoculate seed cultures into 500 ml Erlenmeyer flasks containing 100 ml of Luria Bertani medium broth supplemented with 30 µg/ml
30 of kanamycin. Seed flasks are cultured for 5 hours at 28°C. 50 ml of culture is used to inoculate 2 liter tanks. The base media is 0.3% K₂HPO₄, 0.2% KH₂PO₄, 0.2% MgSO₄, 0.05% (NH₄)₂SO₄, 0.003% FeSO₄, 0.001% MnSO₄, 0.3% yeast extract, and 30 µg/ml of kanamycin. The pH is 6.8-7.2. The
35 tanks are run in a pH-regulated feeding profile. The tanks are supplemented with 20% NZ amine, 20% glucose, and

15 Shake the ultrafiltered broth with UOP beads
overnight at 10°C. Wash beads with water and store at 4°C.

(2) Immobilization to Diacalite

4% Triton X-100, 5% Biocryl and isopropanol to 30%
were added to the ultrafiltered broth and the mixture
20 stirred for 1 hour and filtered. To the filtrate 1%
Speedplus was added and 50% PEG was added to a final
concentration of 15%, the mixture was stirred for 15
minutes and 50% glutaraldehyde was added to a final
concentration of 0.4%. The immobilization was allowed to
25 proceed for 15 minutes at room temperature. The enzyme was
filtered and washed with water until the wash was
colorless. The pH was maintained between 7.2 and 7.6
throughout the procedure.

30

Example 5

Assay of Hydrolytic Activity of Penicillin G acylases

(1) Assay with the commercial substrate, 6-nitro-3-
(phenyl-acetamido) benzoic acid.

20 µl samples of cell culture are added to wells of
35 a 96-well microtiterplate which contains 0.1% of the
substrate in 0.2M potassium phosphate buffer, pH 7.4. The
reaction is followed spectrophotometrically at 405nm.

2. Assay with p-Dimethyl-Aminobenzaldehyde (p-DAB):
Sonicate 1ml cell culture sample and add 1 ml 4.5% K
penicillin G in 200 mM potassium phosphate buffer (pH 7.5).
Incubate 15 minutes at 37°C with shaking. Add 1 ml 99.0%
5 acetonitrile and 1.0% acetic acid. Mix and centrifuge. To
1 ml of supernatant add 3 ml p-DAB reagent. (To prepare p-
DAB Reagent; combine 1 part 10 mg/ml p-DAB and 6 parts
sodium acetate buffer). Incubate 4 minutes and read at 415
nm. Calculate IU/ml using a standard factor of 100 µg/ml
10 of 6APA.

Example 6

Assay of Synthetic Activity of Penicillin G acylases

(1) Cefadroxil:

15 Dissolve 10.5g hydroxyethyl salt of p-
hydroxyphenylglycine in 37.5 ml water. Adjust pH to 8.0
with ammonium hydroxide. Add 4.8 g 7-ADCA and dissolve (pH
7.5). Adjust pH to 7.0 with 6N HCl. Bring volume to 60
ml. Divide reaction mix into 12 equal parts of 5 ml each.
20 Add immobilized penicillin G acylase to a final
concentration of 40 IU/ml. Remove aliquots at designated
times for HPLC assay.

(2) Cefproxil Synthesis

Add 4.5 g ester salt to 60 ml of water, pH to 8.26.
25 Add 3.6 g 7-PACA. Add 1.72 ml ammonium hydroxide to pH
8.26. Add 4.5 g ester, pH to 7.56. Divide reaction mix
into 12 equal parts. Add immobilized Penicillin G acylase
to a final concentrations of 40 IU/ml.
Remove aliquots at designated times for HPLC assay

30 (3) Amoxicillin Assay

Add 3.5g of ester salt to 12.5 ml of water. pH to
8.0 with ammonium hydroxide. Add 1.6 g 6-APA, dissolve
and pH to 7.0. Bring volume to 20 ml. Divide reaction mix
into 4 equal parts of 5 ml. Add immobilized penicillin G
35 acylase to a final concentration of 40 IU/ml. Remove
aliquots at designated times for HPLC assay.

and Amoxicillin	18, 30 cm x 0.25 inch , Waters Associates	hydroxide 0.00693M tetrabutylammonium hydroxide 10% methanol, pH 7.0
Cefprozil	Phenomenex Phenosphere ODS % micron, 4.6 mm x 5.0 cm	24% acetonitrile, 0.16% KH ₂ PO ₄ , 0.2% NaSDS pH 2.6

15 The mutant of penicillin G acylase with an alanine
 substituted for phenylalanine 24 on the beta subunit was
 found to demonstrate superior synthesis for β -lactam
 antibiotics although it exhibited 25% of the hydrolytic
 activity. This mutant penicillin G acylase will be
 designated as F1. These results are demonstrated in Tables
 2-12.

20

Table 2

Synthesis vs Hydrolysis activity of wild-type and mutant Penicillin G acylases

MUTANT	HYDROLYSIS	SYNTHESIS
Wild-type	100%	100%
Met 142-Ala	10%	0%
Val 56-Thr	4%	127%
Phe146-Ala	5%	0%
Phe24-Ala	25%	330%
Phe24-Val	36%	3%
Phe24-Leu	80%	229%

5 25 ml cultures were inoculated into Luria-Bertaini broth containing 30 µg/ml kanamycin and shaken overnight at 28°C. Cultures were induced with 800 µM IPTG for 4 hours. Cells were concentrated 10 fold and sonicated.

Hydrolysis was determined by microtitre plate assay.

10 The substrate was 0.1% nitro-(phenyl-acetamido) benzoic acid in 0-0.2 M potassium phosphate buffer. The data is expressed as % of wild-type.

 Synthesis was determined by formation of cefadroxil after 4 hours of incubation with hydroxy ethyl ester and 7
15 ADCA. The assay was performed by HPLC. The activity is expressed as % of wild-type.

Serine	39%	23 %
Tyrosine	2%	0
Arginine	7%	4%
Asparagine	8%	6%
Glutamic acid	7%	0
Glutamine	3%	28%
Isoleucine	8%	4%
Threonine	20%	6%
Tryptophan	9%	9%
Glycine	27%	19%
Cysteine	26%	0%
Alanine + Val(B)56-Thr	0%	0%
Leucine + Val(B)56-Thr	6%	0%

Table 4
Effects of Temperature on the Synthetic Yield of Cefprozil

The reaction is run at pH 7.5 with 2.3 molar equivalents of ester to 7-PACA
The percent conversion of 7PACA to cefprozil in 120 minutes is reported.

Temperature	Wild-Type PGA	F1 Mutant PGA
37°C	80%	99%
Room Temperature	85%	98%
10°C	90%	100%

Table 5
Effects of Temperature on the Synthetic Yield of Cefadroxil

The reaction is run at pH 7.0 with 1.9 molar equivalents of ester to 7-ADCA
The percent conversion of 7-ADCA to cefadroxil in 120 minutes is reported.

Temperature	Wild-Type PGA	F1 Mutant PGA
37°C	79%	95%
Room Temperature	86%	91%
10°C	83%	95%

Table 6
Effects of Enzyme Concentrations on the Synthetic Yield of Cefprozil

The reaction is run at pH 7.5, room temperature, with 2.3 molar equivalents of ester to 7-PACA.
 The percent conversion of 7-PACA to cefprozil in 120 minutes is reported.

Gram of immobilized enzyme	Wild-Type PGA	F1 Mutant PGA
0.4 g	88%	100%
0.2 g	90%	100%
0.1 g	90%	98%

Table 7
Effects of Enzyme Concentrations on the Synthetic Yield of Cefadroxil

The reaction is run at pH 7.0, room temperature, with 1.9 molar equivalents of ester to 7-ADCA.
 The percent conversion of 7-PACA to cefprozil in 120 minutes is reported.

Gram of immobilized enzyme	Wild-Type PGA	F1 Mutant PGA
0.7 g	89%	95%
0.35 g	81%	97%
0.175 g	79%	97%

Table 8
Effects of Acyl Donor Concentration on the Synthetic Yield of Cefprozil

The reaction is run at pH 6.5 and at room temperature.
0.2 g of immobilized enzyme is added to reactions.
The percent conversion of 7-PACA to cefprozil in 120 minutes is reported.

Molar equivalent of ester to 7-PACA	Wild-Type PGA	F1 Mutant PGA
2.3	91%	100%
1.5	86%	99%
1.45	83%	99%
1.38	83%	99%
1.3	85%	99%
1.2	80%	96%

Table 9
Effects of Acyl Donor Concentration on the Synthetic Yield of Cefadroxil

The reaction is run at pH 7.0 and at room temperature.
0.2 g of immobilized enzyme is added to reactions.
The percent conversion of 7-ADCA to cefadroxil in 120 minutes is reported.

Molar equivalent of ester to 7-ADCA	Wild-Type PGA	F1 Mutant PGA
1.89	81%	96%
1.63	73%	90%

Table 10
Wild-type vs Mutant Penicillin G acylase semisynthesis of Amoxicillin

WT PGA		F1 PGA	
% 6APA Remaining	% Ester Remaining	% 6APA Remaining	% Ester Remaining
58	62	52	58
30	27	12	4
20	17	9	1

Table 11
Optimized Conditions for the Semisynthesis of Cefprozil

Temperature	Room temperature
pH	6.5
Ester Concentration	1.2 -1.3 molar equivalents
Enzyme Concentration	0.2 g in a 5 ml volume

Table 12
Optimized Conditions for the Semisynthesis of Cefadroxil

Temperature	Room temperature
pH	7.0
Ester Concentration	1.89 molar equivalents
Enzyme Concentration	0.2 g in a 5 ml volume

What Is Claimed Is:

1. A mutant prokaryotic penicillin G acylase having an amino acid substitution at one or more of the positions
5 corresponding to A142, A146, B24, B56, or B177.
2. The mutant acylase of Claim 1 originating from E. coli.
- 10 3. The mutant acylase of Claim 2, where said amino acid substitution is one or more of the following:
Met at A142 to Ala;
Phe at A146 to Ala;
Phe at B24 to
15 Ala, Leu, Val, Pro, Tyr, Met, Ser, Cys, Gly, Asp,
Lys, Arg, Tyr, Thr, Ile, Glu, Gln, Asn
or His;
Val at B56 to Ser or Thr;
Ile at B177 to Phe.
- 20 4. The mutant acylase of Claim 1 wherein said amino acid substitution is Met at A142 to Ala.
5. The mutant acylase of Claim 1 wherein said amino
25 acid substitution is Phe at A146 to Ala.
6. The mutant acylase of Claim 1 wherein said amino acid substitution is Phe at B24 to
Ala, Leu, Val, Pro, Tyr, Met, Ser, Cys, Gly,
30 Asp, Lys, Arg, Tyr, Thr, Ile, Glu, Gln,
Asa or Hist.
7. The mutant acylase of Claim 1 wherein said amino acid substitution is Val at B56 to Ser or Thr.
- 35 8. The mutant acylase of Claim 1 wherein said amino acid substitution is Ile at B177 to Phe.



9. The mutant acylase of Claim 1 wherein said amino acid substitution is Phe at B24 to Ala.

5 10. A mutant type II E. coli penicillin G acylase having an amino acid substitution of PheB24-Ala.

10 11. The mutant acylase of Claim 1 wherein the N-terminus of the alpha subunit is truncated by 1 or 2 amino acids; the C-terminus of the alpha subunit is truncated by 10 to 15 amino acids; or both the N-terminus of the alpha subunit is truncated by 1 or 2 amino acids and the C-terminus of the alpha subunit is truncated by 10 to 15 amino acids.

15 12. The mutant acylase of Claim 11 wherein the C-terminus of the alpha subunit is truncated by 12 or 13 amino acids.

20 13. A nucleic acid sequence encoding the mutant acylase of Claim 1.

 14. The nucleic acid sequence of Claim 13 encoding the mutant acylase of Claim 2

25 15. The nucleic acid sequence of Claim 13 encoding the mutant acylase of Claim 3.

30 16. The nucleic acid sequence of Claim 13 encoding the mutant acylase of Claim 4.

 17. The nucleic acid sequence of Claim 13 encoding the mutant acylase of Claim 5.

35 18. The nucleic acid sequence of Claim 13 encoding the mutant acylase of Claim 6.



19. The nucleic acid sequence of Claim 13 encoding the mutant acylase of Claim 7.

20. The nucleic acid sequence of Claim 13 encoding
5 the mutant acylase of Claim 8.

21. The nucleic acid sequence of Claim 13 encoding the mutant acylase of Claim 9.

10 22. The nucleic acid sequence of Claim 13 encoding the mutant acylase of Claim 10.

23. The nucleic acid of Claim 13 encoding the mutant acylase of Claim 11.
15

24. The nucleic acid of Claim 13 encoding the mutant acylase of Claim 12.

25. The nucleic acid sequence of Claim 13 which is
20 DNA.

26. An expression vector comprising a nucleic acid sequence as defined in Claim 13 operably linked to a promoter sequence capable of directing its expression in a
25 host cell.

27. A host cell comprising the expression vector of Claim 26.

30 28. A process for producing a mutant penicillin G acylase comprising culturing the host cell of Claim 27 under conditions suitable for production of the mutant acylase.



29. A process for producing a semi-synthetic 6-acylated penicillanic acid, a 7-acylated cephalosporanic acid or a salt or ester thereof which comprises contacting a corresponding 6-amino β -lactam or salt or ester thereof, respectively, 7-ACA and an acylating agent with a mutant acylase as defined in Claim 1 under conditions suitable for acylation to occur.

30. The process of Claim 29 wherein said mutant acylase is immobilized.

31. The process of Claim 30 wherein said mutant acylase has the amino acid substitution at B24(Phe) to Ala.