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(54) Title: ALTERED ACYLTRANSFERASE POLYPEPTIDES AND RELATED POLYNUCLEOTIDES

(57) Abstract: The present invention relates to novel acyltransferase polypeptides, as well as the polynucleotides that encode them, that are particularly useful in the production of beta-lactam intermediates and antibiotic compounds having adipoyl side chains. The present invention is also directed to related vectors, host cells, and methods for making and using the novel acyltransferase polypeptides.



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ALTERED ACYLTRANSFERASE POLYPEPTIDES AND RELATED POLYNUCLEOTIDES

Field of the Invention

The present invention relates generally to novel acyltransferase polypeptides that are useful in the production of beta-lactam intermediates and antibiotic compounds.

Background of the invention

The most important classes of the beta-lactam antibiotics are the penicillins (penams) and the cephalosporins (ceph-3-ems). Penicillins are produced by filamentous fungi only (*Penicillium chrysogenum* or *Aspergillus nidulans*), whereas cephalosporins are produced by filamentous fungi (*Acremonium chrysogenum*) as well as bacteria (e.g. *Streptomyces clavuligerus*). All beta-lactam antibiotics share the common structural feature of a four-member beta-lactam ring. The naturally occurring penams and ceph-3-ems are synthesized from the same three amino acid precursors: L-alpha-aminoadipic acid (L-alpha-AAA), L-cysteine, and L-valine. The first two steps in the biosynthesis pathways of penicillins and cephalosporins are the same. In the first step, the amino acid precursors are condensed to the tripeptide delta-L-alpha-aminoadipyl-L-cysteinyl-D-valine (ACV). The requisite reaction cycle (e.g., recognition, activation and formation of peptide bonds) is catalyzed by the multifunctional enzyme ACV synthetase (ACVS). In the second step the resulting linear tripeptide is cyclized by oxidative ring closure leading to the formation of a bicyclic ring structure, i. e., the four-member beta-lactam ring fused to the five-membered thiazolidine ring which is characteristic of all penams. Cyclization is catalyzed by the enzyme isopenicillin N synthase (IPNS). The resulting compound represents the first bioactive intermediate and is referred to as isopenicillin N (IPN). See generally, e.g., EP 0 422 790 (Miller et al.).

After IPN synthesis, the biosynthetic pathways leading to the production of the penicillins and cephalosporins diverge. In some fungi, for example in *P. chrysogenum* and in *A. nidulans*, the hydrophilic L-alpha-AAA side chain of IPN can be replaced by a hydrophobic acyl group in a third and final exchange step. The side chain may be of intracellular origin (e.g., hexonic acid or octenoic acid) or supplied exogenously. The

only directly fermented penicillins of industrial importance are penicillin V and penicillin G, produced by adding the exogenous side chain precursors phenoxyacetic or phenylacetic acid, respectively. The exchange is catalyzed by acyl coenzyme A (CoA):isopenicillin N acyltransferase (AT) which is encoded by the penDE gene.

5 In contrast, the ceph-3-ems are produced by the isomerization of the L-alpha-AAA side chain of IPN to the D enantiomer by IPN epimerase to produce penicillin N which is the precursor of antibiotics comprising the ceph-3-em nucleus. For example, in the fungus *A. chrysogenum*, the alpha-aminoadipic acid side chain of IPN is isomerized to produce penicillin N, after which the five-membered thiazolidine ring of the penicillin is
10 "expanded" by deacetoxycephalosporin synthetase (DAOCS) expandase activity to produce deacetoxycephalosporin C (DAOC) which comprises the six-membered dihydrothiazine ring that is characteristic of the cephalosporins.

Many of the so-called natural beta-lactams (e.g., penicillin F, isopenicillin N, cephalosporin C, etc.) are of limited utility as antibiotics because they are unstable,
15 difficult to purify from fermentation broth, have only limited antibiotic effect, and/or are produced in low yield. Replacing the side chains of these beta-lactams with other side chains leads to the formation of semisynthetic penicillins and cephalosporins, such as amoxycillin, ampicillin and cephalixin, which are more stable, easier to isolate, and have a higher antibiotic activity.

20 The large variety of side chains found in commercially significant beta-lactam compounds has placed increased importance on achieving more economic and efficient methods of preparing key intermediates for synthesis of various beta-lactam compounds.

The cephalosporin intermediate 7-ADCA is an important intermediate for the
25 synthesis of many semisynthetic cephalosporins. It is currently produced by either chemical derivatization of penicillin G or by a bioprocess as described in EP 0532341.

In EP 0532341 it is shown that adipyl-7-ADCA is formed by a *Penicillium chrysogenum* strain modified to express expandase and fed with the side chain precursor adipic acid. Subsequent removal of the adipyl side chain with a suitable
30 enzyme leads to formation of 7-ADCA. Although the AT enzyme thus is capable of accepting other side chains than phenyl- or phenoxyacetic acid, it is unpredictable whether or not the capacity of the AT enzyme to accept other side chains than phenyl- or phenoxyacetic acid can be improved or its substrate specificity modified.

Figures legends

Figure 1 is a schematic of a 6343 bp vector (pET-penDE Pc) containing a T7 promoter, a T7 terminator, and a kanamycin resistance gene.

Figure 2 is an alignment display which provides the amino acid sequences of the acyltransferase enzymes disclosed herein.

Summary of the invention

The present invention provides polypeptides having acyltransferase activity with altered substrate specificity as compared to the native *Penicillium chrysogenum* acyltransferase. In particular, the present invention provides acyltransferase polypeptides with an improved capacity as compared to the native *P. chrysogenum* acyltransferase to form adipoyl-6-APA from alpha-amino adipoyl-6-APA. More particularly, said improved capacity is at least 1.5 times the activity of the native *P. chrysogenum* acyltransferase, preferably at least 2 times, more preferably at least 5 times, more preferably at least 10 times, most preferably at least 30 times.

In a further aspect, the present invention is directed to an isolated polypeptide having acyltransferase activity, wherein the isolated polypeptide is selected from the group consisting of:

(a) a polypeptide having an amino acid sequence that is at least 96% identical to SEQ ID NO: 38;

(b) a polypeptide having an amino acid sequence that is at least 95% identical to SEQ ID NO: 42;

(c) a polypeptide having an amino acid sequence that is at least 95% identical to SEQ ID NO: 52;

(d) a polypeptide having an amino acid sequence that is at least 96% identical to SEQ ID NO: 54 and having at least one residue selected from the group consisting of Arg at position 97, Val at position 221, Pro at position 251, Glu at position 254, Ile at position 259, Gly at position 277, Glu at position 291, and Gly at position 299; and

(e) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions over substantially the entire length of a nucleotide sequence that encodes an amino acid sequence selected from the group consisting of SEQ ID NO: 38 and SEQ ID NO: 42 and SEQ ID NO: 52.

In still a further aspect, the present invention is directed to polynucleotides that encode acyltransferase polypeptides.

In other embodiments, the present invention is directed to expression vectors, host cells, and methods that are useful for producing beta-lactam compounds using the acyltransferase polypeptides of the present invention.

Detailed description of the invention

In a first aspect, the present invention provides novel polypeptides having acyltransferase ("AT") activity selected from the group consisting of:

In a further aspect, the present invention is directed to an isolated polypeptide having acyltransferase activity, wherein the isolated polypeptide is selected from the group consisting of:

(a) a polypeptide having an amino acid sequence that is at least 96% identical to SEQ ID NO: 38;

(b) a polypeptide having an amino acid sequence that is at least 95% identical to SEQ ID NO: 42;

(c) a polypeptide having an amino acid sequence that is at least 95% identical to SEQ ID NO: 52;

(d) a polypeptide having an amino acid sequence that is at least 96% identical to SEQ ID NO: 54 and having at least one residue selected from the group consisting of Arg at position 97, Val at position 221, Pro at position 251, Glu at position 254, Ile at position 259, Gly at position 277, Glu at position 291, and Gly at position 299; and

(e) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions over substantially the entire length of a nucleotide sequence that encodes an amino acid sequence selected from the group consisting of SEQ ID NO: 38 and SEQ ID NO: 42 and SEQ ID NO: 52.

Acyltransferase polypeptides of the present invention are useful in the production of beta-lactam compounds, such as, for example, penicillin antibiotic compounds having adipoyl side chains. As used herein, the terms "acyltransferase polypeptide," "AT polypeptide," "acyltransferase enzyme," and "AT enzyme" are used interchangeably herein to refer to a polypeptide that exhibits acyltransferase activity. The term "acyltransferase activity" is used herein to refer to the ability of an enzyme to catalyze at

least the conversion of 6-aminopenicillanic acid ("6-APA") and adipoyl-coenzyme A ("Ad-CoA") to a detectable amount of adipoyl-6-aminopenicillanic acid ("Ad-6-APA") using the assay described in Example 3. Ad-6-APA is a useful intermediate that can be converted to a penicillin antibiotic having an adipoyl side chain or to the 6-APA nucleus.

5 Alternatively, ad-6-APA is a useful intermediate that can be expanded to adipoyl-7-ADCA as described in e.g. EP 0 532 341. The AT enzyme is also able to catalyze the hydrolysis of IPN, whereby the alpha-AAA side chain is cleaved off, resulting in the formation of 6-APA. The terms "acyltransferase polynucleotides" and "AT polynucleotides" are used interchangeably herein to refer to polynucleotides that encode

10 acyltransferase polypeptides. It is to be understood that the reference to adipoyl should not be considered as a limitation, but only as a means to characterize at least one of the catalytic capacities of the enzyme.

As used herein, the term "isolated" refers to a polynucleotide or polypeptide that is substantially free of other material with which it is normally found in nature, and thus is

15 substantially free of other naturally occurring cellular material, as well as culture medium. For example, an "isolated polynucleotide" is largely free of sequences that flank the polynucleotide in its native genomic location.

The terms "polynucleotide," "nucleic acid," and "nucleic acid molecule" are used interchangeably herein to refer to DNA, RNA, or synthetic analogues thereof. As used

20 herein, the terms "polypeptide" and "protein" are used interchangeably herein to refer to a polymer of amino acids.

The terms "percent identity" and "% identity" are used interchangeably herein to refer to the percent amino acid sequence identity that is obtained by Clustal W analysis (version W 1.8 available from European bioinformatics Institute, Cambridge, UK),

25 counting the number of identical matches in the alignment and dividing such number of identical matches by the length of the reference sequence, and using the following default Clustal W parameters to achieve slow/accurate pairwise alignments – Gap Open Penalty:10; Gap Extension Penalty:0.10; Protein weight matrix: Gonnet series; DNA weight matrix: IUB; Toggle Slow/Fast pairwise alignments = SLOW or FULL Alignment.

30 The present invention also provides acyltransferase polypeptides that have an amino acid sequence at least 97% identical to SEQ ID NO: 38, typically at least 98% identical to SEQ ID NO: 38, and in some embodiments, at least 99% identical to SEQ ID NO: 38. In another embodiment, the present invention provides acyltransferase polypeptides that have an amino acid sequence that is at least 96% identical to SEQ ID

NO: 52. The present invention includes AT polypeptides having an amino acid sequence that is at least 97% identical to SEQ ID NO: 52, as well as those that are at least 98% and at least 99% identical to SEQ ID NO: 52.

5 In a further embodiment, the present invention provides acyltransferase polypeptides that have an amino acid sequence that is at least 96% identical to wild type acyltransferase from *P. chrysogenum* (SEQ ID NO: 54), and which also has in its sequence, at least one residue selected from the group consisting of Arg at position 97, Val at position 221, Pro at position 251, Glu at position 254, Ile at position 259, Gly at position 277, Glu at position 291, and Gly at position 299. In certain embodiments, the
10 amino acid sequence of the AT polypeptide has at least two residues selected from this group, and sometimes at least four residues, often up to about six residues (e.g., Pro at position 251, Glu at position 254, Ile at position 259, Gly at position 277, Glu at position 291, and Gly at position 299). The amino acid positions recited herein are the positions that correspond to SEQ ID NO: 54 upon optimal alignment of the AT polypeptide
15 sequence with SEQ ID NO: 54.

The term "optimally aligned" refers to the alignment created using the Clustal W algorithm (Nucleic Acid Research, 22(22): 4673-4680 (1994)). With respect to an amino acid sequence that is optimally aligned with a reference sequence, an amino acid residue "corresponds to" the position in the reference sequence with which the residue
20 is paired in the alignment.

In a further embodiment of the present invention, acyltransferase polypeptides of the present invention are provided that have higher acyltransferase activity than that of the wild type *P. chrysogenum* acyltransferase (SEQ ID NO: 54) as measured in the assay described in Example 3. Certain acyltransferase polypeptides of the present
25 invention exhibit acyltransferase activity that is at least about 1.6 fold greater than that of *P. chrysogenum* wild type acyltransferase (SEQ ID NO: 54). In specific embodiments, acyltransferase polypeptides of the present invention may exhibit acyltransferase activity that is at least about 2 fold, sometimes at least about 5 fold, and in some embodiments at least about 10 fold or 20 fold greater up to about 30 fold or 50 fold greater than that of
30 *P. chrysogenum* wild type acyltransferase.

In another embodiment, the present invention provides acyltransferase polypeptides that are encoded by a polynucleotide that hybridizes under stringent conditions over substantially the entire length of a nucleotide sequence that encodes an

amino acid sequence selected from the group consisting of SEQ ID NO: 38 and SEQ ID NO: 52.

As used herein, the term "stringent hybridization and/or wash conditions" in the context of nucleic acid hybridization experiments, such as Southern and Northern hybridizations, are sequence dependent, and are different under different environmental parameters. An extensive guide to the hybridization of nucleic acids is found in Tijessen (1993) "Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes," Part I, Chapter 2 (Elsevier, New York).

For purposes of the present invention, "highly stringent" hybridization and/or wash conditions are generally selected to be about 5°C or less lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH (as noted below, highly stringent conditions can also be referred to in comparative terms). The T_m is the temperature (under defined ionic strength and pH) at which 50% of the test sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe.

Stringent and highly stringent hybridization and wash conditions can be readily determined empirically for any nucleic acid. For example, in determining highly stringent hybridization and wash conditions, the hybridization and wash conditions are gradually increased (e.g., by increasing temperature, decreasing salt concentration, increasing detergent concentration and/or increasing the concentration of organic solvents, such as formamide, in the hybridization or wash), until a selected set of criteria is met.

An example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or northern blot is 50% formamide with 1 mg of heparin at 42°C, with the hybridization being carried out overnight. An example of stringent wash conditions are 0.2x SSC wash at 65°C for 15 minutes (see Sambrook, et al., "Molecular Cloning – A Laboratory Manual" (1989) Cold Spring Harbor laboratory (Cold Spring Harbor, New York) for a description of SSC buffer). Often the high stringency wash is preceded by a low stringency wash to remove background probe signal. An example low stringency wash is 2x SSC at 40°C for 15 minutes.

Specific acyltransferase polypeptides of the present invention include those having an amino acid sequence corresponding to SEQ ID NOS: 2 (H1), 4 (H2), 6 (H3), 8 (H4), 10 (H5), 12 (H6), 14 (H7), 16 (H14), 18 (H25), 20 (H26), 22 (H27), 24 (H28), 26 (H29), 28 (H30), 30 (H31), 32 (H32), 34 (H33), 36 (H34), 38 (H35), 40 (H36), 42 (H37),

44 (H38), 46 (H40), 48 (H41), 50 (H42), and 52 (H43). The acyltransferase activities for these polypeptides are described in Example 3. The present invention also provides acyltransferase polypeptides that are variants of these polypeptides having a substitution, deletion, and/or insertion of one to six amino acid residues.

5 As used herein, the term "variant" refers to a sequence that has a high percent identity with respect to the reference sequence. Variant acyltransferase polypeptides of the present invention may be naturally occurring or non-naturally occurring. Variant acyltransferase polypeptides may have a substitution, deletion, and/or insertion of one to six amino acid residues. Such substitution, deletion and/or insertion may occur at more
10 than one site in the polypeptide and may occur at the N-terminal and/or C-terminal end of the polypeptide as well as at one or more sites internal to the polypeptide. Variant polypeptides encompassed by the present invention have acyltransferase activity.

Conservative variants can be readily generated by making conservative substitutions such as those within the group of basic amino acids (arginine, lysine and
15 histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagines), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (glycine, alanine, serine, threonine, proline, cysteine and methionine).

Variants of the acyltransferase polypeptides of the present invention may also be
20 generated using mutagenesis and directed evolution methods that are well known to those having ordinary skill in the art. Libraries created by such methods can be screened for variants having acyltransferase activity as measured using the assay described in Example 3.

In a further embodiment, the present invention is directed to a fragment of an AT
25 polypeptide of the present invention that exhibits AT activity in the assay described in Example 3. Fragments can be readily prepared using commercially available exonucleases and endonucleases according to known methods. As used herein, the term "fragment" refers to a polypeptide having a deletion of 1 to 15 amino acid residues from either or both the carboxy and/or amino terminus. In specific embodiments, the
30 deletion is of 1 to 10 amino acid residues, and in some instances 1 to 5 amino acid residues. AT fragments of the present invention have AT activity that is at least 1.6 fold greater than that of wild type *P. chrysogenum* AT activity using the assay described in Example 3. In some embodiments, AT fragments of the present invention have AT

activity that is at least 2 fold, and typically up to about 30 fold greater than that of wild type *P. chrysogenum* AT activity in the assay of Example 3.

In a second aspect, the present invention provides polynucleotides that encode acyltransferase polypeptides of the present invention. In one embodiment, the present invention provides an isolated polynucleotide that encodes a polypeptide having acyltransferase activity, wherein the isolated polypeptide is selected from the group consisting of:

(a) a polypeptide having an amino acid sequence that is at least 96% identical to SEQ ID NO: 38;

(b) a polypeptide having an amino acid sequence that is at least 95% identical to SEQ ID NO: 42;

(c) a polypeptide having an amino acid sequence that is at least 95% identical to SEQ ID NO: 52;

(d) a polypeptide having an amino acid sequence that is at least 96% identical to SEQ ID NO: 54 and having at least one residue selected from the group consisting of Arg at position 97, Val at position 221, Pro at position 251, Glu at position 254, Ile at position 259, Gly at position 277, Glu at position 291, and Gly at position 299; and

(e) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions over substantially the entire length of a nucleotide sequence that encodes an amino acid sequence selected from the group consisting of SEQ ID NO: 38 and SEQ ID NO: 42 and SEQ ID NO: 52.

Specific acyltransferase polynucleotides of the present invention include those having an polynucleotides sequence corresponding to SEQ ID NO: 1 (H1), 3 (H2), 5 (H3), 7 (H4), 9 (H5), 11 (H6), 13 (H7), 15 (H14), 17 (H25), 19 (H26), 21 (H27), 23 (H28), 25 (H29), 27 (H30), 29 (H31), 31 (H32), 33 (H33), 35 (H34), 37 (H35), 39 (H36), 41 (H37), 43 (H38), 45 (H40), 47 (H41), 50 (H41), and 51 (H43). SEQ ID NOS: 13 and 15 are polynucleotide variants that encode the same acyltransferase polypeptide (i.e., SEQ ID NOS: 14 and 16 are the same). Those having ordinary skill in the art will readily appreciate that due to the degeneracy of the genetic code, a multitude of nucleotide sequences encoding acyltransferase polypeptides of the present invention exist and can be readily determined using codon tables.

One of ordinary skill in the art will recognize that each codon in a nucleic acid (except ATG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified by standard techniques to encode a functionally identical polypeptide. Accordingly, each silent variation of a nucleic acid that encodes a polypeptide is implicit in any described sequence. Acyltransferase polynucleotides may be codon optimized for expression in a particular host organism by modifying the polynucleotides to conform to the optimum codon usage of the desired host organism. Those having ordinary skill in the art will recognize that tables and other references providing preference information for a wide range of organisms are readily available.

Polynucleotides of the present invention can be prepared using methods that are well known in the art. See, e.g., Carruthers, et al., Cold Spring Harbor Symp. Quant. Biol., 47:411-418 (1982) and Adams, et al., J. Am. Chem. Soc., 105:661 (1983). Typically, oligonucleotides of up to about 100 bases are individually synthesized, then enzymatically or chemically ligated together to form the desired sequence.

In a third aspect, the present invention provides an expression cassette comprising an acyltransferase polynucleotide of the present invention operatively linked to a regulatory sequence, such as a promoter. Expression cassettes of the present invention provide for the genetic transfer of acyltransferase polynucleotides, as well as expression and production of the acyltransferase polypeptides that they encode. The regulatory sequences of the construct operate to drive transcription and translation of the acyltransferase polynucleotides.

As used herein, the term "construct" refers herein to a single- or double-stranded nucleic acid molecule made up of DNA, RNA, synthetic analogues thereof, or a combination of two or more of these. In addition to an expression cassette, the nucleic acid construct may optionally contain other nucleic acid segments that may provide other functions useful in genetic transfer and/or expression, such as, for example, selectable marker genes.

The nucleic acid construct may be derived from a number of different sources, including bacterial, fungal, and plant. Suitable fungal sources include *Aspergillus*, and *Penicillium* (e.g., *Penicillium chrysogenum*). The nucleic acid construct may be in the form of a vector that is suitable for manipulation, transformation, and/or expression of the acyltransferase polynucleotides of the present invention. For example, the nucleic

acid construct may be selected and/or designed in accordance with the characteristics of the desired host organism into which it is to be introduced. Thus, the construct may be autonomously replicating, i.e., it may exist normally as an extrachromosomal entity, e.g., a plasmid. In another embodiment, the nucleic construct may be a molecule which, when introduced into a host cell, is integrated into the genome.

Suitable regulatory sequences employed in the practice of the present invention may be any polynucleotide that exhibits appropriate activity in the host cell desired, and may be natural or synthetic in origin. Promoters that are suitable for use in filamentous fungal host cells include, for example, the *Aspergillus nidulans* trpC promoter (Yelton, M.M., et al., Proc. Natl. Acad. Sci. USA, 81:1470-1474 (1984); Mullaney, E.J. et al., Mol. Gen. Genet., 199:37-45 (1985)), the *Aspergillus nidulans* glyceraldehydes-3-phosphate dehydrogenase (gpd) promoter (Punt, P.J., et al., Gene, 56(1):117-24 (1987); Punt, P.J., et al., Gene, 69(1):49-57 (1988)), the *Aspergillus nidulans* ADH3 promoter (McKnight et al., EMBO J., 4:2093-2099 (1985)), the *Aspergillus awamori* glucoamylase gene promoter (Nunberg, J.H., et al., Mol. Cell Biol., 4(11):2306-15 (1984)), and the *Aspergillus oryzae* transcription elongation factor gene promoter (Kitamoto, N., et al., Appl. Microbiol. Biotechnol., 50(1):85-92 (1998)). Other suitable promoters include those from the penicillin biosynthetic pathway itself, such as the *Penicillium chrysogenum* pcbC (IPNS) promoter (Barredo, J.L., et al., Mol. Gen. Genet., 216(1):91-98 (1989)) or the *Penicillium chrysogenum* penDE (AT) promoter (Diez, B., et al., Mol. Gen. Genet., 218(3):572-6 (1989)). Where production of more than one polypeptide is desired, it may be advantageous to use identical or similar promoters to regulate synchronized production of the polypeptides. For example, when it is desired to transform multiple polynucleotides into a host cell encoding enzymes utilized in the beta-lactam antibiotic biosynthetic pathway, it may be desirable to synchronize production of intermediates in the pathway to improve the efficiency of production.

Nucleic acid constructs of the present invention may also comprise a selectable marker, e.g., a polynucleotide encoding a marker that facilitates the selection of the cell that contains the nucleic acid construct. Those having ordinary skill in the art will recognize that many selectable markers are suitable for use in expression vectors of the present invention. Suitable selectable markers include kanamycin resistance genes, chloramphenicol resistance genes, as well as other antibiotic resistance-conferring genes. Preferred selectable markers for use in filamentous fungi include genes encoding acetamidase (Corrick, C.M. et al., Gene, 53(1):63-71 (1987)), hygromycin

resistance (Kaster, K.R., et al., Nucleic Acids Res., 11(19):6895-911 (1983); Punt, P.J., et al., Gene, 56(1):11724 (1987)), phleomycin resistance (Drocourt, D., et al., Nucleic Acids Res., 18(13):4009 (1990)), benomyl resistance (Orbach, M.J., et al., Mol. Cell. Biol., 6(7):2452-61 (1986)). Other genes that are useful as selection markers are those that encode factors for auxotrophic complementation, such as, for example, PyrG (Cantoral, J.M., et al., Nucleic Acids Res., 16(16):8177 (1988)), ArgB (Upshall, A., et al., Mol. Gen. Genet., 204(2):349-54 (1986)), niaD (Johnstone, I.L., et al., Gene, 90(2):181-92 (1990)), and TrpC (Penalva, M.A., Nucleic Acids Res., 15(4):1874 (1987)). In *Penicillium* species, nucleic acid constructs containing the ampicillin resistance gene are less useful when beta-lactam production is the reaction of interest because the gene encodes beta-lactamase, which, if expressed, will degrade the beta-lactam. Instead, a marker such as phleomycin resistance gene (phIR) can be used in *Penicillium* species when beta-lactam production is the reaction of interest.

Nucleic acid constructs of the present invention may also contain a targeting signal or secretory signal sequence to direct expression of the acyltransferase polypeptide to a desired location within the host cell or into the fermentation media. The targeting signal and/or secretory signal sequence is joined to the acyltransferase polynucleotide so that both are in the same reading frame. These signal sequences may be native to the host cell, or they may be of synthetic or foreign origin. Where the host organism is a filamentous fungus, the signal peptide may be derived from a fungal enzyme, for example, *Aspergillus spp.* amylase. The procedures used to create a construct having the desired properties, including manipulation of the various nucleotide fragments and alignment of reading frames, etc., are well known to persons skilled in the art. See, for example, Sambrook, et al. (1989), "Molecular Cloning: A Laboratory Manual," 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

The host cell into which the construct is introduced may be any cell having the desired properties. For example, it may be a cell that can produce large amounts of the acyltransferase polypeptide of the present invention. It may also be a host cell that is capable of producing a beta-lactam compound, i.e. a host cell that contains the machinery to produce beta-lactam compounds. In some embodiments the host cell is a bacterium, a yeast or a filamentous fungus. A filamentous fungus may be, for example, *Aspergillus spp.* or *Penicillium chrysogenum*. The use of *Aspergillus spp.* for the expression of proteins is described in, e.g., EP 272 277, EP 238 023 and EP 184 438.

The use of a genetically modified *P. chrysogenum* strain for the production of adipyl-7-ADCA is described in, e.g., EP 532 341.

The transformed host cell containing the construct is cultured in a suitable nutrient medium under conditions permitting the expression of the polypeptide, after
5 which the polypeptide may be recovered. Recovery may be accomplished, for example, by separation of the host cells from the medium by centrifugation or filtration. Where the polypeptide is produced intracellular, the cells are recovered from the fermentation media by centrifugation or filtration and the cells are homogenized to release the polypeptide. Proteinaceous components may then be precipitated from the media or
10 supernatant by means of a salt (e.g., ammonium sulfate) and purified by a variety of chromatographic and/or electrophoretic procedures (e.g., ion exchange chromatography, gel filtration chromatography, isoelectric focusing, affinity chromatography, and the like). Recovery techniques are often dependent on the characteristics of the particular polypeptide. The polypeptide may then be further used.

15 Alternatively or additionally, the transformed host cell containing the construct according to the invention is cultured in a suitable nutrient medium under conditions permitting the expression of a beta-lactam compound of interest.

In a fourth aspect, the present invention provides a process for the production of a beta-lactam compound comprising culturing the transformed host cell containing the
20 construct according to the invention under conditions conducive to the production of an N-acylated beta-lactam compound; optionally deacylating the produced N-acylated beta-lactam compound to produce the corresponding N-deacylated compound; and recovering the N-acylated or N-deacylated beta-lactam compound. The skilled person will immediately recognize that the nature of the beta-lactam compound that is produced
25 depends on the transformed host that is used. If the transformed host containing the construct according to the invention is a genetically modified *P. chrysogenum* strain that is modified to express expandase, the N-deacylated beta-lactam compound is 7-ADCA. Alternatively, if the transformed host cell containing the construct according to the invention is an *Acremonium chrysogenum* strain naturally producing expandase, the N-
30 deacylated beta-lactam compound is 7-ACA.

The N-acyl side chain precursor to be used in the process performed under conditions conducive to the production of a N-acylated beta-lactam compound preferably is adipic acid or a salt thereof. Alternatively, other side chain precursors may

be used, for instance those mentioned in international applications WO95/04148, WO95/04149, WO98/48034 or WO 98/48035.

The techniques required generating the array of vectors and transformed host cells discussed above are familiar to one skilled in the art, and all such embodiments are within the scope of the present invention. In preparing the expression cassette, the various polynucleotide elements may be manipulated such that they are in the proper orientation and in the proper reading frame. Toward this end, adapters or linkers may be employed to join the polynucleotide elements. Likewise, various known procedures may be employed to introduce convenient restriction sites, remove superfluous nucleotides, remove restriction sites, and the like.

Polynucleotide sequences of the present invention can be used to identify and/or isolate corresponding sequences from other organisms, such as other fungi. Methods known in the art, such as PCR, hybridization, and the like, can be used to identify sequences based on their degree of sequence identity to the sequences of the present invention, and sequences isolated based on their sequence identity to sequences set forth herein are encompassed by the present invention. Appropriate methods for such identification are known in the art and described in, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). For example, fragments of the nucleotide sequences disclosed herein may be used as probes. Such probes would be capable of specifically hybridizing to corresponding nucleotide sequences as well as messenger RNAs and could be used, for example, to distinguish single base polymorphisms in different candidate sequences in an SSCP assay. To achieve specific hybridization under a variety of conditions, such probes include sequences that differ between the candidate sequences. Such probes may be used to amplify corresponding acyltransferase sequences from a chosen organism by PCR; this technique may be used to isolate further sequences from an organism or as a diagnostic assay to determine the presence or expression level of coding sequences in an organism.

The following examples are offered not by way of limitation but rather by way of illustration.

EXAMPLE 1

CLONING AND EXPRESSION OF ACYLTRANSFERASE GENES

Acyl-coenzyme A:isopenicillin N acyltransferases (acyltransferases) have been identified in several penicillin-producing organisms: *Penicillium chrysogenum* ATCC9480 (Tobin et al. 1990, J.Bacteriol. 172(10): 5908), *Aspergillus nidulans* ATCC38163 (Tobin et al. 1990, J.Bacteriol. 172(10): 5908), *Penicillium nalgiovense* ATCC10472 (Laich et al. 1999, Appl. And Env. Micorbiol. 65(3): 1236) and *Penicillium griseofulvum* ATCC10120 (Laich et al. 2002, Appl. And Env. Micorbiol. 68(3): 1211). Such genes may be cloned by culturing of the organisms, extracting the total RNA, purifying the mRNA and constructing a cDNA library using reverse transcriptase. Standard procedures for DNA techniques are described in, for example, Sambrook et al. (Sambrook, J., Fritsch, E. F. and Maniatis, T.: Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989). Standard conditions for growth and manipulation of *E. coli* strains are described by Miller (Miller, J.: Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1972).

The genes can be isolated by designing and synthesizing PCR primers that anneal to the 5' and 3' termini of the acyltransferase genes, amplifying the specific gene from the cDNA library and cloning in an expression vector. Alternatively the present example describes a method by which acyltransferase genes of known sequence may be synthesized starting from short oligonucleotides. The assembly of such synthetic genes has been widely described (Stemmer WP et al. 1995, Gene 164: 49; Jayaraman K et al. 1989, Nucleic Acids Res. 17(11): 4403; Holowachuk EW et al. 1995, PCR Methods Appl. 4(5): 299; Prodromou C et al. 1992, Protein Eng. 5(8): 827).

For example, to synthesize the acyltransferase (*penDE*) from *Penicillium chrysogenum* ATCC 9480, the following overlapping oligonucleotides, which collectively encode both strands of the *penDE* gene, as well as 5' and 3' termini sequences encoding recognition sites for commercially available restriction endonucleases, are synthesized (USA-QIAGEN Inc., Valencia, CA, USA):

oligo:1	tctagacatatgcttcacatcctctgtcaaggcactccc	(SEQ ID NO: 57)
oligo:2	tttgaaatcggtacgaacatggctctgctgccaagcc	(SEQ ID NO: 58)
oligo:3	gtgatagccagaagcattgacttcgccgtgatctcatccga	(SEQ ID NO: 59)
oligo:4	gggaaaacgaagaagacggacgaagagcttaaacaggta	(SEQ ID NO: 60)
oligo:5	ctctcgcaactggggcgctgatcgaggaaagatggccc	(SEQ ID NO: 61)
oligo:6	aaatactacgaggagattcgcggtattgcaaagggcgctgaa	(SEQ ID NO: 62)

	oligo:7	cgcgatgtctccgagattgtcatgcttaatacccgcacg	(SEQ ID NO: 63)
	oligo:8	gaattgcatacgggctcaaggcagcccgtgatggctgc	(SEQ ID NO: 64)
	oligo:9	accactgcctattgtcaacttccaaatggagccctccagggc	(SEQ ID NO: 65)
	oligo:10	caaaactgggatttctttctgccaccaagagaacctg	(SEQ ID NO: 66)
5	oligo:11	atccggtaacgatccgtcaggccggacttcccaccatc	(SEQ ID NO: 67)
	oligo:12	aaattcataaccgaggctggaatcatcggaaggttgattt	(SEQ ID NO: 68)
	oligo:13	aacagtgcgggctgcgcgtcaattacaacgcccttcac	(SEQ ID NO: 69)
	oligo:14	cttcagggtcttcgaccaccggagttccttcgcatatt	(SEQ ID NO: 70)
	oligo:15	gccctccgcatagcgctcgaagcacttctcctcccaggcc	(SEQ ID NO: 71)
10	oligo:16	tatgaccggatcgtggagcaaggcgaatggccgccagc	(SEQ ID NO: 72)
	oligo:17	gctttatcatgtgggcaatgggcacgaggcatttgg	(SEQ ID NO: 73)
	oligo:18	ttggaatttccccaccagcatccgaaagcaggtgctcgac	(SEQ ID NO: 74)
	oligo:19	gcgaatggtaggatggtgcacaccaaccactgcttgctt	(SEQ ID NO: 75)
	oligo:20	cagcacggcaaaaatgagaaagagctcgatcccttaccg	(SEQ ID NO: 76)
15	oligo:21	gactcatggaatcgccaccagcgtatggagtctcctcgac	(SEQ ID NO: 77)
	oligo:22	gggttcgacggcaccaaacaggcatttggccagctctgg	(SEQ ID NO: 78)
	oligo:23	gccgacgaagacaattatccctttagcatctgccgcgt	(SEQ ID NO: 79)
	oligo:24	tacgaggagggaagagcagaggcgactctgttcaatatc	(SEQ ID NO: 80)
	oligo:25	atctacgaccatgcccgtagagaggcaacggtgcggctt	(SEQ ID NO: 81)
20	oligo:26	ggccggccgaccaaccctgatgagatgttgcacgcgg	(SEQ ID NO: 82)
	oligo:27	tttgacgaggaggacgagaggctcgcgtcaacgccaggctt	(SEQ ID NO: 83)
	oligo:28	atgttcgtagccgatttcaaaggagtgcttgacagaggat	(SEQ ID NO: 84)
	oligo:29	gtcaatgcttctggctatcacggctttggcagcagagcc	(SEQ ID NO: 85)
	oligo:30	cgtcttctcgttttccctcgatgagatcgacggcgaa	(SEQ ID NO: 86)
25	oligo:31	cacgcgccccagttgcgagagtacctgttaagctcttcgtc	(SEQ ID NO: 87)
	oligo:32	gcgaatctcctcgtagtatttgggccatcttctcgtat	(SEQ ID NO: 88)
	oligo:33	aatctcggagacatcgcggtcagcgcccttgcaatacc	(SEQ ID NO: 89)
	oligo:34	cttgagcccgtatgcaaattccgtgcgggtattaagcatgac	(SEQ ID NO: 90)
	oligo:35	aagttgacaataggcagtggtgcagccatcacgggctgc	(SEQ ID NO: 91)
30	oligo:36	aaagaaatcccagtttggccctggagggtccatttgg	(SEQ ID NO: 92)
	oligo:37	ctgacggatcgttaaccggatcaggttctcttgggtggcaga	(SEQ ID NO: 93)
	oligo:38	tccagcctcggttatgaattgatggtgggaagtcggc	(SEQ ID NO: 94)
	oligo:39	ggcgacgcccgcactgttaaatccaaccttccgatgat	(SEQ ID NO: 95)
	oligo:40	ggtgggtcgaagaccctgaagggtgaaggcggtgaattgac	(SEQ ID NO: 96)

	oligo:41	ttcgagcgctatgcggagggcaatatgcgaaggaactcc	(SEQ ID NO: 97)
	oligo:42	ctccacgatccggtcataggcctgggaaggagaagtgt	(SEQ ID NO: 98)
	oligo:43	attgcccaccatgataaaagcgctggcgccattccgccttg	(SEQ ID NO: 99)
	oligo:44	gctggtgggggagaattccaaacaaatgcctcgtgccc	(SEQ ID NO: 100)
5	oligo:45	caccatcctaccattcgctcgagcacctgctttcggat	(SEQ ID NO: 101)
	oligo:46	tttctcatTTTTGCCGTGCTGAAGCAAGCAGTGTTGGTGTG	(SEQ ID NO: 102)
	oligo:47	ctggtggcgattccatgagtcgggaaggatcgagctc	(SEQ ID NO: 103)
	oligo:48	tttggtgccgtcgaacccgtcgaggaggaactccatacg	(SEQ ID NO: 104)
	oligo:49	gggataattgtcttcgtcgcccgagctgggcaaatagcctg	(SEQ ID NO: 105)
10	oligo:50	tctgctcttGCCCTCCTCGTAAGCGCGGCAGATGCTAAA	(SEQ ID NO: 106)
	oligo:51	acgggcatggtcgtagatgatattgaacagagtcgcgcc	(SEQ ID NO: 107)
	oligo:52	atcaggggtggtcggccggccaagccgcaccgttgctctct	(SEQ ID NO: 108)
	oligo:53	cctctcgtcctcctcgtcaaaccgcatgacaaacatctc	(SEQ ID NO: 109)
	oligo:54	cgtcatgaagagccttcaaagcctggcgttgagcgcaga	(SEQ ID NO: 110)

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The oligonucleotides are mixed together at a uniform concentration of 250 μ M. The mixture is diluted 100-fold in 20 μ l PCR mix containing 10mM Tris-HCl pH 9.0/2.2 mM $MgCl_2$ /50 mM KCl/ 0.1% Triton X-100 [1x Cloned *Pfu* buffer (Stratagene, La Jolla, CA, USA)]/0.2 mM each deoxynucleotide (Stratagene, La Jolla, CA, USA)/1 u of Taq DNA polymerase (Promega, Madison, WI, USA)/0.02 u of Pfu DNA polymerase (Stratagene, La Jolla, CA, USA). The gene is assembled in a thermocycler [MJ Research PTC-150 minicycler (MJ Research, Inc., Reno, NV, USA)] set to the following program of 55 cycles at 94°C for 30 seconds, 52°C for 30 seconds and 72°C for 30 seconds. The resulting gene assembly mixture is diluted 40-fold in 100 μ l PCR mix containing 10mM Tris-HCl pH 9.0/2.2 mM $MgCl_2$ /50 mM KCl/ 0.1% Triton X-100 [1x Cloned *Pfu* buffer (Stratagene, La Jolla, CA, USA)]/0.2 mM each deoxynucleotide (Stratagene, La Jolla, CA, USA)/5 u of Taq DNA polymerase (Promega, Madison, WI, USA)/0.1 u of Pfu DNA polymerase (Stratagene, La Jolla, CA, USA)/2 outside primers at a concentration of 1 μ M. The 2 outside primers can be the same as the two oligonucleotides representing the 5' ends of the plus and minus strand, in this example the outside primer that anneals to the 5'-end of the plus strand adds an *NdeI* recognition site (the ATG of the second half of the recognition sequence overlaps the initiation codon of the acyltransferase gene) and the outside primer that anneals to the 5'-end of the minus strand adds an *NotI* recognition site. The gene is amplified in a thermocycler

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[MJ Research PTC-150 minicycler (MJ Research, Inc., Reno, NV, USA)] set to the following program of 23 cycles at 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 60 seconds. The final gene is purified using the QIAquick PCR Purification Kit (USA-QIAGEN Inc., Valencia, CA, USA), digested with *NdeI* and *NotI* (New England Biolabs, Beverly, MA, USA) and the resulting 1.0-kb fragment is purified using the QIAquick Gel Extraction kit (USA-QIAGEN Inc., Valencia, CA, USA). The synthesized *penDE* gene from *Penicillium chrysogenum* ATCC9480 can be cloned into an appropriate expression vector. All other acyltransferase genes described herein may be constructed using a similar procedure by introduction of the relevant changes to the oligonucleotide sequences (reflecting the changes in sequence compared to the *penDE* gene from *Penicillium chrysogenum* ATCC9480).

Plasmid pET24B is one of the pET series of vectors created by Studier (Moffatt, B.A. et al. 1986, J.Mol.Biol. 189:113) and further developed by Novagen (Novagen, Inc., Madison, WI, USA) to facilitate cloning, detection, and purification of recombinant proteins in *E.coli*. These vectors typically carry the colicin E1 replicon of pBR322 and confer resistance to ampicillin or kanamycin. There are two general categories of vectors in the pET series, transcription and translation. The translation vectors, which contain a highly efficient ribosomal binding site from phage T7, are designed to express a gene without its own ribosomal binding site. Alternatively, transcription vectors are designed for expressing target gene with its own prokaryote's ribosomal binding site. The utilization of plasmid pET24B (a translation vector) for cloning of the acyltransferase gene from *Penicillium chrysogenum* ATCC9480 illustrates how expression of an active acyltransferase enzyme may be performed. The Multiple Cloning Site (MCS) of pET24B comprises both an *NdeI* and *NotI* recognition sequences, allowing the directional cloning of the synthesized acyltransferase gene. pET24B is digested with *NdeI* and *NotI* (New England Biolabs, Beverly, MA, USA) and purified using the QIAquick Gel Extraction kit (USA-QIAGEN Inc., Valencia, CA, USA) generating an appropriate expression vector. The complementary cohesive termini of the two fragments (pET24B vector and acyltransferase insert) are ligated using a T4 DNA ligase kit (New England Biolabs, Beverly, MA, USA). Once the acyltransferase fragment is ligated into the pET24B plasmid, generating a new plasmid named pET-penDE Pc (Figure 1), replication and expression of the gene can be confirmed by transforming the recombinant plasmid into *E.coli* BL21 cells (Novagen, Inc., Madison, WI, USA) and then selecting cells based upon the expression of antibiotic resistant gene carried on the plasmid. For pET24B,

kanamycin is used as a selection marker for cells, containing an intact and functional plasmid, which may or may not carry the acyltransferase gene. A small number of clones are picked, grown in liquid cultures and DNA is prepared using a QIAprep Spin Miniprep Kit (USA-QIAGEN Inc., Valencia, CA, USA). The DNA is sequenced on an ABI 3700 DNA Analyzer (Applied Biosystems, Inc, Foster City, CA, USA) using pET sequencing primers (Novagen, Inc., Madison, WI, USA) and one clone with the correct acyltransferase sequence is selected. Characterization of acyltransferase activity is described in Example 2.

EXAMPLE 2

EXPRESSION OF ACYLTRANSFERASE

Plasmid pET-penDE Pc is transformed into chemically competent E.coli BL21(DE3) cells (Novagen, Inc., Madison, WI, USA) and plated on LB media agar (EM Science, Gibbstown, NJ, USA) containing 1% glucose (Sigma, St. Louis, MO, USA) and chloramphenicol (30ug/mL) (Sigma, St. Louis, MO, USA). Individual colonies formed on the plate are picked and used to inoculate 2xYT media (EM Science, Gibbstown, NJ, USA) containing 0.5% glucose and chloramphenicol. The inclusion of glucose in the media is important to reduce premature gene expression, which may be detrimental to cell growth. The culture is grown overnight in a shaking incubator at 37°C. A small aliquot of the resultant culture diluted into fresh LB media containing 0.5% glucose and chloramphenicol to a cell density of approximately 0.05 A₆₀₀, and the culture is grown to early log phase (an approximate cell density of at least 0.5 A₆₀₀). Expression of the acyltransferase gene is induced by the addition of 0.2mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma, St. Louis, MO, USA), and the culture is further incubated for 16h, shaking at 28°C. Cells are harvested by centrifugation at 4000 rpm at 4°C and washed at least three times with 5mM morpholine (Sigma, St. Louis, MO, USA) buffer pH 7.5 to remove any undesirable salt residue. The washed cells are resuspended in 100uL lysis buffer (2mg/mL polymixin B sulfate (PMBS) (Amersham Biosciences, Newark, NJ, USA), 10U/μl lysozyme (Epicentre, Madison, WI, USA.), 1U/μl RNase (Epicentre, Madison, WI, USA.), and 10U/μl DNase (Epicentre, Madison, WI, USA.)) and shaken at room temperature.

EXAMPLE 3

CHARACTERIZATION OF ACYLTRANSFERASE ACTIVITY

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This assay is used to evaluate acyltransferase specific activity that converts 6-APA and Ad-CoA substrates into Ad-6-APA product. Separate stock solutions of 1.5mM 6-APA, 50mM DTT, and 0.6mM Ad-CoA are prepared freshly by dissolving each in 5mM morpholine buffer, pH 7.5. 6-APA and DTT are available from Sigma (St. Louis, MO, USA). Adipoyl-Coenzyme A is obtained as follows: Adipoyl chloride (0.14 ml; 6.95 mmol) was added to a well-stirred and ice-water cooled suspension of Co-enzyme A sodium salt (400 mg; 0.48 mmol) in acetone (60 ml) + water (0.6ml) + 0.2M KHCO₃ (to raise the pH) under an atmosphere of nitrogen. The pH of the reaction contents was brought to ~7.7 and stirred further for about 60 min. Thereafter acetone was removed partially under reduced pressure, the product dissolved in cold water and this was freeze-dried. Finally the product was subjected to ultrafiltration. The yield was 0.9 g. Assay 70%. The water used was treated with a stream of nitrogen gas to avoid oxidation of co-enzyme A.

8mL of 1.5mM 6-APA, 4mL of 0.6 mM Ad-CoA, and 4mL of 50mM DTT are combined to create a master solution of 0.75mM 6-APA, 0.15mM Ad-CoA, 12.5mM DTT, 5mM morpholine buffer, pH7.5. After lysing cells from Example 2 for 30 minutes at room temperature, 300µL of 5mM morpholine buffer pH 7.5 is added to the solution. To start the acyltransferase reaction, 155µL of 5mM morpholine buffer is transferred into a tube or a well of the 96-well plate. 30µL of the fresh master solution (0.75mM 6-APA, 0.15mM Ad-CoA, 12.5mM DTT, 5mM morpholine buffer, pH7.5) and 20µL of the 1:4 diluted acyltransferase enzymes from the whole lysate are added. The enzyme and master solution are gently mixed by pipetting 2 to 3 times. The reaction is allowed to proceed for 3 minutes at room temperature. 60µL of the reaction mix is transferred to a fresh microtiter plate or container, which contains 60µL of ice cold methanol, and mixed well to stop the reaction. Optimally, the sample is filtered, for example, through a Whatman custom filter plate (1 micron pore size) (Whatman Inc., Clifton, NJ, USA) and the filtrate is collected in a Nunc V-bottom polystyrene microtiter plate (Nunc, Rochester, NY, USA). The plate is sealed with regular aluminum foil.

Ad-6-APA product can be detected using mass spectrometry. Electrospray ionization mass spectrometry (ESI/MS) analyses may be performed on a Quattro Ultima triple-quadrupole mass spectrometer (Micromass, Manchester, UK) with an electrospray ion source. Typically, samples are dissolved in methanol/water (1:1) as described in example 2. Adipoyl-6-APA will yield intense deprotonated molecules $[M-H]^-$ in the negative-ion mode. The capillary voltage is 3.0kV, source temperature is 120°C, and desolvation temperature is 250°C. Adipoyl-6-APA is monitored by mass transition 343.0 to 265.0, under cone energy of 15V, collision energy of 15eV. Ad-6-APA can be quantified by comparing the area of the sample peak to a standard curve. The standard curve is computed by injecting ad-6-APA samples of known concentration and measuring the peak area.

For flow injection (FIA) analyses, an 1100 series (Agilent, San Jose, CA) LC system equipped with a HTP pal (CTC analytics, Zwingen, Switzerland) autosampler is used to deliver the mobile phase, at a flow rate of 0.5mL/min. Samples of 5 μ L are injected. The mobile phase is isocratic elution at 70% methanol and 30% water.

Acyltransferase polypeptides corresponding in sequence to SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, and 52 all exhibited acyltransferase activity (corresponding to amount of Ad-6-APA product generated) greater than that of wild type acyltransferase from *Penicillium chrysogenum* (SEQ ID NO: 54) in this assay.

Results are provided in Table I:

Table I. Acyltransferase Activity Relative to *P. chrysogenum* AT (SEQ ID NO: 54)

AT Polypeptide	Activity In Fold Improvement Relative to <i>P. chrysogenum</i> AT
H01 (SEQ ID NO: 2)	*
H02 (SEQ ID NO: 4)	**
H03 (SEQ ID NO: 6)	**
H04 (SEQ ID NO: 8)	**
H05 (SEQ ID NO: 10)	*
H06 (SEQ DI NO: 12)	*
H07 (SEQ ID NO: 14)	**
H25 (SEQ ID NO: 18)	**
H26 (SEQ ID NO: 20)	**

	H27 (SEQ ID NO: 22)	**
	H28 (SEQ ID NO: 24)	**
	H29 (SEQ ID NO: 26)	**
	H30 (SEQ ID NO: 28)	**
5	H31 (SEQ ID NO: 30)	**
	H32 (SEQ ID NO: 32)	**
	H34 (SEQ ID NO: 36)	**
	H35 (SEQ ID NO: 38)	***
	H36 (SEQ ID NO: 40)	**
10	H37 (SEQ ID NO: 42)	**
	H38 (SEQ ID NO: 44)	***
	H40 (SEQ ID NO: 46)	**
	H41 (SEQ ID NO: 48)	***
	H42 (SEQ ID NO: 50)	***
15	H43 (SEQ ID NO: 52)	***

Key: * 1 < fold improvement < 2
 ** 2 ≤ fold improvement < 10
 *** 10 ≤ fold improvement ≤ 30

20 All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. Subheadings in the specification
 25 document are included solely for ease of review of the document and are not intended to be a limitation on the contents of the document in any way.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended
 30 claims.

CLAIMS

1. An isolated polypeptide having acyltransferase activity selected from the group consisting of:
 - 5 (a) a polypeptide having an amino acid sequence that is at least 96% identical to SEQ ID NO: 38;
 - (b) a polypeptide having an amino acid sequence that is at least 95% identical to SEQ ID NO: 42;
 - (c) a polypeptide having an amino acid sequence that is at least 95% identical to SEQ ID NO: 52;
 - 10 (d) a polypeptide having an amino acid sequence that is at least 96% identical to SEQ ID NO: 54 and having at least one residue selected from the group consisting of Arg at position 97, Val at position 221, Pro at position 251, Glu at position 254, Ile at position 259, Gly at position 277, Glu at position 291, and Gly at position 299; and
 - 15 (e) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions over substantially the entire length of a nucleotide sequence that encodes an amino acid sequence selected from the group consisting of SEQ ID NO: 38 and SEQ ID NO: 42 and SEQ ID NO: 52.
 - 20
2. The isolated polypeptide of claim 1, wherein the isolated polypeptide has an amino acid sequence that is at least 96% identical to SEQ ID NO: 38.
- 25 3. The isolated polypeptide of claim 1, wherein the isolated polypeptide has an amino acid sequence that is at least 95% identical to SEQ ID NO: 42.
4. The isolated polypeptide of claim 1, wherein the isolated polypeptide has an amino acid sequence that is at least 95% identical to SEQ ID NO: 52.
- 30 5. The isolated polypeptide of claim 1, wherein the isolated polypeptide has an amino acid sequence that is at least 96% identical to SEQ ID NO: 54 and has at least one residue selected from the group consisting of Arg at position 97, Val at

position 221, Pro at position 251, Glu at position 254, Ile at position 259, Gly at position 277, Glu at position 291, and Gly at position 299.

- 5 6. The isolated polypeptide of claim 5, wherein the amino acid sequence comprises Arg at position 97.
7. The isolated polypeptide of claim 5, wherein the amino acid sequence comprises Val at position 221.
- 10 8. The isolated polypeptide of claim 5, wherein the amino acid sequence comprises at least two residues selected from the group consisting of Arg at position 97, Val at position 221, Pro at position 251, Glu at position 254, Ile at position 259, Gly at position 277, Glu at position 291, and Gly at position 299.
- 15 9. The isolated polypeptide of claim 8, wherein the amino acid sequence comprises at least four residues selected from the group consisting of Arg at position 97, Val at position 221, Pro at position 251, Glu at position 254, Ile at position 259, Gly at position 277, Glu at position 291, and Gly at position 299.
- 20 10. The isolated polypeptide of claim 5, wherein the amino acid sequence comprises at least one residue selected from the group consisting of Pro at position 251, Glu at position 254, Ile at position 259, Gly at position 277, Glu at position 291, and Gly at position 299.
- 25 11. The isolated polypeptide of claim 1, wherein the isolated polypeptide has at least 1.5 times the acyltransferase activity of wild type *P. chrysogenum* acyltransferase corresponding to SEQ ID NO: 54.
- 30 12. The isolated polypeptide of claim 8, wherein the isolated polypeptide has at least 2 times the acyltransferase activity of wild type *P. chrysogenum* acyltransferase corresponding to SEQ ID NO: 54.

13. The isolated polypeptide of claim 8, wherein the isolated polypeptide has at least 5 times the acyltransferase activity of wild type *P. chrysogenum* acyltransferase corresponding to SEQ ID NO: 54.
- 5 14. An isolate polypeptide comprising a fragment of an acyltransferase polypeptide, wherein said acyltransferase polypeptide is selected from the group consisting of:
- (a) a polypeptide having an amino acid sequence that is at least 96% identical to SEQ ID NO: 38;
 - 10 (b) a polypeptide having an amino acid sequence that is at least 95% identical to SEQ ID NO: 42;
 - (c) a polypeptide having an amino acid sequence that is at least 95% identical to SEQ ID NO: 52;
 - 15 (d) a polypeptide having an amino acid sequence that is at least 96% identical to SEQ ID NO: 54 and having at least one residue selected from the group consisting of Arg at position 97, Val at position 221, Pro at position 251, Glu at position 254, Ile at position 259, Gly at position 277, Glu at position 291, and Gly at position 299; and
 - 20 (e) a polypeptide encoded by a polynucleotide that hybridizes under stringent conditions over substantially the entire length of a nucleotide sequence that encodes an amino acid sequence selected from the group consisting of SEQ ID NO: 38 and SEQ ID NO: 42 and SEQ ID NO: 52.
- 25 15. A polynucleotide encoding the polypeptide of claim 1.
16. An expression cassette comprising the polynucleotide of claim 15 operatively linked to a promoter.
- 30 17. A host cell transformed to express the polynucleotide of claim 15.
18. A method of making an acyltransferase polypeptide of claim 1, comprising cultivating a host cell comprising a nucleic acid construct comprising a nucleic acid sequence encoding the polypeptide under conditions conducive to production of the polypeptide; and, optionally, recovering the polypeptide.

19. A method of producing a beta-lactam compound of interest, comprising cultivating a host cell capable of producing a beta-lactam compound comprising a nucleic acid construct comprising a nucleic acid sequence encoding the acyltransferase polypeptide of claim 1 under conditions conducive to production of the beta-lactam compound; and, recovering the beta-lactam compound.
- 21 The method of claim 20, further comprising N-deacylating the beta-lactam compound and recovering the N-deacylated beta-lactam compound.
- 22 The method of claim 20, wherein the beta-lactam compound of interest is adipoyl-7-ADCA.

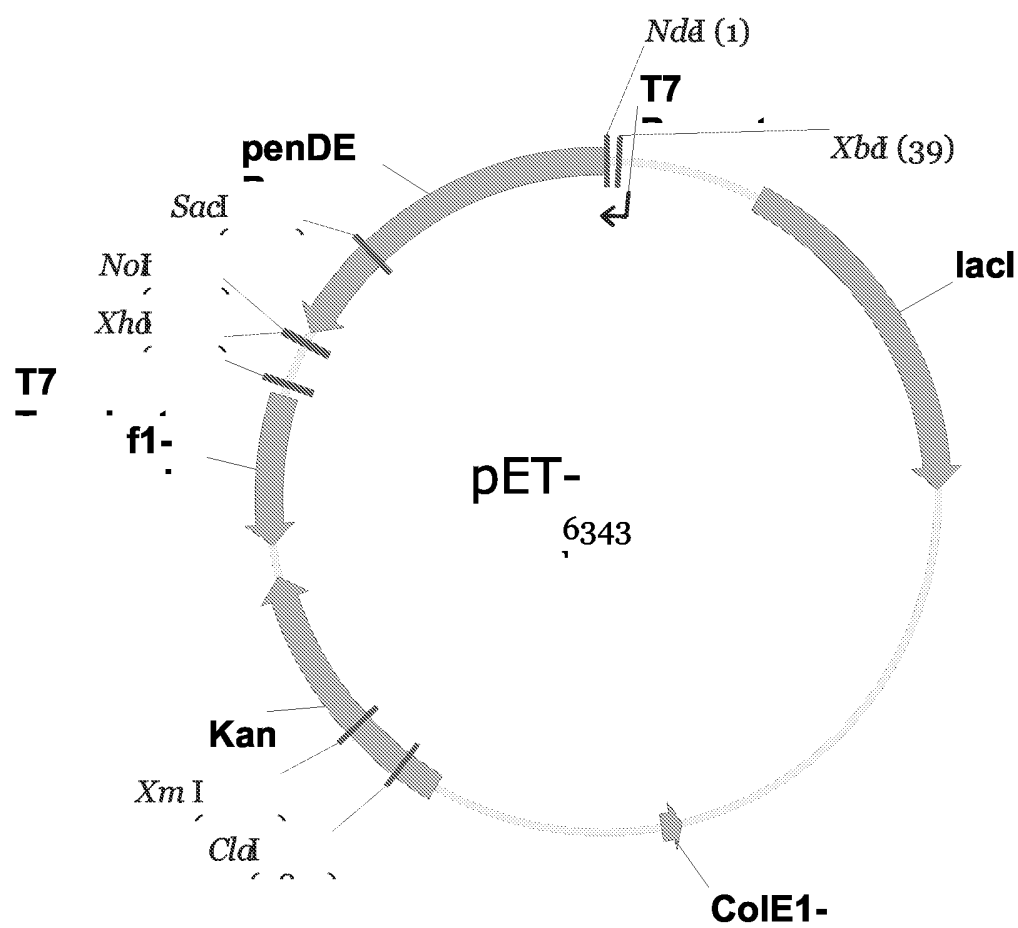


Fig. 1

acid comparisons of modified acyltransferases, compared to *Penicillium chrysogenum*, *Penicillium* *var. roqueforti* and *Aspergillus nidulans* acyltransferase enzymes.

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aa no:1
| NO:54) penDE_Pc   (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO:56) penDE_An   (1) MLHVTCTGTFSEIGYHHGSAAKGEIAKAIDFATGLIHGKTKKTQAELEQLLRELEQVMKQRPWPRYYEEICGIAGAE
| NO: 2) Hybrid     (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO: 4)           H02 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO: 6)           H03 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO: 8)           H04 (1) MLHILCQGTFPEIGYQHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO:10)          H05 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO:12)          H06 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO:14)          H07 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO:16)          H14 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO:18)          H25 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO:20)          H26 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO:22)          H27 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO:24)          H28 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO:26)          H29 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO:28)          H30 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO:34)          H33 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO:36)          H34 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO:38)          H35 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO:40)          H36 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO:42)          H37 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO:44)          H38 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEICGIAGAE
| NO:46)          H40 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO:48)          H41 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEIRGIAGAE
| NO:50)          H42 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEICGIAGAE
| NO:52)          H43 (1) MLHILCQGTFPEIGYEHGSAAKAVIARSIDFAVDLIRGKTKKTDEELKQVLSQLGRVIEERWPKYYEEICGIAGAE

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Fig. 2A

81

160

[illegible]

Fig. 2B

[illegible]

Fig. 2C

241

320

[illegible]

Fig. 2D

321 358

(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNARL-
(1) HVGRKATVRLGRPNNPDETFVMTFSNLDTKSAIQANI-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNARL-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNARL-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNARL-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNAKL-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNARL-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNARL-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNARL-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNARL-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNARL-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNAKL-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNARL-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNARL-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNARL-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNAKL-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNAKL-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNARL-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNAKL-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNARL-
(1) HARREATVRLGRPTNPDETFVMRFDEEDERSALNARL-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNAKL-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNARL-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNAKL-
(1) HARREATVRLGRPTNPDEMFMRFDEEDERSALNARL-

Fig. 2E