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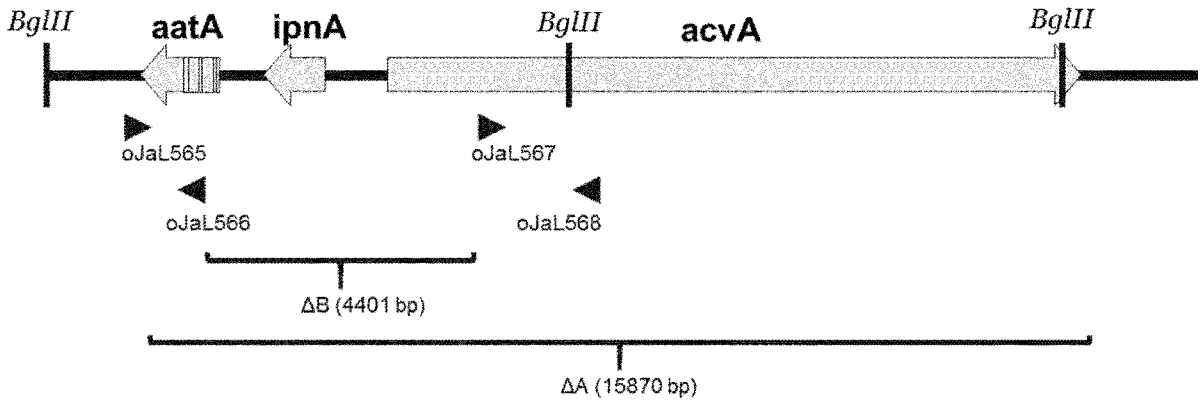
(54) Title: IMPROVED FILAMENTOUS FUNGAL HOST CELL

Figure 1



(57) Abstract: The present invention relates to penicillin-inactivated filamentous fungal cells producing a polypeptide of interest and methods of producing a polypeptide of interest in said cells as well as methods of producing said cells.

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Improved Filamentous Fungal Host Cell

Reference to sequence listing

This application contains a Sequence Listing in computer readable form. The computer readable form is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to genetically modified improved filamentous fungal cells and to methods for producing such cells as well as methods of producing polypeptides of interest therein.

BACKGROUND OF THE INVENTION

Filamentous fungal host cells are widely employed in the industrial manufacture of polypeptides of interest, such as, enzymes. The host cells are constantly modified to improve an array of characteristics, including, product yield as well as overall production economy.

SUMMARY OF THE INVENTION

The present invention is directed to genetically modified improved filamentous fungal host cells in which one or more gene involved in synthesis of penicillin G has been inactivated. Inactivation of the one or more gene involved in synthesis of penicillin G may be done by any suitable gene inactivation method known in the art. An example of a convenient way to inactivate one or more gene involved in synthesis of penicillin G is based on the techniques of gene replacement or gene interruption.

The inactivation of one or more gene involved in synthesis of penicillin G in a filamentous fungal host cell has several potential benefits, such as, improved product yield of a polypeptide of interest, improved carbon-utilization, reduced fermentation broth viscosity, reduced allergenicity of the fermentation broth due to the absence of any trace amount of penicillin G etc.

Accordingly, in a first aspect, the invention relates to filamentous fungal host cells comprising a polynucleotide encoding a polypeptide of interest and comprising at least one inactivated gene which in its active form encodes a delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase having at least 80% amino acid sequence identity with SEQ ID NO:2, an isopenicillin-N synthase having at least 80% amino acid sequence identity with SEQ ID NO:4, and/or an isopenicillin N-acyltransferase having at least 80% amino acid sequence identity with SEQ ID NO:6, whereby the host cell is deficient in the production of penicillin.

In a second aspect, the invention relates to methods of producing a polypeptide of interest, said methods comprising the steps of:

a) cultivating a filamentous fungal host cell comprising a polynucleotide encoding a polypeptide of interest and comprising at least one inactivated gene which in its active

form encodes a delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase having at least 80% amino acid sequence identity with SEQ ID NO:2, an isopenicillin-N synthase having at least 80% amino acid sequence identity with SEQ ID NO:4, and/or an isopenicillin N-acyltransferase having at least 80% amino acid sequence identity with SEQ ID NO:6, whereby the host cell is deficient in the production of penicillin and, optionally,

b) recovering the polypeptide of interest.

In a final aspect, the invention relates to methods of producing an improved filamentous fungal host cell producing a polypeptide of interest, said method comprising the following steps in no particular order:

a) transforming a filamentous fungal host cell with a polynucleotide encoding the polypeptide of interest; and

b) inactivating at least one gene which in its active form encodes a delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase having at least 80% amino acid sequence identity with SEQ ID NO:2, an isopenicillin-N synthase having at least 80% amino acid sequence identity with SEQ ID NO:4, and/or an isopenicillin N-acyltransferase having at least 80% amino acid sequence identity with SEQ ID NO:6, whereby the host cell becomes deficient in the production of penicillin.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a map of the *Aspergillus oryzae* penicillin G gene cluster. The grey arrows indicate the three genes *aatA* (isopenicillin N-acyltransferase); *ipnA* (isopenicillin-N synthase) and *acvA* (delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase). The black arrowheads indicate primer locations. ΔA and ΔB indicate the deletions tested with indication of the size of the deletion. *Bgl*II indicates the location of restriction sites.

DEFINITIONS

cDNA: The term "cDNA" means a DNA molecule that can be prepared by reverse transcription from a mature, spliced, mRNA molecule obtained from a eukaryotic or prokaryotic cell. cDNA lacks intron sequences that may be present in the corresponding genomic DNA. The initial, primary RNA transcript is a precursor to mRNA that is processed through a series of steps, including splicing, before appearing as mature spliced mRNA.

Coding sequence: The term “coding sequence” means a polynucleotide, which directly specifies the amino acid sequence of a polypeptide. The boundaries of the coding sequence are generally determined by an open reading frame, which begins with a start codon such as ATG, GTG, or TTG and ends with a stop codon such as TAA, TAG, or TGA. The coding sequence may be a genomic DNA, cDNA, synthetic DNA, or a combination thereof.

Control sequences: The term “control sequences” means nucleic acid sequences necessary for expression of a polynucleotide encoding a mature polypeptide of the present

invention. Each control sequence may be native (*i.e.*, from the same gene) or foreign (*i.e.*, from a different gene) to the polynucleotide encoding the polypeptide or native or foreign to each other. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the polynucleotide encoding a polypeptide.

Expression: The term “expression” includes any step involved in the production of a polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

Expression vector: The term “expression vector” means a linear or circular DNA molecule that comprises a polynucleotide encoding a polypeptide and is operably linked to control sequences that provide for its expression.

Host cell: The term "host cell" means any cell type that is susceptible to transformation, transfection, transduction, or the like with a nucleic acid construct or expression vector comprising a polynucleotide of the present invention. The term “host cell” encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication.

Isolated: The term “isolated” means a substance in a form or environment that does not occur in nature. Non-limiting examples of isolated substances include (1) any non-naturally occurring substance, (2) any substance including, but not limited to, any enzyme, variant, nucleic acid, protein, peptide or cofactor, that is at least partially removed from one or more or all of the naturally occurring constituents with which it is associated in nature; (3) any substance modified by the hand of man relative to that substance found in nature; or (4) any substance modified by increasing the amount of the substance relative to other components with which it is naturally associated (*e.g.*, recombinant production in a host cell; multiple copies of a gene encoding the substance; and use of a stronger promoter than the promoter naturally associated with the gene encoding the substance).

Mature polypeptide: The term “mature polypeptide” means a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc. It is known in the art that a host cell may produce a mixture of two of more different mature polypeptides (*i.e.*, with a different C-terminal and/or N-terminal amino acid) expressed by the same polynucleotide. It is also known in the art that different host cells process polypeptides differently, and thus, one host cell expressing a polynucleotide may produce a different mature polypeptide (*e.g.*, having a different C-terminal and/or N-terminal amino acid) as compared to another host cell expressing the same polynucleotide.

Mature polypeptide coding sequence: The term “mature polypeptide coding sequence” means a polynucleotide that encodes a mature polypeptide

Nucleic acid construct: The term "nucleic acid construct" means a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or is modified to contain segments of nucleic acids in a manner that would not otherwise exist in nature or which is synthetic, which comprises one or more control sequences.

Operably linked: The term “operably linked” means a configuration in which a control sequence is placed at an appropriate position relative to the coding sequence of a polynucleotide such that the control sequence directs expression of the coding sequence.

Sequence identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter “sequence identity”. For purposes of the present invention, the sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled “longest identity” (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

(Identical Residues x 100)/(Length of Alignment – Total Number of Gaps in Alignment)

For purposes of the present invention, the sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *supra*), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled “longest identity” (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

(Identical Deoxyribonucleotides x 100)/(Length of Alignment – Total Number of Gaps in Alignment)

DETAILED DESCRIPTION OF THE INVENTION

Host Cells

The present invention relates to recombinant host cells comprising a polynucleotide of the present invention operably linked to one or more control sequences that direct the production and secretion of a heterologous polypeptide of interest.

A construct or vector comprising a polynucleotide is introduced into a host cell so that the construct or vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector as described earlier. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication. The choice of a host cell will to a large extent depend upon the gene encoding the polypeptide and its source.

The host cell may be a fungal cell. “Fungi” as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota as well as the Oomycota and all mitosporic fungi (as defined by Hawksworth *et al.*, *In*, *Ainsworth and Bisby's Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK).

The fungal host cell of the invention is a filamentous fungal cell. “Filamentous fungi” include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth *et al.*, 1995, *supra*). The filamentous fungi are generally characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic.

The filamentous fungal host cell may be an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes*, or *Trichoderma* cell.

For example, the filamentous fungal host cell may be an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Bjerkandera adusta*, *Ceriporiopsis aneirina*, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvescens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermispora*, *Chrysosporium inops*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium merdarium*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*, *Chrysosporium zonatum*, *Coprinus cinereus*, *Coriolus hirsutus*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Phlebia radiata*, *Pleurotus eryngii*, *Thielavia terrestris*, *Trametes villosa*, *Trametes versicolor*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*.

Suitable procedures for transformation of *Aspergillus* and *Trichoderma* host cells are described in EP 238023, Yelton *et al.*, 1984, *Proc. Natl. Acad. Sci. USA* 81: 1470-1474, and Christensen *et al.*, 1988, *Bio/Technology* 6: 1419-1422. Suitable methods for transforming *Fusarium* species are described by Malardier *et al.*, 1989, *Gene* 78: 147-156, and WO 96/00787.

In a first aspect, the invention relates to methods of producing an improved filamentous fungal host cell producing a polypeptide of interest, said method comprising the following steps in no particular order:

c) transforming a filamentous fungal host cell with a polynucleotide encoding the polypeptide of interest; and

d) inactivating at least one gene which in its active form encodes a delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase having at least 80% amino acid sequence identity with SEQ ID NO:2, an isopenicillin-N synthase having at least 80% amino acid sequence identity with SEQ ID NO:4, and/or an isopenicillin N-acyltransferase having at least 80% amino acid sequence identity with SEQ ID NO:6, whereby the host cell becomes deficient in the production of penicillin.

In another aspect, the invention relates to the resulting host cells; filamentous fungal host cells comprising a polynucleotide encoding a polypeptide of interest and comprising at least one inactivated gene which in its active form encodes a delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase having at least 80% amino acid sequence identity with SEQ ID NO:2, an isopenicillin-N synthase having at least 80% amino acid sequence identity with SEQ ID NO:4, and/or an isopenicillin N-acyltransferase having at least 80% amino acid sequence identity with SEQ ID NO:6, whereby the host cell is deficient in the production of penicillin.

Preferably, the least one gene encodes a delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase which has at least 85%, 90%, 95%, 97% or 98% amino acid sequence identity with SEQ ID NO:2, an isopenicillin-N synthase which has at least 85%, 90%, 95%, 97% or 98% amino acid sequence identity with SEQ ID NO:4, and/or an isopenicillin N-acyltransferase which has at least 85%, 90%, 95%, 97% or 98% amino acid sequence identity with SEQ ID NO:6.

Preferably the at least one inactivated gene in its active form comprises or consists of a polynucleotide encoding a delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase and having at least 80%, 85%, 90%, 95%, 97% or 98% sequence identity with SEQ ID NO:1, a polynucleotide encoding an isopenicillin-N synthase and having at least 80%, 85%, 90%, 95%, 97% or 98% sequence identity with SEQ ID NO:3, and/or a polynucleotide encoding an isopenicillin N-acyltransferase and having at least 80%, 85%, 90%, 95%, 97% or 98% sequence identity with SEQ ID NO:5.

In a preferred aspect of the invention, at least two genes are inactivated which in their active form encodes a delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase having at least 80% amino acid sequence identity with SEQ ID NO:2, an isopenicillin-N synthase having at least 80% amino acid sequence identity with SEQ ID NO:4, and/or an isopenicillin N-

acyltransferase having at least 80% amino acid sequence identity with SEQ ID NO:6. Even more preferably all three genes are inactivated.

In a preferred embodiment of the aspects of the invention, the filamentous fungal host cell is of a genus selected from the group consisting of *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes* and *Trichoderma*; even more preferably the filamentous fungal host cell is an *Aspergillus* cell; preferably an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger* or an *Aspergillus oryzae* cell.

Preferably, the polypeptide of interest is homologous or heterologous; more preferably the homologous or heterologous the polypeptide of interest is an enzyme; preferably the enzyme is a hydrolase, isomerase, ligase, lyase, oxidoreductase, or transferase, *e.g.*, an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, endoglucanase, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phospholipase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, xylanase, or beta-xylosidase.

In a preferred embodiment the polypeptide of interest is a secreted polypeptide.

Nucleic Acid Constructs

The present invention also relates to nucleic acid constructs comprising a polynucleotide of the present invention operably linked to one or more control sequences that direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences.

The polynucleotide may be manipulated in a variety of ways to provide for expression of the polypeptide. Manipulation of the polynucleotide prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotides utilizing recombinant DNA methods are well known in the art.

The control sequence may be a promoter, a polynucleotide that is recognized by a host cell for expression of a polynucleotide encoding a polypeptide of the present invention. The promoter contains transcriptional control sequences that mediate the expression of the polypeptide. The promoter may be any polynucleotide that shows transcriptional activity in the host cell including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

Examples of suitable promoters for directing transcription of the nucleic acid constructs of the present invention in a filamentous fungal host cell are promoters obtained from the genes for *Aspergillus nidulans* acetamidase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Aspergillus oryzae* TAKA amylase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Fusarium oxysporum* trypsin-like protease (WO 96/00787), *Fusarium venenatum* amyloglucosidase (WO 00/56900), *Fusarium venenatum* Daria (WO 00/56900), *Fusarium venenatum* Quinn (WO 00/56900), *Rhizomucor miehei* lipase, *Rhizomucor miehei* aspartic proteinase, *Trichoderma reesei* beta-glucosidase, *Trichoderma reesei* cellobiohydrolase I, *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei* endoglucanase I, *Trichoderma reesei* endoglucanase II, *Trichoderma reesei* endoglucanase III, *Trichoderma reesei* endoglucanase V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* xylanase III, *Trichoderma reesei* beta-xylosidase, and *Trichoderma reesei* translation elongation factor, as well as the NA2-tpi promoter (a modified promoter from an *Aspergillus* neutral alpha-amylase gene in which the untranslated leader has been replaced by an untranslated leader from an *Aspergillus* triose phosphate isomerase gene; non-limiting examples include modified promoters from an *Aspergillus niger* neutral alpha-amylase gene in which the untranslated leader has been replaced by an untranslated leader from an *Aspergillus nidulans* or *Aspergillus oryzae* triose phosphate isomerase gene); and mutant, truncated, and hybrid promoters thereof. Other promoters are described in U.S. Patent No. 6,011,147.

The control sequence may also be a transcription terminator, which is recognized by a host cell to terminate transcription. The terminator is operably linked to the 3'-terminus of the polynucleotide encoding the polypeptide. Any terminator that is functional in the host cell may be used in the present invention.

Preferred terminators for filamentous fungal host cells are obtained from the genes for *Aspergillus nidulans* acetamidase, *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* glucoamylase, *Aspergillus niger* alpha-glucosidase, *Aspergillus oryzae* TAKA amylase, *Fusarium oxysporum* trypsin-like protease, *Trichoderma reesei* beta-glucosidase, *Trichoderma reesei* cellobiohydrolase I, *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei* endoglucanase I, *Trichoderma reesei* endoglucanase II, *Trichoderma reesei* endoglucanase III, *Trichoderma reesei* endoglucanase V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* xylanase III, *Trichoderma reesei* beta-xylosidase, and *Trichoderma reesei* translation elongation factor.

The control sequence may also be an mRNA stabilizer region downstream of a promoter and upstream of the coding sequence of a gene which increases expression of the gene.

The control sequence may also be a leader, a nontranslated region of an mRNA that is important for translation by the host cell. The leader is operably linked to the 5'-terminus of the

polynucleotide encoding the polypeptide. Any leader that is functional in the host cell may be used.

Preferred leaders for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase and *Aspergillus nidulans* triose phosphate isomerase.

The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3'-terminus of the polynucleotide and, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence that is functional in the host cell may be used.

Preferred polyadenylation sequences for filamentous fungal host cells are obtained from the genes for *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* glucoamylase, *Aspergillus niger* alpha-glucosidase *Aspergillus oryzae* TAKA amylase, and *Fusarium oxysporum* trypsin-like protease.

The control sequence may also be a signal peptide coding region that encodes a signal peptide linked to the N-terminus of a polypeptide and directs the polypeptide into the cell's secretory pathway. The 5'-end of the coding sequence of the polynucleotide may inherently contain a signal peptide coding sequence naturally linked in translation reading frame with the segment of the coding sequence that encodes the polypeptide. Alternatively, the 5'-end of the coding sequence may contain a signal peptide coding sequence that is foreign to the coding sequence. A foreign signal peptide coding sequence may be required where the coding sequence does not naturally contain a signal peptide coding sequence. Alternatively, a foreign signal peptide coding sequence may simply replace the natural signal peptide coding sequence in order to enhance secretion of the polypeptide. However, any signal peptide coding sequence that directs the expressed polypeptide into the secretory pathway of a host cell may be used.

Effective signal peptide coding sequences for filamentous fungal host cells are the signal peptide coding sequences obtained from the genes for *Aspergillus niger* neutral amylase, *Aspergillus niger* glucoamylase, *Aspergillus oryzae* TAKA amylase, *Humicola insolens* cellulase, *Humicola insolens* endoglucanase V, *Humicola lanuginosa* lipase, and *Rhizomucor miehei* aspartic proteinase.

The control sequence may also be a propeptide coding sequence that encodes a propeptide positioned at the N-terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to an active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding sequence may be obtained from the genes for *Bacillus subtilis* alkaline protease (*aprE*), *Bacillus subtilis* neutral protease (*nprT*), *Myceliophthora thermophila* laccase (WO 95/33836), *Rhizomucor miehei* aspartic proteinase, and *Saccharomyces cerevisiae* alpha-factor.

Where both signal peptide and propeptide sequences are present, the propeptide sequence is positioned next to the N-terminus of a polypeptide and the signal peptide sequence is positioned next to the N-terminus of the propeptide sequence.

It may also be desirable to add regulatory sequences that regulate expression of the polypeptide relative to the growth of the host cell. Examples of regulatory sequences are those that cause expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. In filamentous fungi, the *Aspergillus niger* glucoamylase promoter, *Aspergillus oryzae* TAKA alpha-amylase promoter, and *Aspergillus oryzae* glucoamylase promoter, *Trichoderma reesei* cellobiohydrolase I promoter, and *Trichoderma reesei* cellobiohydrolase II promoter may be used. Other examples of regulatory sequences are those that allow for gene amplification. In eukaryotic systems, these regulatory sequences include the dihydrofolate reductase gene that is amplified in the presence of methotrexate, and the metallothionein genes that are amplified with heavy metals. In these cases, the polynucleotide encoding the polypeptide would be operably linked to the regulatory sequence.

Expression Vectors

The present invention also relates to recombinant expression vectors comprising a polynucleotide of the present invention, a promoter, and transcriptional and translational stop signals. The various nucleotide and control sequences may be joined together to produce a recombinant expression vector that may include one or more convenient restriction sites to allow for insertion or substitution of the polynucleotide encoding the polypeptide at such sites. Alternatively, the polynucleotide may be expressed by inserting the polynucleotide or a nucleic acid construct comprising the polynucleotide into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

The recombinant expression vector may be any vector (*e.g.*, a plasmid or virus) that can be conveniently subjected to recombinant DNA procedures and can bring about expression of the polynucleotide. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vector may be a linear or closed circular plasmid.

The vector may be an autonomously replicating vector, *i.e.*, a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one that, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids that together contain the total DNA to be introduced into the genome of the host cell, or a transposon, may be used.

The vector preferably contains one or more selectable markers that permit easy selection of transformed, transfected, transduced, or the like cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like.

Selectable markers for use in a filamentous fungal host cell include, but are not limited to, *adeA* (phosphoribosylaminoimidazole-succinocarboxamide synthase), *adeB* (phosphoribosyl-aminoimidazole synthase), *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), *hph* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenyltransferase), and *trpC* (anthranilate synthase), as well as equivalents thereof. Preferred for use in an *Aspergillus* cell are *Aspergillus nidulans* or *Aspergillus oryzae amdS* and *pyrG* genes and a *Streptomyces hygroscopicus bar* gene. Preferred for use in a *Trichoderma* cell are *adeA*, *adeB*, *amdS*, *hph*, and *pyrG* genes.

The selectable marker may be a dual selectable marker system as described in WO 2010/039889. In one aspect, the dual selectable marker is an *hph-tk* dual selectable marker system.

The vector preferably contains an element(s) that permits integration of the vector into the host cell's genome or autonomous replication of the vector in the cell independent of the genome.

For integration into the host cell genome, the vector may rely on the polynucleotide's sequence encoding the polypeptide or any other element of the vector for integration into the genome by homologous or non-homologous recombination. Alternatively, the vector may contain additional polynucleotides for directing integration by homologous recombination into the genome of the host cell at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, 400 to 10,000 base pairs, and 800 to 10,000 base pairs, which have a high degree of sequence identity to the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding polynucleotides. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. The origin of replication may be any plasmid replicator mediating autonomous replication that functions in a cell. The term "origin of replication" or "plasmid replicator" means a polynucleotide that enables a plasmid or vector to replicate *in vivo*.

Examples of origins of replication useful in a filamentous fungal cell are AMA1 and ANS1 (Gems *et al.*, 1991, *Gene* 98: 61-67; Cullen *et al.*, 1987, *Nucleic Acids Res.* 15: 9163-9175;

WO 00/24883). Isolation of the AMA1 gene and construction of plasmids or vectors comprising the gene can be accomplished according to the methods disclosed in WO 00/24883.

More than one copy of a polynucleotide of the present invention may be inserted into a host cell to increase production of a polypeptide. An increase in the copy number of the polynucleotide can be obtained by integrating at least one additional copy of the sequence into the host cell genome or by including an amplifiable selectable marker gene with the polynucleotide where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the polynucleotide, can be selected for by cultivating the cells in the presence of the appropriate selectable agent.

The procedures used to ligate the elements described above to construct the recombinant expression vectors of the present invention are well known to one skilled in the art (see, *e.g.*, Sambrook *et al.*, 1989, *supra*).

Inactivation of one or more gene

The present invention also relates to methods of producing a mutant of a parent cell, which comprises inactivating, disrupting or deleting a polynucleotide, or a portion thereof, which results in the mutant cell producing less of the encoded polypeptide than the parent cell when cultivated under the same conditions.

The mutant cell may be constructed by reducing or eliminating expression of the polynucleotide or a homologue thereof using methods well known in the art, for example, insertions, disruptions, replacements, or deletions. In a preferred aspect, the polynucleotide is inactivated. The polynucleotide to be modified or inactivated may be, for example, the coding region or a part thereof essential for activity, or a regulatory element required for expression of the coding region. An example of such a regulatory or control sequence may be a promoter sequence or a functional part thereof, *i.e.*, a part that is sufficient for affecting expression of the polynucleotide. Other control sequences for possible modification include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, signal peptide sequence, transcription terminator, and transcriptional activator.

Modification or inactivation of the polynucleotide may be performed by subjecting the parent cell to mutagenesis and selecting for mutant cells in which expression of the polynucleotide has been reduced or eliminated. The mutagenesis, which may be specific or random, may be performed, for example, by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the mutagenesis may be performed by use of any combination of these mutagenizing agents.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG),

O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues.

When such agents are used, the mutagenesis is typically performed by incubating the parent cell to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions, and screening and/or selecting for mutant cells exhibiting reduced or no expression of the gene.

Modification or inactivation of the polynucleotide or homologue thereof may be accomplished by insertion, substitution, or deletion of one or more nucleotides in the gene or a regulatory element required for transcription or translation thereof. For example, nucleotides may be inserted or removed so as to result in the introduction of a stop codon, the removal of the start codon, or a change in the open reading frame. Such modification or inactivation may be accomplished by site-directed mutagenesis or PCR generated mutagenesis in accordance with methods known in the art. Although, in principle, the modification may be performed *in vivo*, *i.e.*, directly on the cell expressing the polynucleotide to be modified, it is preferred that the modification be performed *in vitro* as exemplified below.

Methods for deleting or disrupting a targeted gene are described, for example, by Miller, et al (1985. Mol. Cell. Biol. 5:1714-1721); WO 90/00192; May, G. (1992. Applied Molecular Genetics of Filamentous Fungi. J. R. Kinghorn and G. Turner, eds., Blackie Academic and Professional, pp. 1-25); and Turner, G. (1994. Vectors for Genetic Manipulation. S. D. Martinelli and J. R. Kinghorn, eds., Elsevier, pp. 641-665).

An example of a convenient way to eliminate or reduce expression of a polynucleotide is based on techniques of gene replacement, gene deletion, or gene disruption. For example, in the gene disruption method, a nucleic acid sequence corresponding to the endogenous polynucleotide is mutagenized *in vitro* to produce a defective nucleic acid sequence that is then transformed into the parent cell to produce a defective gene. By homologous recombination, the defective nucleic acid sequence replaces the endogenous polynucleotide. It may be desirable that the defective polynucleotide also encodes a marker that may be used for selection of transformants in which the polynucleotide has been modified or destroyed. In an aspect, the polynucleotide is disrupted with a selectable marker such as those described herein.

The polypeptide-deficient mutant cells are particularly useful as host cells for expression of heterologous secreted polypeptides.

The methods used for cultivation and purification of the product of interest may be performed by methods known in the art.

Methods of Production

The host cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods known in the art. For example, the cells may be cultivated by shake flask cultivation, or small-scale or large-scale fermentation (including continuous, batch, fed-batch, or

solid state fermentations) in laboratory or industrial fermentors in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (*e.g.*, in catalogues of the American Type Culture Collection). If the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it can be recovered from cell lysates.

The polypeptide may be detected using methods known in the art that are specific for the polypeptides. These detection methods include, but are not limited to, use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. For example, an enzyme assay may be used to determine the activity of the polypeptide.

The polypeptide may be recovered using methods known in the art. For example, the polypeptide may be recovered from the nutrient medium by conventional procedures including, but not limited to, collection, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation. In one aspect, a fermentation broth comprising the polypeptide is recovered.

The polypeptide may be purified by a variety of procedures known in the art including, but not limited to, chromatography (*e.g.*, ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (*e.g.*, preparative isoelectric focusing), differential solubility (*e.g.*, ammonium sulfate precipitation), SDS-PAGE, or extraction (see, *e.g.*, *Protein Purification*, Janson and Ryden, editors, VCH Publishers, New York, 1989) to obtain substantially pure polypeptides.

In an alternative aspect, the polypeptide is not recovered, but rather a host cell of the present invention expressing the polypeptide is used as a source of the polypeptide.

One aspect of the invention relates to methods of producing a polypeptide of interest, said methods comprising the steps of:

a) cultivating a filamentous fungal host cell comprising a polynucleotide encoding a polypeptide of interest and comprising at least one inactivated gene which in its active form encodes a delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase having at least 80% amino acid sequence identity with SEQ ID NO:2, an isopenicillin-N synthase having at least 80% amino acid sequence identity with SEQ ID NO:4, and/or an isopenicillin N-acyltransferase having at least 80% amino acid sequence identity with SEQ ID NO:6, whereby the host cell is deficient in the production of penicillin and, optionally,

b) recovering the polypeptide of interest.

Preferably, the least one inactivated gene in its active form encodes a delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase which has at least 85%, 90%, 95%, 97% or 98% amino acid sequence identity with SEQ ID NO:2, an isopenicillin-N synthase which has at least 85%, 90%, 95%, 97% or 98% amino acid sequence identity with SEQ ID NO:4, and/or an

isopenicillin N-acyltransferase which has at least 85%, 90%, 95%, 97% or 98% amino acid sequence identity with SEQ ID NO:6.

Preferably the at least one inactivated gene in its active form comprises or consists of a polynucleotide encoding a delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase and having at least 80%, 85%, 90%, 95%, 97% or 98% sequence identity with SEQ ID NO:1, a polynucleotide encoding an isopenicillin-N synthase and having at least 80%, 85%, 90%, 95%, 97% or 98% sequence identity with SEQ ID NO:3, and/or a polynucleotide encoding an isopenicillin N-acyltransferase and having at least 80%, 85%, 90%, 95%, 97% or 98% sequence identity with SEQ ID NO:5.

In a preferred aspect of the invention, at least two genes are inactivated which in their active form encode a delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase having at least 80% amino acid sequence identity with SEQ ID NO:2, an isopenicillin-N synthase having at least 80% amino acid sequence identity with SEQ ID NO:4, and/or an isopenicillin N-acyltransferase having at least 80% amino acid sequence identity with SEQ ID NO:6. Even more preferably all three genes are inactivated.

In a preferred embodiment, the filamentous fungal host cell is of a genus selected from the group consisting of *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes* and *Trichoderma*; even more preferably the filamentous fungal host cell is an *Aspergillus* cell; preferably an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger* or an *Aspergillus oryzae* cell.

Preferably, the polypeptide of interest is homologous or heterologous; more preferably the homologous or heterologous polypeptide of interest is an enzyme; preferably the enzyme is a hydrolase, isomerase, ligase, lyase, oxidoreductase, or transferase, *e.g.*, an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, endoglucanase, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, xylanase, or beta-xylosidase. Even more preferably, the polypeptide of interest is secreted.

In a preferred embodiment, the at least one inactivated gene in its active form comprises or consists of a polynucleotide encoding a delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase and having at least 80% sequence identity with SEQ ID NO:1, a polynucleotide encoding an isopenicillin-N synthase and having at least 80% sequence identity with SEQ ID

NO:3, and/or a polynucleotide encoding an isopenicillin N-acyltransferase and having at least 80% sequence identity with SEQ ID NO:5.

EXAMPLES

Methods

General methods of PCR, cloning, ligation nucleotides etc. are well-known to a person skilled in the art and may for example be found in in "Molecular cloning: A laboratory manual", Sambrook et al. (1989), Cold Spring Harbor lab., Cold Spring Harbor, NY; Ausubel, F. M. et al. (eds.); "Current protocols in Molecular Biology", John Wiley and Sons, (1995); Harwood, C. R., and Cutting, S. M. (eds.); "DNA Cloning: A Practical Approach, Volumes I and II", D.N. Glover ed. (1985); "Oligonucleotide Synthesis", M.J. Gait ed. (1984); "Nucleic Acid Hybridization", B.D. Hames & S.J. Higgins eds (1985); "A Practical Guide To Molecular Cloning", B. Perbal, (1984).

PCR amplification

All PCR amplifications was performed in a volume of 100 microL containing 2.5 units Taq po-lymerase, 100 ng of pSO2, 250 nM of each dNTP, and 10 pmol of each of the two primers described above in a reaction buffer of 50 mM KCl, 10 mM Tris-HCl pH 8.0, 1.5 mM MgCl2.

Amplification was carried out in a Perkin-Elmer Cetus DNA Termal 480, and consisted of one cycle of 3 minutes at 94°C, followed by 25 cycles of 1 minute at 94°C, 30 seconds at 55°C, and 1 minute at 72°C.

Aspergillus transformation

Aspergillus transformation was done as described by Christensen et al.; Biotechnology 1988 6 1419-1422. In short, *A.oryzae* mycelia were grown in a rich nutrient broth. The mycelia were separated from the broth by filtration. The enzyme preparation Glucanex® (Novozymes) was added to the mycelia in osmotically stabilizing buffer such as 1.2 M $MgSO_4$ buffered to pH 5.0 with sodium phosphate. The suspension was incubated for 60 minutes at 37degrees C with agitation. The protoplast was filtered through mira-cloth to remove mycelial debris. The protoplast was harvested and washed twice with STC (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5). The protoplasts were finally re-suspended in 200-1000 microl STC.

For transformation, 5 microg DNA was added to 100 microl protoplast suspension and then 200 microl PEG solution (60% PEG 4000, 10 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5) was added and the mixture is incubated for 20 minutes at room temperature. The protoplast were harvested and washed twice with 1.2 M sorbitol. The protoplast was finally re-suspended 200 microl 1.2 M sorbitol. Transformants containing the amdS gene were selected for its ability to used acetamide as the sole source for nitrogen on minimal plates (Cove D.J. 1966. Biochem. Biophys. Acta.

113:51-56) containing 1.0 M sucrose as carbon source, 10 mM acetamide as nitrogen source. After 5-7 days of growth at 37 degrees C, stable transformants appeared as vigorously growing and sporulating colonies. Transformants were purified twice through conidiospores.

Shake flask fermentation

Shake flask containing 10 ml YPM medium (2 g/l yeast extract, 2 g/l peptone, and 2% maltose) were inoculated with spores from a transformant/heterokaryon or diploid strain and incubated at 30 degrees C, 200 rpm for 4 days.

Genes

acvA: This gene codes for delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase, an enzyme involved in the biosynthesis of penicillin G.

ipnA: This gene codes for isopenicillin N synthetase, an enzyme involved in the biosynthesis of penicillin G.

aatA: This gene codes for Isopenicillin N-acyltransferase, an enzyme involved in the biosynthesis of penicillin G.

pyrG: This gene codes for orotidine-5'-phosphate decarboxylase, an enzyme involved in the biosynthesis of uridine.

amdS: This gene codes for acetamidase, an enzyme involved in the metabolism of acetamide.

Plasmids

pCR-4 Blunt-TOPO is from Invitrogen.

pHUda797 is described in patent US2013095525, example 1

pJaL554 is described in patent WO07045248, example 9

pJaL1123 is described in patent WO2012160093, example 10

Strains

Aspergillus oryzae NBRC4177: available from Institute for fermentation, Osaka; 17-25 Juso Hammachi 2-Chome Yodogawa-Ku, Osaka, Japan.

COls454 is described in patent WO2012160093, example 16

RUNG237 is described in patent WO20150226, example 1

JaL1844 is described in example 1

JaL1877 is described in example 2

JaL1898 is described in example 3

JaL1903 is described in example 4

Sequences

SEQ ID NO:1: DNA sequence of *acvA*

SEQ ID NO:2: Amino acid sequence of AcvA encoded by SEQ ID NO:1

SEQ ID NO:3: DNA sequence of *ipnA*

SEQ ID NO:4: Amino acid sequence of IpnA encoded by SEQ ID NO:3

SEQ ID NO:5 DNA sequence of *aatA*

SEQ ID NO 3: Amino acid sequence of AatA encoded by SEQ ID NO:5

SEQ ID NO:7: Primer oJaL113 5'- gagctgctggatttggctg

SEQ ID NO:8: Primer oJaL114 5'- ccaacagccgactcaggag

SEQ ID NO:9: Primer oJaL565 5'- cggttctacagtccgccc

SEQ ID NO:10: Primer oJaL566 5'- cgtccacgcggggattatgctgatcgccaaatctattaac

SEQ ID NO:11: Primer oJaL567 5'- cgataagctccttgacggggtgactgggcaacaccacgaag

SEQ ID NO:12: Primer oJaL568 5'- ggtcatagtccgccagttg

SEQ ID NO:13: Primer X1111CO7 5'- gcataatccccgcgtggacg

SEQ ID NO:14: Primer X1111CO8 5'- ccccgtcaaggagcttatcg

Example 1. Construction of a *ligD* minus *A. oryzae* strain, JaL1844.

For deletion of the ligD gene (AO090120000322) involved in non-homologous-end-joining plasmid pJaL1123 was linearized with SpeI and used to transform A. oryzae RUNG237 and transformants were selected on minimal medium supplemented 0.6 mM 5-fluoro-2'-deoxyuridine (FdU) as described in WO 0168864. A number of transformants were re-isolated twice and genomic DNA was prepared. The chromosomal DNA from each of the transformants was digested with Asp718 and analyzed by Southern blotting, using the 1102 bp 32P-labelled DNA EcoRI – BamHI fragment from pJaL1123 containing the 5' flanks of the A. oryzae ligD gene as the probe. Strains of interest were identified by the disappearance of a 3828 kb Asp718 band and the appearance of a 2899 kb Asp718 band. One transformant having the above characteristics was named JaL1844.

Example 2. Isolation of a *pyrG* minus *A. oryzae* strain, JaL1877

The *A. oryzae* strain JaL1844 was screened for resistance to 5-flouro-orotic acid (FOA) to identify spontaneous pyrG mutants on minimal plates (Cove D.J. 1966. Biochem. Biophys. Acta. 113:51-56) supplemented with 1.0 M sucrose as carbon source, 10 mM sodiumnitrate as nitrogen source, and 0.5 mg/ml FOA. One strain, JaL1877, was identifying as being pyrG minus. JaL1877 is uridine dependent, therefore it can be transformed with the wild type pyrG gene and transformants selected by the ability to grow in the absence of uridine.

Example 3. Construction of a penG minus *A. oryzae* strain, JaL1898.

The three genes *acvA* (AO090038000543; SEQ ID NO:1 encoding SEQ ID NO:2) , *ipnA* (AO090038000544; SEQ ID NO:3 encoding SEQ ID NO:4) and *aatA* (AO090038000545; SEQ ID NO: 5 encoding SEQ ID NO:6) are involved in synthesis of penicillin G (penG) which is clustered

on chromosome 6 in *A. oryzae* strains. Fig. 1 shows the orientation of these three 3 genes and location of primers used for PCR amplification of flanks used for deletion of part of the penG gene cluster. First it was tried to deleted the entire penG gene cluster (all three genes, ΔA in Fig. 1), but was not able to obtained any *Aspergillus oryzae* clones having this entire penG gene cluster deletion, so therefore only part of the penG gene cluster was tried deleted with success. The 4401 bp deletion includes the first 132 bp (encoding the first 44 amino acids) of the *aatA* gene, the entire *ipnA* gene, the first 1515 bp (encoding the first 505 amino acids) of the *avcA* gene and the promoters for all three genes as indicated in Fig. 1. The strategy used for the deletion is as described in Nielsen M. L. *et al.* (2006), *Efficient PCR-based gene targeting with a recyclable marker for Aspergillus nidulans*, Fungal Genetics and Biology vol. 43: 54–64. It was done in the following way:

First the *aatA* flank and a partially N-terminal pyrG gene was fused by overlap extension PCR of two PCR generated fragments 1) an 1418 bp fragment (aatA flank) using primers oJaL565 (SEQ ID NO:9) and oJaL566 (SEQ ID NO:10) on genomic DNA from RUNG237 and 2) an 1129 bp fragment (encoding part of the A. oryzae pyrG gene) using primers oJaL114 (SEQ ID NO:8) and X1111C07 (SEQ ID NO:13) on plasmid pJaL554. The two fragments was mix and PCR amplification with primers oJaL565 and oJaL114 was done giving a fragment on 2527 bp, which was purified over a 1% agarose gel.

Second the *acvA* flank and a partially C-terminal *pyrG* gene was fused by overlap extension PCR of two PCR generated fragments 1) an 2003 bp fragment (acvA flank) using primers oJaL567 (SEQ ID NO:11) and oJaL568 (SEQ ID NO:12) on genomic DNA from RUNG237 and 2) an 1445 bp fragment (encoding part of the *A. oryzae pyrG* gene) using primers oJaL113 (SEQ ID NO:7) and X1111C08 (SEQ ID NO:14) on plasmid pJaL554. The two fragments was mix and PCR amplification with primers oJaL568 and oJaL113 was done giving a fragment on 3428 bp, which was purified over a 1% agarose gel.

The above two fragments (1 µg of each) was mix and transformed into RUNG237. A number of transformants were re-isolated twice and genomic DNA was prepared. The chromosomal DNA from each of the transformants was digested with Asp718 and analyzed by Southern blotting, using 32P-labelling of the above 1418 bp PCR product containing the aatA flank as the probe. Strains of interest were identified by the disappearance of an 8520 kb BglII band and the appearance of a 6161 kb BglII band. One transformant having the above characteristics was named JaL1898.

Example 4. Isolation of a pyrG minus *A. oryzae* strain, JaL1903

The *A. oryzae* strain JaL1898 was screened for resistance to 5-flouro-orotic acid (FOA) to identify spontaneous *pyrG* mutants on minimal plates (Cove D.J. 1966. Biochem. Biophys. Acta. 113:51-56) supplemented with 1.0 M sucrose as carbon source, 10 mM sodiumnitrate as nitrogen source, and 0.5 mg/ml FOA. One strain, JaL1903, was identifying as being pyrG minus.

JaL1903 is uridine dependent, therefore it can be transformed with the wild type pyrG gene and transformants selected by the ability to grow in the absence of uridine. The loss of the pyrG gene was confirmed by Southern blotting analysis, using 32P-labelling of the above 1418 bp PCR product containing the aatA flank as the probe. Strains of interest were identified by the disappearance of a 6161 kb BglII band and the appearance of a 4554 kb BglII band. One transformant having the above characteristics was named JaL1903. This strain can then be used for further gene deletions.

CLAIMS

1. A filamentous fungal host cell comprising a polynucleotide encoding a polypeptide of interest and comprising at least one inactivated gene which in its active form encodes a delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase having at least 80% amino acid sequence identity with SEQ ID NO:2, an isopenicillin-N synthase having at least 80% amino acid sequence identity with SEQ ID NO:4, and/or an isopenicillin N-acyltransferase having at least 80% amino acid sequence identity with SEQ ID NO:6, whereby the host cell is deficient in the production of penicillin.

2. The host cell of claim 1 which is of a genus selected from the group consisting of *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes* and *Trichoderma*.

3. The host cell of claim 2 which is an *Aspergillus* cell; preferably an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger* or an *Aspergillus oryzae* cell.

4. The host cell of any preceding claim, wherein the polypeptide of interest is homologous or heterologous; preferably the homologous or heterologous polypeptide of interest is an enzyme; preferably the enzyme is a hydrolase, isomerase, ligase, lyase, oxidoreductase, or transferase, *e.g.*, an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, endoglucanase, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, xylanase, or beta-xylosidase.

5. The host cell of claim 4, wherein the polypeptide of interest is a secreted polypeptide.

6. The host cell of any preceding claim, wherein the at least one inactivated gene in its active form comprises or consists of a polynucleotide encoding a delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase and having at least 80% sequence identity with SEQ ID NO:1, a polynucleotide encoding an isopenicillin-N synthase and having at least 80% sequence identity

with SEQ ID NO:3, and/or a polynucleotide encoding an isopenicillin N-acyltransferase and having at least 80% sequence identity with SEQ ID NO:5.

7. A method of producing a polypeptide of interest, said method comprising the steps of:

a) cultivating a filamentous fungal host cell comprising a polynucleotide encoding a polypeptide of interest and comprising at least one inactivated gene which in its active form encodes a delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase having at least 80% amino acid sequence identity with SEQ ID NO:2, an isopenicillin-N synthase having at least 80% amino acid sequence identity with SEQ ID NO:4, and/or an isopenicillin N-acyltransferase having at least 80% amino acid sequence identity with SEQ ID NO:6, whereby the host cell is deficient in the production of penicillin and, optionally,

b) recovering the polypeptide of interest.

8. The method of claim 7, wherein the filamentous fungal host cell is of a genus selected from the group consisting of *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes* and *Trichoderma*.

9. The method cell of claim 8, wherein the filamentous fungal host cell is an *Aspergillus* cell; preferably an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger* or an *Aspergillus oryzae* cell.

10. The method of any of claims 7-9, wherein the polypeptide of interest is homologous or heterologous; preferably the homologous or heterologous polypeptide of interest is an enzyme; more preferably the enzyme is a hydrolase, isomerase, ligase, lyase, oxidoreductase, or transferase, *e.g.*, an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, endoglucanase, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, xylanase or beta-xylosidase.

11. The method of claim 10, wherein the polypeptide of interest is a secreted polypeptide.

12. The method of any of claims 7-11, wherein the at least one inactivated gene in its active form comprises or consists of a polynucleotide encoding a delta-(L-alpha-aminoadipyl)-L-

cysteinyl-D-valine synthetase and having at least 80% sequence identity with SEQ ID NO:1, a polynucleotide encoding an isopenicillin-N synthase and having at least 80% sequence identity with SEQ ID NO:3, and/or a polynucleotide encoding an isopenicillin N-acyltransferase and having at least 80% sequence identity with SEQ ID NO:5.

13. A method of producing an improved filamentous fungal host cell producing a polypeptide of interest, said method comprising the following steps in no particular order:

e) transforming a filamentous fungal host cell with a polynucleotide encoding the polypeptide of interest; and

f) inactivating at least one gene which in its active form encodes a delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase having at least 80% amino acid sequence identity with SEQ ID NO:2, an isopenicillin-N synthase having at least 80% amino acid sequence identity with SEQ ID NO:4, and/or an isopenicillin N-acyltransferase having at least 80% amino acid sequence identity with SEQ ID NO:6, whereby the host cell becomes deficient in the production of penicillin.

14. The method of claim 13, wherein the filamentous fungal host cell is of a genus selected from the group consisting of *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes* and *Trichoderma*.

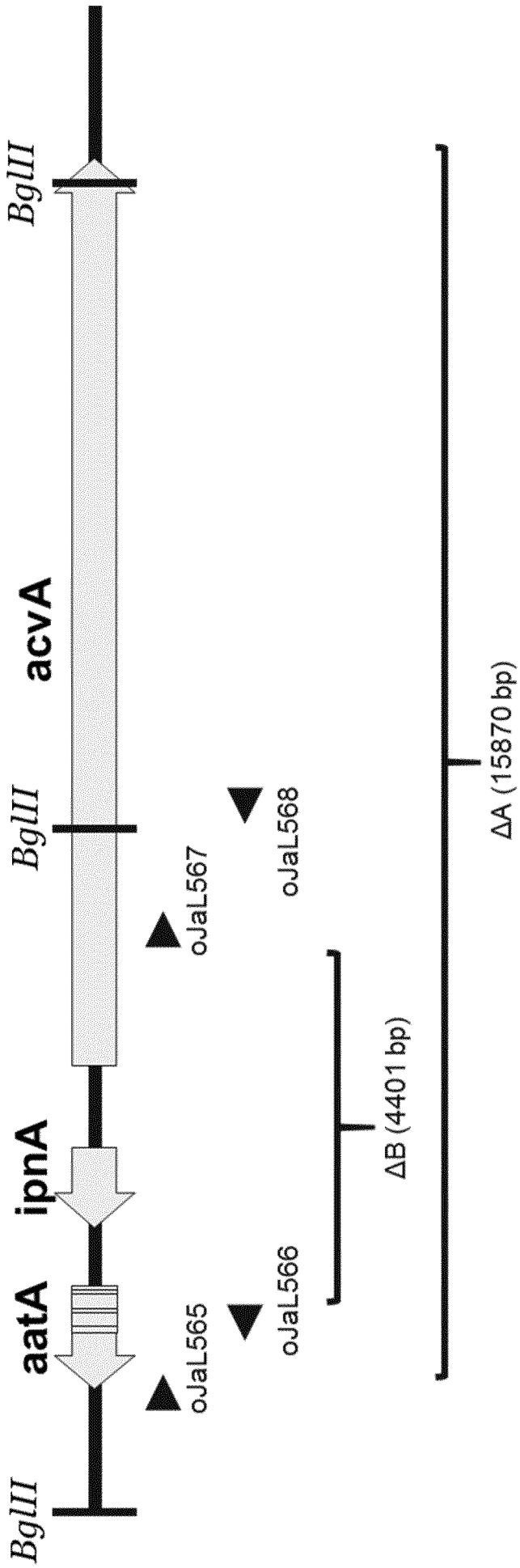
15. The method cell of claim 14, wherein the filamentous fungal host cell is an *Aspergillus* cell; preferably an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger* or an *Aspergillus oryzae* cell.

16. The method of any of claims 13-15, wherein the polypeptide of interest is homologous or heterologous; preferably the homologous or heterologous polypeptide of interest is an enzyme; more preferably the enzyme is a hydrolase, isomerase, ligase, lyase, oxidoreductase, or transferase, *e.g.*, an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, endoglucanase, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phospholipase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, xylanase or beta-xylosidase.

17. The method of claim 16, wherein the polypeptide of interest is a secreted polypeptide.

18. The method of any of claims 13-17, wherein the at least one inactivated gene in its active form comprises or consists of a polynucleotide encoding a delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase and having at least 80% sequence identity with SEQ ID NO:1, a polynucleotide encoding an isopenicillin-N synthase and having at least 80% sequence identity with SEQ ID NO:3, and/or a polynucleotide encoding an isopenicillin N-acyltransferase and having at least 80% sequence identity with SEQ ID NO:5.

Figure 1


BglII
acvA
BglII
oJaL567
oJaL568
ΔA (15870 bp)
ipnA
ΔB (4401 bp)
aatA
oJaL566
oJaL565
BglII

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/056406

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P21/00 C12N9/58
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/122249 A1 (DSM IP ASSETS BV [NL]; BOVENBERG ROELOF ARY LANS [NL]; VAN DEN BERG MA) 1 November 2007 (2007-11-01) claims 1-5 page 7, lines 15-18 page 3, lines 27-33 page 5, line 8 page 4, lines 20-24 page 22, line 18 - page 23, line 12 page 11, lines 9-13 ----- -/--	1-18

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 April 2018	24/04/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Herrmann, Klaus

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CANTORAL J M ET AL: "Biochemical characterization and molecular genetics of nine mutants of Penicillium chrysogenum impaired in penicillin biosynthesis", JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY, US, vol. 268, no. 1, 5 January 1993 (1993-01-05), pages 737-744, XP002400770, ISSN: 0021-9258 abstract page 742, left-hand column, paragraph 2	1-18
X	PETRA SPRÖTE ET AL: "Identification of the novel penicillin biosynthesis gene aatB of Aspergillus nidulans and its putative evolutionary relationship to this fungal secondary metabolism gene cluster", MOLECULAR MICROBIOLOGY., vol. 70, no. 2, 15 September 2008 (2008-09-15), pages 445-461, XP055467474, GB ISSN: 0950-382X, DOI: 10.1111/j.1365-2958.2008.06422.x page 445, left-hand column	1-18
A	FIERRO F ET AL: "Mutants blocked in penicillin biosynthesis show a deletion of the entire penicillin gene cluster at a specific site within a conserved hexanucleotide sequence", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER, DE, vol. 44, no. 5, 1 January 1996 (1996-01-01), pages 597-604, XP009072972, ISSN: 0175-7598, DOI: 10.1007/S002530050605 abstract	1-18

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2018/056406

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007122249 A1	01-11-2007	EP 2010559 A1	07-01-2009
		US 2009233287 A1	17-09-2009
		WO 2007122249 A1	01-11-2007