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(54) Title: A NITRILE-METABOLISING ENZYME AND A PROCESS FOR PRODUCING AN ACID USING SAME

(57) Abstract: The present invention relates to an isolated nucleotide sequence and corresponding polypeptide derived from the nitrile-metabolising Pantoea strain deposited under NCIMB 41854. Said isolated polypeptide acts as a nitrilase and the invention extends to a process for producing a carboxylic acid using said isolated polypeptide to metabolise nitriles such as 3-hydroxyglutaronitrile, 3-hydroxybutyronitrile and 3-hydroxy-phenylpropionitrile to form corresponding carboxylic acids.

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A Nitrile-Metabolising Enzyme and a Process for Producing an Acid using Same

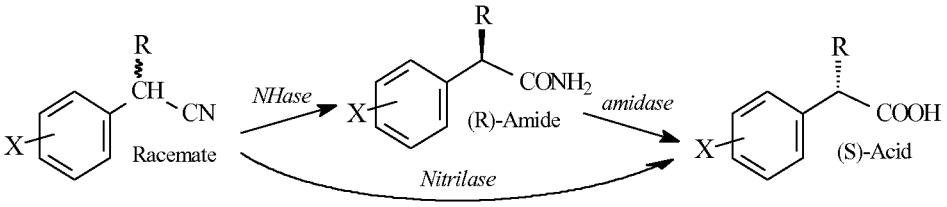
Field of the invention

The present invention relates to a nitrile-metabolising enzyme and to use of same in the production of carboxylic acids. In particular, the present invention relates to a nitrile-metabolising enzyme obtainable from culturing a *Pantoea* strain and a process for producing acids using said nitrile-metabolising enzyme.

Background to the Invention

The pharmaceutical industry requires amides and acids for use as intermediates in the manufacture of many drugs and chemicals. These may be obtained by traditional chemical methods, but this approach has problems. The chemical methods for producing these require extreme/harsh reaction conditions. In addition, undesirable by-products are produced and enantioselectivity and regioselectivity are low.

An alternative to the use of traditional chemical methods is the use of nitrile-metabolising enzymes. Certain bacterial cells contain a nitrile-metabolizing gene. When the corresponding enzyme is incubated in a reaction mixture containing a nitrile, the nitrile-metabolizing enzyme catalyses the conversion of the nitrile to the corresponding amide or acid. Hydrolysis of nitriles may occur as a one-step or two-step process as shown below. Nitrile hydratase and isonitrile hydratase enzymes catalyse conversion of nitriles or isonitriles to amides. Amidase enzymes catalyse conversion of the amides to acids. Nitrilases catalyse the conversion of nitriles to acids. The resulting amide or acid may then be extracted from the reaction mixture. Ammonia will be produced as a by-product of the conversion of nitrile to acid. The ammonia may be used by the cell as a nitrogen source.



The introduction of an enzyme-based step in the manufacturing process of a pharmaceutical drug can reduce costs significantly. It would be desirable if novel nitrile-metabolising strains and enzymes could be provided having improved characteristics over known nitrile-metabolising strains and enzymes.

Summary of the invention

The present inventors have isolated a novel nitrile-metabolising strain having a novel nitrile-metabolising gene encoding a nitrilase enzyme which can be used to produce acids.

Accordingly, according to a first aspect of the present invention there is provided an isolated polypeptide comprising SEQ ID NO:2 or a variant amino acid sequence having at least 95%, preferably 98% sequence identity with SEQ ID NO:2 wherein said polypeptide is a nitrilase.

In certain embodiments, the variant amino acid sequence has at least 98.5, 99 or 99.5% sequence identity with SEQ ID NO:2.

In certain embodiments, the variant amino acid sequence differs from SEQ ID NO:2 due to the presence of one or more conservative amino acid substitutions. Typically, the variant amino acid sequence differs from SEQ ID NO:2 due to the presence of less than 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3 or 2 conservative amino acid substitutions. In certain embodiments, the only differences or sole differences between the variant amino acid sequence and SEQ ID NO:2 are conservative amino acid substitutions, that is, no non-conservative amino acid residue alterations are present.

In certain embodiments, the variant amino acid sequence has a threonine residue at a position corresponding to position 56 of SEQ ID NO:2 when the variant amino acid sequence and SEQ ID NO:2 are aligned for maximum sequence identity. In certain embodiments, the variant amino acid sequence has a valine residue at a position corresponding to position 99 of SEQ ID NO:2 when the variant amino acid sequence and SEQ ID NO:2 are aligned for maximum sequence identity.

In certain embodiments, the isolated polypeptide comprises SEQ ID NO:2 or the variant amino acid sequence. In certain embodiments, the isolated polypeptide includes SEQ ID NO:2 or the variant amino acid sequence. In certain embodiments, the isolated polypeptide consists essentially of SEQ ID NO:2 or the variant amino acid sequence. In certain embodiments, the isolated polypeptide consists of SEQ ID NO:2 or the variant amino acid sequence.

In certain embodiments, the isolated polypeptide is derived from *Pantoea* sp. SS-17 NCIMB 41854. In certain embodiments, the isolated polypeptide is obtainable by culturing *Pantoea* sp. SS-17 NCIMB 41854.

In certain embodiments, the nitrilase is a wild type nitrilase.

The invention extends to a fragment of the isolated polypeptide wherein said fragment is a nitrilase.

The invention further extends to an isolated nucleotide sequence encoding for the isolated polypeptide of the invention.

Accordingly, according to a second aspect of the invention there is provided an isolated nucleotide sequence comprising SEQ ID NO:l or a variant nucleotide sequence which has at least 95%, preferably 98% sequence identity to

(a) SEQ ID NO: 1, or

(b) a nucleotide sequence that is capable of hybridising to SEQ ID NO:l under stringent conditions,

wherein said isolated nucleotide sequence encodes an amino acid sequence having nitrile-metabolising activity.

In certain embodiments, the variant nucleotide sequence has at least 98.5, 99, 99.5 or 100% sequence identity to

(a) SEQ ID NO: 1, or

(b) a nucleotide sequence that is capable of hybridising to SEQ ID NO:l under stringent conditions.

In certain embodiments, the isolated nucleotide sequence has 100% sequence identity to a nucleotide sequence that is capable of hybridising to SEQ ID NO:l under stringent conditions.

In certain embodiments, the isolated nucleotide sequence comprises, includes, consists essentially of or consists of SEQ ID NO:l or the variant nucleotide sequence.

In certain embodiments, the isolated nucleotide sequence is derived from *Pantoea* sp. SS-17 NCIMB 41854. In certain embodiments, the isolated nucleotide sequence is obtainable by culturing *Pantoea* sp. SS-17 NCIMB 41854.

The invention extends to a fragment of the isolated nucleotide sequence wherein said fragment encodes an amino acid sequence having nitrile-metabolising activity.

Typically, said amino acid sequence having nitrile-metabolising activity is a nitrilase. Suitably therefore the isolated nucleotide sequence of the invention encodes a nitrilase.

The invention further provides a gene construct comprising or including an isolated nucleotide sequence according to the invention and a control sequence, for example

a promoter. There is further provided a vector including an isolated nucleotide sequence according to the invention and a promoter which is operably linked to said nucleotide sequence. Suitable vectors include viruses (e.g. Vaccinia virus, adenovirus, baculovirus etc), yeast vectors, phage, chromosomes, artificial chromosomes, plasmids or cosmid DNA. In certain embodiments, the vector is introduced into an organism such as *E. coli.*

The present invention further extends to a process for making an organism suitable for metabolising nitriles to carboxylic acids comprising the step of introducing an isolated nucleotide sequence of the invention encoding a nitrilase into the organism. In certain embodiments, the organism is *E. coli.*

The present invention further extends to a recombinant organism comprising an isolated nucleotide sequence of the present invention wherein the isolated nucleotide sequence encodes a nitrilase. In certain embodiments, the organism is *E. coli.*

Suitably, the nucleotide sequences of the present invention may be expressed to provide polypeptides.

According to a further aspect of the invention there is provided a method of producing a polypeptide encoded by a nucleotide sequence of the invention, the method comprising the steps of:

(a) contacting a bacterial cell and / or an insect cell and / or a yeast cell and / or a plant cell with a vector as described herein, and

(b) cultivating said bacterial cell and / or insect cell and / or yeast cell and / or plant cell under conditions suitable for the production of the polypeptide.

Suitably the bacterial cell may be *Escherichia coli.* Suitably the polypeptide is encoded by a nucleotide sequence comprising SEQ ID NO: 1. In certain

embodiments, the polypeptide is encoded by a nucleotide sequence derived from or obtainable by culturing NCIMB strain 41854.

The invention further provides a polypeptide produced substantially from the above method. As will be understood by those of skill in the art, such a polypeptide may be isolated or substantially purified from the mixture in which it is expressed. Suitably a polypeptide of the invention will have nitrile-metabolising activity. Suitably a polypeptide of the invention will be a nitrilase.

The invention further extends to an isolated polypeptide encoded by an isolated nucleotide sequence of the invention. Preferably there is provided a polypeptide sequence encoded by a nucleotide sequence comprising, consisting essentially of or consisting of SEQ ID NO: 1. Suitably a polypeptide of the invention will have nitrile-metabolising activity. Suitably a polypeptide of the invention will be a nitrilase.

According to a further aspect of the present invention there is provided an isolated *Pantoea* strain wherein said strain is that deposited under NCIMB 41854. The strain may be designated *Pantoea* sp. SS-17.

It is understood that the strain of the present invention is not limited to the deposited strain since mutants and variants of the strain, for example, cell fusion strains or recombinant bacteria strains, may also be used in the process of the invention.

Accordingly, in certain embodiments, the strain is an acid-producing mutant of the strain NCIMB 41854. In certain embodiments, the strain is an amide-producing mutant of the strain NCIMB 41854. The term "mutant" is understood herein to refer to a microorganism which is derived from strain NCIMB 41854 by one or more mutations. The mutant should retain the nitrile-metabolising capability of NCIMB 41854 or have improved nitrile-metabolising capability over that of NCIMB 41854. These mutants can be related bacteria isolates that have either arisen

spontaneously or under selection conditions designed to isolate the mutants. For example, commercial kits are available that generate random mutations following which all generated mutants can be screened for nitrile-metabolising capability and/or enantioselectivity.

In certain embodiments, the strain is an acid-producing variant of the strain NCIMB 41854. In certain embodiments, the strain is an amide-producing variant of the strain NCIMB 41854. The term "variant" is understood herein to refer to a microorganism which comprises the nitrile-metabolising enzyme of strain NCIMB 41854, wherein the variant of the strain NCIMB 41854 retains the nitrile-metabolising capability of NCIMB 41854 or has improved nitrile-metabolising capability over that of NCIMB 41854. These variants can be related bacteria isolates that have either arisen spontaneously or under selection conditions designed to isolate the variants. The variants can also be recombinant bacteria which express the nitrilase gene of NCIMB 41854 or a variant thereof and which can be constructed using genetic engineering methods which would be well known to those skilled in the art. All generated variants may be easily screened for nitrile-metabolising capability and/or enantioselectivity.

The present invention extends to an isolated nucleotide sequence, or a fragment thereof, obtainable by culturing, or derived from, the strain of the invention. In certain embodiments, the isolated nucleotide sequence encodes a nitrilase. In certain embodiments, the isolated nucleotide sequence comprises, consists essentially of or consists of SEQ ID NO: 1 as set forth below, or a variant thereof. In certain embodiments, the isolated nucleotide sequence encodes an amidase. Suitably the isolated nucleotide sequence or fragment will have nitrile-metabolising activity.

The present invention further extends to an isolated polypeptide, or a fragment thereof, obtainable by culturing, or derived from, the strain of the invention, for example NCIMB 41854. In certain embodiments, the polypeptide is a nitrilase. In

certain embodiments, the polypeptide is an amidase. Suitably the polypeptide or fragment will have nitrile-metabolising activity. In certain embodiments the polypeptide comprises, consists essentially of or consists of SEQ ID NO: 2.

According to a further aspect of the present invention there is provided a process for producing a carboxylic acid, the process comprising the step of treating a nitrile with the nitrile-metabolising polypeptide of the invention to produce the carboxylic acid.

Suitably the nitrile-metabolising polypeptide is derived from or obtainable by culturing a strain according to the invention, in particular *Pantoea* strain NCIMB 41854. It is understood that the process of the present invention is not limited to the use of the nitrile-metabolising polypeptide of the deposited strain since nitrile-metabolising polypeptides derived from or obtainable by culturing mutants and variants of the strain, for example, cell fusion strains or recombinant bacteria strains, may also be used in the process of the invention.

Suitably the nitrile-metabolising polypeptide is encoded by an isolated nucleotide sequence of the invention. Suitably the nitrile-metabolising polypeptide is the polypeptide of the invention as described above.

In certain embodiments, the process includes a step of purifying or extracting the carboxylic acid. In certain embodiments, ammonia is also produced.

In certain embodiments, the nitrile is selected from the group consisting of acetonitrile, benzonitrile, adiponitrile, mandelonitrile, acrylonitrile and phenylacetonitrile. In certain embodiments, the nitrile is benzonitrile.

In certain embodiments, the nitrile is 3-hydroxyglutaronitrile and the acid is (R)-4-cyano-hydroxybutyric acid, a precursor for the cholesterol-lowering drug Lipitor. The

enzyme has been shown to have very high activity towards 3-hydroxyglutaronitrile. This nitrile is of significant pharmaceutical importance.

In certain embodiments, the nitrile is 3-hydroxy-butyronitrile and the acid is a beta-hydroxy carboxylic acid, which is an intermediate for drugs such as beta-blockers (blood pressure medication) and atomoxetine. The enzyme has been shown to have activity towards 3-hydroxy-butyronitrile.

In certain embodiments, the nitrile is 3-hydroxy-phenylpropionitrile and the acid is beta-hydroxy carboxylic acid, which is an intermediate for drugs such as beta-blockers (blood pressure medication) and atomoxetine. In certain embodiments, the nitrile is mandelonitrile and the acid is mandelic acid, which is used in many cosmetics and as many pharmaceutical and agricultural intermediates. In certain embodiments, the acid is 2-arylpropionic acid, which is used as an intermediate in ibuprofen.

In certain embodiments, the process is carried out at a temperature of 15 to 30°C, preferably 15 to 27°C, more preferably 20 to 27°C and even more preferably about 25°C. In certain embodiments, the process is carried out at a temperature of 25°C. The isolate demonstrated the highest activity at 25°C and the effect of temperature on the enantioselectivity of biotransformation was optimum at 25°C. In certain embodiments, the process is carried out at a temperature of 15°C. The isolate demonstrated optimum growth at 15°C. In certain such embodiments, the nitrile is 3-hydroxybutyronitrile.

In certain embodiments, the nitrile-metabolising polypeptide is comprised within whole cells of the microorganism, for example, *Pantoea* strain NCIMB 41854. In certain embodiments, the nitrile-metabolising polypeptide is extracted from the microorganism, for example, *Pantoea* strain NCIMB 41854. In certain embodiments, the nitrile-metabolising polypeptide is produced in *E. coli*

According to a further aspect of the present invention there is provided a process for producing a carboxylic acid, the process comprising the step of incubating a nitrile with a strain of the invention.

Suitably the strain is NCIMB 41854.

In certain embodiments, the process includes a step of purifying or extracting the carboxylic acid. In certain embodiments, ammonia is also produced.

Detailed description of the invention

The nitrile-metabolising enzyme of the present invention displays a different substrate/activity/enantioselective profile to other known nitrilases wherein this profile is of high potential in the production of acids. In particular, the nitrile-metabolising enzyme identified by the inventors has increased activity and speed over known nitrile-metabolising enzymes. No significant influence of reaction temperatures on the activity of the isolates was observed when the isolates were assayed at various temperatures ranging from 15°C to 30°C, suggesting that the activity is robust over a range of temperatures. Furthermore, the identified nitrile-metabolising enzyme has improved enantioselectivity and/or regioselectivity over known nitrile-metabolising enzymes and can be used for the resolution of racemic mixtures. The generation of unwanted by-products is reduced. The nitrile-metabolising enzyme of the present invention is therefore suitable for converting nitriles to corresponding carboxylic acids on an industrial scale. The process of the present invention uses said strain or enzyme, or variants thereof, to convert nitriles to acids. The process of the invention allows the use of milder reaction conditions than traditional chemical methods and reduces the manufacturing costs of drugs.

A comparison of the 16S rRNA gene sequence of the isolated strain with other 16S rRNA gene sequences in the sequence databases resulted in the strain being determined to be a novel strain of the *Pantoea* sp., or more specifically *Pantoea* sp. SS-17. *Pantoea* is a genus of Enterobacteriaceae bacteria containing mostly plant

pathogenic species. The isolate was deposited with NCIMB Ltd, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen, AB21 9YA, Scotland, UK by Chemical and Life Science Department, Waterford Institute of Technology, Cork Road, Waterford, Ireland on 29 June 2011 and assigned the accession number NCIMB 41854.

The nitrilase gene from NCIMB strain 41854 was sequenced in part and was found to differ from any other known nitrilase genes. The sequence is set forth below as SEQ ID NO: 1. The nitrile-metabolising gene was found to differ from any other known nitrile-metabolising genes.

SEQ ID NO: 1 - *Pantoea* sp. SS-17 partial nitrilase gene sequence:

GCTGGCGCGCTACAGCCTGATGACGCAGCACGAGGAGATCCACTGCAGCCAGTTCCCCGG
CTCGCTGGTGGGGCCGATTTTCGCGGAGCAGATGGATGTCACCATCCGTCATCATGCGCT
GGAGTCCGGCTGTTTTGTCATCAACGCCACCGGCTGGCTGACGGAAACGCAAATCAATGA
ATTAACATCCGATCCCGCCCTGCAGAAGGGGCTACGCGGCGGGTGCAATACCGCGATTAT
TTCGCCGGAAGGCCGCCATCTGGTGCCGCCGCTGACGGAAGGGGAAGGGATCCTGGTGGC
CGATCTGGATATGGCGCTGATCACC

SEQ ID NO: 2 is the partial amino acid sequence of a nitrilase enzyme from *Pantoea* sp. SS-17:

LARYSLMTQHEEIHCSQFPGSLVGPIFAEQMDVTIRHHALESGCFVINATGWLTETQINEL
TSDPALQKGLRGGCNTAIISPEGRHLVPPLTEGEGILVADLDMALIT

In relation to sequences provided by the invention, sequence identity is determined using a suitable mathematical algorithm. Computer implementations of such mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to, CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California), the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA and TFASTA in

the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wisconsin, USA)). Suitably alignments using these programs may be performed using the default parameters.

As used herein, "sequence identity" or "identity" in the context of two nucleotide or polypeptide sequences makes reference to a specified percentage of residues in the two sequences that are identical when aligned for maximum correspondence over a specified comparison window, as measured by sequence comparison algorithms or by visual inspection. Suitably, a specified comparison window is selected from a sequence encoding or representing at least 50, at least 75, at least 80, at least 85, at least 90, at least 95, at least 100, at least 105, at least 106, at least 107, at least 150, at least 200, at least 250 or most preferably all of the amino acids of a specified polypeptide being aligned. In certain embodiments, the specified comparison window is all of the residues of the sequences, e.g. 108 amino acid residues or 325 nucleotides. When percentage of sequence identity is used in reference to proteins it will be understood by those of skill in the art that residue positions which are not identical often differ by conservative amino acid substitutions, i.e. wherein amino acids are substituted with amino acids which have similar chemical properties to those amino acids which are replaced. The percent sequence identity may be adjusted upwards to correct for the conservative nature of a substitution.

Amino acids may be grouped according to the properties of their side chains, for examples as follows: (1) non-polar: Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M); (2) uncharged polar: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gin (Q); (3) acidic: Asp (D), GIu (E); and (4) basic: Lys (K), Arg (R), His(H). Alternatively, naturally occurring residues may be divided into groups based on common side-chain properties, for example: (1) hydrophobic: Norleucine, Met, Ala, Val, Leu, lie; (2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gin; (3) acidic: Asp, GIu; (4) basic: His, Lys, Arg; (5) residues that influence chain orientation: Gly, Pro; and (6) aromatic: Trp, Tyr, Phe. Conservative substitutions entail exchanging a member of one of these classes for another member of the same class, that is an amino acid

residue with side chains having similar biochemical properties to the amino acid residue being substituted. Preferably when the amino acid sequences of the invention are modified by way of conservative substitution of any of the amino acid residues contained therein, these changes have no effect on the functional activity of the resulting polypeptide when compared to the unmodified polypeptide comprising SEQ ID NO:2. The effect (if any) on function of a change in an amino acid residue may be easily assessed by a person skilled in the art, for example, by generating a mutation library, screening for changes in function and sequencing the mutants.

Hybridisation refers to the binding, duplexing or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (e.g., total cellular) DNA or RNA.

Stringent hybridisation occurs when a nucleic acid binds the target nucleic acid with minimal background. Typically, to achieve stringent hybridisation, temperatures of around 1°C to about 20°C, more preferably 5°C to about 20°C below the Tm (melting temperature at which half the molecules dissociate from their partner) are used. However, it is further defined by ionic strength and pH of the solution.

In certain embodiments of the present invention, the stringent conditions are selected from highly stringent conditions, medium stringent conditions, stringent conditions and low stringent conditions. An example of highly stringent wash conditions is 0.15 M NaCI at 72 C for about 15 minutes. An example of a stringent wash condition is a 0.2X SSC wash at 65°C for 15 minutes (see, Sambrook and Russell, infra, for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example of a medium stringency wash for a duplex of, for example, more than 100 nucleotides, is I X SSC at 45°C for 15 minutes. An example of a low stringency wash for a duplex of, for example, more than 100 nucleotides, is 4-6X SSC at 40°C for 15 minutes. For short probes (for example about 10 to 50 nucleotides), stringent

conditions typically involve salt concentrations of less than about 1.5 M, more preferably about 0.01 to 1.0 M, Na ion concentration (or other salts) at pH 7.0 to 8.3, and the temperature is typically at least about 30°C and at least about 60°C for long probes (for example, > 50 nucleotides).

Polypeptides of the present invention may comprise a variant amino acid sequence of the polypeptide comprising SEQ ID NO: 2. Said variant amino acid sequence may include one or more, preferably less than 3 and more preferably less than 2, truncations, substitutions, deletions or insertions wherein a polypeptide comprising said variant amino acid sequence is capable of metabolising nitriles similar to, or better than, the polypeptide comprising SEQ ID NO: 2. Advantageously, variations may be made to the polypeptide to enhance the efficacy of the polypeptide in metabolising nitrile.

Typically, the polypeptide comprises at least 105, 106 or 107 contiguous amino acids encoded by SEQ ID NO: 1. A nitrile-metabolising variant may be generated using, for example, C terminal deletion of the polynucleotide sequence of SEQ ID NO:l and said C. terminal deletion construct may then be inserted into suitable prokaryotic or eukaryotic expression plasmids. The nitrile-metabolising activity of the expression products derived from the polynucleotide may then be tested by assaying nitrile-metabolising activity with various nitriles using known methods, for example, reverse phase high performance liquid chromatography or gas chromatography.

Alternatively, synthetic peptides could be generated using SEQ ID NO:l or fragments thereof. The peptides or fragments thereof can be assayed for nitrile metabolising activity, for example, using reverse phase high performance liquid chromatography or gas chromatography.

Suitably the invention provides a polypeptide wherein between 1 to 2, 1 to 3, 1 to 5, 1 to 10, 1 to 15 or 1 to 20 amino acid residues are deleted, substituted and/or added

to the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 1 and wherein said polypeptide has nitrile-metabolising activity, e.g. similar to SEQ ID NO: 2.

Suitably the invention provides a polypeptide comprising an amino acid sequence wherein 1 to 2, 1 to 3, 1 to 5, 1 to 10, 1 to 15 or 1 to 20 amino acid residues are deleted, substituted, and/or added to the amino acid sequence of SEQ ID NO: 2 wherein said polypeptide has nitrile-metabolising activity, e.g. similar to SEQ ID NO: 2.

In particular embodiments the nucleotide sequence of the invention encodes a polypeptide consisting of an amino acid sequence of SEQ ID NO: 2, or a protein consisting of an amino acid sequence wherein 1 to 2, 1 to 3, 1 to 5, 1 to 10, 1 to 15 or 1 to 20 amino acid residues amino acid residues are deleted, substituted, and/or added to the amino acid sequence of SEQ ID NO: 2 wherein said protein has a similar nitrile-metabolising activity as SEQ ID NO: 2.

Nucleotide sequences may be codon-optimised or otherwise modified to increase the efficiency of expression of the polypeptides.

Nitrile-metabolising activity may be assayed using methods which would be well known to persons skilled in the art, for example, reverse phase high performance liquid chromatography or gas chromatography.

Certain compounds may exist in one or more particular geometric, optical, enantiomeric, diasteriomeric, epimeric, atropic, stereoisomeric, tautomeric, conformational or anomeric forms, including, but not limited to, cis- and trans-forms; E- and Z- forms; c-, t-, and r- forms; endo- and exo-forms; R-, S- and meso forms; D- and L- forms; d- and l- forms; (+) and (-) forms; keto-, enol- and enolate-forms; syn- and anti- forms; syndrical- and anticlinical- forms; *a*- and β- forms; axial and equatorial forms; boat-, chair-, twist-, envelope-, and halfchair- forms; and

combinations thereof, herein collectively referred to as "isomers" (or "isomeric forms"). Unless otherwise specified, a reference to a particular compound includes all such isomeric forms, including (wholly or partially) racemic and other mixtures thereof. Methods for the preparation (e.g. asymmetric synthesis) and separation (e.g. fractional crystallization and chromatographic means) of such isomeric forms are either known in the art or are readily obtained by adapting the methods taught herein, or known methods, in a known manner.

Definitions

As used herein, the term "isolated" refers to an *in vitro* preparation, isolation and / or purification of a peptide, polypeptide, protein, bacteria or nucleic acid molecule of the invention, such that it is not associated with *in vivo* substances or is substantially purified from *in vivo* substances or is present outside its naturally occurring environment.

The term "nitrile-metabolising" as used herein to refer to an enzyme is understood to refer to an enzyme which is capable of metabolising nitriles. The term includes nitrilases, nitrile hydratases and amidases. The term "nitrilase" as used herein is understood to refer to an enzyme which is capable of metabolising nitriles to carboxylic acids.

As used herein, a nitrile-metabolising polypeptide can be considered to be "derived from" a strain if the polypeptide originates directly or indirectly from the strain. The polypeptide may be encoded by a part of the genome of the strain and may therefore be obtainable directly from culturing the strain, including for example being expressed in the strain, for example in microbial whole cells, being present in the cytosol thereof, being present in a cell culture thereof or being present in a cell lysate thereof. The polypeptide may also be synthetically prepared from a gene which is endogenous to the strain of the invention following isolation of the gene from the strain and sequencing of the gene. For example, the polypeptide may be synthetically prepared, and/or be obtained using recombinant DNA technology,

such as from a genetically engineered plasmid/host cell system in which the plasmid includes a nucleic acid polymer which encodes the polypeptide.

As used herein the terms "nucleic acid" or "nucleotide sequence" includes genomic DNA, cDNA or RNA.

The term "activity" is understood herein to refer to the rate of metabolic/catalytic conversion.

The phrase "consists essentially of" or "consisting essentially of" as used herein means that a polypeptide or nucleotide sequence may have additional features or elements beyond those described provided that such additional features or elements do not materially affect the ability of the polypeptide to metabolise nitriles. For example, a polypeptide consisting essentially of a specified sequence may contain one, two or three additional, deleted or substituted amino acids, at either end or at both ends of the sequence provided that these amino acids do not interfere with its function. Similarly, a polypeptide of the invention may be chemically modified with one or more functional groups provided that such functional groups do not interfere with its function.

The terms "polypeptide", "peptide", or "protein" are used interchangeably herein to designate a linear series of amino acid residues connected one to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues. The amino acid residues are usually in the natural "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide.

Unless otherwise defined, all technical and scientific terms used herein have the meaning commonly understood by a person who is skilled in the art in the field of the present invention.

Throughout the specification, unless the context demands otherwise, the terms "comprise" or "include", or variations such as "comprises" or "comprising", "includes" or " including" will be understood to imply the inclusion of a stated integer or group of integers, but not the exclusion of any other integer or group of integers.

The present invention will now be exemplified with reference to the following non-limited figure and examples which are provided for the purpose of illustration and are not intended to be construed as being limiting on the present invention. Other embodiments of this invention will be apparent to those of ordinary skill in the art in view of this description.

Figure 1 shows a comparison between the 16S ribosomal (rRNA) gene sequence of the isolate and the 16S rRNA gene sequence of other bacteria using BLAST software.

Example 1 - Isolation and Identification of *Pantoea* sp. SS-17

Environmental soil sampling and culture enrichment

The strain was isolated from soil based on its ability to metabolise benzonitrile. Environmental soil samples were collected from various suburban and agricultural sites in Co. Waterford and Co. Kilkenny, Ireland, with landowners' permission. A 0.5 g sample of each soil was incubated in 50 ml M9 liquid media (Sambrook et al. 1989) containing 0.2% (w/v) glucose as the carbon source and 10 mM nitrile as the nitrogen source. Each soil was incubated in six flasks, each with a different nitrile: acetonitrile, benzonitrile, adiponitrile, mandelonitrile, acrylonitrile and phenylacetonitrile. Flasks were incubated for 28 days at 25°C and 175 rpm. Soil cultures were sampled on day 3, 7, 11, 14, 21 and 28. Flasks were allowed to stand for 2 hours before sampling to allow larger soil particles to settle. Two 1 ml samples were withdrawn from the top of the culture and dispensed in sample tubes. These 1 ml portions were centrifuged for 1 min at 1,000$ to pellet remaining large soil particles. 0.8 ml portions of the resulting supernatant were transferred to fresh

sample tubes for immediate further analysis, or storage at -70°C after addition of glycerol to a final concentration of 30% (v/v). Soil supernatants were serially diluted (from 10^{-1} to 10^{-8}) and all dilutions were plated onto CMM agar containing glucose and the same nitrile as used for enrichment, at the same concentration as the sole nitrogen source. Single colonies were picked and serially streaked to fresh CMM agar (containing glucose and nitrile) up to ten times to obtain pure isolates.

Nitrilase gene screening and degenerate clade-specific PCR primer design

Degenerate primers were designed from ClustalW alignments of nitrilase genes from the database. For the sequence clade targets 1A, IB, 2, 3, 4, 5A, 5B and 6 (Robertson et al. 2004), a total of 38, 32, 52, 5, 18, 14 and 27 sequences were used in ClustalW alignments. The forward and reverse primer sequences are shown in Table 1. It should be noted that there are two different 2A clade reverse primers, each targeting a different sub-group within the clade.

Table 1. Sequences of Forward and Reverse Primers.

Primer Name	**Sequence 5'-3'**
1B F (SEQ ID NO: 4)	TGYTGGGARCAYTACAAYCC
1B R (SEQ ID NO: 5)	TCCATCATBCKYTTKCGYTT
2A F (SEQ ID NO: 6)	GSVYTBTGCTGYTGGGARCA
2A R1 (SEQ ID NO: 7)	RTARTGVCCRGCVGGRTC
2A R2 (SEQ ID NO: 8)	GARTARTGSCCGRCSGGRTC

Each 15 µï PCR reaction mixture contained 7.5 µï GoTaq Green Master Mix (Promega, UK), 15 pmoles of each primer and cells of individual isolates adjusted to O.D.@6oo = 0.04. Each isolate was subjected to PCR screening with each primer pair.

The 16S ribosomal DNA from all environmental isolates that yielded positive results for nitrilase gene detection was amplified using primers 63f and 1387r and PCR conditions described in Marchesi et al. 1998. The touchdown PCR conditions for all primer sets consisted of 1 cycle at 95°C for 5 min, 16 cycles of 95°C for 1 min, 58-51°C for 1 min, 72°C for 40 s, with a reduction of annealing temperature by 1°C

every 2 cycles, 20 cycles of 95°C for 1 min, 50°C for 1 min, 72°C for 40 s, followed by 1 cycle of 72°C for 8 min. All PCR products were analysed by DNA gel electrophoresis.

Identification of isolates and nitrile metabolising genes

All positive PCR products were first purified using the DNA Clean and Concentrator-5 kit (Zymo Research, CA, USA), as per the manufacturer's instructions. Purified nitrilase PCR products were first cloned using the PCR cloning kit (Qiagen, Germany) and NovaBlue Gigasingles competent cells (Novagen, Germany), before plasmid extractions were prepared using the Genelute plasmid mini-prep kit (Sigma, UK), as per the manufacturer's instructions. The nitrilase gene was detected from SS-17 using primers specific for clade IB.

Part of the 16S rRNA gene was screened for identification purposes (a ~1300 base pair region from the full ~1550 bp). The sequencing of 16S rRNA gene PCR products or nitrilase-bearing plasmids was performed using an ABI Prism 310 Genetic Analyser (Applied Biosystems, CA, USA) as per the manufacturer's instructions. The primers used for the sequencing reactions of the 16S rRNA genes were those used in the PCR amplifications, while primers specific for the cloning vector (T7 promoter and T7 terminator) were used to sequence the nitrilase genes.

The partial DNA sequence of the *Pantoea* sp. SS-17 16S rRNA gene is shown below as SEQ ID NO: 3:

AATGTCTGGGAAACTGCCCGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCG
CATAACGTCTTCGGACCAAAGTGGGGGACCTTCGGGCCTCACACCATCGGATGTGCCCAG
ATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTG
AGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAG
TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAG
GCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGATGAGGTTAATAACCTTAT
CGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATA

CGGAGGGTGCAACCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCA
AGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTGAAACTGGCAGGCTTGA
GTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGG
AATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGG
GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGACTTGGAGG
TTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTAC
GGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGT
GGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGACATCCACAGAACTTTCCA
GAGATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCG
TGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGC
GATTCGGTCGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGA
CGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGCGCATACAA
AGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATCGGA
GTCTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGTAGATCAGAA

Nucleotide sequences were analysed using blastn or blastx software (Altschul et al. 1990) (http://www.ncbi.nlm.nih.gov/BLAST/) from the GenBank (NCBI) database and DNASTAR Lasergene Pro (http://www.dnastar.com/).

The genus/species of the isolate was determined by comparing its 16S ribosomal (rRNA) gene sequence to the 16S rRNA gene sequence of other bacteria. The comparison results obtained using BLAST software are shown in Figure 1. The highest identity seen between SS-17 and other known strains was 99%. The isolate was therefore determined to be a novel strain of the *Pantoea* sp. or more specifically *Pantoea* sp. SS-17. The isolate was deposited at the National Collection of Industrial and Marine Bacteria (NCIMB) on 29 June 2011 where it was assigned the accession number NCIMB 41854.

The nitrilase gene from this strain was sequenced in part and was found to differ from any other known nitrilase genes. The partial DNA sequence of the nitrilase gene is set forth as SEQ ID NO:l and the partial amino acid sequence of the nitrilase

gene is set forth as SEQ ID NO: 2. The gene sequence may be synthesised commercially and inserted into a host such as *E.coli.* for activity analyses.

Example 2 - Initial activity screening of *Pantoea* sp. SS-17 isolates

Isolates were inoculated to 600 µl M9 minimal media broth in 96-well microplate blocks containing 0.2% (w/v) glucose as the carbon source and 10 mM nitrile as the N source and incubated at 25°C with shaking at 200 rpm. The nitriles used were 3-hydroxyglutaronitrile, 3-hydroxyphenylpropionitrile and 3-hydroxybutyronitrile. Isolates were subcultured to fresh media to ensure induction of nitrile-metabolising enzymes. Whole-cell activity assays of induced, subcultured clones on the same nitriles were carried out using the Nesslerisation method for ammonia determination, as described previously (Snell and Colby 1999).

The activity or speed of the nitrile conversion of the isolate, although not optimised, was found impressive. In particular, the enzyme was shown to have activity towards 3-hydroxy-butyronitrile. The activity of SS-17 towards 3-hydroxy-butyronitrile was 2.42 U/ml with a specific activity of 62.24 U/mg dry cells. Activity may be further optimised by, for example, changing the temperature.

Example 3 - Instrumentation-based activity and enantioselectivity analyses on isolates and recombinant clones

The required quantity of washed induced cells will be suspended in buffer to a fixed total volume and pH. The substrate will be added to the suspension in the required quantity and reaction mixtures will be incubated /shaken/gently stirred at the optimum temperature for the required time. Reactions will be quenched using a suitable acid (or reagent) and the biomass will be removed. Nitrile conversion to acid will be monitored in some cases directly on the supernatant using reverse phase, high performance liquid chromatography. In other cases, extraction using a suitable solvent and sample derivatisation will be required. Derivatisation transforms the unreacted nitrile into a product of similar structure which can be analyzed more efficiently by chromatography. In this case, the products dissolved in

a suitable organic solvent will be stirred with the required derivatising reagent for the required reaction time. After work-up, aliquots will be analysed using reverse phase high performance liquid chromatography or gas chromatography.

To measure enantioselectivity, the supernatant post biomass removal will be extracted with a suitable solvent. Aliquots of the extract will be analysed by normal phase high performance liquid chromatography employing a column with chiral stationary phase or gas chromatography with a suitable chiral column. Again, in some cases, sample derivitisation will be required as outlined in the paragraph above prior to analysis by chiral chromatography.

Example 4 - characterisation of activity of *Pantoea* sp. SS-17 towards 3-Hydroxybutyronitrile

Method

A solution of potassium phosphate buffer (0.1M, pH= 7.2) containing induced cells @OD600nm was activated at 25°C for 30 minutes with orbital shaking. Racemic nitrile (10 mM) was added in one portion to the flask and the mixture incubated using an orbital shaker (250 rpm). The reaction was quenched after 24 hours by removal of the biomass by centrifugation at 3,000 g. The resulting aqueous solution was acidified by the addition of 1M HC1 (200 µL). The aqueous portion was then extracted with ethyl acetate, the extracts were dried over $MgSO_4$ and the solvent removed under vacuum. Silver oxide (1 equiv, 0.06 mmol, 13.6 mg), benzylbromide (4 equiv, 0.24 mmol, 28 µL) and dichloromethane (2 mL) were added and the mixture stirred in the dark for 24 hours. The reaction mixture was diluted with acetone and filtered through a 0.45 µm filter and removal of the solvent under vacuum. 1 mL of mobile phase (90% hexane : 10% IPA) was added before the solution was injected on the Chiral HPLC system. Chiralcel AD-H and OJ-H columns (all from Daicel Chemical Industries) were used for chiral analysis. Chiralcel AD-H was used for the resolution of /Miydroxyacids. Analytical conditions applied: 90 % hexane, 10% IPA and 0.1% TFA, with a flow rate of 0.8 mL/min and a detection wavelength of 215 nm. Chiralcel OJ-H was used for the resolution of β-

hydroxyamides and nitriles using the same mobile phase conditions with the exception of TFA. The biotransformation products of 3-hydroxybutyronitrile were first derivatised to their corresponding /?-benzyloxyethers before analysis.

Results

No significant influence of reaction temperature on the activity of *Pantoea* sp. SS-17 was observed when the isolates were assayed at various temperatures ranging from 15°C to 30°C, suggesting that activity is robust over a range of temperatures. The isolate demonstrated the highest activity at 25°C (Table 3). However, optimum growth was observed at lower temperatures (Table 2). The effect of temperature on the enantioselectivity of the biotransformation was examined at 15°C and 25°C with an optimum enantioselectivity of 15.6% after incubation at 15°C for 24 hours (Table 3).

Table 2. Effect of temperature on growth.

Isolate	Growth				Optimum temperature
	15°C	20°C	25°C	30°C	
SS-17	1.87	1.1	0.5	0.61	15°C

Table 3. Effect of temperature on activity and enantioselectivity.

Isolate	Time (Hours)	Temperature (°C)	Activity (mmol)	Acid EE %	Nitrile EE%
SS-17	6	25	0.26	8.2%	2.4%
SS-17	24	25	0.37	12.4%	6.3%
SS-17	24	15	0.28	15.6%	8.2%

The invention now being fully described, it will be apparent to one of ordinary skill in the art that changes and modifications may be made thereto without departing from the scope of the claims.

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Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Wade WG (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Environ Microbiol 64:795-799.

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Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning, 628 2nd edn. CSHL Press.

Snell D, Colby J (1999) Enantioselective hydrolysis of racemic ibuprofen amide to s-(+)-ibuprofen by *Rhodococcus* AJ270. Enzyme Microb Technol 24:160-163.

Claims

1. An isolated polypeptide comprising SEQ ID NO:2 or a variant amino acid sequence having at least 98% sequence identity with SEQ ID NO:2 wherein said polypeptide is a nitrilase.

2. The isolated polypeptide as claimed in claim 1 wherein the variant amino acid sequence has at least 99% sequence identity with SEQ ID NO:2.

3. The isolated polypeptide as claimed in claim 1 or 2 wherein the variant amino acid sequence differs from SEQ ID NO:2 solely due to the presence of one or more conservative amino acid substitutions.

4. The isolated polypeptide as claimed in claim 3 wherein the variant amino acid sequence differs from SEQ ID NO:2 solely due to the presence of three or less conservative amino acid substitutions.

5. The isolated polypeptide as claimed in claim 1 wherein the polypeptide comprises SEQ ID NO:2.

6. The isolated polypeptide as claimed in any one of claims 1 to 5 wherein the polypeptide is obtainable by culturing *Pantoea* sp. SS-17 NCIMB 41854.

7. An isolated nucleotide sequence comprising SEQ ID NO:l or a variant nucleotide sequence which has at least 98% sequence identity to

(a) SEQ ID NO: 1, or

(b) a nucleotide sequence that is capable of hybridising to SEQ ID NO:l under stringent conditions,

wherein said isolated nucleotide sequence encodes an amino acid sequence having nitrile-metabolising activity.

8. The isolated nucleotide sequence as claimed in claim 7 wherein the variant nucleotide sequence has at leat 99% sequence identity to

(a) SEQ ID NO: 1, or

(b) a nucleotide sequence that is capable of hybridising to SEQ ID NO:l under stringent conditions.

9. The isolated nucleotide sequence as claimed in claim 7 wherein the variant nucleotide sequence has 100% sequence identity to

(a) SEQ ID NO: 1, or

(b) a nucleotide sequence that is capable of hybridising to SEQ ID NO:l under stringent conditions.

10. The isolated nucleotide sequence as claimed in claim 7 wherein the isolated nucleotide sequence comprises SEQ ID NO:l.

11. The isolated nucleotide sequence as claimed in any one of claims 7 to 10 wherein the isolated nucleotide sequence is obtainable by culturing *Pantoea* sp. SS-17 NCIMB 41854.

12 A gene construct comprising the isolated nucleotide sequence as claimed in any one of claims 7 to 11 and a control sequence.

13. A vector comprising an isolated nucleotide sequence as claimed in any one of claims 7 to 11 and a promoter which is operably linked to said nucleotide sequence.

14. A process for making an organism suitable for metabolising nitriles to carboxylic acids comprising the step of introducing the isolated nucleotide sequence as claimed in any one of claims 7 to 11 into the organism.

15. A method of producing a polypeptide encoded by a nucleotide sequence as claimed in any one of claims 7 to 11, the method comprising the steps of:

(a) contacting a bacterial cell and / or an insect cell and / or a yeast cell and / or a plant cell with a vector as claimed in claim 13, and

(b) cultivating said bacterial cell and / or insect cell and / or yeast cell and / or plant cell under conditions suitable for the production of the polypeptide.

16. An isolated polypeptide encoded by the isolated nucleotide sequence as claimed in any one of claims 7 to 11, wherein the polypeptide is a nitrilase.

17. An isolated *Pantoea* strain wherein said strain is that deposited under NCIMB 41854.

18. An isolated nitrilase polypeptide obtainable by culturing *Pantoea* sp. SS-17 NCIMB 41854.

19. A process for producing a carboxylic acid, the process comprising the step of treating a nitrile with the polypeptide as claimed in any one of claims 1 to 6, 16 or 18 to produce the carboxylic acid.

20. The process as claimed in claim 19 wherein the polypeptide is the polypeptide as claimed in claim 3.

21. The process as claimed in claim 19 wherein the polypeptide is the polypeptide as claimed in claim 4.

22. The process as claimed in claim 19 wherein the polypeptide is the polypeptide as claimed in claim 5.

23. The process as claimed in claim 19 wherein the polypeptide is the polypeptide as claimed in claim 6.

24. The process as claimed in claim 19 wherein the polypeptide is the polypeptide as claimed in claim 16.

25. The process as claimed in claim 19 wherein the polypeptide is the nitrilase as claimed in claim 18.

26. The process as claimed in any one of claims 19 to 25 wherein the nitrile is 3-hydroxyglutaronitrile and the carboxylic acid is (R)-4-cyano-hydroxybutyric acid.

27. The process as claimed in any one of claims 19 to 25 wherein the nitrile is 3-hydroxybutyronitrile and the carboxylic acid is a beta-hydroxy carboxylic acid.

28. The process as claimed in any one of claims 19 to 25 wherein the nitrile is 3-hydroxy-phenylpropionitrile and the carboxylic acid is beta-hydroxy carboxylic acid.

29. The process as claimed in any one of claims 19 to 28 wherein the process is carried out at a temperature of about 25°C.

30. The process as claimed in any one of claims 19 to 28 wherein the process is carried out at a temperature of about 15°C.

31. A process for producing a carboxylic acid, the process comprising the step of incubating a nitrile with the strain deposited as NCIMB 41854.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU463165.1	Uncultured bacterium clone molerat_aai70h06 16S ribosomal RNA ge	2219	2219	100%	0.0	99%
FM958439.1	Uncultured gamma proteobacterium partial 16S rRNA gene, clone M4	2197	2197	100%	0.0	99%
HM556680.1	Uncultured bacterium clone BICH1477 16S ribosomal RNA gene, parti	2177	2177	100%	0.0	98%
AM419023.1	Pantoea sp. NJ-87 partial 16S rRNA gene, strain NJ-87	2177	2177	100%	0.0	98%
HM556674.1	Uncultured bacterium clone BICH1466 16S ribosomal RNA gene, parti	2176	2176	100%	0.0	98%
HM556855.1	Uncultured bacterium clone BICH614 16S ribosomal RNA gene, partia	2168	2168	100%	0.0	98%
HM556354.1	Uncultured bacterium clone TIBW777 16S ribosomal RNA gene, partia	2168	2168	100%	0.0	98%
HM556315.1	Uncultured bacterium clone TIBW646 16S ribosomal RNA gene, partia	2168	2168	100%	0.0	98%
HM556753.1	Uncultured bacterium clone BICH452 16S ribosomal RNA gene, partia	2165	2165	100%	0.0	98%
HM556370.1	Uncultured bacterium clone TIBW843 16S ribosomal RNA gene, partia	2165	2165	100%	0.0	98%
HM556264.1	Uncultured bacterium clone TIBW424 16S ribosomal RNA gene, partia	2165	2165	100%	0.0	98%
HM556916.1	Uncultured bacterium clone BICH710 16S ribosomal RNA gene, partia	2161	2161	100%	0.0	98%
HM556802.1	Uncultured bacterium clone BICH529 16S ribosomal RNA gene, partia	2161	2161	100%	0.0	98%
HM556749.1	Uncultured bacterium clone BICH448 16S ribosomal RNA gene, partia	2161	2161	100%	0.0	98%
HM557047.1	Uncultured bacterium clone BICH916 16S ribosomal RNA gene, partia	2159	2159	100%	0.0	98%
HM556925.1	Uncultured bacterium clone BICH723 16S ribosomal RNA gene, partia	2159	2159	100%	0.0	98%
HM556543.1	Uncultured bacterium clone BICH1237 16S ribosomal RNA gene, parti	2159	2159	100%	0.0	98%
HM556445.1	Uncultured bacterium clone BICH1067 16S ribosomal RNA gene, parti	2159	2159	100%	0.0	98%
HM556415.1	Uncultured bacterium clone BICH1020 16S ribosomal RNA gene, parti	2159	2159	100%	0.0	98%
HM556317.1	Uncultured bacterium clone TIBW651 16S ribosomal RNA gene, partia	2159	2159	100%	0.0	98%
HM556276.1	Uncultured bacterium clone TIBW470 16S ribosomal RNA gene, partia	2159	2159	100%	0.0	98%
HM582879.1	Erwinia tasmaniensis strain AR_PINLTS5 16S ribosomal RNA gene, pa	2159	2159	100%	0.0	98%
HM557022.1	Uncultured bacterium clone BICH879 16S ribosomal RNA gene, partia	2158	2158	100%	0.0	98%
HM556891.1	Uncultured bacterium clone BICH672 16S ribosomal RNA gene, partia	2158	2158	100%	0.0	98%
HM556851.1	Uncultured bacterium clone BICH609 16S ribosomal RNA gene, partia	2158	2158	100%	0.0	98%
HM556600.1	Uncultured bacterium clone BICH1341 16S ribosomal RNA gene, parti	2158	2158	100%	0.0	98%
HM556219.1	Uncultured bacterium clone TIBW1388 16S ribosomal RNA gene, part	2158	2158	100%	0.0	98%

Figure 1

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/063321

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/78 C12P7/40
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)
C12N C12P

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, EMBASE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/106662 A2 (VERENIUM CORP [US] ; DESANTIS GRACE [US] VERENIUM CORP [US] ; DESANTIS G) 4 September 2008 (2008-09-04) page 92 - page 93; sequences 9, 10	1-31

X	WO 03/000840 A2 (DIVERSA CORP [US] ; DESANTIS GRACE [US] ; CHAPLIN JENNIFER ANN [ZA] ; WEI) 3 January 2003 (2003-01-03) page 107; sequences 183, 184 page 114 page 138 page 231	1-31

	-/- ·	

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

"E" earlier application or patent but published on or after the international filing date

"L" documentwhich may throw doubts on priority claim(s) orwhich is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 August 2012	09/10/2012

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	Weinberg, Suzanna

1

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.
A	COFFEY LEE ET AL: "Isolation of identical nitrilase genes from multiple bacterial strains and real-time PCR detection of the genes from soils provides evidence of horizontal gene transfer. ", ARCHIVES OF MICROBIOLOGY OCT 2009 LNKD-PUBMED: 19730817 , vol . 191 , no. 10, October 2009 (2009-10) , pages 761-771 , XP002682457 , ISSN : 1432-072X abstract ----	1-31

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/063321

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2008106662 A2	04-09-2008	CA 2679503 Al	04-09-2008
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		EP 2327766 Al	01-06-2011
		EP 2327767 Al	01-06-2011
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