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Alfred [DE/DE]; Universität Bielefeld, Universitätsstrasse  
25, 33615 Bielefeld (DE).

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(74) Agent: TARUTTIS, Stefan; Aegidientorplatz 2b, 30159  
Hannover (DE).

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(71) Applicants (for all designated States except US):  
HELMHOLTZ-ZENTRUM FÜR INFESTIONS-  
FORSCHUNG GMBH [DE/DE]; Inhoffenstrasse 7,  
38124 Braunschweig (DE). UNIVERSITÄT DES  
SAARLANDES [DE/DE]; Campus, 66123 Saarbrücken  
(DE). UNIVERSITÄT BIELEFELD [DE/DE]; Univer-  
sitätsstrasse 25, 33615 Bielefeld (DE).

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(71) Applicants (for US only): GERTH, Klaus [DE/DE];  
Inhoffenstr. 7, 38124 Braunschweig (DE). PERLOVA,  
Lena [DE/DE]; Universität des Saarlandes, Campus,  
66123 Hannover (DE). MÜLLER, Rolf [DE/DE]; Uni-  
versität des Saarlandes, Campus, 66123 Saarbrücken (DE).  
SCHNEIKER, Susanne [DE/DE]; Universität Bielefeld,  
Universitätsstr. 25, 33615 Bielefeld (DE). PÜHLER,

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(54) Title: GENES ENCODING THE BIOSYNTHETIC PATHWAY FOR ETNANGIEN PRODUCTION

(57) Abstract: The present invention provides the genes encoding the biosynthetic pathway enzymes and the biosynthetic pathway enzymes for producing in a host micro-organism the compound etnangien. The invention provides the coding sequences and the respective translation products obtainable therefrom, which constitute the coding sequences and the catalytically active translation products obtainable therefrom, necessary for the biosynthetic pathway for the production of etnangien in micro-organisms.

### **Genes encoding the biosynthetic pathway for etnangien production**

The present invention relates to biosynthetic pathway coding sequences, the translation products of which are part of or constitute the biosynthetic pathway metabolism of etnangien, a polyketide compound having antibiotic activity.

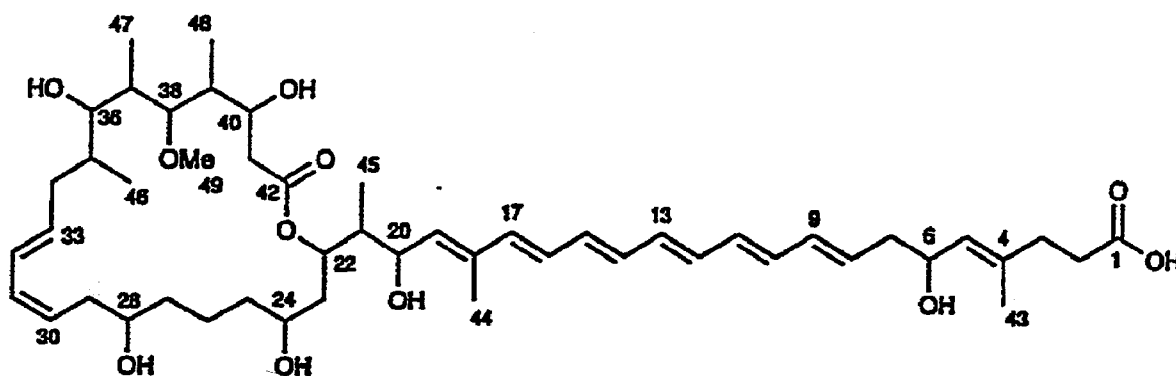
In accordance with the provision of the biosynthetic pathway coding sequences or genes encoding the enzymes constituting the biosynthetic pathway involved in the production of etnangien, the present invention provides a process for the production of etnangien, making use of the biosynthetic pathway genes and their translation products, as well as microorganisms, especially bacteria, which are genetically manipulated to contain at least one of the biosynthetic pathway genes, for the production of etnangien.

Further, the present invention provides a production process including an isolation method for separating the novel compound from an aqueous mixture, e.g. from a fermentation broth.

## State of the art

DE 19630980 A1 describes an antibiotic compound termed etnangien, which is obtained by fermentation of *Sorangium cellulosum* (DSM 11028) without genetic manipulation, adsorbed to Amberlite XAD adsorber resin and eluted from the adsorber resin using methanol. The methanol eluate can further be purified by extraction with acetic acid ester, dissolving in diethylether, extracting with aqueous sodium hydrogencarbonate, acidifying, and extracting with dichloromethane. After removal of dichloromethane, the residue can be purified by reverse phase chromatography using methanol/water as the eluent, extracting the aqueous phase with acetic acid ester, and drying.

The structure given for etnangien is:



The antibacterial activity that has been found for etnangien is indicated to be based on the inhibition of bacterial DNA- dependent RNA polymerase. However, during the development of the present invention, a compound having the known structure could not be found.

## Objects of the invention

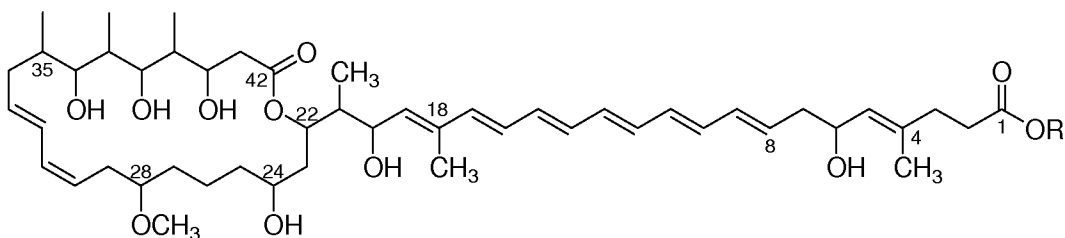
In view of the fermentation process for etnangien using a wild-type isolate bacterium as described in the above prior art, the present invention seeks to provide the coding sequences, the translation products of which constitute the biosynthetic pathway enzymes for the production of etnangien, e.g. for use in the genetic manipulation of bacteria.

Further, the present invention seeks to provide microorganisms, e.g. bacteria that are genetically manipulated to contain at least one of the biosynthetic pathway genes, and the use

of the said genetically manipulated bacteria for the production of etnangien by a fermentation process involving the genetically manipulated micro-organism.

### General description of the invention

The present invention attains the above-mentioned objects by providing the genes encoding the biosynthetic pathway enzymes and the biosynthetic pathway enzymes for producing in a host micro-organism the compound etnangien according to formula I having antibiotic activity:



(I),

wherein R is hydrogen. Although the core structure of the compounds according to the present invention differs in a hydroxyl group at C-38 instead of the methoxy group at C-38 in the compound according to DE 19630980 A1, the compound produced according to the present invention is presently termed etnangien.

In a first embodiment, the present invention provides the coding sequences and the respective translation products obtainable therefrom, which constitute the coding sequences and the catalytically active translation products obtainable therefrom, necessary for the biosynthetic pathway for the production of etnangien in micro-organisms.

In a second embodiment, the present invention provides genetically modified microorganisms, preferably bacteria, which have been provided with at least one of the coding sequences encoding the biosynthetic pathway enzymes for the production of etnangien, with the coding sequences introduced by genetic manipulation being contained in expression cassettes. The expression cassettes containing the coding sequences for the biosynthetic pathway of etnangien can contain a single coding sequence each, also referred to

as open reading frame (orf), or multi-cistronic expression cassettes containing two or more of the coding sequences of the invention.

Since it has been found that biosynthesis of etnangien in bacteria requires presence of the translation products of each orf 1 to orf 25 as identified in the present invention, the coding sequences of the invention in a first aspect can be used for genetic manipulation of bacteria that naturally contain all of orf 1 to orf 25, e.g. for duplication or further increase of copy number of certain orfs by introducing the respective coding sequences in addition to the natural genome.

In a second aspect, the coding sequences of the invention can be used for complementation of an incomplete natural set of the biosynthetic pathway genes, namely by introducing the coding sequence missing in the original bacterial strain. Accordingly, the resultant micro-organism contains a complete set of coding sequences for the biosynthetic pathway enzymes for etnangien synthesis, containing both original coding sequences and coding sequences selected from orf 1 to orf 25 of the invention introduced by genetic manipulation.

In a third aspect, the complete set of coding sequences of the invention, namely orf 1 to orf 25, can be used for introduction into a micro-organism originally devoid of these coding sequences in order to establish the biosynthetic pathway for etnangien for the first time in the respective micro-organism. Accordingly, the resultant micro-organism is provided with the entire set of orf 1 to orf 25 of the invention as additional orfs to the natural genome.

Preferably, the genetically manipulated micro-organism of the invention contains one or more copies of the coding sequences encoding the biosynthetic pathway enzymes for etnangien in order to enhance production rates of etnangien. Micro-organisms can be selected from the group comprising myxobacteria, lysobacteria, streptomyces spp. and pseudomonas spp. In the case of bacteria of the genus Sorangium, also referred to as Polyangium, genetically manipulated bacteria of the invention contain at least one copy of the least one coding sequence of the pathway enzymes for the production of etnangien in addition to naturally occurring copies of genes encoding biosynthetic pathway enzymes, e.g. as described for the first aspect.

It has been found that in the Sorangium strain analysed, designated So ce56, the biosynthetic

pathway genes are arranged as consecutive orfs in multicistronic expression cassettes. Accordingly, it is preferred that the microorganisms genetically manipulated according to the invention contain at least two of orf 1 to orf 25 in a multi-cistronic expression cassette that is introduced by genetic manipulation. Especially preferred expression cassettes are a first multi-cistronic expression cassette containing a promoter sequence and, arranged downstream thereof, orf 1 (Seq.-ID No. 1), orf 2 (Seq.-ID No. 2), orf 3 (Seq.-ID No. 3), orf 4 (Seq.-ID No. 4), orf 5 (Seq.-ID No. 5), orf 6 (Seq.-ID No. 6), orf 7 (Seq.-ID No. 7), orf 8 (Seq.-ID No. 8), orf 9 (Seq.-ID No. 9), orf 10 (Seq.-ID No. 10), orf 11 (Seq.-ID No. 11), orf 12 (Seq.-ID No. 12), orf 13 (Seq.-ID No. 13), and a terminator sequence; and/or, a second multi-cistronic expression cassette, containing a promoter sequence, and arranged downstream thereof, orf 15 (Seq.-ID No. 15), orf 16 (Seq.-ID No. 16), orf 17 (Seq.-ID No. 17), orf 18 (Seq.-ID No. 18), orf 19 (Seq.-ID No. 19), orf 20 (Seq.-ID No. 20), orf 21 (Seq.-ID No. 21), followed by a terminator sequence., and/or a third multi-cistronic expression cassette containing a promoter sequence, and arranged downstream thereof, orf 22 (Seq.-ID No. 22), orf 23 (Seq.-ID No. 23), followed by terminator, and/or a fourth multi-cistronic expression cassette, containing a promoter sequence, and arranged downstream thereof, orf 24 (Seq.-ID No. 24, orf 25 (Seq.-ID No. 25), and/or a fifth monocistronic expression cassette, containing a promoter sequence, and arranged downstream thereof, orf 14 (Seq.-ID No. 14), followed by a terminator sequence.

In a preferred embodiment, the host micro-organism harbouring by genetic manipulation at least one copy of an orf encoding a biosynthetic pathway enzyme for the production of etnangien, in addition contains a mutation in the *rpsL* gene coding for the S12 ribosomal protein, causing a lysine to arginine mutation at amino acid No. 88, which can e.g. be caused by an A to G transition at nucleotide 263 in the S12 protein encoding exon.

The biosynthetic pathway genes encoding the biosynthetic pathway enzymes involved in the production of etnangien comprise the coding sequences listed in following table 1, designated orf 1 to orf 25. For each orf, the DNA sequence is given.

Accordingly, the present invention provides the biosynthetic pathway genes encoded by coding sequences for the etnangien biosynthetic pathway enzymes selected from the group comprising Seq.-ID No 1 to Seq.-ID No 14 and complementary sequences Seq.-ID No 15 to Seq.-ID No 25. Preferably, the biosynthetic pathway enzymes are encoded by Seq.-ID No 1 to

Seq.-ID No 14 and complementary sequences Seq.-ID No 15 to Seq.-ID No 25, i.e. the biosynthetic pathway enzymes comprise the amino acid sequences of Seq.-ID No 26 to Seq.-ID No. 50.

Table 1: Coding sequences and their translation products constituting the biosynthetic pathway enzymes for etnangien biosynthesis

nucleotide sequence	amino acid sequence	protein/gene	length, bp/aa	Description
<b>upstream region</b>				
Seq.-ID No 1	Seq.-ID No 26	orf 1	315/104	hypothetical protein
Seq.-ID No 2	Seq.-ID No 27	orf 2	918/305	malonyl CoA-acyl carrier protein transacylase
Seq.-ID No 3	Seq.-ID No 28	orf 3	1902/633	asparagine synthetase
Seq.-ID No 15 (complementary)	Seq.-ID No 40	orf 15	1380/459	putative FMN-dependent oxidoreductase
Seq.-ID No 16 (complementary)	Seq.-ID No 41	orf 16	251/82	acyl carrier protein
Seq.-ID No 17 (complementary)	Seq.-ID No 42	orf 17	1233/410	ketosynthase-like decarboxylase
Seq.-ID No 18 (complementary)	Seq.-ID No 43	orf 18	1260/419	HMG-CoA synthase
Seq.-ID No 19 (complementary)	Seq.-ID No 44	orf 19	768/255	Enoyl-CoA hydratase
Seq.-ID No 20 (complementary)	Seq.-ID No 45	orf 20	750/249	Enoyl-CoA hydratase
Seq.-ID No 21 (complementary)	Seq.-ID No 46	orf 21	3336/1111	hypothetical protein
Seq.-ID No 22 (complementary)	Seq.-ID No 47	orf 22	366/121	hypothetical protein
Seq.-ID No 23 (complementary)	Seq.-ID No 48	orf 23	1410/469	putative amidase

Table 1 continued:

Seq.-ID No 24 (complementary)	Seq.-ID No 49	orf 24	789/262	conserved hypothetical protein
Seq.-ID No 25 (complementary)	Seq.-ID No 50	orf 25	3577118	conserved hypothetical protein
<b>PKS region</b>				
Seq.-ID No 4	Seq.-ID No 29	orf 4	15438/5146	PKS
Seq.-ID No 5	Seq.-ID No 30	orf 5	11484/3828	PKS
Seq.-ID No 6	Seq.-ID No 31	orf 6	10884/3628	PKS
Seq.-ID No 7	Seq.-ID No 32	orf 7	15210/5070	PKS
Seq.-ID No 8	Seq.-ID No 33	orf 8	10734/3578	PKS
Seq.-ID No 9	Seq.-ID No 34	orf 9	17919/5973	PKS
<b>downstream region</b>				
Seq.-ID No 10	Seq.-ID No 35	orf 10	1374/458	MATE efflux family protein
Seq.-ID No 11	Seq.-ID No 36	orf 11	2076/692	acyltransferase/ oxidoreductase
Seq.-ID No 12	Seq.-ID No 37	orf 12	1041/347	-
Seq.-ID No 13	Seq.-ID No 38	orf 13	1656/552	Rhs family protein
Seq.-ID No 14	Seq.-ID No 39	orf 14	534/178	-

The descriptions for enzymatic activities given in table 1 are inferred from computer-assisted analysis of the sequences. Therefore, the coding sequences and their respective translation products define the biosynthetic pathway enzymes of the invention, which may have different activities than indicated in table 1.

It has been found during the preparation of this invention that etnangien produced by fermentation is preferably isolated in the presence of a stabiliser, e.g. 4-tert.-butyl-o-dihydroxybenzene to avoid degradation.

Optionally, the fermentation process is performed in the presence of an adsorber resin, followed by the isolation of cell mass and of the optional adsorber resin, extraction of etnangien from cell mass and/or of the adsorber resin.

Preferably, further extractive and/or chromatographic processing steps follow for purification of etnangien, optionally followed by derivatisation reactions to generate derivatives of etnangien.

Preferably, an adsorber resin having aromatic side chains, e.g. a divinylbenzene copolymer, is present during the fermentation or contacted with the fermentation broth following cultivation. The adsorber resin is preferably isolated from the fermentation broth and transferred to a chromatography column, e.g. an open or expanded bed column. The resin is preferably washed with water to remove adherent cells. For extraction of etnangien, the resin is preferably extracted with an alcohol, e.g. methanol. The alcoholic extract is buffered with aqueous ammonium acetate and concentrated, e.g. by removal of the alcohol. The residue is extracted with an organic solvent, e.g. ethyl acetate (EtOAc), which solution is preferably concentrated by removal of the organic solvent, preferably by evaporation in the presence of toluene, allowing mild evaporation conditions. This first residue can be re-dissolved in the organic solvent and extracted with an aqueous carbonate solution, e.g. sodium hydrogen carbonate. The aqueous phase is acidified, e.g. to pH 5, and extracted with an organic solvent, e.g. EtOAc. The organic phase is preferably partitioned with aqueous buffer solution, e.g. ammonium acetate to shift the pH to a range of 6.5. to 7.3, preferably to pH 6.8 before removal of the organic solvent, e.g. by evaporation to obtain a second residue. Preferably, evaporation is performed in the presence of toluene. The second residue contains etnangien and can be purified further, e.g. by reverse phase chromatography.

Generally, it is preferred to isolate etnangien at temperatures below 10 °C, more preferred below 5 °C, most preferred at about 0 °C. Further, it is generally preferred to add 1,2-dihydroxy-4-tert.-butylbenzene to etnangien containing preparations for enhanced stability. For storage, an alcoholic solution of etnangien, e.g. in methanol, is preferred.

For production of etnangien, a production process is preferred comprising the cultivation of a producer micro-organism, e.g. *Sorangium cellulosum*, genetically manipulated to contain at least one additional copy of at least one of the genes encoding the biosynthetic pathway enzymes for the production of etnangien.

### Detailed description of the invention

The present invention is now described by way of examples with reference to the figures, wherein

- Figure 1 schematically shows the natural arrangement of coding sequences orf 1 to orf 25,
- Figure 2 schematically shows catalytic activities and the respective etnangien biosynthesis steps,
- Figure 3 A schematically shows the integration of a DNA fragment resulting in a knock-out of etnangien production, Figure 3 B shows the analysis of PCR products of the resultant knock-out mutants in comparison to the wild-type genome,
- Figure 4 shows an HPLC chromatogramme of a knock-out mutant and a wild-type etnangien producer, and
- Figure 5 A schematically shows the integration of a DNA fragment resulting in a knock-out of etnangien production and Figure 5 B shows a PCR analysis of respective knock-out mutants.

#### Example 1: Identification of coding sequences participating in the biosynthesis of etnangien

Using knock-out mutagenesis by conjugational transfer according to Kopp et al. (J. Biotechnology 29-40 (2004)) of a natural etnangien producer *Sorangium cellulosum* strain, e.g. strain So ce56, subcloning and sequencing resulted in the identification of coding sequences of the biosynthetic pathway enzymes for etnangien.

A schematic overview of the natural arrangement of coding sequences is given in Figure 1. In So ce56 the biosynthetic pathway enzymes are encoded in a first cluster comprising orf 1 to orf 13 and it is presently assumed that orf 1 to orf 13 are contained in one common operon, i.e. in one expression cassette functionally arranged downstream of one promoter and arranged upstream of one terminator. Using computer-assisted analysis of orf 1 to orf 13, it was concluded that these have polyketide synthase activity and, accordingly, this arrangement can be referred to as a polyketide synthase expression cassette or cluster. Orf 1 to orf 13 have been found to be arranged on one DNA strand. In orf 11, computer-assisted analysis led to the identification of QQGHS LGRFHNQV (Seq.-ID No. 51) as the active site motif.

The catalytic activities of the orfs as derived from computer-assisted analysis and the respective deduced etnangien biosynthesis steps are schematically shown in Figure 2. In the figures, KS indicates ketosynthase, PKS indicates polyketide synthase, DH indicates  $\beta$ -hydroxydehydratase, KR indicates  $\beta$ -ketoacyl reductase, ACP indicates acyl carrier protein, *O*-MT indicates *O*-methyltransferase and MT indicates methyltransferase activity, respectively.

Figure 2 shows that in module 1 biosynthesis of etnangien starts with succinate being loaded to one of the ACPs, possibly assisted by one of the AT domains. Incorporation of the methylgroup at C4 could be carried out by the HMG-CoA synthase encoded by orf 18 and modifying enzymes ACP encoded by orf 16, a KS-like (KS<sup>S</sup>) decarboxylase encoded by orf 17 and two ECHs encoded by orfs 19 and 20, respectively.

The intermediary product is transferred to modules 2 to 5 having KS-DH-KR-ACP activity to catalyze the incorporation of acetate units from malonyl-CoA and the formation of double bonds. Module 6 could cooperate with HMG-CoA synthase and modifying enzymes as well as being involved in methylation at C18.

However, as the intermediate product predicted from the PKS modules and loaded onto the ACP of module 6 is shorter than the one expected for subsequent methylation, modules 2 to 5 could be active iteratively to incorporate additional malonyl-CoA units before methylation by HMG-CoA. Modules 7 to 10 catalyze the substitution with additional four malonyl-CoA units. The ER-activity participating in the catalytic steps associated with module 10 could be provided by the ER of module 16, probably involving the enoyl reduction step.

Presumably, biosynthesis continues on module 12, containing an *O*-methyltransferase domain which could catalyze the *O*-methylation at C28. Incorporation of further malonyl-CoA units is likely carried out by modules 13 and 15 to 19. From this it would follow that DH domains are missing in modules 13 and 15, whereas DH domains were identified in modules 16 and 18. As DH activity is not required in modules 16 and 18, it could be concluded that these DH domains participate in synthesis steps catalysed by modules 13 and 15.

In close spatial relationship and arranged on the opposite strand to orfs 1 to 13, orf 15 to orf 23 have been identified in a polycistronic arrangement, presumably as one common operon.

From computer-assisted analysis, it is at present concluded that orfs 15 to 20 belong to the so-called 3-hydroxymethyl-3-glutaryl (HMG)-CoA synthase cassette protein encoding sequences, including two enoyl-CoA hydratases (ECH) by orf 19 and orf 20, which are probably involved in incorporation of the methyl groups at C4 and C18 of etnangien. From computer-assisted analysis, orf 15 is assumed to contain an 2-nitropropane dioxygenase (NPD) domain associated with S-malonyltransferase with a flavin/adenin binding domain (FABD), an acyl-carrier protein.

Due to the close arrangement of orf 20 and orf 21, which are separated by 41 bp only, it is at present assumed that orf 21, orf 22 and orf 23 are contained in one expression cassette, e.g. are arranged in one operon.

In detail, the gene cluster comprising the biosynthetic pathway genes for etnangien were identified using an initial knock-out mutant that was generated by plasmid integration containing a PCR amplificate that was generated using primers OP73: 5' – aagcttGATC CCGAAGCGATTGTAGA (Seq.-ID No. 52) and OP74: 5' – ggatcCTCAGCAGGA TGTCGAACAA (Seq.-ID No. 53) on chromosomal So ce56 DNA, which amplificate was introduced by conjugational transfer according to Kopp et al. (J. Biotechnology 29-40 (2004)) of plasmid termed pSUPHyg containing the amplificate, now termed pOPB45, into the wild-type producer.

The resultant knock-out mutant is termed So ce56::pOPB45. A schematic representation of the arrangement of the integrating plasmid pOPB45 within the chromosomal biosynthetic gene cluster is shown in Figure 3A. Arrows with numbers indicate the position of the primers used in PCR. For verification of the integration, DNA of the knock-out mutant was analysed by PCR using primers OP105: 5' – GAGAAGCTCCGCTACCTGA (Seq.-ID No. 54) and OP77: 5' – TGAAGTCAGCCCCATACGAT (Seq.-ID No. 55), OP106: 5' – GGATGCCG TAGTTG AGAAGG (Seq.-ID No. 56), and OP78: 5' - GGTGATGTCGGCGATATAGG (Seq.-ID No. 57) as indicated in Figure 3B showing the resultant amplicates on mutant (m) DNA and wild-type (wt) DNA. From the position of primers OP77 and OP78 within the integrated DNA fragment pSUPHyg and the position of primers OP105 and OP106 within adjacent chromosomal DNA, integration of the fragment into the chromosomal etnangien gene cluster is verified.

Figure 4 shows an HPLC chromatogram of culture extracts obtained from the wild-type (wt) *Sorangium cellulosum* in comparison to the knock-out mutant (m). The peak indicating etnangien missing in the mutant extract demonstrates that the integration interrupted the etnangien biosynthetic pathway, and therefore shows that the gene cluster of the present invention is coding for the biosynthetic pathway enzymes of etnangien production in *Sorangium cellulosum*.

The function of HMG-CoA synthase was verified using gene inactivation by insertion of a PCR amplificate of a fragment of orf 18 using primers OP158: 5' – CGGAGATCTATCGTATCGAG (Seq.-ID No. 58) with introduced *Bgl*III (underlined) restriction site and OP159: 5' – aagcttCGAGCCGTACGAG AAATACC (Seq.-ID No. 59) with a *Hind*III (underlined) restriction site, which 779 bp amplificate was cloned into a conjugational transfer vector pSUPHyg. The integration of the vector containing the amplificate was confirmed by PCR using primer pair OP188: 5' – acgaggatcctgtgacgttc (Seq.-ID No. 60) and OP77, and primer pair OP189: 5' - cgtactctgcatcgacaac (Seq.-ID No. 61) and OP78.

The positions of the primers are shown in Figure 5 A; PCR amplicates are analysed by gel electrophoresis shown in Figure 5 B, wherein L designates a 1 kb ladder, lanes 1, 5, 9, and 13 are amplicates for the hygromycin gene of pSUPHyg, lanes 2, 6, 10, and 14 are from primer pair OP188-OP77, lanes 3, 7, 11, 15 are from primer pair OP189-OP78, and lanes 4, 8, 12, and 16 are from primer pair OP188-OP189, for pSUPHyg vector DNA, wild-type (So ce56 wt), and knock-out mutants (Mutants #14 and #16), respectively.

#### Example 2: Production of etnangien in an original non-producer strain after introduction of biosynthetic pathway coding sequences

As an example for an originally non-producer of etnangien, a *Sorangium cellulosum* was provided with expression cassettes containing orf 1 to orf 25 in the arrangement that had been identified in So ce56. Transformation was done by conjugational transfer according to Kopp et al. (J. Biotechnology 29-40 (2004)) using transposons containing the expression cassettes, or by electroporation according to EP 1 619 241 using linear nucleic acids containing the expression cassettes.

The identity of etnangien produced by transformants was confirmed by HPLC, MS and H-NMR analyses.

Example 3: Enhanced production of etnangien from coding sequences orf 1 to orf 25 in heterologous bacteria having a mutation in S12 ribosomal protein

One of the transformants obtained in Example 2 was subjected to random mutagenesis using UV irradiation. Among the mutants, screening identified a strain showing increased etnangien production rates.

Genetic analysis of the mutated transformant revealed that it carried an A to G transition mutation in the *rpsL* gene encoding the S12 ribosomal protein, resulting in a lysine to arginine exchange at amino acid No. 88.

## Claims

1. Synthetic pathway enzymes for the biosynthesis of etnangien having catalytic activities of translation products obtainable from coding sequences selected from the group containing Seq.-ID No 1 to Seq.-ID No 14, sequences complementary to Seq.-ID No 15 to Seq.-ID No 25, and sequences homologous thereto.
2. Synthetic pathway enzymes according to claim 1, comprising each translation product obtainable from Seq.-ID No 1 to Seq.-ID No 14 and sequences complementary to Seq.-ID No 15 to Seq.-ID No 25.
3. Micro-organism containing the pathway enzymes for the biosynthesis of etnangien, characterized by containing at least one artificially introduced DNA sequence encoding at least one translation product having enzymatic activity obtainable from coding sequences selected from the group containing Seq.-ID No 1 to Seq.-ID No 14 and complementary sequences Seq.-ID No 15 to Seq.-ID No 25.
4. Micro-organism according to claim 3, characterized in that it originally is devoid of at least one coding sequence homologous to one of the sequences contained in the group comprising Seq.-ID No 1 to Seq.-ID No 14 and complementary sequences Seq.-ID No 15 to Seq.-ID No 25.
5. Micro-organism according to one of claims 2 and 3, characterized in that at least two coding sequences artificially introduced are arranged between a promoter and a terminator in one expression cassette.
6. Micro-organism according to one of claims 2 to 4, characterized in that it is selected from the group comprising myxobacteria, lysobacteria, streptomyces spp., pseudomonas spp., and *Sorangium*, including natural etnangien producer strains and etnangien non-producer strains.
7. Micro-organism according to one of claims 2 to 4, characterized in that it carries a mutation in the gene encoding the S12 ribosomal protein causing an amino acid exchange from lysine to arginine at amino acid number 88.

8. Process for producing etnangien, characterized by the fermentation of a micro-organism obtained by genetic manipulation introducing at least one coding sequence selected from the group containing Seq.-ID No 1 to Seq.-ID No 14, complementary sequences Seq.-ID No 15 to Seq.-ID No 25, and sequences homologous thereto.
9. Process according to claim 8, characterized in that the micro-organism is obtained by genetically manipulating a micro-organism originally lacking at least one coding sequence encoding a catalytic activity of the pathway biosynthesis of etnangien by introducing a coding sequence selected from the group containing Seq.-ID No 1 to Seq.-ID No 14, complementary sequences Seq.-ID No 15 to Seq.-ID No 25, and sequences homologous thereto, which coding sequence encodes a translation product complementing the originally lacking catalytic activity.
10. Process according to claim 8 or 9, characterized in that it carries a mutation in the gene encoding the S12 ribosomal protein causing an amino acid exchange from lysine to arginine at amino acid number 88.

Figures

Figure 1

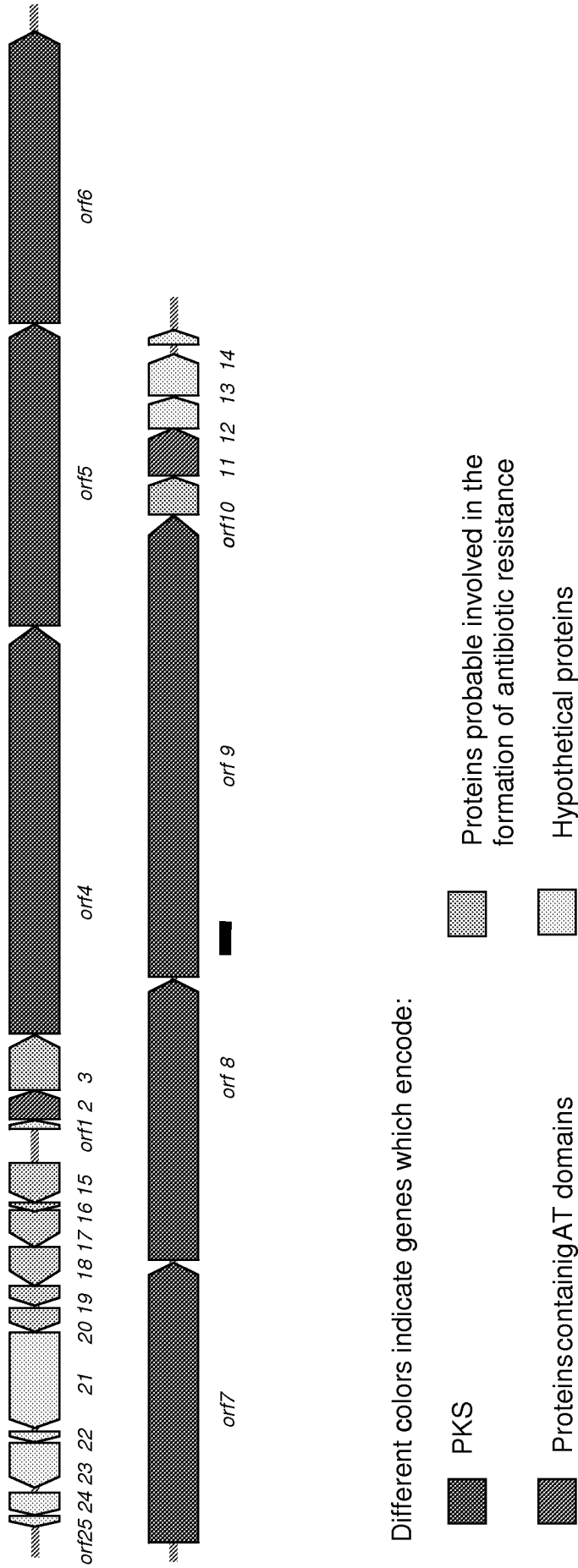






Figure 3 A

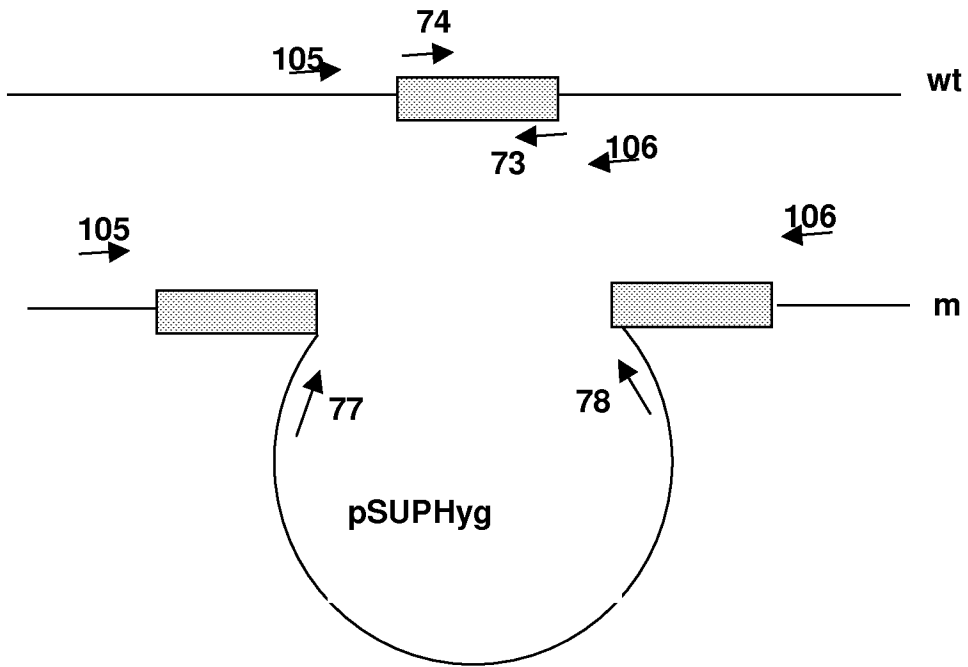


Figure 3 B

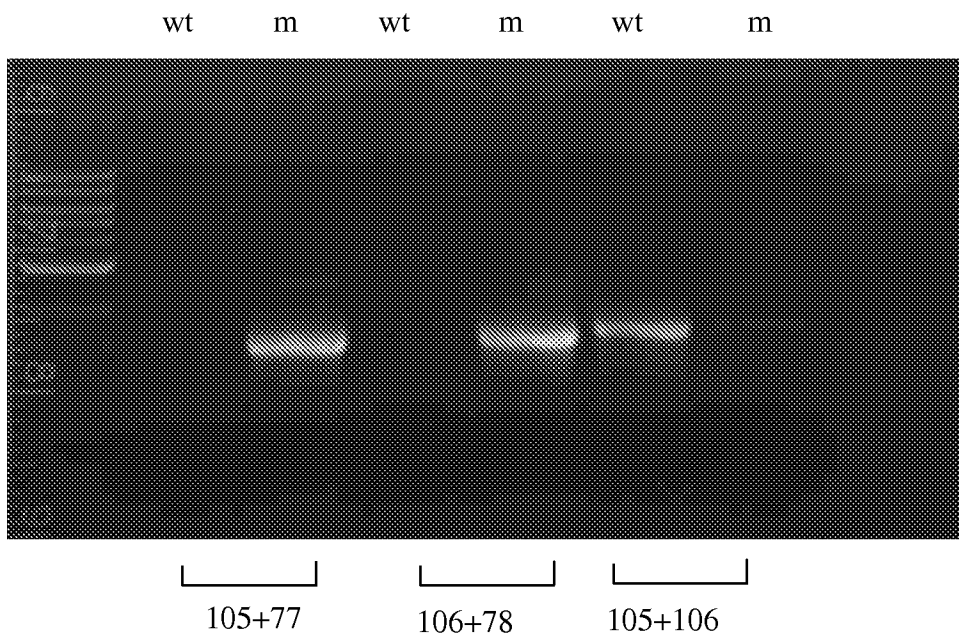


Figure 4

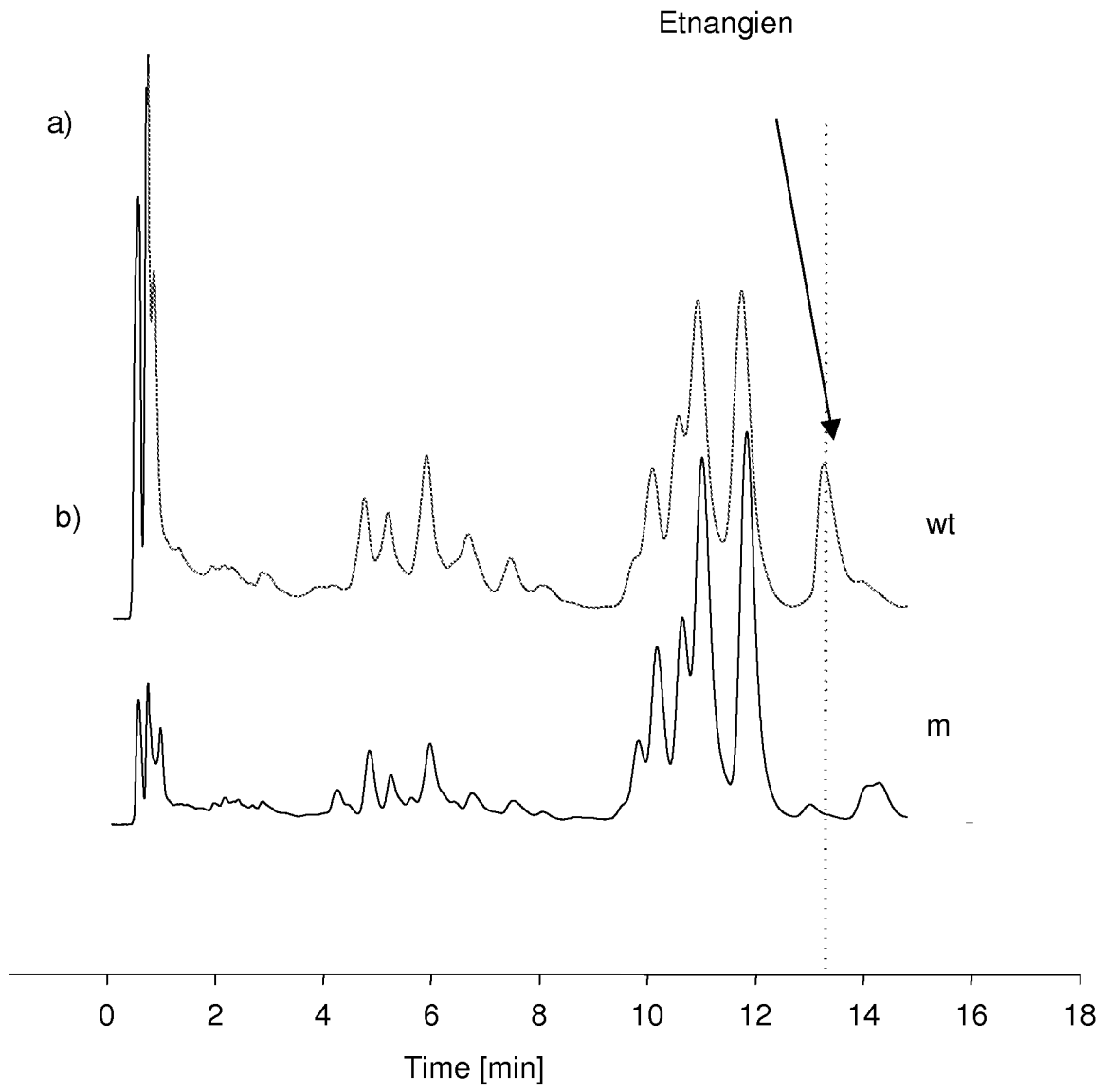


Figure 5 A

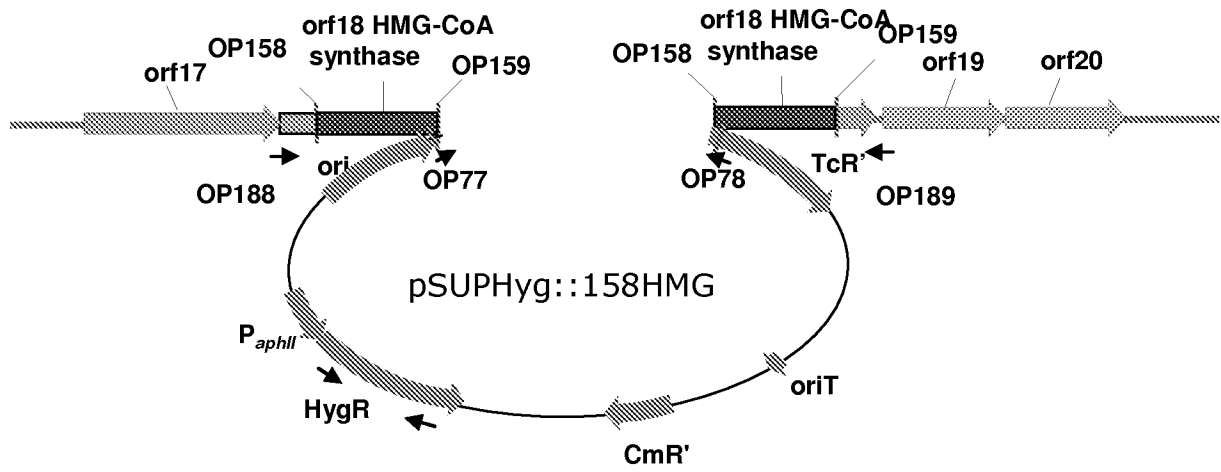
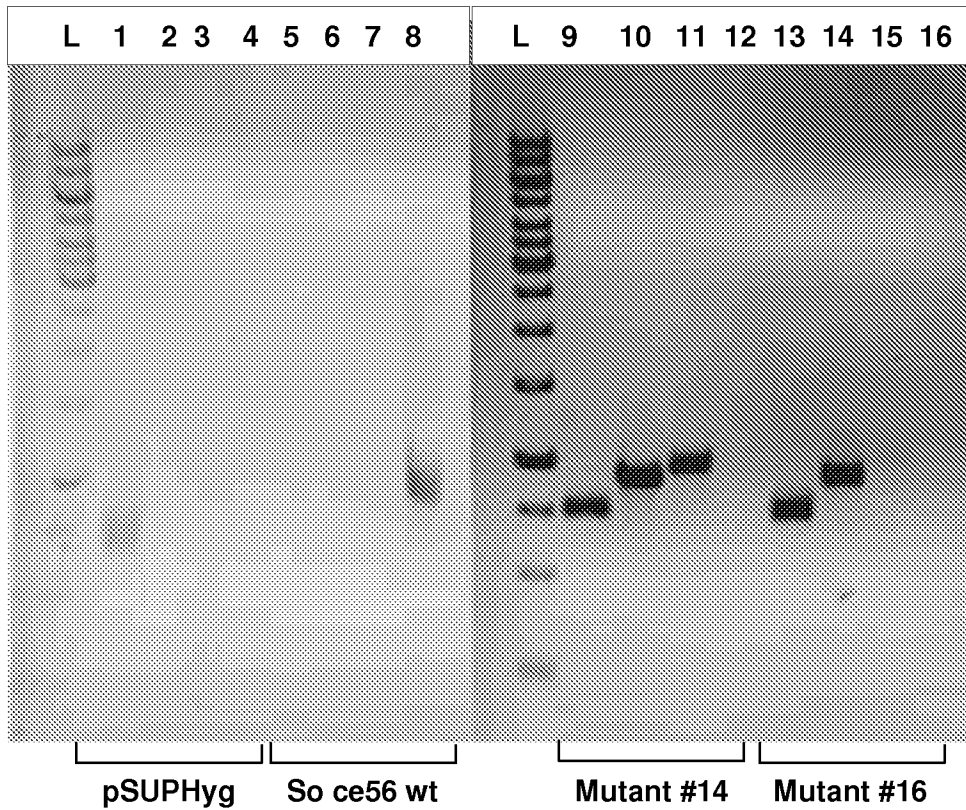


Figure 5 B



## INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2008/060276A. CLASSIFICATION OF SUBJECT MATTER  
INV. C12P17/08 C12N15/52  
ADD. C12R1/01

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 C12R 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 practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, FSTA, EMBASE, COMPENDEX

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ACHUPP T ET AL: "A SORANGIUM CELLULOSUM (MYXOBACTERIUM) GENE CLUSTER FOR THE BIOSYNTHESIS OF THE MACROLIDE ANTIBIOTIC SORAPHEN A: CLONING, CHARACTERIZATION, AND HOMOLOGY TO POLYKETIDE SYNTHASE GENES FROM ACTINOMYCETES" JOURNAL OF BACTERIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 177, no. 13, 1 July 1995 (1995-07-01), pages 3673-3679, XP000654849 ISSN: 0021-9193 the whole document  ----- -/--	1,8,9

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

\* Special categories of cited documents:

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Date of the actual completion of the international search

5 November 2008

Date of mailing of the international search report

21/11/2008

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

Authorized officer

Bassias, Ioannis

## INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2008/060276

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>PERLOVA ET AL: "Identification and analysis of the chivosazol biosynthetic gene cluster from the myxobacterial model strain Sorangium cellulosum So ce56"            JOURNAL OF BIOTECHNOLOGY, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL,            vol. 121, no. 2,            24 January 2006 (2006-01-24), pages 174-191, XP005236110            ISSN: 0168-1656            the whole document</p>	1,8,9
P,X	<p>SCHNEIKER SUSANNE ET AL: "Complete genome sequence of the myxobacterium Sorangium cellulosum"            NATURE BIOTECHNOLOGY,            vol. 25, no. 11, November 2007 (2007-11), pages 1281-1289, XP002502475            ISSN: 1087-0156            the whole document</p>	1-6,8,9
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## INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2008/060276

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>KOPP MAREN ET AL: "Critical variations of conjugational DNA transfer into secondary metabolite multiproducing Sorangium cellulosum strains So cel2 and So ce56: development of a mariner-based transposon mutagenesis system" JOURNAL OF BIOTECHNOLOGY, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 107, no. 1, 8 January 2004 (2004-01-08), pages 29-40, XP002355734 ISSN: 0168-1656</p>	

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Information on patent family members

International application No  
PCT/EP2008/060276

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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