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(54) Title: PRODUCTION OF CEPHALOSPORIN INTERMEDIATES IN *PENICILLIUM CHRYSOGENUM*

(57) Abstract: The invention is concerned with a bioprocess for the production of a ceph-3-em compound selected from the group consisting of desacetoxycephalosporin C, deacetylcephalosporin C and cephalosporin C in *P. chrysogenum.* The invention is also concerned with a strain of *P. chrysogenum,* which by virtue of genetic transformation is capable to produce said ceph-3-em compound. This strain of *P. chrysogenum* is characterized by the capability to convert isopenicillin N into penicillin. In particular, this capability was established by the introduction of genes encoding an acyl-CoA synthetase and a CoA racemase of *A. chrysogenum.*

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PRODUCTION OF CEPHALOSPORIN INTERMEDIATES IN *PENICILLIUM CHRYSOGENUM*

The present invention is concerned with a process for the production of a ceph-3-em compound selected from the group consisting of desacetoxycephalosporin C (DAOC), deacetylcephalosporin C (DAC) and cephalosporin C (CPC) in *Penicillium chrysogenum* (*P. chrysogenum*). The invention is also concerned with a strain of *P. chrysogenum*, which by virtue of genetic transformation is capable to produce said ceph-3-em compound.

Wild-type *P. chrysogenum* is capable of producing penicillin compounds, however this organism is unable to produce cephalosporin compounds.

Ceph-3-em compounds (such as CPC) are produced by organisms like *Acremonium chrysogenum* (*A. chrysogenum*) (Aharonowitz, Y., Cohen, G., Martin, J.F. *Annu. Rev. Microbiol.* 46: 461-495)

Penicillin compounds have a penam core structure characterized by a five-membered ring, whereas cephalosporin compounds are characterized by a six-membered ring in the ceph-3-em core. The key enzyme needed to convert a penam core into the corresponding ceph-3-em core is DAOC synthase, which enzymatically expands the five-membered ring into a six-membered ring.

This DAOC synthase enzyme is lacking in wild-type *P. chrysogenum*.

An essential step in the production of CPC in *A. chrysogenum* is the conversion of isopenicillin N into penicillin N by the socalled isopenicillin N isomerase enzyme system . This latter enzyme system is lacking in *P. chrysogenum* as well. Ullán et al. recently have reported the identification of the isopenicillin N isomerase of *A. chrysogenum* (Ullán RV, Casqueiro J, Banuelos O, Fernandez FJ, Gutierrez S, Martin JF. 2002. J Biol Chem **277**(48):46216-25). According to this report, the isopenicillin N isomerase activity of *A. chrysogenum* resides in the joint action of a number of gene products (Fig. 1). Two of the relevant genes were identified as *cefD1* and *cefD2*. Blocking of the expression of either of these two genes impaired the formation of penicillin N in *A. chrysogenum*.

The structure of these genes has been elucidated as well (Ullán *et al*, 2002, *supra*).

The enzyme encoded by the *cefD1* gene shows high similarity to long chain acyl-CoA synthetases and is considered to be responsible for the activation of isopenicillin N to isopenicillyl-CoA.

The enzyme encoded by the *cefD2* gene has high similarity to α-methylacyl-racemases and 2-arylpropionyl-CoA epimerases suggesting that after activation of isopenicillin N to isopenicillyl-CoA epimerisation to penicillyl-CoA occurs via a similar mechanism.

However, a thioesterase which would establish the hydrolysis of penicillyl-CoA to form penicillin N was not identified by Ullán *et al.* In view of the required energy efficiency of the cells this should be expected to be an enzyme specific for the stereochemical configuration of penicillyl-CoA.

Hence, it seems that further information on a third enzyme essential for the conversion of isopenicillin N into penicillin N in *A. chrysogenum* is still lacking.

In view thereof it is highly surprising that according to the present invention it was found that a full epimerase activity could be established in *P. chrysogenum* by transforming this filamentous fungus by DNA encoding the enzymes cefD1 and cefD2 of the isopenicillin N epimerase system of *A. chrysogenum*.

According to a preferred embodiment of the present invention *P. chrysogenum* strains are transformed with the genes encoding the cefD1 and cefD2 enzymes of *A. chrysogenum*. Thus transformed strains are capable to produce penicillin N.

In a further embodiment of the present invention *P. chrysogenum* strains are transformed with the genes encoding the cefD1 and cefD2 enzymes of *A.* chrysogenum as well as with a gene encoding an enzyme with DAOC synthase activity. Thus transformed strains are capable to produce DAOC.

In a further embodiment of the present invention *P. chrysogenum* strains are transformed with the genes encoding the cefD1 and cefD2 enzymes of *A. chrysogenum* as well as with a gene or genes encoding enzyme with DAOC synthase activity and DAC synthase activity. Thus transformed strains are capable to produce DAC.

In a still further embodiment of the present invention *P. chrysogenum* strains are transformed with the genes encoding the cefD1 and cefD2 enzymes of *A. chrysogenum*, with a gene or genes encoding enzymes with DAOC synthase activity and DAC synthase activity as well as with a gene encoding an enzyme having acetyltransferase activity. Thus transformed strains are capable to produce cephalosporin C (CPC)

A further embodiment of the present invention relates to a bioprocess for preparing a cephalosporin derivative comprising the steps of

a) maintaining in a culture medium capable of sustaining its growth a strain of *P. chrysogenum* which produces isopenicillin N;

b) carrying out the following enzymatic conversion by *in situ* expression of the corresponding at least one gene:

i) the isopenicillin N is *in situ* converted into penicillin N by isopenicillin N epimerase enzyme, wherein said strain of *P. chrysogenum* has been transformed by DNA encoding the isopenicillin N epimerase enzyme system comprising the $cefD_1$ and $cefD_2$ genes of *A. chrysogenum* capable of accepting said isopenicillin N as a substrate, whereupon as a result of its expression, said isopenicillin N produced by said strain is also thereafter *in situ* converted into penicillin N

ii) the penicillin N is *in situ* ring-expanded to form the corresponding desacetoxycephalosporin C (DAOC) by DAOC synthase enzyme, wherein said strain of *P. chrysogenum* has been transformed by DNA encoding the DAOC synthase enzyme capable of accepting said penicillin N as a substrate, whereupon as a result of its expression, said penicillin N produced by said strain is also thereafter *in situ* ring-expanded to form DAOC.

A further embodiment of the present invention relates to a bioprocess for preparing a cephalosporin derivative comprising the steps of

a) maintaining in a culture medium capable of sustaining its growth a strain of *P. chrysogenum* which produces isopenicillin N;

b) carrying out the following enzymatic conversion by *in situ* expression of the corresponding at least one gene:

i) the isopenicillin N is *in situ* converted into penicillin N by isopenicillin N epimerase enzyme, wherein said strain of *P. chrysogenum* has been transformed by DNA encoding the isopenicillin N epimerase enzyme system comprising the $cefD_1$ and $cefD_2$ genes of *A. chrysogenum* capable of accepting said isopenicillin N as a substrate, whereupon as a result of its expression, said isopenicillin N produced by said strain is also thereafter *in situ* converted into penicillin N

ii) the penicillin N is *in situ* ring-expanded to form the corresponding DAOC by DAOC synthase enzyme, wherein said strain of *P. chrysogenum* has been transformed by DNA encoding the DAOC synthase enzyme capable of accepting said penicillin N as a substrate, whereupon as a result of its

expression, said penicillin N produced by said strain is also thereafter *in situ* ring-expanded to form DAOC

iii) the 3-methyl side chain of said DAOC is *in situ* hydroxylated to yield DAC by DAC synthase enzyme, wherein said strain of *P. chrysogenum* has been transformed by DNA encoding the DAC synthase enzyme capable of accepting said DAOC as a substrate, whereupon as a result of its expression, said DAOC produced by said strain is also thereafter *in situ* hydroxylated to form DAC.

A further embodiment of the present invention relates to a bioprocess for preparing cephalosporin derivative comprising the steps of

a) maintaining in a culture medium capable of sustaining its growth a strain of *P. chrysogenum* which produces isopenicillin N;

b) carrying out the following enzymatic conversion by *in situ* expression of the corresponding at least one gene:

i) the isopenicillin N is *in situ* converted into penicillin N by isopenicillin N epimerase enzyme, wherein said strain of *P. chrysogenum* has been transformed by DNA encoding the isopenicillin N epimerase enzyme system comprising the $cefD_1$ and $cefD_2$ genes of *A. chrysogenum* capable of accepting said isopenicillin N as a substrate, whereupon as a result of its expression, said isopenicillin N produced by said strain is also thereafter *in situ* converted into penicillin N

ii) the penicillin N is *in situ* ring-expanded to form the corresponding DAOC by DAOC synthase enzyme, wherein said strain of *P. chrysogenum* has been transformed by DNA encoding the DAOC synthase enzyme capable of accepting said penicillin N as a substrate, whereupon as a result of its expression, said penicillin N produced by said strain is also thereafter *in situ* ring-expanded to form DAOC

iii) the 3-methyl side chain of said DAOC is *in situ* hydroxylated to yield DAC by DAC synthase enzyme, wherein said strain of *P. chrysogenum* has been transformed by DNA encoding the DAC synthase enzyme capable of accepting said DAOC as a substrate, whereupon as a result of its expression, said DAOC produced by said strain is also thereafter *in situ* hydroxylated to form DAC

iv) DAC is in situ acetylated to yield CPC, by acetyltransferase enzyme, wherein said strain of P. chrysogenum has been transformed by DNA encoding the activity of the acetyltransferase enzyme capable of accepting

said DAC as a substrate, whereupon as a result of its expression, said DAC produced by said strain is also thereafter *in situ* acetylated to form CPC.

Preferably the *P.chrysogenum* strain used in the instant bioprocess has a non-functional acyltransferase

In a preferred bioprocess the DNA encoding the activity of the DAOC synthase, DAC synthase and/or acetyl-CoA:DAC acetyltransferase enzymes is derived from *A. chrysogenum.*

Alternatively in a bioprocess according to the present invention a single bifunctional DAOC synthase/DAC synthase enzyme is used. Such a bifunctional enzyme may be derived e.g. from *A. chrysogenum.*

A further embodiment of the present invention is a genetically transformed *P. chrysogenum* comprising DNA encoding an enzyme having isopenicillyl-CoA synthase activity, and DNA encoding an enzyme having isopenicillyl-CoA-racemase activity and DNA encoding an enzyme having DAOC synthase activity, and optionally comprising DNA encoding an enzyme having DAC synthase activity, and further optionally comprising DNA encoding an enzyme having acetyl-CoA: DAC acetyltransferase activity.

Preferably this *P.chrysogenum* strain has a non-functional acyltransferase

This transformed *P. chrysogenum* strain can be transformed with DNA encoding the cefD1 and cefD2 enzymes of *A. chrysogenum* as well as with DNA encoding an enzyme with DAOC synthase activity, optionally also with DNA encoding an enzyme with DAC synthase activity and optionally also with DNA encoding an enzyme with acetyl-CoA:DAC acetyl transferase activity.

The DNA encoding the DAOC synthase enzyme, DAC synthase enzyme, or acetyl-CoA:DAC acetyl transferase enzyme for use according to the present invention can be obtained from micro-organisms reported to contain this DNA and which are available from culture collections or it may be obtained from micro-organisms isolated from appropriate natural sources.

Examples of microorganisms reported to contain the DAOC synthase enzyme are *A. chrysogenum* (cefEF), *Streptomyces clavuligerus* (cefE), *Nocardia lactamdurans* (cefE), *Lysobacter lactamgenus* (cefE).

Examples of microorganisms reported to contain the DAC synthase enzyme are *A. chrysogenum* (cefEF), *S. clavuligerus* (cefF), *N. lactamdurans* (cefF), *Lysobacter L.* (cefF).

An example of a microorganism reported to contain the acetyl-CoA:DAC acetyl transferase enzyme is *A. chrysogenum* (cefG).

The DNA for use according to the present invention can be obtained from the source organism by methods known in the art.

Transformation of host cells, for example of *P. chrysogenum* or other fungi can, in general, be achieved by different means of DNA delivery, like PEG-Ca mediated protoplast uptake, electroporation or particle gun techniques, and selection of transformants. See for example Van den Hondel and Punt, "Gene and Transfer and Vector Development for Filamentous Fungi", in: Applied Molecular Genetics of Fungi (Peberdy, Laten, Ogden, Bennett, eds.), Cambridge University Press (1991). The application of dominant and non-dominant selection markers has been described (Van den Hondel *et al.*, supra). Selection markers of both homologous (*P. chrysogenum* derived) and heterologous (non-*P. chrysogenum* derived) origin have been described (Gouka *et al.*, J. Biotechnol. 20 (1991) 189-200).

The application of various homologous or heterologous transformant selection markers, in the presence or absence of vector sequences, physically linked or not to the non-selectable DNA, in the selection of transformants is well known.

The DNA sequence encoding the DAOC synthase activity, the DAC synthase activity and the acetyl-CoA:DAC acetyl transferase activity are introduced into and expressed in this way in *P. chrysogenum*, for instance in strain Wisconsin 54-1255 (deposited at ATCC under accession number 28089). Other strains of *P. chrysogenum*, including mutants of strain Wisconsin 54-1255, having an improved beta-lactam yield, are also suitable. Examples of such high-yielding strains are the strains CBS 455.95, Panlabs P2 and ASP-78.

Furthermore, the *cef*G gene together with the *cef*E *and cef*F *or cef*EF gene are placed under the transcriptional and translational control of heterologous or homologous control elements, preferably under control of fungal gene control elements. Those elements can be obtained from cloned fungal genes like the *P.chrysogenum* IPNS or *pcb*C gene, the β-tubulin gene, the *Aspergillus nidulans gpd*A gene, or the *A. niger gla*A gene.

The *cefD1* and *cefD2* genes can come to expression under their respective native transcriptional and translational control elements, however, other control elements suitable for expression in *P. chrysogenum* can be employed as well. Preferably use is made of fungal transcriptional and translational control elements, such as those from cloned fungal genes like the *P.chrysogenum* IPNS or *pcb*C gene, the β-tubulin gene, the *A. nidulans gpd*A gene, or the *A. niger gla*A gene.

The cephalosporin derivatives prepared according to the process of the present invention can be isolated and further purified according to methods known in the art.

Brief description of the Figures:

Fig. 1.: Schematic representation of the enzymatic conversion of isopenicillin N into penicillin N in *A. chrysogenum*.

Fig. 2.: Detailed map of the p43EFG plasmid. K, *Kpn*I; X, *Xho*I; S, *Sal*I; EV, *Eco*RV; E, *Eco*RI; P, *Pst*I; H, *Hind*III; Xb, *Xba*I; Scl, *Sac*I. The *ble* (phleomycin/bleomycin resistance) cassette is expressed from the *pcb*C promoter (pr *pcb*C). tcyc1, transcriptional terminator of the *S. cerevisiae cyc*1 gene. pBSK(+) corresponds to pBluescript SK(+).

Fig. 3.: Presence of intact copies of p43EFG in different *P. chrysogenum* transformants. (A) Map of the p43EFG plasmid; the bands of non-reorganized copies of the *cefEF* and *cefG* genes are shown. (B) Southern blot hybridization of genomic DNAs digested with *Sal*I using a probe of the *cef*G gene: lane 1, TA98; Lane 2 TA2. (C) Southern blot hybridization of genomic DNAs digested with *Xba*I using a probe of the *cefEF* gene: lane 1, TA98; lane 2 TA2. The size of the hybridization bands (in kb) is indicated on the right.

Fig. 4.: Detailed map of the pCD1+2 plasmid. K, *Kpn*I; X, *Xho*I; S, *Sal*I; EV, *Eco*RV; E, *Eco*RI; P, *Pst*I; H, *Hind*III; Xb, *Xba*I; Scl, *Sac*I. Other gene designations are as in the legend of Fig. 2.

Fig. 5.: Presence of intact copies of pCD1+2 in different *P. chrysogenum* transformants. (A) Map of the pCD1+2 plasmid showing the bands of non-reorganized copies of the *cefD1* and *cefD2* genes. (B) Southern blot hybridization of genomic DNAs digested with *Spe*I/*Cla*I using a probe of the bidirectional promoter of the *cefD1-cefD2* genes: lane 1, TA98; lane 2, TA64; lane 3, TA 71 and lane 4, TA2. The size (in kb) of the hybridizing band is shown on the right side.

Fig. 6.: (A) Production of total β-lactams in cultures of TA98, TA2 and Wis 54-1255. (B) Production of cephalosporins in cultures of TA98, TA2 and Wis 54-1255.

Fig. 7.: HPLC chromatograms for determination of DAC and CPC in cell-free extracts of strains TA2 (upper panel) and TA98 (lower panel) grown in CPM medium for 72 hours. The peaks corresponding to DAC and CPC in transformant TA98 are indicated by arrows

Example 1

Transformation of a *P. chrysogenum* strain with the genes *cefD1*, *cefD2*, *cefEF* and *cefG*

A. MATERIALS AND METHODS

a. Strains and media

P. chrysogenum Wisconsin 54-1255 is a low penicillin production strain containing a single copy of the penicillin gene cluster. *P. chrysogenum npe*6 *pyr*G is a Wis 54-1255 derivative obtained by nitrosoguanidine treatment that lacks acyl-CoA:isopenicillin N acyltransferase (Fernández FJ, Gutiérrez S, Velasco J, Montenegro E, Marcos AT, Martín JF (1994) Molecular characterization of three loss-of-function mutations in the isopenicillin N-acyltransferase gene (*penDE*) of *P. chrysogenum*. *J. Bacteriol.* 176:4941-4948.) and is also a *pyrG* mutant obtained as described previously (Díez B, Alvarez E, Cantoral JM, Barredo JL, Martín JF (1987) Isolation and characterization of *pyr*G mutants of *P. chrysogenum* by resistance to 5′-fluoroorotic acid. *Curr. Genet.* 12:277-282.). *P. chrysogenum npe*10 is a deletion mutant that lacks the penicillin gene cluster (Fierro et al., 1995 PNAS 92: 6200-6204 "The penicillin gene cluster is amplified in tandem repeats linked by conserved hexanucleotide sequences"; Fierro F, Montenegro E, Gutiérrez S, Martín JF (1996a) Mutants blocked in penicillin biosynthesis show a deletion of the entire penicillin gene cluster at a specific site within a conserved hexanucleotide sequence. *Appl. Microbiol. Biotechnol.* 44:597-604)

P. chrysogenum spores were obtained from plates of PW medium (Fierro et al 1996a, *supra*) grown for 5 days at 28°C. Seed cultures were initiated by inoculating fresh spores during 18-24 h in CIM (Complex Inoculum Medium): corn steep solids 20 g/L; sucrose 20 g/L; yeast extract 10 g/L; $CaCO_3$ 5 g/L. Cultures in CPM medium (Complex Production Medium: Pharmamedia 20 g/L; lactose 50 g/L $(NH_4)_2SO_4$ 4 g/L; $CaCO_3$ 5 g/L); were inoculated with 5% of seed cultures and incubated in a orbital shaker (250 rpm, 25°C).

b. Transformation of *P. chrysogenum* protoplasts

Protoplasts of *P. chrysogenum* were obtained as described by Fierro et al. (Fierro F, Gutiérrez S, Díez B, Martín JF (1993) Resolution of four chromosomes in penicillin-producing filamentous fungi: the penicillin gene cluster is located on chromosome II (9.6 Mb) in *P. notatum* and chromosome I (10.4 Mb) in *P. chrysogenum*. *Mol. Gen. Genet.* 241:573-578). Transformation was performed according to the procedures of Cantoral et al. (Cantoral JM, Díez B, Barredo JL,

Alvarez E, Martín JF (1987) High-frequency transformation of *P. chrysogenum. Bio/Technology* 5:494-497) and Díez et al. (*supra*). Transformant clones were selected by complementation of the uridine auxotrophy.

c. Nucleic acid isolation and Southern blotting

Small amounts of total DNA from *P. chrysogenum* were isolated from mycelium grown in MPPY medium (Fierro *et al*, 1993, *supra*), following the protocol described by Casqueiro et al. (Casqueiro J, Bañuelos O, Gutiérrez S, Hijarrubia MJ, Martín JF (1999) Intrachromosomal recombination in *P. chrysogenum*: gene conversion and deletion events. *Mol. Gen. Genet.* 261:994-1000). Total RNA was isolated with the RNeasy Kit (Qiagen). Total DNA digested with restriction enzymes were separated by agarose gel electrophoresis and blotted onto Nylon membranes (Hybond NX, Amersham) (Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual (2nd edn). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). Probes were labeled with [α^{32}P] dCTP by nick-translation and purified by filtration through Wizard minicolumns (Promega). Hybridizations were carried out as described by Sambrook et al. (*supra*).

B. Construction of *P. chrysogenum* TA2: integration of the *cefEF* and *cefG* genes of *A. chrysogenum*

Protoplasts of *P. chrysogenum npe6 pyrG* were transformed with the integrative plasmid p43EFG (Figure 2). This plasmid bears the *cefEF* under the control of its own promoter. The p43EFG plasmid also bears the cDNA encoding region of the *cefG* under the control of the *pcbC* gene promoter from *P. chrysogenum* and the *ble* gene conferring resistance to phleomycin as selection marker. After transformation, a Southern blot hybridization (Fig. 3) was performed to select one strain with non-reorganized copies of the exogenous added copies of the *cefEF* and *cefG* genes. To study the presence of the *cefG* gene the genomic DNA of the transformants was digested with *Sal*I. Results showed that transformant *P. chrysogenum* TA2 (Fig 3, lane 2) showed a 2.1 kb hybridization band that corresponds to a non-reorganized copy of the *cefG* gene. To study the integration of *cefEF* gene the genomic DNA of the transformants was digested with *Xba*I. Results showed that transformant TA2 (Fig 3, lane 2) showed a 2.7 kb hybridization band that corresponds to a non-reorganized copy of the *cefEF* gene.

C. Construction of *P. chrysogenum* TA98: integration of *cefD1* and *cefD2* genes of *A. chrysogenum*

TA2 is a *pyrG*$^-$ strain. TA2 was co-transformed with pCD1+2 (Fig 4), that contains the *cefD1* and *cefD2* genes under its own promoter, and pBG that bears the *pyrG* gene of *P. chrysogenum*. Transformants were selected by protothrophy (complementation of the *pyrG* mutation in the host strain) in Czapek medium.

A Southern blot with the genomic DNA of some of the transformants was performed to select one with non-reorganized copies of the *cefD1* and *cefD2* genes. The genomic DNA was digested with *ClaI*/*SpeI*. Results (Fig 5) showed that transformants *P. chrysogenum* TA64, TA71 and TA98 had a non-reorganized copy of he *cefD1* and *cefD2* genes. The TA98 transformant was selected for further studies.

D. Production of β-lactams and cephalosporins in TA98

Fermentations in complex penicillin production medium CPM (without phenylacetic acid) were performed with *P. chrysogenum* strains Wis 54-1255, TA2 and TA98. Results showed (Fig. 6) that TA2 produced a low level of β-lactams while both Wis 54-1255 and TA98 showed a similar level in the production of β-lactams. The quantification of cephalosporins titers by bioassay showed that only strain TA98 containing all cephalosporin biosynthesis genes was able to produce cephalosporins while in *P. chrysogenum* strain Wis 54-1255 and TA2 no detectable amounts of cephalosporins (CPC, DAC nor DAOC) were formed.

E. HPLC analysis of culture broth of *P. chrysogenum* TA98

The intracellular production of DAC and CPC in *P. chrysogenum* TA2 and *P. chrysogenum* TA98 grown in CPM (without phenylacetic acid) was studied. Results showed (Fig. 7) formation of DAC and CPC in transformant TA98 inside the cells.

CLAIMS

1) A bioprocess for preparing a ceph-3-em compound selected from the group consisting of deacetoxycephalosporin C (DAOC), deacetylcephalosporin C (DAC) and cephalosporin C (CPC), comprising the steps of
   a) maintaining in a culture medium capable of sustaining its growth a strain of *Penicillium chrysogenum* (*P. chrysogenum*) which produces isopenicillin N;
   b) carrying out the following enzymatic conversion by *in situ* expression of the corresponding at least one gene:
      i) the isopenicillin N is *in situ* converted into penicillin N by isopenicillin N epimerase enzyme, wherein said strain of *P. chrysogenum* has been transformed by DNA encoding the isopenicillin N epimerase enzyme system comprising the $cefD_1$ and $cefD_2$ genes of *Acremonium chrysogenum* (*A. chrysogenum*) capable of accepting said isopenicillin N as a substrate, whereupon as a result of its expression, said isopenicillin N produced by said strain is also thereafter *in situ* converted into penicillin N
      ii) the penicillin N is *in situ* ring-expanded to form DAOC by DAOC synthase enzyme, wherein said strain of *P. chrysogenum* has been transformed by DNA encoding the DAOC synthase enzyme capable of accepting said penicillin N as a substrate, whereupon as a result of its expression, said penicillin N produced by said strain is also thereafter *in situ* ring-expanded to form DAOC;
   c) whereafter optionally the 3-methyl side chain of said DAOC is *in situ* hydroxylated to yield DAC by DAC synthase enzyme, wherein said strain of *P. chrysogenum* has been transformed by DNA encoding the DAC synthase enzyme capable of accepting said DAOC as a substrate, whereupon as a result of its expression, said DAOC produced by said strain is also thereafter *in situ* hydroxylated to form DAC, and
   d) whereafter optionally DAC is *in situ* acetylated to yield CPC, by acetyltransferase enzyme, wherein said strain of *P. chrysogenum* has been transformed by DNA encoding the activity of the acetyltransferase enzyme capable of accepting said DAC as a substrate, whereupon as a result of its expression, said DAC produced by said strain is also thereafter *in situ* acetylated to form CPC

2) A bioprocess according to claim 1 wherein the strain of *P. chrysogenum* has a non-functional acyltransferase.

3) A bioprocess according to claim 1 wherein the DNA encoding the activity of the DAOC synthase, DAC synthase and/or acetyltransferase enzymes is derived from *A. chrysogenum.*

A bioprocess according to claim 1 wherein a bifunctional DAOC synthase / DAC synthase enzyme is used.

4) A bioprocess according to claim 1 wherein the DNA encoding the bifunctional DAOC synthase / DAC synthase enzyme is derived from *A. chrysogenum.*

5) A genetically transformed *P. chrysogenum* comprising DNA encoding an isopenicillin N epimerase system comprising the *cefD*$_1$ and *cefD*$_2$ genes of *A. chrysogenum* and DNA encoding an enzyme having DAOC synthase activity, and optionally comprising DNA encoding an enzyme having DAC synthase activity, and further optionally comprising DNA encoding an enzyme having acetyl-CoA:DAC acetyltransferase activity.

6) A genetically transformed *P. chrysogenum* according to claim 5 characterised by a non-functional acyltransferase.

7) A genetically transformed *P. chrysogenum* according to claim 5 wherein the DNA encoding the activity of the DAOC synthase, DAC synthase and/or acetyl-CoA:DAC acetyltransferase enzymes is derived from *A. chrysogenum.*

8) A genetically transformed *P. chrysogenum* according to claim 7 wherein a bifunctional DAOC synthase / DAC synthase enzyme is used.

9) A genetically transformed *P. chrysogenum* according to claim 7 wherein the DNA encoding the bifunctional DAOC synthase / DAC synthase enzyme is derived from *A. chrysogenum.*

Fig. 1

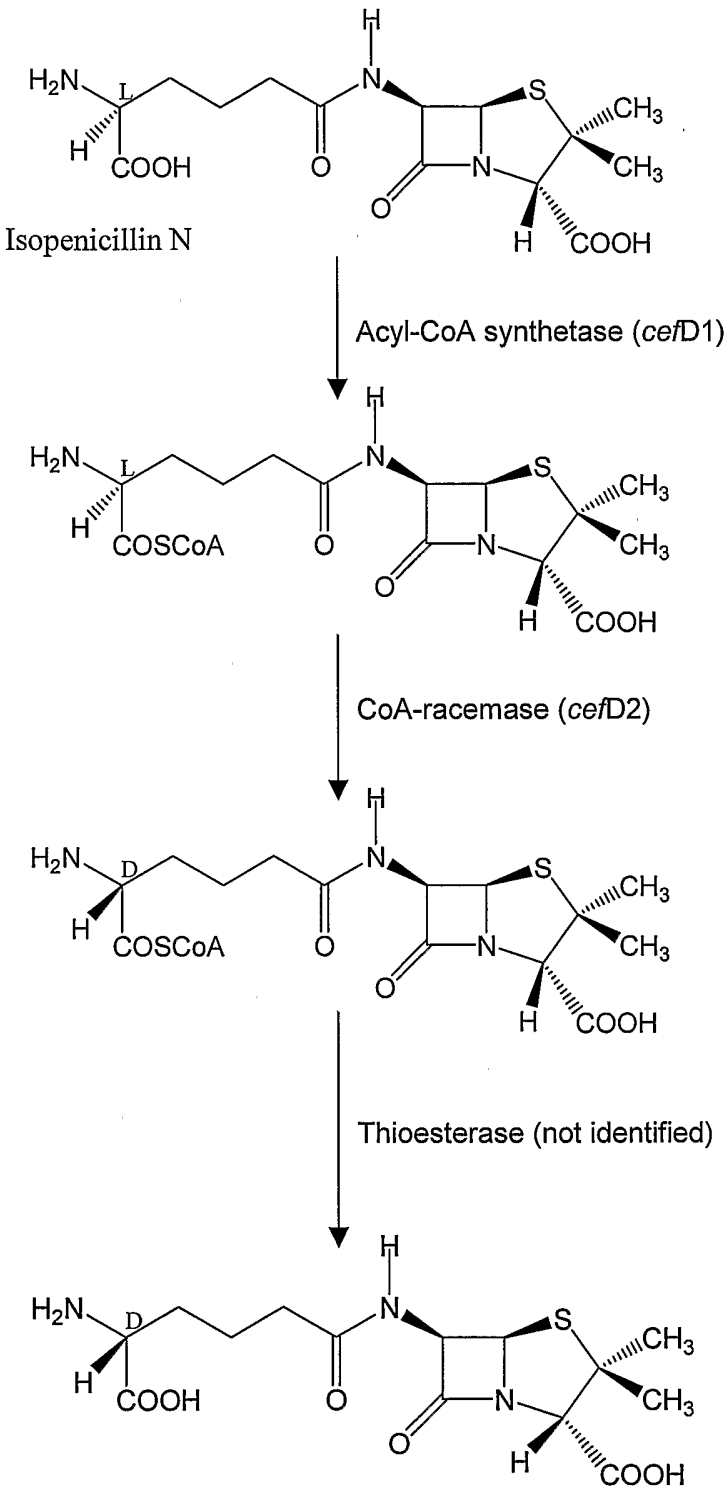

Isopenicillin N
Acyl-CoA synthetase (cefD1)
CoA-racemase (cefD2)
Thioesterase (not identified)

Fig. 2

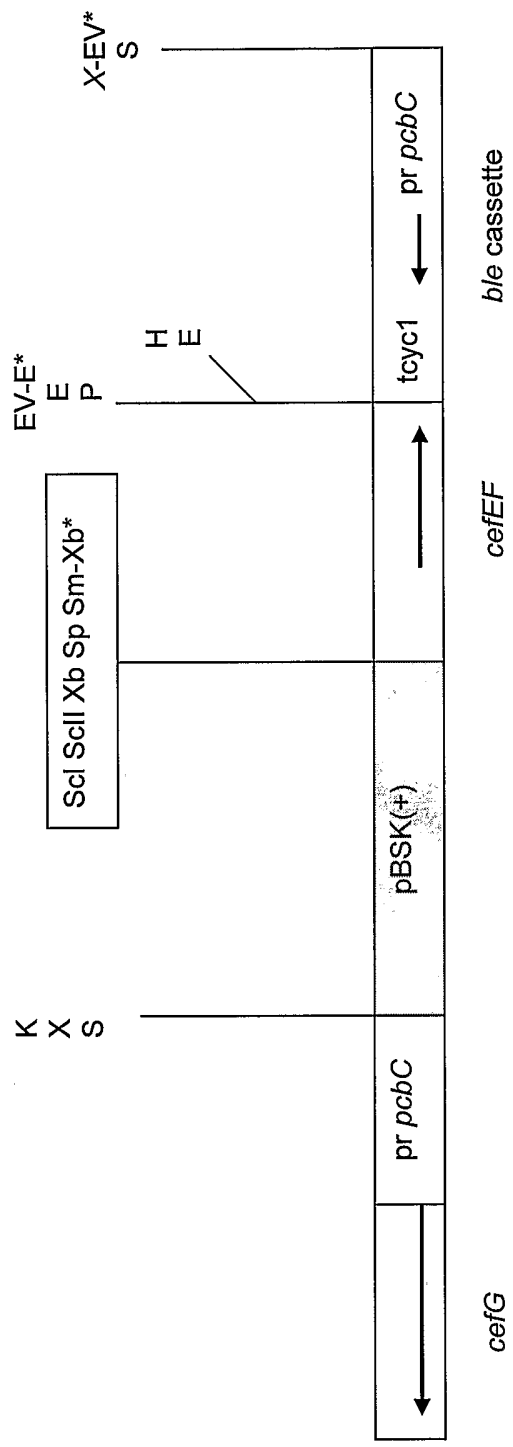

X-EV*
S
EV-E*
E
P
H
E
Scl SclI Xb Sp Sm-Xb*
K
X
S
pBSK(+)
pr pcbC
tcyc1
pr pcbC
ble cassette
cefEF
cefG

Fig. 3

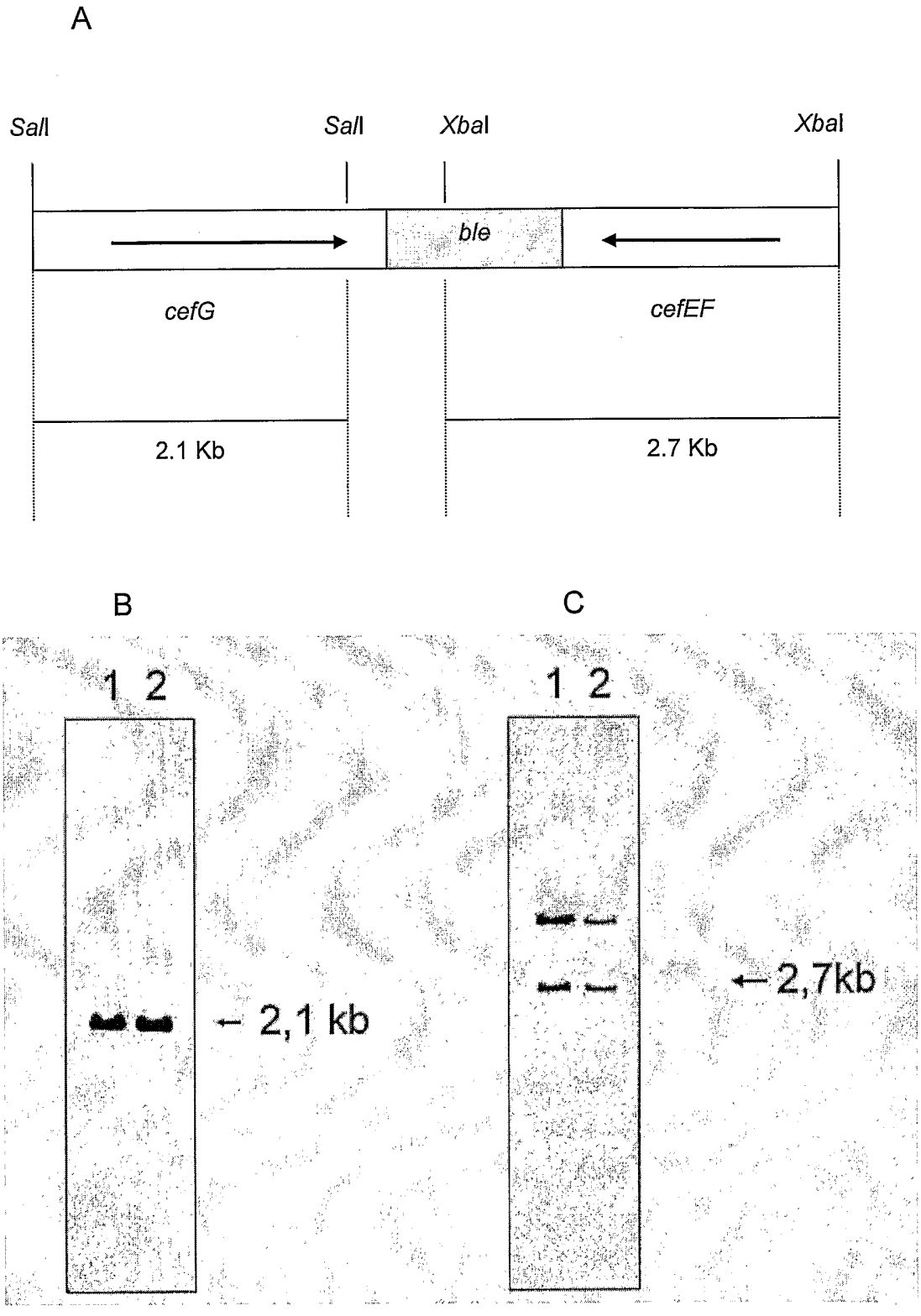

A
SalI
SalI
XbaI
XbaI
ble
cefG
cefEF
2.1 Kb
2.7 Kb
B
C
1 2
1 2
← 2,1 kb
← 2,7kb

Fig. 4

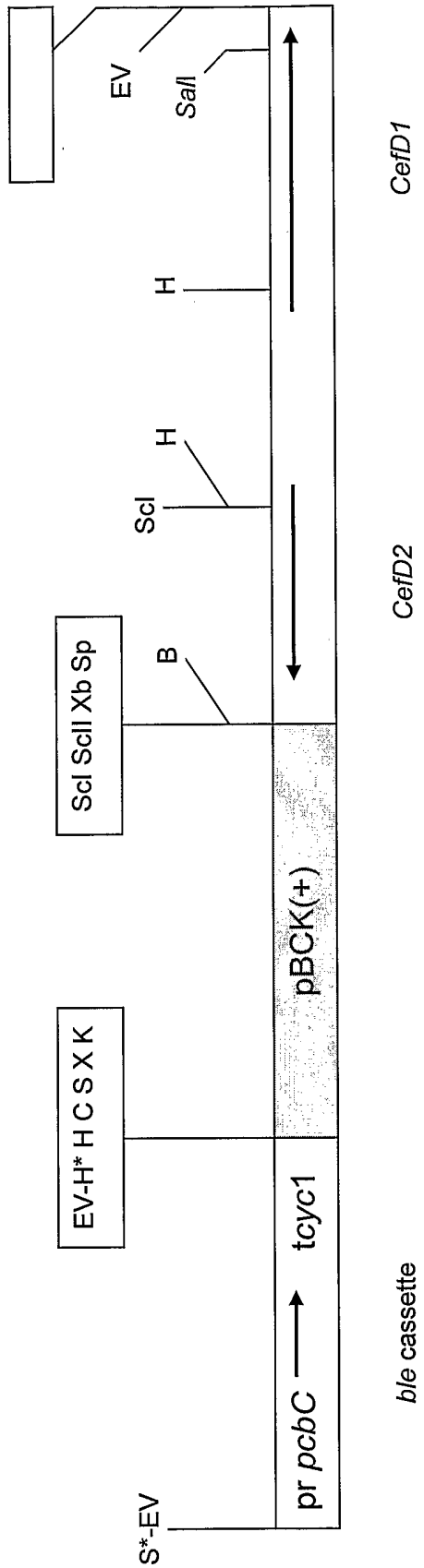
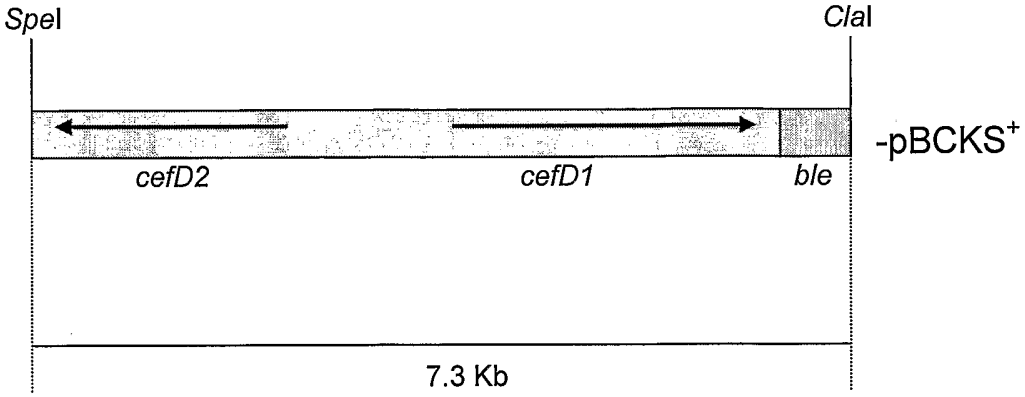

EV
SalI
H
ScI
H
B
CefD1
CefD2
ScI ScII Xb Sp
EV-H* H C S X K
pBCK(+)
pr pcbC
tcyc1
ble cassette
S*-EV

Fig. 5

A



B

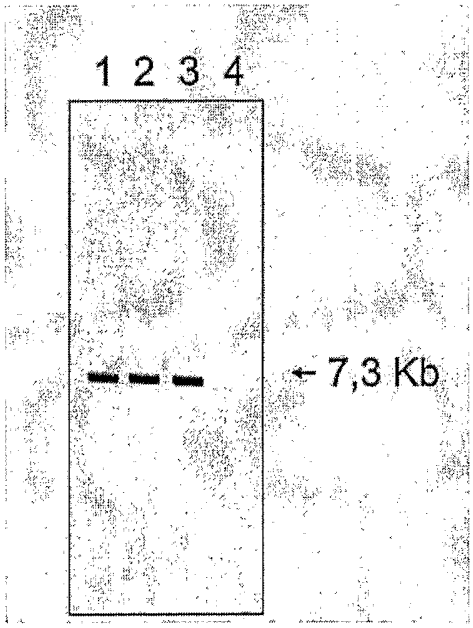


Fig. 6

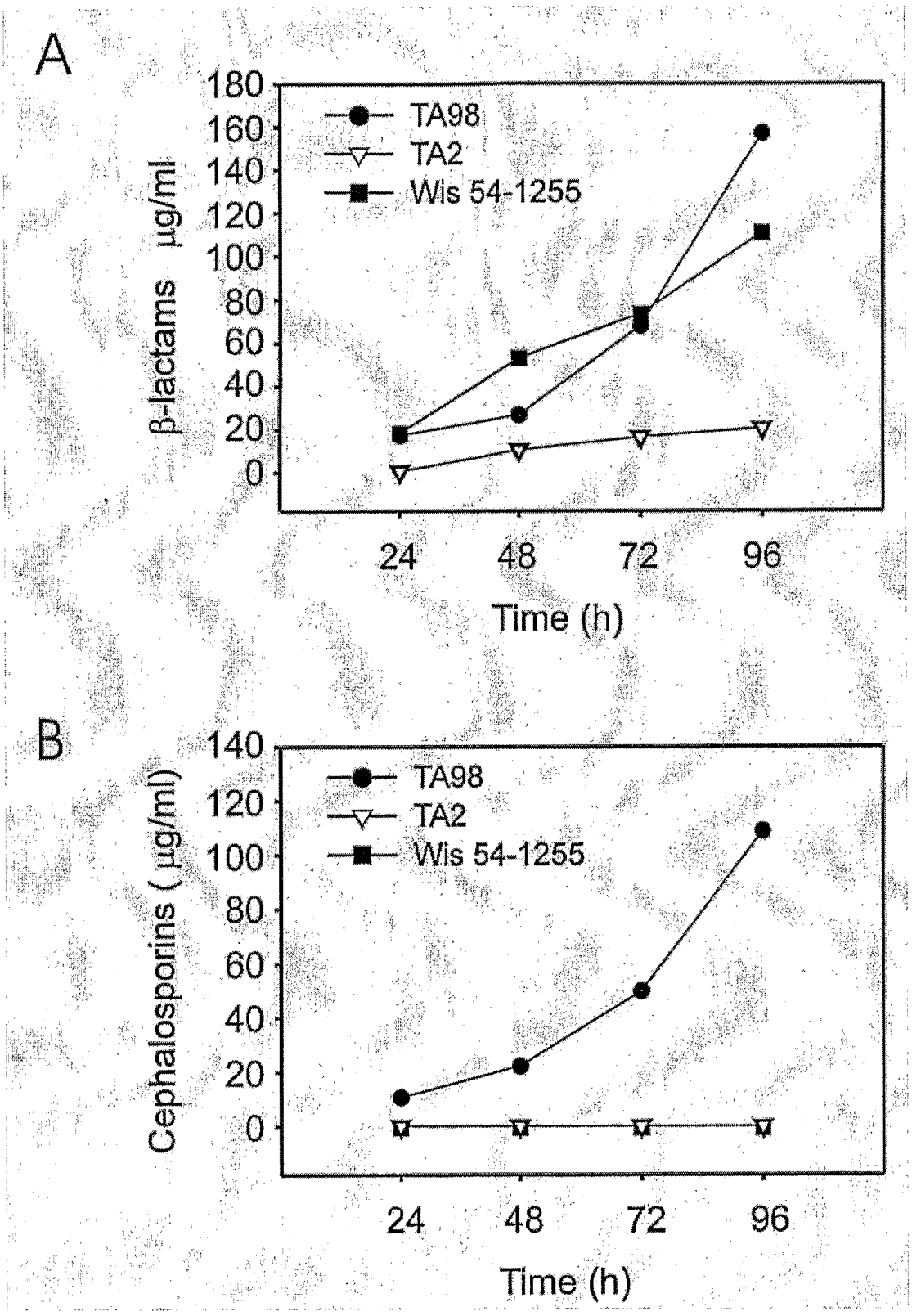
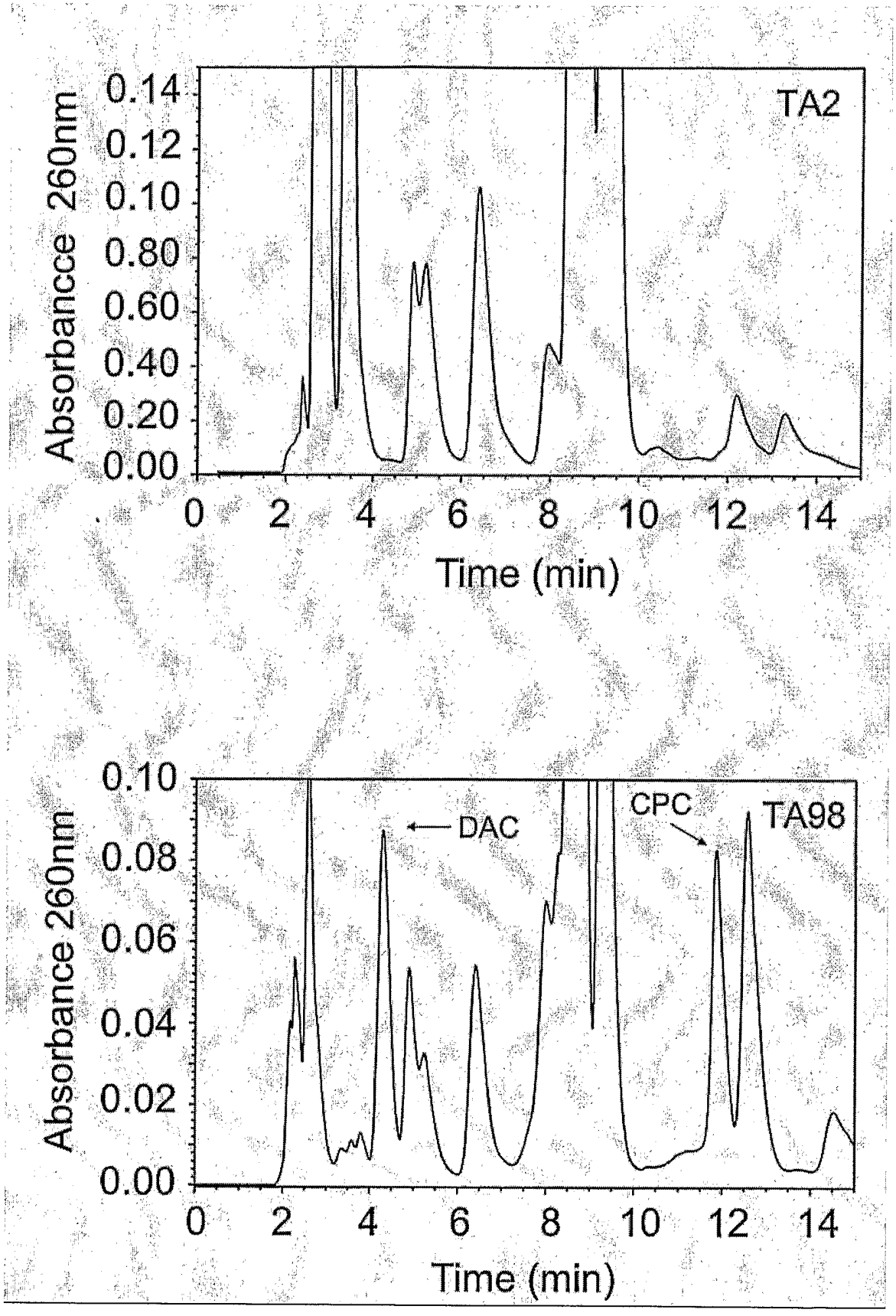

A
β-lactams µg/ml
180
160
140
120
100
80
60
40
20
0
TA98
TA2
Wis 54-1255
24
48
72
96
Time (h)
B
Cephalosporins (µg/ml)
140
120
100
80
60
40
20
0
TA98
TA2
Wis 54-1255
24
48
72
96
Time (h)

Fig. 7


TA2
Absorbancce 260nm
0.14
0.12
0.10
0.80
0.60
0.40
0.20
0.00
0
2
4
6
8
10
12
14
Time (min)
TA98
DAC
CPC
Absorbance 260nm
0.10
0.08
0.06
0.04
0.02
0.00
0
2
4
6
8
10
12
14
Time (min)

INTERNATIONAL SEARCH REPORT

Application No
./NL2004/000433

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N1/15 C12P35/06 C12N9/90 C12N9/02 C12N9/10
C12N15/53 C12N15/54 C12N15/61
//(C12P35/06,C12R1:82)

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)
IPC 7 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 practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, FSTA, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 566 897 A (HOECHST AG) 27 October 1993 (1993-10-27) the whole document	1-9
Y	ULLAN RICARDO V ET AL: "A novel epimerization system in fungal secondary metabolism involved in the conversion of isopenicillin N into penicillin N in Acremonium chrysogenum." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 277, no. 48, 29 November 2002 (2002-11-29), pages 46216-46225, XP002261579 ISSN: 0021-9258 cited in the application the whole document	1-9

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

[X] Patent family members are listed in annex.

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"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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 September 2004	14/09/2004

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, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Devijver, K

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INTERNATIONAL SEARCH REPORT

ıational Application No
Γ/NL2004/000433

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CANTWELL C ET AL: "ISOLATION OF DEACETOXYCEPHALOSPORIN C FROM FERMENTATION BROTHS OF PENICILLIUM CHRYSOGENUM TRANSFORMANTS: CONSTRUCTION OF A NEW FUNGAL BIOSYNTHETIC PATHWAY" PHILOSOPHICAL TRANSACTIONS. ROYAL SOCIETY OF LONDON. BIOLOGICAL SCIENCES, ROYAL SOCIETY, LONDON, GB, vol. 248, no. 1323, 22 June 1992 (1992-06-22), pages 283-289, XP002049879 ISSN: 0962-8436 the whole document	1-9
A	FERNANDEZ FRANCISCO J ET AL: "Molecular characterization of three loss-of-function mutations in the isopenicillin N-acyltransferase gene (penDE) of Penicillium chrysogenum" JOURNAL OF BACTERIOLOGY, vol. 176, no. 16, 1994, pages 4941-4948, XP008024600 ISSN: 0021-9193 cited in the application the whole document	1-9
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A	EP 0 281 391 A (LILLY CO ELI) 7 September 1988 (1988-09-07)	
P,X	WO 2004/026902 A (KUERNSTEINER HUBERT ; SANDOZ AG (AT); FRIEDLIN ERNST (AT)) 1 April 2004 (2004-04-01) the whole document	1-9

INTERNATIONAL SEARCH REPORT

ıtional Application No
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