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(54) **PROCEDE DE GENIE GENETIQUE POUR LA PRODUCTION DE S-(+)-2,2-DIMETHYLCYCLOPROPANECARBOXAMIDE AU MOYEN DE MICROORGANISMES**

(54) **GENETIC ENGINEERING PROCESS FOR THE PRODUCTION OF S-(+)-2,2-DIMETHYLCYCLOPROPANECARBOXAMIDE BY MICROORGANISMS**

(57) A genetic engineering process is disclosed for the production of S-(+)-2,2-dimethylcyclopropanecarboxamide. For this purpose, a new DNA that codes for a stereospecific hydrolase is isolated from a microorganism. This DNA is then ligated in an expression vector, and a hybrid plasmid results, which is used to transform microorganisms. These microorganisms are then able to biotransform the R-(-) isomer in racemic R,S-(±)-2,2-dimethylcyclopropanecarboxamide into R-(-)-2,2-dimethylcyclopropanecarboxylic acid. Optically active S-(+)-2,2-dimethylcyclopropanecarboxamide is thus obtained.

ABSTRACT OF THE DISCLOSURE

A genetic engineering process is disclosed for the production of S-(+)-2,2-dimethylcyclopropanecarboxamide. For this purpose, a new DNA that codes for a stereospecific hydrolase is isolated from a microorganism. This DNA is then ligated in an expression vector, and a hybrid plasmid results, which is used to transform microorganisms. These microorganisms are then able to biotransform the R-(-) isomer in racemic R,S-(±)-2,2-dimethylcyclopropanecarboxamide into R-(-)-2,2-dimethylcyclopropanecarboxylic acid. Optically active S-(+)-2,2-dimethylcyclopropanecarboxamide is thus obtained.

The present invention relates to a new process for the production of S-(+)-2,2-dimethylcyclopropane-carboxamide using new microorganisms suitable for that process. These microorganisms have been transformed with a new gene, which forms a stereospecific hydrolase, and is thus able to biotransform the R-(-)-isomer in racemic R,S-(±)-2,2-dimethylcyclopropanecarboxamide into the corresponding acid. Optically active S-(+)-2,2-dimethyl-cyclopropane carboxamide is thus obtained.

Hereinafter, 2,2-dimethylcyclopropanecarboxamide may be abbreviated as 2,2-DMCPCA and 2,2-dimethylcyclo-propanecarboxylic acid may be abbreviated as 2,2-DMCPCS.

Optically pure S-(+)-2,2-DMCPCA is used as the initial material for the production of the dehydropeptidase inhibitor cilastatin, which is administered, in treatment, together with penem or carbapenem antibiotics, to prevent deactivation of the antibiotics by a renal dehydropeptidase in the kidneys (see European Published Patent Application No. 048301).

Examples of microorganisms known in the art which produce a stereospecific hydrolase for the R-(-)-2,2-DMCPCA are microorganisms of the species Comamonas acidovorans A:18 (DSM No. 6315), Bacterium sp. VIII:II (DSM No. 6316), Pseudomonas sp. NSAK:42 (DSM No. 6433) and Comamonas acidovorans TG 308 (DSM No. 6552) as well as the descendants and mutants thereof. These microorganisms have already been described in detail in European Published

Patent Application No. 92103780.0.

The main object of the present invention is to provide new microorganisms as production strains, by recombinant DNA techniques, in which the catalytic capability as well as the expression of the above-mentioned hydrolase gene can be considerably increased as compared to the results achievable by known processes.

Accordingly, the present invention provides a genetic engineering process for the production of S-(+)-2,2-dimethylcyclopropanecarboxamide, wherein R-(-)-2,2-dimethylcyclopropanecarboxamide in racemic R,S-(+)2,2-dimethylcyclopropanecarboxamide is biotransformed, by microorganisms which have been transformed, with a gene that forms a stereospecific hydrolase, to form R-(-)-2,2-dimethylcyclopropanecarboxylic acid, and optically active S-(+)-2,2-dimethylcyclopropanecarboxamide is thus obtained and is then isolated.

The present invention also provides a DNA coding for a stereospecific hydrolase characterized by the restriction map which is represented in Figure 1.

The present invention further provides a DNA fragment coding for a polypeptide with stereospecific hydrolase activity whose amino acid sequence is represented in Figure 3.

Another aspect of the present invention further provides a DNA fragment that hybridizes with the DNA

fragment represented in Figure 3 and which codes for a polypeptide with stereospecific hydrolase activity.

The invention involves a genetic engineering process for the production of S-(+)-2,2-dimethylcyclopropanecarboxamide. According to the process of the present invention, R-(-)-2,2-dimethylcyclopropanecarboxamide in racemic R,S-(+)-2,2-dimethylcyclopropanecarboxamide is biotransformed by means of microorganisms transformed with a gene that forms a stereospecific hydrolase to R-(-)-2,2-dimethylcyclo-propanecarboxylic acid. Optically active S-(+)-2,2-dimethylcyclopropanecarboxamide is thus obtained and can then be isolated.

Preferably, the biotransformation of the present invention is performed with microorganisms which have been transformed with a gene that is characterized by the restriction map represented in Figure 1. Preferably, the biotransformation is performed with microorganisms, which have been transformed with a DNA fragment, which codes for a polypeptide with stereospecific hydrolase activity whose amino acid sequence is represented in Figure 3. Preferably, the biotransformation is performed with microorganisms, which have been transformed with a DNA fragment that hybridizes with the DNA fragment represented in Figure 3 and which codes for a polypeptide with stereospecific hydrolase activity. Preferably, the biotransformation is performed with microorganisms of genus Escherichia, Pseudomonas, Comamonas, Acinetobacter,

Rhizobium or Agrobacterium. Preferably, the biotransformation is performed with microorganisms of the species Escherichia coli. Preferably, the biotransformation is performed with microorganisms of the species Escherichia coli XL1-Blue* (DSM No. 6551) or their descendants and mutants, which have been transformed with the hybrid plasmid pCAR6. Preferably, the biotransformation is performed with microorganisms of the species Escherichia coli DH5* (DSM No. 7053) or their descendants and mutants, which have been transformed with the hybrid plasmid pCAR6. Preferably, the biotransformation is performed with an immobilized stereospecific hydrolase. Preferably, the biotransformation is performed in a medium containing racemic R,S-(±)-2,2-dimethylcyclopropane-carboxamide in an amount of 0.2 to 5 percent by weight. Preferably, the biotransformation is performed at a pH of 6 to 11 and a temperature of 15° to 55°C.

The invention involves a DNA coding for a stereospecific hydrolase characterized by the restriction map which is represented in Figure 1. The invention also involves a DNA fragment coding for a polypeptide with stereospecific hydrolase activity whose amino acid sequence is represented in Figure 3. The invention further involves a DNA fragment that hybridizes with the DNA fragment which is represented in Figure 3 and codes for a polypeptide with stereospecific hydrolase activity. Preferably, the DNA or the DNA fragment in hybrid plasmid pCAR6, according to any

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of the above, is deposited in Escherichia coli XL1-Blue* (DSM No. 6551). Preferably, any of the above DNA or DNA fragments in hybrid plasmid pCAR6 is deposited in Escherichia coli DH5* (DSM No. 7053).

The invention involves a hybrid plasmid composed of an expression vector with any of the above DNA or DNA fragments inserted in it. Preferably, the hybrid plasmid is hybrid plasmid pCAR6 composed of any of the above DNA or DNA fragments and expression vector pBLUESCRIPT-KS+*, deposited in Escherichia coli XL1-Blue* (DSM No. 6551). Preferably, the hybrid plasmid is hybrid plasmid pCAR6 composed of any of the above DNA or DNA fragments and expression vector pBLUESCRIPT-KS+*, deposited in Escherichia coli DH5* (DSM No. 7053).

The invention involves microorganisms that have been transformed with any of the above hybrid plasmids. Preferably, the microorganisms are of the species Escherichia coli XL1-Blue* (DSM No. 6551) and the descendants and mutants thereof that have been transformed with hybrid plasmid pCAR6. Preferably, the microorganisms are of the species Escherichia coli DH5* (DSM No. 7053) and the descendants and mutants thereof that have been transformed with hybrid plasmid pCAR6.

The present invention is further illustrated by reference to the accompanying drawings in which:

Figure 1 is the restriction map of the gene of the present invention;

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Figure 2 is a diagram of hybrid plasmid pCAR6;

Figure 3 shows both the amino-acid sequence and the DNA sequence of the gene which codes for the stereospecific hydrolase;

Figure 4 shows the DNA-oligomer mixture based on the N-terminal peptide sequence of the hydrolase; and

Figure 5 shows the DNA-"antisense" oligomer mixture based on the N-terminal peptide sequence of the hydrolase.

According to the invention, the process is performed so that R-(-)-2,2-DMCPCA in racemic R,S-(±)-2,2-DMCPCA is biotransformed, by microorganisms transformed with a gene that forms a stereospecific hydrolase, to R-(-)-2,2-DMCPCS. Optically active S-(+)-2,2-DMCPCA is thus obtained and than then be isolated.

Productions of the Transformed Microorganisms

The production of the microorganisms, according to the present invention, which form a stereospecific hydrolase, takes place such that:

(A) a DNA coding for hydrolase according to the present invention is isolated;

(B) this specific gene sequence is introduced in an expression vector, and a hybrid plasmid results. Optionally it can be advantageous to perform further modifications in the hybrid plasmid to achieve more effective expression of the gene sequence thus introduced.

(C) this hybrid plasmid is introduced by transformation [transformation takes place] in a suitable microorganism (host strain) and this transformed microorganism then forms the production strain for the biotransformation process according to the present invention.

(A) Isolation of Stereospecific Hydrolase DNA

As a source of the hydrolase DNA, which is designated below as hydrolase DNA (rad) or hydrolase gene (rad), for example, the chromosomal DNA of microorganisms Comamonas acidovorans A:18 (DSM No. 6315) or Comamonas acidovorans TG 308 (DSM No. 6552), which has already been described in European Published Patent Application No. 92103780.0, can be used. Preferably, Comamonas acidovorans A:18 is used as the source. The hydrolase DNA can be isolated from a linear gene bank of Comamonas acidovorans A:18 in Escherichia coli (E. Coli) XL1-Blue* with BLUESCRIPT* (BLUESCRIPT-KS+ or BLUESCRIPT-SK+) (available from the Stratagene Co., supplier Genofit SA, Geneva, Switzerland) as a commercially available gene bank vector).

For this purpose, for example, first chromosomal DNA of Comamonas acidovorans A:18 is isolated following the method of R.H. Chesney et al [J. Mol. Biol., 130, (1979), pages 161 to 173]. This DNA can then be cut by the usual molecular biological methods with the restriction enzyme EcoRI and then inserted in the previously likewise cut

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expression vector DNA pBLUESCRIPT-KS+*. Then this ligated DNA (hybrid plasmid mixture) can be transformed, for example, according to the method of S. Fiedler and R. Wirth [Analyt. Biochem., 170, (1988), pages 38 to 44], in the appropriate commercially available microorganisms E. coli XL1-Blue*.

The "screening" of the gene bank can also take place according to methods known in the art. In this connection, the hybrid plasmid clones can advantageously be examined for their growth capability in a suitable medium with R,S-(±)-2,2 DMCPCA as the sole N-source, a usual C-source, a suitable inductor and a suitable antibiotic. After this "screening" the hybrid plasmid clones can be selected which contain the active hydrolase gene (rad) on the hybrid plasmid DNA and, thus, are, preferably, capable of using R-(-)-2,2-DMCPCA as the sole N-source. These hybrid plasmid clones are then able to hydrolyse the R-(-)-2,2-DMCPCA to the corresponding acid.

Suitably, the location of the hydrolase gene (rad) then takes place in the hybrid plasmid pCAR1 (selected from the hybrid plasmid clones) which consists of the expression vector pBLUESCRIPT-KS+* and an EcoRI-"insert" of about 23 kb.

The actual location of the hydrolase gene (rad) can then take place using the "Southern-Blot" hybridization technique known in the art [Current Protocols in Molecular Biology, John Wiley and Sons, New York, (1987), section

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2.9]. Preferably, for this purpose hybrid plasmid pCAR1 is first digested with the restriction enzymes BamHI, PstI, PvuII and EcoRI. The DNA fragments thus developed can then be hybridized against radioactively labelled DNA oligomers which correspond to the N-terminal protein sequences of the hydrolase. In this way a 2.3 kb EcoRi-BamHI DNA section or a 1.85 kb PvuII-BamHI DNA section can be labelled on the hybrid plasmid pCAR1.

The DNA oligomers for the hybridization can be obtained according to prior art methods, for example, by the stereospecific hydrolase being chromotographically enriched and the N-terminal amino acid determined after which the corresponding DNA sequence can be synthesized and radioactively labelled. The DNA section which codes for the stereospecific hydrolase (rad) and whose restriction map is represented in Figure 1 is also a novel feature of the present invention. Subsequently, the aforesaid DNA section can be isolated with the restriction enzymes BamHI and EcoRI or BamHI and PvuII from the hybrid plasmid pCAR1 according to methods known in the art, i.e., to determine the entire amino acid sequence by analysis of the nucleotide sequence comprising the genetic code and to produce the transformed microorganisms suitable for the process of the present invention.

Therefore, the present invention provides both a DNA fragment which codes for a polypeptide with stereospecific hydrolase activity, whose amino acid

sequence is represented in Figure 3, and a DNA fragment which hybridizes with the DNA fragment represented in Figure 3 and codes for this polypeptide.

(B) Ligation of the Specific Gene Sequence (Hydrolase Gene; rad) in Expression Vectors

The gene sequence thus obtained can be ligated by standard molecular biological techniques with a previously likewise cut expression vector DNA to provide a hybrid plasmid.

Expression vectors usually contain a suitable, in most cases adjustable, promoter (expression control sequence). At best there are one or more singular cutting sites for the restriction enzymes behind this promoter in the direction of transcription. Usually the gene section intended to be expressed is then inserted in these cutting sites.

For the process according to the present invention either expression vectors with a broad host range can be applied or, for example, the commercially available expression vector pBLUESCRIPT-KS+* can be applied. Preferably, pBLUESCRIPT-KS+* with promoter P_{Lac} (lactose promoter) can be used as the expression vector. Suitably, the expression vector pBLUESCRIPT-KS+* is cut with the restriction enzymes EcoRI and BamHI or with PvuII and BamHI. The restriction ends that are thus developed are ligated with the isolated DNA section (EcoRI-BamHI or

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PvuII-BamHI) which codes for the stereospecific hydrolase, for example, by using T4-DNA-ligase.

(C) Hybrid Plasmids

The invention further relates to the hybrid plasmids thus developed which contain the stereospecific hydrolase gene sequence (rad).

Basically, all hybrid plasmids which are able to replicate and express the DNA sequence coding for the hydrolase according to the invention in the selected microorganism (production strain) are suitable. Suitable hybrid plasmids contain, from their original expression vector, an intact replicon and a labelling gene which makes possible the selection and identification of the microorganisms transformed with the hybrid plasmid on the basis of a phenotypic feature. A suitable labelling gene provides, for example, resistance to the microorganism against antibiotics.

To achieve effective expression in a hybrid plasmid, it is suitable for the hydrolase gene (rad) to be placed, correctly, in "phase" with the promoter.

Examples of such hybrid plasmids that are suitable for the expression of the hydrolase gene in an E. coli strain are the hybrid plasmids pCAR5 and pCAR6, having the labelling gene bla (which provides resistance to ampicillin; Ap*) and the promotor P_{Lac}. Suitably, the hybrid plasmid pCAR5 consists of the 2.3 kb EcoRI-BamHI DNA fragment (the restriction map in Figure 1) and the

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expression vector pBLUESCRIPT-KS+*. Preferably, the hybrid plasmid pCAR6 consists of the 1.85 kb PvuII-BamHI DNA fragment in Figure 1 (restriction map) and the expression vector pBLUESCRIPT-KS+*.

Suitably, the hybrid plasmid pCAR6 is used with promoter P_{Lac} and the expression of the hydrolase gene (rad), depending on the host strain, is induced with isopropylthiagalactoside.

The hybrid plasmid pCAR6 was deposited both on June 6, 1991, under DSM No. 6551 in E. coli XL1-Blue* and on April 21, 1992, under DSM No. 7053 in E. coli DH5*, in the Deutsche Sammlund fur Mikroorganismen und Zellkulturen GmbH [German Collection for Microorganisms and Cell Structures GmbH], Mascheroderweg 1b, D-3300 Brunswick, Germany.

Figure 2 shows a diagram of the hybrid plasmid pCAR6.

(D) Host Strains

The hybrid plasmids thus obtained are advantageously used in host strains.

Preferably in the case of "broad-host-range" hybrid plasmids, host strains with high substrate and precursor tolerance are used, such as, microorganisms of genus Pseudomonas, Comamonas, Acinetobacter, Rhizobium, Agrobacterium or Escherichia.

In the case of hybrid plasmids which have a limited host range, such as, the hybrid plasmid pCAR6, usually the specific host strains in which they replicate

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are used. Accordingly, microorganisms of the genus Escherichia, especially those of species E. coli which are listed in Table 1, are preferably used as the host strains for the hybrid plasmid pCAR6.

(E) Transformation

The transformation of the host strains takes place with the hybrid plasmids of the present invention according to known processes. Preferably, the isolation of the transformed host strains then takes place from a selective nutrient medium to which an antibiotic is added, against which the labelling gene contained in the hybrid plasmid provides resistance. Preferably, the hybrid plasmid pCAR6 which contains the bla gene is used, ampicillin is accordingly added to the nutrient medium.

(F) Production Strain

All host strains which are transformed with the hybrid plasmid which contains the stereospecific hydrolase gene can be used as production strains according to the present invention. Preferably, the microorganisms of the species E. coli that are listed in Table 1 and the descendants and mutants thereof, and that are transformed with hybrid plasmid pCAR6 are used as production strains. The microorganisms E. coli XL1-Blue* (DSM No. 6551) and E. coli DH5* (DSM No. 7053) were deposited as described above.

If, for example, from Table 1 E. coli MC4100 [described in Mol. Gen. Genet., 192, (1983), pages 293 and 294] is used as the host strain, the expression of the

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stereoselective hydrolase gene (rad) takes place under the permanent control of promotor P_{Lac}, because of a deletion in the lac-operon (lactose operon) [deletion of (argF - lac) U169]. Accordingly, the lac repressor-gene lacI is not formed (repressor gene negative microorganism). If, for example, from Table 1, E. coli K12 (obtainable under DSM No. 498) or E. coli HB101 [H.W. Boyer and D. Roulland-Dussoix, J. Mol. Biol., 41, (1969), pages 459 to 472] is used as the host strain, the expression of hydrolase gene (rad) with IPTG is induced because of the presence of the repressor gene lacI (repressor gene positive microorganism).

(G) Biotransformation

All microorganisms (production strains) can be used for the biotransformation of the present invention which have been transformed with a gene that forms a stereospecific hydrolase and, thus, hydrolize stereospecifically R-(-)-2,2-DMCPCA into acid.

Preferably, the biotransformation is performed with microorganisms which have been transformed with a hydrolase gene (rad) whose restriction map is represented in Figure 1. Microorganisms which have been transformed with a DNA fragment that codes for a polypeptide with stereospecific hydrolase activity whose amino acid sequence is represented in Figure 3 can also be used. Also suitable are microorganisms which have been transformed with a DNA fragment that hybridizes with the DNA fragment represented

in Figure 3 and that codes for a polypeptide with stereospecific hydrolase activity. Especially preferred for the process of the present invention are, as described above, the microorganisms of the species E. coli (Table 1) transformed with the hybrid plasmid pCAR5 or pCAR6, especially those transformed with the hybrid plasmid pCAR6. The cell-free enzymes (the stereospecific hydrolases) from these microorganisms are also suitable. These cell-free enzymes can be obtained by breaking down the microorganism cells using prior art methods. For this purpose, for example, the ultrasonic, the French press or the lysozyme methods can be used. In carrying out the process of the present invention, these cell-free enzymes can then be immobilized on a suitable support material according to methods known in the art.

Preferably, the process is performed with dormant microorganism cells (not growing cells) which previously have been induced in accordance with their expression system. This means that, if repressor gene-positive microorganisms are used for the process, such as, E. coli XL1-Blue* or E. coli DH5* that have been transformed with hybrid plasmid pCAR6, the induction takes place with IPTG. If, for example, repressor gene-negative (i.e., absence of the repressor gene) microorganisms of species E. coli, such as, E. coli MC4100, are used for the process, the expression of the hydrolase gene (rad) takes place permanently.

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In as especially preferred embodiment, the specific hydrolase activity of the microorganisms is increased with C_1-C_4 alcohols. For example, methanol, ethanol, propanol, isopropanol or butanol can be used as the C_1-C_4 alcohols. Preferably methanol or ethanol is used.

As a medium for the process of the present invention, media commonly used in the art can be selected, such as, low-molecular phosphate buffers, a mineral salt medium according to Kulla et al. [Arch. Microbiol., 135, (1983), pages 1 to 7] or HEPES-buffer (N-2-hydroxyethylpiperazine-2'-ethanesulfonic acid). Preferably, the process is performed in a low-molecular phosphate buffer.

Preferably the medium for the biotransformation contains racemic R,S-(±)-2,2-DMCPCA in an amount of 0.2 to 5 percent by weight, preferably 0.2 to 2 percent by weight. The biotransformation can be performed in a range of pH 6 to 11, preferably in a range of pH 6.5 to 10. The temperature for the biotransformation can be between 15° and 55°C, preferably between 20° and 40°C. After a usual reaction time of 1 to 30 hours, preferably 5 to 25 hours, R-(-)-2,2-DMCPCA is completely converted to the corresponding acid and optically pure S-(+)-2,2-DMCPCA is obtained. The S-(+)-2,2-DMCPCA thus obtained can then be isolated, for example, by extraction, electrodialysis or drying.

Deposit and taxonomic information for Comamonas acidovorans A:18 (DSM No. 6315), Comamonas acidovorans TG308 (DSM No. 6552), Pseudomonas sp. NSAK:42 (DSM No. 6433), and microorganism Bacterium sp. VIII:II (DSM No. 6316) is set out below.

The strains of DSM Nos. 6315 and 6316 were deposited on January 29, 1991, those of DSM No. 6433 on March 25, 1991, and those of DSM No. 6552 on April 6, 1991, with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures GmbH), Mascherodeweg 1B, D-3300 Brunswick, Germany.

The scientific (taxonomic) description of Comamonas acidovorans A:18 (DSM No. 6315), is:

cell shape	rods
width micron	0.5 to 0.7
length micron	1.5 to 3.0
mobility	+
flagella	polar > 1
Gram reaction	-
lysis by 3% KOH	+
aminopeptidase (Cerny)	+
spores	-
oxidase	+
catalase	+
growth	

anaerobic	-
37°/41°C	+/-
pH 5.6	+
MacConkey agar	+
SS agar	+
cetrimide agar	+
pigments	
nondiffusing	-
diffusing	-
fluorescent	-
pyocyanin	-
acid from (OF test)	
glucose, aerobic	-
glucose, anaerobic	-
gas from glucose	-
acid from	
glucose	-
fructose	+
xylose	-
mannitol	+
glycerol	+
ONPG	-
ADH	-
VP	-
indole	-
NO_2 from NO_3	+

denitrification	-
phenylalaninedesaminase	-
levan from saccharose	-
lecithinase	-
urease	-
hydrolysis of	
starch	-
gelatin	-
casein	-
DNA	-
Tween 80 *	-
aesculin	-
tyrosine	
catabolism	-
use of substrate	
acetate	+
adipate	+
caprate	+
citrate	+
glycolate	+
laevulinate	+
malate	+
malonate	-
phenyl acetate	+
L-arabinose	-
fructose	+

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glucose	-
mannose	-
maltose	-
xylose	-
inositol	-
mannitol	+
gluconate	+
N-acetylglucosamine	-
L-serine	-
L-tryptophan	+
acetamide	+
mesaconate	+
citraconate	+
L-tartrate	+
N-source	
NH_4^+	++
R,S-(±)-2,2-DMCPCA	+
butyramide	++
acetamide	+
propionamide	+
formamide	±
benzamide	+
nicotinamide	+

API ZONE --- Cs.acidovorans 99.0 percent

The scientific (taxonomic) description of Comamonas acidovorans TG 308 (DSM No. 6552) is:

cell shape	rods
Gram reaction (KOH test)	-
Gram stain	-
spores	-
mobility	+
°C growth	
37°C	+
41°C	-
45°C	-
catalase	+
oxidase	+
fermentation in	
glucose (OF test)	-
	isolates
	TG308
nitrate reduction	+
indole production	-
acid from glucose	-
arginine dehydrolase	-
urease	-
aesculin hydrolysis	-
gelatin hydrolysis	-
β-galactosidase	-
glucose assimilation	-

arabinose assimilation	-
mannose assimilation	-
mannitol assimilation	+
N-acetyl-glucosamine assimilation	-
maltose assimilation	-
gluconate assimilation	+
caprate assimilation	+
adipate assimilation	+
malate assimilation	+
citrate assimilation	-
phenyl acetate assimilation	+
cytochrome oxidase	+
NO_2 from NO_3	+
hydrolysis from urea	-
use of fructose	+
alkalization of acetamide	+
alkalization of tartrate	+
alkalization of Simmon's citrate	+
alkalization of malonate	(+)

(+) weakly positive

The scientific (taxonomic) description of Pseudomonas sp. NSAK: 42 (DSM No. 6433) is:

cell shape	rods
width micron	0.6 to 0.8
length micron	1.5 to 3.0
mobility	+

Gram reaction	-
lysis by 3% KOH	+
aminopeptidase (Cerny)	+
spores	-
oxidase	+
catalase	+
growth	
anaerobic	-
37°/41°C	+/-
pH 5.6	+
MacConkey agar	+
SS agar	-
cetrimide agar	-
pigments	yellow
acid from (OF test)	
glucose, aerobic	-
glucose, anaerobic	-
gas from glucose	-
acid from	
glucose	-
fructose	-
xylose	-
ONPG	-
ODC	-

ADH	-
VP	-
indole	-
NO_2 from NO_3	-
denitrification	-
phenylalaninedesaminase	-
levan from saccharose	-
lecithinase	-
urease	-
hydrolysis of	
starch	-
gelatin	-
casein	-
DNA	-
Tween 80*	-
aesculin	-
tyrosine catabolism	-
growth material requirement	-
use of substrate	
acetate	+
caprate	+
citrate	+
glycolate	+
lactate	+
laevulinate	+
malate	+

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malonate	+
phenyl acetate	+
suberate	+
L-arabinose	-
fructose	+
glucose	-
mannose	-
maltose	-
xylose	-
mannitol	-
gluconate	+
2-ketogluconate	+
N-acetylglucosamine	-
L-serine	+
L-histidine	+
hydroxybutyrate	+
N-source	
NH_4^+	++
R,S-(±)-2,2-DMCPCA	+

The scientific description of Bacterium sp. VIII:II (DSM No. 6316), is:

Gram stain	+
Gram reaction (KOH test)	-
oxidase	-
catalase	-
nitrate reduction	-

tryptophan ---> indole	-
glucose (anaerobic)	-
arginine	-
urease	-
aesculin	+
gelatin	-
β-galactosidase	+
glucose	+
arabinose	-
mannose	(+)
mannitol	+
N-acetylglucosamine	-
maltose	+
gluconate	-
caprate	-
adipate	-
malate	-
citrate	-
phenyl acetate	-

The following examples further illustrate the present invention:

Example 1

1.1 Preparation of the Chromosomol DNA of Comamonas Acidovorans A:18

The chromosomal DNA of a fresh overnight culture of Comamonas acidovorans A:18 (100 ml of nutrient yeast broth,

30°C) was isolated according to the modified methods of R.H. Chesney et al. [J. Mol. Biol., 130, (1979), pages 161 to 173].

After being centrifuged-off (15 min., 6'500 X g, 4°C) the cells were resuspended in tris-HCl-buffer (2.25 ml, 0.05 mol/1), pH 8.0, 10 percent (w/v) saccharose. Subsequently, 375 µl of lysozyme solution (10 mg/ml, 0.24 mol/l tris-MCl-buffer, pH 8.0) and 900 µl of 0.1 mol/1 EDTA, pH 8.0 was added and the suspension was cooled on ice for 10 minutes. Then following the addition of 450 µl of 5 percent (w/v) SDS and 50 µl of ribonuclease (10 mg/ml H_2O) an incubation was carried out at 37°C for 30 min. After the addition of a spatula tip full of proteinase K and 400 µl of pronase (20 ml/ml H_2O) the incubation was continued for 2 hours. The suspension was centrifuged after mixing with 4.3 g of CsCl (30 min., 40'000 X g, 20°C), and then mixed with 250 µl of ethidium bromide (10 mg/ml), and centrifuged in an ultracentrifuge (VTi 65.2-tube) (more than 8 hours, 246'000 X g, 20°C). The DNA band was suctioned off from the tube by means of longwave UV light. After the addition of a 4-fold volume of TE-buffer (10 mmol/1 tris-HCl, pH 8.0, 1 mmol/1 EDTA), the ethidium bromide was extracted three times with n-butanol saturated with water. The DNA was precipitated with isopropanol, taken up in TE-buffer and incubated for 15 minutes at 65°C. The preparation could be stored at 4°C.

1.2 Restriction and Ligation of Chromosomal DNA

5 µg of Comamonas acidovorans A:18 (DSM No. 6315)-DNA and 4.5 µg of Vector-DNA (pBLUESCRIPT-KS+*) were each cut (6.5 hours at 37°C) with 20 units of restriction enzyme EcoRI in a total volume of restriction buffer of 100 µl. The DNAs were precipitated with ethanol and dried in a Speed Vac* concentrator. The precipitates were taken up in the ligation buffer [20 mmol/l tris-buffer, 10 mmol/l DTT (dithiothreitol), 10 mmol/l $MgCl_2$, 0.6 mol/l ATP (adenosine triphosphate, pH 7.2], and combined (ligation volume 100 µl). After the addition of 1 unit of T4-DNA-ligase, it was incubated over night at 13°C. The DNA of the ligation mixture was precipitated with isopropanol and taken up in 30 µl of water for transformation.

1.3 Transformation of E. coli XL1-Blue* and Selection

Competent cells of E. coli XL1-Blue* were transformed by electroporation with the ligation mixture according to the method described by S. Fieldler and R. Wirth [Analyt. Biochem., 170, (1988), pages 38 to 44]. For plasmid detection nutrient agar with ampicillin (100 µg/ml) was selected and for "insert" detection with 0.5 mmol/l IPTG (isopropyl-β-D-thiogalactoside) and x-Gal (30 µg/ml, 5-bromo-4-chloro-3-idnolyl-β-D-galactopyranoside) was used. Incubation was carried out at 37°C. At a transformation sequence of 1.7 X 10^8 cfu/ml ("colony forming units" = active cells), almost all of the clones had an EcoRI-"insert".

* - Trade-mark

Example 2

2. Screening of Comamonas Acidovorans A:18-Gene Bank According to the R-specific Amidase Gene

Clones with hybrid plasmids (EcoRI-"insert") were examined for their growth capabilities on minimal medium agar according to H. Kulla et al. [Arch. Microbiol., 135, (1983), pages 1 to 7] with 0.2 percent (v/v) of glycerol as the C-source, 0.15 percent (w/v) of R,S-(±)-2,2-DMCPCA as the sole N-source, and 0.5 mmol/l of IPTG as the inductor of the lac promoter, as well as ampicillin (5 µg/ml) for plasmid stabilization. Only clones, which contained the intact hydrolase gene rad on the DNA "insert" in the plasmid, were able to use R-(-)-2,2-DMCPCA as the N-source, to react the latter into the desired R-acid and to grow on this minimal medium. Clones so selected all contained a hybrid plasmid from the pBLUESCRIPT-KS+® vector with an EcoRI-"insert" of about 23 kb. The plasmid pCAR1 was isolated and more closely characterized.

Example 3

3.1 Isolation of the R-specific Hydrolase from Comamonas acidovorans A:18 and N-terminal Peptide Analysis

(a) Preparation of Cell-Free Extract

16 liters of a cell suspension of Comamonas acidovorans A:18 (DSM No. 6315) was concentrated to 700 ml (OD_{650} = 33.5) with a hydrolase activity at 37°C of 0.6 g of R-(-)-2,2-DMCPCS/l/h/optical density at 650 nm (OD_{650}) = 1, that was

previously induced with R,S-(±)-DMCPCA. Then the cells were centrifuged several times, resuspended in HEPES-buffer, and then taken up in HEPES-buffer (40 ml). The volume of the total cell suspension was then 95 ml (OD_{650} = 210). The hydrolase activity was determined at 30°C and was 0.34 g of product/l/h/OD_{650} = 1. Then the cells were broken down twice in the French press at a pressure of 1200 bars. To obtain a cell-free extract, this suspension was centrifuged at 20000 X g for 20 min. 50 ml of extract was obtained with a protein amount (measured according to the Bradford method) of 39.3 mg/ml and with a hydrolase activity at 30°C of 12.5 g of R-(-)-2,2-DMCPCS/l/h/mg of protein.

(b) Chromatography

This crude-cell extract (50 ml) was applied to a column filled with Q-Sepharose* (Pharmacia) which was equilibrated against a HEPES-buffer (0.1 mol/l, pH 7.5). This column was flushed twice more with the same buffer and then the proteins were eluted with a HEPES-buffer-gradient (0.1-1 mol/l). Altogether 140 ml of protein solution with hydrolase activity was eluted with HEPES-buffer (1 mol/l) that was then concentrated by ultrafiltration (Amicon membrane YM10*). The amount of protein of this enzyme solution was 131 mg/ml and the hydrolase activity was 1 μmol/min/ng of protein. Then 2 ml of this protein solution was applied to a column with Superose-12* (Pharmacia) which had been equilibrated against a HEPES-buffer (0.1 mol/1, pH 7.5). With this buffer altogether

* - Trade-mark

36 ml of protein solution was eluted. The latter was also concentrated by ultrafiltration (Amicon membrane YM10*). The amount of protein was 20.1 mg/ml and the hydrolase activity was 1.2 μmol/min/ng of protein. The protein solution thus obtained was then applied to a column with anion exchanger Li Chirospher 2000 TMAE (trimethylammoniumethyl salt) (Merck) which was equilibrated against a HEPES-buffer (0.1 mol/l, pH 7.5). After flushing of the column with the same buffer, the protein solution was eluted with a NaCl gradient (0-1 mol/l) in the same buffer. The protein concentration was 15 mg/ml and the hydrolase activity was 1.2 μmol/min/ng of protein.

(c) Identification of the Hydrolase by 1- and 2-Dimensional Electrophoresis

In the crude-cell extract the hydrolase protein was identified by SDS-PAGE. At the same time non-induced cell extract was compared with induced cell extract on the SDS-PAGE (induction with R,S-(±)-2,2-DMCPCA). A protein band with a molecular weight around 46000 was detected in the induced cell extract. The protein fractions obtained by chromatography with hydrolase activity were also analyzed by SDS-PAGE. The protein with a molecular weight of about 46000 was concentrated by this chromatographic purification and it was concentrated after the third chromatography to about 80 percent. This 80 percent pure sample was then analyzed by biodimensional electrophoresis (2-D SDS-PAGE). Using this method

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a protein "spot" with a molecular weight of about 46000, which corresponded to the hydrolase, could be detected.

(d) Sequencing

The protein "spot" obtained by 2-D SDS-PAGE was then blotted on a PVDF (polyvinylidene difluoride) membrane and cut out from the membrane. Then this protein was directly sequenced according to the method of Hochstrasser et al. [Applied and Theoretical Electrophoresis, 1, (1988), pages 73 to 76, "HDL particle-associated proteins in plasma and cerebrospinal fluid"]. 21 amino acids (AS) of the N-terminal amino acid sequence was identified by this method.

Example 4

4. Localization of the Hydrolase Gene (rad) of the Cloned EcoRI Fragment

4.1 Rough Restriction Map of pCAR1

A rough restriction map of pCAR1 relative to EcoRV, PVuII, KspI, SmaI, PstI, StuI and BamHI was made by restriction analysis according to a conventional process [Current Protocols Molecular Biology, John Wiley and Sons, New York, (1987), section 2].

4.2 Formulation of Mixed DNA-oligomers Based on the N-terminal Peptide Sequence of the Hydrolase

By virtue of the nature of the genetic code, two mixed DNA oligomers could be formulated for the N-terminal peptide sequence of Comamonas acidovorans A:18 hydrolase and synthesized with a DNA-synthesis machine.

DNA-oligomer (mixture)

See Figure 4.

DNA-"Antisense" oligomer (mixture)

See Figure 5.

4.3 "Southern Blot-Hybridization" of Restriction Fragments Of Plasmid pCAR1

The DNA fragments separated by agarose gel electrophoresis (0.6 percent), which were obtained according to various restrictions (BamHI, PstI, EcoRI) or pCAR1, were transferred by the known "Southern Blot process" to nitrocellulose [Current Protocols in Molecular Biology, John Wiley and Sons, New York, (1987), section 2.9 ff].

The DNA-oligomers were likewise end-labeled with [^{32}P]-gamma-ATP:

400 ng of DNA-oligomer, 22 μCi ^{32}P-Gamma-ATP, and 11 units of polynucleotide kinase phosphate-free, in a total of 25 μl of polynucleotide kinase-buffer (0.05 mol/l tris-HCl, pH 7.5, 0.01 mol/l $MgCl_2$, 5 mmol/l DTT) were incubated for 30 minutes at 37°C.

Purification was then effected by Sephadex G-25* gel filtration (Pharmacia) and hybridization against the "Southern Blots" according to the process disclosed in the above-mentioned reference.

By hybridization against the nucleotide-oligomers corresponding to the N-terminal protein sequence, a 2.3 kb of EcoRI-BamHI DNA fragment of a 1.85 kb or PvuII-BamHI DNA

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fragment could be labeled on hybrid plasmid pCAR1.

4.4 Subclonings of the Hydrolase Gene (rad)

The 2.3 kb EcoRI-BamHI DNA fragment of the 1.85 kb PvuII-BamHI DNA fragment, which codes for the R-specific hydrolase from Comamonas acidovorans A:18, was inserted in likewise digested vector-DNA pBLUESCRIPT-KS+® or pBLUESCRIPT-SK+®. The desired hydrolase activity only shows an orientation of the "insert" toward promoter P_{Lac} in the clones after IPTG induction. The vector pBLUESCRIPT-KS+® with the 2.3 kb EcoRI-BamHI DNA fragment was designated as hybrid plasmid pCAR6. Vector pBLUESCRIPT-KS+® with the 1.85 kb PvuII-BamHI DNA fragment was designated hybrid plasmid pCAR5.

Example 5

5. Determination of Activity of R-(-)-2,2-DMCPCA Hydrolase

The microorganism suspension was adjusted to an optical density of 0.5 at 650 nm for the determination of the hydrolase activity. A phosphate buffer (10 mmol/l), pH 7.0, with 0.2 percent by weight of R-(±)-2,2-DMCPCA served as the medium. This suspension was incubated for 4 hours at 30°C with shaking. The NH^+_4 released by the hydrolase or the R-(-)-2,2-DMCPCS was measured and the activity was expressed as g of R-(-)-2,2-DMCPCA reacted per 1/h/optical density at 650 nm, provided that 1 mmol of formed NH^+_4 = 1 mmol corresponds to the reacted R-(-)-2,2-DMCPCA.

Example 6

Production of S-(+)-2,2-DMCPCA

E. coli K12 with hybrid plasmid pCAR6, in the mineral salt medium containing 0.2 percent (v/v) of glycerol and 0.15 percent by weight of R,S-(±)-2,2-DMCPCA, showed a specific hydrolase activity of 1.2 g of R-(-)-2,2-DMCPCS/l/h/OD_{650} after IPTG-induction. The reaction of R-(-)-2,2-DMCPCA to R-(-)-acid was confirmed by NH^{+}_{4} release and GC analysis. The target product S-(+)-2,2-DMCPCA remained unchanged in the racemic mixture.

Corresponding to E. coli K12, the microorganisms listed in Table 1 were cultivated and the results of the reaction were shown in Table 1.

TABLE 1

Stereospecific Hydrolase Activity In Various E. Coli Strains

Strain	Specific Activity g/l/h/OD	Factor	Stability in % (4)	Max. OD_{650} nm	Total Activity g/l/h
Comamonas acidovorans A:18 (1) (not according to the invention)	0.5	1	-	6	3.0
E. coli K12/pCAR6 (2)	1.2	2.4	90	nt	-
E. coli HB101/pCAR6 (2)	0.25	0.5	nt	nt	-
E. coli MC4100/pCAR6 (3)	0.53	1	89	nt	-
E. coli XL1BLUE/pCAR6 (5) (DSM No. 6551)	0.5	1	90	30	15.0
E. coli DH5/pCAR6 (5) (DSM No. 7053)	2.1	4.2	100	30	63.0

Notes:

(1) induction with amide
(2) induction with IPTG
(3) constitutive
(4) plasmid stability after 24 hours with selection of antiobiotics; nt = not tested
(5) without induction

Example 7

Activity Test With C_1-C_4 alcohols

The activity tests were performed first with Comamonas acidovorans A:18 at 37°C with 0.5 percent R,S-2,2-DMCPCA in 10 mM of potassium phosphate buffer at pH 7.0. The control was without a solvent; the test studies were with 5 to 16 volume percent of solvent. The computation of the specific activity took place as described in Example 5.

Solvent (Volume percent)	Activity for the reaction of g R-2,2-DMCPCA/l/h/OD_{650} nm
---	0.64
Ethanol (10)	1.24
Isopropanol (10)	1.85
Methanol (5)	1.79
Methanol (10)	1.54
Methanol (16)	1.66

In biotransformations in the 20 l-fermenter with 2 to 3 percent R,S-2,2-DMCPCA (37°C, 10 mM, potassium phosphate buffer, pH 7.0) with the addition of 5 to 7.5 volume percent of methanol or ethanol, a shortening of the reaction time and a higher yield of S-(+)-2,2-DMCPCA (selectivity enhancement) was achieved. The same effect was observed in the E. coli-strain XL1-Blue* with the hydrolase gene. The results are compiled in Table 2.

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Table 2

[The same activities (in water) were used for each strain.]

Strain	R,S-2,2-DMCPCA (%)	Solvent (Vol. %)	Time (h)	ee (%)	Yield (%)*
A:18	2.0	--	22	99	41.5
A:18	2.3	Methanol (7.5)	15	99.2	47
XL1/pCAR6	2.8	---	24	100	36
XL1/pCAR6	2.8	Methanol (7.5)	7	98.2	46
XL1/pCAR6	2.8	Ethanol (5)	7	98.6	44

Note:

* of S-(+)-2,2-DMCPCA relative to the R,S-DMCPCA used.

Example 8

Immobilization of the Stereospecific Hydrolase of E. coli XL1-Blue*/pCAR6

The cell-free extract (288 ml) of E. coli XL1-Blue*/pCAR6 containing 28 mg of protein/ml with a hydrolase activity at 37°C of 0.29 μmol R-(-)-2,2-DMCPCA/min•mg of protein was first prepurified by column chromatography on Q-Sepharose* (Pharmacia). In this connection the hydrolase protein was eluted with a NaCl-gradient (0-1 mol/l) in a tris-HCl buffer (0.1 molar, pH 7.5). The protein with hydrolase activity, which had been eluted between 40 percent and 80 percent of the NaCl-gradient, was then concentrated by ultrafiltration (Amicon membrane YM10*) and desalinated by gel filtration

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(PD-10, Sephadex G-25M*, Pharmacia LKB). The end volume was then 67 mg of protein/ml containing 47 ml in potassium phosphate buffer (0.1 molar, pH 7.0) with a hydrolase activity at 37°C of 0.69 μmol R-(-)-2,2-DMCPCA/min•mg of protein. Then this prepurified stereospecific hydrolase was immobilized on Eupergit C as the carrier material (Rohm Pharma GmbH, Weiterstadt, FRG). In this connection the oxirane groups of the insoluble carrier material were covalently bound to the free amino groups of the hydrolase protein. The immobilization was performed for 90 hours at room temperature in potassium phosphate-buffer (1 molar, pH 8.5). 10.2 mg of immobilized protein/g moist weight of Eupergit C with a hydrolase activity at 37°C of 1.5 μmol of R-(-)-2,2-DMCPCA/min•g moist weight of Eupergit C was obtained. The stability of the immobilized hydrolase at 37°C in the potassium phosphate buffer (10 molar, pH 8.5), containing 0.5 percent by weight of R,S-(±)-2,2-DMCPCA, is represented in Table 3.

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Table 3

Time, hours	Activity of the immobilized hydrolase [μmol R-(-)-2,2-DMCPCA/min•g moist weight of Eupergit C]
0 - 90	1.5
90 - 185	0.68

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A genetic engineering process for the production of S-(+)-2,2-dimethylcyclopropanecarboxamide, wherein R-(-)-2,2-dimethylcyclopropanecarboxamide in racemic R,S-(+)-2,2-dimethylcyclopropanecarboxamide is biotransformed, by microorganisms which have been transformed with a gene that encodes a stereospecific hydrolase, and which is characterized by the restriction map which is represented in Figure 1, to form R-(-)-2,2-dimethylcyclopropanecarboxylic acid, and optically active S-(+)-2,2-dimethylcyclopropanecarboxamide is thus obtained and is then isolated.

2. A process according to Claim 1, wherein the biotransformation is performed with microorganisms which have been transformed with a DNA fragment whose nucleotide sequence is represented in Figure 3 and which codes for a polypeptide with stereospecific hydrolase activity.

3. A process according to Claim 1, wherein the biotransformation is performed with microorganisms which have been transformed with a DNA fragment that codes for a polypeptide with stereospecific hydrolase activity and whose amino acid sequence is represented in Figure 3.

4. A process according to Claim 1, 2 or 3, wherein the biotransformation is performed with microorganisms which have been transformed with a DNA fragment that hybridizes under conditions effective to achieve such hybridization and has at least a homology with the DNA fragment represented by the nucleotide sequence in Figure 3 of 90 percent and which codes for a polypeptide with stereospecific hydrolase activity.

5. A process according to Claim 1, 2 or 3, wherein the biotransformation is performed with microorganisms of the genus Escherichia, Pseudomonas, Comamonas, Acinetobacter, Rhizobium or Agrobacterium.

6. A process according to Claim 1, 2 or 3, wherein the biotransformation is performed with microorganisms of the species Escherichia coli.

7. The process according to Claim 1, 2 or 3, wherein the biotransformation is performed with microorganisms of the species Escherichia coli XL1-Blue* (DSM No. 6551) or a descendant thereof, or a mutant thereof, which have been transformed with the hybrid plasmid pCAR6.

8. A process according to Claim 1, 2 or 3, wherein the biotransformation is performed with microorganisms of the species Escherichia coli DH5* (DSM No. 7053) or with a

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descendant thereof, or a mutant thereof, which have been transformed with the hybrid plasmid pCAR6.

9. A process according to Claim 1, 2 or 3, wherein the biotransformation is performed with an immobilized stereospecific hydrolase.

10. A process according to Claim 1, 2 or 3, wherein the biotransformation is performed in a medium containing racemic R,S-(±)-2,2-dimethylcyclopropanecarboxamide in an amount of 0.2 to 5 percent by weight.

11. A process according to Claim 1, 2 or 3, wherein the biotransformation is performed at a pH of 6 to 11 and a temperature of 15° to 55°C.

12. A DNA coding for a stereospecific hydrolase characterized by the nucleotide sequence which is represented in Figure 3.

13. A DNA fragment coding for a polypeptide with stereospecific hydrolase activity whose amino acid sequence is represented in Figure 3.

14. A DNA fragment that hybridizes under conditions effective to achieve such hybridization and has at least a homology with the DNA fragment represented by the nucleotide sequence in Figure 3 of 90 percent and which codes for a polypeptide with stereospecific hydrolase activity.

15. The DNA or the DNA fragment according to Claim 12, 13 or 14, in hybrid plasmid pCAR6, deposited in Escherichia coli XL1-Blue* (DSM No. 6551).

16. The DNA or the DNA fragment according to Claim 12, 13 or 14, in hybrid plasmid pCAR6, deposited in Escherichia coli DH5* (DSM No. 7053).

17. A hybrid plasmid consisting of an expression vector with the DNA or the DNA fragment according to Claim 12, 13 or 14 inserted in it.

18. Hybrid plasmid pCAR6 consisting of the DNA or the DNA fragment according to Claim 12, 13 or 14 and expression vector pBLUESCRIPT-KS+*, deposited in Escherichia coli XL1-Blue* (DSM No. 6551).

19. Hybrid plasmid pCAR6 consisting of the DNA or the DNA fragment according to Claim 12, 13 or 14 and expression vector pBLUESCRIPT-KS+*, deposited in Escherichia coli DH5* (DSM No. 7053).

20. A microorganism that has been transformed with a hybrid plasmid selected from the group consisting of: a hybrid plasmid consisting of an expression vector with the DNA or the DNA fragment according to Claim 12, 13 or 14 inserted

* - Trade-mark

in it, a hybrid plasmid pCAR6 consisting of the DNA or the DNA fragment according to Claim 12, 13 or 14 and expression vector pBLUESCRIPT-KS+*, deposited in Escherichia coli XL1-Blue* (DSM No. 6551), and a hybrid plasmid pCAR6 consisting of the DNA or the DNA fragment according to Claim 12, 13 or 14 and expression vector pBLUESCRIPT-KS+*, deposited in Escherichia coli DH5* (DSM No. 7053).

21. A microorganism according to Claim 20 of the species Escherichia coli XL1-Blue* (DSM No. 6551) or a descendant thereof or a mutant thereof, that has been transformed with hybrid plasmid pCAR6.

22. A microorganism according to Claim 20 of the species Escherichia coli DH5* (DSM No. 7053) or a descendant thereof or a mutant thereof, that has been transformed with hybrid plasmid pCAR6.

* - Trade-mark

Fig. 1

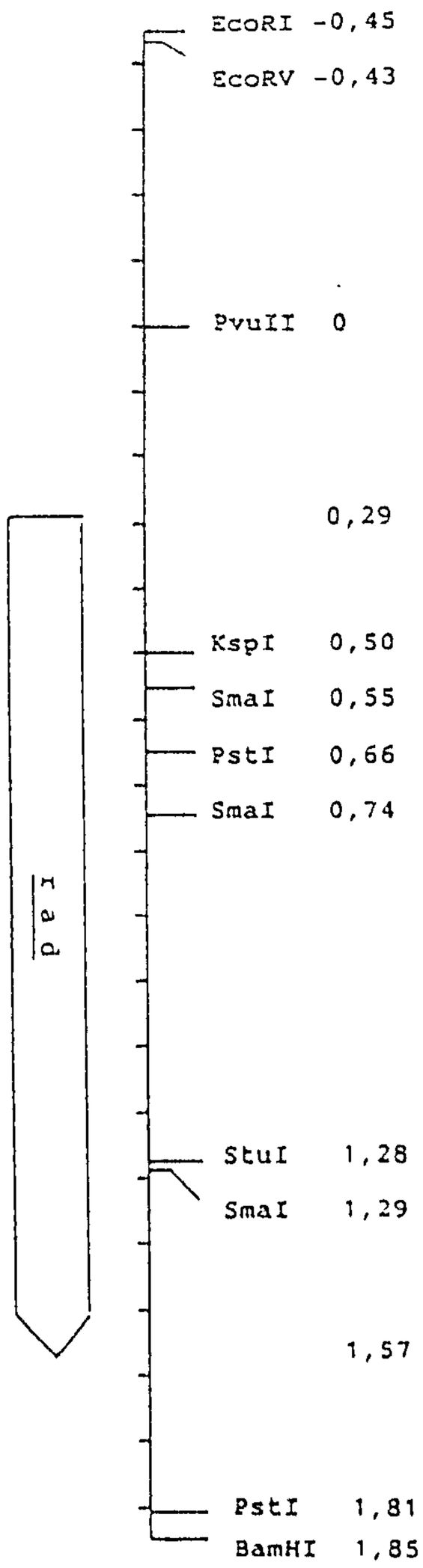

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Fig. 2

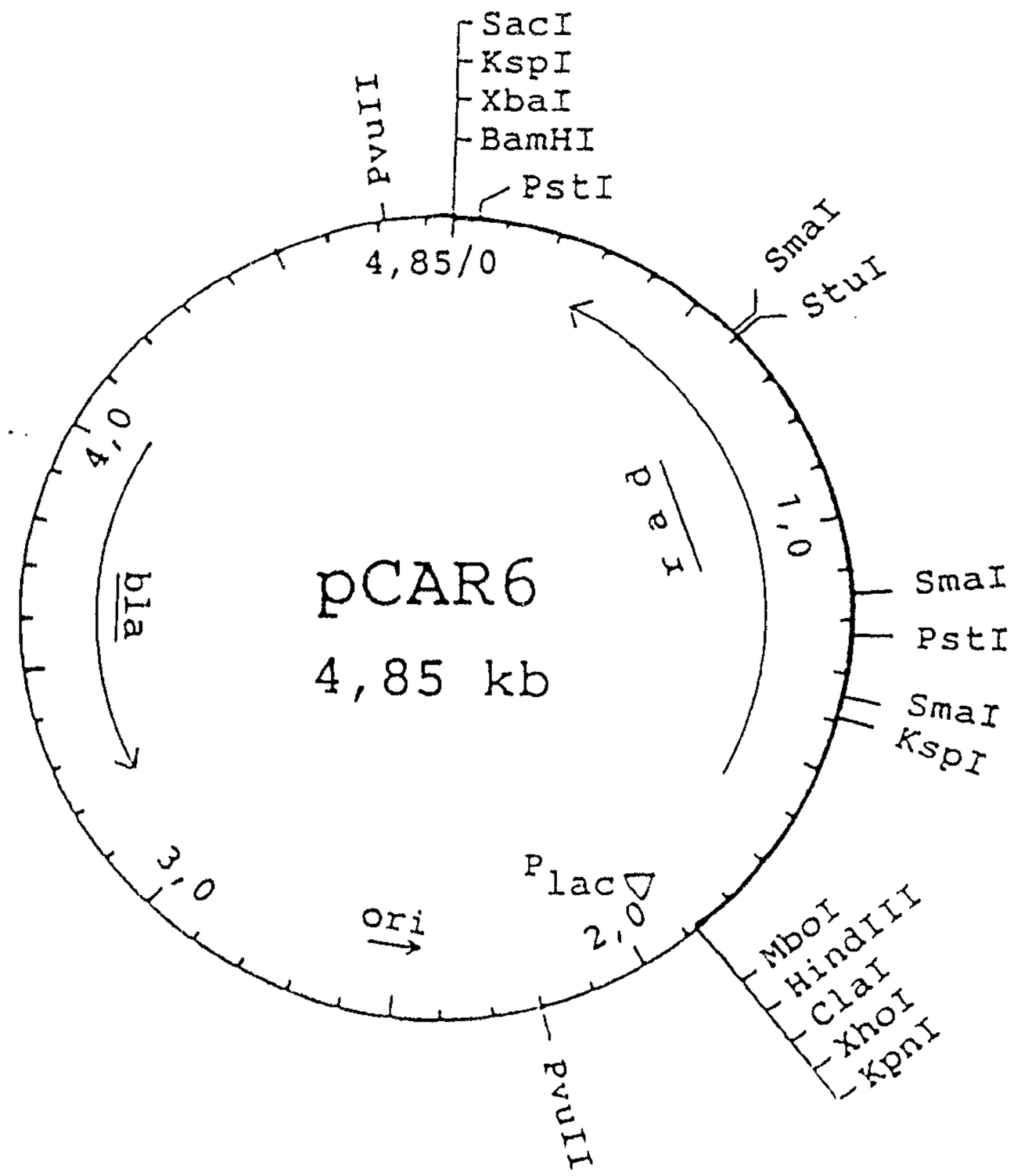

Fig. 3

```
                                                              PvuII
                                                              c agc tgc ggt tgc agg cca gcc gcc agc gcc tga tgc agg ggc

gct gct cca gcg tga ccg agg cgg cct tcg cgc atg ggt tct ccg atg cgg cgc act tca gcc gag cct ttc gca agg cgt tcg gct gca

cgc ccc gca gcc tgc tgg ccg cct gag caa ggt cgc tga cct cac gaa caa gcg tcg gcg ccg ccg gct ccc tag cat ggg cct gcc gtg

cgt ccc gtg cgg ctt ttc cag tgc aac aac ggt ggc gcc ccg gag ccg ggc gcc ggg aga cat gcc ATG AAT GAC AGC GAA CTG CAC CAT
                                                                                        MET Asn Asp Ser Glu Leu His His

CTC GAA CTG CTG GAG GTG GGG CGC GAG ATC CAG TCC CGC CGC ATT TCG TCG GAA GAG GTG ACC CGC CAC ATG CTG GCG CGC ATC GAG GCC
Leu Glu Leu Leu Glu Val Gly Arg Glu Ile Gln Ser Arg Arg Ile Ser Ser Glu Glu Val Thr Arg His MET Leu Ala Arg Ile Glu Ala

GTG GAC GCG CGG CTG CAC AGC TAC GTG ACG GTG ATG GCG CAG CAG GCG ATG GAG GAC GCC CGC CGC GCC GAC GCC GAG ATC GCG CAG GGC
Val Asp Ala Arg Leu His Ser Tyr Val Thr Val MET Ala Gln Gln Ala MET Glu Asp Ala Arg Arg Ala Asp Ala Glu Ile Ala Gln Gly
   KspI                                                         SmaI
GCC GCC GCG GTG CGC TGC ACG GCG TGC CGT GGC GCT CAA GGA CCT GCT GTG GAC CCG GGG CGT CCC CAC CAC GCA TGG AAT GAC GCT GCA
Ala Ala Ala Val Arg Cys Thr Ala Cys Arg Gly Ala Gln Gly Pro Ala Val Asp Pro Gly Arg Pro His His Ala Trp Asn Asp Ala Ala
                                                                                                PstI
CCG CGA CCA TCG CCC CAC GGA AGA TGC CAC CGT GGT GCG CAG GCT GCG CGA GGC CGG CGC CGT CAT CCT GGG CAA GCT GCA GCA GAC CGA
Pro Arg Pro Ser Pro His Gly Arg Cys His Arg Gly Ala Gln Ala Ala Arg Gly Arg Arg Arg His Pro Gly Gln Ala Ala Ala Asp Arg
                                                                                        SmaI
AGG CGC CTT CGC CGA CCA CCA TCC CGA GAT CAC TGC CCC CGT CAA CCC CTG GAG CGC CCA GCT ATG GCC CGG GGC CTC GTC CAG CGG CTC
Arg Arg Leu Arg Arg Pro Pro Ser Arg Asp His Cys Pro Arg Gln Pro Leu Glu Arg Pro Ala MET Ala Arg Gly Leu Val Gln Arg Leu

GGG CGT GGC CAC GGC GGC GGG GCT GTG CTT CGG ATC GCT GGG CAC GGA CAC CGG GGG CTC CAT CCG CTT TCC ATC GGC CGC CAA CGG CAT
Gly Arg Gly His Gly Gly Gly Ala Val Leu Arg Ile Ala Gly His Gly His Arg Gly Leu His Pro Leu Ser Ile Gly Arg Gln Arg His

CAC GGG GCT CAA GCC CAC CTG GGG CAG GGT GAG CCG CCA CGG CGC CTT CGA ACT GGC CGC GTC CCT GGA CCA CAT AGG CCC GAT GGC GCG
His Gly Ala Gln Ala His Leu Gly Gln Gly Glu Pro Pro Arg Arg Leu Arg Thr Gly Arg Val Pro Gly Pro His Arg Pro Asp Gly Ala

CAG TGC TGC CGA TGC CGC AGC CAT GCT CGC GGC CAT CGC CGG GGC GGA CCC GCT GGA CCC TAC GGC CAG CCA GTG CAG CGT GCC CGA CTA
Gln Cys Cys Arg Cys Arg Ser His Ala Arg Gly His Arg Arg Gly Gly Pro Ala Gly Pro Tyr Gly Gln Pro Val Gln Arg Ala Arg Leu

TCT GGC CAT GAT GAC GCG CGG ATT CTC CGG CCT GCG CCT GGG CAT GGA CCG GCA ATG GGC ACT GGA CGG CGT GGA TGC CCC CTC CCG CCA
Ser Gly His Asp Asp Ala Arg Ile Leu Arg Pro Ala Pro Gly His Gly Pro Ala MET Gly Thr Gly Arg Arg Gly Cys Pro Leu Pro Pro

GGC GGT GGA GCA GGC CCT GGC GGT GGC GCA GCG CCT GGG GGC CAG CGT GCA GGA GGT CCG CTT TCC CGA TGC CAC CCA GGC GGT GGA GGA
Gly Gly Gly Ala Gly Pro Gly Gly Gly Ala Ala Pro Gly Gly Gln Arg Ala Gly Gly Pro Leu Ser Arg Cys His Pro Gly Gly Gly Gly
                                                                                     StuI        SmaI
CTG GCC GGC GCT GTG CGC GGT GGA GAC CGC CGT GGC GCA CGG CGC CAC GTT CCC TGC ACG GCG CGA GGC CTA TGG CCC CGG GCT CGC CGG
Leu Ala Gly Ala Val Arg Gly Gly Asp Arg Arg Gly Ala Arg Arg His Val Pro Cys Thr Ala Arg Gly Leu Trp Pro Arg Ala Arg Arg

GTT GAT CGA CCT GGG GCT GGG CCT GTC CGC CAC CGA CTA CCA GCG GCT GCT GCT GCG CCG CGC GGA CTT CAC GGG CCG GGT GCG TGC ACT
Val Asp Arg Pro Gly Ala Gly Pro Val Arg His Arg Leu Pro Ala Ala Ala Ala Ala Pro Arg Gly Leu His Gly Pro Gly Ala Cys Thr

CTT CGC GCA GGT GGA TCT GCT GCT GGT CCC CGC CAC GGC CTT TGC GGC CCC CAC GCT GCA ACG CAT GGC GCA TTT CGG CTC CGA TGC CGA
Leu Arg Ala Gly Gly Ser Ala Ala Gly Pro Arg His Gly Leu Cys Gly Pro His Ala Ala Thr His Gly Ala Phe Arg Leu Arg Cys Arg

GCT GTT CTC GGG CAT GCT GCG CTA CAC CTG CCC GTT CGA CCT CAC GGG CAG CCC CAC GAT CAC GCT GCC CGG CGG ACG CAC TTC TGA ggg
Ala Val Leu Gly His Ala Ala Leu His Leu Pro Val Arg Pro His Gly Gln Pro His Asp His Ala Ala Arg Arg Thr His Phe ---

cgc gcc cgt ggc ctt cca gtt cgt ggc ccc cga ctt ccg cga aga cct gct ggt gcg cgc ggg ctg ggc gtt cca gca ggc cac gga ctc

gca cag aca gca ccc tgc tgc ctg agc tgc ctg agc cgc cag gcc ggt ggc gcg aca cgg gcc tgt cac aca gcc ttc cta gac tgg cgt
                                                                        PstI                                       BamHI
gat gtc ctt gat cga gat gga agg tgt cgc caa gtc ctg ggg cgg cac cac ggc gct gca ggc gct gga tct gcg cat tga acc cgg atc

c
```

FIGURE 4

DNA-oligomer (mixture)

			T	T	TCT	A	T T	T	
5′	ATG	AAC	GAC	AGC	GAG	CTC	CAC	CA	3′
				C		C			
				A		A			
AS	Met	Asn	Asp	Ser	Glu	Leu	His		AS

FIGURE 5

DNA-"Antisense" oligomer (mixture)

		A	C	T	T	T	T	
5′	TG	GAT	TTC	CCG	CCC	CAC	CTC	3′
				G	G	G		
					A	A		
AS		Ile	Glu	Arg	Gly	Val	Glu	AS