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(54) Titre : PROCEDE D'OBTENTION DE CIS-3-HYDROXY-L-PROLINE
(54) Title: PROCESS FOR PRODUCING CIS-3-HYDROXY-L-PROLINE

(57) **Abrégé/Abstract:**

The present invention relates to industrial methods for producing cis-3-hydroxy-L-proline which is useful as a starting compound for medicines and an additive to foods, genes which code for L-proline 3-hydroxylases and which are useful for the above-mentioned process, recombinant DNA containing the gene, transformants containing the recombinant DNA, methods for producing L-proline 3-hydroxylases using the transformants and the enzyme.



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Abstract

The present invention relates to industrial methods for producing *cis*-3-hydroxy-L-proline which is useful as a starting compound for medicines and an additive to foods, genes which code
5 for L-proline 3-hydroxylases and which are useful for the above-mentioned process, recombinant DNA containing the gene, transformants containing the recombinant DNA, methods for producing L-proline 3-hydroxylases using the transformants and the enzyme.

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SPECIFICATION

PROCESS FOR PRODUCING CIS-3-HYDROXY-L-PROLINE

5 Field of the Invention

The present invention relates to industrial methods for producing cis-3-hydroxy-L-proline which is useful as a starting compound for medicines and an additive to foods, genes which code for a protein having the enzymatic activity of hydroxylating the 3-position of L-proline and which are useful for the above-mentioned process (hereinafter referred to as L-proline 3-hydroxylase genes), transformants containing the gene, and methods for producing L-proline 3-hydroxylases using the transformants.

15 Background of the Invention

Heretofore, chemosynthetic methods of producing cis-3-hydroxy-L-proline are known [J. Amer. Chem. Soc., 84, 3980 (1962); J. Amer. Chem. Soc., 85, 2824 (1963); Nature 289, 310 (1981); J. Org. Chem., 54, 1866 (1989); Acta Chemica Scandinavica, 43, 290 (1989)].

No chemosynthetic or biological method of producing cis-3-hydroxy-L-proline by hydroxylating L-proline both regio-selectively and stereo-selectively, had been reported yet.

The conventional chemosynthetic methods for producing cis-3-hydroxy-L-proline are not satisfactory for industrial production, because of (1) the expensive raw materials, (2) too many the reaction steps, (3) the complicated procedures for isolating and purifying the product and/or (4) the lower productivity of cis-3-hydroxy-L-proline.

30 Industrial methods for advantageously producing cis-3-hydroxy-L-proline by using L-proline 3-hydroxylases with high activity are desired.

The object of the present invention is to provide methods for efficiently producing cis-3-hydroxy-L-proline from inexpensive and easily-available L-proline by using L-proline 3-hydroxylases, for which, in order to more industrially

advantageously produce cis-3-hydroxy-L-proline, L-proline 3-hydroxylase genes and transformants containing the gene are provided. L-proline 3-hydroxylases are produced in large quantities by using the genes and the transformants, and cis-3-hydroxy-L-proline is industrially produced at low costs by using the transformants or the hydroxylases.

DISCLOSURE OF THE INVENTION

The present invention relates to novel, microorganism-derived L-proline 3-hydroxylase genes, to recombinant DNAs containing the gene, to transformants containing the recombinant DNA, to methods for producing L-proline 3-hydroxylases using the transformants, to the hydroxylases, and to methods for producing cis-3-hydroxy-L-proline using the transformants or the hydroxylases.

The L-proline-3-hydroxylases of the present invention are enzymes which hydroxylate free L-proline in the presence of 2-ketoglutaric acid and divalent iron ions to produce cis-3-hydroxy-L-proline.

The present invention encompasses any and every protein having the enzymatic activity of hydroxylating the 3-position of L-proline, which includes, for example, a protein having the amino acid sequence indicated by Sequence No. 1 or 2, a fused protein having an amino acid sequence that results from the protein or a protein having a partial amino acid sequence of the protein as bonded to a peptide having a partial amino acid sequence of an Escherichia coli-derived β -galactosidase protein, a fused protein having an amino acid sequence that results from the protein having the amino acid sequence indicated by Sequence No. 1 or 2 or a protein having a partial amino acid sequence of the protein as bonded to a peptide having a partial amino acid sequence of an E. coli-derived maltose-binding protein, etc. Examples of the fused proteins include proteins having the amino acid sequence as indicated by Sequence No. 15, 16 or 17, etc.

The amino acid sequence indicated by Sequence No. 1, 2, 15, 16 or 17 includes proteins having an amino acid sequence with one

or more amino acids substituted, deleted or added and having the enzymatic activity of hydroxylating the 3-position of L-proline. The substitution, the deletion and the addition of amino acids can be conducted in accordance with the methods described in Nucleic
 5 Acids Research, Vol. 10, pp. 6487-6500 (1982); Proc. Natl. Acad. Sci., USA, Vol. 79, pp. 6409-6413 (1982); Proc. Natl. Acad. Sci., USA, Vol. 81, pp. 5662-5666 (1984); Science, Vol. 224, pp. 1431-1433 (1984); PCTWO85/00817 (1985); Nature, Vol. 316, pp. 601-605 (1985); Gene, Vol. 34, pp. 315-323 (1985); Nucleic Acids Research, Vol.
 10 13, pp. 4431-4442 (1985); Current Protocols in Molecular Biology, Chap. 8, Mutagenesis of Cloned DNA, John Wiley & Sons, Inc. (1989), etc.

The present invention encompasses any and every L-proline-3-hydroxylase genes of a DNA fragment containing a gene
 15 that codes for a protein having the enzymatic activity of hydroxylating the 3-position of L-proline, and this may include, for example, genes coding for the protein having the amino acid sequence as indicated by Sequence No. 1, 2, 15, 16, or 17, and also genes which code for a protein that has an amino acid sequence
 20 corresponding to the amino acid sequence as indicated by Sequence No. 1, 2, 15, 16 or 17 and derived therefrom by substitution, deletion or addition of at least one amino acid and which have the enzymatic activity of hydroxylating the 3-position of L-proline. Concretely mentioned are DNAs indicated by Sequence Nos. 3, 4, 13
 25 and 14.

The L-proline-3-hydroxylases genes of the present invention include the DNAs as defined hereinabove and also DNAs as derived therefrom by mutation, such as substituting mutation, deleting mutation, inserting mutation or the like, to be conducted to the
 30 extent that the mutated DNAs do not lose the L-proline-3-hydroxylases activity, for example, DNAs with homology to Sequence No. 3, 4, 13 or 14. Such homologous DNAs are those to be obtained by colony hybridization or plaque hybridization using, as a probe, the DNA having the nucleotide sequence as indicated by Sequence
 35 No. 3, 4, 13 or 14. These treatments can be conducted in accordance with known in vitro recombination techniques [see Molecular

Cloning: A Laboratory Manual, 2nd Ed., edited by Sambrook, Fritsch, Maniatis, published by Cold Spring Harbor Laboratory Press, 1989].

DNA fragments containing the L-proline-3-hydroxylases gene can be obtained from microorganisms having the ability of hydroxylating L-proline to produce cis-3-hydroxy-L-proline. As the microorganisms, any microorganism having the ability of hydroxylating L-proline to produce cis-3-hydroxy-L-proline can be employed in the present invention. As preferable example of such a microorganism, microorganisms belonging to the genus Streptomyces or Bacillus and having the enzymatic activity of hydroxylating the 3-position of L-proline can be mentioned. More preferable examples thereof include Streptomyces canus ATCC12647, Streptomyces canus ATCC12646, Streptomyces sp. TH1 (FERM BP-4399), Bacillus sp. TH2 (FERM BP-4397), Bacillus sp. TH3 (FERM BP-4398), or mutants or derivatives of these strains.

Methods for obtaining L-proline-3-hydroxylases genes derived from microorganisms having the ability of producing L-proline-3-hydroxylases are described below.

Chromosomal DNA is prepared from a microorganism having the ability of producing L-proline-3-hydroxylase through a usual DNA isolation method, for example, a phenol method [see Biochim. Biophys. Acta, 72, 619-629 (1963)]. The thus-obtained chromosomal DNA is cleaved with suitable restriction enzymes, then the resulting fragments are inserted into vector DNAs to construct chromosomal DNA libraries for the chromosomes of the microorganisms. Using the chromosomal DNA library, host microorganisms can be transformed. The transformants containing the L-proline-3-hydroxylase gene are selected from the obtained transformants by the hybridization method. DNAs containing the intended gene can be obtained from the thus-selected transformants.

The process comprising a series of such steps can be conducted in accordance with known in vitro recombination method (molecular Cloning, A Laboratory Manual, 2nd edition, edited by Sambrook, Fritsch and Maniatis, Cold Spring Harbor Laboratory Press, 1989).

As the vector DNAs that are used to construct the chromosomal DNA library of the microorganism having the ability of producing

L-proline-3-hydroxylase, phage vectors and plasmid vectors can be used if they can be replicated autonomously in Escherichia coli K12 strain. Preferable examples of the vector DNA include λ ZAPII*, pUC18 and pBluescript* (commercially available from STRATAGENE Co.).

As the host microorganisms that are used to construct the chromosomal DNA library of the microorganism having the ability of producing L-proline-3-hydroxylase, any of the microorganisms belonging to the genus Escherichia can be used. Preferable examples of the host microorganisms include E. coli XL1-Blue*, E. coli XL2-Blue*, E. coli DH1, E. coli MC1000, etc.

Based on the information about the amino acid sequence of L-proline-3-hydroxylase, DNA primers are synthesized. Using the DNA primers, DNA fragments are prepared through polymerase chain reaction (hereinafter referred to as PCR). Using the thus-obtained DNA fragments, transformants containing an L-proline-3-hydroxylase gene can be selected by the hybridization method.

The information on the amino acid sequences of L-proline-3-hydroxylases can be obtained through analysis of pure L-proline-3-hydroxylases using ordinary amino acid sequencers, such as Protein Sequencer Model PPSQ-10 (produced by Shimadzu Seisakusho K.K.). As the information on the amino acid sequences thus obtained, concretely mentioned are the amino acid sequences as indicated by Sequence Nos. 5 to 7, etc.

The DNA primers can be synthesized by means of an ordinary DNA synthesizers, for example, 380A DNA Synthesizer (produced by Applied Biosystems Co.), etc.

As the probes for the hybridization, usable are partial fragments of L-proline-3-hydroxylases genes, which can be obtained through PCR. For example, a DNA as indicated by Sequence No. 8 in the Sequence List (this corresponds to a sense chain DNA coding for from the first to the sixth amino acids in the amino acid sequence of Sequence No. 1) and a DNA as indicated by Sequence No. 10 in the Sequence List (this corresponds to an anti-sense chain DNA coding for from the 21st to 25th amino acids in the amino acid sequence of Sequence No. 1) are chemically synthesized. Through

* Trade-mark

PCR using these partial fragment as DNA primers, obtained is a DNA fragment of 74 bp as indicated by Sequence No. 11. The thus-obtained DNA fragment can be used as the probe for the hybridization.

The DNA which is obtained from the transformant as selected through the hybridization and which contains an L-proline-3-hydroxylases gene is cleaved with suitable restriction enzymes, such as PstI or the like, and then cloned into plasmids, such as pBluescript KS(+) (commercially available from STRATAGENE Co.). The nucleotide sequence of the above-mentioned gene can be determined by ordinary base sequencing methods, such as the dideoxy chain termination method of Sanger et al [see Proc. Natl. Acad. Sci., USA, 74, 5463 (1977)]. The determination of the nucleotide sequence can be conducted using automatic DNA sequencers, such as 373A•DNA Sequencer (produced by Applied Biosystems Co.) or the like.

As the thus-determined nucleotide sequences of L-proline-3-hydroxylases genes, for example the nucleotide sequences as indicated by Sequence Nos. 3, 4, 13 and 14 can be mentioned.

As the plasmid containing the DNA that codes for an L-proline-3-hydroxylases of the present invention, for example, pTH30, pTH71, pTH75, etc. can be mentioned. Escherichia coli XL2-Blue/pTH30 which is an Escherichia coli strain containing pTH30 and Escherichia coli XL2-Blue/pTH75 which is an Escherichia coli strain containing pTH75 were deposited at the National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology (which is located at 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305, Japan) as of March 2, 1995 under FERM BP-5026 for the strain XL2-Blue/pTH30 and as of February 22, 1996 under FERM BP-5409 for the strain XL2-Blue/pTH75, both in terms of the Budapest Treaty.

To express the thus-obtained L-proline-3-hydroxylase genes in hosts, the DNA fragment containing the L-proline-3-hydroxylase gene is first cleaved with a restriction enzyme or deoxyribonucleases (DNases) to form a DNA fragment of a suitable length containing the L-proline-3-hydroxylases gene. The thus-formed DNA fragment is inserted into an expression vector at the

downstream position of the promoter therein, and thereafter the expression vector having the thus-inserted DNA therein is introduced into a host cell suitable for the expression vector.

As the host, usable is any one capable of being expressed in the intended gene. For example, the host includes microorganisms belonging to the genus Escherichia, Serratia, Corynebacterium, Brevibacterium, Pseudomonas, Bacillus, etc., as well as yeast strains, animal cell hosts, etc.

As the expression vector, usable is one which is autonomously replicable in the above-mentioned hosts or capable of being inserted into their chromosomes and which contains a promoter at the position at which the L-proline-3-hydroxylases gene can be transcribed.

When the microorganisms such as Escherichia coli or the like are used as the host, it is desirable that the L-proline-3-hydroxylases expression vector is replicable autonomously in the microorganisms and is composed of a promoter, a ribosome-binding sequence, an L-proline-3-hydroxylase gene and a transcription terminator sequence. A regulatory gene may be contained therein.

As examples of the expression vector, mentioned are pBTrp2*, pBTac1*, pBTac2* (all commercially available from Boehringer Mannheim Co.); pKYP10 (see Japanese Published Unexamined Patent Application No. 58-110600); pKYP200 [see Agric. Biol. Chem., Vol. 48, pp. 669-675 (1984)]; pLSA1 [see Agric. Biol. Chem., Vol. 53, p. 277 (1989)]; pGEL1 [see Proc. Natl. Acad. Sci., USA, Vol. 82, p. 4306 (1985)]; pBluescript (produced by STRATAGENE Co.); pTrs30 [prepared from Escherichia coli JM109/pTrs30 (FERM BP-5407)]; pTrs32 [prepared from Escherichia coli JM109/pTrs32 (FERM BP-5408)], etc.

As the promoter, usable is any one capable of being expressed in hosts such as Escherichia coli, etc. For example, mentioned are promoters derived from Escherichia coli, phage, etc., such as trp promoter (P_{trp}), lac promoter (P_{lac}), P_L promoter and P_R promoter. Also usable are artificially designed and modified promoters, such as P_{trpx2} to be prepared by connecting two P_{trp}'s in series, as well as tac promoter.

* Trade-mark

As the ribosome-binding sequence, any one capable of being expressed in hosts such as Escherichia coli can be used. However, it is desirable to use plasmids having a ribosome-binding sequence and an initiation codon as spaced at suitable intervals therebetween
 5 (for example, by from 6 to 18 bases).

The L-proline-3-hydroxylase gene may be any and every gene that codes for an L-proline-3-hydroxylase. However, it is desirable that the bases constituting the DNA sequence of the gene are suitably substituted in order that the substituted DNA sequence
 10 can be constituted of codon most suitable for expression in the host microorganisms to be used.

Transcription terminator sequences are not always necessary for the expression of the genes of the present invention. However, it is desirable that a transcription terminator sequence is arranged
 15 just after the structural gene.

Examples of the hosts usable in the present invention include Escherichia coli XL1-Blue, Escherichia coli XL2-Blue, Escherichia coli DH1, Escherichia coli MC1000, Escherichia coli KY3276, Escherichia coli W1485, Escherichia coli JM109, Escherichia coli HB101, Escherichia coli No. 49, Escherichia coli W3110, Escherichia coli NY49, Bacillus subtilis, Bacillus amyloliquefaciens, Brevibacterium immariophilum ATCC14068, Brevibacterium saccharolyticum ATCC14066, Brevibacterium flavum ATCC14067, Brevibacterium lactofermentum ATCC13869, Corynebacterium glutamicum ATCC13032, Corynebacterium acetoacidophilum ATCC13870,
 20 Microbacterium ammoniophilum ATCC15354, etc.

When yeast strains are used as the host, for example, YEp13 (ATCC37115), YEp24 (ATCC37051), YCp50 (ATCC37419), etc. can be used as the expression vector.

30 As the promoter, any one that can be expressed in the host of yeast strains can be used. For example, usable are promoters in genes of glycolases, such as hexosekinase, etc., as well as other promoters such as gal 1 promoter, gal 10 promoter, heat-shock protein promoters, MF α 1 promoter, CUP 1 promoter, etc.

As examples of the host cells, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces lactis, Trichosporon pullulans, Schwanniomyces alluvius, etc. can be mentioned.

When animal cells are used as the host, for example, pcDNA I/Amp*, pcDNA I*, pcDM8* (all commercially available from Funakoshi Co.), etc. can be used as the expression vector. As the promoter, any one that can be expressed in the host of animal cells can be used. For example, usable are promoters such as those in human CMV IE (immediate early) genes. An enhancer of human CMV IE genes can be used along with the promoter.

As examples of the host cells, Namalwa cells, HBT 5637 cells (see Japanese Published Unexamined Patent Application No. 299/88), COS cells, CHO cells, etc. can be used.

To introduce DNA into animal cells, any and every method capable of introducing DNA into animal cells can be employed herein. For example, employable are electroporation methods [see Miyaji et al., Cytotechnology, 3, 133 (1990)], calcium phosphate methods (see Japanese Published Unexamined Patent Application No. 227075/90), lipofection methods [see Philip L. Felgner, et al., Proc. Natl. Acad. Sci., USA, 84, 7413 (1987)], etc. The resulting transformants can be collected and cultivated in accordance with the methods described in Japanese Published Unexamined Patent Application Nos. 227075/90 and 257891/90.

The transformants obtained in the manner mentioned above can be cultivated according to ordinary incubation methods.

As the media for cultivating the transformants obtained by using the hosts of microorganisms such as Escherichia coli, yeast strains, etc., both natural and synthetic media can be employed so long as they properly contain carbon sources, nitrogen sources, inorganic salts and other materials capable of being assimilated by the microbial hosts and in which the transformants can be efficiently cultivated.

Any carbon sources that can be assimilated by the microorganisms may be used. Examples of the carbon source include carbohydrates such as glucose, fructose, sucrose, molasses containing these components, starch and starch hydrolyzates;

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organic acids such as acetic acid and propionic acid; and alcohols such as ethanol and propanol.

As the nitrogen sources, ammonia, ammonium salts of inorganic and organic acids such as ammonium chloride, ammonium sulfate, ammonium acetate and ammonium phosphate, other nitrogen-containing compounds, peptone, meat extracts, yeast extracts, corn steep liquor, casein hydrolyzates, soybean cakes, soybean cake hydrolyzates, cultured fermented cells, their digested products, etc. may be used.

As inorganic salts, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate, calcium carbonate, etc. may be used.

The cultivation is conducted under aerobic conditions, for example, with shaking culture or submerged-aerial stirring culture. The temperature for the cultivation is 15 to 40°C. The period for the cultivation is usually 16 to 96 hours. During the cultivation, the pH of the medium is kept at 3.0 to 9.0. The pH is adjusted using inorganic or organic acids, alkaline solutions, urea, calcium carbonate, ammonia or the like.

L-Proline is suitably added to the media in such a manner that its concentration may be from 5 to 1000 mM, preferably from 20 to 200 mM, whereby the intended L-proline-3-hydroxylases can be produced more efficiently.

Antibiotics such as ampicillin, tetracycline or the like may be added to the medium during the cultivation, if required.

For the cultivation of the microorganisms which are transformed with the expression vector using the inducible promoter, inducers may be added to the medium, if required. For example, in cultivation of microorganisms transformed with the expression vector using lac promoter, isopropyl-β-D-thiogalactopyranoside (IPTG) may be added to the medium. In cultivation of microorganisms transformed with the expression vector using trp promoter, indoleacrylic acid (IAA) may be added to the medium.

As the medium for cultivating the transformants which are obtained by using the animal cells as a host cell, RPMI1640 medium

and Eagle's MEM medium which are generally used or these culture media containing a fetal bovine serum can be used.

The cultivation of the cells is conducted in the presence of 5% CO₂. The temperature for the cultivation is preferably 35 to 37°C, and the period for the cultivation is usually 3 to 7 days.

L-Proline is suitably added to the media in such a manner that its concentration may be from 5 to 1000 mM, preferably from 20 to 200 mM, whereby the intended L-proline-3-hydroxylases can be produced more efficiently.

Antibiotics such as kanamycin, penicillin or the like may be added to the medium during the cultivation, if required.

A considerable amount of L-proline-3-hydroxylase is produced and accumulated in the thus-cultivated transformants in comparison to the microorganism strain used as the gene source, such as Streptomyces sp. TH1 or the like. Thus, the isolation and purification of the enzyme or the production of cis-3-hydroxy-L-proline from L-proline using the enzyme can be performed far more efficiently in comparison to the production of cis-3-hydroxy-L-proline from L-proline using the non genetically-engineered microorganism as the enzyme source, such as Streptomyces sp. TH1 or the like.

The production of L-proline-3-hydroxylase in the transformants can be carried out by adding the culture, the cells or the treated cells to an aqueous medium suitable for the enzymatic reaction together with L-proline, a divalent iron ion and 2-ketoglutaric acid, and adding a surfactant or an organic solvent, if required, to determine cis-3-hydroxy-L-proline produced. With respect to the activity of the L-proline-3-hydroxylase of which the formation is confirmed in the cell, the activity of the enzyme for producing 1 nmol of cis-3-hydroxy-L-proline for 1 minute under the following conditions is defined as 1 unit (U). The microorganism cells and the animal cells are here called cells.

Measurement of L-proline-3-hydroxylase activity:

The cells, the treated cells or the enzyme preparation are added to 200 mM TES [N-tris(hydroxymethyl)methyl-2-

aminoethanesulfonic acid] buffer containing 12 mM L-proline, 24 mM 2-ketoglutaric acid, 4 mM ferrous sulfate and 8 mM L-ascorbic acid to make 250 μ l in total. The mixture is kept at 35°C for 10 minutes. The reaction mixture is heated at 100°C for 2 minutes to stop the reaction, and the amount of cis-3-hydroxy-L-proline produced in the reaction mixture is determined by high-performance liquid chromatography (hereinafter referred to as HPLC).

To determine the amount of cis-3-hydroxy-L-proline formed, any method capable of determining it can be employed. For example, employable are a method of separating and eluting cis-3-hydroxy-L-proline from the reaction mixture by means of HPLC using a ligand exchange chromatography column, such as SUMICHIRAL OA5000 (produced by Sumika Analysis Center Co.), or the like, followed by reacting it with 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (hereinafter referred to as NBD) to give its derivative after the column and detecting the resulting derivative (post-column derivative method); a method of previously reacting the product in the reaction mixture with NBD to give its derivative therein followed by isolating and detecting the resulting derivative with NBD by means of reversed-phase chromatography with HPLC (pre-column derivative method) [see William J. Lindblad and Robert F. Diegelmann, Analytical Biochemistry, Vol. 138, pp. 390-395, 1984], etc. The detection of the derivative with NBD is conducted by measuring its luminescence (at the excited wavelength of 503 nm and the fluorescent wavelength of 541 nm) in both cases.

The enzyme may be isolated and purified in a usual manner from the culture of the transformant in which the formation of L-proline-3-hydroxylase is confirmed in the cultivated cell as mentioned above. For instance, the culture broth of the transformant is centrifuged to collect the cultivated cells therefrom, and the cells are washed and then disrupted by an ultrasonic cell disrupter, a French press, a Manton-Gauline homogenizer, a Dyno mill or the like to obtain a cell-free extract. The purified enzyme preparation can be obtained by ammonium sulfate precipitation, anion exchange chromatography such as diethylaminoethyl (DEAE) Sepharose* or the like, hydrophobic

* Trade-mark

chromatography such as butyl-Sepharose, phenyl-Sepharose or the like, gel filtration, electrophoresis such as isoelectric point electrophoresis, and so on from the supernatant of the cell-free extract obtained by centrifugation.

5 The cultivated transformant cells that have been identified to contain the L-proline-3-hydroxylase as formed therein can be cultivated under the same conditions as above, under which the transformant was cultivated, to thereby make the cells produce and accumulate cis-3-hydroxy-L-proline in the cells, and the thus-
10 produced cis-3-hydroxy-L-proline can be collected from the culture to obtain it.

 If the transformant cells derived from host cells which have the ability of producing L-proline from saccharide sources and accumulating it in the cultures and where such cells are used, it
15 is possible to produce cis-3-hydroxy-L-proline even if L-proline is not added to the media during the cultivation of the cells therein. However, it is desirable to suitably add to the media L-proline at a concentration of from 5 to 1000 mM, preferably from 20 to 200 mM, whereby the intended L-proline-3-hydroxylases can be produced
20 more efficiently.

 If the transformant cells have the ability of producing 2-ketoglutaric acid from saccharide sources and accumulating it in the cultures and where such cells are used, it is possible to produce cis-3-hydroxy-L-proline even if 2-ketoglutaric acid is not
25 added to the media during the cultivation of the cells therein. Where such transformant cells are used, saccharide sources such as glucose, etc. may be suitably added to the media to make the cells produce and accumulate 2-ketoglutaric acid in the cultures, whereby the intended L-proline-3-hydroxylases can be produced more
30 efficiently. Where, on the other hand, transformant cells not having the ability of producing 2-ketoglutaric acid from saccharide sources are used, 2-ketoglutaric acid may be added to the media during the cultivation of the cells, if desired.

 If desired, divalent iron ions may be added to the media during
35 the cultivation of the transformant cells.

To produce cis-3-hydroxy-L-proline, also employable is another method to be mentioned below using, as the enzyme source, the cultures of the transformant cells where the formation of L-proline-3-hydroxylases has been identified, the cells isolated
5 from the cultures, or the products as obtained by processing the cells.

The method to produce cis-3-hydroxy-L-proline is as follows: The cultures of the transformant cells, the cells isolated from the culture, or the products as obtained by processing the cells
10 are added to aqueous media suitable for enzymatic reaction, along with L-proline, divalent iron ions and 2-ketoglutaric acid and optionally with surfactants and organic solvents, thereby converting L-proline into cis-3-hydroxy-L-proline, and thereafter the resulting cis-3-hydroxy-L-proline is collected from the
15 reaction mixtures.

As examples of the processed cells, dried cells, lyophilized cells, surfactant-treated cells, enzymatically-treated cells, ultrasonically-treated cells, mechanically-ground cells, mechanically-compressed cells, solvent-treated cells,
20 fractionated cell proteins, immobilized cells, immobilized materials obtained by processing their cells, etc. can be used. The enzyme preparations obtained by extraction from the cells having L-proline-3-hydroxylase activity, purified products of these enzyme preparations, and immobilized products thereof can also be
25 used.

As examples of the aqueous medium, water, buffers such as phosphates, carbonates, acetates, borates, citrates and tris-buffers, alcohols such as methanol and ethanol, esters such as ethyl acetate, ketones such as acetone, and amides such as acetamide can
30 be mentioned.

As examples of the surfactant, cationic surfactants such as polyoxyethylene-stearylamine (for example, Nymeen^{*} S215 produced by Nippon Oils & Fats Co.), cetyltrimethylammonium bromide, Cation FB, Cation F2-40E, etc.; anionic surfactants such as sodium
35 oleylamidosulfate, Newrex^{*} TAB, and Rapizole^{*} 80.; ampholytic surfactants such as polyoxyethylene-sorbitan monostearate (for

* Trade-mark

example, Nonion ST221) or the like; and also other tertiary amines PB, hexadecyldimethylamine, etc. can be mentioned. Any and every surfactant that promotes the reaction may be employed. The concentration of the surfactant is usually from 0.1 to 50 mg/liter, preferably from 1 to 20 mg/liter.

As examples of the organic solvent, toluene, xylene, aliphatic alcohols, benzene and ethyl acetate can be mentioned. The concentration of the organic solvent is usually from 0.1 to 50 μ l/ml, preferably from 1 to 20 μ l/ml.

The reaction may be conducted during the cultivation of the transformant having the activity of L-proline-3-hydroxylase, or may also be conducted after the completion of the cultivation, in the aqueous medium using the cells, the treated cells, the purified enzyme or the crude enzyme prepared from the culture.

The activity of the enzyme added to the reaction mixture is determined depending on the amount of the substrate used. It is usually from 1,000 to 10,000,000 U/liter, preferably from 10,000 to 3,000,000 U/liter. In case of using the cells or the treated cells of the microorganism, the concentration of wet cells is usually from 1 to 300 g/liter.

The reaction is usually conducted at a temperature from 15 to 50°C at a pH from 6.0 to 9.0 for 1 to 96 hours.

The concentration of L-proline used in the reaction may be from 1 mM to 2 M. L-Proline can be supplied by adding L-proline itself to the reaction mixture, or adding the culture of the microorganism which can produce and accumulate L-proline from sugar source. Further, if a microorganism having the ability of producing L-proline from a sugar source is used as the host microorganism of the transformant, L-proline produced from a sugar source by the host microorganism can be used in the reaction.

The divalent iron ion is required for the reaction. This divalent iron ion is ordinarily used in a concentration of from 1 to 100 mM. Any divalent iron ion can be used so long it does not inhibit the reaction. As examples of the divalent iron ion, sulfates such as ferrous sulfate; chlorides such as ferrous chloride; ferrous carbide; and organic acid salts such as citrates, lactates and fumarates can be mentioned. When the divalent iron ion is contained in the cells, the treated cells or the reaction mixture, the divalent iron ion need not be added.

2-Ketoglutaric acid itself may be added to the reaction mixture or may be supplied from a compound which can be converted into 2-ketoglutaric acid by the metabolic activity of the cells or the treated cells used. As examples of such a compound, saccharides such as glucose; amino acids such as glutamic acid; and organic acids such as succinic acid can be mentioned. These compounds may be used singly or in combination.

Cis-3-hydroxy-L-proline is recovered from the culture or the aqueous medium by any ordinary separation method, for example, column chromatography using an ion-exchange resin, crystallization, etc.

The structure of the thus-recovered cis-3-hydroxy-L-proline can be identified by ordinary analytical method such as ^{13}C -NMR spectrum, ^1H -NMR spectrum, mass spectrum, specific rotation or the like.

The cis-3-hydroxy-L-proline produced by the present invention can be determined quantitatively by the above-mentioned post-column derivatization method or pre-column derivatization method.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the steps of constructing plasmid pTH30 and plasmid pTH40.

In this, the thick, solid black lines each indicate a cloned Streptomyces sp. TH1 chromosome site. Ap indicates a pBR322-derived ampicillin-resistant gene. Plac indicates an Escherichia coli lactose promoter; lacZ indicates a β -galactosidase structural

gene; and MCS indicates a multi-cloning site. In this, only the restriction enzyme sites having relation to the construction of the plasmids are shown.

Fig. 2 shows plasmid pTH71 and plasmid pTH75.

5 In this, the thick, solid black lines each indicate a cloned Streptomyces sp. TH1 chromosome site. At the part of the thick, solid black line as drawn along with an arrow, the site has the base sequence corresponding to Sequence No. 14. The direction of each arrow indicates the direction toward the terminal of the
10 sequence of Sequence No. 14 from the head thereof. Ap indicates a pBR322-derived ampicillin-resistant gene. Plac indicates an Escherichia coli lactose promoter; lacZ indicates a β -galactosidase structural gene; and MCS indicates a multi-cloning site. In this, only the restriction enzyme sites having relation to the
15 construction of the plasmids are shown.

Fig. 3 shows the steps of constructing plasmid pTH50.

In this, the thick, solid black lines each indicate a part containing a Streptomyces sp. TH1 chromosome site-derived L-proline-3-hydroxylase gene. Ap indicates a pBR322-derived
20 ampicillin-resistant gene. Plac indicates an Escherichia coli lactose promoter; lacZ indicates a β -galactosidase structural gene; and MCS indicates a multi-cloning site. In this, only the restriction enzyme sites having relation to the construction of the plasmid are shown.

25 Fig. 4 shows the steps of constructing plasmid pEX1-5.

In this, the thick, solid black lines each indicate a part containing a Streptomyces sp. TH1 chromosome site-derived L-proline-3-hydroxylase gene. Ap indicates a pBR322-derived ampicillin-resistant gene. Plac indicates an Escherichia coli
30 lactose promoter; lacZ indicates a β -galactosidase structural gene; and MCS indicates a multi-cloning site. In this, only the restriction enzyme sites having relation to the construction of the plasmid are shown.

Fig. 5 shows the steps of constructing plasmid pTH60.

35 In this, the thick, solid black lines each indicate a part containing a Streptomyces sp. TH1 chromosome site-derived L-

proline-3-hydroxylase gene. Ap indicates a pBR322-derived
ampicillin-resistant gene. Plac indicates an Escherichia coli
lactose promoter; lacZ indicates a β -galactosidase structural gene;
and MCS indicates a multi-cloning site. In this, only the
5 restriction enzyme sites having relation to the construction of
the plasmid are shown.

Fig. 6 shows the steps of constructing plasmid pTH70.

In this, the thick, solid black lines each indicate a part
containing a Streptomyces sp. TH1 chromosome site-derived L-
10 proline-3-hydroxylase gene. Ap indicates a pBR322-derived
ampicillin-resistant gene. Plac indicates an Escherichia coli
lactose promoter; lacZ indicates a β -galactosidase structural gene;
and MCS indicates a multi-cloning site. In this, only the
restriction enzyme sites having relation to the construction of
15 the plasmid are shown.

Fig. 7 shows the steps of constructing plasmid pTH80.

In this, the thick, solid black lines each indicate a part
containing a Streptomyces sp. TH1 chromosome site-derived L-
proline-3-hydroxylase gene. Ap indicates a pBR322-derived
20 ampicillin-resistant gene. Plac indicates an Escherichia coli
lactose promoter; lacZ indicates a β -galactosidase structural gene;
and MCS indicates a multi-cloning site. In this, only the
restriction enzyme sites having relation to the construction of
the plasmid are shown.

25

BEST MODES OF CARRYING OUT THE INVENTION

Example 1: Preparation of partial DNA fragment of the gene encoding
L-proline-3-hydroxylase protein derived from Streptomyces sp. TH1:

(1) Isolation of Chromosomal DNA of Streptomyces sp. TH1:

30 Chromosomal DNA of Streptomyces sp. TH1 was isolated in
the usual manner as follows. SK#2 medium (comprising 0.25 % glucose,
1.0 % soluble starch, 0.25 % yeast extract, 0.25 % peptone, 0.15 %
meat extract, 0.01 % potassium dihydrogen phosphate and 0.03 %
magnesium sulfate, and adjusted to a pH 7.6 with 6 N NaOH) containing
35 5 % mannitol and 0.05 % glycine, was put in test tubes in an amount
of 10 ml each, and sterilized at 120°C for 20 minutes. One loopful

of cells of Streptomyces sp. THI which had grown in HT-agar plate medium (comprising 1% soluble starch, 0.2% NZ amine, 0.1% yeast extract, 0.1% meat extract and 1.5% agar, and adjusted to pH 7.2 with 6 N NaOH) was inoculated in the above-mentioned SK#2 medium,
5 and cultivated at 28°C for 3 days with shaking.

The culture was centrifuged, and the obtained cells were washed with 10 ml of a 10.3 % sucrose solution, and suspended in 6 ml of TS comprising 10.3 % sucrose, 50 mM tris-HCl (pH 8.0) and 25 mM EDTA. One milliliter of a lysozyme solution (50 mg/ml·TS) was
10 added thereto, and the mixture was incubated at 37°C for 60 minutes.

Subsequently, 0.6 ml of a Proteinase K (produced by Sigma Co.) solution (2 mg/ml·TS) was added to the lysozyme-treated solution, and gently stirred. Further, 3.6 ml of a 3.3% (w/v) SDS
15 solution was added thereto while gently mixing, and the mixture was incubated at 37°C for 60 minutes. The mixture was heated at 50°C for 30 minutes, and then cooled with water. An equal amount of TE [containing 10 mM tris-HCl (pH 8.0) and 1 mM EDTA] saturated phenol-chloroform (1/1, v/v) was added thereto, and the mixed
20 solution was moderately shaken for 30 minutes. After the centrifugation, the upper layer was taken, and again subjected to extraction with the mixture of TE saturated phenol-chloroform. The extract was centrifuged, and an equal amount of chloroform was then added to the upper layer, and mixed. The mixture was recentrifuged.

25 The upper layer was taken, and 20 µl of an RNase A aqueous solution (10 mg/ml) heat-treated at 100°C for 10 minutes were added to the upper layer. The mixture was incubated at 37°C for 45 minutes. To the RNase A-treated solution were added 1/10 volume of a 5 M NaCl aqueous solution and 1/4 volume of 50% PEG6000, and
30 gently mixed. The mixture was allowed to stand overnight while being cooled with ice. After the mixed solution was centrifuged at 12,000 rpm for 10 minutes, the supernatant was discarded completely, and the precipitate was dissolved in 5 ml of TE. After 1/10 volume of a 3 M sodium acetate solution and 1/30 volume of a
35 66 mM magnesium chloride solution were added thereto and mixed, 2.2 volumes of cold ethanol were added, and gently mixed. After the

mixed solution was centrifuged at 10,000 rpm for 10 minutes, the supernatant was discarded, and the precipitate was washed twice with 70% cold ethanol. The precipitate containing 250 µg of chromosomal DNA was dissolved in TE and used in the subsequent experiment as chromosomal DNA.

(2) Determination of Partial Amino Acid Sequence of Streptomyces sp. TH1-derived L-proline-3-hydroxylase Protein:

L-proline-3-hydroxylase produced by Streptomyces sp. TH1 was isolated and purified according to the method described in Reference Example 1, and the N-terminal amino acid sequence of the protein of the purified enzyme was analyzed, using Protein Sequencer Model PPSQ-10 (produced by Shimadzu Seisakusho Co.), to determine the N-terminal amino acid sequence thereof as indicated by Sequence No. 5.

Furthermore, the purified enzyme obtained in Example 5 was treated with lysyl endopeptidase in 0.1 M Tris-HCl with 4 M urea (pH 9), at 37°C for 6 hours, and the thus-obtained peptide fragments were collected through HPLC. Their amino acid sequences were also analyzed in the same manner as above to determine the partial amino acid sequences as indicated by Sequence Nos. 6 and 7.

(3) Preparation of Partial DNA of L-proline-3-hydroxylase Gene:

A sense chain mix DNA primer as indicated by Sequence No. 8, which corresponds to the sequence of from the 1st to 6th amino acids in the amino acid sequence as indicated by Sequence No. 5, and an anti-sense chain mix DNA primer as indicated by Sequence No. 9, which corresponds to the sequence of from the 24th to 28th amino acids in the amino acid sequence as indicated by Sequence No. 5, were synthesized, using 380A DNA Synthesizer (produced by Applied Biosystems Co.).

Using the above-synthesized DNAs as primers and the Streptomyces sp. TH1 chromosome DNA as template, the PCR was conducted using DNA Thermal Cycler* 480 (produced by Perkin Elmer Cetus Co.).

The reaction was conducted using 20 µl of a reaction mixture having the following formulation.

* Trade-mark

Formulation of the reaction mixture:: 21 ng/ μ l Streptomyces sp. TH1 chromosome DNA, 10 μ M sense chain mix DNA primer, 10 μ M anti-sense chain mix DNA primer, 0.125 U/ μ l Pfu* DNA polymerase (produced by STRATAGENE Co.), 10% DMSO, 20

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* Trade-mark

mM Tris-HCl (pH 8.2), 10 mM KCl, 6 mM ammonium sulfate,
2 mM magnesium chloride, 0.1 % Triton^{*}X-100, 10 ng/ μ l bovine
serum albumin.

After the completion of an incubation at 95°C for 5 minutes,
5 a three step incubation, namely at 95°C for 0.5 minute, at 25°C
for 0.5 minute and at 75°C for 1 minute was repeated for a total
of 40 times. The reaction mixture was subjected to electrophoresis
with 15% polyacrylamide gel (PAGE^{*}L NPU-15L produced by Atto Co.),
and the amplified 83 bp DNA was recovered using da Vinci Kun (Pen
10 Touch Recovery NB-7000 Model) manufactured by Nippon Eido K.K.

Using the recovered DNA fragment (83 bp) as a template, the
sense strand mix DNA primer indicated in Sequence No. 8 as a primer
and the anti-sense strand mix DNA primer indicated in Sequence No.
10 corresponding to amino acids Nos. 21st to 25th of an amino acid
15 sequence indicated in Sequence No. 5 as a primer, the PCR was
conducted again in the same manner, and the amplified 74 bp DNA
was recovered. The thus-recovered DNA fragment was inserted into
a Sma I site of pUC18 using Sure Clone^{*}Ligation Kit (produced by
Pharmacia Co.), and the nucleotide sequence was determined by a
20 nucleotide sequencing kit (Taq DyeDeoxyTM Terminator Cycle
Sequencing Kit produced by Applied Biosystems).

The nucleotide sequences thus determined are indicated by
Sequence Nos. 11 and 12. Thus, the two nucleotide sequences were
determined. The amino acid sequence to be presumed from the
25 nucleotide sequence of Sequence No. 11 completely corresponded to
the N-terminal amino acid sequence of the pure enzyme as indicated
by Sequence No. 5, except for the 2nd amino acid that could not
be identified in the sequencing of the N-terminal amino acid
sequence. The amino acid sequence to be presumed from the
30 nucleotide sequence of Sequence No. 12 exhibited high homology to
the N-terminal amino acid sequence of the pure enzyme as indicated
by Sequence No. 5.

Example 2: Cloning of a DNA fragment containing L-proline-3-
35 hydroxylase gene:

(1) Preparation of a digoxigeniated (DIGated) probe:

* Trade-mark

The two 74 bp fragments obtained in (3) of Example 1 and indicated by Sequence Nos. 11 and 12 each were inserted into the Sma I site of pUC18. Using each of these two plasmids thus obtained as a template, each plasmid was subjected to PCR in the same manner as in (3) of Example 1, using 50 µl of a reaction mixture containing 10 µM of the sense chain synthetic DNA as indicated by Sequence No. 8 and 10 µM of the anti-sense chain synthetic DNA as indicated by Sequence No. 10. The resulting reaction mixture each were subjected to 12.5 % polyacrylamide gel electrophoresis to identify the formation of an amplified 74 bp fragment. From each gel, the amplified fragment was recovered in the same manner as in (3) of Example 1.

The 74 bp fragment was digoxigeniated (DIGated), using PCR DIG Labeling Kit (produced by Boehringer Mannheim Co.).

Using each of the thus-recovered two 74 bp fragments as a template, each fragment was subjected to PCR, using 2.5 U of Pfu DNA polymerase (produced by STRATAGENE Co.), 5 µl of $\times 10$ buffer for Pfu DNA polymerase (produced by STRATAGENE Co.), 5 µl of DMSO, 5 µl of $\times 10$ PCR DIG mix (produced by Boehringer Mannheim Co.) and 50 µl of a reaction mixture comprising 10 µM of the sense chain synthetic DNA of Sequence No. 8 and 10 µM of the anti-sense chain synthetic DNA of Sequence No. 10.

After the incubation at 95°C for 5 minutes, the step of the incubation at 95°C for 0.5 minute, at 50°C for 0.5 minute and at 75°C for 1 minute was repeated 40 times. After the reaction, each reaction mixture was subjected to 12.5 % polyacrylamide gel electrophoresis to identify the formation of an amplified 74 bp fragment. From each gel, was recovered the amplified fragment in the same manner as in (3) of Example 1. The resulting two amplified fragments were used as probes. The probe derived from the nucleotide sequence of Sequence No. 11 is referred to as probe A, and that from the nucleotide sequence of Sequence No. 12 as probe B.

(2) Southern Hybridization:

Twenty units of a restriction enzyme Pst I (produced by Takara Shuzo Co., LTD) was added to 20 µg of Streptomyces sp. TH1

chromosome DNA and reacted at 37°C for 2 hours to cleave the DNA, which was then subjected to agarose gel electrophoresis. Using the probe A as obtained in (1) of Example 2, Southern hybridization was conducted with DIG Luminescent Detection Kit (produced by
 5 Boehringer Mannheim Co.) according to the method as described in the description attached to the kit.

Precisely, after the completion of the agarose gel electrophoresis, the gel was gently shaken in 0.25 N HCl for 20 minutes and then blotted onto Hybond-N⁺*membrane (produced by
 10 Amersham Co.) in 0.4 M sodium hydroxide solution, while being sucked at 7.5 mmHg using Genopirator Pump AE-6680P (produced by ATTO Co.) and Genopirator AE-6680C (produced by ATTO Co.). The thus-blotted membrane was dried in air. The resulting membrane was dipped in 10 ml of a hybridization buffer of DIG Luminescent Detection Kit
 15 [comprising 50 % v/v formamide, 2 % blocking reagent, 0.1 % w/v N-laurylsarcosine and 0.02 % w/v SDS in x5 SSC (x1 SSC is comprised of 150 mM sodium chloride and 15 mM sodium citrate)] at 42°C for 1 hour and thereafter dipped in a probe solution [prepared by adding 3 µl of the probe A as obtained in (1) of Example
 20 2 to 200 µl of the hybridization buffer, then heating the mixture at 95°C for 2 hours and thereafter adding thereto the hybridization buffer until the total volume was 1.5 ml], at 42°C overnight. The thus-obtained membrane was washed twice each with 25 ml of x2 SSC containing 0.1 % SDS for 5 minutes at room temperature and then
 25 twice each with 25 ml of x0.1 SSC containing 0.1 % SDS for 15 minutes at 68°C.

After this, the membrane was treated with a washing buffer [buffer 1 (0.1 M maleic acid, 0.15 M sodium chloride, pH 7.5) containing 0.3 % w/v Tween^{*}-20] for 1 to 5 minutes at room
 30 temperature, then with 50 ml of buffer 2 (buffer 1 containing 1 % blocking reagent) for 30 minutes at room temperature, then with 10 ml of buffer 2 containing 1 µl of anti-digoxigenin-AP Fab for 30 minutes at room temperature, then twice each with 50 ml of buffer 2 for 30 minutes at room temperature, then with 10 ml of
 35 buffer 3 (0.1 M Tris-HCl, 0.1 M sodium chloride, 50 mM magnesium chloride, pH 9.5) for from 2 to 5 minutes at room temperature, and
 * Trade-mark

then with 5 ml of buffer 3 containing 50 μ l of Lumigen^{*} PPD for 5 minutes at room temperature, in that order. Subsequently, water was removed from the film quickly over a filter paper, then the film was wrapped with a wrapping film, Saran Wrap^{*} (poly vinylidene chloride film) and thereafter allowed to stand at 37°C for 15 minutes. Using Hyperfilm^{*} ECL (produced by Amersham Co.), the resulting film was exposed at room temperature for 30 minutes.

A DNA fragment as strongly hybridized with the probe was detected at the position of approximately 6 kb.

The same process as in (2) of Example 2 was repeated, while using the probe B as obtained in (1) of Example 2 and while using 20 U of a restriction enzyme KpnI (produced by Takara Shuzo Co., LTD) in place of the restriction enzyme PstI. As a result, a DNA fragment as strongly hybridized with the probe was detected at the position of approximately 5 kb.

(3) Fractionation of Chromosome DNA:

Twenty units of a restriction enzyme PstI (produced by Takara Shuzo Co., LTD) was added to 20 μ g of Streptomyces sp. TH1 chromosome DNA and the mixture was allowed to stand at 37°C for 2 hours to cleave the DNA, which was then mixed with the same amount, as that of the reaction mixture, of TE-saturated phenol/chloroform. The resulting mixture was centrifuged, and the upper layer was taken out. To the layer was added 2.2 times volume of cold ethanol and then gently mixed. This mixture was centrifuged at 10,000 rpm for 10 minutes, the supernatant was removed, and the precipitate was washed twice with 70 % cold ethanol to obtain an ethanol precipitate. The process of obtaining the ethanol precipitate by using TE-saturated phenol/chloroform and cold ethanol is hereinafter referred to as an ethanol precipitation. The ethanol precipitate was dissolved in 120 μ l of TE and subjected to agarose gel electrophoresis. After the completion of the electrophoresis, a DNA fraction at approximately 6 kb was extracted from the agarose gel and purified by the use of Prep-A-Gene^{*} (produced by Biorad Co.). Thus approximately 0.5 μ g of a PstI-cleaved chromosome DNA fraction was obtained.

The same process as in (3) of Example 2 was repeated, except

* Trade-mark

using 20 U of a restriction enzyme KpnI in place of PstI. Thus was obtained approximately 0.5 µg of a KpnI-cleaved chromosome DNA fraction at approximately 5 kb.

(4) Formation of Plasmid Library:

5 1) After 1 µg of pBluescriptII KS(+) (produced by STRATAGENE Co.) was cleaved with restriction enzyme PstI (produced by Takara Shuzo Co., LTD) and then dephosphorylated with an alkaline phosphatase (derived from calf intestines) (produced by Takara Shuzo Co., LTD) in accordance with the method described in the
10 description attached to the product. After the completion of the reaction, the reaction mixture was subjected to the ethanol precipitation to obtain an ethanol precipitate. The precipitate was dissolved in 5 µl of TE. The thus-obtained, PstI-cleaved pBluescriptII KS(+) DNA was reacted with 0.1 µg of PstI-cleaved
15 chromosome DNA as obtained in (3) of Example 2, using a ligation kit (TAKARA Ligation Kit, produced by Takara Shuzo Co., LTD), for 2.5 hours at 26°C, whereby the two DNAs were ligated to each other. Using the thus-ligated DNA, E. coli XL2-Blue was transformed in a usual manner. The resulting transformant were spread on LB-agar
20 medium containing 50 µg/ml ampicillin and cultivated thereon overnight at 37°C to obtain about 240 colonies.

 2) After 1 µg of pBluescriptII KS(+) (produced by STRATAGENE Co.) was cleaved with a restriction enzyme KpnI (produced by Takara Shuzo Co., LTD), dephosphorylation was conducted with
25 an alkaline phosphatase (derived from calf intestines) (produced by Takara Shuzo Co., LTD) in accordance with the method described in the description attached to the product. After the completion of the reaction, the reaction mixture was subjected to the ethanol precipitation to obtain an ethanol precipitate. The precipitate
30 was dissolved in 5 µl of TE. The thus-obtained, KpnI-cleaved pBluescriptII KS(+) DNA was reacted with 0.1 µg of KpnI-cleaved chromosome DNA as obtained in (3) of Example 2, using a ligation kit (TAKARA Ligation Kit, produced by Takara Shuzo Co., LTD), for 2.5 hours at 26°C, whereby the two DNAs were ligated to each other.
35 Using the thus-ligated DNA, E. coli XL2-Blue was transformed in a usual manner. The resulting transformants were spread on LB-

agar medium containing 50 µg/ml of ampicillin and cultivated thereon overnight at 37°C to obtain about 200 colonies.

(5) Selection of Intended Clone:

The colonies having the desired clone was selected from the above-obtained colonies as follows.

Precisely, the colonies appearing on the LB-agar medium were transferred onto a nylon membrane (Nytran*, produced by Schleicher & Schuell Co.) that has been previously washed with x5 SSC, and the membrane was put on filter paper immersed with 0.5 M NaOH for 5 minutes. Subsequently, the film was put on filter paper immersed with 1.0 M Tris-HCl (pH 8.0) for 5 minutes and then on filter paper immersed with 1.5 M sodium chloride-1.0 M Tris-HCl (pH 8.0) for 5 minutes. After the film was lightly washed with x2 SSC, then crosslinking was conducted by exposure to ultraviolet rays (120 mJ/cm²). Then the surface of the film was wiped with x2 SSC containing 0.1% of SDS, and the membrane was dried in air.

The detection was conducted using the DIGated probes as obtained in (1) of Example 2, and DIG Luminescent Detection Kit (produced by Boehringer Mannheim Co.) in accordance with the process as described in (2) of Example 2, and positive colonies were detected on the membrane.

Precisely, by the colony hybridization between about 240 colonies as obtained in (1) of Example 2 (4) and the probe A as obtained in (1) of Example 2, one positive colony having the intended clone was detected. By the colony hybridization between about 200 colonies as obtained in (2) of Example 2 (4) and the probe B as obtained in (1) of Example 2, five positive colonies each having the intended clone were detected.

From these colonies, plasmids were extracted in a usual manner. Thus were obtained pTH30, pTH71 and pTH75.

The structures of the plasmids were identified through digestion with restriction enzymes. The structure of pTH30 was such that a PstI-cleaved DNA fragment of approximately 6 kb was inserted into the PstI site of pBluescriptII KS(+) (see Fig. 1); while the structures of pTH71 and pTH75 were such that a KpnI-cleaved DNA fragment of approximately 5 kb was inserted into the KpnI site of pBluescriptII KS(+) in the opposite directions (see

Fig. 2).

(6) Determination of Nucleotide Sequence:

1) Using a deletion kit for kilosequences (produced by Takara Shuzo Co., LTD), plasmid pTH30 was processed to give various
 5 deletion mutant plasmids. The reagents and the process as indicated in the description attached to the kit were employed. The nucleotide sequences of the deletion plasmids were analyzed, using a nucleotide sequencing kit (Taq DyeDeoxyTM Terminator Cycle Sequencing Kit, produced by Applied Biosystems Co.), and the
 10 nucleotide sequence of 1081 b KpnI-EcoRI fragment as indicated by Sequence No. 13 was identified.

In the nucleotide sequence thus identified, the nucleotide sequence indicated by Sequence No. 3 that codes for the protein composed of 290 amino acid residues indicated by Sequence No. 1
 15 was present. The amino acid sequence comprises the N-terminal amino acid sequence indicated by Sequence No. 5, which was determined by using the sequence of the pure L-proline-3-hydroxylase, and the internal amino acid sequences indicated by Sequence Nos. 6 and 7, which revealed the existence of the intended L-proline-3-
 20 hydroxylase gene in the PstI fragment of approximately 6 kb obtained hereinabove. The gene is hereinafter referred to as L-proline-3-hydroxylase I-type gene.

2) The nucleotide sequence of the Streptomyces-derived insertion fragment terminal in each of plasmids pTH71 and pTH75
 25 was analyzed, using a base sequencing kit (Taq DyeDeoxyTM Terminator Cycle Sequencing Kit, produced by Applied Biosystems Co.). On the basis of the thus-sequenced nucleotide sequence, a sequencing primer was synthesized using 380A Model DNA Synthesizer (produced by Applied Biosystems Co.). Using the primer, the nucleotide
 30 sequence of a part of each of pTH71 and pTH75 existing downstream from the Streptomyces-derived insertion fragment was analyzed. On the basis of the thus-sequenced nucleotide sequence, another sequencing primer was synthesized. Using the primer, a part of each of the plasmids existing further downstream was analyzed. The base
 35 sequencing operation comprising the above-mentioned steps was repeated, by which the NruI-KpnI fragment of 1826 bp was sequenced

as in Sequence No. 14.

The nucleotide sequence of the fragment thus sequenced comprised the nucleotide sequence indicated by Sequence No. 4 that codes for the protein composed of 290 amino acid residues as indicated by Sequence No. 2. The nucleotide sequence of Sequence No. 4 comprised the nucleotide sequence indicated by Sequence No. 12. The nucleotide sequence indicated by Sequence No. 4 was homologous at 78.5 % to the L-proline-3-hydroxylase I-type gene. The gene indicated by Sequence No. 4 is hereinafter referred to as L-proline-3-hydroxylase II-type gene.

Example 3: Construction of L-proline-3-hydroxylase Expression Plasmid:

(1) Construction of L-proline-3-hydroxylase I-type Gene Expression Plasmid:

Plasmid pTH30 as obtained in Example 2 was cleaved with a restriction enzyme EcoRI, and then self-ligated using a ligation kit (produced by Takara Shuzo Co., LTD). E. coli XL1-Blue MRF' strain was transformed with the DNA resulting from the self-ligation in a usual manner. A plasmid was extracted from the transformant in a usual manner, and the structure of the plasmid was identified through digestion with restriction enzyme.

As a result of the above, obtained was plasmid pTH40, from in which the EcoRI fragment of approximately 3 kb existing in the 3'-side of L-proline-3-hydroxylase gene was removed (see Fig. 1).

Subsequently, plasmid pTH40 was cleaved with KpnI and SmaI, then a fragment of approximately 0.95 kbp containing L-proline-3-hydroxylase I-type gene was identified through agarose gel electrophoresis, and the fragment was recovered from the agarose gel in a usual manner. The KpnI-SmaI-cleaved fragment was inserted into the KpnI-SmaI-cleaved site of pBluescriptII KS(+), using a ligation kit (produced by Takara Shuzo Co., LTD). With the resulting DNA, E. coli XL1-Blue MRF' strain was transformed in a usual manner. The resulting transformant was spread on LB-agar medium containing 50 µg/ml ampicillin and cultivated thereon

overnight at 37°C. A plasmid was extracted from the colonies of the transformant thus grown in a usual manner, and its structure was identified through digestion with restriction enzyme.

As a result of the above, obtained was plasmid pTH50, into which the DNA fragment coding for L-proline-3-hydroxylase I-type gene was inserted in the same direction as that for transcription of lac promoter (see Fig. 3).

(2) Construction of L-proline-3-hydroxylase II-type Gene Expression Plasmid:

Plasmid pTH75 as obtained in Example 2 was cleaved with restriction enzymes NruI and SacI, and thus-cleaved fragment of approximately 1900 bp containing L-proline-3-hydroxylase II-type gene was recovered through agarose gel electrophoresis. The NruI-SacI-cleaved fragment was inserted into the HincII-SacI-cleaved site of pBluescriptII KS(+), using a ligation kit (produced by Takara Shuzo Co., LTD). With the resulting DNA, E. coli XL2-Blue MRF' strain was transformed in a usual manner. The resulting transformant was spread on LB-agar medium containing 50 µg/ml ampicillin and cultivated thereon overnight at 37°C. A plasmid was extracted from the colonies of the transformant thus grown in a usual manner, and its structure was identified through digestion with restriction enzyme.

As a result of the above, obtained was plasmid pEX1-5, into which the DNA fragment coding for L-proline-3-hydroxylase II-type gene was inserted in the direction same as that for transcription of lac promoter (see Fig. 4).

Example 4: Production of L-proline-3-hydroxylase by Transformants:

(1) Production of L-proline-3-hydroxylase by Transformant Having L-proline-3-hydroxylase I-type Gene:

E. coli ATCC12435 was transformed with plasmid pTH50 as obtained in Example 3. The resulting transformant was inoculated in 3 ml of an LB medium containing 50 µg/ml ampicillin and cultivated therein overnight at 30°C by shaking. The resulting culture was centrifuged and the thus-obtained wet cells were frozen

at -20°C.

After the frozen cells were thawed, thus-obtained cells were added to 250 µl of a reaction mixture [containing 12 mM L-proline, 24 mM 2-ketoglutaric acid, 4 mM ferrous sulfate and 8 mM L-ascorbic acid in 200 mM TES buffer (pH 7.5)] at 4 % (w/v) in terms of the wet cells, and the mixture was allowed to stand for 10 minutes at 35°C. The reaction mixture was heated at 100°C for 2 minutes to stop the reaction.

The resulting reaction mixture was centrifuged, and 100 µl of 0.3 M borate buffer (pH 10.7), 4 µl of 10 % (v/v) mercaptoethanol aqueous solution and 16 µl of 5 % (w/v) OPA in ethanol were added to 100 µl of the resulting supernatant and left at 60°C for 30 seconds, and then 50 µl of 2 % (w/v) NBD in ethanol was added thereto. The mixture was allowed to stand at 60°C for 40 minutes. Then 30 µl of 1 N hydrochloric acid was added to the reaction mixture to stop the reaction. The resulting reaction mixture was centrifuged and filtered through a filter to remove the precipitate therefrom, and the resulting filtrate was analyzed through HPLC by which the product cis-3-hydroxy-L-proline produced was quantitatively determined.

HPLC was conducted under the following conditions.

Mobile Phase: 10 mM citric acid (pH 4.0)/methanol = 3/1 (v/v).

Flow Rate: 1 ml/min.

Column: YMC Pack* ODS AQ-312

(produced by YMC Co., 6 x 150 mm).

Column Temperature: 50°C.

Detection: fluorophotometry

excitation wavelength : 503 nm

emission wavelength : 541 nm.

As is shown in Table 1 below, the transformant E. coli ATCC12435/pTH50 produced L-proline-3-hydroxylase more by approximately 34 times/cell than the Streptomyces sp. TH1 strain which had been used as the gene source.

* Trade-mark

Table 1

L-proline-3-hydroxylase Activity Produced by Transformant

Strain	Cell Activity ¹⁾	Relative Activity ²⁾
<u>E. coli</u> ATCC12435/pTH50	1.94	34.0
<u>E. coli</u> ATCC12435/pBluescriptII KS(+)	Not detected.	-
<u>Streptomyces</u> sp. TH1 ³⁾	0.057	1

5 1) Cell activity indicates the enzymatic activity per mg of wet cells (U/mg wet cells). One U indicates the enzymatic activity of producing 1 nmol of cis-3-hydroxy-L-proline per minute (nmol/min).

10 2) Relative activity is based on the enzymatic activity produced by Streptomyces sp. TH1 strain of being 1 (one).

3) This is referred to in Reference Example 2.

(2) Production of L-proline-3-hydroxylase by Transformant Having L-proline-3-hydroxylase II-type Gene:

15 E. coli ATCC12435 was transformed with plasmid pEX1-5 as obtained in Example 3. The resulting transformant was inoculated in 3 ml of a TB medium containing 50 µg/ml ampicillin and cultivated therein overnight at 30°C by shaking. The resulting culture was centrifuged, and the cells were collected. If desired,
20 the cells were frozen and stored at -20°C, and the frozen cells were thawed before use.

The cells were added to 500 µl of a reaction mixture [containing 25 mM L-proline, 50 mM 2-ketoglutaric acid, 4 mM ferrous sulfate and 8 mM L-ascorbic acid in 200 mM TES buffer (pH 7.5)]
25 at 2.2 % (w/v) in terms of the wet cells, and reacted for 15 minutes at 35°C. The reaction mixture was heated at 100°C for 2 minutes to stop the reaction.

The resulting reaction mixture was centrifuged, and 100 µl of 0.3 M borate buffer (pH 10.7), 4 µl of 10 % (v/v) mercaptoethanol
30 aqueous solution and 16 µl of 5 % (w/v) OPA in ethanol were added to 100 µl of the resulting supernatant and left at 60°C for 30 seconds, and thereafter the mixture was neutralized. This treatment resulted in modification of primary amino acids with OPA but not

5 Mobile Phase: aqueous solution of 1 mM copper sulfate.
Flow Rate: 1 ml/min.
Column: SUMICHIRAL OA-5000 (produced by Sumika Chemical
Analysis Service Co., 4.6 x 250 mm)
Column Temperature: 38°C.

10 The eluate as optically resolved under the conditions
mentioned above was on-line mixed with reactants mentioned below
under the conditions mentioned below, in which the eluate was
modified with NBD in a reaction box CRB-6A (produced by Shimadzu
Co.) and the NBDated-secondary amino acid was quantitatively -
15 determined through fluorophotometry.

aqueous solution of 300 mM boric acid and
25 mM EDTA (pH 9.6) 0.2 ml/min.
1 g/liter methanol of NBD: 0.5 ml/min.

20 Reaction Temperature: 60°C.
 Detection: fluorophotometry

excitation wavelength 503 nm
emission wavelength 541 nm

32

Table 2

L-Proline-3-hydroxylase Activity Produced by Transformant

Strain	Cell Activity ¹⁾	Relative Activity ²⁾
<u>E. coli</u> ATCC12435/pEX1-5	4.42	77.5
<u>E. coli</u> ATCC12435/pBluescriptII KS(+)	Not detected.	-
<u>Streptomyces</u> sp. TH1 ³⁾	0.057	1

- 1) Cell activity indicates the enzymatic activity per mg of wet cells (U/mg wet cells). One U indicates the enzymatic activity of producing 1 nmol of cis-3-hydroxy-L-proline per minute (nmol/min).
- 2) Relative activity is based on the enzymatic activity produced by Streptomyces sp. TH1 strain of being 1 (one).
- 3) This is referred to in Reference Example 2.

Example 5: Construction of Expression Plasmid for a fused protein with a β -galactosidase protein fragment:

(1) Construction of pTH60 Plasmid:

Four micrograms of pTH40 DNA was cleaved with MluI and subjected to ethanol precipitation to obtain an ethanol precipitate. The thus-obtained ethanol precipitate (DNA fragment) was dissolved in 36 μ l of TE, and the both terminals of the DNA fragment were blunted using Takara DNA Blunting Kit (produced by Takara Shuzo Co., LTD). The resulting DNA fragment was recovered through ethanol precipitation. The DNA was cleaved with EcoRI and subjected to agarose gel electrophoresis, a fragment of approximately 1 kb containing the structural gene of L-proline-3-hydroxylase was extracted from the agarose gel and recovered in a usual manner using Prep-A-Gene (produced by Biorad Co.). The DNA fragment was then dissolved in 10 μ l of TE.

On the other hand, 2.4 μ g of plasmid pBluescriptII KS(+) DNA was cleaved with ApaI and subjected to agarose gel electrophoresis. The DNA fragment was then recovered from the gel and purified in a usual manner. Using Takara DNA Blunting Kit (produced by Takara Shuzo Co., LTD), the both terminals of the DNA fragment were blunted. The thus-blunted DNA fragment was cleaved with EcoRI and then subjected to ethanol precipitation to obtain an ethanol precipitate.

The ethanol precipitate was dissolved in 5 μ l of TE.

The above-obtained MluI-EcoRI fragment of approximately 1 kb containing the structural gene of L-proline-3-hydroxylase was ligated to the ApaI-EcoRI-cleaved pBluescriptII KS(+), using a
5 ligation kit (produced by Takara Shuzo Co., LTD).

With the ligated DNA, E. coli XL1-Blue MRF' strain was transformed in a usual manner. The resulting transformant was spread on LB-agar medium containing 50 μ g/ml ampicillin and then cultivated thereon overnight at 37°C. A plasmid was extracted from
10 the grown colonies of the transformant in a usual manner, and its structure was identified through digestion with restriction enzyme.

As a result of the above, obtained was plasmid pTH60, into which had been inserted the structural gene of L-proline-3-
15 hydroxylase as fused with the N-terminal amino acid sequence of β -Gal, in the direction same as that for transcription of lac promoter (see Fig. 5). The structure of the amino acid sequence of the fused protein thus constructed hereinabove was such that the N-terminal sequence of β -Gal comprised of 21 amino acids has
20 been fused to the L-proline-3-hydroxylase protein via arginine (22nd) as newly formed at the DNA binding site. However, methionine (GTG) which is the first amino acid of the L-proline-3-hydroxylase protein is translated as valine. The amino acid sequence of the fused protein is indicated by Sequence No. 15.

25 (2) pTH70 Plasmid:

pBluescriptII KS(+) DNA (2.4 μ g) was cleaved with AccI and subjected to ethanol precipitation to obtain an ethanol precipitate (DNA fragment). The both terminals of the DNA fragment were blunted using Takara DNA Blunting Kit (produced by Takara Shuzo Co., LTD).
30 The resulting DNA fragment was cleaved with EcoRI and then subjected to ethanol precipitation to obtain an ethanol precipitate. The ethanol precipitate was dissolved in 5 μ l of TE.

The MluI-EcoRI fragment of approximately 1 kb containing the structural gene of L-proline-3-hydroxylase, which had been
35 obtained in (1) of Example 5, was ligated to the AccI-EcoRI-cleaved pBluescriptII KS(+), using a ligation kit (produced by Takara Shuzo

Co., LTD).

With the ligated DNA, *E. coli* XL1-Blue MRF' strain was transformed in a usual manner. The resulting transformant was spread on LB-agar medium containing 50 µg/ml ampicillin and then
 5 cultivated thereon overnight at 37°C. A plasmid was extracted from the grown colonies of the transformant in a usual manner, and its structure was identified through digestion with restriction enzyme.

As a result of the above, obtained was plasmid pTH70, into
 10 which had been inserted the structural gene of L-proline-3-hydroxylase as fused with the N-terminal amino acid sequence of β-Gal in the direction same as that for transcription of lac promoter (see Fig. 6). The structure of the amino acid sequence of the fused protein thus constructed hereinabove was such that the N-terminal
 15 sequence of β-Gal comprised of 27 amino acids has been fused to the L-proline-3-hydroxylase via arginine (28th) as newly formed at the DNA binding site. However, methionine (GTG) which is the first amino acid of the L-proline-3-hydroxylase protein is translated as valine. The amino acid sequence of the fused protein
 20 is indicated by Sequence No. 16.

(3) pTH80 Plasmid:

Four micrograms of pTH40 DNA was cleaved with MluI and subjected to ethanol precipitation to obtain an ethanol precipitate. The ethanol precipitate (DNA fragment) was dissolved in 36 µl of
 25 TE. The both terminals of the DNA fragment were blunted using Takara DNA Blunting Kit (produced by Takara Shuzo Co., LTD). The resulting DNA fragment cleaved with SacII and then subjected to agarose gel electrophoresis. After the electrophoresis, a fragment of approximately 0.95 kb containing the structural gene of L-
 30 proline-3-hydroxylase was extracted from the gel and recovered in a usual manner using Prep-A-Gene (produced by Biorad Co.). The thus-recovered fragment was then dissolved in 10 µl of TE.

On the other hand, 2.4 µg of plasmid pBluescriptII KS(+) DNA was cleaved with PstI and subjected to ethanol precipitation to
 35 obtain an ethanol precipitate (DNA fragment). This DNA fragment was dissolved in 36 µl of TE, and the both terminals of the DNA

fragment were blunted using Takara DNA Blunting Kit (produced by Takara Shuzo Co., LTD). The resulting DNA fragment was then cleaved with SacII and subjected to ethanol precipitation to obtain an ethanol precipitate (DNA fragment). The DNA fragment was then
 5 dissolved in 5 μ l of TE.

The MluI-SacII fragment of approximately 1 kb containing the structural gene of L-proline-3-hydroxylase, which had been obtained in the above, was ligated to the PstI-SacII-cleaved pBluescriptII KS(+), using a ligation kit (produced by Takara Shuzo
 10 Co., LTD).

With the ligated DNA, E. coli XL1-Blue MRF' strain was transformed in a usual manner. The resulting transformant was spread on LB-agar medium containing 50 μ g/ml ampicillin and then cultivated thereon overnight at 37°C. A plasmid was extracted from
 15 the grown colonies of the transformant in a usual manner, and its structure was identified through digestion with restriction enzyme.

As a result of the above, obtained was plasmid pTH80, into which had been inserted the structural gene of L-proline-3-hydroxylase as fused with the N-terminal amino acid sequence of β -Gal in the direction same as that for transcription of lac promoter (see Fig. 7). The structure of the amino acid sequence of the fused protein thus constructed hereinabove was such that the N-terminal
 20 sequence of β -Gal comprised of 37 amino acids has been fused to the L-proline-3-hydroxylase protein via arginine (38th) as newly formed at the DNA binding site. However, methionine (GTG) which is the first amino acid of the L-proline-3-hydroxylase protein is translated as valine. The amino acid sequence of the fused protein is indicated by Sequence No. 17.

30
 Example 6: Production of L-proline-3-hydroxylase by Transformant Having Expression Plasmid for Fused Protein:

E. coli ATCC12435 was transformed with any one of plasmids pTH60, pTH70 and pTH80 as obtained in Example 5. In the same manner
 35 as in Example 4, the resulting transformant was cultivated, and the productivity of L-proline-3-hydroxylase by the transformant

cells was examined.

As shown in Table 3 below, the transformant produced L-proline-3-hydroxylase in an amount of 46 to 121 times per cell in comparison to Streptomyces sp. TH1 strain which had been used as
5 the gene source.

Table 3

L-proline-3-hydroxylase Activity Produced by Transformants

Strain	Cell Activity ¹⁾	Relative Activity ²⁾
<u>E. coli</u> ATCC12435/pTH60	5.19	91.1
<u>E. coli</u> ATCC12435/pTH70	6.90	121.1
<u>E. coli</u> ATCC12435/pTH80	2.63	46.1
<u>E. coli</u> ATCC12435/pBluescriptII KS (+)	Not detected.	-
<u>Streptomyces</u> sp. TH1 ³⁾	0.057	1.0

1) Cell activity indicates the enzymatic activity per mg of wet cells (U/mg wet cells). One U indicates the enzymatic activity of producing 1 nmol of cis-3-hydroxy-L-proline per minute (nmol/min).
10

2) Relative activity is based on the enzymatic activity produced by Streptomyces sp. TH1 strain of being 1 (one).

3) This is referred to in Reference Example 2.
15

Example 7: Production of Cis-3-hydroxy-L-proline by Transformant:

(1) Production of Cis-3-hydroxy-L-proline by Transformant E. coli ATCC12435/pTH70:

Cis-3-hydroxy-L-proline was produced using the transformant
20 E. coli ATCC12435/pTH70 as obtained in Example 6.

Precisely, the transformant of E. coli ATCC12435/pTH70 was inoculated in 3 ml of a TB medium (comprising 12 g of bactotryptone, 24 g of bacto yeast extract, 4 g of glycerol, 2.3 g of KH₂PO₄ and 12.5 g of dipotassium phosphate in one liter of distilled water
25 and sterilized at 120°C for 20 minutes) containing 100 µg/ml ampicillin, and cultivated therein at 30°C for 16 hours by shaking culture. The resulting culture was centrifuged, and the amount of cis-3-hydroxy-L-proline in the supernatant thus separated was quantitatively determined.

30 As a result, 620 µM (81.1 mg/liter) of cis-3-hydroxy-L-

proline was formed in the supernatant of the culture of E. coli ATCC12435/pTH70.

On the other hand, cis-3-hydroxy-L-proline was not detected in the supernatant of the culture of the E. coli ATCC12435 which had been used as the host.

(2) Production of Cis-3-hydroxy-L-proline by Transformant E. coli ATCC12435/pTH70:

The transformant of E. coli ATCC12435/pTH70 was inoculated in 50 ml of a Med4 medium containing 100 µg/ml ampicillin, and cultivated therein at 30°C for 16 hours by shaking.

The culture was used as a seed culture, which was inoculated in a 5-liter jar fermenter containing therein 2 liters of a Med6 medium. 200 mM L-proline and 100 µg/ml ampicillin were added thereto, and the transformant was cultivated in the fermenter under the condition of 400 rpm and 1vvm, at 30°C.

During the cultivation, glucose and L-proline were suitably added to the medium in such a manner that glucose was always present in the medium and L-proline could be at about 50 mM therein, and the lowermost limit of the pH of the medium was controlled at 6.5 by adding NH₄OH to the medium.

The culture was centrifuged, and the amount of cis-3-hydroxy-L-proline in the supernatant separated was quantitatively determined. Seventy two hours after the start of the incubation, 115 mM (15 g/liter) cis-3-hydroxy-L-proline was produced and accumulated in the supernatant of the culture of E. coli ATCC12435/pTH70.

On the other hand, free cis-3-hydroxy-L-proline was not detected in the supernatant of the culture of E. coli ATCC12435 which had been used as the host.

Example 8: Conversion of L-proline into Cis-3-hydroxy-L-proline with Transformant cells:

Using the transformant E. coli ATCC12435/pTH70 as obtained in Example 6, L-proline was converted into cis-3-hydroxy-L-proline.

Precisely, the transformant was inoculated in 10 ml of an

LB medium containing 50 µg/ml ampicillin and cultivated therein overnight at 30°C by shaking. Then 7 ml of the culture was centrifuged to collect the cells (wet cells). If desired, the cells were frozen and stored at -20°C and thawed before use.

5 After 10 % (w/v) of the wet cells were added to 500 µl of a reaction mixture [comprising 24 mM L-proline, 24 mM 2-ketoglutaric acid, 4 mM ferrous sulfate and 8 mM L-ascorbic acid in 200 mM TES buffer (pH 7.5)], reaction was conducted at 35°C for 60 minutes. The amount of cis-3-hydroxy-L-proline as formed in the reaction
10 mixture was quantitatively determined. As a result, 17.7 mM (2.3 g/liter) of cis-3-hydroxy-L-proline was formed in the reaction mixture.

Reference Example 1: Isolation and Purification of L-proline-
15 3-hydroxylase:

(1) Preparation of Frozen Cells of Streptomyces sp. TH1:

Ten milliliters of an SR3 medium (comprising 1.0 % glucose, 1.0 % soluble starch, 0.5 % yeast extract, 0.5 % tryptone, 0.3 % meat extract and 0.05 % magnesium phosphate and adjusted
20 at pH 7.2 with 6 N NaOH) was put into a test tube and sterilized at 120°C for 20 minutes. One loopful of cells of Streptomyces sp. TH1, that had grown in HT-agar plate medium (comprising 1% soluble starch, 0.2% NZ amine, 0.1% yeast extract, 0.1% meat extract and 1.5% agar, adjusted to pH 7.2 with 6N NaOH, and sterilized at 120°C
25 for 20 minutes) was inoculated into the above-mentioned SR3 medium in each test tube and cultivated at 28°C for 2 days by shaking. The resulting culture was used as the seed culture in the following step.

Df3 medium comprising 5% soluble starch, 3% corn steep liquor, 0.05% potassium dihydrogen phosphate, 0.05% magnesium sulfate 7
30 hydrate and 0.5% calcium carbonate, and adjusted to pH 7.0 with 6N NaOH, was put in 5 liter-jar fermenters in an amount of 2 liters each and sterilized at 120°C for 20 minutes. The above-mentioned seed culture was inoculated in the DF3 medium in each jar fermenter
35 under germ-free condition and cultivated under the condition of 700 rpm and 1 vvm, at 28°C for 2 days. During the cultivation,

the pH of the medium was not adjusted. The thus-obtained culture was subjected to centrifugation at 15,000 x g for 10 minutes at 4°C and 75g of the wet cells thus separated were obtained per liter of the culture. The wet cells were washed with a physiological saline at 4°C and then recentrifuged. The thus-obtained wet cells
5 were frozen and stored at -80°C before use.

(2) Preparation of Cell-free Extract:

Thirty grams of the wet cells obtained in (1) was suspended in 200 ml of Buffer (A) [20 mM piperazine buffer, pH was
10 adjusted to 5.3 with 6N HCl, containing 1 mM dithiothreitol (DTT), 0.2 mM EDTA, 0.1% (v/v) Tween 20 and 10% (v/v) glycerol] while cooling with ice. The cells in the resulting suspension was disrupted by means of an ultrasonic cell disruptor while cooling with ice. The thus-disrupted cell suspension was subjected to
15 centrifugation at 30,000 x g at 4°C for 30 minutes to separate a supernatant.

The subsequent operations were conducted under cooling with ice at a temperature of 4°C or lower.

(3) Isolation and Purification by Column Chromatography:

20 (3)-1 Acid Treatment

The pH of the supernatant obtained in the previous step was adjusted at 4.5 with 6 N HCl, and the precipitate formed was removed therefrom through centrifugation as conducted at 15,000 g for 30 minutes to obtain a supernatant.

25 (3)-2 Resource Q Column Chromatography (I):

The supernatant obtained in the previous step was passed through a RESOURCETM Q column (6 ml; produced by Pharmacia Co.) that had been equilibrated with Buffer (A). The column was washed with Buffer (A), and the fraction containing the desired enzyme was
30 eluted with Buffer (A) having a linear concentration gradient of NaCl of 0 to 0.3M.

(3)-3 Resource Q Column Chromatography (II):

The active fraction obtained in the previous step was diluted 3-fold with Buffer (B) [20 mM TES buffer (pH 7.5) containing
35 1 mM DTT, 0.2 mM EDTA and 10% (v/v) of glycerol] and was passed through a RESOURCETM Q column (1 ml; produced by Pharmacia Co.) that

had been equilibrated with Buffer (B). After the column was washed with Buffer (B), the fraction containing the enzyme was eluted with Buffer (B) having a linear concentration gradient of NaCl of 0 to 0.3 M.

5 3)-4 Phenyl Sepharose Column Chromatography:

NaCl was added to the active fraction obtained in the previous step until a NaCl concentration was 2M. The mixture was applied to a Phenyl Sepharose column (1 ml; Phenyl Sepharose HP HiLoad) that had been equilibrated with Buffer (B) containing 2M NaCl. The column was washed with Buffer (B) containing 2M NaCl, and the fraction containing the desired enzyme was eluted with Buffer (B) containing 0.1% (v/v) of Tween 20.

(3)-5 Resource Q Column Chromatography (III):

The active fraction obtained in the previous step was desalted using a PD-10 column, and the resulting mixture was passed through a RESOURCETH Q column (1 ml; produced by Pharmacia Co.) that had been equilibrated with Buffer (A). After the column was washed with Buffer (A), the fraction containing the desired enzyme was eluted with Buffer (A) having a linear concentration gradient of NaCl of 0 to 0.3 M.

(3)-6 Resource Q Column Chromatography (IV):

The active fraction obtained in the previous step was diluted 3-fold with Buffer (B) containing 0.1% (v/v) Tween 20 and passed through a RESOURCETH Q column (1 ml; produced by Pharmacia Co.) that had been equilibrated with Buffer (B). The column was washed with Buffer (B), and the fraction containing the desired enzyme was eluted with Buffer B having a linear concentration gradient of NaCl of 0 to 0.3m.

The foregoing steps for the isolation and purification of the L-proline-3-hydroxylase are summarized in Table 4.

Table 4

Summary of Isolation and Purification of L-proline-3-Hydroxylase

Fraction	Total Protein (mg)	Total Activity (U)	Relative Activity (U/mg protein)	Yield (%)
Cell-free Extract	542	3540	6.5	100
Acid-treatment Supernatant, pH 4.5	106	1800	17	56
Resource Q(I), pH 5.3	7.5	1553	207	44
Resource Q(II), pH 7.5	3.3	1036	306	29
Phenyl-Sepharose	0.43	188	437	5.3
Resource Q(III), pH 5.3	0.16	90.5	566	2.6
Resource Q(IV), pH 7.5	0.035	60.3	1723	1.7

5 Reference Example 2: Production of L-proline-3-hydroxylase by Streptomyces sp.:

SR3 medium was put into a test tube in an amount of 10 ml and sterilized at 120°C for 20 minutes. One loopful of cells of Streptomyces sp. TH1, that had grown in HT-agar plate medium, was
 10 inoculated into the above-mentioned SR3 medium in each test tube and cultivated at 28°C for 2 days by shaking. The resulting culture was used as the seed culture in the following step.

Df3 medium was put into a test tube in an amount of 10 ml each and sterilized at 120°C for 20 minutes. The above-mentioned
 15 seed culture was inoculated in the Df3 medium in each test tube under germ-free condition and cultivated at 28°C for 2 days with shaking. The thus-obtained culture was subjected to centrifugation at 8,000 rpm for 10 minutes at 4°C. The cells thus separated were suspended in 80 mM TES (N-tris (hydroxymethyl)
 20 methyl-2-aminoethanesulfonic acid) buffer (pH 7.5), washed and then centrifuged to separate the wet cells.

One gram of the thus-obtained wet cells was suspended in 10 ml of a reaction mixture [prepared by adding 1.4% (v/v) of Nymeen solution (prepared by dissolving 4g of Nymeen S-215 (produced by

Nippon Oils & Fats Co.) in 10 ml of xylene) to 100 mM TES buffer (pH 7.5) containing 5 mM L-proline, 5 mM α -ketoglutaric acid, 5 mM L-ascorbic acid and 1 mM ferrous sulfate] and the mixture was allowed to stand at 30°C for 30 minutes. After the reaction, the
5 cells were removed from the reaction mixture by centrifugation, and the amount of cis-3-hydroxy-L-proline formed in the supernatant was quantitatively determined.

The results are shown in Table 1.

10 INDUSTRIAL APPLICABILITY OF THE INVENTION

According to the present invention, there are provided industrial methods for producing cis-3-hydroxy-L-proline which is useful as a starting compound for medicines and an additive to foods, genes which code for a protein having the enzymatic activity of
15 hydroxylating the 3-position of L-proline and which are useful for the above-mentioned process, transformants containing the gene, methods for producing L-proline-3-hydroxylases using the transformants and L-proline-3-hydroxylases.

SEQUENCE LISTING

SEQUENCE NO.:1

SEQUENCE LENGTH: 290

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: protein

ORIGINAL SOURCE

ORGANISM: Streptomyces sp.

STRAIN: TH1

SEQUENCE:

```

Met Arg Ser His Ile Leu Gly Arg Ile Glu Leu Asp Gln Glu Arg Leu
 1           5           10           15
Gly Arg Asp Leu Glu Tyr Leu Ala Thr Val Pro Thr Val Glu Glu Glu
           20           25           30
Tyr Asp Glu Phe Ser Asn Gly Phe Trp Lys Asn Ile Pro Leu Tyr Asn
           35           40           45
Ala Ser Gly Gly Ser Glu Asp Arg Leu Tyr Arg Asp Leu Glu Gly Ser
           50           55           60
Pro Ala Gln Pro Thr Lys His Ala Glu Gln Val Pro Tyr Leu Asn Glu
           65           70           75           80
Ile Ile Thr Thr Val Tyr Asn Gly Glu Arg Leu Gln Met Ala Arg Thr
           85           90           95
Arg Asn Leu Lys Asn Ala Val Val Ile Pro His Arg Asp Phe Val Glu
           100          105          110
Leu Asp Arg Glu Leu Asp Gln Tyr Phe Arg Thr His Leu Met Leu Glu
           115          120          125
Asp Ser Pro Leu Ala Phe His Ser Asp Asp Asp Thr Val Ile His Met
           130          135          140
Arg Ala Gly Glu Ile Trp Phe Leu Asp Ala Ala Ala Val His Ser Ala
           145          150          155          160
Val Asn Phe Ala Glu Phe Ser Arg Gln Ser Leu Cys Val Asp Leu Ala
           165          170          175
Phe Asp Gly Ala Phe Asp Glu Lys Glu Ala Phe Ala Asp Ala Thr Val
           180          185          190
Tyr Ala Pro Asn Leu Ser Pro Asp Val Arg Glu Arg Lys Pro Phe Thr
           195          200          205
Lys Glu Arg Glu Ala Gly Ile Leu Ala Leu Ser Gly Val Ile Gly Arg
           210          215          220
Glu Asn Phe Arg Asp Ile Leu Phe Leu Leu Ser Lys Val His Tyr Thr
           225          230          235          240
Tyr Asp Val His Pro Gly Glu Thr Phe Glu Trp Leu Val Ser Val Ser
           245          250          255
Lys Gly Ala Gly Asp Asp Lys Met Val Glu Lys Ala Glu Arg Ile Arg
           260          265          270
Asp Phe Ala Ile Gly Ala Arg Ala Leu Gly Glu Arg Phe Ser Leu Thr
           275          280          285
Thr Trp
           290

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SEQUENCE NO.:2

SEQUENCE LENGTH: 290

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: protein

ORIGINAL SOURCE

ORGANISM: Streptomyces sp.
STRAIN: TH1
SEQUENCE:

SEQUENCE NO.:3
SEQUENCE LENGTH: 870
SEQUENCE TYPE: nucleic acid
STRANDEDNESS: double
MOLECULE TYPE: genomic DNA
ORIGINAL SOURCE
ORGANISM: Streptomyces sp.
STRAIN: TH1
SEQUENCE:

GTGCGCTCGC	ACATACTCGG	CCGCATTGAA	CTCGACCAGG	AACGCTTAGG	CAGGGACCTC	60
GAATATCTCG	CCACGGTGCC	CACCGTGGAA	GAGGAGTACG	ACGAGTTCAG	CAACGGGTTC	120
TGGAAGAACA	TCCCGCTGTA	CAACGCGAGC	GGCGGCAGCG	AGGACCGGCT	GTACCGCGAC	180
CTCGAGGGCT	CACCGGCGCA	GCCCACCAAA	CACGCCGAGC	AGGTTCCGTA	CCTCAACGAG	240

ATCATCACCA	CGGTCTACAA	CGGCGAGCGG	CTCCAGATGG	CGCGTACGCG	GAACCTGAAG	300
AACGCCGTCG	TCATCCCGCA	CCGCGACTTC	GTGGAGCTCG	ACCGCGAACT	CGACCAGTAC	360
TTCCGCACCC	ATTTGATGCT	TGAGGACAGC	CCGCTGGCCT	TCCACTCGGA	CGACGACACC	420
GTCATCCACA	TGCGGGCCGG	CGAGATCTGG	TTCCTCGACG	CGGCCGCCGT	CCACTCGGCC	480
GTCAACTTCG	CCGAGTTCAG	CAGGCAGTCG	CTCTGCGTCG	ACCTCGCCTT	CGACGGCGCG	540
TTCGACGAGA	AGGAAGCCTT	CGCGGACGCC	ACGGTCTACG	CCCCGAACCT	CAGCCCCGAC	600
GTCCGCGAAC	GCAAGCCGTT	CACCAAGGAG	CGGGAGGCCG	GGATCCTCGC	CCTGTCCGGC	660
GTGATCGGAC	GCGAGAACTT	CCGGGACATC	CTCTTTCTGC	TGTCCAAGGT	CCACTACACC	720
TACGACGTCC	ATCCGGGTGA	AACCTTCGAG	TGGCTCGTGA	GCGTCTCCAA	GGGTGCGGGA	780
GACGACAAGA	TGGTGGAGAA	GGCCGAGCGG	ATCAGGGACT	TCGCCATCGG	CGCACGGGCA	840
CTCGGCGAGC	GTTTCTCGCT	GACCACCTGG				870

SEQUENCE NO.:4

SEQUENCE LENGTH: 870

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: double

MOLECULE TYPE: genomic DNA

ORIGINAL SOURCE

ORGANISM: Streptomyces sp.

STRAIN: TH1

SEQUENCE:

ATGCGCTCGC	ACATCCTTGG	CAAGATCGAA	CTCGATCAGA	CCCGGTTGGC	GCCGGATCTC	60
GCATACCTCG	CCGCAGTTCC	GACCGTGGAG	GAGGAGTACG	ACGAGTTCAG	CAACGGGTTC	120
TGGAAGCACG	TGCCCCTGTG	GAACGCCTCC	GGGGACAGCG	AGGACCGGCT	CTACCGCGAC	180
CTCAAGGACG	CCGCCGCACA	GCCGACCGCG	CACGTGGAGC	ACGTCCCCTA	CCTCAAGGAG	240
ATCGTGACCA	CGGTCTTCCA	CGGCACGCAC	CTGCAGATGG	CGCGGAGCCG	GAACCTGAAG	300
AACGCCATCG	TCATCCCGCA	CCGCGACTTC	GTGGAGCTGG	ACCGCGAAGT	CGACCGGTAC	360
TTCCGCACGT	TCATGGTGCT	GGAGGACAGC	CCGCTCGCCT	TCCACTCGAA	CGAGGACACC	420
GTCATCCACA	TGCGCCCGGG	CGAAATATGG	TTCCTGGACG	CGGCGACGGT	GCACTCCGCG	480
GTCAACTTCT	CGGAAATCAG	CCGTCAGTCC	CTGTGCGTCG	ACTTCGCCTT	CGACGGTCCC	540
TTCGACGAGA	AGGAGATCTT	CGCGGACGCC	ACCCTCTACG	CTCCGGGCTC	CACGCCCCGAC	600
CTGCCCCGAGC	GCCGCCCTT	CACCGCGGAG	CACCGGCGGC	GGATCCTCTC	CCTGGGCCAG	660
GTGATCGAGC	GGGAGAACTT	CCGGGACATT	CTGTTCTTGC	TGTCCAAGGT	GCACTACAAG	720
TACGACGTGC	ACCCAGCGA	GACGTACGAC	TGGCTGATCG	AGATCTCGAA	ACAGGCCGGC	780
GACGAGAAGA	TGGTCGTGAA	GGCGGAGCAG	ATCAGGGACT	TCGCCGTCTGA	GGCCCGCGCC	840
CTGAGCGAGC	GCTTCTCCCT	GACCTCCTGG				870

SEQUENCE NO.:5

SEQUENCE LENGTH: 29

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

FRAGMENT TYPE: N-terminal fragment

ORIGINAL SOURCE

ORGANISM: Streptomyces sp.

STRAIN: TH1

SEQUENCE:

Met	Arg	Ser	His	Ile	Leu	Gly	Arg	Ile	Glu	Leu	Asp	Gln	Glu	Arg	Leu
1				5				10					15		

Gly	Arg	Asp	Leu	Glu	Tyr	Leu	Ala	Thr	Val	Pro	Thr	Val
			20					25				

SEQUENCE NO.:6

SEQUENCE LENGTH: 25

SEQUENCE TYPE: amino acid
 TOPOLOGY: linear
 MOLECULE TYPE: peptide
 FRAGMENT TYPE: internal fragment
 ORIGINAL SOURCE
 ORGANISM: Streptomyces sp.
 STRAIN: TH1
 SEQUENCE:

Glu Ala Phe Ala Asp Ala Thr Val Tyr Ala Pro Asn Leu Ser Pro Asp
 1 5 10 15
 Val Arg Glu Arg Lys Pro Phe Thr Lys
 20 25

SEQUENCE NO.:7
 SEQUENCE LENGTH: 25
 SEQUENCE TYPE: amino acid
 TOPOLOGY: linear
 MOLECULE TYPE: peptide
 FRAGMENT TYPE: internal fragment
 ORIGINAL SOURCE
 ORGANISM: Streptomyces sp.
 STRAIN: TH1
 SEQUENCE:

Asn Ile Pro Leu Tyr Asn Ala Ser Gly Gly Ser Glu Asp Arg Leu Tyr
 1 5 10 15
 Arg Asp Leu Glu Gly Ser Pro Ala Gln
 20 25

SEQUENCE NO.:8
 SEQUENCE LENGTH: 17
 SEQUENCE TYPE: nucleic acid
 STRANDEDNESS: single
 MOLECULE TYPE: other nucleic acid, synthetic DNA
 SEQUENCE:

ATGTGYTCNC AYATHYT

17

SEQUENCE NO.:9
 SEQUENCE LENGTH: 14
 SEQUENCE TYPE: nucleic acid
 STRANDEDNESS: single
 MOLECULE TYPE: other nucleic acid, synthetic DNA
 SEQUENCE:

GTNGGNACNG TWGC

14

SEQUENCE NO.:10
 SEQUENCE LENGTH: 14
 SEQUENCE TYPE: nucleic acid
 STRANDEDNESS: single
 MOLECULE TYPE: other nucleic acid, synthetic DNA
 SEQUENCE:

GTNGCNAGRT AYTC

14

SEQUENCE NO.:11
 SEQUENCE LENGTH: 74
 SEQUENCE TYPE: nucleic acid
 STRANDEDNESS: double
 MOLECULE TYPE: other nucleic acid, synthetic DNA
 SEQUENCE:

ATG TGT TCG CAT ATA CTC GGC CGC ATT GAA CTC GAC CAG GAA CGC TTA	48
Met Cys Ser His Ile Leu Gly Arg Ile Glu Leu Asp Gln Glu Arg Leu	
1 5 10 15	
GGC AGG GAC CTC GAA TAC CTC GCC AC	74
Gly Arg Asp Leu Glu Tyr Leu Ala	
20	

SEQUENCE NO.:12
 SEQUENCE LENGTH: 74
 SEQUENCE TYPE: nucleic acid
 STRANDEDNESS: double
 MOLECULE TYPE: other nucleic acid, synthetic DNA
 SEQUENCE:

ATG TGC TCG CAT ATC CTT GGC AAG ATC GAA CTC GAT CAG ACC CGG TTG	48
Met Cys Ser His Ile Leu Gly Lys Ile Glu Leu Asp Gln Thr Arg Leu	
1 5 10 15	
GCG CCG GAT CTC GAG TAC CTC GCC AC	74
Ala Pro Asp Leu Glu Tyr Leu Ala	
20	

SEQUENCE NO.:13
 SEQUENCE LENGTH: 1081
 SEQUENCE TYPE: nucleic acid
 STRANDEDNESS: double
 MOLECULE TYPE: genomic DNA
 ORIGINAL SOURCE
 ORGANISM: Streptomyces sp.
 STRAIN: TH1
 SEQUENCE:

GGTACCCATT	CCGTCACCGA	AAGCTGAACT	CGACACCAGG	AGGACGACGC	GTGCGCTCGC	60
ACATACTCGG	CCGCATTGAA	CTCGACCAGG	AACGCTTAGG	CAGGGACCTC	GAATATCTCG	120
CCACGGTGCC	CACCGTGGAA	GAGGAGTACG	ACGAGTTCAG	CAACGGGTTC	TGGAAGAACA	180
TCCCGCTGTA	CAACGCGAGC	GGCGGCAGCG	AGGACCGGCT	GTACCGCGAC	CTCGAGGGCT	240
CACCGGCGCA	GCCCACCAA	CACGCCGAGC	AGGTTCCGTA	CCTCAACGAG	ATCATCACCA	300
CGGTCTACAA	CGGCGAGCGG	CTCCAGATGG	CGCGTACGCG	GAACCTGAAG	AACGCCGTCG	360
TCATCCCGCA	CCGCGACTTC	GTGGAGCTCG	ACCGCGAACT	CGACCAGTAC	TTCCGCACCC	420
ATTTGATGCT	TGAGGACAGC	CCGCTGGCCT	TCCACTCGGA	CGACGACACC	GTCATCCACA	480
TGCGGGCCGG	CGAGATCTGG	TTCCTCGACG	CGGCCGCCGT	CCACTCGGCC	GTCAACTTCG	540
CCGAGTTCAG	CAGGCAGTCG	CTCTGCGTCG	ACCTCGCCTT	CGACGGCGCG	TTCGACGAGA	600
AGGAAGCCTT	CGCGGACGCC	ACGGTCTACG	CCCCGAACCT	CAGCCCCGAC	GTCCGCGAAC	660
GCAAGCCGTT	CACCAAGGAG	CGGGAGGCCG	GGATCCTCGC	CCTGTCCGGC	GTGATCGGAC	720
GCGAGAACTT	CCGGGACATC	CTCTTTCTGC	TGTCCAAGGT	CCACTACACC	TACGACGTCC	780
ATCCGGGTGA	AACCTTCGAG	TGGCTCGTGA	GCGTCTCCAA	GGGTGCGGGA	GACGACAAGA	840
TGGTGGAGAA	GGCCGAGCGG	ATCAGGGACT	TCGCCATCGG	CGCACGGGCA	CTCGGCGAGC	900
GTTTCTCGCT	GACCACCTGG	TAGACGGCCG	AAGAACGGGG	CCCGGGGGAA	CAGTGATCGA	960
GGAGATACTT	CCCGTCGACG	TGATGTCCGC	GGAGGCGTTC	GACGACGATG	CGGACATCCA	1020
GCTCTTCGCC	GAGGAACGCG	CGGCCGTCGC	CGATGCCGTA	CCGCGGCGCC	GACGGGAATT	1080

C

SEQUENCE NO.:14
 SEQUENCE LENGTH: 1826
 SEQUENCE TYPE: nucleic acid
 STRANDEDNESS: double
 MOLECULE TYPE: genomic DNA
 ORIGINAL SOURCE
 ORGANISM: Streptomyces sp.
 STRAIN: TH1
 SEQUENCE:

TCGCGACCTG	GAGGGTCGGA	TGCGCTCGCA	CATCCTTGGC	AAGATCGAAC	TCGATCAGAC	60
CCGGTTGGCG	CCGGATCTCG	CATACCTCGC	CGCAGTTCCG	ACCGTGGAGG	AGGAGTACGA	120
CGAGTTCAGC	AACGGGTTCT	GGAAGCACGT	CCCCCTGTGG	AACGCCTCCG	GGGACAGCGA	180
GGACCGGCTC	TACCGCGACC	TCAAGGACGC	CGCCGCACAG	CCGACCGCGC	ACGTGGAGCA	240
CGTCCCCTAC	CTCAAGGAGA	TCGTGACCAC	GGTCTTCGAC	GGCACGCACC	TGCAGATGGC	300
GCGGAGCCGG	AACCTGAAGA	ACGCCATCGT	CATCCCGCAC	CGCGACTTCG	TGGAGCTGGA	360
CCGCGAAGTC	GACCGGTACT	TCCGCACGTT	CATGGTGCTG	GAGGACAGCC	CGCTCGCCTT	420
CCACTCGAAC	GAGGACACCG	TCATCCACAT	GCGCCCGGGC	GAAATATGGT	TCCTGGACGC	480
GGCGACGGTG	CACTCCGCGG	TCAACTTCTC	GGAAATCAGC	CGTCAGTCCC	TGTGCGTCGA	540
CTTCGCCTTC	GACGGTCCCT	TCCGACGAGAA	GGAGATCTTC	GCGGACGCCA	CCCTCTACGC	600
TCCGGGCTCC	ACGCCCAGAC	TGCCCCGAGCG	CCGCCCCTTC	ACCGCGGAGC	ACCGGCGGCG	660
GATCCTCTCC	CTGGGCCAGG	TGATCGAGCG	GGAGAACTTC	CGGGACATTC	TGTTCTCTGCT	720
GTCCAAGGTG	CACTACAAGT	ACGACGTGCA	CCCCAGCGAG	ACGTACGACT	GGCTGATCGA	780
GATCTCGAAA	CAGGCCGGCG	ACGAGAAGAT	GGTCGTGAAG	GCGGAGCAGA	TCAGGGACTT	840
CGCCGTTCGAG	GCCCGCGCCC	TGAGCGAGCG	CTTCTCCCTG	ACCTCCTGGT	AATGGCGCGA	900
CCTCAGCCCG	CGTGACAACA	GTTCGGCCCC	GGTGGCGCGG	NGCGTCCCGG	ACGCGCGCCG	960
GNCGCTACCG	GACACCGGGG	CACGTAGGTG	GTGTACCGNC	TGNCGGTCTC	CGGCAGTCAG	1020
TTGCTGGTTC	GACTCCAGCT	GCCCCGACCG	CCTCGCGCTC	GGACGCGACC	CCGGGCACCT	1080
CCGNGGGCAC	CGCGTTCGCA	TCACGGACAC	CCCACCCACA	CCAGCCGGGC	CCACCCGAGG	1140
AGCCAGTTCA	CATGTCCAGT	GACATACACG	CGGCGGCATC	CAGACCGATT	CCGCCGAAGC	1200
CCGCCGCATC	GACGGAGGTC	GCGCCGCGCA	CGCTGGTCAG	CCGGCTGCCC	TCGCTGACCG	1260
GCCTGCGCTT	CCCGGCGGCG	TTCATCGTGT	TCCTCTTCCA	CGCCTCGCTG	CCGTTTCCCG	1320
CGGTGCGCCT	GTTTCGCCGAC	GACGGGGTGG	AACACCGCTA	CGGGTGGGCC	CTCGGCCCGA	1380
GCGGCGCACT	CGGCGTGACG	TTCTTCTTCG	CGCTCAGCGG	ATTCGTGCTG	ACGTGGTCGG	1440
CTCCCGCCGG	CGACACCGCA	CCGTCCCTTCT	GGCGCCGGCG	CTTCGTCAAG	ATCCTGCCCA	1500
ACTACGTCTG	CGCGTGGGTC	CTGGCGATGG	TGCTGTACGC	GGCCGCGACG	CCCGTCCTGC	1560
CCGCGCTCGG	CGCCCTTTTC	ATGCTCCAGG	TGTGGACGCC	GTACTTCACC	GAGCACCTCC	1620
CGGTGAACCC	ACCGAGCTGG	TCGCTGNCCG	TGGAAGCCGT	CTTCTATCTG	GCCTTCCCGT	1680
TCCTGCTGGC	CGGGATCAGA	CGGATACCGG	CCGCCCGGCT	GAAGTACTGG	ATCGCCGGCA	1740
CGGTGGCCCG	CGTCTTCGCC	ACGCCGCTGA	TCACCTACCT	CCTGGTGCCG	GCGGGCCCGC	1800
ACGTGATGCC	GGGCACGGGC	GGTACC				1826

SEQUENCE NO.:15
 SEQUENCE LENGTH: 312
 SEQUENCE TYPE: amino acid
 TOPOLOGY: linear
 MOLECULE TYPE: protein
 ORIGINAL SOURCE:
 ORGANISM: Streptomyces sp.
 STRAIN: TH1
 SEQUENCE FEATURE:
 NAME/KEY: peptide
 location: 23 to 312
 IDENTIFICATION METHOD: S
 ORIGINAL SOURCE:
 ORGANISM: Escherichia coli

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IMMEDIATE SOURCE: pBluescriptIIS+

SEQUENCE FEATURE:

NAME/KEY: peptide

location: 1 to 21

IDENTIFICATION METHOD: S

SEQUENCE:

Met	Thr	Met	Ile	Thr	Pro	Ser	Ala	Gln	Leu	Thr	Leu	Thr	Lys	Gly	Asn	1	5	10	15
Lys	Ser	Trp	Val	Pro	Arg	Val	Arg	Ser	His	Ile	Leu	Gly	Arg	Ile	Glu	20	25	30	
Leu	Asp	Gln	Glu	Arg	Leu	Gly	Arg	Asp	Leu	Glu	Tyr	Leu	Ala	Thr	Val	35	40	45	
Pro	Thr	Val	Glu	Glu	Glu	Tyr	Asp	Glu	Phe	Ser	Asn	Gly	Phe	Trp	Lys	50	55	60	
Asn	Ile	Pro	Leu	Tyr	Asn	Ala	Ser	Gly	Gly	Ser	Glu	Asp	Arg	Leu	Tyr	65	70	75	80
Arg	Asp	Leu	Glu	Gly	Ser	Pro	Ala	Gln	Pro	Thr	Lys	His	Ala	Glu	Gln	85	90	95	
Val	Pro	Tyr	Leu	Asn	Glu	Ile	Ile	Thr	Thr	Val	Tyr	Asn	Gly	Glu	Arg	100	105	110	
Leu	Gln	Met	Ala	Arg	Thr	Arg	Asn	Leu	Lys	Asn	Ala	Val	Val	Ile	Pro	115	120	125	
His	Arg	Asp	Phe	Val	Glu	Leu	Asp	Arg	Glu	Leu	Asp	Gln	Tyr	Phe	Arg	130	135	140	
Thr	His	Leu	Met	Leu	Glu	Asp	Ser	Pro	Leu	Ala	Phe	His	Ser	Asp	Asp	145	150	155	160
Asp	Thr	Val	Ile	His	Met	Arg	Ala	Gly	Glu	Ile	Trp	Phe	Leu	Asp	Ala	165	170	175	
Ala	Ala	Val	His	Ser	Ala	Val	Asn	Phe	Ala	Glu	Phe	Ser	Arg	Gln	Ser	180	185	190	
Leu	Cys	Val	Asp	Leu	Ala	Phe	Asp	Gly	Ala	Phe	Asp	Glu	Lys	Glu	Ala	195	200	205	
Phe	Ala	Asp	Ala	Thr	Val	Tyr	Ala	Pro	Asn	Leu	Ser	Pro	Asp	Val	Arg	210	215	220	
Glu	Arg	Lys	Pro	Phe	Thr	Lys	Glu	Arg	Glu	Ala	Gly	Ile	Leu	Ala	Leu	225	230	235	240
Ser	Gly	Val	Ile	Gly	Arg	Glu	Asn	Phe	Arg	Asp	Ile	Leu	Phe	Leu	Leu	245	250	255	
Ser	Lys	Val	His	Tyr	Thr	Tyr	Asp	Val	His	Pro	Gly	Glu	Thr	Phe	Glu	260	265	270	
Trp	Leu	Val	Ser	Val	Ser	Lys	Gly	Ala	Gly	Asp	Asp	Lys	Met	Val	Glu	275	280	285	
Lys	Ala	Glu	Arg	Ile	Arg	Asp	Phe	Ala	Ile	Gly	Ala	Arg	Ala	Leu	Gly	290	295	300	
Glu	Arg	Phe	Ser	Leu	Thr	Thr	Trp									305	310		

SEQUENCE NO.: 16

SEQUENCE LENGTH: 318

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: protein

ORIGINAL SOURCE:

ORGANISM: Streptomyces sp.

STRAIN: TH1

SEQUENCE FEATURE:

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NAME/KEY: peptide
 location: 29 to 318
 IDENTIFICATION METHOD: S
 ORIGINAL SOURCE:
 ORGANISM: Escherichia coli
 IMMEDIATE SOURCE: pBluescriptIIKS+
 SEQUENCE FEATURE:
 NAME/KEY: peptide
 location: 1 to 27
 IDENTIFICATION METHOD: S
 SEQUENCE:

Met	Thr	Met	Ile	Thr	Pro	Ser	Ala	Gln	Leu	Thr	Leu	Thr	Lys	Gly	Asn	1	5	10	15
Lys	Ser	Trp	Val	Pro	Gly	Pro	Pro	Ser	Arg	Ser	Arg	Val	Arg	Ser	His	20	25	30	
Ile	Leu	Gly	Arg	Ile	Glu	Leu	Asp	Gln	Glu	Arg	Leu	Gly	Arg	Asp	Leu	35	40	45	
Glu	Tyr	Leu	Ala	Thr	Val	Pro	Thr	Val	Glu	Glu	Glu	Tyr	Asp	Glu	Phe	50	55	60	
Ser	Asn	Gly	Phe	Trp	Lys	Asn	Ile	Pro	Leu	Tyr	Asn	Ala	Ser	Gly	Gly	65	70	75	80
Ser	Glu	Asp	Arg	Leu	Tyr	Arg	Asp	Leu	Glu	Gly	Ser	Pro	Ala	Gln	Pro	85	90	95	
Thr	Lys	His	Ala	Glu	Gln	Val	Pro	Tyr	Leu	Asn	Glu	Ile	Ile	Thr	Thr	100	105	110	
Val	Tyr	Asn	Gly	Glu	Arg	Leu	Gln	Met	Ala	Arg	Thr	Arg	Asn	Leu	Lys	115	120	125	
Asn	Ala	Val	Val	Ile	Pro	His	Arg	Asp	Phe	Val	Glu	Leu	Asp	Arg	Glu	130	135	140	
Leu	Asp	Gln	Tyr	Phe	Arg	Thr	His	Leu	Met	Leu	Glu	Asp	Ser	Pro	Leu	145	150	155	160
Ala	Phe	His	Ser	Asp	Asp	Asp	Thr	Val	Ile	His	Met	Arg	Ala	Gly	Glu	165	170	175	
Ile	Trp	Phe	Leu	Asp	Ala	Ala	Ala	Val	His	Ser	Ala	Val	Asn	Phe	Ala	180	185	190	
Glu	Phe	Ser	Arg	Gln	Ser	Leu	Cys	Val	Asp	Leu	Ala	Phe	Asp	Gly	Ala	195	200	205	
Phe	Asp	Glu	Lys	Glu	Ala	Phe	Ala	Asp	Ala	Thr	Val	Tyr	Ala	Pro	Asn	210	215	220	
Leu	Ser	Pro	Asp	Val	Arg	Glu	Arg	Lys	Pro	Phe	Thr	Lys	Glu	Arg	Glu	225	230	235	240
Ala	Gly	Ile	Leu	Ala	Leu	Ser	Gly	Val	Ile	Gly	Arg	Glu	Asn	Phe	Arg	245	250	255	
Asp	Ile	Leu	Phe	Leu	Leu	Ser	Lys	Val	His	Tyr	Thr	Tyr	Asp	Val	His	260	265	270	
Pro	Gly	Glu	Thr	Phe	Glu	Trp	Leu	Val	Ser	Val	Ser	Lys	Gly	Ala	Gly	275	280	285	
Asp	Asp	Lys	Met	Val	Glu	Lys	Ala	Glu	Arg	Ile	Arg	Asp	Phe	Ala	Ile	290	295	300	
Gly	Ala	Arg	Ala	Leu	Gly	Glu	Arg	Phe	Ser	Leu	Thr	Thr	Trp			305	310	315	

SEQUENCE NO.: 17
 SEQUENCE LENGTH: 328
 SEQUENCE TYPE: amino acid
 TOPOLOGY: linear

MOLECULE TYPE: protein
 ORIGINAL SOURCE:
 ORGANISM: Streptomyces sp.
 STRAIN: TH1
 SEQUENCE FEATURE:
 NAME/KEY: peptide
 location: 38 to 328
 IDENTIFICATION METHOD: S
 ORIGINAL SOURCE:
 ORGANISM: Escherichia coli
 IMMEDIATE SOURCE: pBluescriptIIKS+
 SEQUENCE FEATURE:
 NAME/KEY: peptide
 location: 1 to 37
 IDENTIFICATION METHOD: S
 SEQUENCE:

Met	Thr	Met	Ile	Thr	Pro	Ser	Ala	Gln	Leu	Thr	Leu	Thr	Lys	Gly	Asn	1	5	10	15
Lys	Ser	Trp	Val	Pro	Gly	Pro	Pro	Ser	Arg	Ser	Thr	Val	Ser	Ile	Ser	20	25	30	
Leu	Ile	Ser	Asn	Ser	Arg	Val	Arg	Ser	His	Ile	Leu	Gly	Arg	Ile	Glu	35	40	45	
Leu	Asp	Gln	Glu	Arg	Leu	Gly	Arg	Asp	Leu	Glu	Tyr	Leu	Ala	Thr	Val	50	55	60	
Pro	Thr	Val	Glu	Glu	Glu	Tyr	Asp	Glu	Phe	Ser	Asn	Gly	Phe	Trp	Lys	65	70	75	80
Asn	Ile	Pro	Leu	Tyr	Asn	Ala	Ser	Gly	Gly	Ser	Glu	Asp	Arg	Leu	Tyr	85	90	95	
Arg	Asp	Leu	Glu	Gly	Ser	Pro	Ala	Gln	Pro	Thr	Lys	His	Ala	Glu	Gln	100	105	110	
Val	Pro	Tyr	Leu	Asn	Glu	Ile	Ile	Thr	Thr	Val	Tyr	Asn	Gly	Glu	Arg	115	120	125	
Leu	Gln	Met	Ala	Arg	Thr	Arg	Asn	Leu	Lys	Asn	Ala	Val	Val	Ile	Pro	130	135	140	
His	Arg	Asp	Phe	Val	Glu	Leu	Asp	Arg	Glu	Leu	Asp	Gln	Tyr	Phe	Arg	145	150	155	160
Thr	His	Leu	Met	Leu	Glu	Asp	Ser	Pro	Leu	Ala	Phe	His	Ser	Asp	Asp	165	170	175	
Asp	Thr	Val	Ile	His	Met	Arg	Ala	Gly	Glu	Ile	Trp	Phe	Leu	Asp	Ala	180	185	190	
Ala	Ala	Val	His	Ser	Ala	Val	Asn	Phe	Ala	Glu	Phe	Ser	Arg	Gln	Ser	195	200	205	
Leu	Cys	Val	Asp	Leu	Ala	Phe	Asp	Gly	Ala	Phe	Asp	Glu	Lys	Glu	Ala	210	215	220	
Phe	Ala	Asp	Ala	Thr	Val	Tyr	Ala	Pro	Asn	Leu	Ser	Pro	Asp	Val	Arg	225	230	235	240
Glu	Arg	Lys	Pro	Phe	Thr	Lys	Glu	Arg	Glu	Ala	Gly	Ile	Leu	Ala	Leu	245	250	255	
Ser	Gly	Val	Ile	Gly	Arg	Glu	Asn	Phe	Arg	Asp	Ile	Leu	Phe	Leu	Leu	260	265	270	
Ser	Lys	Val	His	Tyr	Thr	Tyr	Asp	Val	His	Pro	Gly	Glu	Thr	Phe	Glu	275	280	285	
Trp	Leu	Val	Ser	Val	Ser	Lys	Gly	Ala	Gly	Asp	Asp	Lys	Met	Val	Glu	290	295	300	
Lys	Ala	Glu	Arg	Ile	Arg	Asp	Phe	Ala	Ile	Gly	Ala	Arg	Ala	Leu	Gly	305	310	315	320

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Glu Arg Phe Ser Leu Thr Thr Trp
325

CLAIMS

1. A DNA coding for a protein having an amino acid sequence selected from amino acid sequences of Sequence Nos. 1, 2, 15, 16 and 17.

2. A DNA having a nucleotide sequence selected from nucleotide sequences of Sequence Nos. 3, 4, 13 and 14.

3. A DNA coding for a protein having an enzymatic activity of hydroxylating the 3-position of L-proline and which acts on free L-proline in the presence of 2-ketoglutaric acid and divalent iron ions to produce cis-3-hydroxy-L-proline, and hybridizing with the DNA having a nucleotide sequence selected from SEQ ID Nos:3, 4, 13, and 14 in a solution comprising 50% v/v of formamide, 2% of a blocking reagent, 0.1% w/v of N-laurylsarcosine and 0.02% w/v of SDS, 750mM sodium chloride and 75mM sodium citrate at 42°C overnight, the hybridizing including further steps of; washing twice with a solution containing 0.1% SDS, 300mM sodium chloride and 30mM sodium citrate at room temperature for 5 minutes; washing twice with a solution containing 0.1% SDS, 15mM sodium chloride and 1.5mM sodium citrate at 68°C for 15 minutes each.

4. The DNA according to any one of claims 1 to 3, wherein the DNA is derived from microorganisms belonging to the genus *Streptomyces* or *Bacillus*.

5. The DNA according to claim 4, wherein the microorganisms are selected from those of *Streptomyces*

canus ATCC12647, Streptomyces canus ATCC12646, Streptomyces sp. TH1 (FERM BP-4399), Bacillus sp. TH2 (FERM BP-4397) and Bacillus sp. TH3 (FERM BP-4398).

6. A recombinant DNA constructed by inserting into a vector a DNA fragment according to any one of claims 1 to 5.

7. A transformant obtainable by introducing a recombinant DNA according to claim 6 into an isolated host cell.

8. The transformant according to claim 7, which is selected from Escherichia coli XL2-Blue/pTH30 (FERM BP-5026) and Escherichia coli XL2-Blue/pTH75 (FERM BP-5409).

9. A protein encoded by the DNA according to claim 2 or 3.

10. A protein having an amino acid sequence selected from amino acid sequences of Sequence Nos. 1, 2, 15, 16.

11. A method for producing L-proline-3-hydroxylase, which comprises cultivating in a medium a transformant according to claim 7 or 8, thereby producing and accumulating L-proline-3-hydroxylase, followed by collecting the L-proline-3-hydroxylase from the resulting culture.

12. The method for producing L-proline-3-hydroxylase according to claim 11, wherein L-proline is added to the medium.

13. A method for producing cis-3-hydroxy-L-proline, which comprises cultivating in a medium a transformant according to claim 7 or 8, thereby producing and accumulating cis-3-hydroxy-L-proline, followed by collecting the cis-3-hydroxy-L-proline from the resulting culture.

14. The method for producing cis-3-hydroxy-L-proline according to claim 13, wherein the transformant has the ability of producing L-proline from the saccharide sources in the medium and accumulating cis-3-hydroxy-L-proline in the culture.

15. The method for producing cis-3-hydroxy-L-proline according to claim 13, wherein the transformant has the ability of producing 2-ketoglutaric acid from the saccharide sources in the medium and accumulating cis-3-hydroxy-L-proline in the culture.

16. The method for producing cis-3-hydroxy-L-proline according to claim 13, wherein L-proline is added to the medium.

17. The method for producing cis-3-hydroxy-L-proline according to claim 13, wherein L-proline, 2-ketoglutaric acid and divalent iron ions are added to the medium.

18. A method for producing cis-3-hydroxy-L-proline, which comprises:

(a) cultivating in a medium a transformant according to claim 7 or 8

(b) converting L-proline into cis-3-hydroxy-L-proline in the presence of 2-ketoglutaric acid and

divalent iron ions in an aqueous medium by using the culture of the transformant, cells isolated from the culture or a product to be prepared by processing the cells as the enzymatic source, and

(c) collecting the resulting cis-3-hydroxy-L-proline from the aqueous medium.

19. A method for producing cis-3-hydroxy-L-proline, which comprises:

(a) cultivating in a medium a transformant according to claim 7 or 8

(b) converting L-proline into cis-3-hydroxy-L-proline during the cultivation, and

(c) collecting cis-3-hydroxy-L-proline from the culture medium.

20. The method for producing cis-3-hydroxy-L-proline according to claim 18 or 19, wherein the transformant has the ability of producing L-proline from the saccharide sources in the medium and accumulating L-proline in the culture.

21. The method for producing cis-3-hydroxy-L-proline according to claim 18 or 19, wherein the transformant has the ability of producing 2-ketoglutaric acid, from the saccharide sources in the medium and accumulating 2-ketoglutaric acid in the culture.

22. The method for producing cis-3-hydroxy-L-proline according to claim 18, wherein the product to be prepared by processing the cells cultivated is selected from dried cells, lyophilized cells, surfactant-treated

cells, enzymatically-treated cells, ultrasonically-treated cells, mechanically-ground cells, mechanically-compressed cells, solvent-treated cells, fractionated cell proteins, immobilized cells, immobilized materials obtained by processing their cells, enzymes having the enzymatic ability of hydroxylating the 3-position of L-proline to be extracted from the cells, pure products to be prepared by purifying the enzymes and immobilized enzymes.

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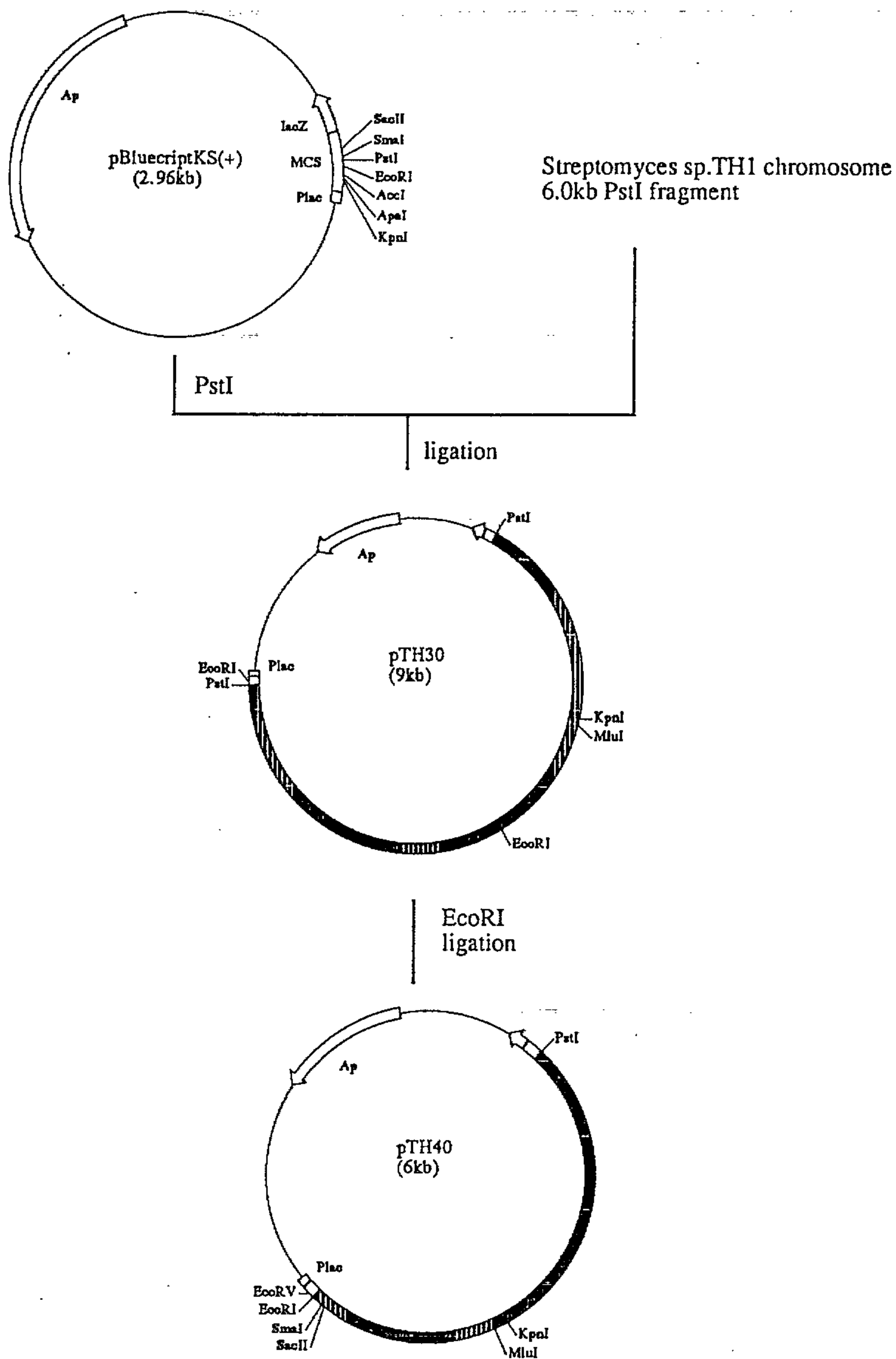


Fig. 1

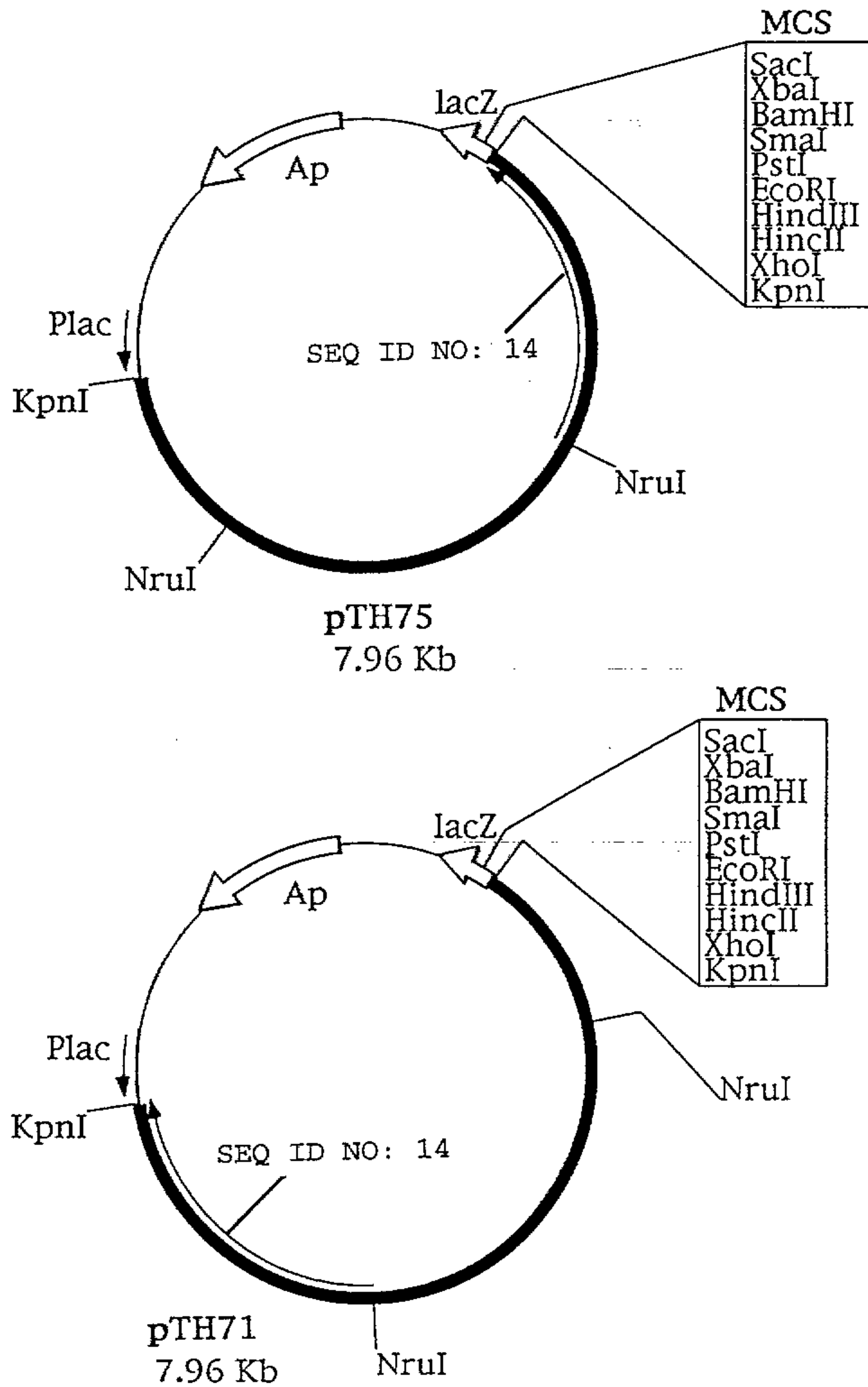


Fig. 2

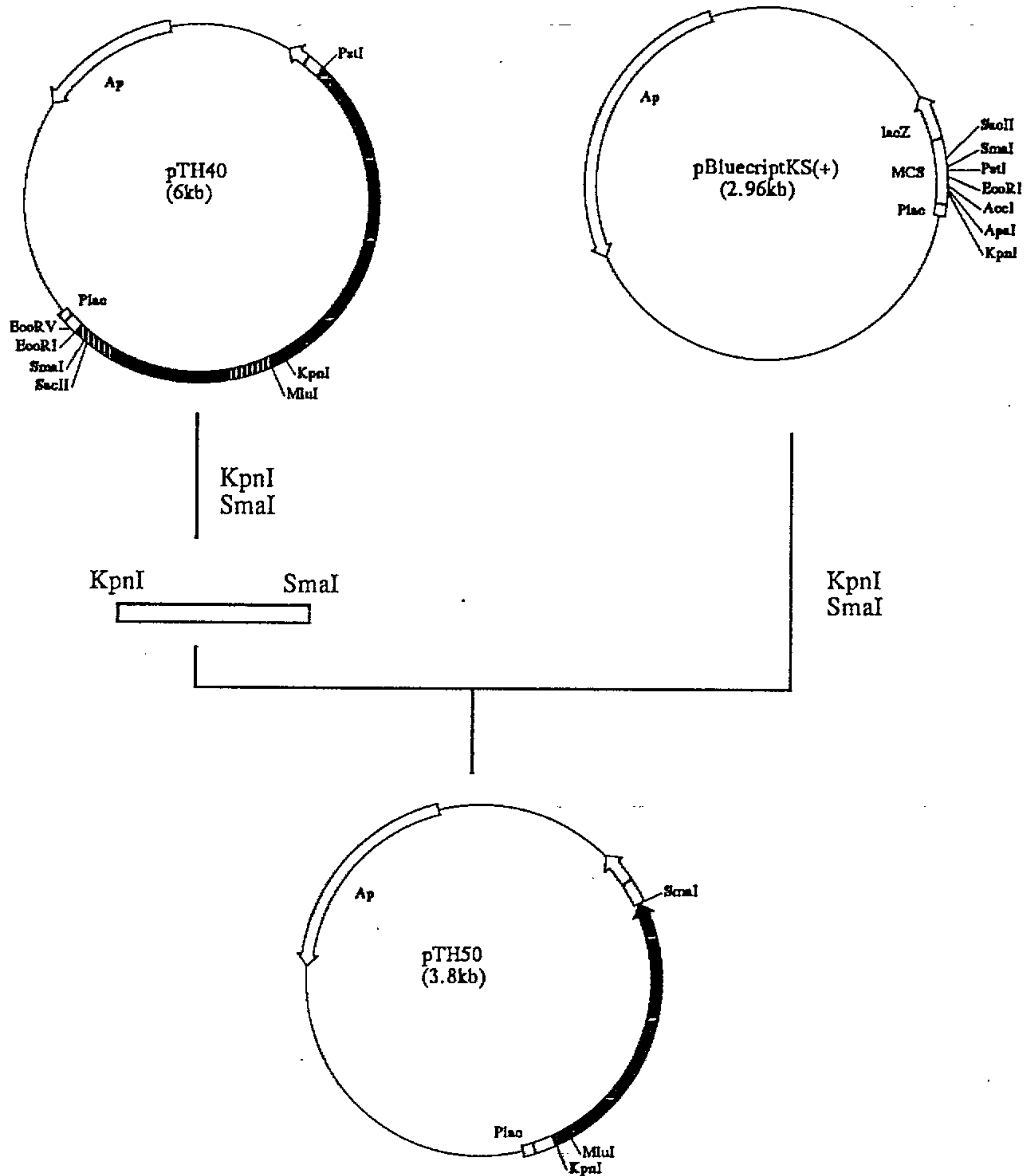


Fig. 3

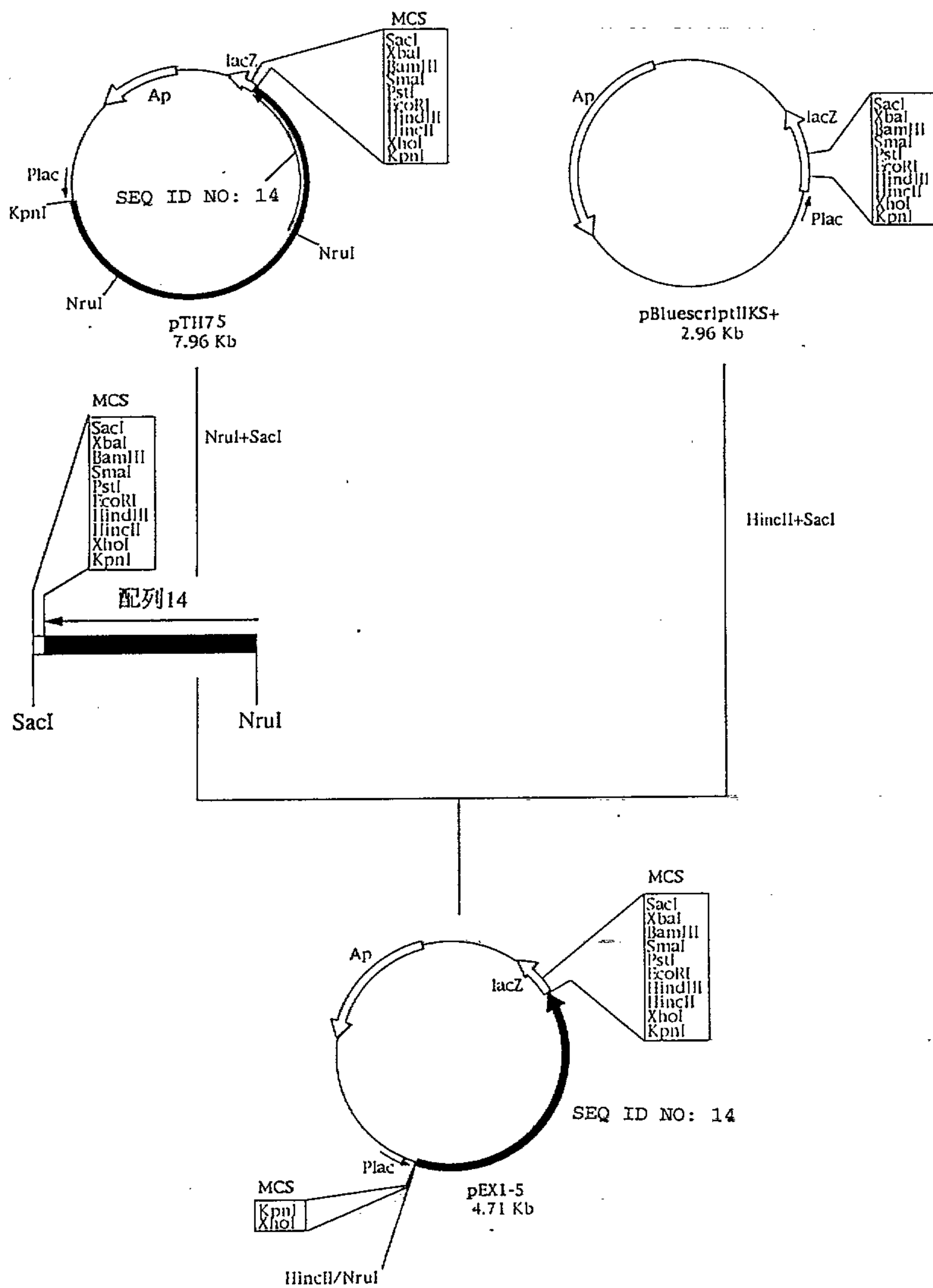


Fig. 4

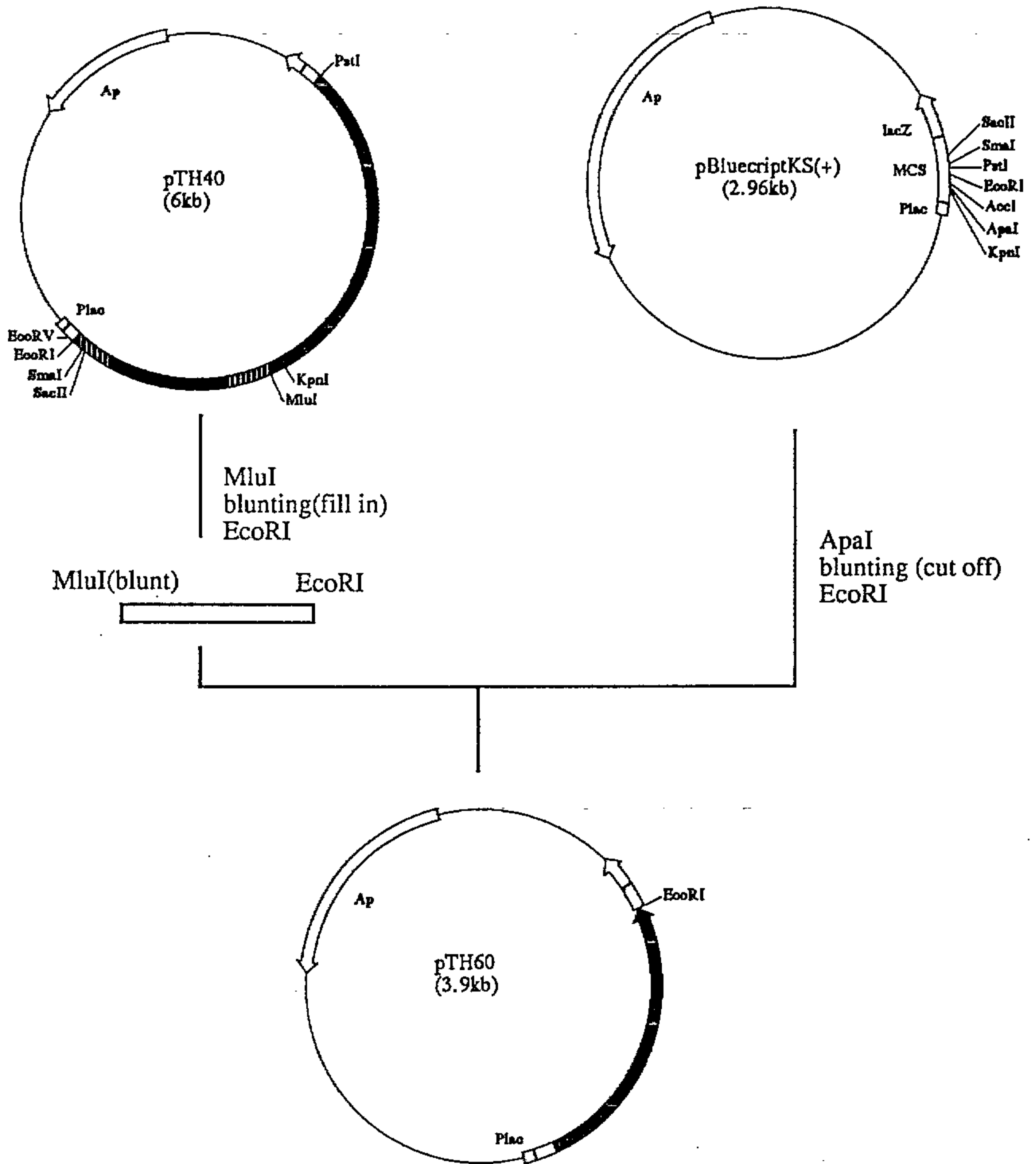


Fig. 5

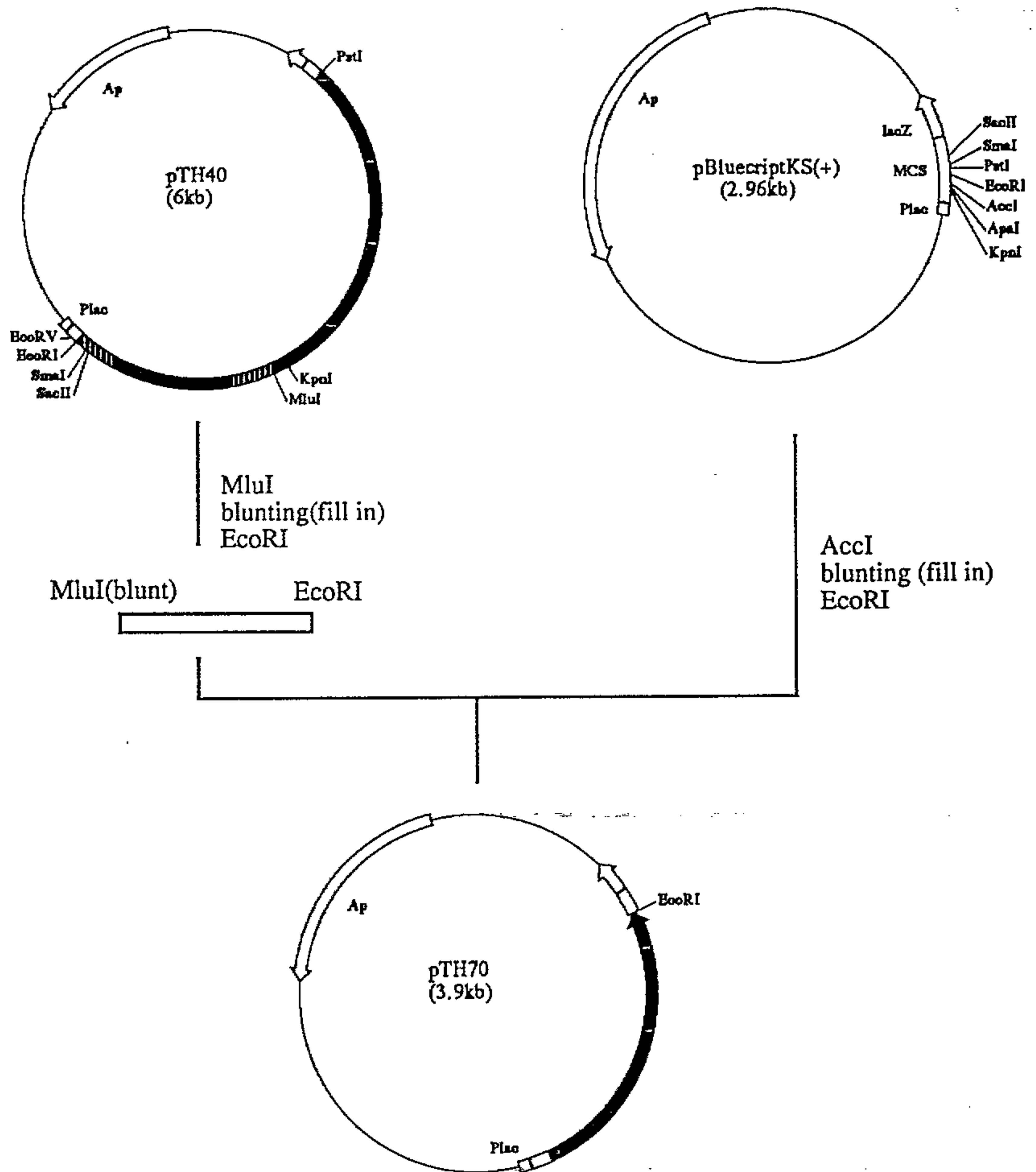


Fig. 6

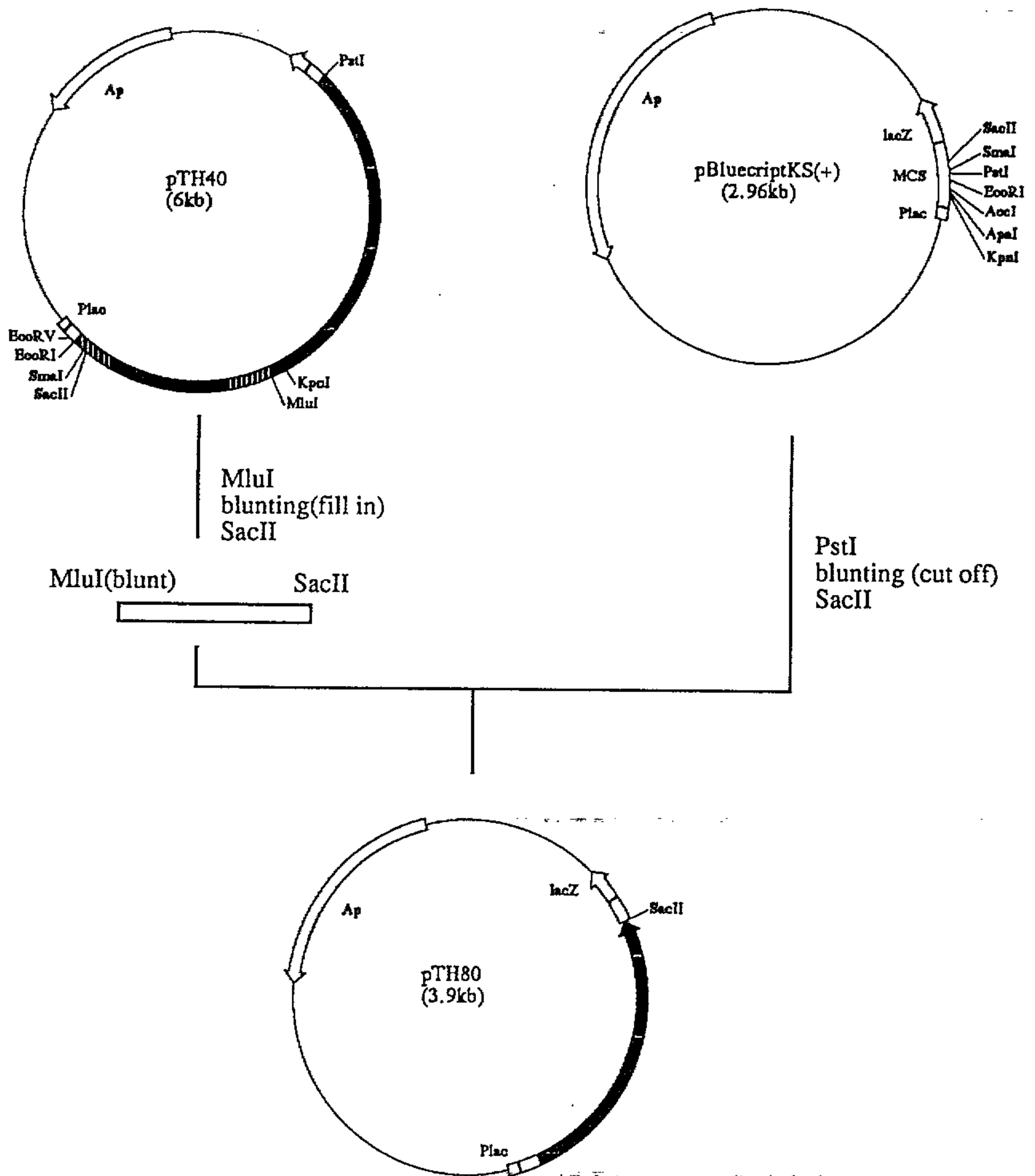


Fig. 7