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(54) **Titre** : GENE DE LA CEPHALOSPORINE-ACETYLHYDROLASE ET PROTEINE ENCODEE PAR LEDIT GENE
(54) **Title**: CEPHALOSPORIN ACETYLHYDROLASE GENE AND PROTEIN ENCODED BY SAID GENE

(57) **Abrégé/Abstract**:

The invention relates to a cephalosporin acetylhydrolase gene, a recombinant DNA molecule prepared by introducing the gene into a vector used in an E. coli host-vector system, E. coli cells transformed with the recombinant DNA molecule, a protein having the amino acid sequence encoded by the gene, an enzyme which is a multimer of the protein, and a process for preparing the protein or enzyme. The cephalosporin acetylhydrolase is an enzyme useful for converting cephalosporins, e.g. cephalosporin C and 7-ACA, into deacetylated ones, e.g. deacetylcephalosporin C and deacetyl 7-ACA, which are useful as intermediates in the preparation of a variety of derivatized cephalosporin antibiotics.

Abstract

The invention relates to a cephalosporin acetylhydrolase gene, a recombinant DNA molecule prepared by introducing the gene into a vector used in an *E. coli* host-vector system, *E. coli* cells transformed with the recombinant DNA molecule, a protein having the amino acid sequence encoded by the gene, an enzyme which is a multimer of the protein, and a process for preparing the protein or enzyme. The cephalosporin acetylhydrolase is an enzyme useful for converting cephalosporins, e.g. cephalosporin C and 7-ACA, into deacetylated ones, e.g. deacetylcephalosporin C and deacetyl 7-ACA, which are useful as intermediates in the preparation of a variety of derivatized cephalosporin antibiotics.

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Cephalosporin Acetylhydrolase Gene and Protein Encoded by said Gene

The present invention relates to a cephalosporin acetylhydrolase gene, a recombinant DNA molecule prepared by introducing said gene into a vector used in an Escherichia coli host-vector system, E. coli cells transformed with said recombinant DNA molecule, a protein having an amino acid sequence encoded by said gene, an enzyme comprising a multimer, preferably tetramer or octamer, of said protein, and a process for preparing said protein or enzyme.

Cephalosporins, e.g. cephalosporin C and 7-aminocephalosporanic acid (hereinafter referred to as 7-ACA), were hitherto derived by eliminating the acetyl group bonded to the hydroxymethyl group at their 3-position (hereafter referred to as deacetylation) to deacetylated compounds, e.g. deacetylcephalosporin C and deacetyl 7-ACA. These compounds are useful as starting materials to synthesize a variety of cephalosporin antibiotics including those already on the market.

As a method for deacetylation of cephalosporins, there exists chemical and enzymatic methods. Of these methods, the latter is believed to be advantageous partly because it can be performed at approximately neutral pH and at a mild temperature, and partly because it is accompanied by fewer side reactions. Several enzymatic methods have already been disclosed (for example, Japanese Patent Publication Nos. 108,790/1984, 132,294/1974 and 67,489/1986, and U.S. Patent No. 3,304,236).

The present inventors have found that a strain of Bacillus subtilis isolated from soil produces cephalosporin acetylhydrolase and efficiently produces deacetylcephalosporins by deacetylation of cephalosporins. This finding has led the inventors to the idea that the construction of a microorganism capable of preferentially producing the cephalosporin acetylhydrolase by recombinant DNA technology would be industrially very advantageous for preparing deacetylcephalosporins. Such a microorganism remarkably producing only cephalosporin acetylhydrolase, is presently not known.

The present inventors have made an extensive study, and have succeeded in isolating a DNA fragment carrying a cephalosporin acetylhydrolase gene from B. subtilis newly isolated from soil, and in cloning of said DNA fragment in E. coli. They also found that E. coli transformed with a plasmid DNA vector, into which said fragment had been inserted, produced a remarkable amount of cephalosporin acetylhydrolase. The present invention has been completed on the basis of the above findings.

Thus, the present invention provides a cephalosporin acetylhydrolase gene, a recombinant DNA molecule containing said gene, E. coli cells transformed with said recombinant DNA molecule, a protein having an amino acid sequence encoded by said gene, an enzyme comprising a multimer of said protein, and a process for preparing said protein or enzyme.

The newly isolated strain, Bacillus subtilis SHS0133, from which the cephalosporin acetylhydrolase gene was obtained according to the present invention, was deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, 1-3, Higashi 1 chome, Tsukuba-shi, Ibaraki-ken, 305, Japan under the Budapest Treaty with the accession number FERM BP-2755 (Date: February 15, 1990).

The invention is described in detail below with reference to the accompanying drawings, in which:

Fig. 1 depicts the amino acid sequence of cephalosporin acetylhydrolase.

Fig. 2 depicts the base sequence of cephalosporin acetylhydrolase gene (the lower row) and the amino acid sequence deduced therefrom (the upper row).

Fig. 3 depicts the partially determined amino acid sequence (34 amino acid residues) of cephalosporin acetylhydrolase.

Fig. 4 depicts four synthetic oligonucleotide probes including all of the genetically possible DNA base sequences deduced from the underlined amino acid sequence in Fig. 3.

Fig. 5 depicts the restriction enzyme cleavage map of recombinant plasmid pCAH01 and the position at which the DNA probe hybridizes.

Fig. 6 depicts the restriction enzyme cleavage map of recombinant plasmid pCAH03.

Fig. 7 depicts the DNA sequence of the cloned cephalosporin acetylhydrolase gene and flanking regions.

Fig. 8 depicts the construction of miniaturized plasmid pCAH10 which corresponds to plasmid pCAH03 but deletes a region downstream of cephalosporin acetylhydrolase gene.

Fig. 9 depicts the construction of expression plasmid pCAH21 used in E. coli.

Fig. 10 depicts the construction of expression plasmids pCAH211 and pCAH212 used in E. coli.

Characteristics of B. subtilis SHS0133

1. G+C mole percent (%) of chromosomal DNA : 43.4
2. Morphological characteristics

The strain is a gram-positive, short rod-shaped bacterium of 0.7-0.8 x 1.8-2.6μm in size, and is peritrichous. This strain grows well under an aerobic condition and also grows weakly in a natural medium containing glucose under an anaerobic condition. Spore, 0.8 x 1.3-1.7μm in size, is formed in the central part of the cell.

3. Cultural characteristics

(1) Meat extract agar plate culture (at 30°C, for 7 days)

Colony formation : 24 hours after inoculation
Shape : irregular
Surface : gilled
Margin : undulate
Elevation : convex
Colour : cream
Lustre : dull
Optical property : opaque

(2) Meat extract agar slant culture (at 30°C, for 7 days)

Growth : good
Shape : filiform
Surface : gilled

Colour : cream
Luster : dull
Optical property : opaque
Consistency : butyrous

(3) Meat extract broth culture (at 30°C, for 7 days)
Growth on surface : thick film is formed
Clouding : slight
Odour : slightly aromatic
Sediment : visid
Amount of sediment : scanty

(4) Meat extract gelatin stab culture (at 24°C, for 30 days)
Growth : grows uniformly along the stab line
Line of puncture : filiform
Mode of liquefaction : liquefied on 7th day at 24°C to form 0.5 mm of liquefied layer (stratiform)

(5) Litmus milk culture

Litmus is reduced. Casein is digested rapidly without coagulation.

4. Biochemical characteristics

(1) Reduction of nitrate : positive
(2) Denitrification : positive
(3) MR test : negative
(4) VP test : positive
(5) Formation of indole : negative
(6) Formation of H_2S : lead acetate paper : negative
TSI : negative
Krigler : negative
(7) Hydrolysis of starch : positive
(8) Utilization of citrate : Koser : positive
Christensen : positive
(9) Utilization of inorganic nitrogen source :
nitrate : positive
ammonium : positive
(10) Formation of pigment : brown pigment was formed
(11) Urease test : positive
(12) Oxidase test : positive

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(13) Catalase test : positive

(14) pH : growth range : 1.5-8.8
optimum : 6.0-8.8

(15) Temperature : growth range : 16.5°C-50.5°C
optimum : 26.0°C-36.0°C

(16) OF test :
D-glucose : produces acids fermentatively without generating gas
lactose : produces acids fermentatively without generating gas

(17) Formation of acids and gas from sugars :

i) produces acids and no gas from L-arabinose, D-xylose, D-glucose, D-mannose, D-fructose, maltose, sucrose, trehalose, D-mannitol, glycerol, and starch;

ii) produces neither acids nor gas from D-galactose, lactose, D-sorbitol, and inositol.

(18) Nutritional requirement : none

(19) Degradation of pectin : positive

(20) Degradation of hippuric acid : negative

(21) Formation of levan (from sucrose, raffinose) : positive

(22) Arginine hydrolase : negative

(23) Lecithinase : negative

(24) Growth under anaerobic condition : grows weakly in natural medium containing D-glucose.

From the above results, the strain was identified to be one strain of *B. subtilis* on the basis of Bergey's Manual of Systematic Bacteriology, Vol. II, 1986, and designated as *Bacillus subtilis* SHS0133.

The present invention encompasses a process for preparing cephalosporin acetylhydrolase using a recombinant microorganism transformed with a recombinant DNA molecule prepared by introducing into a vector used in an *E. coli* host-vector system an isolated DNA base sequence encoding the amino acid sequence depicted in Fig. 1, preferably the DNA base sequence depicted in Fig. 2. This process may be realized by following the next seven steps which represent the history of the development of the present invention, although the process

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can be achieved by a simpler procedure as the DNA base sequence encoding the cephalosporin acetylhydrolase is revealed by this invention.

(1) Bacillus subtilis (FERM BP-2755) is cultured in an appropriate medium and allowed to produce cephalosporin acetylhydrolase, the produced cephalosporin acetylhydrolase is separated from the medium and purified. The purified enzyme is digested with an appropriate protease used in fragmentation of a protein, the resultant peptide fragments are separated, and the amino acid sequence of one of the peptide fragments is determined from the amino terminus.

(2) Possible DNA base sequences corresponding to a part of the amino acid sequence determined are deduced, a pool of oligonucleotides having the deduced base sequences are chemically synthesized, and the 5'-terminus of the oligonucleotides labelled with ^{32}P. The labelled oligonucleotides are used as probes for gene cloning.

(3) Chromosomal DNA is extracted and purified from B. subtilis (FERM BP-2755), digested with various restriction enzymes, electrophoresed on agarose gel, and the separated DNA fragments are transferred onto a nitrocellulose membrane from the gel. Southern hybridization is then conducted using the nitrocellulose membrane and the ^{32}P-labelled probes prepared in the above step (2) to select DNA fragments showing homology to the probes. Of the DNA fragments, a somewhat larger fragment as compared with the size of the desired gene expected from the molecular weight of cephalosporin acetylhydrolase protein is selected, the relevant region on agarose gel containing the DNA fragment is excised, and the DNA is extracted.

(4) The DNA fragment from the above step (3) is inserted into an E. coli cloning vector and the resultant recombinant plasmid is introduced into E. coli cells by transformation. The transformants are plated on an agar medium to form colonies, and colony hybridization is conducted using the ^{32}P-labelled probes. The colony of E. coli showing homology with the probe is selected and isolated.

(5) The recombinant plasmid DNA is extracted from the selected E. coli cells, the restriction enzyme cleavage map is constructed, and the subsequent region irrelevant to cephalosporin acetylhydrolase gene is eliminated. The base sequence of the B. subtilis-derived DNA fragment encoding the cephalosporin acetylhydrolase is determined. The amino acid sequence deduced from the determined DNA base sequence is then compared with the partial amino acid sequence, molecular weight, terminal amino acid analysis and amino acid composition analysis of cephalosporin acetyhydrolase, and the structural gene for the cephalosporin acetylhydrolase is determined.

(6) The DNA fragment containing the cephalosporin acetylhydrolase gene is properly modified and then introduced into a gene expression vector for E. coli so that the structural gene is connected after a promoter derived from E. coli, to construct a recombinant plasmid for expression.

(7) The recombinant expression plasmid obtained above is introduced into an E. coli host by transformation to prepare a novel E. coli strain producing cephalosporin acetylhydrolase.

The procedures employed in the above steps are known to those skilled in the art and can be readily carried out according to an experimental protocol disclosed in standard text books, for example, "Molecular Cloning", T. Maniatis et al., Cold Spring Harbor Laboratory (1982). All of the materials used, e.g. enzyme are reagents, are commercially available and may be used according to the supplier's instructions.

E. coli used as a host may be a strain of E. coli K-12 derivatives, e.g. HB101, DH1, C600, JM103, JM105 and JM109. As an E. coli vector used in cloning, a plasmid vector, e.g. pUC13, pBR322 and pAT153, as well as a phage vector, e.g. λgt10, can be exemplified. The above-mentioned hosts and vectors are commercially available and easily obtainable.

In the above step (1), the amino acid sequencing of a protein is known (for example, a commercially available automated amino acid sequencer may be used). In the above step (2), the synthesis of the oligonucleotides can be carried out using a commercially available DNA synthesizer according to the supplier's protocol. In the above step (5), the determination of the DNA base sequence can be performed according to the method by Sanger *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, *74*, 5463-5467 (1977), where a known M13 vector system is employed.

In the above step (6), the construction of a plasmid to direct efficient expression of the desired gene in *E. coli* may be carried out by inserting the DNA fragment containing the desired cephalosporin acetylhydrolase structural gene into an expression vector (pKK223-3, pBS, pDR540, pPL-lambda, etc.) containing a suitable promoter (Lac, Tac, Trc, Trp, P_L, etc.) functional in a host and Shine-Dalgarno (SD) sequence, or into an ATG vector (pKK233-2, etc.) which additionally contains the translational initiation condon ATG. Introduction of the expression plasmid into a suitable host (for example, such a strain as *E. coli* JM103, JM109, HB101 and C600) yields a microorganism efficiently expressing cephalosporin acetylhydrolase.

The expressed cephalosporin acetylhydrolase may be purified according to a conventional purification method, for example, by combining centrifugation, column chromatography and the like.

The present invention is further illustrated by the following Example, but not limited thereto.

Example 1

1. Separation and purification of cephalosporin acetylhydrolase and determination of partial amino acid sequence

(1) Separation and purification of cephalosporin acetylhydrolase

A medium (20L) composed of 2.5% glucose, 0.75% corn steep liquor, 1.0% amino acids mixture, 0.3% KH_2PO_4, and 0.8 ppm $MnSO_4.4H_2O$, pH 7.0 was charged into a 30L volume jar-fermenter. After sterilization, *B. subtilis* (FERM BP-2755) which had been precultured in a medium composed of 0.5% glucose, 0.75% corn steep liquor, 0.5% amino acids mixture, and 0.02% KH_2PO_4, pH 7.0, was poured into the medium so as to obtain 6% of inoculum size. After 48 hours of cultivation at 28°C, activated charcoal was added to the cultured fluid to 1%, and stirred for 2 hours. Subsequent filtration gave a crude enzyme solution as filtrate. To the crude enzyme solution, DEAE Sephadex A-50* (Pharmacia) was added to 0.7%, and the mixture was adjusted to pH 8.0 using 2N NaOH and then stirred for one hour. After filtration, DEAE Sephadex A-50*, on which cephalosporin acetylhydrolase activity was adsorbed, was washed with 50mM Tris-HCl buffer (pH 8.0) (4L) containing 0.1M NaCl, and the activity was then eluted with the same buffer containing 0.4M NaCl. After concentration and desalting by an ultrafiltration apparatus (Tosoh), the activity was adsorbed onto a column filled with DEAE Sepharose CL-6B* (Pharmacia) which had been previously equilibrated with the same buffer. The column was washed with the same buffer and subsequently that containing 0.15M NaCl, and the activity was then eluted with the same buffer containing 0.2M NaCl. After concentration and desalting by ultrafiltration, purification with high performance liquid chromatography (hereafter referred to as HPLC) was performed. DEAE Toyopearlpak 650M* (Tosoh) was used as the column. The activity was eluted by a concentration gradient elution method where salt concentration was sequentially raised. Thus, starting with the same buffer containing 0.15M NaCl, the concentration of NaCl was sequentially raised to 0.5M. Fractions containing the eluted activity were collected, concentrated by ultrafiltration, and then purified by HPLC using a molecular sieve column. TSK-G3000* (Tosoh) was used as the column and 0.2M phosphate buffer (pH 7.0) was used as the mobile phase. The eluted active fractions were collected, concentrated by ultrafiltration, and then purified by HPLC using a reversed phase column. Microbondapak C_{18}* (Waters) was used as the column and the elution was carried out by concentration

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gradient elution method where acetonitrile concentration was sequentially raised. Thus, starting with an aqueous system containing 0.1% trifluoracetic acid, the acetonitrile concentration was raised to the final concentration of 98%. The fractions containing the eluted cephalosporin acetylhydrolase were concentrated under reduced pressure at 50°C and the residue was dissolved in 0.5M Tris-HCl buffer (pH 8.0) containing 6M quanidine hydrochloride. To the solution, EDTA (ethylenediaminetetraacetic acid) was added to obtain a 2mM concentration, a 200-fold mole amount of 2-mercaptoethanol relative to cephalosporin acetylhydrolase was added to the solution, and the reduction was performed at 37°C for 4 hours under a nitrogen atmosphere. Subsequently, a 190-fold mole amount of sodium iodoacetate relative to cephalosporin acetylhydrolase was added to the solution and reacted at 37°C for 10 minutes in the dark to perform reductive carboxymethylation. Then, purification by HPLC using a reversed phase column was conducted again according to the above method. The resulting cephalosporin acetylhydrolase fractions were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the procedure of Laemmli, *Nature*, *227*, 680-685 (1970). Only a single band was detected at a position of molecular weight 35kD, implying that purification was achieved homogeneously. In addition, the facts that the purified cephalosporin acetylhydrolase solubilized in 0.1M Tris-HCl buffer (pH 8.0) containing 7M urea was reactivated by 10-fold dilution with 0.1M phosphate buffer (pH 7.0), and that the activity was eluted at a position of molecular weight of 280kD by a molecular sieve column chromatography using the above-mentioned HPLC, suggested that the cephalosporin acetylhydrolase in a natural form is the octamer consisting of homogeneous subunits.

On the other hand, the molecular weight determined using the procedure of Hedrick *et al.*, *Arch. Biochem. Biophys.*, *126*, 155-164 (1968) was about 150kD and the activity was detected at the relevant position, which suggested that the tetramer is also active.

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Amino acid terminal analysis of the purified cephalosporin acetylhydrolase by Edman degradation and hydrazine hydrolysis identified the amino terminus to be methionine and the carboxy terminus to be glycine.

(2) Determination of partial amino acid sequence of cephalosporin acetylhydrolase

The purified cephalosporin acetylhydrolase (1mg) was solubilized in 0.1M Tris-HCl buffer (pH 8.8)(1.0ml) containing 2M urea, lysylendopeptidase (0.01mg) was added to the solution, and the mixture was reacted at 37°C for 4 hours. The reaction mixture was then separated by HPLC. Microbondapak C_{18}* (Waters) was used as the column and elution was carried out by concentration gradient elution method, sequentially raising the acetonitrile concentration. Thus, starting with an aqueous system containing 0.1% trifluoroacetic acid, the acetonitrile concentration was raised to a final concentration of 98%. The detection was carried out at 214nm. Of the separated peaks, distinctive fractions having a long retention time were collected and again purified using the same reversed phase column, and the amino acid sequence of the peptide was analyzed using a gas phase automated amino acid sequencer (Applied Biosystems) to determine the amino acid sequence from amino terminus to 34th amino acid of the peptide fragment, as shown in Fig. 3.

2. <u>Synthesis of DNA probes and labelling the 5′-termini</u>

The sequence underlined in the amino acid sequence in Fig. 3 obtained by the above step 1 was selected and a pool of oligonucleotides corresponding to all of the genetically possible DNA base sequences deduced from this amino acid sequence were synthesized. As shown in Fig. 4, 4 groups of the oligonucleotides, which were designated as DNA probes CAH-RM1, CAH-RM2, CAH-RM3, CAH-RM4, were synthesized. Synthesis of the oligonucleotides was conducted using automated DNA synthesizer ZEON-GENET A-II* (Nihon Zeon).

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5'-Termini of the resulting DNA probes were labelled using T4 polynucleotide kinase and [λ-^{32}P]ATP according to the procedure of Wallace et al., Nucleic Acids Res., 6, 3543-3557(1979).

3. Extraction and purification of chromosomal DNA from B. subtilis and Southern hybridization

Cells of B. subtilis (FERM BP-2755) were treated with lysozyme followed by sodium N-lauroylsarcosinate, and the chromosomal DNA was extracted and purified from the resulting lysate by CsCl-ethidium bromide equilibrium density gradient ultracentrifugation according to the procedure of Harris-Warrick et al., Proc. Natl. Acad. Sci. U.S.A., 72, 2207-2211(1975). The DNA (1μg) was digested with various restriction enzymes (each about 10 units) under suitable conditions, and the reactants were electrophoresed on 0.8% agarose gel. After the analysis of the restriction enzyme cleavage pattern, the gel was subjected to Southern hybridization according to the procedure of Southern et al., J. Mol. Biol., 98, 503-517(1975). The gel was treated with 1.5M NaCl solution containing 0.5N NaOH at room temperature for 1 hour to denature DNA, and then neutralized in 1M Tris-HCl buffer (ph 7.0) containing 1.5M NaCl at room temperature for 1 hour. A nitrocellulose membrane was then placed on the gel to transfer the DNA from the gel to the membrane. Using the DNA-transferred nitrocellulose membrane, the hybridization with the labelled probes was carried out. The hybridization was conducted at 38°C overnight using 4-fold concentration of SSC (1 x SSC: 0.15M NaCl, 0.015M sodium citrate, pH 7.0), 10-fold concentration of Denhardt's solution (1 x Denhardt's solution : 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), and about 1 x 10^6 cpm/ml of the labelled probes. The membrane was washed several times with a 4-fold concentration of SSC at room temperature, and then subjected to autoradiography. In addition, the washing temperature of the membrane was raised stepwise, and the membrane was provided for autoradiography repeatedly in each case. It was found that several DNA bands show positive signals even at high washing temperatures, e.g. 48°C and 53°C, and that the sizes of the hybridizing bands differ from each other depending on the restriction enzymes employed. Out of the DNA fragments showing positive signals, 2.5-3kb DraI digested and 4-4.5kb HindIII digested fragments were preferable for gene cloning because their sizes were larger then the desired gene.

4. <u>Cloning of cephalosporin acetylhydrolase gene</u>

(1) Construction of gene library

To the chromosomal DNA (12μg) from <u>B</u>. <u>subtilis</u> extracted and purified in the above step 3, restriction enzyme DraI (about 120 units) was added and incubated at 37°C for 90 minutes. The reactant was then extracted with an equal volume of phenol, and ethanol was added to the resulting aqueous layer to precipitate DNA. The resulting DNA was dissolved in TE buffer [10mM Tris-HCl, 1mM EDTA, pH 8.0] (60μl), the resultant solution was electrophoresed on 0.8% agarose gel, and a region of the gel corresponding to a size of 2-4kb was excised. DNA fragments were eluted and recovered from the gel using a commercially available kit (Bio 101, GENECLEAN*), and dissolved in TE buffer (30μl).

On the other hand, pUC13 was used as a vector for constructing a gene library. pUC13 (10μg) was mixed with restriction enzyme SmaI (about 100 units), incubated at 37°C for 140 minutes, and then treated at 65°C for 10 minutes. Alkaline phosphatase (BAP)(about 20 units) was added to the mixture and further reacted at 65°C for 80 minutes. Phenol extraction followed by ethanol precipitation of DNA was carried out according to the above procedure and the DNA was finally dissolved in TE buffer (50μl).

The solution containing the DraI digested fragments of <u>B</u>. <u>subtilis</u> chromosome (7.5μl) and the solution containing the SmaI-BAP treated fragments of vector pUC13 (2.5μl) were combined, and the mixture was incubated with T4 DNA ligase at 6°C for 20 hours to ligate the fragments, forming a

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recombinant DNA. The resultant recombinant DNA was used to transform *E. coli* HB101 strain according to the procedure of Hanahan *et al.*, *J. Mol. Biol.*, *166*, 557-580 (1983). Subsequently, colonies were allowed to form on a nitrocellulose membrane placed on L-broth [1% bacto-tryptone, 0.5% yeast extract, 0.5% NaCl (pH 7.3)] agar medium containing 40μg/ml ampicillin. These colonies were designated as the gene library of *B. subtilis*.

(2) Selection of cephalosporin acetylhydrolase-positive clone by colony hybridization

The colonies formed on the nitrocellulose membrane in the above step (1) were replicated on another nitrocellulose membrane. The replica membrane was placed on L-broth agar medium containing 40μg/ml ampicillin and incubated at 37°C for 3 hours. Then, the membrane was transferred on L-broth agar medium containing 250μg/ml chloramphenicol and incubated at 37°C overnight. Colony hybridization was then carried out substantially according to the procedure of Grunstein *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, *72*, 3961-3965(1975). Thus, the membrane was treated with 0.5N NaOH (for 10-15 minutes) to effect lysis of colonies and denaturation of DNA. Subsequently, the membrane was neutralized with 1M Tris-HCl buffer (pH 7.2) for 5-10 minutes and further treated with 1M Tris-HCl buffer (pH 8.0) containing 1.5M NaCl for 10-15 minutes. After the membrane was baked under reduced pressure at 80°C for 2 hours to immobilize DNA to the membrane, the immobilized DNA was hybridized with the labelled probe CAH-RM2. The reaction was carried out in a solution containing 4-fold concentration of SSC, 10-fold concentration of Denhardt's solution and about 2×10^6 cpm/ml of the labelled probe at 38°C for 16 hours. Subsequently, the membrane was washed 3-4 times with 4-fold concentration of SSC at room temperature, then washed at 38°C for 2 minutes, and subjected to autoradiography (exposure condition : -80°C, 3 hours). In order to examine the correlation between washing temperature and intensity of signal on the autoradiography, the washing temperature was further raised stepwise and the autoradiography was conducted in each case. The washing was carried out at 43°C, 48°C and 53°C each for 2 minutes.

As a result, it was found that 3 of about 30,000 colonies showed positive signals even at the high washing temperature. These colonies were liquid-cultured in L-broth (5ml) containing 40μg/ml ampicillin at 37°C overnight and recombinant plasmids were prepared [procedure of Birnboim <u>et al</u>., <u>Nucleic Acids Res.</u>, <u>7</u>, 1513-1523(1979)]. These plasmids were cleaved and fragmented with a restriction enzyme, e.g. EcoRI, HindIII and PvuII for which the vector DNA (pUC13) has a cleavage site. Then, electrophoresis was carried out on agarose gel and Southern hybridization with the labelled probe was carried out. As a result, it was found that two of three recombinant plasmids contained a restriction enzyme digested fragment clearly hybridizing with the DNA probe. The size of the hybridizing fragments differed between the two positive plasmids, and therefore, the DNA fragments inserted into the vector DNA appeared different from each other. Accordingly, respective recombinant plasmids were distinguished and designated as pCAH01 and pCAH02.

5. <u>Identification of positive clones and determination of base sequence</u>

(1) Identification of positive clones

In the course of attempts to prepare restriction enzyme cleavage maps for the recombinant plasmids pCAH01 and pCAH02 by cleaving them with various restriction enzymes, it was found that plasmid pCAH01 has two different DraI digested fragments and plasmid pCAH02 has at least three different DraI digested fragments inserted into the SmaI site on the vector DNA, and that the DNA probe hybridizes with one of these DraI fragments. Consequently, the recombinant plasmid pCAH01 was used in the subsequent procedures. The restriction enzyme cleavage map of plasmid pCAH01 was depicted in Fig. 5 together with the position at where the DNA probe hybridized.

Since plasmid pCAH01 contains two exogenous DraI fragments derived from the chromosomal DNA of <u>B</u>. <u>subtilis</u> (1.8kb and 2.5kb) and only the 2.5kb fragment hybridizes with the DNA probe, the 1.8kb DraI fragment was deleted from the plasmid. DraI-PstI fragment (2.6kb) containing the 2.5kb fragment was removed from plasmid pCAH01 and inserted into SmaI-PstI site of vector plasmid pUC13 to obtain a miniaturized recombinant plasmid pCAH03 (5.3kb). The restriction enzyme cleavage map of the recombinant plasmid pCAH03 is shown in Fig. 6.

(2) Determination of base sequence

From the recombinant plasmid pCAH03, 0.24kb EcoRi-HindIII fragment to which the DNA probe had hybridized was removed and its DNA base sequence was determined according to the procedure of Sanger *et al*., *Proc. Natl. Acad. Sci. U.S.A*, *74*, 5463-5467(1977). As a result, a base sequence corresponding to the partial amino acid sequence of cephalosporin acetylhydrolase obtained in step 1 above was found in the EcoRI-HindIII fragment, revealing that the fragment contains a part of the cephalosporin acetylhydrolase gene. By subsequent determination of the base sequence between DraI and HindIII sites (0.38 kb) as well as between EcoRI and EcoRI sites (1.45kb) on the basis of the restriction enzyme cleavage map of plasmid pCAH03, the base sequence of about 2kb between DraI and EcoRI sites was revealed as shown in Fig. 7. This proved the presence of a base sequence encoding a protein composed of 318 amino acid residues containing methionine encoded by translational initiation condon ATG. In addition, the putative protein encoded by the above base sequence showed a good coincidence with the cephalosporin acetylhydrolase with respect to their molecular weight, amino-terminal and carboxy-terminal amino acids, as well as amino acid composition of lysylendopeptidase digested fragments and therefore, it was believed that this protein must be cephalosporin acetylhydrolase. Thus, it was proved that the structural gene of cephalosporin acetylhydrolase was entirely contained in the *B. subtilis*-derived DNA fragment on plasmid pCAH03.

6. Construction of expression plasmid

The cloned *B. subtilis*-derived DNA fragment on plasmid pCAH03 contains a region other than the cephalosporin

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acetylhydrolase gene, and therefore, the region considered to be irrelevant to phenotypic expression of the gene was deleted according to the process depicted in Fig. 8.

In order to drive high expression of a heterologous gene in E. coli, it is generally effective to construct an expression plasmid in which a desired structural gene is connected immediately after a sequence consisting of a promoter having a high expression efficiency, SD sequence and translational initiation condon ATG. Thus, in order to produce a large amount of cephalosporin acetylhydrolase in E. coli, an expression plasmid was constructed using a vector containing a promoter, SD sequence and ATG, according to the process depicted in Fig. 9. In the process of the construction, the cephalosporin acetylhydrolase structural gene can be prepared as follows. A region entirely containing the desired gene is cloned into a vector of M13mp series to obtain a single-stranded DNA. According to a primer extension method, using a primer as an oligonucleotide specifying several amino acids excluding methionine on the amino terminal sequence of cephalosporin acetylhydrolase, a DNA fragment in which only the cephalosporin acetylhydrolase structural gene moiety is double-stranded, is obtained.

Fig. 10 shows a process for constructing a modified expression plasmid which shows increased copy number as compared with the expression plasmid prepared by the process in Fig. 9, and which bears a tetracycline (Tc^r) resistance marker instead of an ampicillin (Ap^r) resistance marker.

Following is a more detailed description of each step.

(1) Construction of miniaturized plasmid

In order to shorten the downstream region of the cephalosporin acetylhydrolase structural gene (abbreviated as CAH in Fig. 8), plasmid pCAH03 was cleaved with EcoRV and BamHI, about 4.1kb DNA fragment obtained was purified, and then the 3′ recessed termini created by BamHI digestion were filled using DNA polymerase Klenow fragment (Fig. 8). After further purification, ligation was carried out using T4 DNA

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ligase to construct miniaturized plasmid pCAH10 containing the cephalosporin acetylhydrolase gene.

(2) Preparation of single-stranded DNA for construction of expression plasmid

The miniaturized plasmid pCAH10 prepared in the above step (1) was cleaved with SacI and SalI and about 1.5kb fragment obtained was purified and recovered as a SacI-SalI fragment. On the other hand, double-stranded phage M13mp11 DNA was cleaved with SacI and SalI. Then, to the latter was added the above SacI-SalI fragment and ligated with T4 DNA ligase to construct double-stranded phage M13-CAH DNA which contained the complete cephalosporin acetylhydrolase gene. Subsequently, its single-stranded DNA was prepared according to the procedure of Messing, *Methods in Enzymology*, *101*, 20-78(1983).

(3) Primer extension

A region containing cephalosporin acetylhydrolase structural gene was prepared by eliminating a protein-noncoding region derived from *B*. *subtilis*, which locates upstream the initiation site (ATG) of cephalosporin acetylhydrolase, in substantial accordance with the procedure of Goeddel *et* *al*., *Nucleic Acids Res.,* *8*, 4057-4074(1980). An oligonucleotide having a base sequence corresponding to the 2nd amino acid glutamine to the 7th amino acid proline from the amino terminus of cephalosporin acetylhydrolase was synthesized as a primer to be used in primer extension and designated as CAH-P1. The primer CAH-P1 (3pmole) was then added to the single-stranded DNA (7.5μg) of phage M13-CAH prepared in step (2) above, and the mixture was heated to 60°C for 20 minutes and then allowed to stand at room temperature. Subsequently, to the mixture were added dATP, dCTP, dGTP and dTTP (each 0.25mM) as well as DNA polymerase Klenow fragment (2 units), and the primer extension was carried out at 37°C for 2 hours in a reaction mix (20μl) of 7mM Tris-HCl buffer (pH 7.5), 7mM $MgCl_2$, 0.1 mM EDTA, and 20 mM NaCl. After the reaction, phenol extraction and ethanol precipitation were conducted. The DNA was dissolved in a small amount of

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distilled water, to which S1 nuclease (4 units) was added and incubated at 37°C for 30 minutes in a reaction mix (40μl) of 30mM sodium acetate (pH4.6), 100mM NaCl and 1mM $ZnSO_4$ to digest the remaining single-stranded DNA. The solution containing the double-stranded DNA fragment obtained was subjected to phenol extraction followed by ethanol precipitation, and the DNA was treated with DNA polymerase Klenow fragment according to the above procedure for repair reaction of the termini.

(4) Construction of expression plasmid

The vector pKK233-2*(ampicillin resistance) used in the present example for constructing an expression plasmid is commercially available from Pharmacia. The vector is a member of the ATG vectors, which contains a Trc promoter and can be cleaved immediately after initiation codon ATG by digestion with restriction enzyme NcoI and filling of the 3′ recessed termini. As depicted in Fig. 9, the DNA fragment obtained in step (3) above, which contains the cephalosporin acetylhydrolase structural gene, was cleaved with PstI, and 1.27kb DNA fragment was separated and purified. On the other hand, pKK233-2 containing Trc promoter was cleaved with NcoI and treated with DNA polymerase Klenow fragment. The resulting fragment was then cleaved with PstI to obtain about 4.6kb DNA fragment. Subsequently, the above two fragments were mixed and ligated to each other with T4 DNA ligase, the resultant mixture was used to transform E. coli JM103 strain, and colonies formed on L-broth agar medium containing 40μg/ml ampicillin were selected. These colonies were liquid-cultured in L-broth overnight, cells were collected, plasmid DNA was extracted from the cells, and the base sequence near the junction of the ATG vector and the fragment containing cephalosporin acetylhydrolase gene was determined. As a result, an expression plasmid, in which the structural gene of cephalosporin acetylhydrolase excluding amino-terminal methionine had been inserted immediately after the ATG codon, was obtained and designated as pCAH21. Also, E. coli

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harbouring the expression plasmid was designated as *E. coli* JM103/pCAH21.

The replication system of the expression plasmid pCAH21 is derived from pBR322. Accordingly, in order to enhance the copy number of this plasmid to raise the expression level in *E. coli*, its replication region (ori) was changed to that derived from pAT153. Simultaneously, its drug resistance marker was changed from ampicillin resistance (Ap^r) to tetracycline resistance (Tc^r). A process for modifying the plasmid was depicted in Fig. 10. Plasmid pCAH21 was first cleaved with BamHI and treated with DNA polymerase Klenow fragment. Then, the resulting fragment was cleaved with ScaI to obtain about 2.4kb DNA fragment containing Trc promoter, cephalosporin acetylhydrolase gene and T_1T_2 terminator of 5S ribosomal RNA (5SrrnBT_1T_2). Commercially available plasmid pAT153 was used as the vector plasmid. Plasmid pAT153 was cleaved with EcoRI, treated with DNA polymerase Klenow fragment, and then cleaved with DraI. Furthermore, in order to prevent self-ligation of the vector, alkaline phosphatase treatment was conducted, and about 2.5kb DNA fragment containing the replication region and tetracycline resistance gene from pAT153 was prepared. Then, the above two DNA fragments were mixed and ligated with T4 DNA ligase, the resultant mixture was used to transform *E. coli* JM103 strain, and colonies formed on L-broth agar medium containing 20μg/ml tetracycline were selected. After these colonies were cultured in L-broth overnight, cells were collected, and the plasmid DNA was extracted from the cells and analyzed by restriction enzyme cleavage. As a result, two recombinant plasmids which differ in ligation orientation were obtained. One plasmid in which the orientation of the cephalosporin acetylhydrolase gene was identical with that of tetracycline resistance gene was designated as pCAH211 and another plasmid in which these genes were reversely inserted was designated as pCAH212. Also, *E. coli* strains harbouring these recombinant expression plasmids were designated as *E. coli* JM103/pCAH211 and *E. coli* JM103/pCAH212, respectively.

7. Expression of cephalosporin acetylhydrolase gene in *E. coli*

(1) Expression of cephalosporin acetylhydrolase gene *E. coli* JM103/pCAH211 or *E. coli* JM103/pCAH212 was inoculated on 2-fold concentration of L-broth (50ml) containing 20μg/ml tetracycline (in 0.5L volume flask) and cultured at 37°C for 24 hours with shaking. An aliquot (0.5ml) of the cultured fluid was centrifuged to collect cells. The cells were suspended in 0.1M phosphate buffer (pH 7.0)(0.5ml) and disrupted with an ultrasonicator. The supernatant obtained by centrifuging the solution was used as a sample solution containing the desired enzyme. On the other hand, the supernatant obtained by centrifuging the cultured fluid of *B. subtilis* (FERM BP-2755) obtained in step 1 above was used as an enzyme solution for comparison. Cephalosporin acetylhydrolase acts not only on cephalosporin C and 7-ACA but also on p-nitrophenyl acetate (hereafter abbreviated to pNPA) to form a coloured substance, p-nitrophenol (hereafter abbreviated to pNP). The pNP is detectable spectrophotometrically, and therefore, the procedure in which pNPA is used as a substrate was adopted as a simple determination method for cephalosporin acetyhydrolase activity. Reaction was carried out in a mixture (3ml) containing 0.02% pNPA, 0.1M phosphate buffer (pH 6.8) and the above-mentioned enzyme solution at 30°C, and the enzyme activity was determined by measuring absorbence at 400nm using a spectrophotometer. The amount of the enzyme producing 1μmole of pNP per minute under the condition of pH 6.8 and 30°C of temperature was defined as one unit(U). As a result, it was found that the enzyme activities per cultured fluid of *E. coli* JM103/pCAH211 and *E. coli* JM103/pCAH212 were 9.9U/ml and 12.4U/ml, respectively. On the other hand, the enzyme activity of *B. subtilis* was 0.36U/ml.

Furthermore, a plasmid was constructed in which Trc promoter and SD-ATG sequence of expression plasmid pCAH211 were replaced by Trp promoter and SD-ATG sequence derived from E. coli tryptophan operon. After the plasmid was introduced into E. coli JM109 by transformation, the resulting transformant was cultured in a similar manner as described above, and 75.5U/ml of enzyme activity per cultured fluid was obtained.

The amount of cephalosporin acetyhydrolase produced can be increased by growing E. coli harbouring these expression plasmids in a suitable medium under a suitable culture condition using a large scale culture apparatus, e.g. a jar-fermenter.

(2) Deacetylation of cephalosporin C and 7-ACA

Deacetylation by an enzyme solution from E. coli JM103/pCAH212 was carried out using cephalosporin C or 7-ACA as a substrate. The reaction was conducted at 37°C for 40 minutes after adding the enzyme solution (0.2ml) to 0.1M phosphate buffer (pH 7.0)(1.0ml) containing 10mM of the substrate, and terminated by addition of 0.2M acetate buffer (pH 4.0)(1.2ml). The resulting solution was subjected to HPLC, and deacetylcephalosporin C or deacetyl 7-ACA was measured. Cosmosil $5C_8$*(Nacalai tesque) was used as a column and the elution was carried out by concentration gradient elution method where methanol concentration was sequentially raised. Thus, using a solution containing 20mM NaH_2PO_4, and 5mM tetra-n-butylammonium hydroxide (TBAH), the methanol concentration was raised to 20%. Detection of the deacetylated products was carried out at 254nm. Activity of cephalosporin acetylhydrolase was defined as follows. The amount of the enzyme producing 1 μmole of the product per minute under the condition of pH 7.0 and a temperature of 37°C is defined as one unit(U). As a result, it was found that the activity per cultured fluid of the enzyme solution of E. coli JM103/pCAH212 was 7.4U/ml for both cephalosporin C and 7-ACA.

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(3) Structure of recombinant cephalosporin acetylhydrolase

Determination of molecular weights of the active form and subunit of cephalosporin acetylhydrolase produced in *E. coli* gave the same results as obtained in step 1 above, suggesting that the recombinant cephalosporin acetylhydrolase also exists in an octamer form similar to the natural form.

Furthermore, the recombinant cephalosporin acetylhydrolase was purified by HPLC using a reversed phase column. Terminal analysis by Edman degradation and hydrazine hydrolysis revealed that the amino and carboxy termini of the enzyme were methionine and glycine, respectively, and identical with those of natural form. In addition, determination of the amino terminal sequence using an automated amino acid sequencer revealed that the amino acid sequence to 25th amino acid was entirely identical with that deduced from the structural gene (Fig. 2).

As described in the above Example in detail, the present inventors have confirmed that a very efficient production of cephalosporin acetylhydrolase is possible by cloning a gene encoding cephalosporin acetylhydrolase produced by *B. subtilis* and constructing a recombinant plasmid containing the gene by the use of a vector expressible in *E. coli*. This provides a premise for extensive application of this enzyme. In addition, the DNA fragment containing the cloned cephalosporin acetylhydrolase gene provides an extremely powerful means of advantageously utilizing the function of cephalosporin acetylhydrolase.

Claims:

1. A DNA base sequence encoding the amino acid sequence depicted below:

Gln-Leu-Phe-Asp-Leu-Pro-Leu-Asp-Gln-Leu-Gln-Thr-Tyr-Lys-Pro-Glu-Lys-Thr-Ala-Pro-Lys-
Asp-Phe-Ser-Glu-Phe-Trp-Lys-Leu-Ser-Leu-Glu-Glu-Leu-Ala-Lys-Val-Gln-Ala-Glu-Pro-Asp-Leu-
Gln-Pro-Val-Asp-Tyr-Pro-Ala-Asp-Gly-Val-Lys-Val-Tyr-Arg-Leu-Thr-Tyr-Lys-Ser-Phe-Gly-Asn-
Ala-Arg-Ile-Thr-Gly-Trp-Tyr-Ala-Val-Pro-Asp-Lys-Gln-Gly-Pro-His-Pro-Ala-Ile-Val-Lys-Tyr-
His-Gly-Tyr-Asn-Ala-Ser-Tyr-Asp-Gly-Glu-Ile-His-Glu-Met-Val-Asn-Trp-Ala-Leu-His-Gly-Tyr-
Ala-Ala-Phe-Gly-Met-Leu-Val-Arg-Gly-Gln-Gln-Ser-Ser-Glu-Asp-Thr-Ser-Ile-Ser-Leu-His-Gly-
His-Ala-Leu-Gly-Trp-Met-Thr-Lys-Gly-Ile-Leu-Asp-Lys-Asp-Thr-Tyr-Tyr-Tyr-Arg-Gly-Val-Tyr-
Leu-Asp-Ala-Val-Arg-Ala-Leu-Glu-Val-Ile-Ser-Ser-Phe-Asp-Glu-Val-Asp-Glu-Thr-Arg-Ile-Gly-
Val-Thr-Gly-Gly-Ser-Gln-Gly-Gly-Gly-Leu-Thr-Ile-Ala-Ala-Ala-Ala-Leu-Ser-Asp-Ile-Pro-Lys-
Ala-Ala-Val-Ala-Asp-Tyr-Pro-Tyr-Leu-Ser-Asn-Phe-Glu-Arg-Ala-Ile-Asp-Val-Ala-Leu-Glu-Gln-
Pro-Tyr-Leu-Glu-Ile-Asn-Ser-Phe-Phe-Arg-Arg-Asn-Gly-Ser-Pro-Glu-Thr-Glu-Val-Gln-Ala-Met-
Lys-Thr-Leu-Ser-Tyr-Phe-Asp-Ile-Met-Asn-Leu-Ala-Asp-Arg-Val-Lys-Val-Pro-Val-Leu-Met-Ser-
Ile-Gly-Leu-Ile-Asp-Lys-Val-Thr-Pro-Pro-Ser-Thr-Val-Phe-Ala-Ala-Tyr-Asn-His-Leu-Glu-Thr-
Glu-Lys-Glu-Leu-Lys-Val-Tyr-Arg-Tyr-Phe-Gly-His-Glu-Tyr-Ile-Pro-Ala-Phe-Gln-Thr-Glu-Lys-
Leu-Ala-Phe-Phe-Lys-Gln-His-Leu-Lys-Gly.

2. A recombinant DNA molecule prepared by introducing the DNA base sequence defined in Claim 1 into a vector used in an E. coli host-vector system.

3. An E. coli cell transformed with the recombinant DNA molecule of Claim 2.

4. A protein having the amino acid sequence depicted in Claim 1.

5. A protein having the amino acid sequence further containing methionine at the amino-terminus of the amino acid sequence depicted in Claim 1.

6. A protein according to Claim 4 produced by the E. coli cell of Claim 3.

7. A protein according to Claim 5 produced by the E. coli cell of Claim 3.

8. An enzyme composed of a multimer of the protein of Claim 4.

9. An enzyme composed of a multimer of the protein of Claim 5.

10. An enzyme composed of a multimer of the protein of Claim 6.

11. An enzyme composed of a multimer of the protein of Claim 7.

12. An enzyme according to Claim 8 which is a tetramer or octamer of said protein.

13. An enzyme according to Claim 9 which is a tetramer or octamer of said protein.

14. An enzyme according to Claim 10 which is a tetramer or octamer of said protein.

15. An enzyme according to Claim 11 which is a tetramer or octamer of said protein.

16. A process for preparing the protein of Claim 4, characterized in that the *E. coli* cell of claim 3 is cultured in a medium and said protein is recovered from the cultured fluid.

17. A process for preparing the protein of Claim 5, characterized in that the *E. coli* cell of Claim 3 is cultured in a medium and said protein is recovered from the cultured fluid.

18. A process for preparing the enzyme of Claim 8, characterized in that the *E. coli* cell of Claim 3 is cultured in a medium and said enzyme is recovered from the cultured fluid.

19. A process for preparing the enzyme of claim 9, characterized in that the *E. coli* cell of Claim 3 is cultured in a medium and said enzyme is recovered from the cultured fluid.

20. A process for preparing the enzyme of Claim 12, characterized in that the *E. coli* cell of Claim 3 is cultured in a medium and said enzyme is recovered from the cultured fluid.

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21. A process for preparing the enzyme of Claim 13, characterized in that the *E. coli* cell of Claim 3 is cultured in a medium and said enzyme is recovered from the cultured fluid.

Gln-Leu-Phe-Asp-Leu-Pro-Leu-Asp-Gln-Leu-Gln-Thr-Tyr-Lys-Pro-Glu-Lys-Thr-Ala-Pro-Lys-
Asp-Phe-Ser-Glu-Phe-Trp-Lys-Leu-Ser-Leu-Glu-Glu-Leu-Ala-Lys-Val-Gln-Ala-Glu-Pro-Asp-Leu-
Gln-Pro-Val-Asp-Tyr-Pro-Ala-Asp-Gly-Val-Lys-Val-Tyr-Arg-Leu-Thr-Tyr-Lys-Ser-Phe-Gly-Asn-
Ala-Arg-Ile-Thr-Gly-Trp-Tyr-Ala-Val-Pro-Asp-Lys-Gln-Gly-Pro-His-Pro-Ala-Ile-Val-Lys-Tyr-
His-Gly-Tyr-Asn-Ala-Ser-Tyr-Asp-Gly-Glu-Ile-His-Glu-Met-Val-Asn-Trp-Ala-Leu-His-Gly-Tyr-
Ala-Ala-Phe-Gly-Met-Leu-Val-Arg-Gly-Gln-Gln-Ser-Ser-Glu-Asp-Thr-Ser-Ile-Ser-Leu-His-Gly-
His-Ala-Leu-Gly-Trp-Met-Thr-Lys-Gly-Ile-Leu-Asp-Lys-Asp-Thr-Tyr-Tyr-Tyr-Arg-Gly-Val-Tyr-
Leu-Asp-Ala-Val-Arg-Ala-Leu-Glu-Val-Ile-Ser-Ser-Phe-Asp-Glu-Val-Asp-Glu-Thr-Arg-Ile-Gly-
Val-Thr-Gly-Gly-Ser-Gln-Gly-Gly-Gly-Leu-Thr-Ile-Ala-Ala-Ala-Ala-Leu-Ser-Asp-Ile-Pro-Lys-
Ala-Ala-Val-Ala-Asp-Tyr-Pro-Tyr-Leu-Ser-Asn-Phe-Glu-Arg-Ala-Ile-Asp-Val-Ala-Leu-Glu-Gln-
Pro-Tyr-Leu-Glu-Ile-Asn-Ser-Phe-Phe-Arg-Arg-Asn-Gly-Ser-Pro-Glu-Thr-Glu-Val-Gln-Ala-Met-
Lys-Thr-Leu-Ser-Tyr-Phe-Asp-Ile-Met-Asn-Leu-Ala-Asp-Arg-Val-Lys-Val-Pro-Val-Leu-Met-Ser-
Ile-Gly-Leu-Ile-Asp-Lys-Val-Thr-Pro-Pro-Ser-Thr-Val-Phe-Ala-Ala-Tyr-Asn-His-Leu-Glu-Thr-
Glu-Lys-Glu-Leu-Lys-Val-Tyr-Arg-Tyr-Phe-Gly-His-Glu-Tyr-Ile-Pro-Ala-Phe-Gln-Thr-Glu-Lys-
Leu-Ala-Phe-Phe-Lys-Gln-His-Leu-Lys-Gly

Fig. 1

Fig. 2 (Part 1)

1 Met Gln Leu Phe Asp Leu Pro Leu Asp Gln Leu Gln Thr Tyr Lys Pro 16
1 ATG CAA CTA TTC GAT CTG CCG CTC GAC CAA TTG CAA ACA TAT AAG CCT 48

17 Glu Lys Thr Ala Pro Lys Asp Phe Ser Glu Phe Trp Lys Leu Ser Leu 32
49 GAA AAA ACA GCA CCG AAA GAT TTT TCT GAG TTT TGG AAA TTG TCT TTG 96

33 Glu Glu Leu Ala Lys Val Gln Ala Glu Pro Asp Leu Gln Pro Val Asp 48
97 GAG GAA CTT GCA AAA GTC CAA GCA GAA CCT GAT CTA CAG CCG GTT GAC 144

49 Tyr Pro Ala Asp Gly Val Lys Val Tyr Arg Leu Thr Tyr Lys Ser Phe 64
145 TAT CCT GCT GAC GGA GTA AAA GTG TAC CGT CTC ACA TAT AAA AGC TTC 192

65 Gly Asn Ala Arg Ile Thr Gly Trp Tyr Ala Val Pro Asp Lys Gln Gly 80
193 GGA AAC GCC CGC ATT ACC GGA TGG TAC GCG GTG CCT GAC AAG CAA GGC 240

81 Pro His Pro Ala Ile Val Lys Tyr His Gly Tyr Asn Ala Ser Tyr Asp 96
241 CCG CAT CCG GCG ATC GTG AAA TAT CAT GGC TAC AAT GCA AGC TAT GAT 288

97 Gly Glu Ile His Glu Met Val Asn Trp Ala Leu His Gly Tyr Ala Ala 112
289 GGT GAG ATT CAT GAA ATG GTA AAC TGG GCA CTC CAT GGC TAC GCC GCA 336

113 Phe Gly Met Leu Val Arg Gly Gln Gln Ser Ser Glu Asp Thr Ser Ile 128
337 TTC GGC ATG CTT GTC CGC GGC CAG CAG AGC AGC GAG GAT ACG AGT ATT 384

129 Ser Leu His Gly His Ala Leu Gly Trp Met Thr Lys Gly Ile Leu Asp 144
385 TCA CTG CAC GGT CAT GCT TTG GGC TGG ATG ACG AAA GGA ATT CTT GAT 432

145 Lys Asp Thr Tyr Tyr Tyr Arg Gly Val Tyr Leu Asp Ala Val Arg Ala 160
433 AAA GAT ACA TAC TAT TAC CGC GGT GTT TAT TTG GAC GCC GTC CGC GCG 480

Fig. 2 (Part 2)

```
161  Leu Glu Val Ile Ser Ser Phe Asp Glu Val Asp Glu Thr Arg Ile Gly  176
481  CTT GAG GTC ATC AGC AGC TTC GAC GAG GTT GAC GAA ACA AGG ATC GGT  528

177  Val Thr Gly Gly Ser Gln Gly Gly Gly Leu Thr Ile Ala Ala Ala Ala  192
529  GTG ACA GGA GGA AGC CAA GGC GGA GGT TTA ACC ATT GCC GCA GCA GCG  576

193  Leu Ser Asp Ile Pro Lys Ala Ala Val Ala Asp Tyr Pro Tyr Leu Ser  208
577  CTG TCA GAC ATT CCA AAA GCC GCG GTT GCC GAT TAT CCT TAT TTA AGC  624

209  Asn Phe Glu Arg Ala Ile Asp Val Ala Leu Glu Gln Pro Tyr Leu Glu  224
625  AAC TTC GAA CGG GCC ATT GAT GTG GCG CTT GAA CAG CCG TAC CTT GAA  672

225  Ile Asn Ser Phe Phe Arg Arg Asn Gly Ser Pro Glu Thr Glu Val Gln  240
673  ATC AAT TCC TTC TTC AGA AGA AAT GGC AGC CCG GAA ACA GAA GTG CAG  720

241  Ala Met Lys Thr Leu Ser Tyr Phe Asp Ile Met Asn Leu Ala Asp Arg  256
721  GCG ATG AAG ACA CTT TCA TAT TTC GAT ATT ATG AAT CTC GCT GAC CGA  768

257  Val Lys Val Pro Val Leu Met Ser Ile Gly Leu Ile Asp Lys Val Thr  272
769  GTG AAG GTG CCT GTC CTG ATG TCA ATC GGC CTG ATT GAC AAG GTC ACG  816

273  Pro Pro Ser Thr Val Phe Ala Ala Tyr Asn His Leu Glu Thr Glu Lys  288
817  CCG CCG TCC ACC GTG TTT GCC GCC TAC AAT CAT TTG GAA ACA GAG AAA  864

289  Glu Leu Lys Val Tyr Arg Tyr Phe Gly His Glu Tyr Ile Pro Ala Phe  304
865  GAG CTG AAG GTG TAC CGC TAC TTC GGA CAT GAG TAT ATC CCT GCT TTT  912

305  Gln Thr Glu Lys Leu Ala Phe Phe Lys Gln His Leu Lys Gly ***      319
913  CAA ACG GAA AAA CTT GCT TTC TTT AAG CAG CAT CTT AAA GGC TGA      957
```

Fig. 3

Tyr-His-Gly-Tyr-Asn-Ala-Ser-Tyr-Asp-Gly-
Glu-Ile-His-Glu-Met-Val-Asn-Trp-Ala-Leu-
His-Gly-Tyr-Ala-Ala-Phe-Gly-Met-Leu-Val-
X -Gly-Gln-Gln-

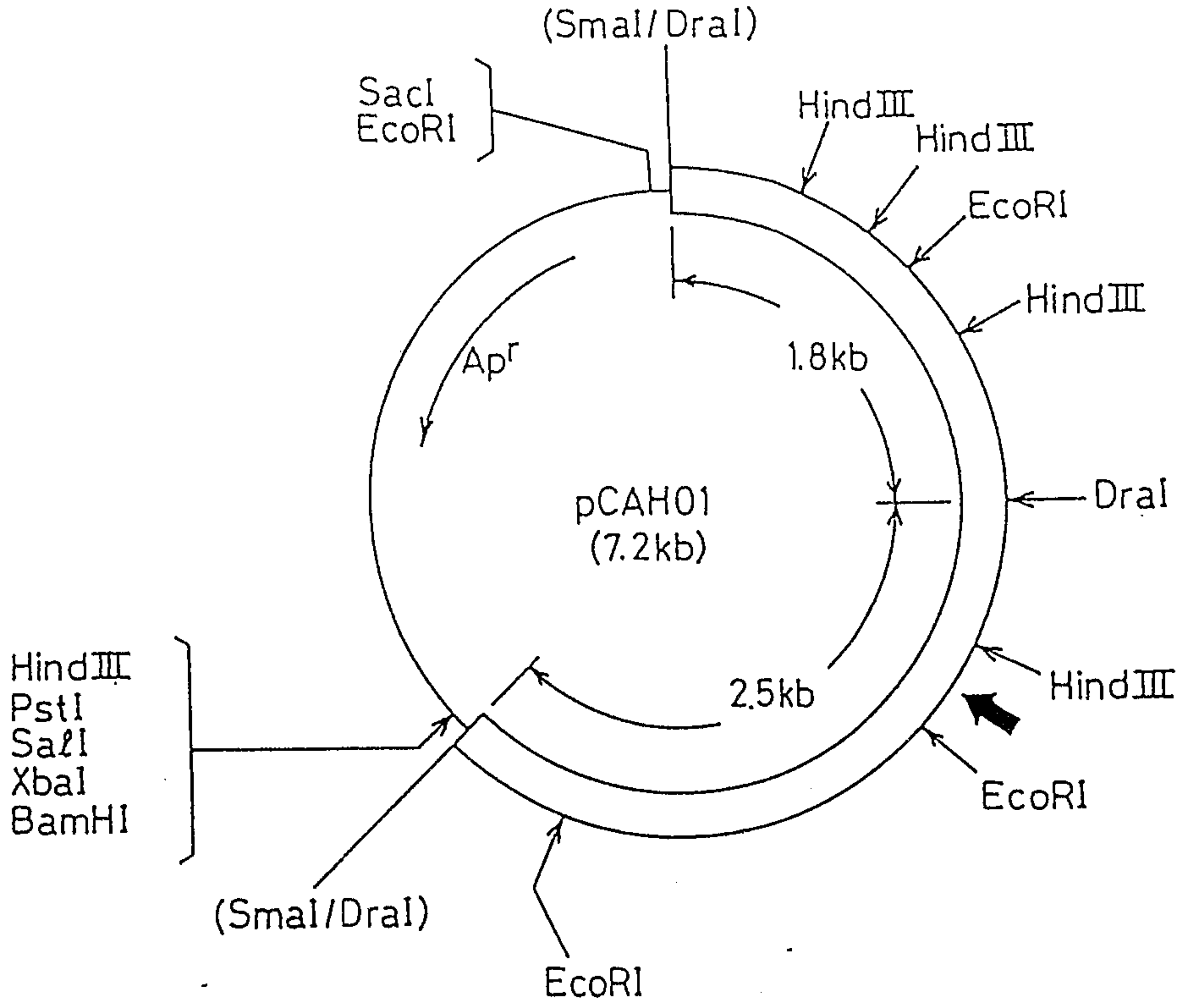
X : not identified

underlined part : sequence used for probe synthesis

amino acid sequence			- G l u -		M e t -	V a l -		A s n -	T r p -	A l a -	
base sequence		5' -	G A	G A	A T G	G T	T A C G	A A	T C	T G G	G C	- 3'
		3' -	C T	C T	T A C	C A	A T G C	T T	A G	A C C	C G	- 5'
synthetic probe	CAH-RM1	3'	C T	C T	T A C	C A	A	T T	A G	A C C	C G	5'
	CAH-RM2	3'	C T	C T	T A C	C A	T	T T	A G	A C C	C G	5'
	CAH-RM3	3'	C T	C T	T A C	C A	G	T T	A G	A C C	C G	5'
	CAH-RM4	3'	C T	C T	T A C	C A	C	T T	A G	A C C	C G	5'

F i g. 4

Fig. 5

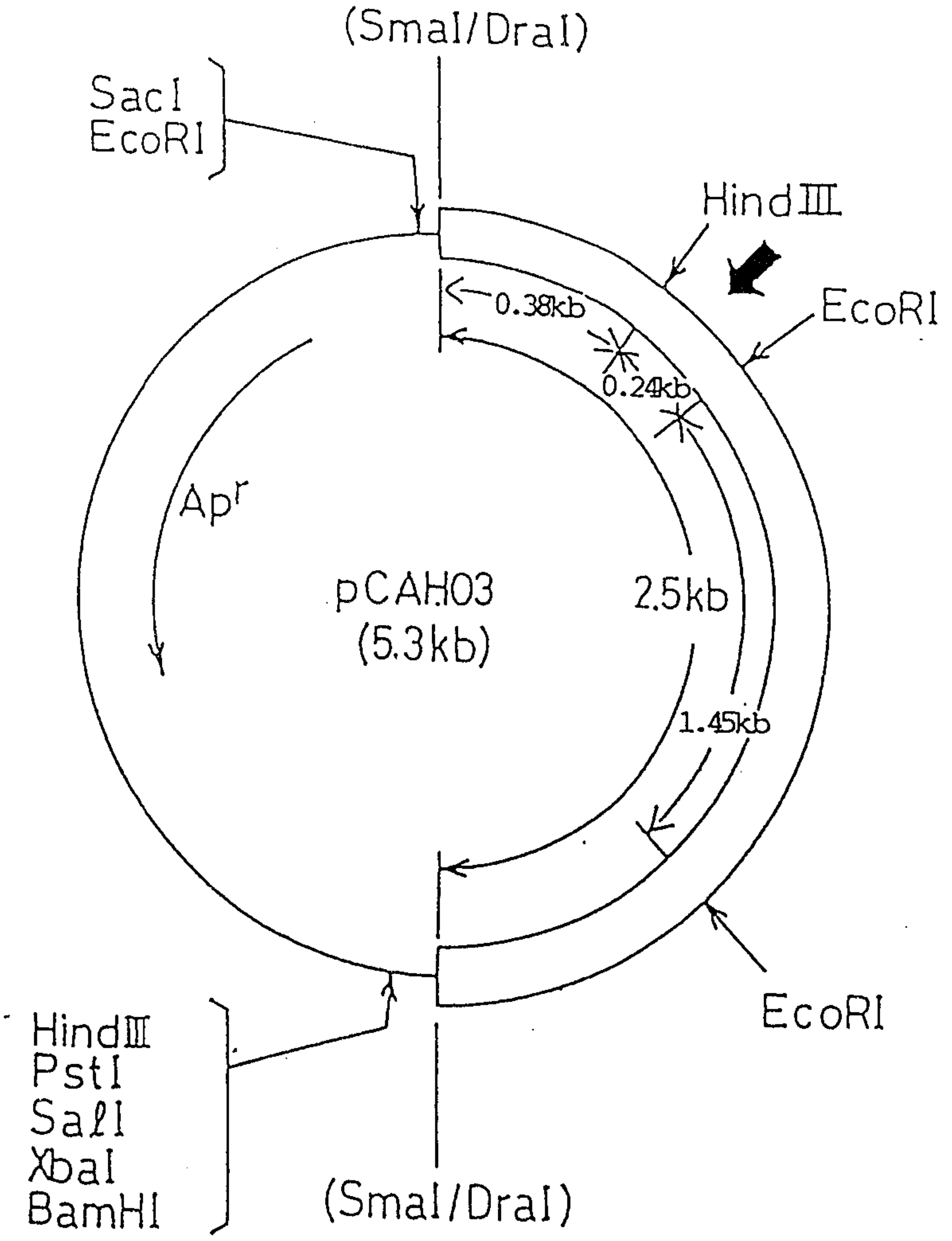


▭ inserted DNA fragment (from B. subtilis FERM BP-2755)

— pUC13

⬅ position at which probe is hybridized

Fig. 6



▭ inserted DNA fragment (from B. subtilis FERM BP-2755)

— pUC13

⬅ position at which probe is hybridized

Fig. 7 (Part 1)

```
(DraI)  10        20        30        40        50        60        70        80        90
AAAGAACCGCTATGTCAGTCTGACGGCCCAGGCCTTTATGGAACTAAGCCGGGAAAGTCTTAAACAAACGTTTGATGAAGGCTGTCTGGG

AAACAAAGATGAAAATATTTAGAAAACAAAGACGAAACGTGGTAGTATAGGAATACAAACTAAATCTTATAAAACAAAGGGGAATAATCG

GAAATGCAACTATTCGATCTGCCGCTCGACCAATTGCAAACATATAAGCCTGAAAAAACAGCACCGAAAGATTTTTCTGAGTTTTGGAAA
   MetGlnLeuPheAspLeuProLeuAspGlnLeuGlnThrTyrLysProGluLysThrAlaProLysAspPheSerGluPheTrpLys

TTGTCTTTGGAGGAACTTGCAAAAGTCCAAGCAGAACCTGATCTACAGCCGGTTGACTATCCTGCTGACGGAGTAAAAGTGTACCGTCTC
LeuSerLeuGluGluLeuAlaLysValGlnAlaGluProAspLeuGlnProValAspTyrProAlaAspGlyValLysValTyrArgLeu

ACATATAAAAGCTTCGGAAACGCCCGCATTACCGGATGGTACGCGGTGCCTGACAAGCAAGGCCCGCATCCGGCGATCGTGAAATATCAT
ThrTyrLysSerPheGlyAsnAlaArgIleThrGlyTrpTyrAlaValProAspLysGlnGlyProHisProAlaIleValLysTyrHis

GGCTACAATGCAAGCTATGATGGTGAGATTCATGAAATGGTAAACTGGGCACTCCATGGCTACGCCGCATTCGGCATGCTTGTCCGCGGC
GlyTyrAsnAlaSerTyrAspGlyGluIleHisGluMetValAsnTrpAlaLeuHisGlyTyrAlaAlaPheGlyMetLeuValArgGly

CAGCAGAGCAGCGAGGATACGAGTATTTCACTGCACGGTCATGCTTTGGGCTGGATGACGAAAGGAATTCTTGATAAAGATACATACTAT
GlnGlnSerSerGluAspThrSerIleSerLeuHisGlyHisAlaLeuGlyTrpMetThrLysGlyIleLeuAspLysAspThrTyrTyr

TACCGCGGTGTTTATTTGGACGCCGTCCGCGCGCTTGAGGTCATCAGCAGCTTCGACGAGGTTGACGAAACAAGGATCGGTGTGACAGGA
TyrArgGlyValTyrLeuAspAlaValArgAlaLeuGluValIleSerSerPheAspGluValAspGluThrArgIleGlyValThrGly

GGAAGCCAAGGCGGAGGTTTAACCATTGCCGCAGCAGCGCTGTCAGACATTCCAAAAGCCGCGGTTGCCGATTATCCTTATTTAAGCAAC
GlySerGlnGlyGlyGlyLeuThrIleAlaAlaAlaAlaLeuSerAspIleProLysAlaAlaValAlaAspTyrProTyrLeuSerAsn
```

Fig. 7 (Part 2)

TTCGAACGGGCCATTGATGTGGCGCTTGAACAGCCGTACCTTGAAATCAATTCCTTCTTCAGAAGAAATGGCAGCCCGGAAACAGAAGTG
PheGluArgAlaIleAspValAlaLeuGluGlnProTyrLeuGluIleAsnSerPhePheArgArgAsnGlySerProGluThrGluVal

CAGGCGATGAAGACACTTTCATATTTCGATATTATGAATCTCGCTGACCGAGTGAAGGTGCCTGTCCTGATGTCAATCGGCCTGATTGAC
GlnAlaMetLysThrLeuSerTyrPheAspIleMetAsnLeuAlaAspArgValLysValProValLeuMetSerIleGlyLeuIleAsp

AAGGTCACGCCGCCGTCCACCGTGTTTGCCGCCTACAATCATTTGGAAACAGAGAAAGAGCTGAAGGTGTACCGCTACTTCGGACATGAG
LysValThrProProSerThrValPheAlaAlaTyrAsnHisLeuGluThrGluLysGluLeuLysValTyrArgTyrPheGlyHisGlu

TATATCCCTGCTTTTCAAACGGAAAAACTTGCTTTCTTTAAGCAGCATCTTAAAGGCTGATAAATGTGAAAAGCCGCCGCATATCATCAG
TyrIleProAlaPheGlnThrGluLysLeuAlaPhePheLysGlnHisLeuLysGly***

GCGGTTTTTTTCTGCAAACTGCCGGAATGAGAACAGACTGGAGACGAATAGATATGAAACAAAGAATCATTAATGAATTAAAACGGATCG
AGCAGTCATACGGAGTCAAAATCGTGTATGCCGTCGAGTCAGGAAGCCGCGCATGGGGATTTCCCTCGCAGGATAGTGATTACGACGTCC
GCTTTATTTATGTGCCGAAAAAGGAGTGGTACTTTTCAATTGAGCAGGAGCGTGATGTCATTGAGGAACCGATTCACGATTTGCTGGATA
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CCTTGCTTGAACGGAAAAGAAGAGGGGAAGAGATTGACCTCGAATCAAAGAACTGATGTAATTCACCAATTCATTGAAACGGAAATCGAA
AGAATCATGGAAGCACAAAAAGAACTGAAGGCAGAGAAAAAAGATATGACATCTGAATTGAACCGTTTACTTTTGAATACGGTTGAAGAA
GTGTGGAAGGATGGAGGAAGCTGATGTTTTTGTCGCTTCCTTTTCTCCTTTATTCGACAGAATTC
EcoRI

＊＊＊: termination codon

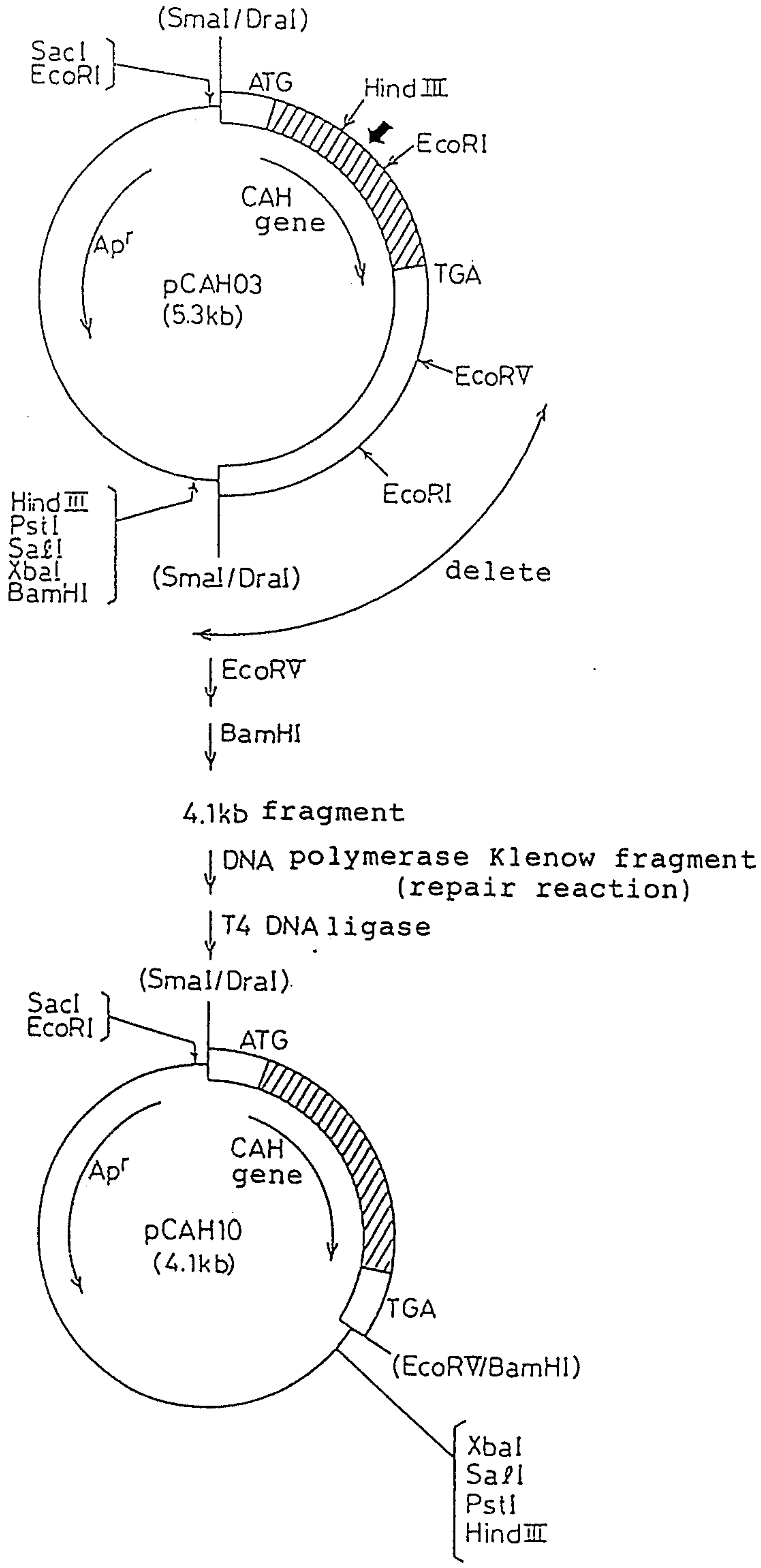
(SmaI/DraI)
SacI
EcoRI
ATG
HindIII
EcoRI
CAH
gene
Apr
pCAH03
(5.3kb)
TGA
EcoRV
EcoRI
HindIII
PstI
SalI
XbaI
BamHI
(SmaI/DraI)
delete
EcoRV
BamHI
4.1kb fragment
DNA polymerase Klenow fragment
(repair reaction)
T4 DNA ligase
(SmaI/DraI)
SacI
EcoRI
ATG
CAH
gene
Apr
pCAH10
(4.1kb)
TGA
(EcoRV/BamHI)
XbaI
SalI
PstI
HindIII

Fig. 8

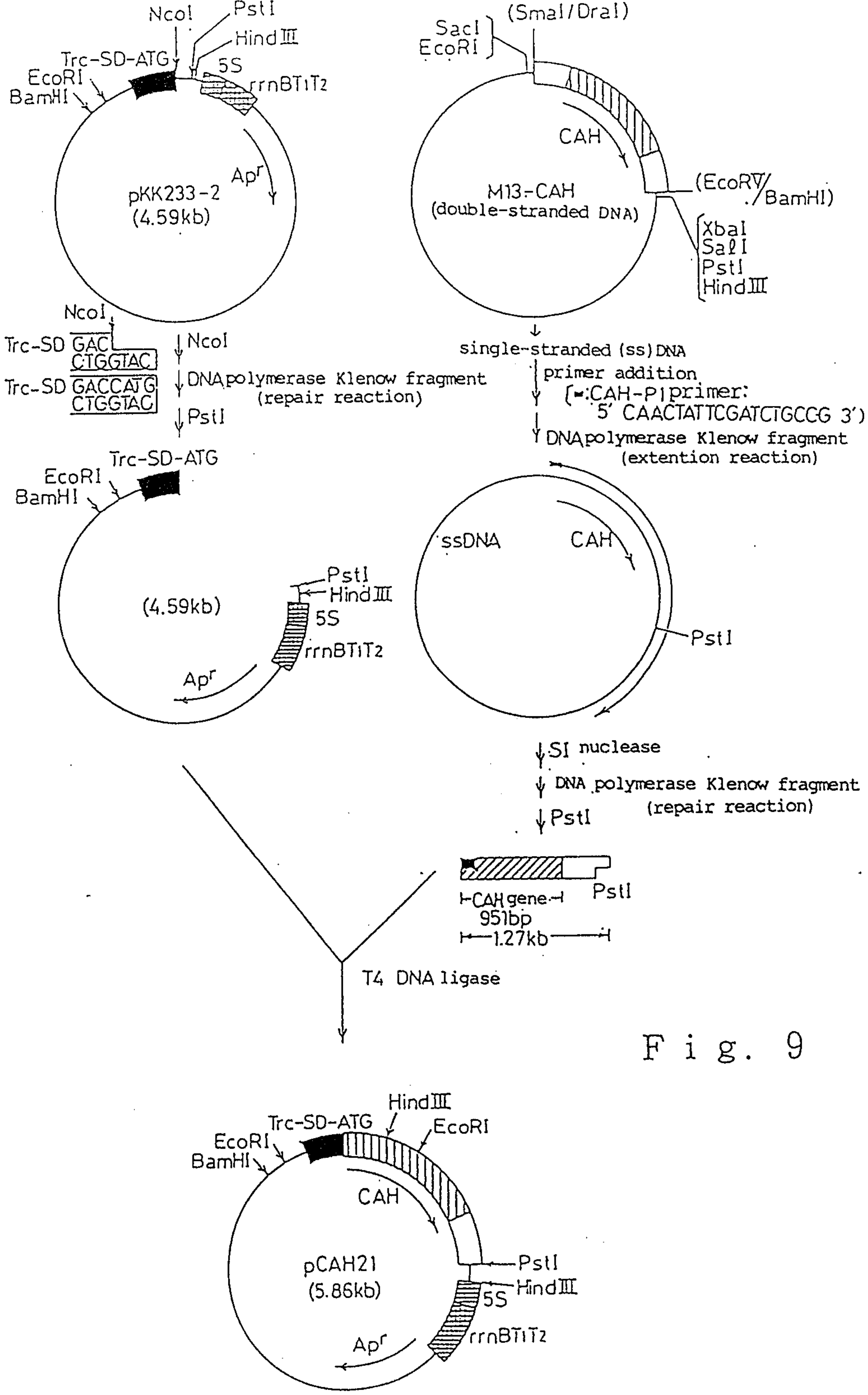
NcoI
PstI
HindIII
Trc-SD-ATG
5S
EcoRI
rrnBT1T2
BamHI
Apr
pKK233-2
(4.59kb)
SacI
EcoRI
(SmaI/DraI)
CAH
M13-CAH
(double-stranded DNA)
(EcoRV/BamHI)
XbaI
SalI
PstI
HindIII
NcoI
Trc-SD GAC
CTGGTAC
Trc-SD GACCATG
CTGGTAC
NcoI
DNA polymerase Klenow fragment
(repair reaction)
PstI
single-stranded (ss) DNA
primer addition
(←:CAH-P1 primer:
5' CAACTATTCGATCTGCCG 3')
DNA polymerase Klenow fragment
(extention reaction)
Trc-SD-ATG
EcoRI
BamHI
(4.59kb)
PstI
HindIII
5S
rrnBT1T2
Apr
ssDNA
CAH
PstI
S1 nuclease
DNA polymerase Klenow fragment
(repair reaction)
PstI
CAH gene
951bp
1.27kb
PstI
T4 DNA ligase
HindIII
Trc-SD-ATG
EcoRI
EcoRI
BamHI
CAH
pCAH21
(5.86kb)
PstI
HindIII
5S
Apr
rrnBT1T2

Fig. 9

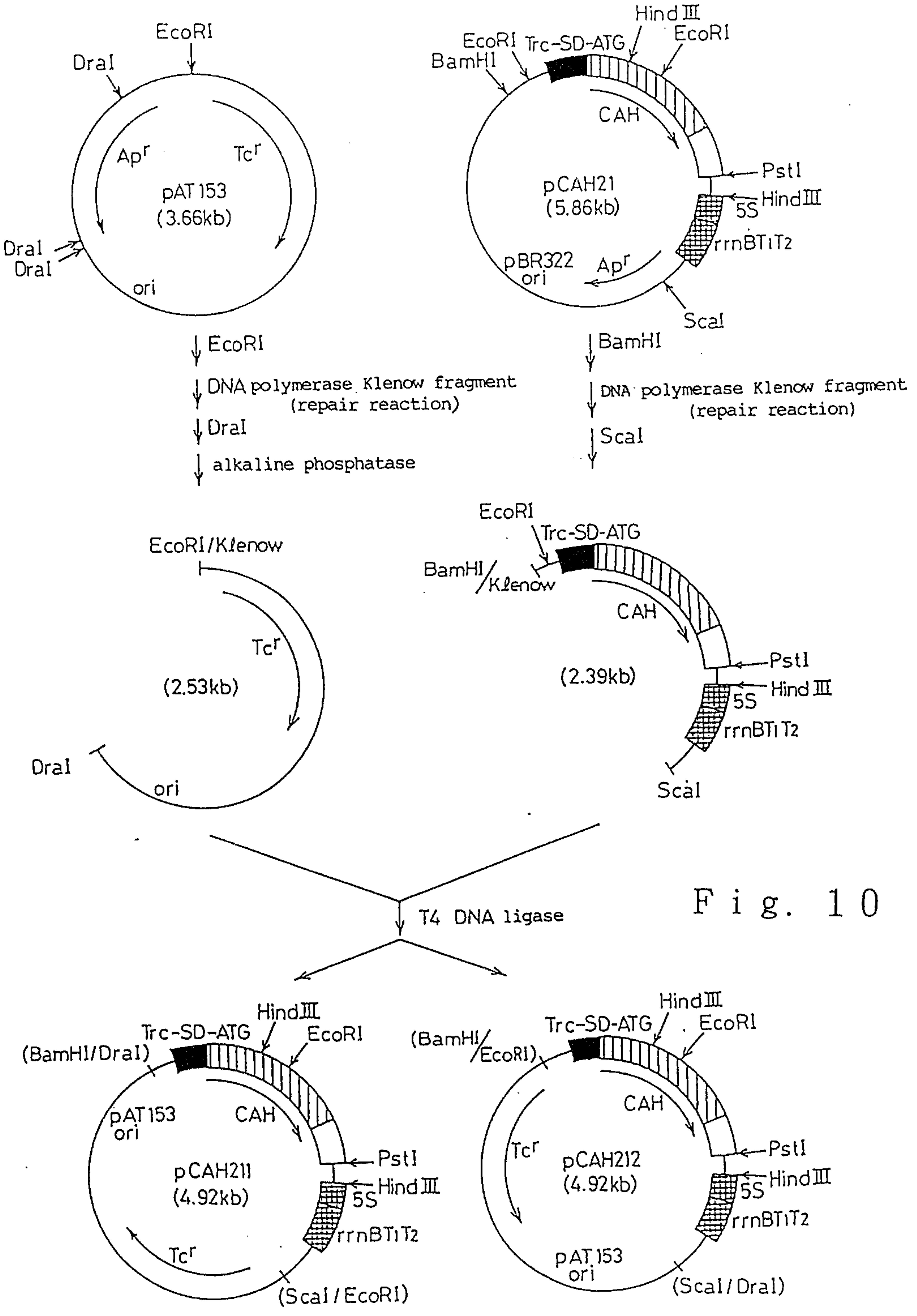
EcoRI
DraI
Apr
Tcr
pAT153
(3.66kb)
DraI
DraI
ori
HindIII
EcoRI
Trc-SD-ATG
EcoRI
BamHI
CAH
PstI
pCAH21
(5.86kb)
5S
HindIII
rrnBT1T2
pBR322
ori
Apr
ScaI
EcoRI
DNA polymerase Klenow fragment
(repair reaction)
DraI
alkaline phosphatase
BamHI
DNA polymerase Klenow fragment
(repair reaction)
ScaI
EcoRI/Klenow
Tcr
(2.53kb)
DraI
ori
EcoRI
Trc-SD-ATG
BamHI/Klenow
CAH
(2.39kb)
PstI
HindIII
5S
rrnBT1T2
ScaI
T4 DNA ligase
HindIII
Trc-SD-ATG
EcoRI
(BamHI/DraI)
pAT153
ori
CAH
PstI
pCAH211
(4.92kb)
HindIII
5S
rrnBT1T2
Tcr
(ScaI/EcoRI)
HindIII
Trc-SD-ATG
EcoRI
(BamHI/EcoRI)
CAH
Tcr
pCAH212
(4.92kb)
PstI
HindIII
5S
rrnBT1T2
pAT153
ori
(ScaI/DraI)

Fig. 10