



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: PCT/GB93/01378 (22) International Filing Date: 30 June 1993 (30.06.93) (30) Priority data: 9213855.1 30 June 1992 (30.06.92) GB 9213857.7 30 June 1992 (30.06.92) GB (71) Applicants (for all designated States except US): SMITH-KLINE BEECHAM P.L.C. [GB/GB]; New Horizons Court, Brentford, Middlesex TW8 9EP (GB). SMITH-KLINE BEECHAM CORPORATION [US/US]; One Franklin Plaza, P.O. Box 7929, Philadelphia, PA 19103 (US). (72) Inventors; and (75) Inventors/Applicants (for US only) : NEAL, Robert, John [GB/GB]; GRIFFIN, Alison, Michelle [GB/GB]; SmithKline Beecham Pharmaceuticals, Clarendon Road, Worthing, West Sussex BN14 8QH (GB). SCOTT, Miller, O'Neill [US/US]; SHATZMAN, Allan, Richard [US/US]; SmithKline Beecham, 709 Swedeland Road, King of Prussia, PA 19406 (US). GORHAM, Hazel, Claire [GB/GB]; Institute of Virology and Environmental Microbiology, Mansfield Road, Oxford OX1 3SR (GB).</p>		<p>(74) Agent: TYRRELL, Arthur, William, Russell; SmithKline Beecham, Corporate Intellectual Property, Great Burgh, Yew Tree Bottom Road, Epsom, Surrey KT18 5XQ (GB). (81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: D-N-CARBAMOYL-AMINO ACID AMIDOHYDROLASE AND HYDANTOINASE</p>		
<p>(57) Abstract</p> <p>Processes are provided for the production of a carbamoylase enzyme which has the capability of converting a D-N-carbamoyl (optionally substituted phenyl) glycine into the corresponding D-(optionally substituted phenyl) glycine by expressing recombinant DNA encoding a carbamoylase gene in a homologous host. Also provided are specific recombinant DNA vectors producing high levels of expression of the carbamoylase enzyme and/or a hydantoinase enzyme in homologous and heterologous hosts and their use in the production of D-α amino acids.</p>		

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D-N-CARBAMOYL-AMINO ACID AMIDOHYDROLASE AND HYDANTOINASE

The present invention relates to DNA molecules and to recombinant vectors for use in the transformation of a microbial host. In particular, the invention relates to DNA
5 encoding a carbamoylase enzyme and to hosts transformed with recombinant vectors comprising the said DNA.

Certain D- α -amino acids are well known pharmaceutical intermediates. For example D(-) p-hydroxyphenylglycine is used as a starting material for the production of the antibiotic amoxycillin. Processes for the preparation of D- α -amino acids are
10 therefore of importance and several enzymatic processes enabling production of D-product from racemic starting material have been reported.

U.K. patents 1,534,426 and 1,564,982, for example, disclose hydantoinase producing organisms which will hydrolyse 5-(substituted phenyl)hydantoins to give intermediate D-N-carbamoyl-(substituted phenyl)glycines. However, to obtain the
15 corresponding D- α -amino acid it is necessary subsequently to hydrolyse the N-carbamoyl compounds formed, for example with nitrous acid.

To overcome the problem of having a first enzymatic and a second chemical step to prepare D- α -amino acids from hydantoin starting material it is possible to convert the N-carbamoyl intermediate into the α -amino acid enzymatically with a
20 carbamoylase enzyme produced by a micro-organism, e.g. an *Agrobacterium* species as described in U.K. Patent No. 2,022,581. A drawback with *Agrobacterium* NRRL B11291 described in U.K. Patent No. 2,022,581, however, is that only limited amounts of carbamoylase enzyme are produced. In addition, the organism exhibits other enzyme activity which may be undesirable in certain circumstances.

PCT application WO 92/10579 (Kanegafuchi) discloses the production of D α amino acids using bacteria transformed with recombinant DNA encoding a carbamoylase enzyme which converts D-N- carbamoyl- α -amino acid to a D- α amino acid.

We have found that surprisingly higher levels of enzyme activity can be
30 obtained when a carbamoylase gene is expressed in a homologous host. By 'homologous host' we mean that the host is the same type of organism from which the gene was originally isolated.

Accordingly the present invention provides a process for the production of a carbamoylase enzyme which has the capability of converting a D-N-carbamoyl
35 (optionally substituted phenyl)glycine into the corresponding D-(optionally substituted phenyl)glycine by expressing recombinant DNA encoding a carbamoylase gene in a homologous host.

Preferably the homologous host is *Agrobacterium* ie the carbamoylase gene is derived from *Agrobacterium*.

We have additionally found that particular constructs lead to high levels of expression in both heterologous and homologous hosts.

Accordingly in a further aspect of the present invention there is also provided a recombinant DNA vector comprising a gene encoding a carbamoylase enzyme
5 which has the capability of converting a D- N-carbamoyl (optionally substituted phenyl)glycine into the corresponding D- (optionally substituted phenyl)glycine wherein the DNA vector is selected from the group consisting of pCAR1, pCAR6, pCAR12, pCAR21, pCAR26, pCAR 27, pCAR28, pCAR29, pGal2789RS3Carb, pCAR31, pCAR32, pCAR36, pCAR44 and pCAR46 as herein described.

10 The advantage of the invention is that particularly large quantities of the carbamoylase enzyme can be produced. Under suitable conditions active carbamoylase enzyme can thus be obtained and used, preferably immobilised on a solid support, to prepare the chosen D- α -amino acid.

Suitable substituents for optionally substituted phenyl groups as described
15 herein include hydroxy, C₍₁₋₆₎alkyl, C₍₁₋₆₎ alkoxy and halogen. Preferred (substituted phenyl)glycines include (p-hydroxyphenyl)glycine and (3,4-dihydroxyphenyl)glycine, especially (p-hydroxyphenyl)glycine.

The carbamoylase gene may be isolated, as described hereinbelow, from total or chromosomal DNA of organisms which produce a carbamoylase enzyme having
20 the said capability.

The carbamoylase gene may comprise further genes, for example regulatory elements, or flanking DNA which has no particular or known function.

In a related aspect the invention provides DNA comprising a gene encoding a hydantoinase enzyme which has the ability to convert a D,L- (optionally substituted
25 phenyl) hydantoin into the corresponding D-N-carbamoyl (optionally substituted phenyl) glycine.

It will of course, be understood that the DNA of the invention has been separated from the majority of such chromosomal DNA and is not in its 'natural' state, i.e. the form in which it occurs in nature. In one aspect, the DNA of the invention is
30 in isolated and substantially purified form and/or consists essentially of a hydantoinase gene encoding the said hydantoinase enzyme.

In a further aspect the invention provides recombinant DNA comprising the DNA of the invention.

Preferably the recombinant DNA encoding the hydantoinase gene comprises a
35 recombinant vector, more preferably a high expression vector capable of expressing high levels of gene transcript.

The invention further provides a process for transforming a host cell with a recombinant vector encoding the carbamoylase or hydantoinase enzyme according to the invention which comprises mixing together the host and recombinant vector under

conventional transformation or electroporation conditions.

In another aspect of the invention there is provided a host cell transformed with a recombinant vector according to the invention. Suitable hosts include *Agrobacterium* and *E. coli.*, for example *E. coli* DH1, *E. coli* JM 101 and *E. coli* HB 101.

The invention also encompasses culturing the transformed host according to the invention so that production of the carbamoylase or hydantoinase enzyme takes place.

In a further aspect of the invention there is provided isolated and recombinant DNA encoding a gene for a carbamoylase enzyme and a gene for a hydantoinase enzyme. Culture of a suitable transformed host can then produce both carbamoylase and hydantoinase activity.

Advantageous results are obtained when the carbamoylase and hydantoinase activity are provided in the same construct for example as in pCAR1, pCAR6, pCAR31, PCAR32 and pCAR36.

In one aspect the DNA of the invention is obtained from a microorganism, preferably from a soil isolate designated '80/44-2A' as described herein. The isolate '80/44-2A' is believed to be an *Agrobacterium* and whilst we do not wish to be bound by this designation the strain will herein be referred to as *Agrobacterium* 80/44-2A. DNA obtained therefrom will similarly be referred to as *Agrobacterium* DNA.

In order to define the invention more clearly reference is made to the accompanying drawings in which:

Figure 1 shows a restriction site and function map of plasmid pCP19;

Abbn:

B = BglII, Ba = BamHI, E = EcoRI, H = HindIII, P = Pst I, C = Cla I, S = Sal I,

Tc^R = Tetracycline resistance gene,

cos = cos site from bacteriophage lambda,

Solid line represents DNA from pCP19;

Figure 2 shows the reaction sequence for conversion of N-carbamoyl-p-hydroxyglycine to D(-)p-hydroxyphenylglycine;

Figure 3 shows a restriction site and function map of plasmid pCAR1;

Abbn:

C = Cla I, B = Bgl II, Ba = Bam HI, E = EcoRI, H = Hind III,

P = Pst I, (Only E,H, and P. enzymes have been used for mapping of the

Agrobacterium DNA), S = Sal I, Tc^R = Tetracycline resistance gene,

cos = cos site from bacteriophage lambda,

Solid line represents DNA from pCP19,

Shaded portion represents DNA from Agrobacterium BO/44-2A

Figure 4 shows a restriction site and function map of plasmid pCAR6;

Abbn:

C = Cla I, B = Bgl II, Ba = Bam HI, H = Hind III, E = EcoRI,

P = Pst I (Only E,H, and P. enzymes have been used mapping the insert in this

plasmid (and Cla I), S = Sal I,

Solid line represents DNA from pCP19,

Shaded portion represents DNA from Agrobacterium

Figure 5 shows a restriction site and function map of plasmid pIJ2925;

Abbn:

Ap^R = ampicillin resistance

Figure 6 shows a restriction site and function map of plasmid pCAR12;

In the regions marked "polylinker", not all of the restriction sites in the pIJ2925 DNA are shown.

Abbn:

Non-shaded portion represents pCP19 DNA (cloned from pCAR6 together with Agrobacterium DNA),

Shaded portion represents Agrobacterium DNA,

Solid line represents pIJ2925 DNA

Figure 7 shows a restriction site and function map of plasmid pCAR21;

In the regions marked "polylinker", not all of the restriction sites in the pIJ2925 DNA are shown.

Abbn:

Non-shaded portion represents pCP19 DNA (cloned from pCAR12 together with Agrobacterium DNA),

Shaded portion represents Agrobacterium DNA,

Solid line represents pIJ2925 DNA

Figure 8 shows a restriction site and function map of plasmid pCAR27;

Abbn:

Solid line represents M13mp 19 DNA,
Non-shaded portion represents pl J2925 DNA,
Shaded portion represents Agrobacterium DNA

Figure 9 shows a restriction site and function map of plasmid pCAR28;

Abbn:

Solid line represents M13mp 19 DNA,
Non-shaded portion represents pl J2925 DNA,
Shaded portion represents Agrobacterium DNA

Figure 10 shows the sequence of the *Agrobacterium* DNA from pCAR27 and pCAR28. Only the "sense" or coding strand of the DNA molecule is shown. The DNA strand is shown 5' → 3' from left to right. The nucleotide sequence is numbered immediately below the sequence. The amino acid sequence (using the three-letter code) of the carbamoylase encoded by the DNA is shown above each line of the nucleotide sequence;

Figure 11 shows a restriction site and function map of pTR550;

Abbn:

rrnB term. represents the transcriptional terminator from a ribosomal RNA operon,
P_{tac} represents the tac promoter of transcription

Figure 12 shows a restriction site and function map of pCAR29;

Abbn:

Solid line represents pTR550 DNA,

Shaded portion represents Agrobacterium DNA

Figure 13 shows a restriction site and function map of pKT210;

Abbn:

A = AccI, E = EcoRI, H = HindIII, Pf = PfIMI, P = Pst I, S = Sal I

Figure 14 shows a restriction site and function map of pCAR26;

Abbn:

A = AccI, B = Bgl II, Ba = Bam HI, E = EcoRI, H = Hind III, P = Pst I, Pf = PfIMI,

S = Sst I, Sa = SalI, Sp = SphI, Sm = SmaI, K = KpnI,

Shaded portion represents Agrobacterium DNA,

Arrow represents carbamoylase coding sequence

The vector DNA has not been mapped for all the enzymes that cut in the polylinker or Agrobacterium DNA. Not all of the Agrobacterium DNA has been mapped for AccI and PfIMI

Figure 15 shows a restriction site and function map of pWOR901;

Abbn:

A = AccI, E = EcoRI, H = Hind III, Pf = PfIMI, P = Pst I, S = Sst I

Figure 16 shows a restriction site and function map of pWOR902;

Abbn:

A = AccI, E = EcoRI, H = Hind III, Pf = PfIMI (no sites in pWOR902), P = Pst I,

S = Sst I;

Figure 17 shows a restriction site and function map of pCAR44;

Abbn:

A = AccI, B = Bgl II, Ba = Bam HI, E = EcoRI, H = Hind III, P = Pst I

S = Sst I, Sa = SalI, Sp = SphI, X = XbaI,

Shaded portion represents Agrobacterium DNA,

Arrow represents carbamoylase coding sequence.

The vector DNA has not been mapped for all the enzymes that cut in the polylinker or Agrobacterium DNA.

Figure 18 shows a restriction site and function map of pWOR903;

Abbn:

A = AvaI, E = EcoRV, P = Pst I, S = Sst I

Figure 19 shows a restriction site and function map of pWOR904;

Abbn:

A = AccI, B = Bal II, Ba = Bam HI, E = EcoRI, H = Hind III, P = Pst I, Sm = SmaI,
S = Sst I, Sa = SalI, Sp = SphI, X = XbaI, V = EcoRV, C = CalI, K = KpnI,
Xh = XhoI, N = NruI

The vector DNA has not been mapped for all the enzymes that cut in the polylinker.

Figure 20 shows a restriction site and function map of pWOR905;

Abbn:

A = AccI, B = Bal II, Ba = Bam HI, E = EcoRI, H = Hind III, P = Pst I, Sm = SmaI,
S = Sst I, Sa = SalI, Sp = SphI, X = XbaI, V = EcoRV, C = CalI, K = KpnI,
Xh = XhoI, N = NruI

The vector DNA has not been mapped for all the enzymes that cut in the polylinker.

Figure 21 shows a restriction site and function map of pCAR46;

Abbn:

A = AccI, B = Bal II, Ba = Bam HI, E = EcoRI, H = Hind III, P = Pst I,
Sm = SmaI, S = Sst I, Sa = SalI, Sp = SphI, X = XbaI, V = EcoRV, C = CalI,
K = KpnI,

Shaded portion represents DNA from Agrobacterium BO/44-2A,

Arrow represents carbamoylase coding sequence

The vector DNA has not been mapped for all the enzymes that cut in the polylinker or
Agrobacterium DNA;

Figure 22 shows a restriction site and function map of pCAR31;

Abbn:

B = Bgl II, Ba = Bam HI, Bs = BspHI, C = ClaI, E = EcoRI, V = EcoRV,
H = Hind III, N = NruI, P = Pst I, S = SstI, Sa = SalI, Sp = SphI,
Sc = ScaI, Tc^R = Tetracycline resistance gene,

cos = cos site from bacteriophage λ ,

Shaded portion represents Agrobacterium DNA

Figure 23 shows a restriction site and function map of pCAR32;

Abbn:

B = Bgl II, Ba = Bam HI, Bs = BspHI, C = ClaI, E = EcoRI, V = EcoRV,
 H = Hind III, N = NruI, P = Pst I, S = SstI, Sa = SalI, Sp = SphI, Sc = ScaI,
 Tc^R = Tetracycline resistance gene, cos = cos site from bacteriophage λ ,
 Shaded portion represents Agrobacterium DNA

Figure 24 shows a restriction site and function map of pCAR36;

Abbn:

A = AccI, B = Bgl II, Ba = Bam HI, Bs = BspHI, C = ClaI, E = EcoRI, V = EcoRV,
 H = Hind III, N = NruI, P = Pst I, Pf = PfiMI, S = SstI, Sa = SalI, Sp = SphI,
 Sc = ScaI, Cm^R = chloramphenicol resistance gene,
 Shaded portion represents Agrobacterium DNA

Figure 25 shows a restriction site and function map of pDAN3;and

Abbn:

B = Bgl II, Ba = Bam HI, E = EcoRI, H = Hind III, P = Pst I, S = SstI, Sa = SalI,
 Sp = SphI, X = XbaI,
 Shaded portion represents Agrobacterium DNA

Figure 26 shows a restriction site and function map of pDAN4.

Abbn:

B = Bgl II, Ba = Bam HI, E = EcoRI, H = Hind III, P = Pst I, S = SstI,
 Sa = SalI, Sp = SphI, X = XbaI, Shaded portion represents Agrobacterium DNA

The DNA of the invention may be ligated to any suitable vector.

Normally the vector is a plasmid, for example a plasmid derived from E. coli, or is a temperate or virulent bacteriophage.

Particular vectors include broad host range vectors such as pCP19 or pKT210 or derivatives thereof. The vectors pIJ2925 and pTR550, described in detail herein, may also advantageously be used.

A particularly useful vector is the mobilisation defective (Mob⁻) vector pWOR902, a derivative of pKT210. pWOR902, obtained by a spontaneous deletion of DNA from the Mob⁻ unstable vector pWOR901, is less promiscuous than pKT210. An advantage of pWOR902 is thus that recombinant strains are safer when transformed by pWOR902 than, for example, pKT210. E. coli HB101 (pWOR902) was deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland on 4th November 1991 under the accession number NCIMB 40451. The deposit was made under the Budapest Treaty on the deposition of micro-organisms for the purposes of patent procedure. pWOR902, derivatives thereof and hosts transformed thereby form a further aspect of this invention.

Particularly useful derivatives of pWOR902 are the plasmids pWOR903,

pWOR904 and pWOR905 described hereinbelow. These may be obtained from pWOR902 by standard methodology.

The recombinant vector according to the invention may be prepared by standard techniques, for example by ligating DNA comprising the carbamoylase gene to the chosen vector via direct combination of cohesive ends, homopolymer tailing or by means of a linker or adapter molecule.

It will be appreciated that recombinant vectors prepared according to the

above methods may contain the insert DNA in one of two possible orientations. Recombinant vectors having the insert DNA in each orientation are included within the scope of the invention.

Particular recombinant vectors include those designated as pCAR1, pCAR6, pCAR12, pCAR21, pCAR26, pCAR27, pCAR28, pCAR29, pGal2789RS3Carb, pCAR31, pCAR32, pCAR36, pCAR44 and pCAR46, the preparation and characterisation of which are described in detail hereinbelow.

In a particularly preferred aspect of the invention, plasmids pCAR26, pCAR44 and pCAR46 are provided. pCAR26, and especially, pCAR44 and pCAR46 give a large amount of carbamoylase activity when host cells, especially *Agrobacterium*, are transformed thereby and are cultured under suitable conditions.

It will be appreciated that the carbamoylase protein coding sequence may be expressed from a heterologous promoter such as the tac promoter. Such expression is included within the scope of the invention.

In a specific aspect the invention provides a host, especially *Agrobacterium*, transformed with pCAR46. In a specific aspect the invention provides NCIMB 40478 deposited under the Budapest Treaty on 14th February 1992.

A process for obtaining DNA encoding the carbamoylase enzyme comprises the steps of:

- a) constructing a gene library from chromosomal DNA fragments obtained from a carbamoylase producing micro-organism;
- b) carrying out one or more hybridisation experiments in order to select from the said library clones which contain the DNA of the invention; and
- c) isolating the DNA of the invention.

The gene library may be prepared by conventional 'shot-gun' methodology by:

- (a) partial digestion of the chromosomal DNA of a carbamoylase producing micro-organism with one or more suitable restriction endonucleases, for example HindIII;
- (b) size fractionation to give fragments of appropriate length;
- (c) ligation of the fragments into a vector to obtain a recombinant vector; and
- (d) transformation or transfection of a suitable host with the recombinant vector.

As described herein, transformation or transfection may be carried out by conventional methods well known in the art.

Size fractionation may suitably be carried out on a sucrose gradient and fragments within chosen size limits may be selected.

International Application No: PCT/ /

MICROORGANISMS	
Optional Sheet in connection with the microorganism referred to on page <u>5</u> , line <u>16</u> of the description *	
A. IDENTIFICATION OF DEPOSIT *	
Further deposits are identified on an additional sheet <input type="checkbox"/> *	
Name of depositary institution *	
NATIONAL COLLECTION OF INDUSTRIAL AND MARINE BACTERIA LIMITED	
Address of depositary institution (including postal code and country) *	
Torry Research Station, P O Box 31, 135 Abbey Road Aberdeen, United Kingdom, AB9 8DG	
Date of deposit *	Accession Number *
14 February 1992	NCIMB 40478
B. ADDITIONAL INDICATIONS * (leave blank if not applicable). This information is continued on a separate attached sheet <input type="checkbox"/>	
In respect of those designations in which a European Patent is sought, a sample of the deposited micro-organism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample.	
C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE * (If the indications are not for all designated States)	
D. SEPARATE FURNISHING OF INDICATIONS * (leave blank if not applicable)	
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<div style="display: flex; align-items: center; justify-content: center;"> <div style="text-align: center; margin-right: 10px;"> <p><i>Kerry A Hadden</i></p> <p>(Authorized Officer)</p> </div> </div>	
<input type="checkbox"/> The date of receipt (from the applicant) by the International Bureau is * <div style="text-align: center; margin-top: 10px;"> <p>was</p> <p>(Authorized Officer)</p> </div>	

In a preferred aspect a 'gene library' may be prepared by selecting fragments of about 15 to 30 kb, in length and ligating the said fragments into a plasmid vector, for example the vector pCP19. A suitable host is *E. coli*, for example *E. coli* DH1.

In order to identify clones in the gene library which contain the carbamoylase gene, it is necessary to screen colonies for the ability to convert
5 N-carbamoyl-4-hydroxyphenylglycine into D(-)4-hydroxy phenylglycine. 'Positive' colonies may conveniently be identified and isolated by testing for the production of ammonia (formed in the above reaction) by standard methods, for example by use of an indicator such as phenol red.

10 To enable the skilled person readily to perform the invention a colony which was positive in this test, specifically an *E. coli* AG1 micro-organism transformed with pCAR1, was deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland on 26th April, 1989 under the Accession Number NCIMB 40133. *E. coli* NCIMB 40133 forms a further aspect of the present invention.

15 The plasmid DNA present in a positive colony, for example *E. coli* NCIB 40133, may be further manipulated as described in the Examples herein to allow determination of the sequence of the carbamoylase enzyme encoded thereby [see Figure 10(b) below].

20 It will be understood that the amino acid sequence of this naturally occurring enzyme may be subject to some variability. These have substantially the same amino acid sequence as shown in Figure 10(b) below and have carbamoylase activity.

In a specific embodiment of the invention there is provided DNA or recombinant DNA comprising the coding sequence from nucleotide 891 to nucleotide 1802 shown in Figure 10(b) below. The DNA sequence encodes a carbamoylase
25 enzyme with a calculated molecular weight of approximately 34,100.

In a further aspect of the invention there is provided a substantially purified carbamoylase enzyme having the amino acid sequence from N- to C-terminus shown in Figure 10(b).

30 In yet a further aspect of this invention there is provided a carbamoylase enzyme as described hereinabove immobilised on a solid support, for example an anion exchange resin such as the phenol-formaldehyde anion exchange resin Duolite A568 (Rohm and Haas).

The DNA coding sequence or amino acid sequence shown in Figure 10(b) can, if desired, be synthesized by conventional means, for example using an
35 automated synthesiser.

Owing to the degenerate nature of the genetic code the amino acid sequence of the carbamoylase enzyme can be encoded by numerous alternative DNA sequences. It will be understood that the DNA of the present invention further comprises such alternative sequences.

International Application No: PCT/ /

MICROORGANISMS	
Optional Sheet in connection with the microorganism referred to on page <u>7</u> , line <u>13</u> of the description *	
A. IDENTIFICATION OF DEPOSIT *	
Further deposits are identified on an additional sheet <input type="checkbox"/> *	
Name of depositary institution *	
NATIONAL COLLECTION OF INDUSTRIAL AND MARINE BACTERIA LIMITED	
Address of depositary institution (including postal code and country) *	
Torry Research Station, P O Box 31, 135 Abbey Road Aberdeen, United Kingdom, AB9 8DG	
Date of deposit *	Accession Number *
26 April 1989	NCIMB 40133
B. ADDITIONAL INDICATIONS * (leave blank if not applicable). This information is continued on a separate attached sheet <input type="checkbox"/>	
In respect of those designations in which a European Patent is sought, a sample of the deposited micro-organism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample.	
C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE * (If the indications are not for all designated States)	
D. SEPARATE FURNISHING OF INDICATIONS * (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")	
E. <input checked="" type="checkbox"/> This sheet was received with the international application when filed (to be checked by the receiving Office)	
<div style="font-family: cursive; font-size: 1.2em; margin: 0;">Kerry A Haddon</div> (Authorized Officer)	
<input type="checkbox"/> The date of receipt (from the applicant) by the International Bureau **	
was _____ (Authorized Officer)	

In yet another aspect the invention provides a DNA compound selected from:

- a) a portion of the DNA insert of pCAR1, said portion being a BspHI - HindIII fragment of 986 base pairs;
 - 5 b) DNA sequences which hybridise to the said portion under stringent conditions and which code for a carbamoylase enzyme capable of converting a D-N-carbamoyl (optionally substituted phenyl)glycine into the corresponding D- (optionally substituted phenyl)glycine.
- 10 In a particular aspect of the invention it has been found that the carbamoylase enzyme may be conveniently produced in *E. coli* despite initial difficulties in getting generation of soluble active enzyme using standard methods. In this aspect of the invention DNA comprising the carbamoylase gene is first cloned into any suitable vector which is, or has been, manipulated so that the gene is expressed in *E. coli* under
- 15 the control of a suitable promoter.

Preferably the vector is a high copy number plasmid, for example pUC 18 or a derivative of the naturally occurring *E. coli* plasmid NR1 (Foster, T.J. *et al.*, *J. Bact.*, 1975, 124, 1153).

- 20 To achieve effective expression of carbamoylase enzyme the promoter is preferably *non-inducible*, i.e. there is no requirement to add chemicals or increase temperature to derepress or induce it. Surprisingly, it has been found that, under these conditions, *E. coli* cells produce, throughout their growth cycle, carbamoylase enzyme which is both soluble and active.

- 25 One preferred promoter for expression of the carbamoylase enzyme in *E. coli* is the galactose promoter as described hereinbelow.

Plasmid DNA present in *E. coli* NCIMB 40133 or derivatives thereof may also be manipulated to give high level expression of hydantoinase as herein described.

The following examples illustrate the invention.

30 Example 1

Culture of *Agrobacterium* 80/44-2A and isolation of chromosomal DNA

- 50ml of AJ-1 broth (20g yeast extract, 1g KH₂PO₄, 1g K₂HPO₄, 0.5g MgSO₄.7H₂O, 0.1g CaCl₂.2H₂O, 15mg MnSO₄.4H₂O, 20mg FeSO₄.7H₂O and 5g glucose per litre, adjusted to pH 7.0 with 5M NaOH) was inoculated with a culture of
- 35 *Agrobacterium* 80/44-2A, (isolated from soil) and incubated in a gyrotory incubator at 25°C for about 24 hours. The culture was centrifuged at approximately 1500 x g for 7 minutes at 10°C. The resulting supernatant was discarded, and the cell pellet was washed in 10ml of saline phosphate buffer (8.5g NaCl, 7.2g KH₂PO₄, 7.0g K₂HPO₄ per litre) and then repelleted. The supernatant was discarded and the cell pellet was

resuspended in 8ml of a lysing solution of 3% SDS (sodium dodecyl sulphate) and 50mM Tris-HCl, pH 8.0, and incubated in a water bath at 65°C for 15 minutes. The solution of lysed cells was allowed to cool to room temperature and was extracted twice with 5ml of buffered phenol/chloroform (a 50:50 mixture of phenol and chloroform buffered to a pH of approximately 7 with Tris-HCl, pH 8.0). The recovered aqueous phase was extracted with 5ml of chloroform. The recovered aqueous phase was precipitated by adding 0.1 volume of 3M sodium acetate (NaOAc), mixing, layering 2.5 volumes of cold (-20°C) ethanol onto the solution and swirling to mix. The clot of precipitated DNA was spooled onto a glass rod and redissolved in 3ml of TE (10mM Tris-HCl, pH 8.0; 1mM EDTA, pH=8.0) containing 40µg/ml Ribonuclease A. The solution was incubated at 37°C for 30 minutes, extracted with buffered phenol/chloroform, extracted with chloroform, and then precipitated with NaOAc and ethanol. The DNA was spooled onto a glass rod and redissolved in 3ml of TE.

15

Example 2

Construction of Genomic Library of *Agrobacterium* 80/44-2A

The plasmid vector pCP19 (Figure 1) is a derivative of the broad host range plasmid pLAFR1 (Friedman et al., Gene 18 (1982) 289-296) and is similar to pCP13 (described by Darzins and Chakrabarty, Journal of Bacteriology 159 (1984) 9-18) but lacks the kanamycin resistance gene present in pCP13. 2µg of plasmid pCP19 were digested with 20 units of restriction enzyme Hind III (Gibco BRL, Paisley, PA3 4EF, Scotland) at 37°C for 3 hours in Hind III digestion buffer (50 mM Tris-HCl, pH 8.0; 10mM MgCl₂; 100mM NaCl). The mixture was extracted with buffered phenol/chloroform, then extracted with chloroform and the recovered aqueous phase was treated with 0.1 volume 3M NaOAc and 2.5 volumes ethanol. The mixture was incubated at -20°C for 1 hour to precipitate the DNA. After centrifugation in microcentrifuge tubes at 12000 x g for 5 minutes, the DNA pellet was redissolved in 50µl of 50mM Tris-HCl (pH 8.0), 0.1mM EDTA (pH 8.0). One unit of calf intestinal alkaline phosphatase (CIAP; from BCL, Boehringer Mannheim House, Lewes, East Sussex, BN7 1LG) was added and the mixture was incubated at 37°C for 30 minutes. 45µl of water and 5µl of a 10% solution of SDS were added and the mixture was incubated at about 68°C for 15 minutes. The mixture was then cooled to room temperature, extracted with buffered phenol/chloroform, extracted with chloroform and precipitated by adding NaOAc and ethanol. The DNA pellet was redissolved in 40µl of TE.

35

25µg of DNA from *Agrobacterium* 80/44-2A (Example 1) were digested with 12 units of Hind III in 150µl of Hind III digestion buffer (50mM Tris-HCl, pH 8.0; 10mM MgCl₂; 100mM NaCl) at 37°C for 10 to 30 minutes to obtain cleavage at

some, but not all, of the recognition sites for the restriction enzyme. The progress of the digestion was monitored by removing aliquots and stopping the reaction by phenol/chloroform extraction. Portions of the DNA samples recovered were fractionated by electrophoresis on a 0.6% agarose (Ultrapure, from Gibco BRL) gel in TBE buffer (89mM Tris base; 89mM boric acid; 2mM EDTA, pH 8.0) containing 0.5µg/ml ethidium bromide, and the DNA bands were visualized using ultraviolet light. The required sizes of partially digested DNA fragments are approximately 15 to approximately 30kbp. These fragments were obtained by choosing suitable aliquots from the partial digestion, combining them, and fractionating them by electrophoresis on a 0.8% low melting point agarose (Ultrapure, from Gibco BRL) gel in TBE buffer containing ethidium bromide. After visualizing the DNA bands with ultraviolet light, the portion of the gel corresponding to fragments of size ca. 15kbp to ca. 30kbp was cut out and melted at 65°C for 15 minutes. The mixture was cooled to about 37°C, and extracted twice with a solution of phenol buffered to neutral pH with Tris-HCl (pH 8.0). The recovered aqueous phase was extracted with buffered phenol/chloroform, extracted with chloroform and the DNA was precipitated by adding NaOAc and ethanol. The DNA was redissolved in 100µl of TE.

About 0.8µg of Hind III-digested, CIAP-treated pCP19 DNA, and about 4µg of Hind III partially digested chromosomal DNA of *Agrobacterium* 80/44-2A were co-precipitated by adding NaOAc and ethanol, and were redissolved in 12µl of DNA ligase buffer (30mM Tris-HCl, pH 8.0; 4mM MgCl₂; 10mM dithiothreitol; 50µg/ml bovine serum albumin, and 0.5mM ATP). One unit of T4 DNA ligase (Gibco BRL) was added and the mixture was incubated at 12°C for about 16 hours. 3µl of the ligated DNA was packaged into lambda bacteriophage particles using the Gigapack Gold Packaging Kit from Stratagene, La Jolla, California 92037 (see also Enquist and Sternberg, *Methods in Enzymology* 68 (1979) 281-298). This gave 500µl of a suspension of bacteriophage particles.

A 10ml culture of *Escherichia coli* AG1 (a derivative of E. coli DH1: Hanahan, *Journal of Molecular Biology* 166 (1983) 557-580), obtained from Stratagene, was grown in L-broth (10g bacto-tryptone, 5g yeast extract, 5g NaCl and 1g glucose per litre), containing 0.4% maltose and 10mM MgSO₄, at 37°C on a gyrotory incubator for approximately 16 hours. The cells were collected by centrifugation at ca. 1500 x g for 7 minutes and resuspended in 10mM MgSO₄ to a final optical density at 600nm of 0.5. Eleven aliquots of 400µl of cells of the E. coli culture were each mixed with 40µl of bacteriophage particles and incubated at 37°C for 15 minutes. Each mixture was added to 800µl of L-broth, incubated on a shaker at 37°C for 1 hour and centrifuged at 12000 x g for 2 minutes to pellet the cells. Each cell pellet was resuspended in 100µl of L-broth and spread on one L-agar (as for L-broth but containing 1.5% agar) plate supplemented with 10µg/ml tetracycline. The plates

were incubated at 37°C for about 16 hours. 800 colonies were picked off to L-agar plates containing tetracycline (10µg/ml) such that each plate had 50 colonies. These plates were incubated at 37°C for about 16 hours, and then each plate was replica-plated to 2 L-agar plates, containing tetracycline, which were incubated at 37°C for about 16 hours. Two sets of 16 plates resulted; these sets were copies of a Genomic Library of *Agrobacterium* 80/44-2A DNA in *E. coli*.

Example 3

Isolation of a clone specifying carbamoylase activity

One set of 800 colonies (Example 2), representing a Genomic Library of *Agrobacterium* 80/44-2A DNA in the plasmid pCP19 in *E. coli* AG1, was screened for the ability to convert N-carbamoyl-p-hydroxyphenylglycine (N-carb.) to D(-)-p-hydroxyphenylglycine (D(-)-HPG). Cells from half of one plate (i.e. from 25 different clones) of the library were scraped off into a microcentrifuge tube containing 800µl of assay buffer. The assay buffer was a solution of 1% N-carb., 10mM phosphate buffer, 0.0012% phenol red (0.6ml of a 2% solution per 100ml of N-carb. solution) adjusted to pH 6.7 with NaOH. This solution was a yellowish colour. The contents of the tube were mixed, and the tube was incubated in a water bath at 42°C for 24 hours. Conversion of N-carb. to D(-)-HPG results in production of ammonia (Figure 2) which raises the pH and can be detected by a change in the colour of the phenol red from yellow to pinkish-red. One of the 32 tubes changed colour to pinkish-red. The 25 colonies corresponding to this tube were restreaked, from the relevant plate of the duplicate library, onto L-agar plates containing tetracycline, which were incubated at 37°C for about 16 hours. A loopful of cells from each restreak was tested for carbamoylase activity using the phenol red test described above. One of the 25 colonies produced a pinkish-red colour. The reaction tube was centrifuged at 12000 x g for 2 minutes to pellet the cells and the supernatant was assayed by HPLC to confirm that D(-)-HPG was present. The clone encoding carbamoylase activity was deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland on 26th April, 1989 under the accession number NCIMB 40133. The deposit was made under the Budapest Treaty on the deposition of micro-organisms for the purposes of patent procedure.

Example 4

Isolation and characterization of pCAR1 from *Escherichia coli* NCIMB 40133

400ml of L-broth containing 10µg/ml tetracycline was inoculated with a culture of *E. coli* NCIMB 40133 (Example 3) and incubated in a gyrotory incubator at 37°C for about 20 hours. The culture was centrifuged at ca. 6000 x g for 10 minutes at 4°C, and the resulting cell pellet was resuspended in 2.5ml of 25% sucrose, 50mM

Tris-HCl (pH 8.0). 0.5ml of a 10mg/ml lysozyme solution was added and the mixture was incubated on ice for 10 minutes. 1ml of 0.25M EDTA (pH 8.0) was added to the lysozyme-treated cells, followed by 4ml of lysing solution (0.25% Triton X-100; 50mM Tris-HCl, pH 8.0; and 62.5mM EDTA, pH 8.0). The solutions were mixed and incubated on ice for a further 5 to 10 minutes to lyse the cells. The lysed cells were centrifuged at approximately 38000 x g for 30 minutes at 4°C and 8.0ml of the supernatant was added to 8.4g CsCl. Once the CsCl had dissolved, 1.0ml of 5mg/ml ethidium bromide solution was added, and the mixture was transferred to a 16 x 76mm polyallomer tube (Beckman Instruments, Palo Alto, California 94304). The tube was sealed, and centrifuged at ca. 185000 x g for 60 hours. The resulting plasmid band was visualized with ultraviolet light, and the DNA was removed using a syringe fitted with a 21-gauge needle. Ethidium bromide was removed from the DNA solution by several extractions with isopropanol that had been saturated with 5M NaCl. The recovered aqueous phase was dialysed against 3 changes of TE buffer for 1 hour each, extracted with buffered phenol/chloroform, extracted with chloroform and the DNA was precipitated by adding NaOAc and ethanol. The plasmid DNA (approximately 50µg) was redissolved in 500µl of TE. This plasmid was designated pCAR1; a restriction site map is shown in Figure 3.

20 Example 5

Construction of Plasmid pCAR6

Approximately 4µg of plasmid pCAR1 (Example 4) were digested with HindIII at 37°C for 3 hours in a HindIII digestion buffer. The digested DNA was fractionated on a 0.9% low-melting point agarose gel (Gibco-BRL) by electrophoresis in TBE buffer. The portion of the gel containing the approximately 12kbp HindIII fragment was excised, melted by heating to 65°C for 10 minutes, cooled to approximately 37°C and extracted twice with buffered phenol (phenol buffered to a pH of approximately 7 with Tris-HCl, pH8.0). The recovered aqueous phase was extracted with buffered phenol/chloroform, extracted with chloroform, and treated with 0.1 volume 3M NaOAc and 2.5 volumes ethanol. The mixture was incubated at -20°C for one hour to precipitate the DNA, which was pelleted by centrifugation in microcentrifuge tubes at 12000 x g for 5 minutes and redissolved in 20µl of TE.

Approximately 0.6 µg of HindIII-digested, CIAP-treated pCP19 DNA (Example 2) and about 0.6µg of the approximately 12kbp HindIII fragment from pCAR1 (see above) were combined in a total volume of 20µl of DNA ligase buffer. One unit of T4 DNA ligase was added and the mixture was incubated at 12°C for about 16 hours.

E. coli HB101 [H. Boyer and D.Roulland-Dussoix, Journal of Molecular Biology 41 (1969) 459] was grown in 50ml of L-broth at 37°C on a gyrotory

incubator to an O.D. at 600nm of approximately 0.5. The cells were pelleted by centrifugation at 1500 x g for 7 minutes at 4°C, resuspended in 4ml 100mM MgCl₂, repelleted, resuspended in 2ml 100mM CaCl₂, repelleted and resuspended again in 2ml 100mM CaCl₂. These cells were kept on ice for 1 hour. Transformation of the

5 *E. coli* cells was performed as follows:- 10µl of the ligation reaction mixture was added to 200µl of cells. The mixture was incubated on ice for 30 minutes, incubated at 42°C for 2 minutes, added to 800µl of L-broth and incubated at 37°C for 30 minutes. Dilutions of this mixture were spread on L-agar plates supplemented with 10µg ml⁻¹ tetracycline, and the plates were incubated at 37°C for 16 hours. 20

10 tetracycline resistant colonies were restreaked on L-agar plates, supplemented with 10µg ml⁻¹ tetracycline, and incubated overnight at 37°C. A loopful of cells was scraped from each plate and tested for carbamoylase activity using the 'phenol red test' described in Example 3. 15 of the 20 isolates gave positive tests for carbamoylase activity.

15 Plasmid DNA was isolated (on a small scale) from each of the 20 isolates as follows: Cells were scraped from a plate into 100µl of 50mM glucose, 25mM Tris HCl pH8.0, 10mM EDTA in a microcentrifuge tube, vortexed to mix and incubated at room temperature for 5 minutes. 200µl of a fresh mixture of 0.2M NaOH and 1% sodium dodecyl sulphate was added, the tube was inverted to mix the contents and

20 was incubated on ice for 5 minutes. 150µl of 3M potassium acetate, adjusted to pH4.8 with acetic acid, was added to the tube, the contents were mixed by inverting the tube and the tube was incubated on ice for 5 minutes. The tube was centrifuged at 12000 x g for 2 minutes, the recovered supernatant was extracted with buffered phenol/chloroform, extracted with chloroform and the recovered aqueous phase was

25 treated with 800µl of cold (-20°C) ethanol. The contents of the tube were mixed and incubated at room temperature for 2 minutes, the tube was centrifuged at 12000 x g for 2 minutes to pellet the nucleic acid. The pellet was dissolved in TE, and portions were used for restriction enzyme digestion to check the restriction map of the plasmid

30 DNA. Of the 20 isolates tested, the 5 isolates that produced no carbamoylase enzyme contained pCP19 DNA, as judged from restriction enzyme digests of the plasmid DNA. All 15 that gave carbamoylase enzyme contained plasmid with the 12kbp HindIII fragment from pCAR1. The plasmid DNA was one of two types, which differed only in the orientation of the 12 kbp HindIII fragment relative to the remainder of the plasmid vector. One of the isolates of E. coli with carbamoylase

35 activity was incubated at 37°C on a gyrotory incubator overnight in 400ml L-broth supplemented with 10µg ml⁻¹ tetracycline. Plasmid DNA was isolated from this culture using the method described in Example 4. This plasmid was designated pCAR6; a restriction site map is shown in Figure 4.

Example 6**Construction of Plasmid pCAR12**

The plasmid vector pIJ2925 (Figure 5) is similar to pUC18 (Norrande et al., Gene 26 (1983) 101-106), but it has a different polylinker at the multiple cloning site near the 5' end of the *lacZ'* gene. 2µg of pIJ2925 DNA were digested with 10 units of BamHI (Gibco-BRL) at 37°C for 3 hours in 100µl of BamHI digestion buffer (50mM Tris-HCl, pH8.0; 10mM MgCl₂; 100mM NaCl). One unit of CIAP was added and the incubation was continued for 30 minutes. 5µl of 10% SDS were added and the mixture was incubated at approximately 68°C for 15 minutes. The mixture was then cooled to room temperature, extracted with buffered phenol/chloroform, extracted with chloroform and precipitated by adding 0.1 volume 3M NaOAc and 2.5 volumes ethanol. After incubating at -20°C for one hour the tube was centrifuged at 12000 x g for 5 minutes and the DNA pellet was redissolved in 10µl TE.

About 20µg of pCAR6 DNA was digested with 12 units of Sau3AI (Gibco-BRL) in Sau3AI digestion buffer (20mM Tris HCl pH7.4, 5mM MgCl₂, 50mM KCl) at 37°C for 1-30 minutes to obtain cleavage at some, but not all, of the recognition sites for the restriction enzyme. The progress of the digestion was monitored by removing aliquots and stopping the reaction by phenol/chloroform extraction. Portions of the DNA samples recovered were fractionated by electrophoresis on a 0.8% agarose gel in TBE buffer containing ethidium bromide and the DNA bands were visualised using ultraviolet light. DNA from one aliquot, in which most of the DNA fragments were in the size range 1 to 5 kbp, was precipitated using NaOAc and ethanol, and redissolved in 20µl TE.

About 0.5µg of BamHI-digested, CIAP-treated pIJ2925 DNA and about 1µg of Sau3AI partially digested DNA of pCAR6 were mixed in 12µl of DNA ligase buffer. One unit of T4 DNA ligase was added and the mixture was incubated at 12°C for 16 hours.

A culture of *E. coli* DH5α (obtained from Gibco-BRL) was grown in a similar way to that described for *E. coli* HB101 in Example 5. The cells from this culture were treated with MgCl₂ and CaCl₂ and then transformed with the ligated DNA. Transformants were selected on L-agar plates containing 50µl ml⁻¹ ampicillin, 12.5µg ml⁻¹ isopropyl-β-D-galactopyranoside (IPTG, from Gibco-BRL) and 40µg ml⁻¹ 5-bromo-4-chloro-3-indolyl-β-D galactopyranoside (X-gal, from Gibco-BRL). Approximately 300 'white' colonies, which failed to convert the X-gal to a blue compound and therefore presumably contain inserts in the polylinker of pIJ2925 which disrupt the *lacZ'* gene on the plasmid, were patched onto L-agar plates containing 50µg ml⁻¹ ampicillin and grown overnight at 37°C. There were 25 patches per plate, and duplicate plates were made. One set of plates was used to test for carbamoylase enzyme activity using the method described in Example 3. Cells

from 25 patches were tested in each tube; one of the 12 tubes gave a positive test. The colonies from the relevant duplicate plate were restreaked, grown overnight and tested individually for carbamoylase activity. One of the 25 clones gave a positive test; 400ml of L-broth supplemented with 50 μ g ml⁻¹ ampicillin was inoculated with a culture of this clone and then grown for about 20 hours in a gyrotory incubator at 37°C. Plasmid DNA was isolated from this culture using the method described in Example 4. This plasmid was designated pCAR12; a restriction map is shown in Figure 6.

10 Example 7

Construction of Plasmid pCAR21

Approximately 2 μ g of plasmid pCAR12 (Example 6) were digested with 5 units of BglII (Gibco BRL) at 37°C for 3 hours in BglII digestion buffer (50mM Tris HC1 pH8.0; 10mM MgCl₂; 100mM NaCl). The digested DNA was fractionated on a 0.9% low-melting point agarose gel by electrophoresis in TBE buffer. The portion of the gel containing the ca. 1.9kbp BglII fragment was excised and the DNA was recovered using the method described in Example 5.

Approximately 0.5 μ g of this DNA fragment was mixed with approximately 0.5 μ g of BamHI-digested, CIAP-treated pIJ2925 DNA (Example 6) in a total volume of 12 μ l of DNA ligase buffer. One unit of T4 DNA ligase was added, and the mixture was incubated at 12°C for about 16 hours and then used to transform *E. coli* DH5 α . The cells were spread on L-agar plates supplemented with ampicillin, IPTG and X-gal (Example 6) and incubated overnight at 37°C. 4 "white" colonies (see Example 6) were restreaked on L-agar plates containing ampicillin, IPTG and X-gal, grown overnight at 37°C and small scale plasmid DNA preparations were performed on some of the cells, using the method described in Example 5. Portions of the DNA were digested with restriction enzymes to check the restriction map of the plasmids. 3 of the 4 clones tested contained a BglII fragment of approximately 2kbp, as expected from the ca. 1.9kbp fragment from pCAR12 plus polylinker sequences from pIJ2925. Cells from these 3 clones were tested for carbamoylase activity using the "phenol red test" (Example 3). All of the tests were positive. 400ml of L-broth containing 50 μ g ml⁻¹ ampicillin was inoculated with a culture of one of the 3 "positive" clones and grown for about 20 hours in a gyrotory incubator at 37°C. Plasmid DNA was isolated from this culture using the method described in Example 4. This plasmid was designated pCAR21; a restriction map is shown in Figure 7.

Example 8

Construction of pCAR27 and pCAR28

Approximately 2 μ g of plasmid pCAR21 were digested with 5 units of HindIII

for 3 hours at 37°C in HindIII digestion buffer. The digested DNA was fractionated on a 0.9% low-melting point agarose gel by electrophoresis in TBE buffer. The portion of the gel containing the ca. 1.9 kbp HindIII fragment was excised, the DNA was extracted using the method described in Example 5 and the DNA was dissolved
5 in 20µl TE.

Approximately 1µg of M13mp19 [Norrander, Kempe and Messing; Gene 26 (1983) 101-106] replicative form (RF) DNA was digested with 5 units of HindIII for 3 hours at 37°C in HindIII restriction buffer. The mixture was extracted with buffered phenol/chloroform, extracted with chloroform and the DNA was precipitated
10 from the recovered aqueous phase using 3M NaOAc and ethanol. The DNA was collected by centrifugation and redissolved in 20µl TE.

About 0.8µg of HindIII-digested M13mp19 DNA and about 0.2µg of the ca. 1.9kbp HindIII fragment from pCAR21 were mixed in 15µl of DNA ligase buffer. One unit of T4 DNA ligase was added and the mixture was incubated at 12°C for 16
15 hours.

A culture of *E. coli* DH5αF' (obtained from Gibco-BRL) was grown in a similar way to that described for E. coli HB101 in Example 5. The cells from this culture were treated with MgCl₂ and CaCl₂ (as described in Example 5) and incubated on ice for 2 hours. Transfection of the *E. coli* cells was performed as
20 follows: 0.5µl or 4µl of the ligation reaction mixture was added to 200µl of cells, incubated on ice for 30 minutes, incubated at 42°C for 2 minutes and incubated at room temperature for 15 minutes. 100µl of an overnight culture of *E. coli* DH5α', 30µl of 2% X-gal and 30µl of 2.5% IPTG were added to the mixture of cells and DNA. This mixture was added to 2.5ml of a 1:1 mixture of L-broth and L-agar (kept
25 molten at 48-50°C), rapidly mixed and then poured onto an L-agar plate to produce a thin layer across the whole plate. The plate was incubated overnight at 37°C.

A mixture of blue (caused by β-galactosidase activity on X-gal) and "white" (i.e. not blue) plaques were visible on a lawn of DH5αF' cells. The HindIII site is within the lacZ' gene of M13mp19. Therefore insertion of DNA at this site should
30 inactivate the lacZ' gene and thus inactivate β-galactosidase activity in DH5αF' cells infected with such M13mp19 derivatives. *E. coli* DH5αF' was grown in L-broth at 37°C for about 16 hours. The resulting culture was diluted 1:100 with L-broth, and "white" plaques were inoculated into 1ml of this diluted culture and incubated on a gyrotory incubator (ca. 300rpm) for 5 hours. The cultures were transferred to
35 microcentrifuge tubes and centrifuged at 12000 x g for 3 minutes to pellet the cells. The supernatants were stored at 4°C; RF DNA was isolated from each sample of cells using the small-scale method for plasmid isolation described in Example 5. Restriction enzyme digests of these plasmids identified two isolates containing the ca. 1.9kbp fragment from pCAR21, inserted in either orientation in M13mp19. 800µl of

the supernatant, from each of the cultures from which these plasmids were isolated, was inoculated separately into 400ml of L-broth containing 4ml of an overnight culture of *E. coli* DH5 α F'. The supernatants contain bacteriophage particles; these may infect the DH5 α F' cells and produce double-stranded RF DNA inside the cells.

5 Each of the 400ml cultures was grown for 20 hours at 37°C and treated as described in Example 4 to purify plasmid (RF) DNA. Restriction enzyme digests of the plasmids confirmed the structures of the plasmids. The plasmid from one culture was designated pCAR27 (Figure 8), and the plasmid from the other culture was designated pCAR28 (Figure 9).

10

Example 9

Determination of the Nucleotide sequence of the *Agrobacterium* DNA in pCAR27 and pCAR28

Plasmids pCAR27 and pCAR28 were treated similarly to produce nested sets
15 of deleted clones and templates for sequencing:-

Approximately 8 μ g of RF DNA was digested with 10 units of KpnI and 10 units of BamHI in 100 μ l of 20mM Tris HCl pH7.4, 5mM MgCl₂, 50mM KCl. The DNA was purified using phenol/chloroform extraction and precipitated using ethanol in the presence of 0.3M NaOAc. The recovered DNA was treated with exonuclease
20 III, using the method described by Henikoff (Gene 28 (1984) 351-359), to delete into the *Agrobacterium* DNA in the plasmids. The ligated products were used to transfect cells of *E. coli* DH5 α F', using the method described in Example 8. Template DNA for use in sequencing reactions was prepared as follows:

"White" plaques were individually inoculated into 1ml of a culture of *E. coli*
25 DH5 α F' (see Example 8), grown for 5-6 hours at 37°C with vigorous shaking (300 rpm) and the cells were then pelleted by centrifugation. 800 μ l of supernatant was removed to a fresh tube containing 150 μ l of 20% polyethyleneglycol (PEG 6000), 2.5M NaCl, mixed and incubated at room temperature for 12 minutes. The tube was centrifuged at 12000 x g for 5 minutes to pellet the bacteriophage particles and the
30 supernatant was discarded. The tube was centrifuged again briefly, all traces of supernatant were removed and the pellet was resuspended in 100 μ l of 10mM Tris-HCl (pH 8.0), 0.1mM EDTA (pH8.0). 50 μ l of phenol (buffered to pH7 with 100mM Tris-HCl pH8.0) was added, mixed and the tube was incubated at room temperature for 5 minutes. The tube was centrifuged at 12000 x g for 3 minutes and
35 the aqueous phase was removed to a fresh tube, extracted with chloroform and treated with NaOAc and ethanol to precipitate the DNA. The DNA was recovered by centrifugation and dissolved in 16 μ l of water. This DNA is single-stranded DNA from M13 bacteriophage particles, and was used as the "template" for sequencing reactions. Sequencing was performed with Sequenase enzyme (United States

Biochemical Corporation, Cleveland, Ohio, 44122, USA) using the method supplied with the kit containing the enzyme. Several templates were sequenced to obtain the complete sequence of the pCAR27 insert. Templates from deletions of pCAR28 were used to determine the sequence of the fragment in the opposite direction. The
5 sequences from both directions were compared using software from DNASTAR Inc., Madison, WI 53715, USA to produce a consensus for the 1880bp of *Agrobacterium* DNA; this consensus is shown in Figure 10, together with the deduced amino acid sequence of the carbamoylase enzyme.

10 Example 10

Construction of pCAR29

Approximately 2µg of RF DNA of pCAR28 (Example 8; Figure 9) was digested with 5 units of BspHI (New England Biolabs, Beverly MA 01915, USA) in 50µl of 20mM Tris HCl pH7.4, 5mM MgCl₂, 50mM KCl. The DNA was purified
15 using phenol/chloroform extraction and chloroform extraction, precipitated using NaOAc and ethanol, recovered by centrifugation and redissolved in 40µl of 10mM Tris HCl pH7.4 10mM MgCl₂, 50mM NaCl, 50µg ml⁻¹ bovine serum albumin, 10mM 2-mercaptoethanol. 1µl of a mixture of 250µM deoxyguanosine-triphosphate, 250µM deoxy-thymidinetriphosphate and 250µM deoxycytidine-triphosphate was
20 added followed by 2 units of "Klenow fragment" of *E. coli* DNA polymerase I (Gibco-BRL). The tube was incubated at 37°C for 10 minutes, extracted with phenol/chloroform and extracted with chloroform. The recovered aqueous phase was treated with NaOAc and ethanol to precipitate the DNA, which was recovered by centrifugation and redissolved in 50µl of 50mM Tris-HCl pH8.0, 10mM MgCl₂ and
25 50mM NaCl. 5 units of each of the restriction enzymes HindIII and EcoRV (Gibco-BRL) were added, the contents of the tube mixed and the tube incubated at 37°C for 3 hours. The digested DNA was fractionated on a 0.9% low melting point agarose gel by electrophoresis in TBE buffer. The portion of the gel containing the 990 bp fragment was excised and the DNA recovered from the agarose segment using
30 the method described in Example 5. The DNA was redissolved in 20µl of water.

pTR550 (Figure 11) is a plasmid vector, constructed by Dr G.M.P. Lawrence (SmithKline Beecham Pharmaceuticals, Great Burgh, Epsom, Surrey, UK), and it is a derivative of pKK223-3 (Pharmacia LKB Biotechnology, S-751 82 Uppsala, Sweden). In pTR550 most of pKK223-3 that is derived from pBR322 has been
35 replaced by DNA from pAT153. Approximately 1µg of pTR550 was digested with 5 units of restriction enzyme SmaI (Gibco-BRL) in 30µl of 20mM Tris HCl pH.7.4, 5mM MgCl₂, 50mM KCl for 3 hours at 37°C. The digested DNA was purified using phenol/chloroform and chloroform extraction, precipitated using NaOAc and ethanol, recovered by centrifugation and redissolved in 30µl of 50mM Tris-HCl

(pH8.0), 10mM MgCl₂, 100mM NaCl containing 5 units of HindIII. The mixture was incubated at 37°C for 3 hours, extracted with phenol/chloroform, extracted with chloroform and the recovered aqueous phase was treated with NaOAc and ethanol to precipitate the DNA. The DNA was collected by centrifugation and redissolved in
5 20µl of water.

Approximately 0.5µg of pTR550 DNA, digested with both SmaI and HindIII, was mixed with approximately 0.2µg of the 990bp fragment (from the BspHI site at the beginning of the carbamoylase gene to the HindIII site at the end of the sequenced DNA) from pCAR28 in 20µl of DNA ligase buffer. One unit of T4 DNA ligase was
10 added and the mixture was incubated at 12°C for about 16 hours.

A culture of E. coli JM101 (Messing, Recombinant DNA Technical Bulletin (NIH) 2 No 2 pp 43-48 (1979)) was grown, and the cells treated with CaCl₂ and MgCl₂, in a similar way to that described for E. coli HB101 in Example 5. The cells were transformed with the ligation mixture DNA as described in Example 5: dilutions
15 of the cells were spread on L-agar plates containing 50µg ml⁻¹ ampicillin and incubated overnight at 37°C. 22 ampicillin-resistant colonies were restreaked on L-agar plates containing 50µg ml⁻¹ ampicillin, incubated at 37°C overnight and then small-scale plasmid DNA preparations (using the method described in Example 5) were performed on cells of each of these isolates. Restriction enzymes digests of the
20 DNA preparations indicated one plasmid of the desired construction. The isolate containing this plasmid was inoculated into 400ml of L-broth containing 50µg ml⁻¹ ampicillin, grown overnight on a gyrotory incubator at 37°C and plasmid DNA was isolated using the method described in Example 4. Restriction enzyme digests confirmed the correct structure of this plasmid; the plasmid was designated pCAR29
25 (Figure 12).

Example 11

Expression of carbamoylase in E. coli JM101 containing pCAR29

E. coli JM101 containing pCAR29 (Example 10) was inoculated into 10ml of
30 L-broth containing 50µg ml⁻¹ ampicillin and was grown overnight at 37°C on a gyrotory incubator. 600µl of this culture was inoculated into 60ml of L-broth containing 50µg ml⁻¹ ampicillin in a 250ml conical flask and incubated on a gyrotory incubator at 37°C. After 2 hours (Optical density at 600nm = ca.0.3) IPTG was added to a final concentration of 1mM and growth was continued for a further 3
35 hours. The E. coli cells in the culture possessed carbamoylase activity. Cells of E. coli JM101 containing pTR550, grown in the way described above, did not possess any carbamoylase activity.

Example 12**Transfer of pCAR1 from *E. coli* to *Agrobacterium***

Plasmid pCAR1 was transferred to *Agrobacterium* from *E. coli* by a triparental mating involving conjugation of the bacteria, with *E. coli* (pRK2013) as the 'helper strain' providing functions necessary for plasmid transfer. *Agrobacterium* 15-10 is a strain derived from *Agrobacterium* 80/44-2A by mutagenesis using ultra-violet light. A 10ml culture of *Agrobacterium* 15-10 was grown in L-broth at 30°C on a gyrotory incubator for approximately 20 hours. A 10ml culture of *E. coli* AG1 (pCAR1) - see Example 3 - was grown in L-broth containing 10µg/ml tetracycline at 37°C on a gyrotory incubator for approximately 16 hours. A 10ml culture of *E. coli* HB101 (pRK2013) - see Figurski and Helinski, Proceedings of the National Academy of Science, USA 76 (1979) 1648-1652 - was grown in L-broth containing 50µg/ml kanamycin sulphate at 37°C on a gyrotory incubator for approximately 16 hours. 800µl of each culture was filtered through one sterile 0.45µm pore-size HA filter (Millipore, Blackmoor Lane, Watford, WD1 8YW, U.K.). The filter, which retains the cells from the cultures, was then placed on the surface of an L-agar plate and was incubated for 24 hours at 30°C. The filter was removed from the surface of the plate to a screw-cap container containing 1ml of sterile saline phosphate buffer and the container was shaken to re-suspend the cells from the filter. 100µl of this solution, and dilutions in saline phosphate buffer, were plated on minimal medium (MM: 1g KH₂PO₄, 1g K₂HPO₄, 0.5g MgSO₄.7H₂O, 0.1g CaCl₂.2H₂O, 15mg MnSO₄.4H₂O, 20mg FeSO₄.7H₂O, 5g glucose, 2g (NH₄)₂SO₄ and 15g agar, adjusted to pH7.0 with 5M NaOH) containing 100µg/ml streptomycin sulphate and 10µg/ml tetracycline. The *E. coli* parent strains should be unable to grow on MM containing 100µg/ml streptomycin. *Agrobacterium* 15-10 should not grow in the presence of 10µg/ml tetracycline. Therefore only pCAR1-containing *Agrobacterium* 15-10 cells should grow on this medium. After 5 days growth at 30°C colonies were re-streaked on L-agar plates containing 25µg/ml streptomycin sulphate and 10µl/ml tetracycline and incubated at 30°C for a further 3 days. Plasmid DNA was prepared from four isolates using the method described in Example 5. Restriction enzyme digestion of portions of the plasmid DNA was used to confirm that the tetracycline resistant ex-conjugants (i.e. progeny from the conjugation) did contain pCAR1.

Example 13**Transfer of pCAR6 from *E. coli* to *Agrobacterium***

The method used for this transfer was very similar to that described for pCAR1 in Example 12. The parents in the mating were *E. coli* HB101 (pCAR6) - see Example 5, *E. coli* HB101 (pRK2013) - see Example 12 - and *Agrobacterium* 15-10 - see Example 12. Since both *E. coli* parent strains are auxotrophs, the progeny from

the mating were plated on MM containing 10µg/ml tetracycline, but no streptomycin, in order to select for *Agrobacterium* 15-10 (pCAR6) colonies. The presence of pCAR6 was confirmed by analysis of restriction enzyme digestion of plasmid DNA isolated from ex-conjugants.

5

Example 14

Transfer of pCP19 from *E. coli* to *Agrobacterium*

The method used for this transfer was the same as that described for pCAR6 in Example 13, except that *E. coli* HB101 (pCP19) was a parent strain in the mating instead of *E. coli* HB101 (pCAR6). The presence of pCP19 in ex-conjugants was confirmed by analysis of restriction enzyme digestion of plasmid DNA isolated from ex-conjugants.

Example 15

15 Demonstration of extra hydantoinase activity associated with pCAR1 and pCAR6

Agrobacterium 15-10 (pCAR1), *Agrobacterium* 15-10 (pCAR6) and *Agrobacterium* 15-10 (pCP19) were each inoculated into 50ml of AJ-1 broth containing 0.1% alanine hydantoin and 10µg/ml chloramphenicol and grown for 24 hours in a gyrotory incubator at 30°C. Each of the cultures was then treated as follows: the cells from 5ml of culture were collected by centrifugation, and the wet weight of cells was measured. The cells were re-suspended in 20ml of 1% D,L-5-(p-hydroxyphenyl) hydantoin in 0.2M Tris in a 50ml screw-capped conical flask and shaken in a water bath at 42°C. Samples were removed after incubation for 10 minutes and after 30 minutes; the cells were spun out by centrifugation and the supernatant was assayed by HPLC for the presence of D-N-carbamoyl-p-hydroxyphenylglycine. The hydantoinase activity, in µmoles of product per g of cells (wet weight) per hour, was calculated using the difference between the amounts of D-N-carbamoyl-p-hydroxyphenylglycine product at 30 minutes and at 10 minutes. The hydantoinase activities for *Agrobacterium* 15-10 containing pCAR1 or pCAR6 were 5-6 times higher than the activity for *Agrobacterium* 15-10 containing pCP19, indicating that pCAR1 and pCAR6 contain the gene encoding hydantoinase activity.

Example 16**Construction of plasmid pCAR26**

The plasmid vector pKT210 (Bagdasarian *et al.*, Gene 16 (1981) pp237-247; map in Figure 13) is a chloramphenicol-resistant derivative of the broad host range
5 plasmid RSF1010. Approximately 2µg of pKT210 DNA were digested with 5 units of EcoRI (Gibco-BRL) at 37°C for 3 hours in EcoRI digestion buffer (50mM Tris-HCl, pH 8.0; 10mM MgCl₂; 100mM NaCl). A portion of the digestion mixture was analysed by electrophoresis; the digestion did not appear to be complete, but one unit of CIAP was added and the incubation was continued for 30 minutes. The digestion
10 mixture was loaded onto a 0.9% low melting point agarose gel and the DNA was fractionated by electrophoresis in TBE buffer. The portion of the gel containing the ca. 11.4 kbp EcoRI fragment, corresponding to linearised plasmid DNA, was excised and the DNA was recovered using the method described in Example 5.

Approximately 2µg of pCAR12 (Example 6; see also Figure 6) were digested
15 with 10 units of EcoRI at 37°C for 3 hours in EcoRI digestion buffer. The digested DNA was fractionated on a 0.9% low melting point agarose gel by electrophoresis in TBE buffer. The portion of the gel containing the ca. 2.7 kbp EcoRI fragment was excised and the DNA was recovered using the method described in Example 5.

Approximately 0.5µg of this ca. 2.7 kbp DNA fragment was mixed with
20 approximately 0.5µg of EcoRI-digested, CIAP-treated pKT210 DNA in a total volume of 12µl of DNA ligase buffer. One unit of T4 DNA ligase was added and the mixture was incubated at 12°C for about 16 hours.

A culture of *E. coli* AG1 (see Example 2) was grown in a similar way to that described for *E. coli* HB101 in Example 5. The cells from this culture were treated
25 with MgCl₂ and CaCl₂ and then with the ligated DNA in a similar way to that described in Example 5. The cell-DNA mixture was spread on L-agar plates supplemented with 25µg ml⁻¹ chloramphenicol, and the plates were incubated at 37°C for 16 hours. 17 chloramphenicol-resistant transformants were restreaked on L-agar plates containing 25µg ml⁻¹ chloramphenicol, and incubated overnight at 37°C.
30 A loopful of cells of each transformant was scraped from the plate and resuspended in 600µl of the "phenol red" assay buffer (Example 3). After incubation for 5 hours at 42°C, 3 of the reactions had developed a pink colour. Plasmid DNA was isolated, using the small-scale method described in Example 5, from the cultures corresponding to the pink coloration in the tests. Restriction enzyme digestion of the
35 DNA preparations showed the presence of the ca. 2.7 kbp fragment from pCAR12. One of the plasmid preparations had two copies of the fragment from pCAR12; this plasmid was designated pCAR26. Further restriction enzyme digests showed that pCAR26 contained all of the *Agrobacterium* DNA from pCAR12 i.e. the fragment from pCAR12 extends beyond the EcoRI site in the carbamoylase gene (see Figures 6 and

10) to the EcoRI site in the polylinker. Therefore the isolated fragment (of ca. 2.7 kbp) must have come from partially digested pCAR12. A map of pCAR26 is shown in Figure 14. Plasmid pCAR26 contains two copies of the complete carbamoylase gene.

5

Example 17

Transfer of pCAR26 from *E. coli* to *Agrobacterium*

Like pCP19-derivatives, pKT210-based plasmids can be transferred by mating using the helper plasmid pRK2013. The method used for transfer of pCAR26 was similar to that described for pCAR1 in Example 12, but with the modifications described below. *E. coli* AG1 (pCAR26) was grown at 37°C for 16 hours in L-broth containing 25µg ml⁻¹ chloramphenicol; *E. coli* HB101 (pRK2013) and *Agrobacterium* 15-10 were grown as described in Example 12. Cells from these 3 cultures were filter-mated and then plated on MM containing 10µg ml⁻¹ chloramphenicol. After 5 days growth colonies were restreaked on L-agar containing 25µg ml⁻¹ streptomycin and 10µg ml⁻¹ chloramphenicol and incubated at 30°C for 3 days. Plasmid DNA was prepared from one isolate using the method described in Example 5. Restriction enzyme digestion of this DNA confirmed the presence of pCAR26.

20 Example 18

Construction of a mobilisation-defective derivative of pKT210

Approximately 2µg of pKT210 were digested with 5 units of PfIMI (New England Biolabs; c/o CP Laboratories P.O. Box 22 Bishop's Stortford, Herts CM23 3DH) in PfIMI buffer (100mM NaCl; 50mM Tris-HCl, pH7.9; 10mM MgCl₂; 1mM dithiothreitol; 100µg ml⁻¹ bovine serum albumin) at 37°C for 16 hours. The digested DNA was fractionated on a 0.75% agarose gel by electrophoresis in TBE buffer. The portion of the gel corresponding to the 10.5 kb fragment was excised, and the DNA was recovered by electroelution using a Model UEA electroeluter (International Biotechnologies Inc. PO Box 9558 New Haven, Connecticut CT 06535), by conditions described in the apparatus manual, followed by precipitation with isopropanol and then centrifugation to collect the DNA. The DNA was redissolved in 32µl of TE, 8µl of a mixture of 150mM sodium acetate (pH5.0), 250mM NaCl, 5mM zinc acetate, 25% glycerol were added, followed by 20 units of mung bean nuclease (New England Biolabs). This mixture was incubated at 37°C for 30 minutes; this should generate blunt ends on the DNA fragment. The digested DNA was fractionated, and the 10.5 kbp fragment isolated, as described above. The DNA was redissolved in 20µl of TE to which 5µl of 5 x ligation buffer (Gibco-BRL) and one unit of T4 DNA ligase were added. The ligation mixture was incubated at 12°C for 16 hours.

E. coli HB101 was transformed with 10 μ l of the ligation mix as described in Example 5. Dilutions of this mixture were spread onto L-agar plates containing 25 μ g ml⁻¹ of chloramphenicol. The plates were incubated at 37°C for 48 hours; only a few transformants were obtained and these grew slowly on the initial selection plates.

5 Sixteen colonies were restreaked onto L-agar plates containing 25 μ g ml⁻¹ chloramphenicol and incubated for 16 hours at 37°C after which the cells were scraped off and small-scale preparations performed using a modified version of the rapid boiling method (Holmes, D.S. and Quigley M. 1981. Anal. Biochem 114:p193). For each transformant, cells were resuspended in 300 μ l STET (8%

10 sucrose w/v; 0.5% Triton X-100 w/v; 50mM EDTA; 50mM Tris-HCl, pH8.0) in a microcentrifuge tube and 10 μ l of 33mg/ml of lysozyme in STET added. The suspension was incubated on ice for 30 minutes, and then placed in a boiling water bath for 3 minutes. The tube was centrifuged for 15 minutes and the pellet removed with a flat sided toothpick. The volume was corrected to 330 μ l with STET and a

15 further 330 μ l of isopropanol was added. The tube was shaken to mix the contents, centrifuged for 10 minutes and the supernatant was discarded. The pellet was allowed to dry, then resuspended in 30 μ l of TE. 2 μ l portions were digested with restriction enzymes to determine the restriction map of each plasmid. DNA was digested with the restriction enzyme AccI (Gibco-BRL), in a total volume of 10 μ l of

20 restriction buffer React 3 (50mM Tris-HCl, pH 8.0; 10mM MgCl₂; 100mM NaCl) containing 5 units of AccI for 2 hours at 37°C. Plasmid DNA from one of the clones appeared to have lost one of the AccI sites present in pKT210 (Figure 13) indicating the loss of the larger (526bp) PfIMI fragment of pKT210 as expected. However on digestion of this plasmid, designated pWOR901, with PfIMI it was found that only

25 one of two PfIMI fragments had been deleted, the smaller fragment remaining (Figure 15). This was not expected and suggested that the fragment isolated from the PfIMI digest must have included a fragment of approximately 10.9 kbp from a partial digest of pKT210 (Figure 13). The frequency of transfer of pWOR901 in triparental matings (performed using a method similar to that described in Example 17) was

30 found to be reduced by approximately 10⁴-10⁵ compared with the frequency of transfer of pKT210. 500ml of L-broth, supplemented with 25 μ g ml⁻¹ chloramphenicol, was inoculated with the isolate of *E. coli* HB101 containing pWOR901 and incubated overnight at 37°C on a gyrotory incubator. Plasmid DNA was isolated using the method in Example 4.

35

Example 19**Electroporation of pWOR901 into *Agrobacterium* 15-10 and Isolation of pWOR902**

Approximately 200ng of pWOR901 DNA (Example 18) was used to
5 transform *Agrobacterium* 15-10 by high voltage electroporation (Wen-jun.S. and Forde
B.G. 1989. Nucleic Acids Research 17: p8385). The greatest efficiency occurred
when a resistance of 600 ohms was applied. Transformants were selected on L-agar
plates containing 10µg ml⁻¹ chloramphenicol, grown at 30°C for 4 days. Twenty-two
10 small scale plasmid preparations were performed from cells of restreaked
transformant colonies using the rapid boiling method described in Example 18.
Restriction digests with 5 units PstI were performed on plasmid DNA in React 2
restriction buffer (50mM Tris-HCl, pH 8.0; 10mM MgCl₂; 50mM NaCl) for 2 hours
at 37°C. From these results various restriction patterns were observed. Examples of
15 the different types of plasmid were used to transform *E. coli* HB101 (using the method
of Example 5). Only one plasmid, when used to transform *E. coli*, gave stable
chloramphenicol resistant colonies. Digests of plasmid DNA prepared from these
chloramphenicol-resistant colonies showed that a further deletion of approximately
500bp had occurred. This deletion completely removed the small PfMI fragment of
20 pWOR901. The new plasmid was designated pWOR902. A large-scale preparation
of DNA was performed on a 400ml culture of *E. coli* HB101 (pWOR902). This DNA
had no sites for PfMI (map shown in Figure 16). After introduction by
electroporation pWOR902 appeared to be stably maintained in *Agrobacterium* 15-10.
Thus pWOR902 is a stable, mobilisation-deficient (Mob⁻) derivative of pKT210,
suitable for use in *E. coli* and in *Agrobacterium*. *E. coli* HB101 (pWOR902) was
25 deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen,
Scotland on 4th November 1991 under the accession number NCIMB 40451. The
deposit was made under the Budapest Treaty on the deposition of micro-organisms
for the purposes of patent procedure.

30 Example 20**Construction of pCAR44 and transfer to *Agrobacterium***

Approximately 2µg of plasmid pCAR21 (Example 7) was digested with 5
units of HindIII at 37°C for 3 hours in HindIII digestion buffer. The 1.9kb fragment
was isolated by electroelution from an agarose gel slice after fractionation by
35 electrophoresis in TBE (Example 18). The recovered DNA was redissolved in 10µl
of TE.

Approximately 0.5µg of pWOR902 (Example 19) was digested with HindIII,
CIAP-treated and redissolved in 10µl of TE. In an attempt to obtain multiple inserts
an excess of fragment (from pCAR21) to vector was ligated: 0.1µl of cut pWOR902

International Application No: PCT/ /

MICROORGANISMS	
Optional Sheet in connection with the microorganism referred to on page <u>26</u> , line <u>26</u> of the description *	
A. IDENTIFICATION OF DEPOSIT :	
Further deposits are identified on an additional sheet <input type="checkbox"/> *	
Name of depositary institution *	
NATIONAL COLLECTION OF INDUSTRIAL AND MARINE BACTERIA LIMITED	
Address of depositary institution (including postal code and country) *	
Torry Research Station, P O Box 31, 135 Abbey Road, Aberdeen, United Kingdom AB9 8DG	
Date of deposit *	Accession Number *
4 November 1991	NCIMB 40451
B. ADDITIONAL INDICATIONS ? (leave blank if not applicable). This information is continued on a separate attached sheet <input type="checkbox"/>	
In respect of those designations in which a European Patent is sought, a sample of the deposited micro-organism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample.	
C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE * (if the indications are not for all designated States)	
D. SEPARATE FURNISHING OF INDICATIONS * (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")	
E. <input checked="" type="checkbox"/> This sheet was received with the international application when filed (to be checked by the receiving Office)	
<div style="font-family: cursive; font-size: 1.2em; margin: 0;">Kerry A Haddan</div> (Authorized Officer)	
<input type="checkbox"/> The date of receipt (from the applicant) by the International Bureau is:	
was _____	
(Authorized Officer)	

and 10 μ l of the 1.9 kb fragment were mixed in a total volume of 15 μ l of ligation buffer (Gibco-BRL) containing 1 unit of T4 DNA ligase for approximately 16 hours at 12°C. 5 μ l of the ligation mixture was used to transform *E. coli* HB101 (using a method similar to that described in Example 5) and the cells were spread on L-agar plates containing 25 μ g ml⁻¹ chloramphenicol and incubated at 37°C for 2 days. 24 transformants were restreaked on L-agar containing 25 μ g ml⁻¹ chloramphenicol, grown overnight at 37°C, and then cells were scraped from the plate and used for small-scale plasmid preparations (Example 18). To determine the number of inserts, and their orientations, an EcoRI digestion was performed on an aliquot of DNA from each preparation. Analysis of the digested DNA showed that 5 of the clones contained double inserts of the fragment from pCAR21; all of these plasmids showed the same insert orientation, and this type of plasmid was designated pCAR44 (map in Figure 17). 16 of the clones contained one insert of the pCAR21 fragment in pWOR902. A large scale plasmid preparation was performed on a 500 ml culture of one of the *E. coli* HB101 (pCAR44) isolates (method of Example 4) and 200ng of this DNA was used to transform *Agrobacterium* 15-10 by electroporation (Example 19). Transformants were subjected to small-scale plasmid preparations; restriction enzyme digests confirmed the presence of pCAR44.

20 Example 21

Demonstration of extra carbamoylase activity associated with pCAR26 and pCAR44 in *Agrobacterium*

Agrobacterium 15-10 was inoculated into 50ml of AJ-1 broth; *Agrobacterium* 15-10 (pCAR26) and *Agrobacterium* 15-10 (pCAR44) were each inoculated into 50 ml of AJ-1 broth containing 10 μ g ml⁻¹ chloramphenicol. All three cultures were grown for 24 hours in a gyrotory incubator at 30°C; then each culture was treated as follows:- 1.36 ml of culture broth was mixed in a microcentrifuge tube with 140 μ l of 1% hexadecyltrimethylammonium bromide (in 65mM Na₂HPO₄, 35mM NaH₂PO₄, pH7.0) and incubated at room temperature for 10 minutes. 50 μ l of this mixture was removed to a microcentrifuge tube containing 1.45ml of 1.5% (w/v) D-N-carbamoyl-p-hydroxyphenylglycine in 65mM Na₂HPO₄ 35mM NaH₂PO₄ (pH7.0; readjusted to pH7.0 after dissolving the reaction substrate). The contents of the tube were mixed and incubated at 48°C for 20 minutes. The cells were collected by centrifugation and the supernatant was analysed by HPLC for the presence of D(-)p-hydroxy phenyl glycine produced by the carbamoylase enzyme. The carbamoylase activities for *Agrobacterium* 15-10 containing pCAR26 or pCAR44, per ml of culture broth, were 10-30 times higher than the activity for *Agrobacterium* 15-10, presumably because the extra copies of the carbamoylase gene in the recombinant strains lead to more carbamoylase enzyme being produced.

Example 22**Construction of pWOR903**

Approximately 2µg of pWOR902 DNA (Example 19) were digested with 10
5 units of Hind III at 37°C for 15 hours in Hind III digestion buffer. The DNA was
purified using phenol/chloroform extraction and chloroform extraction, precipitated
using NaOAc and ethanol, recovered by centrifugation and redissolved in 40µl of
10mM Tris HCl pH7.4 10mM MgCl₂, 50mM NaCl, 50µg ml⁻¹ bovine serum
albumin, 10mM 2-mercaptoethanol. 1µl of a mixture of 250µM
10 deoxyguanosine-triphosphate, 250µM deoxy-thymidinetriphosphate and 250µM
deoxycytidine-triphosphate was added followed by 2 units of "Klenow fragment" of
E. coli DNA polymerase I (Gibco-BRL). The tube was incubated at room temperature
for one hour, extracted with phenol/chloroform and extracted with chloroform. The
recovered aqueous phase was treated with NaOAc and ethanol to precipitate the
15 DNA, which was recovered by centrifugation and redissolved in 15µl of DNA ligase
buffer. One unit of T4 DNA ligase was added and the mixture was incubated at 12°C
for about 16 hours.

5µl of the ligation mixture was used to transform *E. coli* HB101 (using a
method similar to that described in Example 5) and the cells were spread on L-agar
20 plates containing 25µg ml⁻¹ chloramphenicol and incubated at 37°C for 2 days. 16
transformants were restreaked on L-agar containing 25µg ml⁻¹ chloramphenicol,
grown overnight at 37°C, and then cells were scraped from the plate and used for
small-scale plasmid preparations (Example 18).

The object of this experiment was to fill-in the recessed ends generated by
25 Hind III digestion to create blunt-ended DNA fragments. After religation this would
remove the recognition sequence for Hind III. In order to determine whether this had
occurred aliquots of DNA from the plasmid preparations were digested with Hind III.
Further aliquots were digested with EcoRV. Most of the transformants appeared to
contain plasmid with no Hind III sites but with similar EcoRV digestion patterns to
30 pWOR902. A large scale plasmid preparation was performed a 500ml culture of on
one of these isolates (method of Example 4). This plasmid, pWOR903 (map shown
in Figure 18), has a restriction map like that of pWOR902 (Figure 16) but it lacks the
Hind III site.

35 Example 23**Construction of pWOR904 and pWOR905**

Approximately 1µg of pWOR903 DNA (Example 22) was digested with 5
units of EcoRI at 37°C for 3 hours in EcoRI buffer. The mixture was extracted with
phenol/chloroform, extracted with chloroform and the DNA was recovered by

precipitation with NaOAc and ethanol. The recovered DNA was dissolved in 50µl of 50mM Tris-HCl (pH8.0), 0.1mM EDTA (pH8.0) containing 1 unit of CIAP. This phosphatase treatment, and recovery of the DNA, was as described in Example 2. The DNA was redissolved in 10µl of water.

5 The plasmid pIC2OR (Marsh et al Gene 32 (1984) pp481-485) is similar to the pUC plasmids (Vieira and Messing Gene 19 (1982) pp259-268) but with a modified polylinker. Approximately 2µg of pIC2OR DNA were digested with 5 units of EcoRI at 37°C for about 15 hours in EcoRI buffer. The 84bp fragment corresponding to the polylinker segment of pIC2OR was recovered by electroelution
10 from an agarose gel slice after fractionation by electrophoresis in TBE buffer (Example 18). The recovered DNA was redissolved in 10µl of water.

8µl of the 84bp EcoRI fragment of pIC2OR was mixed with 0.5µl of the EcoRI-cut, CIAP-treated pWOR903 in a total volume of 12µl of ligation buffer containing 1 unit of T4 DNA ligase for approximately 16 hours at 12°C. 5µl of the
15 ligation mixture was used to transform *E. coli* JM101 (using a method similar to that described in Example 5) and the cells were spread on L-agar plates containing 25µg ml⁻¹ chloramphenicol and incubated at 37°C for 2 days. 64 transformants were restreaked on L-agar containing 25µg ml⁻¹ chloramphenicol, grown overnight at 37°C, and then cells were scraped from the plate and used for small-scale plasmid
20 preparations (Example 18).

Aliquots of the DNA preparations were digested with HindIII to detect the presence of the polylinker. Further digestions were performed with PstI, SstI, and double digests with BglII and SstII, to determine the orientation of the polylinker. Isolates containing the polylinker in either orientation were obtained; large scale
25 plasmid preparations were performed on 500ml cultures of one of each type of isolate (using the method of Example 4). Restriction maps of the plasmids, designated pWOR904 and pWOR905, are shown in Figures 19 and 20.

Example 24

30 Construction of pCAR46 and transfer to *Agrobacterium*

Approximately 2µg of pCAR29 DNA (Example 10) were digested with 5 units of BamHI and 5 units of HindIII in a total of 50µl of 50mM Tris-HCl (pH8.0), 10mM MgCl₂, 50mM NaCl for 3 hours at 37°C. The 1.35kb fragment was isolated
35 (Example 18). The recovered DNA was redissolved in 10µl TE.

Approximately 2µg of pWOR904 DNA (Example 23) were digested with HindIII, followed by digestion with BglII. The DNA was then treated with CIAP (Example 2) and finally redissolved in 10µl TE.

1µl of the cut pWOR904 was mixed with 4µl of the 1.35kb fragment of

pCAR29 in a total volume of 12 μ l of ligation buffer containing one unit of T4 DNA ligase and incubated at 12°C for approximately 16 hours.

5 5μ l of the ligation mixture was used to transform *E. coli* DH5 α (using a method similar to that described in Example 5) and the cells were spread on L-agar plates containing 25 μ g ml⁻¹ chloramphenicol and incubated at 37°C for 2 days. 15 transformants were restreaked on L-agar containing 25 μ g ml⁻¹ chloramphenicol, grown overnight at 37°C, and then cells were scraped from the plate and used for small-scale plasmid preparations (Example 18).

10 Aliquots of DNA were digested with *Eco*RI, and with *Pst*I, to confirm the presence of the insert derived from pCAR29. A large scale plasmid preparation was performed on a 500ml culture of one of the isolates (using the method of Example 4). A restriction map of the plasmid, designated pCAR46, is shown in Figure 21. 200ng of this DNA was used to transform *Agrobacterium* 15-10 by electroporation (Example 19). Transformants were subjected to small scale plasmid preparations; restriction 15 enzyme digests confirmed the presence of pCAR46. *Agrobacterium* 15-10 (pCAR46) was deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland on 14th February, 1992, under the accession number NCIMB 40478. The deposit was made under the Budapest Treaty on the deposition of micro-organisms for the purposes of patent procedure.

20

Example 25

Demonstration of extra carbamoylase activity associated with pCAR46 in *Agrobacterium*

25 *Agrobacterium* 15-10 was inoculated into 50ml of AJ-1 broth; *Agrobacterium* 15-10 (pCAR46) was inoculated into 50 ml of AJ-1 broth containing 10 μ g ml⁻¹ chloramphenicol. The cultures were grown for 24 hours in a gyrotory incubator at 30°C; then each culture was treated as follows:-

30 1.36 ml of culture broth was mixed in a microcentrifuge tube with 140 μ l of 1% hexadecyltrimethylammonium bromide (in 65mM Na₂HPO₄, 35mM NaH₂PO₄, pH7.0) and incubated at room temperature for 10 minutes. 50 μ l of this mixture was removed to a microcentrifuge tube containing 1.45ml of 1.5% (w/v) D-N-carbamoyl-p-hydroxyphenylglycine in 65mM Na₂HPO₄ 35mM NaH₂PO₄ (pH7.0; readjusted to pH7.0 after dissolving the reaction substrate). The contents of the tube were mixed and incubated at 48°C for 20 minutes. The cells were collected by centrifugation and 35 the supernatant was analysed by HPLC for the presence of D(-)p-hydroxy phenyl glycine produced by the carbamoylase enzyme. The carbamoylase activity for *Agrobacterium* 15-10 containing pCAR46, per ml of culture broth, was 20-30 times higher than the activity for *Agrobacterium* 15-10.

Example 26**Construction of pCAR31 and pCAR32**

Approximately 2 μ g of pCAR6 (Example 5) DNA were digested with 5 units of Cla I in a total of 50ml of 50mM Tris-HCl (pH 8.0), 10mM MgCl₂ for 3 hours at 37°C. The digested DNA was fractionated on a 0.8% low melting point agarose gel by electrophoresis in TBE buffer. The portion of gel containing the ca. 4.7 kb Cla I fragment was excised and the DNA was recovered using the method described in Example 5.

Approximately 0.6 μ g of Cla I digested, CIAP-treated pCP19 DNA (Example 2) and about 0.6 μ g of the 4.7kb Cla I fragment from pCAR6 were combined in a total volume of 20 μ l of DNA ligase buffer. One unit of T4 DNA ligase was added and the mixture was incubated at 4°C for about 16 hours.

10 μ l of the ligation mix was used to transform *E. coli* DH5 α (using a method similar to that described in Example 5) and the cells were spread on L-agar plates supplemented with 10 μ g ml⁻¹ tetracycline and incubated overnight at 37°C. 18 tetracycline resistant colonies were restreaked on L-agar plates containing 10 μ g ml⁻¹ tetracycline, incubated at 37°C overnight and then small scale plasmid DNA preparations (using method described in Example 5) were performed on cells of each of these isolates. Restriction enzyme digests of the DNA preparations indicated clones with both orientations of the Cla I fragment in the vector were obtained. These vectors were designated pCAR31 and pCAR32 (Figures 22 and 23).

500 ml cultures of *E. coli* DH5 α (pCAR31) and *E. coli* DH5 α (pCAR32) were prepared in L-broth containing 10 μ g ml⁻¹ tetracycline. Both cultures were treated as follows:-

The culture was centrifuged at ca 6000 x g for 10 minutes at 4°C and the resulting cell pellet was re-suspended in 10 ml of 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA, 5 mg ml⁻¹ of lysozyme. The mixture was incubated at room temperature for 15 minutes. 10 mls of 0.2 M NaOH, 1% SDS solution was added to the lysozyme treated cells and solutions were mixed and incubated on ice for 20 minutes. 15 mls of ice cold 3M potassium acetate (pH 5.8) was added to the reaction mixture and solutions were mixed and incubated on ice for a further 60 minutes. The lysed cells were centrifuged at approximately 38000 x g for 30 minutes at 4°C. The supernatants were collected and 0.6 volumes of isopropanol added and DNA allowed to precipitate for 15 minutes at room temperature. The reaction mixture was centrifuged at approximately 6000 x g for 30 minutes at room temperature and precipitated DNA re-suspended in 4.0 mls of TE. CsCl gradients were then prepared (as per Example 4) and plasmid

DNA was re-dissolved in 500µl of TE. Further restriction enzyme digestion of this DNA confirmed the correct structure of pCAR31 and pCAR32.

Example 27

5 **Transfer of pCAR31 and pCAR32 from E. coli to *Agrobacterium***

pCAR31 and pCAR32 were transferred to *Agrobacterium* 15-10 by mating using the helper plasmid pRK2013 (as described for pCAR1 in Example 12). Plasmid DNA was prepared from isolates using the method described in Example 5. Restriction enzyme digestion of portions of the plasmid DNA was used to
10 confirm that the tetracycline resistant ex-conjugants did contain either pCAR31 or pCAR32.

Example 28

15 **Demonstration of extra Hydantoinase Activity Associated with pCAR31 and pCAR32**

Agrobacterium 15-10 (pCAR1), *Agrobacterium* 15-10 (pCAR6), *Agrobacterium* 15-10 (pCAR26), *Agrobacterium* 15-10 (pCAR31), *Agrobacterium* 15-10 (pCAR32), and *Agrobacterium* 15-10 were each inoculated into separate flasks containing 50 mls of AJ-1 broth containing 0.1% alanine hydantoin and the appropriate antibiotic. The
20 cultures were grown for 24 hours in a gyrotory incubator at 30°C. The resultant cultures were assayed for hydantoinase activity (as per Example 15). It was confirmed that pCAR1 and pCAR6, but not pCAR26, give increased hydantoinase activity in *Agrobacterium* 15-10. Strains containing pCAR31 or pCAR32 gave hydantoinase activities approximately 20
25 times higher than the activity for *Agrobacterium* 15-10 indicating that pCAR31 and pCAR32 contain the gene encoding hydantoinase activity.

Example 29

Construction of pCAR36

30 Approximately 2µg of pCAR31 (Example 26) DNA were digested with 5 units of Hind III in a total of 50µl of 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 50 mM NaCl for 3 hours at 37°C. The digested DNA was fractionated on a 0.8% low melting point agarose gel by electrophoresis in TBE buffer. The portion of gel containing the ca 4.7 kb Hind III fragment was excised and the DNA was recovered
35 using the method described in Example 5.

Approximately 0.6µg of Hind III digested, CIAP treated pKT210 DNA (Example 16) and about 0.6µg of the 4.7 kb Hind III fragment from pCAR31 were combined in a total volume of 20µl of DNA ligase buffer. One unit of T4 DNA ligase was added and the mixture incubated at 4°C for 16 hours.

10µl of the ligation mix was used to transform *E. coli* HB101 competent cells (using a method similar to that described in Example 5) and the cells were spread on L-agar plates supplemented with 25µg ml⁻¹ chloramphenicol. 17 chloramphenicol resistant colonies were re-streaked on L-agar plates containing
5 25µg ml⁻¹ chloramphenicol, incubated at 37°C overnight and then small scale plasmid DNA preparations (using the method described in Example 5) were performed on cells of each of these isolates. Restriction enzyme digests confirmed the presence of pCAR36. A restriction digest map is shown in Fig. 24.

500 mls of L-broth supplemented with 25µg ml⁻¹ chloramphenicol was
10 inoculated with *E. coli* HB101 (pCAR36) and incubated overnight at 37°C on a gyrotory incubator. Plasmid DNA was isolated using the method in Example 26.

Example 30

15 Transfer of pCAR36 from *E. coli* to *Agrobacterium*

pCAR36 was transferred to *Agrobacterium* by mating using the helper plasmid pRK2013 (as described for pCAR1 in Example 12). Plasmid DNA was prepared from isolates using the method described in Example 5. Restriction enzyme digestion of portions of the plasmid DNA was used to confirm that the
20 chloramphenicol resistant ex-conjugants did contain pCAR36.

Example 31

Demonstration of Extra Hydantoinase Activity associated with pCAR36

Agrobacterium 15-10 and *Agrobacterium* 15-10(pCAR36) were each
25 inoculated into separate flasks containing 50 ml of AJ-1 broth containing 0.1% alanine hydantoin (plus 10µg ml⁻¹ chloramphenicol for *Agrobacterium* 15-10 (pCAR36) culture), and grown for 24 hours in a gyrotory incubator at 30°C. The resultant cultures were assayed for hydantoinase activity (as per Example 15). The hydantoinase activities for *Agrobacterium* 15-10 containing pCAR36 were 6-7
30 times higher than the activity for *Agrobacterium* 15-10 confirming that pCAR36 contains the gene encoding hydantoinase activity.

Example 32

Construction of pDAN3 and pDAN4

35 Approximately 2µg of pCAR36 (Example 29) DNA were digested with 5 units of Sst I (SacI) in a total of 50 µl of 50 mM Tris-HCl (pH8.0), 10 mM MgCl₂, 50 mM NaCl for 3 hours at 37° C. The digested DNA was fractionated on a 0.8% low melting point agarose gel by electrophoresis in TBE buffer. The portion of the gel containing the ca 3.3 kb Sst I fragment was excised and the DNA

was recovered using the method described in Example 5.

Approximately 0.5 μ g of Sst I digested, CIAP treated pWOR902 DNA(Example 19) and 0.5 μ g of the 3.3 kb Sst I fragment from pCAR36 were combined in a total volume of 20 μ l of DNA ligase buffer. One unit of T4 DNA
5 ligase was added and the mixture was incubated at 4°C for 16 hours.

10 μ l of the reaction mix was used to transform *E. coli* HB101 competent cells (using the method described in Example 5) and the cells were spread on L-agar plates supplemented with 25 μ g ml⁻¹ chloramphenicol. 4 chloramphenicol resistant colonies were re-streaked on L-agar plates containing 25 μ g ml⁻¹ chloramphenicol,
10 incubated at 37° C overnight and then small scale plasmid DNA preparations (using the method described in Example 5) were performed on cells of each of these isolates. Restriction enzyme digests of the DNA preparations indicated clones with either orientation of the Sst I fragment in the vector were obtained. These were designated pDAN3 and pDAN4 and restriction maps are shown in
15 Figs. 25 and 26.

500 mls of L-broth supplemented with 25 μ g ml⁻¹ chloramphenicol was inoculated with *E. coli* HB101 (pDAN3) and a further 500 mls of L-broth supplemented with 25 μ g ml⁻¹ chloramphenicol was inoculated with *E. coli* HB101 (pDAN4) and incubated overnight at 37°C on a gyrotory incubator. Plasmid DNA
20 was isolated using the method described in Example 26.

Example 33

Electroporation of pDAN3 and pDAN4 into *Agrobacterium*

Approximately 200 ng of either pDAN3 or pDAN4 was used to
25 transform *Agrobacterium* 15-10 by electroporation (Example 19). Transformants were subjected to small scale plasmid preparations; restriction enzyme digests confirmed the presence of pDAN3 and pDAN4.

Example 34

30 Demonstration of Extra Hydantoinase Activity Associated with pDAN3 and pDAN4

Agrobacterium 15-10 (pCAR36), *Agrobacterium* 15-10 (pWOR902),
Agrobacterium 15-10 (pDAN3) and *Agrobacterium* 15-10 (pDAN4) were each inoculated into separate flasks containing 50 ml of AJ1 medium (Example 1),
35 supplemented with 5g l⁻¹ (NH₄)₂SO₄ and 10 μ g ml⁻¹ chloramphenicol, and grown for 24 hours at 30°C. Each of the cultures were then treated as follows:- 500 μ l of 1M MnSO₄ solution was added to the culture which was then shaken at 30°C for a further 35 minutes. The broth was assayed in the following way:- 1360 μ l of broth either neat or diluted was added to 140 μ l of 1% cetyltrimethylammonium

bromide solution in an eppendorf tube, mixed and incubated at room temperature for 5 minutes. 1 ml of this solution was added to 19 mls of 1% D, L-5-(p hydroxyphenyl) hydantoin in 0.2M Tris in a 50 ml screw capped conical flask and shaken in a water bath at 42° C. Samples were removed after incubation for 5 10 minutes and 30 minutes and assayed using an HPLC (Example 15). The hydantoinase activity in μ moles of product per ml of cells per hour was calculated.

It was demonstrated that pCAR36, pDAN3 and pDAN4 give increased hydantoinase activity in *Agrobacterium* 15-10. Strains containing pDAN4 gave hydantoinase activities approximately 230 times higher than the activity for 10 *Agrobacterium* 15-10 containing pWOR902.

Example 35

Construction of pGal2789RS3Carb

A copy number mutant of the naturally occurring *E. coli* plasmid NR1 was 15 obtained by spontaneous mutation. This plasmid (pRR21) was then reduced in size by deleting un-needed regions to yield pDPT 2789. This is a 125 copy plasmid containing the inc F11 region and Chloramphenicol and Spectinomycin resistance markers. pDPT 2789 was then cut with NdeI, filled in and religated to make pDTP2789 (Nde⁻). pDPT 2789 (Nde⁻) was then digested with StuI and EspI and a 20 StuI-EspI fragment containing a Pgal promoter with a synthetic ribosome binding site downstream was inserted. This vector was called pGal2789RS3V1V2.

pGal2789RS3V1V2 was digested with NdeI, filled-in with Klenow, and then digested with BamHI. pCAR21 (Example 7) was digested with BspHI, filled-in with Klenow, and then digested with BamHI. These digestions released an ~900bp 25 fragment containing the sequences coding for the carbamoylase gene resulting in the plasmid pGal2789RS3Carb that expresses the carbamoylase gene off the Gal promoter.

This plasmid was then transformed into a number of *E. coli* K12 host strains by standard CaCl₂ procedures and production of carbamoylase was monitored during 30 cell growth by Coomassie blue staining of SDS-PAGE gels on which total cell extracts had been run.

CLAIMS

1. A process for the production of a carbamoylase enzyme which has the capability of converting a D-N-carbamoyl (optionally substituted phenyl) glycine into
5 the corresponding D-(optionally substituted phenyl)glycine by expressing recombinant DNA encoding a carbamoylase gene in a homologous host.
2. A process according to claim 1 wherein the homologous host is *Agrobacterium*.
- 10 3. A carbamoylase enzyme obtainable by the process of claim 1 or 2.
4. Recombinant DNA vector comprising a gene encoding a carbamoylase enzyme which has the capability of converting a D-N-carbamoyl (optionally substituted phenyl)glycine into the corresponding D- (optionally substituted phenyl)
15 glycine wherein the DNA vector is selected from the group consisting of pCAR1, pCAR6, pCAR12, pCAR21, pCAR26, pCAR27, pCAR28, pCAR29, pGal2789RS3Carb, pCAR31, pCAR32, pCAR36, pCAR44 and pCAR46.
5. Isolated DNA encoding a gene for a carbamoylase enzyme as defined in claim
20 1 and a gene for a hydantoinase enzyme which has the ability to convert a D,L- (optionally substituted phenyl) hydantoin into the corresponding D-N-carbamoyl (optionally substituted) phenyl glycine.
6. Recombinant DNA comprising the DNA of claim 5.
25
7. A recombinant DNA vector according to claim 6 which is pCAR1, pCAR6, pCAR31, pCAR32, or pCAR36.
8. Isolated DNA encoding a gene for a hydantoinase enzyme which has the
30 ability to convert a D,L - (optionally substituted phenyl) hydantoin into the corresponding D-N-carbamoyl (optionally substituted phenyl) glycine.
9. Recombinant DNA comprising the DNA of Claim 8.
- 35 10. A recombinant DNA vector according to claim 9 which is pDAN3 or pDAN4.
11. A process for transforming a host cell with recombinant DNA according to any one of claims 4, 6, 7, 9 or 10 which comprises mixing together the host and the recombinant DNA under conventional transformation or electroporation conditions.

12. A host cell transformed with a recombinant vector according to any one of claims 4, 6, 7, 9 or 10.
- 5 13. A host cell according to claim 12 which is *Agrobacterium* or *E. coli*.
14. A method of producing carbamoylase and/or hydantoinase activity in a host cell which comprises:
- 10 (a) introducing into said host cell DNA as defined in any one of claims 4 to 10 said DNA positioned for expression from a transcriptional and translational activating sequence functional in said host cell; and
- (b) culturing said host cell transformed in step (a) under conditions which allow for the expression of said DNA.
- 15 15. A method according to claim 14 wherein the DNA is a vector based on pWOR902 or derivatives thereof.

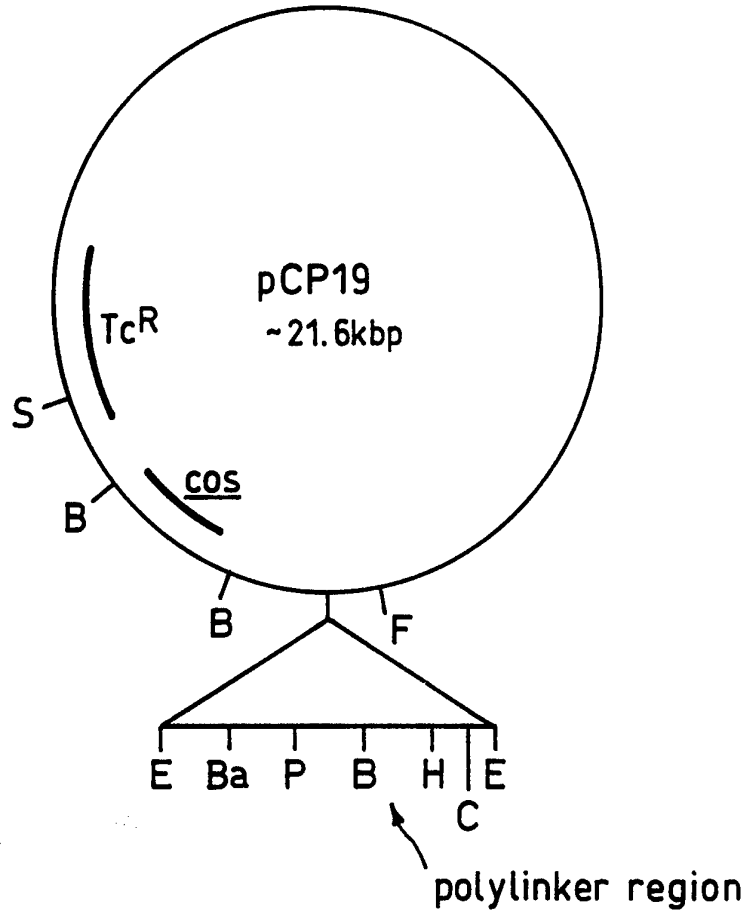
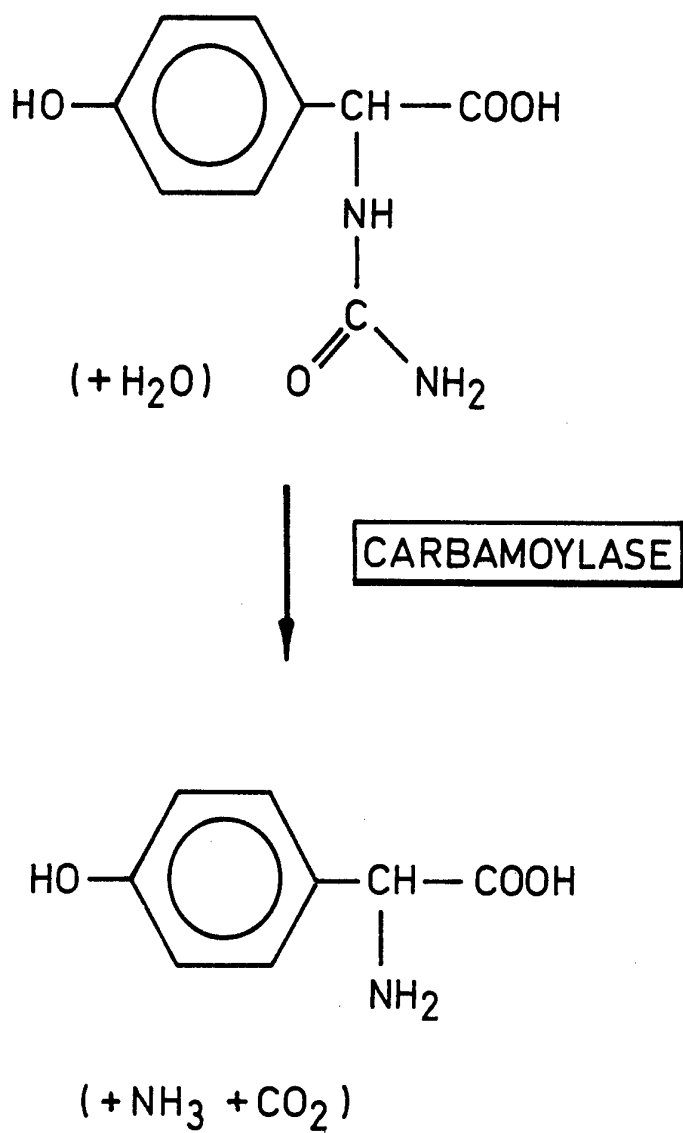


Fig.1

**Fig. 2**

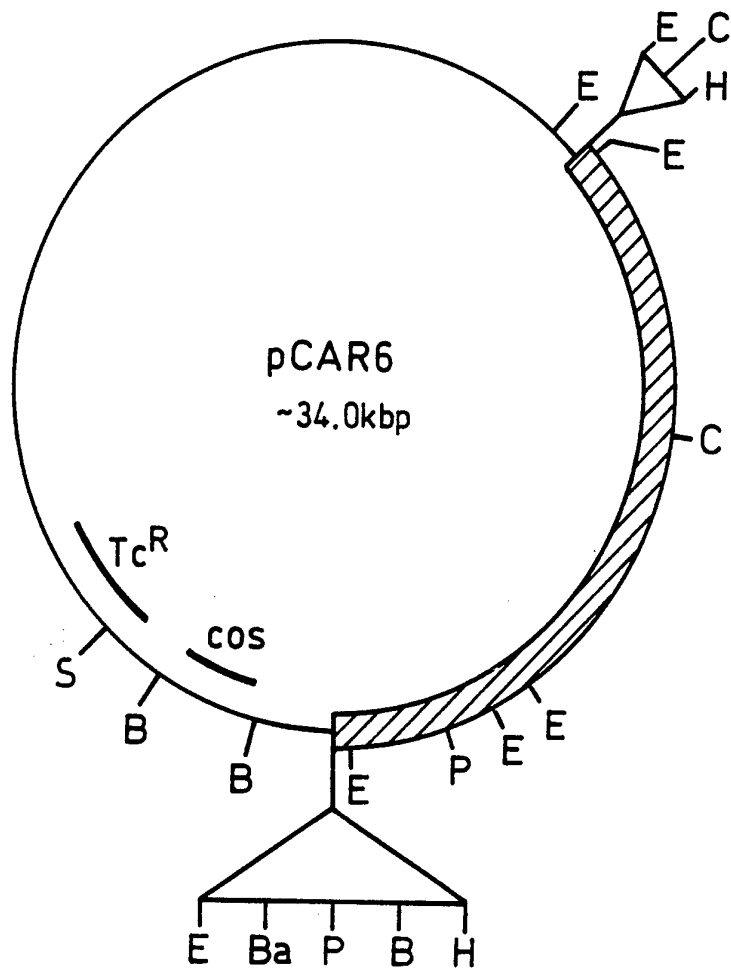


Fig. 4

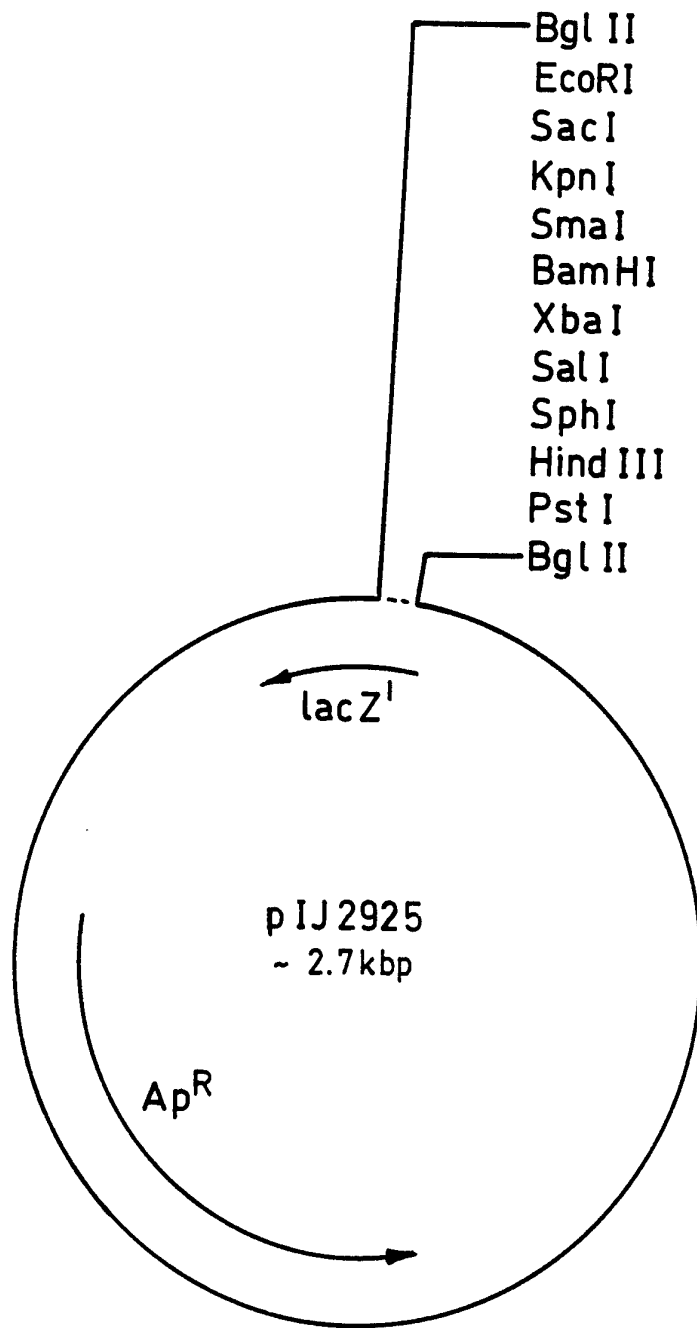


Fig.5

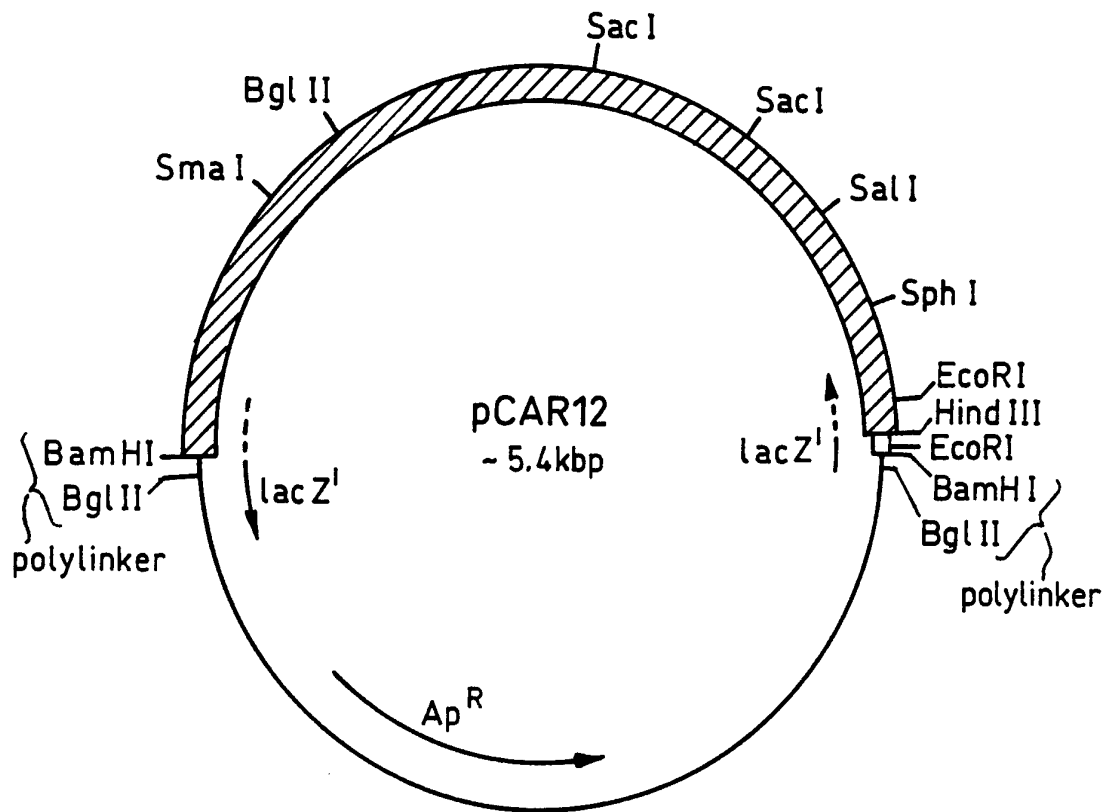


Fig. 6

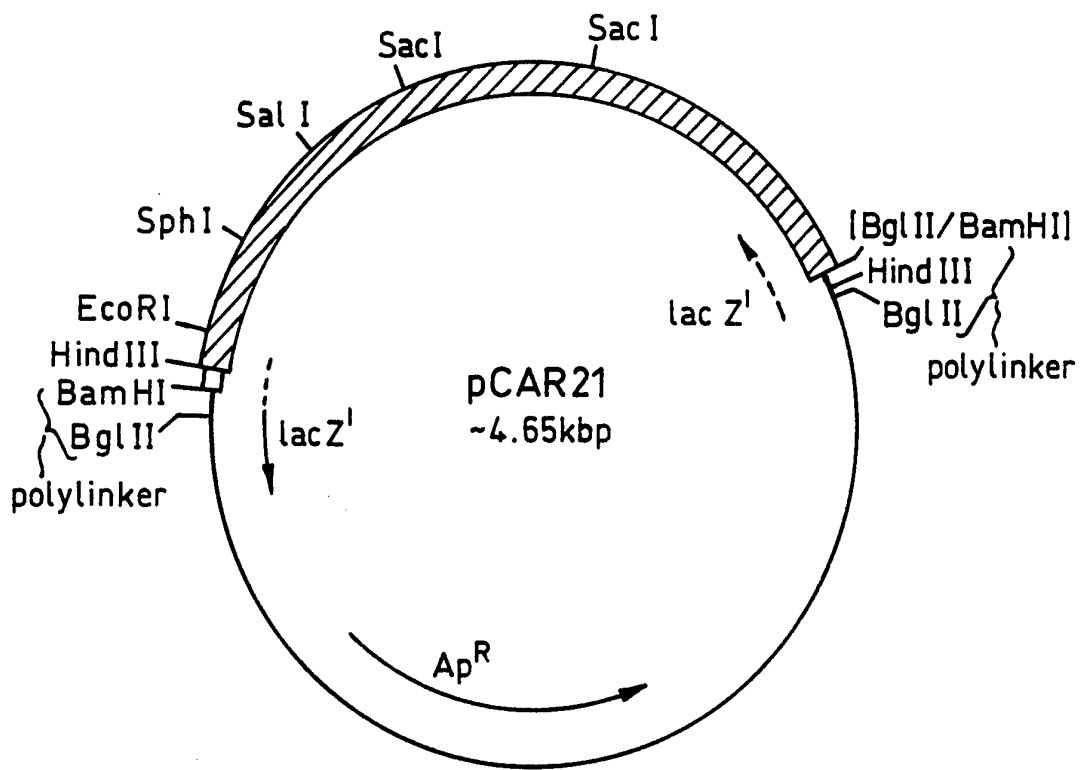


Fig. 7

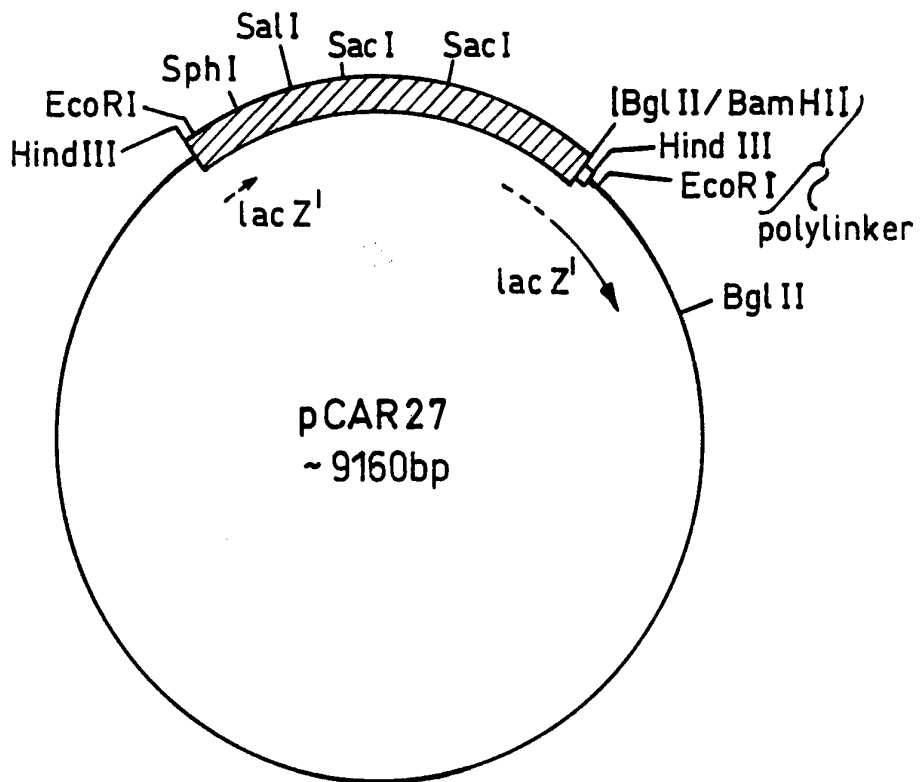


Fig. 8

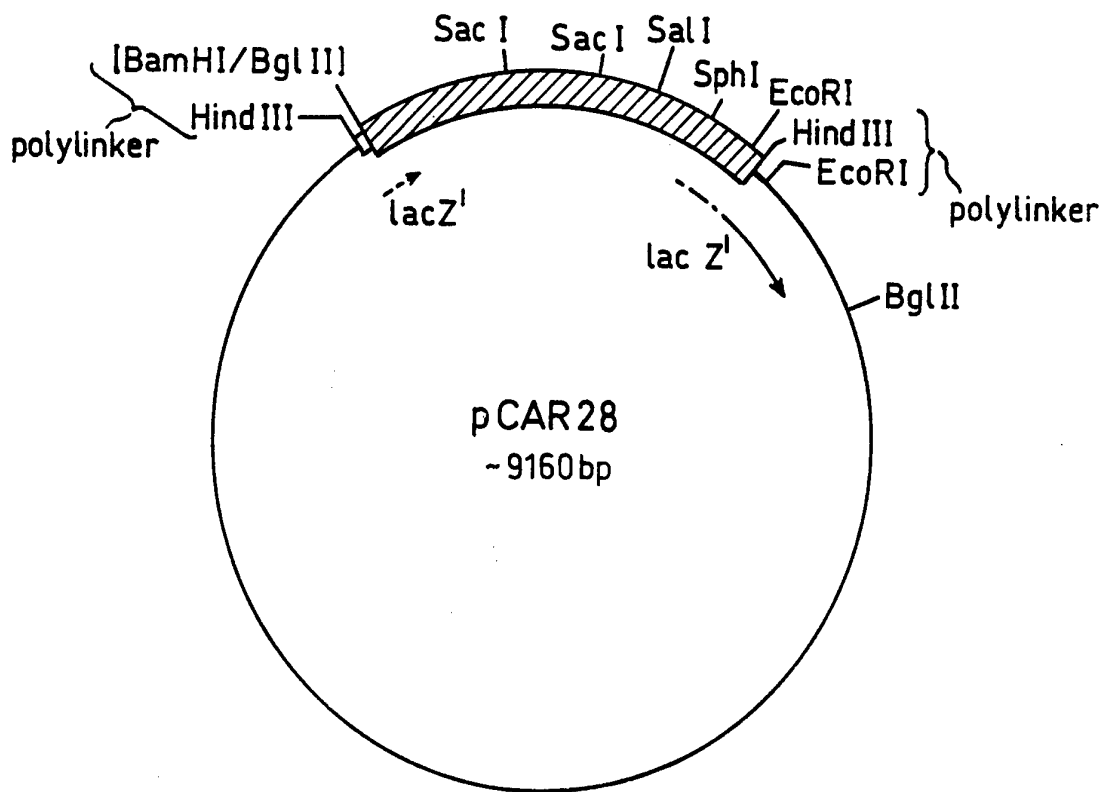


Fig.9

FIG. 10 (a)

```

AGATCTGGCAGTACCTCTAGCTCCTCGATCAGCTATCAGTCCGATCGAGCACGATGATA
  BglII  ^10      ^20      ^30      ^40      ^50      ^60
TGGTAGCCGTAGTCGATCGCCGACTTCCCGCCGGCCATGCCGTCCATTGGCGACCGCC
  ^70      ^80      ^90      ^100     ^110     ^120
TCCCTCAGGCTATGGCCGCGTCTGCTGGCAGAAATCGACGATGGTCGTGCGGCCA
  ^130     ^140     ^150     ^160     ^170     ^180
CAGGCGGCGCGACCGTCGCGGTTGCGAATGTGTCCGCCACTGCGTGTGAAGCTGACCG
  ^190     ^200     ^210     ^220     ^230     ^240
TCTCGACATGCGTATGAACGTCGATGCCGCCCGAAAAACGTAGCGGCCGGAGGCGTCGA
  ^250     ^260     ^270     ^280     ^290     ^300
TTTGTCCGGCCGGCCGGCCGAACGTTCCGCCGATCTGGGCGATCTTGCCATCCTTGATT
  ^310     ^320     ^330     ^340     ^350     ^360
CCGAGATCGGCGGGAGAAATCCCGTCCGCGTTACGATGGTTCCGTTCTTGATGATGATA
  ^370     ^380     ^390     ^400     ^410     ^420
TCCATAAAGCAGCTCTCAGGGTTGATGGATAAATTCTATATCGGGTATGATGTTCTTTAT
  EcoRV  ^430     ^440     ^450     ^460     ^470     ^480
ATAAAGTTTTTCATGTTGCCTTGATCTGTCAAGCGGGAAGGGAAGTTCTCCGGAATCGGC
  ^490     ^500     ^510     ^520     ^530     ^540
GCTGCGAGGGAACGATCGAGTTTCGATTAGACGCGGTTGAAAGCGAGCGGTCATTGAAT
  ^550     ^560     ^570     ^580     ^590     ^600
ACGGAACCTCTGCCAACCTATTCCGCGAGCTGGATTTTTTCTTCTCGTTCGCGAGCTCC
  ^610     ^620     ^630     ^640     ^650     ^660
TAAACGGCGCCGTTCAATCCGGGTGAAAAAGTTCAACCATCGGAAATTTTGACCCTGGT
  SacI   ^670     ^680     ^690     ^700     ^710     ^720
CCTTGACAGATCAAAGTTTTACGCCTGTAGTATGAGTACTGCATGTGGCATTATCCTT
  ^730     ^740     ^750     ^760     ^770     ^780
TTTGTAGAACAATCATTGGCGTGCCAAGCTGAGACGTGTGTTCCCTGAAATGTGCATAGC
  ^790     ^800     ^810     ^820     ^830
    
```

FIG. 10(b)

MetThrArg
 ACCTTCCTCCCGGCCCGAGGCCGGATTAACTATCGAAGGAGCAAAGGTTTCATGACACGT
 849 859 869 879 889 899
 AspHI
 GlnMetIleLeuAlaValGlyGlnGlnGlyProIleAlaArgAlaGluThrArgGluGln
 CAGATGATACCTTGCCTGGACAGCAAGGCCCATCGCGGAGCGGAGACACGCGAACAG
 909 919 929 939 949 959
 ValValGlyArgLeuLeuAspMetLeuThrAsnAlaAlaSerArgGlyValAsnPheIle
 GTGGTTGGCCGCTCCTCGACATGTTGACGAACGCAGCCAGCCGGGGCGTGAACCTTCATC
 969 979 989 999 1009 1019
 ValPheProGluLeuAlaLeuThrThrPhePheProArgTrpHisPheThrAspGluAla
 GTCTTTCCTCGAGCTTGGCGTCACGACCTTCTTCCCGCGTGGCATTTCACCGACGAGGCC
 1029 1039 1049 1059 1069 1079
 GluLeuAspSerPheTyrGluThrGluMetProGlyProValValArgProLeuPheGlu
 GAGCTCGATAGCTTCTATGAGACCGAAATGCCCGGCCCGTGGTCCGCTCCACTCTTTGAG
 SacI 1089 1099 1109 1119 1129 1139
 ThrAlaAlaGluLeuGlyIleGlyPheAsnLeuGlyTyrAlaGluLeuValValGluGly
 ACGGCCCGCAACTCGGGATCGGCTTCAATCTGGGCTACGCCGAAGTCTGTCGTCGAAGGC
 1149 1159 1169 1179 1189 1199
 GlyValLysArgArgPheAsnThrSerIleLeuValAspLysSerGlyLysIleValGly
 GCGTCAAGCGTTCGCTTCAACACGTCCATCTGGTGGATAAGTCAGGCAAGATCGTCCGC
 1209 1219 1229 1239 1249 1259
 LysTyrArgLysIleHisLeuProGlyHisLysGluTyrGluAlaTyrArgProPheGln
 AAGTATCGTAAGATCCATTTGCCGGGTCAACAGGAGTACGAGGCCCTACCGCCGTTCCAG
 1269 1279 1289 1299 1309 1319
 HisLeuGluLysArgTyrPheGluProGlyAspLeuGlyPheProValTyrAspValAsp
 CATCTTGAAAAGCGTTATTTCCGACCGGGCGATCTCGGCTTCCCGGTCTATGACGTCGAC
 1329 1339 1349 1359 1369 SalI 1379
 AlaAlaLysMetGlyMetPheIleCysAsnAspArgArgTrpProGluThrTrpArgVal
 GCCGCGAAAATGGGGATGTTTCATCTGCAACGATCGCCGCTGGCCTGAAACGTGGCGGGTG
 1389 1399 1409 1419 1429 1439
 MetGlyLeuLysGlyAlaGluIleIleCysGlyGlyTyrAsnThrProThrHisAsnPro
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 1509 1519 1529 1539 1549 1559
 SerTyrGlnAsnGlyAlaTrpSerAlaAlaAlaGlyLysValGlyMetGluGluGlyCys
 TCGTACCAAAACGGCGCCTGGTCCGCGCGCGCCGCAAGGTCGGCATGGAGGAGGGGTGC
 1569 1579 1589 1599 1609 SphI 1619
 MetLeuLeuGlyHisSerCysIleValAlaProThrGlyGluIleValAlaLeuThrThr
 ATGCTGCTCGGCCATTCGTGCATCGTGGCGCGACCGCGAAATCGTTGCCCTGACCACG
 1629 1639 1649 1659 1669 1679
 ThrLeuGluAspGluValIleThrAlaAlaValAspLeuAspArgCysArgGluLeuArg
 ACGTTGGAAGACGAGGTGATCACCGCCCGCTCGATCTCGACCGCTGCCGGAACTGCGC
 1689 1699 1709 1719 1729 1739
 GluHisIlePheAsnPheLysAlaHisArgGlnProGlnHisTyrGlyLeuIleAlaGlu
 GAACACATCTTCAATTTCAAAGCCCATCGTCAGCCACAGCACTACGGTCTGATCGCGGAA
 1749 1759 1769 1779 1789 EcoRI 1799
 Phe***
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 1809 1819 1829 1839 1849 1859
 CCTATCCGATCGAGAAAGCTT
 1869 HindIII 1879

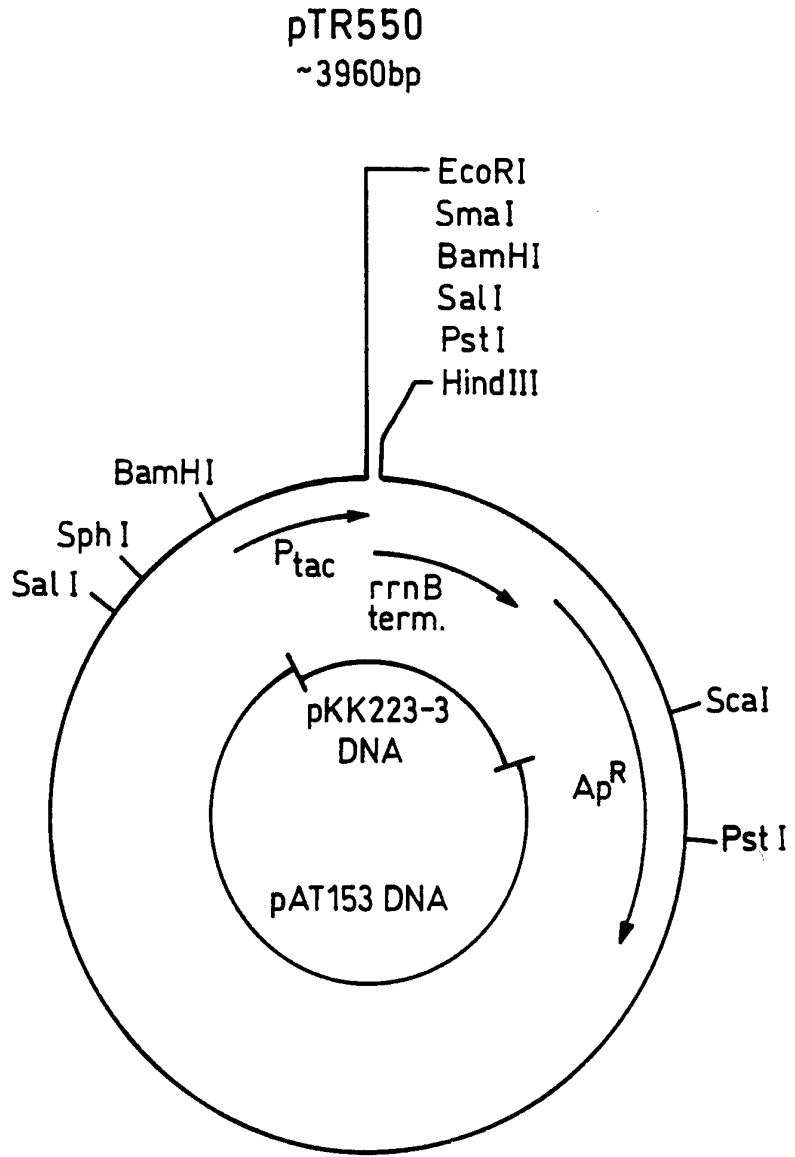


Fig.11

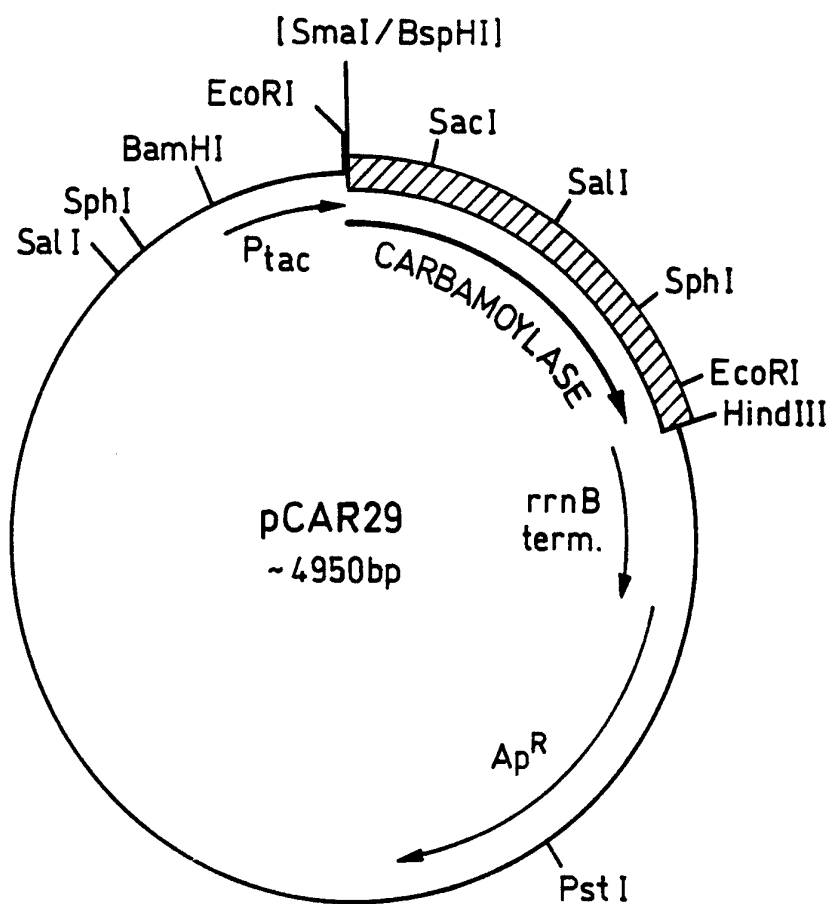


Fig.12

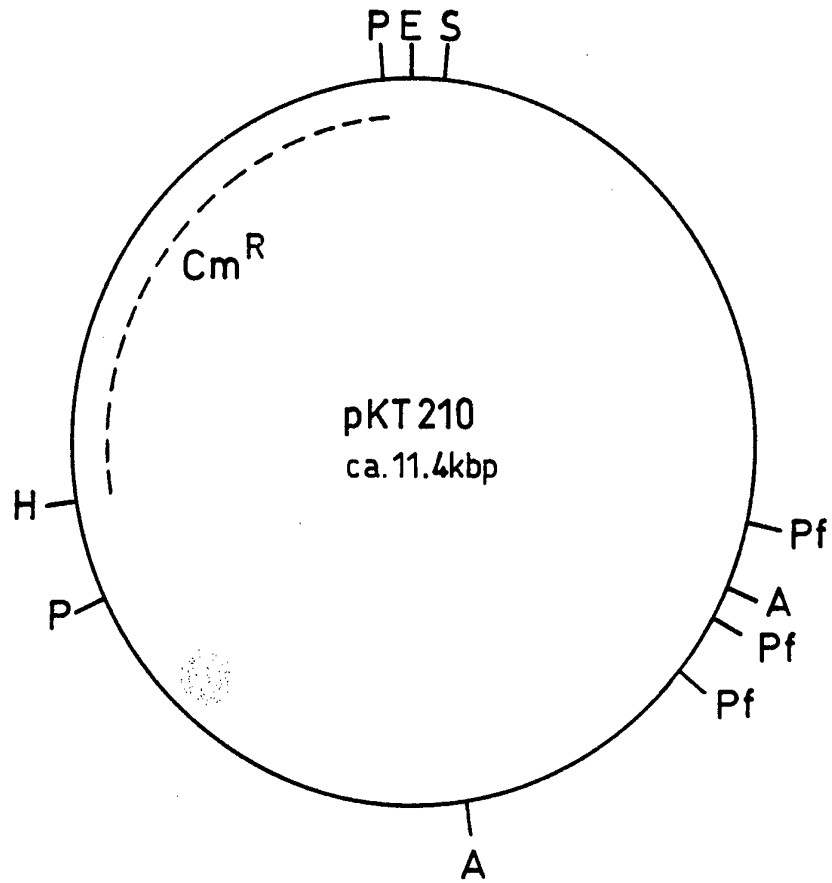


Fig.13

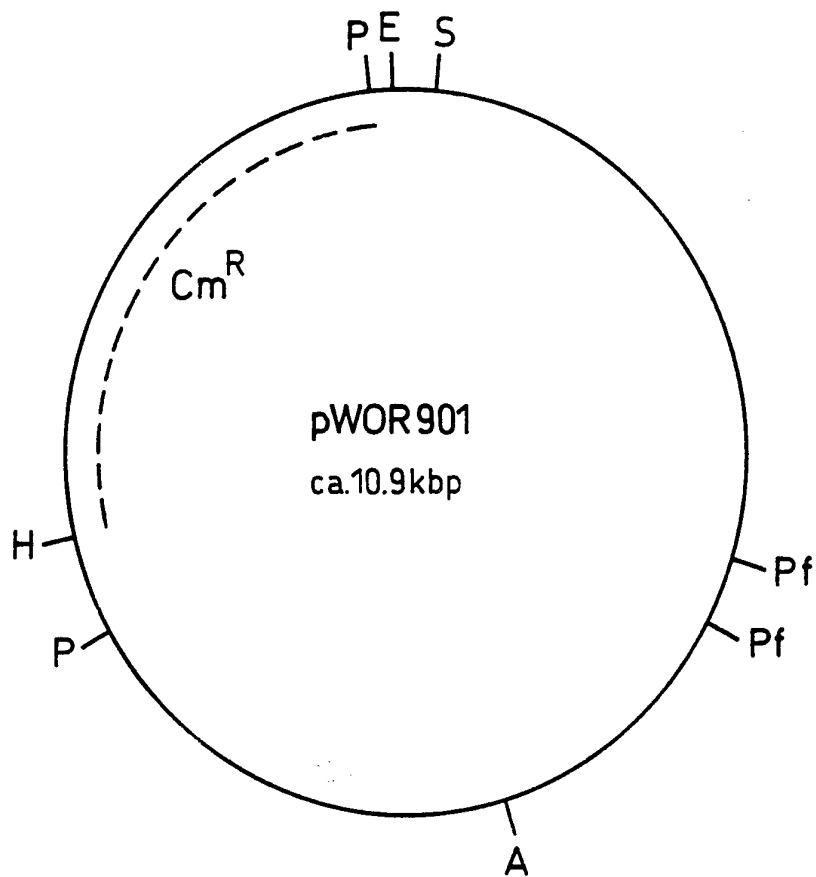


Fig.15

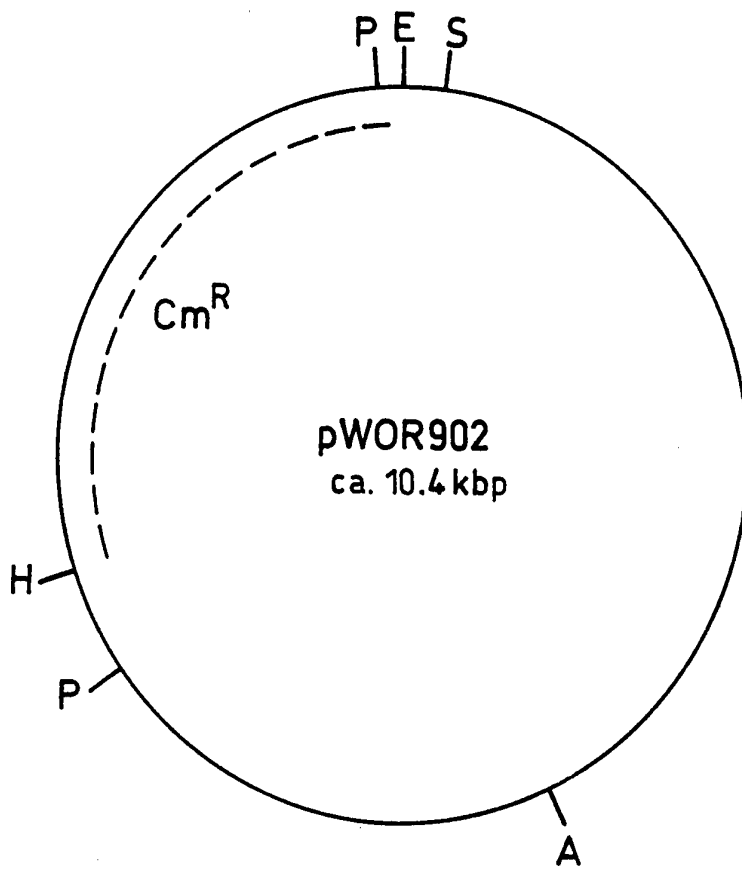


Fig.16

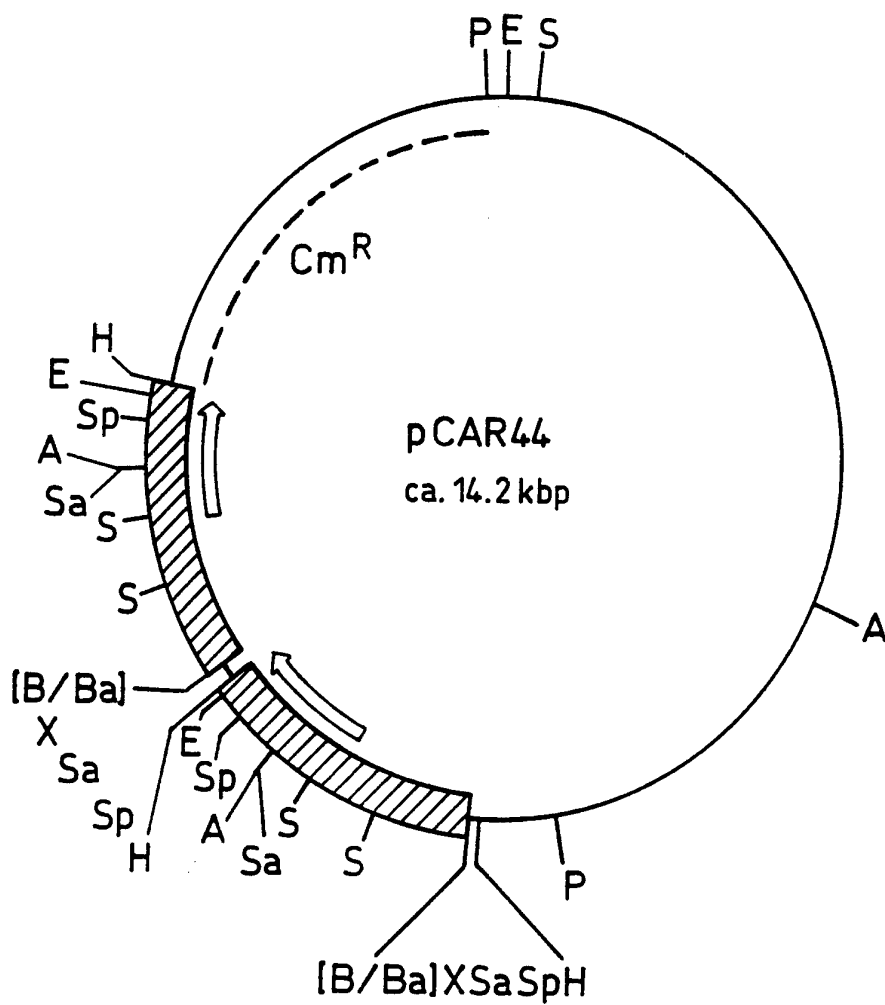


Fig. 17

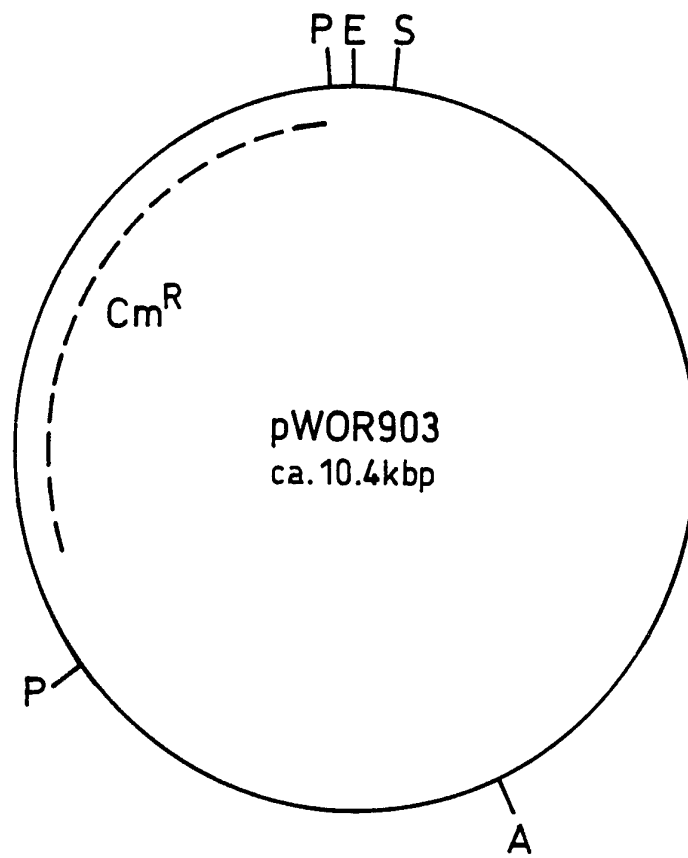


Fig. 18

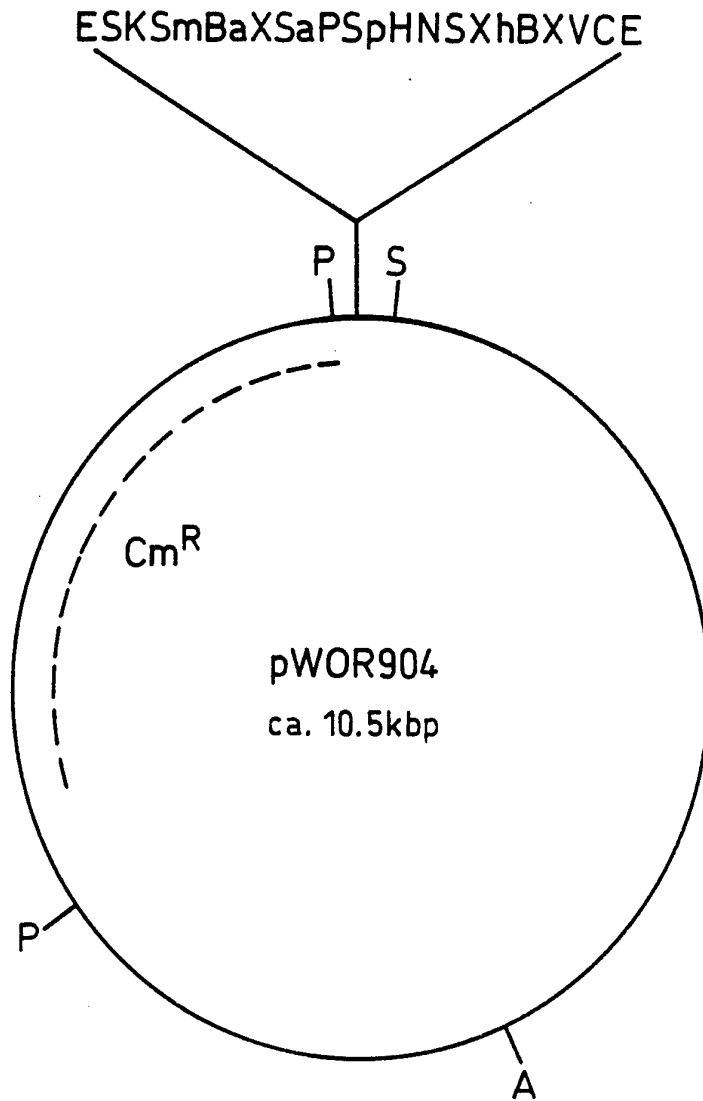


Fig. 19

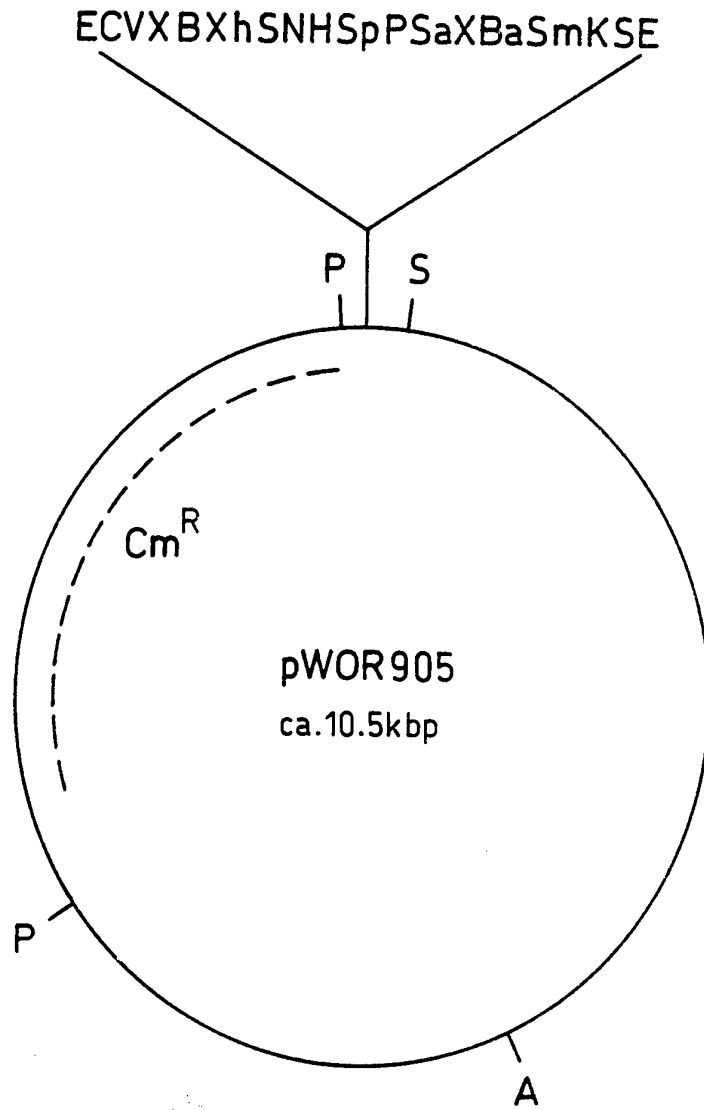


Fig. 20

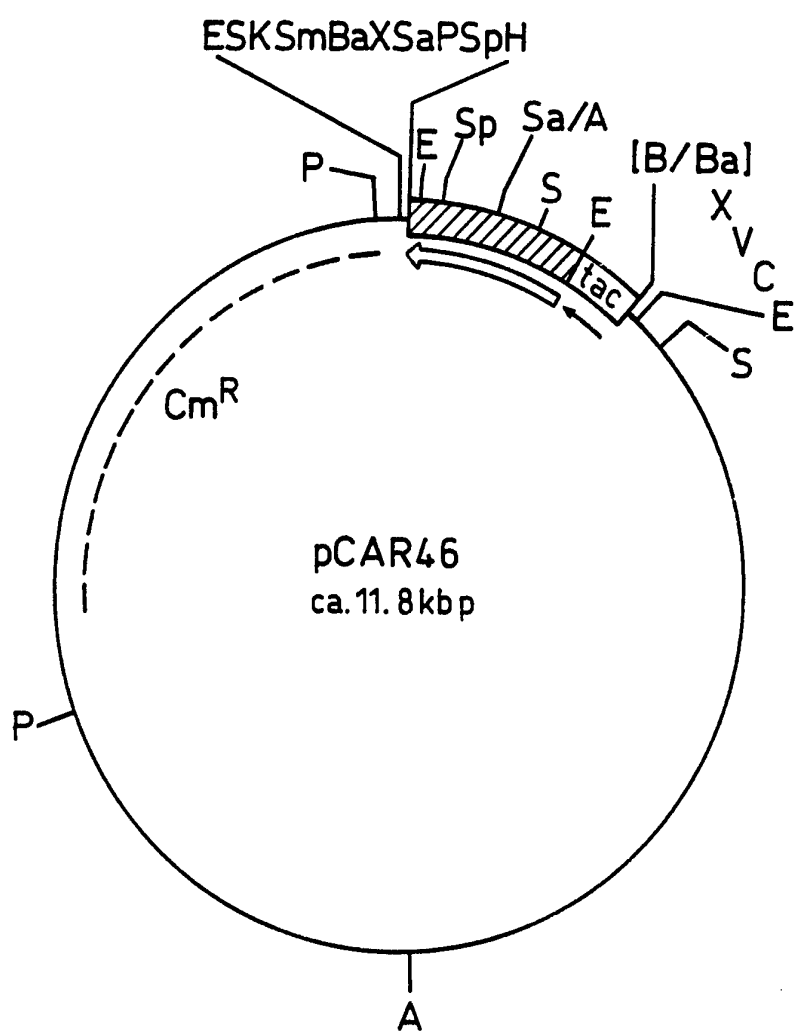


Fig.21

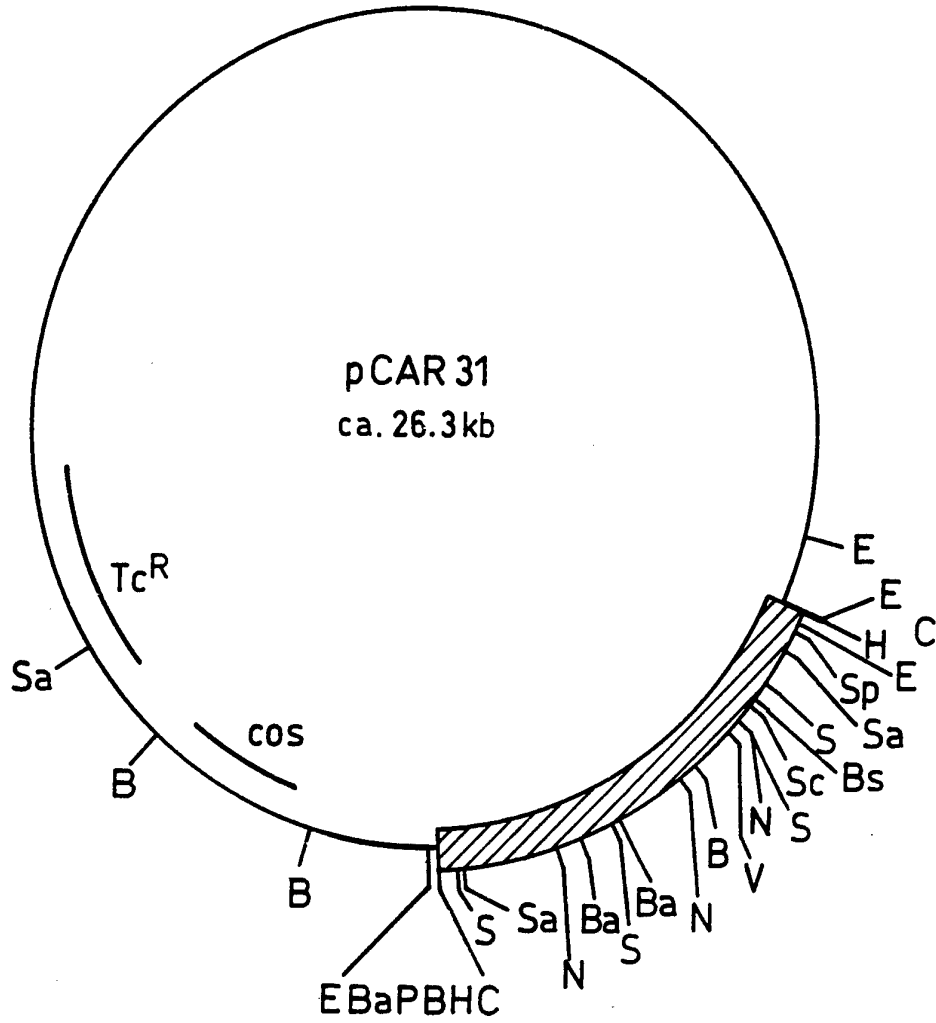


Fig.22

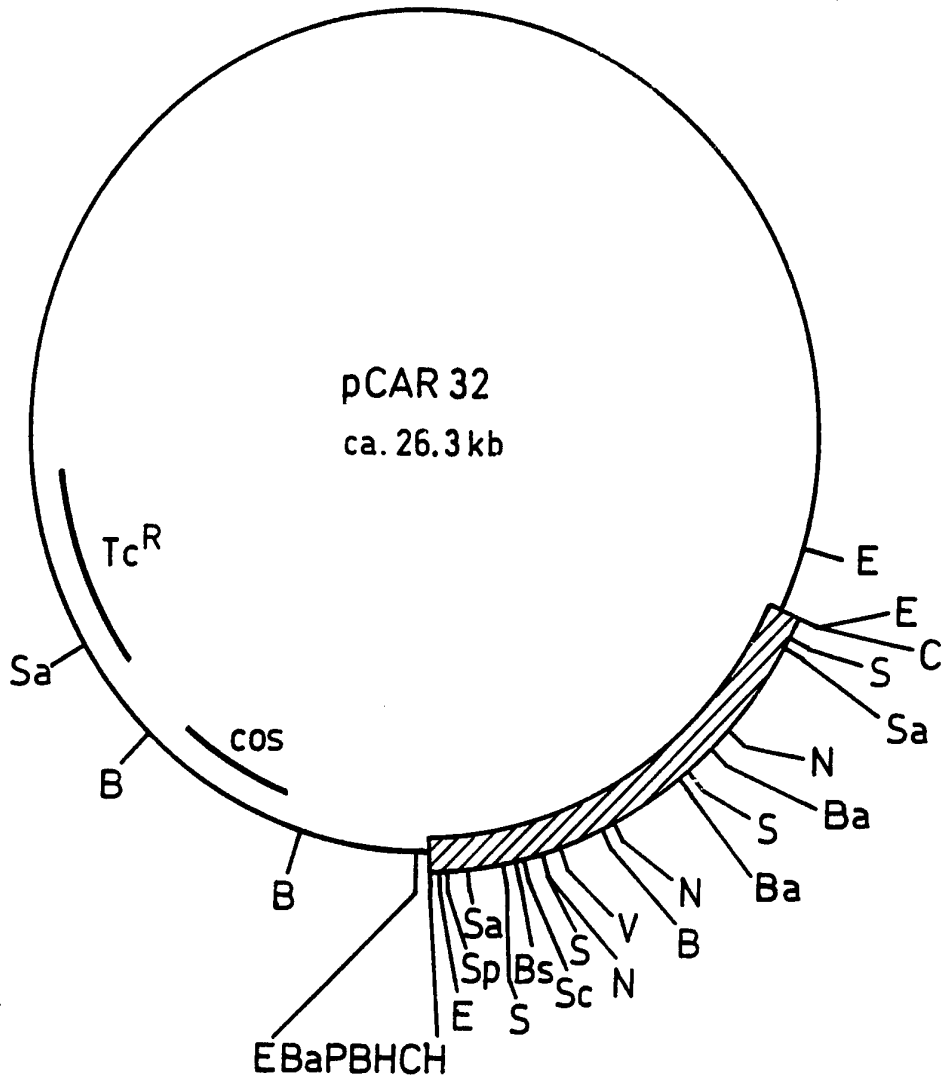


Fig. 23

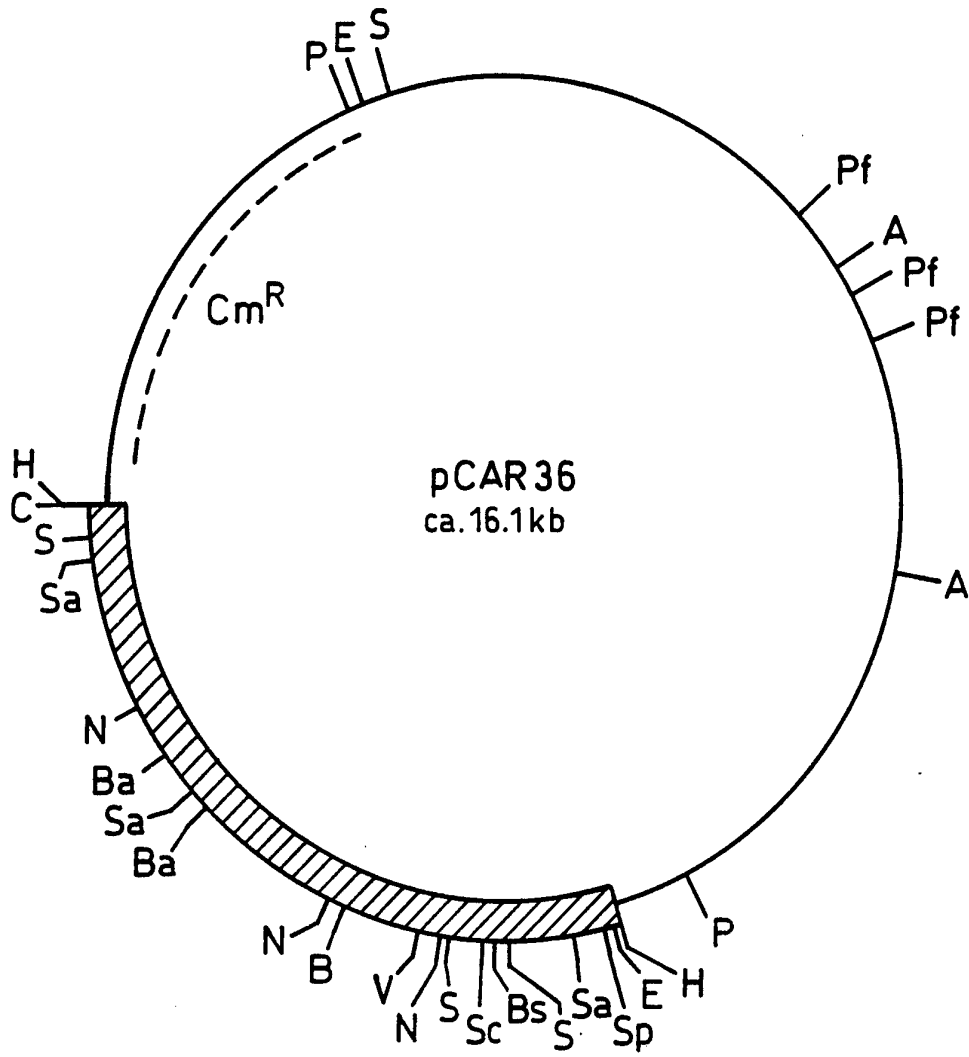


Fig.24

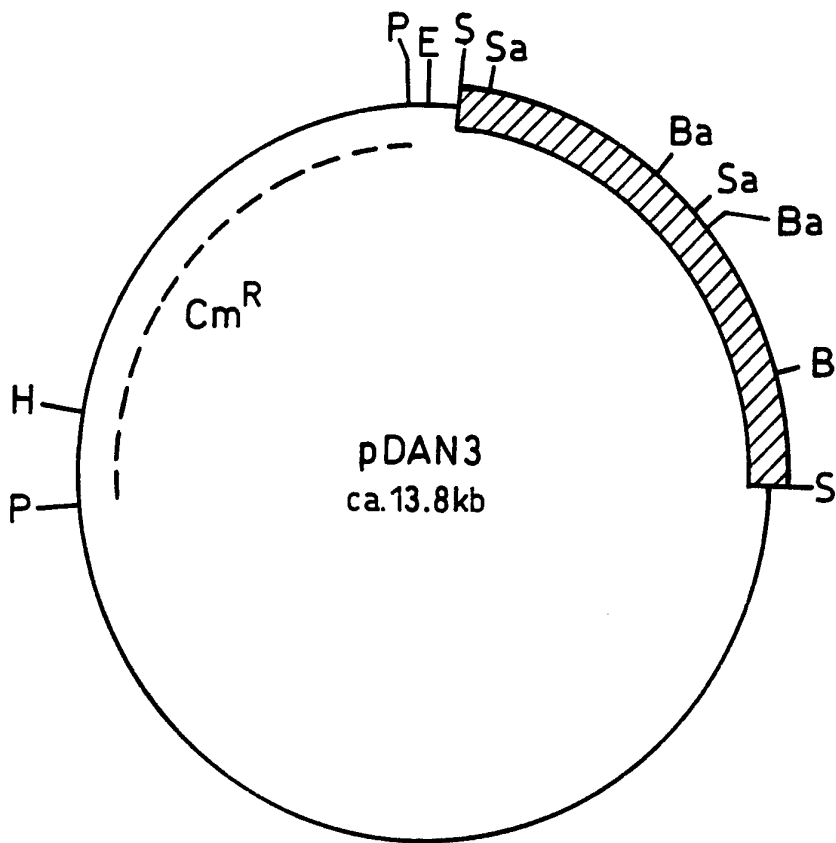


Fig. 25

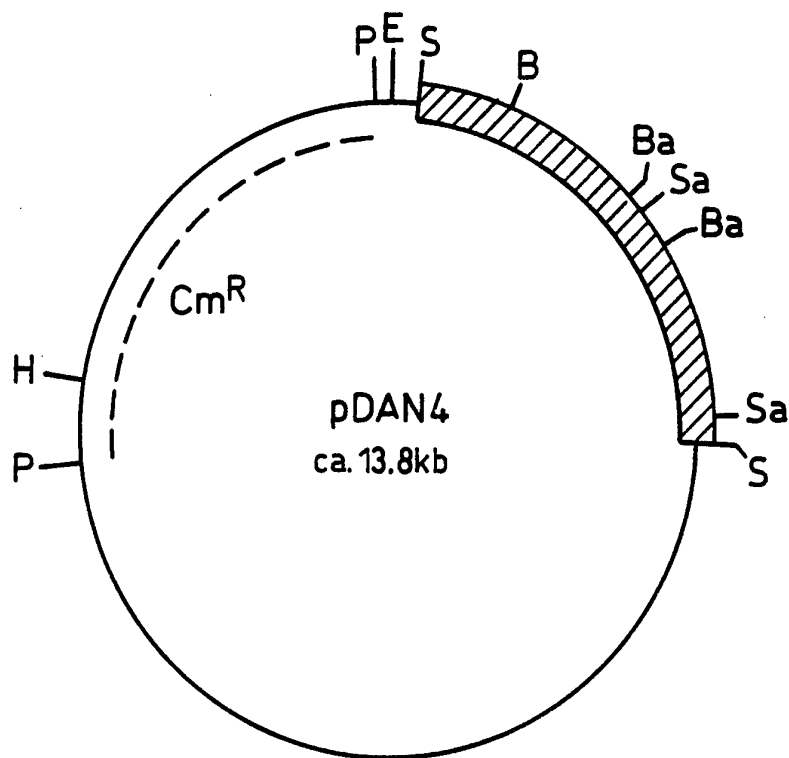


Fig. 26

INTERNATIONAL SEARCH REPORT

Internation. Application No
PCT/GB 93/01378

A. CLASSIFICATION OF SUBJECT MATTER IPC 5 C12N15/55 C12N1/21 C12N9/80 C12N9/86		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) IPC 5 C12N C12P		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 10579 (KANEKAFUCHI KAGAKO KOGYO KABUSHIKI KAISHA) 25 June 1992 cited in the application	1-4, 10-15
Y	& EP,A,0 515 698 ---	5-7
Y	PATENT ABSTRACTS OF JAPAN vol. 12, no. 230 (C-508)(3077) 29 June 1988 & JP,A,63 024 894 (AJINOMOTO CO INC) 2 February 1988 see abstract ---	2, 4-7, 10-15
Y	GB,A,2 022 581 (SNAM PROGETTI) 19 December 1979 cited in the application see the whole document ---	2, 4-7, 10-15
	-/--	
<input checked="" type="checkbox"/>	Further documents are listed in the continuation of box C.	<input checked="" type="checkbox"/>
	Patent family members are listed in annex.	
* Special categories of cited documents :		
<p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
Date of the actual completion of the international search 3 November 1993		Date of mailing of the international search report 03. 12. 93
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016		Authorized officer VAN DER SCHAAL, C

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 93/01378

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOTECHNOLOGY AND BIOENGINEERING. vol. 23, no. 10 , 1981 , NEW YORK US pages 2173 - 2183 R. OLIVIERI ET AL 'Microbial transformation of racemic hydantoins to d-amino acids' see the whole document *see reference 11*	2,4-7, 10-15
X	EP,A,0 219 034 (BASF AG) 22 April 1987	8,9
Y	see the whole document	2,4-7, 10-15
X	CHEMICAL ABSTRACTS, vol. 113, no. 17, 22 October 1990, Columbus, Ohio, US; abstract no. 147669, S. RUNSER AND E. OHLEYER 'Properties of the hydantionase from Agrobacterium' page 318 ; see abstract	8-10
Y	& BIOTECHN. LETT. vol. 12, no. 4 , 1990 pages 259 - 264	4-7, 11-15
X	CHEMICAL ABSTRACTS, vol. 104, no. 23, 9 June 1986, Columbus, Ohio, US; abstract no. 205546, H. KITAGAWA ET AL 'Novel hydantoinase' page 604 ; see abstract & JP-A-60 241888 (DENKI KAGYO KOGYO) 30 Nov 1985	8,9

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