AUSTRALIA 623265

PATENTS ACT 1990

PATENT REQUEST : STANDARD PATENT

I/We being the person(s) identified below as the Applicant(s), request the grant of a patent to the person(s) identified below as the Nominated Person(s), for an invention described in the accompanying standard complete specification.

Full application details follow:

[71/70] Applicant(s)/Nominated Person(s):

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of

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[54] Invention Title:

Cloned N–methylhydantoinase

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[31] Application Number	[33] Country	Code	[32] Date of Application
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DATED this SECOND day of JULY 1991

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a member of the firm of
DAVIES & COLLISON for
and on behalf of the
applicant(s)

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NOTICE OF ENTITLEMENT

We, **Boehringer Mannheim GmbH**, the applicant named in the accompanying Patent Request state the following:-

The Nominated Person is entitled to the grant of the patent because the Nominated Person derives title to the invention from the inventors.

The Nominated Person is entitled to claim priority from the basic application listed on the patent request because the Nominated Person made the basic application, and because the basic application was the first application made in a Convention country in respect of the invention.

DATED this SECOND day of JULY 1991

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a member of the firm of
DAVIES & COLLISON for
and on behalf of the
applicant(s)

(D&C ref: 1415192)

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(56) Prior Art Documents
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(57) Claim

1. DNA, w h e r e i n it has (1) the nucleic acid sequence shown in SEQ ID NO: 1, (2) a sequence corresponding to it within the scope of the degeneracy of the genetic code or (3) has a sequence which hybridizes with a sequence from (1) or/and (2) under stringent conditions and which codes for a protein with N-methylhydantoinase activity.

628265

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COMPLETE SPECIFICATION

NAME OF APPLICANT(S):

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INVENTION TITLE:

Cloned N-methylhydantoinase

The following statement is a full description of this invention, including the best method of performing it known to me/us:-

Description

The enzyme N-methylhydantoinase (NMHase) is required for the determination of the content of creatinine in liquids. The creatinine level is an important parameter for kidney diagnostics. Annually about one thousand million tests are carried out worldwide. Therefore the provision of the enzyme NMHase at a low cost, as well as the possibility of an unproblematic fermentation are basic requirements for the provision of diagnostic kits for the determination of creatinine. The molecular weight of NMHase is 125 kD in an SDS gel. The specific activity is 2 U/mg, the K_M for N-methylhydantoin is 2×10^{-5} mol/l. NMHase is usually isolated from Arthrobacter. However, this process has drawbacks which are related to the microorganism used.

Improved methods of isolation must therefore be developed in order to provide larger amounts of NMHase. This was also the object of the present invention.

The object according to the present invention could be achieved by cloning the gene coding for the NMHase from Arthrobacter and expressing it in a suitable host organism.

The present invention thus provides a DNA which contains (1) the nucleic acid sequence shown in SEQ ID NO: 1, (2) a sequence corresponding to it within the scope of the degeneracy of the genetic code or (3) a sequence which hybridizes with the sequences from (1) or/and (2) under stringent hybridization conditions and which codes for a protein with NMHase activity.

In this connection reference is made to Maniatis et al. (1982) "Molecular Cloning. A laboratory manual", Cold Spring Harbor Laboratory, New York, for the meaning of hybridization under stringent conditions in the present invention.

The DNA according to the present invention codes for a protein with 1288 amino acids whose sequence is shown in SEQ ID NO: 2. The present invention thus also encompasses a protein with NMHase activity and with the amino acid sequence shown in SEQ ID NO: 2 or an amino acid sequence derived therefrom, which is obtained by genetic engineering methods e.g. by expression in a heterologous organism i.e. in an organism in which the gene coding for the protein according to the present invention does not originally occur. On the other hand, it is also possible to achieve an improved expression of the NMHase gene by introducing one or several copies of the DNA according to the present invention into an organism in which a DNA according to the present invention is present.

The present invention in addition provides a recombinant vector which contains one or several copies of a DNA according to the present invention. A recombinant vector according to the present invention can be a vector which is suitable for protein expression in prokaryotic or eukaryotic organisms. It is preferably a prokaryotic vector.

A recombinant vector according to the present invention can be a vector which is present extrachromosomally in a host cell (e.g. plasmid) or is integrated into the genome of the host (e.g. bacteriophage lambda). The recombinant vector according to the present invention is

preferably a plasmid. A suitable plasmid according to the present invention is e.g. the plasmid pBP010.

The DNA which codes for a protein with NMHase activity is located on a recombinant vector according to the present invention and is preferably under the control of a regulatable promoter, which means that an expression of the DNA according to the present invention can be suppressed for example by a repressor and only takes place when the regulatable promoter is specifically induced. This induction can for example take place by a change in temperature or by addition of a chemical inducer (e.g. IPTG for lac promoter derivatives). In a particular preferred embodiment of the present invention the regulatable promoter which is intended to control the NMHase gene is the mgl promoter from Salmonella typhimurium (WO 88/09373) which can be regulated by means of catabolite repression by sugars such as e.g. glucose and fructose.

A suitable vector according to the present invention for the expression of NMHase in gram-negative bacteria, in particular E. coli, is e.g. the plasmid pBP006. In order to construct pBP006, a DNA fragment, which contains the sequence of the mgl promoter from Salmonella typhimurium, was isolated from the plasmid pPZ07-mgl-lac (described in WO 88/09373, Fig. 8) and cloned upstream of a DNA fragment which contains the sequence coding for the NMHase gene from Arthrobacter without its own promoter.

The present invention also provides a cell which is transformed with a DNA according to the present invention or with a recombinant vector according to the

present invention. This cell is preferably a bacterial cell, particularly preferably an E. coli cell.

The DNA according to the present invention is obtained by cloning the NMHase gene. Chromosomal DNA from Arthrobacter was isolated for this by conventional methods and cleaved with suitable restriction enzymes. A gene bank of these DNA fragments was set up in E. coli. However, a cloning of the NMHase gene in the usual manner (screening the gene bank with oligonucleotide probes and selection of the clones by means of NMHase activity) did not succeed. In fact no NMHase activity was found in any of the Arthrobacter DNA fragments used when cloned in E. coli. This finding was surprising since a DNA fragment of the correct length with a start and stop codon could be identified on the basis of hybridization with the oligonucleotide probe. An NMHase activity could only be detected when cloning DNA fragments on which the native NMHase promoter was absent.

The invention also provides a process for the production of a protein with NMHase activity in which a cell is transformed with a DNA according to the present invention or with a recombinant vector according to the present invention, the transformed cells are cultured in a suitable medium and the protein is isolated from the medium or the cells.

E. coli bacteria are preferably used as the host organism for the process according to the present invention. In this connection it is, however, advantageous to culture the transformed cells under suboptimal growth conditions. Suboptimal growth conditions are for example understood as a reduced

temperature during the incubation (30°C or less), a reduction of the oxygen transfer or/and the use of a minimal medium (i.e. a medium which contains certain essential nutrients for the cultured organism in limiting concentrations).

Thus for instance the culture conditions in a process for the isolation of NMHase from E. coli, in which a recombinant vector is used which contains the NMHase gene under the control of the tac promoter, is a minimal medium, an incubation temperature of less than 30°C and an incomplete induction of the tac promoter with 0.8 % lactose.

The particularly preferred expression of the NMHase gene under the control of the mgl promoter of Salmonella typhimurium preferably also takes place at an incubation temperature of 30°C or less, which if desired is coupled with an additional reduction of the oxygen transfer so that the NMHase formed does not accumulate in an inactive form as precipitation bodies. The mgl promoter is regulated by catabolite repression (US patent application 300,357).

In general it is preferred for the process according to the present invention that the induction of the regulatable promoter used in each case is only carried out incompletely which also contributes to a reduced formation of precipitation bodies.

In addition it is particularly preferred for the process according to the present invention that, for the purpose of stabilization and preferably during the isolation of the NMHase from the transformed cells or the medium, the

protein is incubated with the enzyme substrate N-methylhydantoin. Surprisingly the stability of the recombinant NMHase obtained by the process according to the present invention can be substantially increased by the presence of an amount of approximately 3.8 nmol N-methylhydantoin per unit (U) of the enzyme. For this the enzyme is incubated with a N-methylhydantoin solution, preferably at a concentration of 1 to 100 mmol/l, particularly preferably of 10 to 70 mmol/l, most preferably of 50 mmol/l. In this incubation step it is advantageous to increase the temperature to e.g. 55°C. It is especially surprising that the presence of its own substrate stabilizes the enzyme and that at the same time the enzymatic reaction of the recombinant enzyme does not interfere.

The present invention also encompasses a reagent for the determination of the content of creatinine in liquids which contains a protein obtained according to a process according to the present invention in addition to the usual constituents.

The following examples are intended to further elucidate the invention in conjunction with the sequence protocols and figures 1 to 10.

SEQ ID NO: 1 shows the DNA sequence of the NMHase gene,

SEQ ID NO: 2 shows the amino acid sequence of the NMHase derived therefrom,

Fig. 1 shows a 6 kb long EcoRI fragment from Arthrobacter with a ca. 0.6 kb long fragment of the NMHase gene,

Fig. 2 shows a 3.7 kb long SalI fragment from Arthrobacter with a 3.0 kb long region coding for the NMHase gene,

Fig. 3 shows the 3'-terminal region of the NMHase gene,

Fig. 4 shows an EcoRI/AatII linker,

Fig. 5 shows the construction of the plasmid pBP008,

Fig. 6 shows the construction of the plasmid pBP009,

Fig. 7 shows the construction of the NMHase expression plasmid pBP010,

Fig. 8 shows the construction of the plasmid pBP011 with the mgl promoter from Salmonella typhimurium,

Fig. 9 shows the isolation of fragments of the NMHase gene from pBP010,

Fig. 10 shows the construction of the NMHase expression plasmid pBP006.

E x a m p l e 1

Cloning of the NMHase

DNA was isolated according to the usual methods from Arthrobacter spec. DSM 2563 (J. Marmur - A procedure for the isolation of deoxyribonucleic acid from micro-organisms, J.Mol.Biol. 3, 208-218 (1961); S. Visuvanathan et al. - Simple enzymic method for isolation of DNA from diverse bacteria, Journal of Microbiological Methods 10, 59-64 (1989)) and cleaved with the restriction enzymes EcoRI or HindIII. Bacteriophage λgt10 (Boehringer Mannheim GmbH) was used as the cloning vector for the Arthrobacter DNA. The Arthrobacter DNA was cloned in λgt10 according to the instructions of the producer.

The Arthrobacter gene bank obtained was screened with an oligonucleotide probe which was derived from a partial peptide sequence of NMHase.

Partial peptide sequence of NMHase:
Met Lys Arg Ile Gly Val Asp Val Gly Gly Thr Phe Thr Asp Leu Tyr Phe.

The following oligonucleotide probes were derived from this partial peptide sequence:

1. ATG AA(G/A) (C/A)G(G/A) AT(A/C/T) GG(G/A/T/C) GT
2. ATG AA(G/A) (C/A)G(T/C) AT(A/C/T) GG(G/A/T/C) GT
3. ATG AAG CGC ATC GGC GTG GAC GTG GGC GGC ACG TTC ACC GAT CTG TAC TT

Using these oligonucleotide probes a 6 kb long EcoRI fragment was found in the λgt10 gene bank which contains a part of the NMHase gene (ca. 0.6 kb) (Fig. 1).

A part of this fragment (ca. 300 bp between the cleavage sites PstI and EcoRI) was radioactively labelled with ^{32}P. Subsequently Arthrobacter DNA was cleaved with the restriction enzyme SalI, separated on an agarose gel and hybridized in a Southern Blot with the radioactively labelled DNA fragment. The hybridizing DNA region was cut out of the agarose gel and cloned into the SalI restriction cleavage site of the tetracycline resistance gene of pBR328 (Boehringer Mannheim GmbH).

An examination of E. coli cells transformed with this plasmid resulted in a 3.7 kb long DNA fragment which contains a 3.0 kb long region of the NMHase gene (Fig. 2).

The EcoRI/Hind III fragment from this insertion which is marked by a dotted line was labelled with digoxigenin (Boehringer Mannheim, Dig Kit). The λgt10 gene bank already mentioned above was again screened with this probe whereby a 2.7 kb piece was found which contains the 3'-terminal region of the NMHase gene (Fig. 1). This DNA fragment was also cloned into the vector pBR328.

E x a m p l e 2
Expression of NMHase

2.1 Conventional methods

The 3.7 kb long SalI fragment (Fig. 2) was cloned into the commercially available vector pUC19

(Boehringer Mannheim GmbH). Subsequently the NMHase gene was completed by cloning in the EcoRI fragment of Fig. 3. However, such a construct does not lead to the expression of active NMHase.

An attempt to achieve expression of this construct by cloning under the control of an inducible tac promoter and inducing the tac promoter in the usual way (incubation at 37°C, complete medium and complete induction of the tac promoter) also failed. For this the plasmid pKK177-3 (DSM 3062) was cleaved with EcoRI and HindIII and ligated with a polylinker cut out of pUC19 by means of EcoRI and HindIII. The plasmid which forms was denoted pBP177-4. Subsequently the plasmid pBP177-4 was cleaved with EcoRI and KpnI and combined with a 2.5 kb C-terminal NMHase fragment (also cleaved with EcoRI and KpnI) to form the plasmid pBP008 (Fig. 5).

The plasmid pBP008 was cleaved with the enzymes XhoI and EcoRI and the resulting large (5 kb) fragment was combined with a 1.5 kb fragment from the NMHase N-terminus which has the end-cleavage sites AatII and XhoI and with an EcoRI-AatII linker (see Fig. 4) to form plasmid pBP009 (Fig 6). A protein whose molecular weight approximately corresponds to that of NMHase was expressed in E. coli cells which were transformed with this plasmid. However, no enzymatic activity could be detected.

2.2 Process according to the present invention
First a C-terminal extension of the NMHase was carried out. The plasmid pBP009 (Fig. 6) was

cleaved for this with the enzymes XhoI and SmaI and a resulting 5.5 kb DNA fragment is isolated which contains the tac promoter, the N-terminal region of NMHase and the ampicillin resistance gene. This fragment was combined with a C-terminal NMHase fragment from pBR328 which has the end-cleavage sites EcoRI (blunt ends by treatment with Klenow polymerase) and XhoI to form plasmid pBP010 (Fig. 7), pBP010 is able to express NMHase.

In the next step the NMHase gene was brought under the control of the mgl promoter from Salmonella typhimurium (described in WO 88/09373). For this the plasmid pPZ07/mgllac (described in WO 88/09373) was cleaved with the enzymes NcoI and AatII and a 2.9 kb long DNA fragment was isolated therefrom which contains the mgl promoter. This fragment was combined with a NcoI-AatII linker to form plasmid pBP011 (Fig. 8).

Plasmid pBP011 was cleaved with EcoRI, it was treated with Klenow DNA polymerase in order to produce blunt ends and re-cleaved with AatII. Subsequently a resulting 0.8 kb long DNA fragment with a blunt end and an AatII end which contains the mgl promoter and the linker fragment was isolated (Fig. 8).

Plasmid pBP010 was cleaved with NdeI and treated with Klenow DNA polymerase in order to produce blunt fragment ends. Subsequently these fragments were cleaved with XhoI and a 4.9 kb long fragment was isolated which contains the C-terminal region of the NMHase gene (Fig. 9).

Plasmid pBP010 was also cleaved with XhoI and AatII in the process of which a 1.5 kb long fragment could be isolated which contains the N-terminal region of the NMHase gene (Fig. 9).

Both fragments from plasmid pBP010 (4.9 kb and 1.5 kb) were ligated with the 0.8 kb fragment from pBP011 which contains the mgl promoter. The resulting plasmid was denoted pBP006 (Fig. 10) and is capable of expressing NMHase.

E x a m p l e 3

Fermentation and accumulation of recombinant NMHase in E. coli

E. coli HB101 cells (DSM 1607) were transformed with the NMHase expression plasmid pBP010. In order to ensure a better regulatability of the tac promoter the cells were additionally transformed with a plasmid which is compatible with pBP010 and which contains the $lacI^q$ gene.
The $lacI^q$ gene has already been known to one skilled in the art for a long time and is easily obtainable. pACYC 177 (DSM 3693P) or plasmids derived therefrom come into consideration as the plasmid compatible with pBP010.

3.1 Growth and preculture

2x500 ml LB medium with kanamycin and ampicillin in two 2000 ml Erlenmeyer flasks were inoculated with E. coli HB101/$lacI^q$/pBP010 cells. They were then incubated at 37°C and 150 rpm (rotary shaker, Braun Certomat M). The OD at 578 nm was ca. 3.0 to 4.0 in the 10th hour at a pH of ca. 7.6.

Main fermentation:

Nutrient medium and main culture:

glycerol 86%	2500 g
lactose	500 g
NH_4Cl	50 g
$MgSO_4 * 7 H_2O$	50 g
K_2HPO_4	150 g
casein peptone	3000 g
ammonia solution 25% Merck 5432	500 ml
water	100 l

Fermentation course:
After inoculation (1 % inoculum) the culture begins to grow exponentially without delay. The temperature of the fermenter is kept at 28°C up to an OD 578 nm of 1.400. When the desired OD is reached the temperature is decreased to 25°C, the growth slows down. In addition the oxygen transfer can be reduced. These measures are necessary in order to limit the growth and thus to counteract the formation of precipitation bodies (inclusion bodies). The correct time for the temperature shift is important, if it is carried out too soon, growth is delayed for hours, if it is carried out too late, only insoluble protein is obtained.

A further increase in activity is obtained by additionally reducing oxygen. In the fermentation with a shift in temperature the yield is ca. 2500 U/L, max 3000 U/L (150 U/OD) after 30 hours. When the amount of O_2 in the medium is also reduced up to 4000 U/L are obtained after 45 hours.

E x a m p l e 4

Isolation of recombinant NMHase from E. coli

4.1 Measurement of the enzyme activity

The determination of the enzyme activity is carried out by means of a colorimetric test which contains carbamoyl-sarcosine hydrolase, sarcosine oxidase, peroxidase, N-methylhydantoin, 4-aminoantipyrine, tribromo-3-hydroxybenzoic acid, ATP and $MgCl_2$ in phosphate buffer, pH 7.8.

Principle of the measurement :

NMHase converts the N-methylhydantoin which was added to carbamoyl-sarcosine, carbamoyl-sarcosine hydrolase converts this to sarcosine, this is degraded by sarcosine oxidase to form glycine, formaldehyde and hydrogen peroxide. The peroxidase converts the added colour substrates into a dark-violet dye with the aid of the hydrogen peroxide which is formed. The increase in absorbance is measured at a wavelength of 546 nm. The enzyme test is described in detail in US patent 4,816,393.

A unit (U) is defined as µmol of carbamoyl-sarcosine formed per minute at 25°C under measuring conditions in a coupled test with carbamoyl-sarcosine hydrolase, sarcosine oxidase and peroxidase. An activity of 0.16 U/ml is obtained in a 5 ml test culture. This corresponds to an increase by a factor of ca. 20 compared to the original culture (Arthrobacter spec. DSM 2563).

4.2 Enzyme purification

315 g biomass (according to Example 3) resulting

from 10 l fermentation culture with a total activity of 16 KU NMHase were suspended in 2 l 0.1 mol/l potassium phosphate buffer containing 10 % glycerol, pH 8.0 and lysed by treatment with lysozyme and once with 700 bar high pressure dispersion. In order to remove the nucleic acids and cell debris a 10 % polyethyleneimin solution G20 (Luvalgan, MW 20000) was added until no further precipitation occurs and all the NMHase activity remained in the supernatant. For this 3 % v/v G 20 solution was added at room temperature, stirred for 30 minutes and afterwards centrifuged. 8 % v/v in batch wet-pressed DEAE Sephadex was added to the NMHase supernatant and after stirring for 2 hours 95 % of the enzyme had been adsorbed. After filtration the exchanger was washed with phosphate buffer and the NMHase was eluted with 0.5 mol/l ammonium sulphate solution containing 0.1 mol/l $K\text{-}PO_4$ buffer, pH 8.0. The eluate had a specific activity of 1.1 U/mg protein. Subsequently it was heated to 55°C for ten minutes in the presence of 50 mmol/l N-methylhydantoin (final concentration) during which interfering foreign proteins were precipitated. After centrifugation the clear supernatant was further saturated to 2.2 mol/l with ammonium sulphate and the NMHase which thereby precipitates was centrifuged down. This was followed by two crystallizations, the first crystallization takes place at a protein concentration of ca. 60 mg/ml, pH 8.0, 0.1 mol/l $K\text{-}PO_4$ buffer, 1.27 mol/l ammonium sulphate. Prisms form after a short time. After 24 hours the crystallization was complete, only 5 % NMHase remained in the centrifuged supernatant. The NMHase crystals were dissolved in 0.1 mol/l $K\text{-}PO_4$ buffer and after removing undissolved constituents the

enzyme solution was subjected to a second crystallization (1.05 mol/l ammonium sulphate concentration). The enzyme crystals which formed overnight were collected, resuspended in buffer, dialyzed against 20 mmol/l phosphate buffer and 2 parts raffinose were added (with respect to the amount of protein) and lyophilized.
The yield was 5.8 KU NMHase = 34 % of the starting activity with a specific activity of 2.15 U/mg protein.

The enzyme activity was tested according to Example 4.1 after each purification step.

No catalase, creatinase, creatininase and carbamoyl-sarcosine hydrolase activities were measurable. A minimal oxidase activity (= sum of glucose oxidase, pyruvate oxidase, lactate oxidase, uricase and cholesterol oxidase) of 0.002 % was noted.

The properties of the recombinant NMHase concerning the pH optimum, pH stability, temperature dependence, thermal stability, K_M, ATP and magnesium dependence, ammonium dependence and molecular weight corresponded to the properties of the NMHase from Arthrobacter.

E x a m p l e 5
Sequencing of the NMHase gene

Fragments from the gene coding for NMHase were subcloned into the cloning vector M13 and sequenced according to standard techniques. The nucleic acid sequence is shown

in SEQ ID NO: 1. This results in a protein with 1288 amino acids whose sequence is shown in SEQ ID NO: 2.

SEQ ID NO: 1
LENGTH OF SEQUENCE: 3867 base pairs
TYPE OF SEQUENCE: nucleotide sequence

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ATGAAGCGCA TCGGAGTAGA CGTCGGCGGC ACCTTCACCG ACTTGTATTT TTCGGACGAT   60
GACCAGCGCA TCGCTGTGGT CGAGAAGGTT CCCTCGACTC CTCACGACCC GTCCGAGGCC  120
GTGATCAATG GCATTAAGAA GCTCTGTGAG AAGGCGGGAG TGTCTCTGTC AGAGATCGAC  180
CAGCTGGTCC ATGGGACTAC GGTAGCCACC AACACCGCAC TAACGCACAC TGGCGCGGAA  240
GTCGGGATGA TTACTACCGA GGGCTTCCGG GATATCTTGC ATATCGCCAG GCACAAAAAA  300
CCGCATAATT TCTCTCTGCA GCAGGATCTG CCGTGGCAGA CCAAACCACT GATCAAGCGC  360
CGGTATCGGC TCACCGTTAA GGAACGTATC ACCGCGCCGC ACGGTGAGAT CCTGGTCCCT  420
TTGGATGAGG ATGAGGTCCG ACAGAGAGTG CGTGAGCTCA AGACAGCTGG CGTGCAGGCC  480
ATCGCTGTAT GTCTGTTGCA TTCGTATTTG AACCCGGAGC ACGAGCAGCG AATCGGCGAG  540
ATCGTCAATG AGGAATTCCC CGAGGCGTAT CTTTCCCTGT CTTCTGAAAT TGTGCCTCTA  600
TATCGAGAGT ATGAACGATT CTCAACTACC GCATTAAATG CCTACGTTGG CCCTAGGGTC  660
TCGCGCTACC TGCATCGCCT GCAGGAGCAG GCCGAAAATT TGGGGTACCA GCGCGAAATC  720
CTGCTAATGC AGTCTTCAGG CGGCATGGTG CCTATTGGTG AAGCTGCGAA ACGGCCGGTG  780
ACGTTGATGA TGTCCGGTCC AGTGGGAGGT CTGATCGGTG GTATGTGGGC TGCTAAGCAG  840
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GCCTACCAGG GTGAGTTGCG CATGCGCCAC CTGCTGGACA CGAAGATCGG TGATCATCAA  960
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GTCTGTTACG GCCGCGGTGG AACGGAACCA ACGTCAACCG ATGCTCAGGT ACTGCTCGGA 1140
AGGATGCGTC CAGACAGAAT TCTGGCCGGC TCGGGTTTGG ACATGGATCT CGACCGTGCC 1200
CGCGCTGCCA TGCAAGGACT GGCCGACAAG CTCGGCATGT CCATCGAAGA AGCGGCACTG 1260
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ATCATCGCAG GTATCGGGTT GCTGGCCACG GATGAGCAAT ACGAGTTTGT GGCAACCAAC 1500
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GAGGAATACG GCCACCGCTT TAAGGGCGGC ACCGTAGAGG TGATCAATAT CAGGGTGGAA 1800
GCCCGTGCCG TTATGGATGA ACTGCCCACG CCAGAAGCGA CGCAGTCAGG CTCACTTGAA 1860
AATGCGTTGG TGGAGACCCG CCCTGTAACT TTCCAGCAAG CAGGTAAGCC TGTCACCTTG 1920
GACACCGGAT TCTACGACCG GGCCAAGATG GGAATCGGAA CCACGTTCGC CGGACCGGTG 1980
GTCATCGAGC AGTACGACTC CACCACAGTG ATTCCTCCAG GTTTCACCGG GACGGTGGAT 2040
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ACCCCGATTC TCATGCGCGT CATCGGCGGC GCGTTGAACT CGGCGGCCAA AGAAATGGCT 2160
TCGGTGCTTT TCCGCATGTC TTACTCATCG ATCATCCGCG AATCGGAGGA TCTGGGAGCT 2220
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CTGATCGATA ACGGTGGCGC ATTTTCTGGA CTGATGGTAG ATATTCAGGA CGTGCAGTCC 2520
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CGAGACTCGG TTCGTGACGC CGGGCAGTTC TGGATCGATT ATTCAGAGCG TATCGTACGC 2760
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GAGATTACCT ACGACCTCAC AGGATCCTCC GCACAGGTGC CGACGGCCTA CAACTGCGCA 2940
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TTCGAAGGAA CCACTGTCTC GGCGTTCACG TTCATCACCC GCATGATGTT CTTGGATGAG 3000
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GCACCGAAGG GAACTATCTT CAATCCGAAC TACCCGGCGG CTACTTTTAG CAGATTCTCC 3120
CAGGTGCAGC GTGCCGTCGA CCTAGCGTTG CGAGCGCTGG CCCCGGTCAT GCCCGAACGA 3180
GTTACTGCCG GAAACTCGGC CCATATCCAC TTCATGTCCT ACTCTGGCTG GGACGAAAAG 3240
CAAGGTGAGT ACTGGGTCTA TCTGGAAGTC AATGAGGGTT CCTATGGAGC TCGCCAGGAC 3300
TCCGACGGCC CAGATTCGGT TGACAACCTC ATCGCCAACA CCCGCAATAA TCCGATCGAA 3360
GAACTCGAAT GGCGGTTCCC GATGCGTACT GACCGCTACG AGCTACGCGA GGATCCGGCC 3420
GCCGCCGGCG AATACCGTGG CGGAATCGGC ATTGTCCGGG AGAACACCTT CTTGGAGGAT 3480
ACTGCGGTGA CCTGCGAGGG CGAACGTCAC GATTCAGATG TCCCATGGGG CGCCTATGGC 3540
GGCCACGACG GTCTGAATGC GTCCCTGATA AAGAACCCAG GCCGCGACGG GGAAGAGTCC 3600
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CCTAGCGGCG GTGGTTTCGG AGACCCGCTC AAGCGCAACC CATTGCAGGT TCTCGAAGAT 3720
GTGCTCGATG GATTCACCAC CACCGAAGCC GCTTCCAGGG ACTACGGTGT GATTCTCAAA 3780
ACGGTCAATG GTCAACTCAC CGTCGATCTA GCGGCCACCG CTGTAAAACG GGAGAACGCA 3840
GTCTCTGAGC TCAGCCACAC CAACTGA 3867

SEQ ID NO: 2
LENGTH OF SEQUENCE: 1288 amino acids
TYPE OF SEQUENCE: amino acid sequence

Met Lys Arg Ile Gly Val Asp Val Gly Gly Thr Phe Thr Asp Leu Tyr
1 5 10 15
Phe Ser Asp Asp Asp Gln Arg Ile Ala Val Val Glu Lys Val Pro Ser
20 25 30
Thr Pro His Asp Pro Ser Glu Ala Val Ile Asn Gly Ile Lys Lys Leu
35 40 45
Cys Glu Lys Ala Gly Val Ser Leu Ser Glu Ile Asp Gln Leu Val His
50 55 60
Gly Thr Thr Val Ala Thr Asn Thr Ala Leu Thr His Thr Gly Ala Glu
65 70 75 80
Val Gly Met Ile Thr Thr Glu Gly Phe Arg Asp Ile Leu His Ile Ala
85 90 95
Arg His Lys Lys Pro His Asn Phe Ser Leu Gln Gln Asp Leu Pro Trp
100 105 110
Gln Thr Lys Pro Leu Ile Lys Arg Arg Tyr Arg Leu Thr Val Lys Glu
115 120 125
Arg Ile Thr Ala Pro His Gly Glu Ile Leu Val Pro Leu Asp Glu Asp
130 135 140
Glu Val Arg Gln Arg Val Arg Glu Leu Lys Thr Ala Gly Val Gln Ala
145 150 155 160
Ile Ala Val Cys Leu Leu His Ser Tyr Leu Asn Pro Glu His Glu Gln
165 170 175
Arg Ile Gly Glu Ile Val Asn Glu Glu Phe Pro Glu Ala Tyr Leu Ser
180 185 190
Leu Ser Ser Glu Ile Val Pro Leu Tyr Arg Glu Tyr Glu Arg Phe Ser
195 200 205
Thr Thr Ala Leu Asn Ala Tyr Val Gly Pro Arg Val Ser Arg Tyr Leu
210 215 220
His Arg Leu Gln Glu Gln Ala Glu Asn Leu Gly Tyr Gln Arg Glu Ile
225 230 235 240
Leu Leu Met Gln Ser Ser Gly Gly Met Val Pro Ile Gly Glu Ala Ala
245 250 255
Lys Arg Pro Val Thr Leu Met Met Ser Gly Pro Val Gly Gly Leu Ile
260 265 270
Gly Gly Met Trp Ala Ala Lys Gln Ser Gly Phe Glu Asn Val Val Thr
275 280 285
Leu Asp Ile Gly Gly Thr Ser Ala Asp Ile Gly Val Ala Tyr Gln Gly
290 295 300
Glu Leu Arg Met Arg His Leu Leu Asp Thr Lys Ile Gly Asp His Gln
305 310 315 320
Ala Met Val Pro Met Val Asp Ile Asp Thr Ile Gly Ala Gly Gly Gly
325 330 335
Ser Ile Ala Tyr Val Asp Ala Gly Gly Val Phe Arg Val Gly Pro Gln
340 345 350
Ser Ala Gly Ala Val Pro Gly Pro Val Cys Tyr Gly Arg Gly Gly Thr
355 360 365
Glu Pro Thr Ser Thr Asp Ala Gln Val Leu Leu Gly Arg Met Arg Pro
370 375 380
Asp Arg Ile Leu Ala Gly Ser Gly Leu Asp Met Asp Leu Asp Arg Ala
385 390 395 400

SEQ ID NO: 2 (page 2)

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Arg Ala Ala Met Gln Gly Leu Ala Asp Lys Leu Gly Met Ser Ile Glu
                405                 410                 415
Glu Ala Ala Leu Gly Ala Leu Gln Ile Gln Lys Phe Gly Met Thr Gln
            420                 425                 430
Ala Ile Glu Gln Asn Ser Val Arg Arg Gly Tyr Asp Pro Arg Asp Phe
        435                 440                 445
Thr Leu Val Ala Ala Gly Gly Ala Gly Ala Leu Phe Ala Cys Glu Ile
    450                 455                 460
Ala Ala Glu Leu Glu Val Pro His Val Leu Val Pro Ala His Pro Gly
465                 470                 475                 480
Ile Ile Ala Gly Ile Gly Leu Leu Ala Thr Asp Glu Gln Tyr Glu Phe
                485                 490                 495
Val Ala Thr Asn Arg Phe Ser Phe Ala Phe Arg Asp Ala Ala Val Ile
            500                 505                 510
Gln Ala Ser Tyr Glu Gln Leu Glu Arg Glu Arg Asn Ala Gln Leu Asp
        515                 520                 525
Ala Glu Glu Val Pro Ala Glu Arg Arg Lys Ile Val Trp Leu Arg Asp
    530                 535                 540
Ala Arg Tyr Glu Gly Gln Gly Tyr Glu Ile Arg Phe Val Val Pro Glu
545                 550                 555                 560
Gly Pro Val Thr Thr Ala Trp Leu Asp Gln Ala Glu Ala Ala Phe His
                565                 570                 575
Asp Ala His Phe Glu Glu Tyr Gly His Arg Phe Lys Gly Gly Thr Val
            580                 585                 590
Glu Val Ile Asn Ile Arg Val Glu Ala Arg Ala Val Met Asp Glu Leu
        595                 600                 605
Pro Thr Pro Glu Ala Thr Gln Ser Gly Ser Leu Glu Asn Ala Leu Val
    610                 615                 620
Glu Thr Arg Pro Val Thr Phe Gln Gln Ala Gly Lys Pro Val Thr Leu
625                 630                 635                 640
Asp Thr Gly Phe Tyr Asp Arg Ala Lys Met Gly Ile Gly Thr Thr Phe
                645                 650                 655
Ala Gly Pro Val Val Ile Glu Gln Tyr Asp Ser Thr Thr Val Ile Pro
            660                 665                 670
Pro Gly Phe Thr Gly Thr Val Asp Asp Ala Gly Asn Leu Val Ile Ala
        675                 680                 685
Cys Pro Ala Val Thr Gln Thr Val Glu Lys Leu Ala Thr Pro Ile Leu
    690                 695                 700
Met Arg Val Ile Gly Gly Ala Leu Asn Ser Ala Ala Lys Glu Met Ala
705                 710                 715                 720
Ser Val Leu Phe Arg Met Ser Tyr Ser Ser Ile Ile Arg Glu Ser Glu
                725                 730                 735
Asp Leu Gly Ala Gly Leu Phe Asp Lys Asp Gly Asn Val Leu Ala Glu
            740                 745                 750
Ser Asp Ser Thr Pro Met Phe Met Gly Ser Met Pro Lys Ile Val Lys
        755                 760                 765
Gly Val Ile Ser Val Leu Gly Asp Asp Ile His Asp Gly Asp Val Ile
    770                 775                 780
Leu His Asn Asp Pro Tyr Leu Gly Ala Thr His Ser Pro Asp Val Ala
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Ile Ile Glu Pro Ile Phe His Asp Gly Glu Leu Val Gly Phe Ala Gly
                805                 810                 815
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SEQ ID NO: 2 (page 3)

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Ala Ser Gly Gln Leu Ile Asp Asn Gly Gly Ala Phe Ser Gly Leu Met
            820                 825                 830
Val Asp Ile Gln Asp Val Gln Ser Glu Gly Thr Ile Phe Arg Ala Val
        835                 840                 845
Lys Val Tyr Glu Lys Gly Val Arg Gln Glu Ser Leu Ile Arg His Ile
    850                 855                 860
Leu Asn Asn Thr Arg Thr Pro Thr Ser Asn Glu Gly Asp Phe Gln Ala
865                 870                 875                 880
Met Ile Ala Ala Cys Asp Leu Ala Lys Ser Arg Tyr Leu Ala Leu Val
                885                 890                 895
Glu Arg Tyr Gly Arg Asp Ser Val Arg Asp Ala Gly Gln Phe Trp Ile
            900                 905                 910
Asp Tyr Ser Glu Arg Ile Val Arg Gln Glu Ile Ala Lys Ile Pro Asp
        915                 920                 925
Gly Val Tyr Glu Thr Glu Thr Gly Tyr Leu Asp Asp Asp Gly Arg Asn
    930                 935                 940
Tyr Gly Lys Lys Leu Pro Ile Val Val Lys Val Ile Val Glu Gly Asp
945                 950                 955                 960
Glu Ile Thr Tyr Asp Leu Thr Gly Ser Ser Ala Gln Val Pro Thr Ala
                965                 970                 975
Tyr Asn Cys Ala Phe Glu Gly Thr Thr Val Ser Ala Phe Thr Phe Ile
            980                 985                 990
Thr Arg Met Met Phe Leu Asp Glu Val Ala Phe Pro Val Phe Val Pro
        995                1000                1005
Gln Asn Glu Gly Met Leu Lys Ala Leu Lys Val Ile Ala Pro Lys Gly
   1010                1015                1020
Thr Ile Phe Asn Pro Asn Tyr Pro Ala Ala Thr Phe Ser Arg Phe Ser
1025                1030                1035                1040
Gln Val Gln Arg Ala Val Asp Leu Ala Leu Arg Ala Leu Ala Pro Val
                1045                1050                1055
Met Pro Glu Arg Val Thr Ala Gly Asn Ser Ala His Ile His Phe Met
            1060                1065                1070
Ser Tyr Ser Gly Trp Asp Glu Lys Gln Gly Glu Tyr Trp Val Tyr Leu
        1075                1080                1085
Glu Val Asn Glu Gly Ser Tyr Gly Ala Arg Gln Asp Ser Asp Gly Pro
   1090                1095                1100
Asp Ser Val Asp Asn Leu Ile Ala Asn Thr Arg Asn Asn Pro Ile Glu
1105                1110                1115                1120
Glu Leu Glu Trp Arg Phe Pro Met Arg Thr Asp Arg Tyr Glu Leu Arg
                1125                1130                1135
Glu Asp Pro Ala Ala Ala Gly Glu Tyr Arg Gly Gly Ile Gly Ile Val
            1140                1145                1150
Arg Glu Asn Thr Phe Leu Glu Asp Thr Ala Val Thr Cys Glu Gly Glu
        1155                1160                1165
Arg His Asp Ser Asp Val Pro Trp Gly Ala Tyr Gly Gly His Asp Gly
   1170                1175                1180
Leu Asn Ala Ser Leu Ile Lys Asn Pro Gly Arg Asp Gly Glu Glu Ser
1185                1190                1195                1200
Trp Pro Ser Lys Val Thr Gly Arg Gln Leu Gln Ala Gly Asp Ser Leu
                1205                1210                1215
Gln Ile Thr Val Pro Ser Gly Gly Gly Phe Gly Asp Pro Leu Lys Arg
            1220                1225                1230
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SEQ ID NO: 2 (page 4)

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Asn Pro Leu Gln Val Leu Glu Asp Val Leu Asp Gly Phe Thr Thr Thr
      1235                1240                1245
Glu Ala Ala Ser Arg Asp Tyr Gly Val Ile Leu Lys Thr Val Asn Gly
   1250                1255                1260
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1265                1270                1275                1280
Val Ser Glu Leu Ser His Thr Asn
                1285
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Deposit Details:

Microorganism BMTU 2194-1 was deposited at the Deutsche Sammlung von Mikroorganismen, Grisebachstrasse 8, D-3400, Gottingen, Germany on 22nd December, 1982 and accorded Accession No. DSM 2563.

Microorganism BMTU 2859 54-2/pKK 177-3 was deposited at the Deutsche Sammlung von Mikroorganismen, Grisebachstrasse 8, D-3400, Gottingen, Germany on 26th September, 1984 and accorded Accession No. DSM 3062.

AUSTRALIAN PATENT OFFICE SEC 104

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. DNA, w h e r e i n it has (1) the nucleic acid sequence shown in SEQ ID NO: 1, (2) a sequence corresponding to it within the scope of the degeneracy of the genetic code or (3) has a sequence which hybridizes with a sequence from (1) or/and (2) under stringent conditions and which codes for a protein with N-methylhydantoinase activity.

2. Recombinant vector, w h e r e i n it contains one or several copies of a DNA as claimed in claim 1.

3. Recombinant vector as claimed in claim 2, w h e r e i n it is a prokaryotic vector.

4. Recombinant vector as claimed in claim 2 or 3, w h e r e i n it is a plasmid.

5. Plasmid pBP010

6. Recombinant vector as claimed in one of the claims 2 to 4, w h e r e i n the DNA as claimed in claim 1 is under the control of a regulatable promoter.

7. Recombinant vector as claimed in claim 6, w h e r e i n the regulatable promoter is the mgl promoter from Salmonella typhimurium.

8. Plasmid pBP006

9. Cell, w h e r e i n it is transformed with a DNA as claimed in claim 1 or with a vector as claimed in one of the claims 2 to 7.

10. Cell as claimed in claim 9, w h e r e i n it is a bacterial cell.

11. Cell as claimed in claim 10, w h e r e i n it is an E. coli cell.

12. Process for the production of a protein with NMHase activity, w h e r e i n
(1) a cell is transformed with a DNA as claimed in claim 1 or with a vector as claimed in one of the claims 2 to 8,
(2) the transformed cells are cultured in a suitable medium and
(3) the protein is isolated from the medium or the cells.

13. Process as claimed in claim 12, w h e r e i n an E. coli cell is used as the cell.

14. Process as claimed in claim 12 or 13, w h e r e i n a DNA or a vector is used for the transformation in which the gene coding for a protein with NMHase activity is under the control of a regulatable promoter.

15. Process as claimed in one of the claims 12 to 14, w h e r e i n the transformed cell is cultured under suboptimal growth conditions.

16. Process as claimed in claim 15, w h e r e i n the cells are cultured in a minimal medium.

17. Process as claimed in claim 15 or 16, w h e r e i n the cells are incubated at a maximum incubation temperature of 30°C.

18. Process as claimed in one of the claims 15 to 17, w h e r e i n the transfer of oxygen into the medium is reduced.

19. Process as claimed in one of the claims 12 to 18, w h e r e i n the induction of the regulatable promoter is only carried out incompletely.

20. Process as claimed in one of the claims 12 to 19, w h e r e i n approximately 3.8 nmol N-methylhydantoin per unit (U) NMHase is added to the NMHase for stabilization.

21. Process as claimed in claim 20, w h e r e i n the NMHase is incubated with a N-methylhydantoin solution at an increased temperature.

22. Protein with NMHase activity which is obtained according to a process as claimed in one of the claims 12 to 21.

23. Reagent for the determination of the content of creatinine in liquids, w h e r e i n it contains a protein as claimed in claim 22.

24. A product of any one of claims 1 to 11 or a process of claim 12, substantially as hereinbefore described with reference to the Examples and/or drawings.

25. The steps, features, compositions and compounds disclosed herein or referred to or indicated in the specification and/or claims of this application, individually or collectively, and any and all combinations of any two or more of said steps or features.

DATED this SECOND day of JULY 1991

Boehringer Mannheim GmbH

by DAVIES & COLLISON
Patent Attorneys for the applicant(s)

AUSTRALIAN PATENT OFFICE

Fig.1

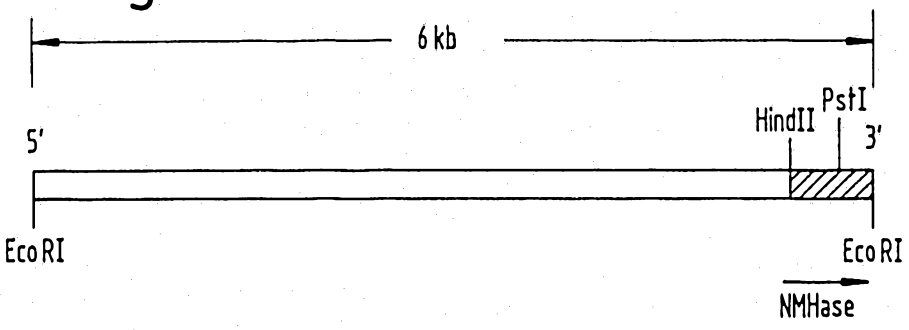


Fig.2

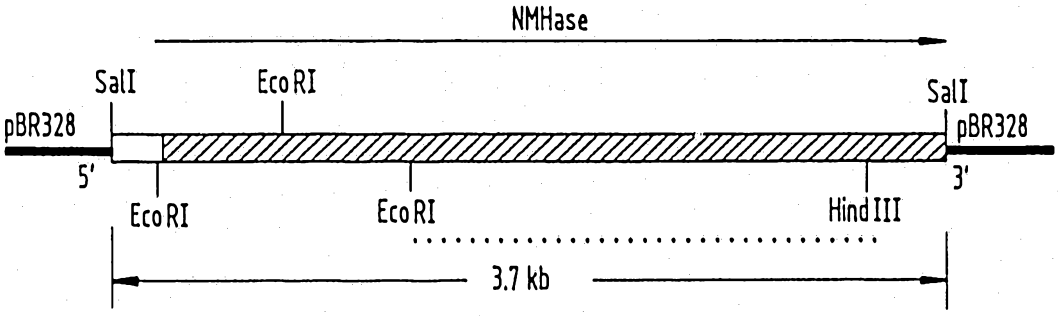


Fig.3

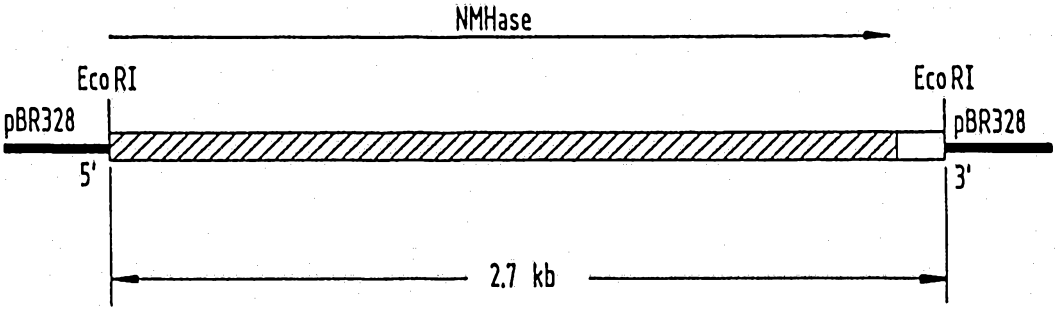


Fig.4

Linker Fragment

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3'-GAATACTTCGCGTAGCCTCATC-5'

Fig.5

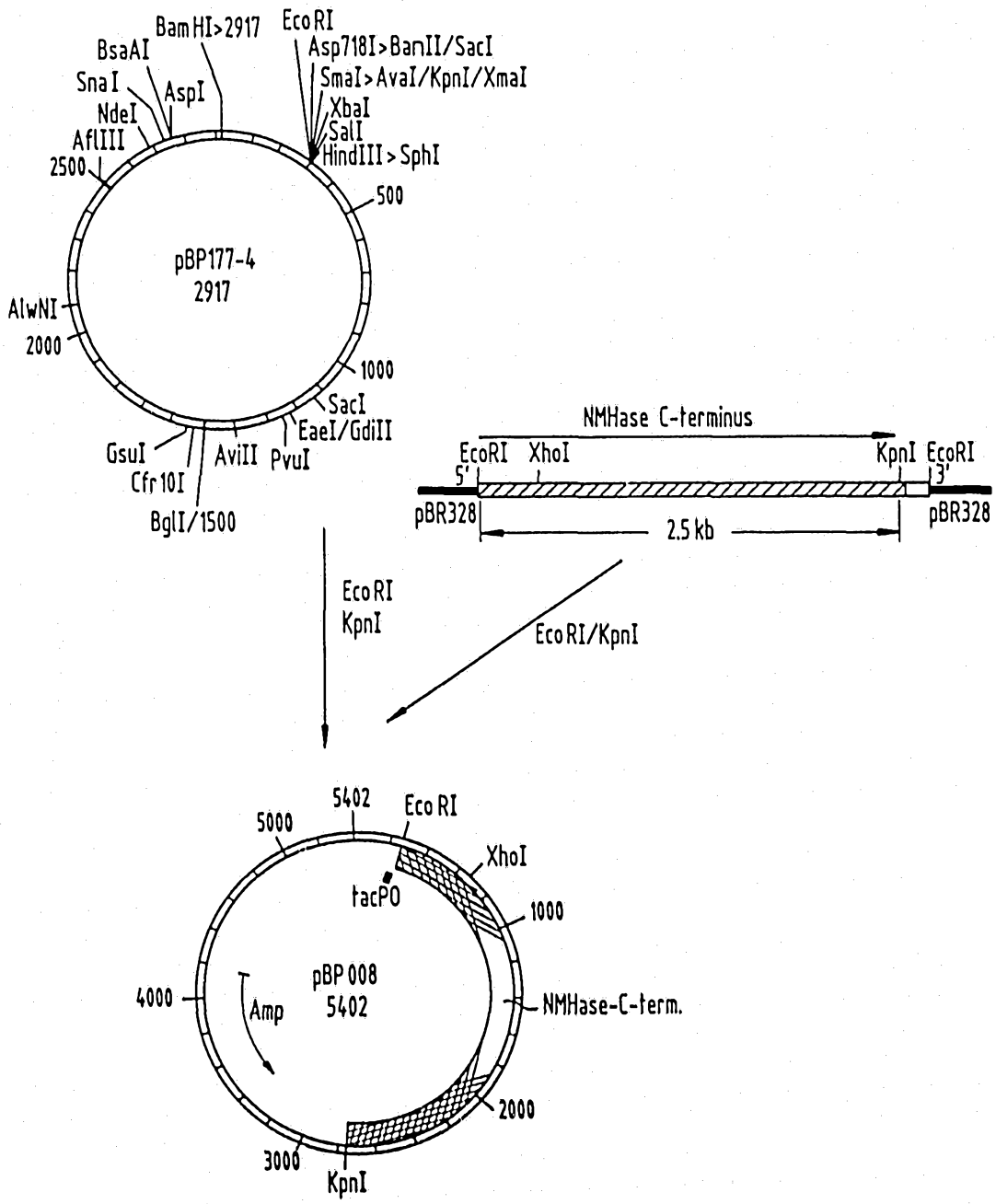

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SmaI>AvaI/KpnI/XmaI
XbaI
SalI
HindIII>SphI
BsaAI
SnaI
AspI
NdeI
AflIII
2500
500
pBP177-4
2917
AlwNI
2000
1000
SacI
EaeI/GdiII
GsuI
AviII
PvuI
Cfr10I
BglI/1500
NMHase C-terminus
EcoRI
XhoI
KpnI
EcoRI
5'
3'
pBR328
2.5 kb
pBR328
EcoRI
KpnI
EcoRI/KpnI
5402
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4000
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Amp
NMHase-C-term.
2000
3000
KpnI

Fig.6

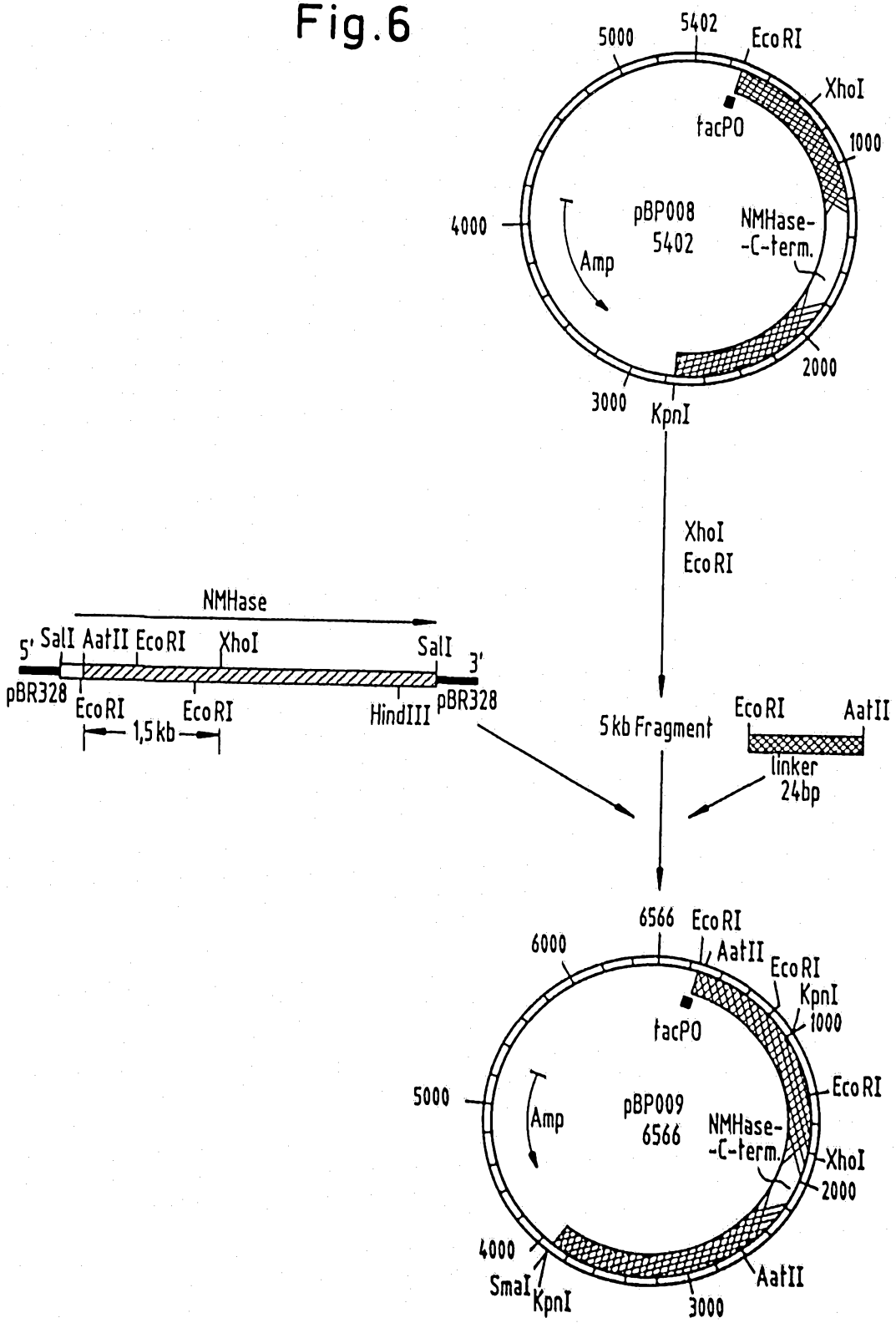

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5402
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XhoI
tacPO
1000
4000
pBP008
5402
NMHase-
-C-term.
Amp
2000
3000
KpnI
XhoI
EcoRI
NMHase
5'
SalI
AatII
EcoRI
XhoI
SalI
3'
pBR328
EcoRI
EcoRI
HindIII
pBR328
1,5 kb
5 kb Fragment
EcoRI
AatII
linker
24bp
6566
EcoRI
6000
AatII
EcoRI
KpnI
tacPO
1000
5000
pBP009
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NMHase-
-C-term.
XhoI
2000
4000
AatII
SmaI
KpnI
3000

Fig.7

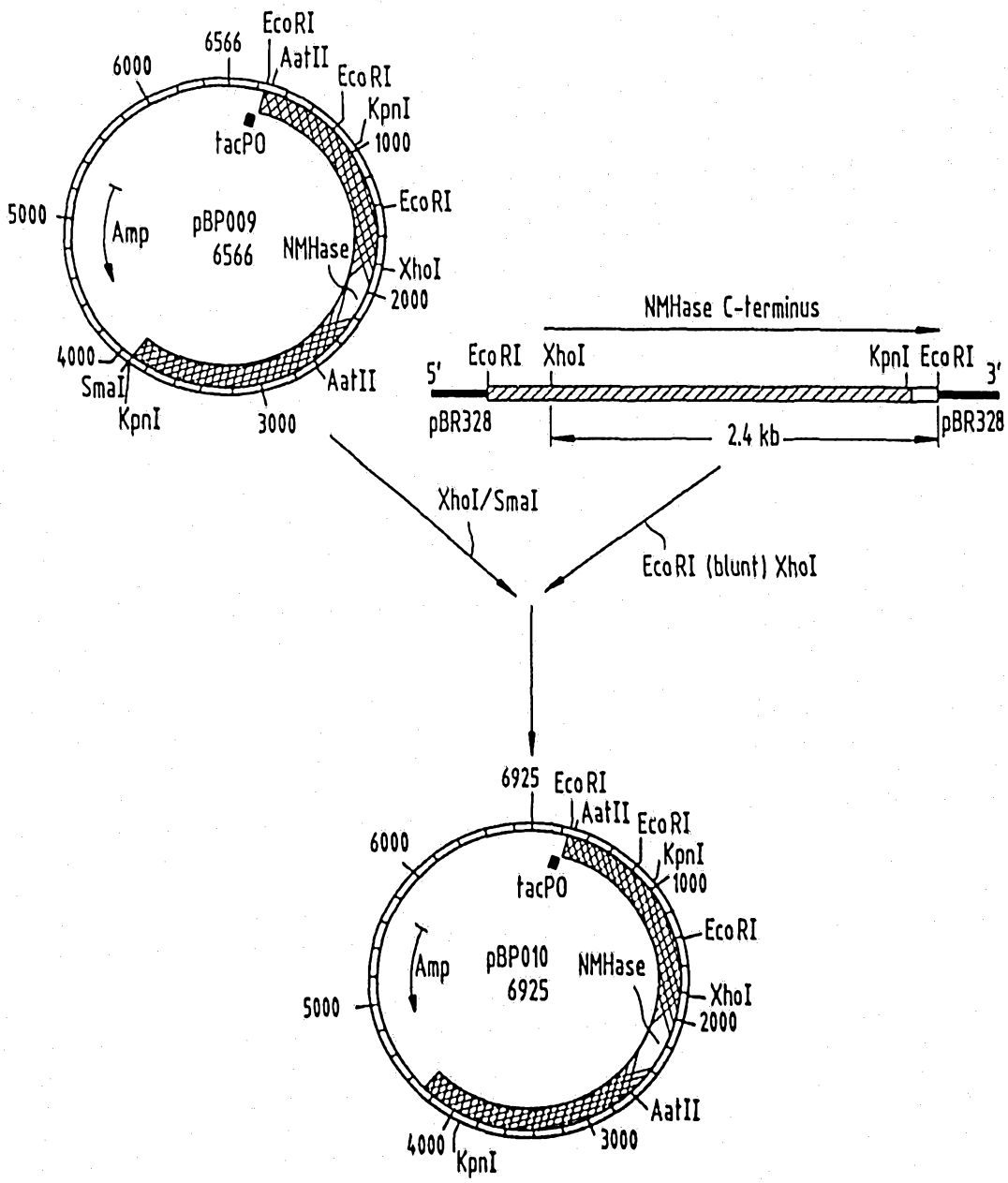

EcoRI
6566
AatII
6000
EcoRI
KpnI
1000
tacPO
5000
Amp
pBP009
6566
NMHase
EcoRI
XhoI
2000
4000
SmaI
KpnI
AatII
3000
NMHase C-terminus
5'
EcoRI
XhoI
KpnI
EcoRI
3'
pBR328
2.4 kb
pBR328
XhoI/SmaI
EcoRI (blunt) XhoI
6925
EcoRI
AatII
EcoRI
6000
KpnI
1000
tacPO
EcoRI
Amp
pBP010
6925
NMHase
XhoI
5000
2000
AatII
4000
KpnI
3000

Fig.8

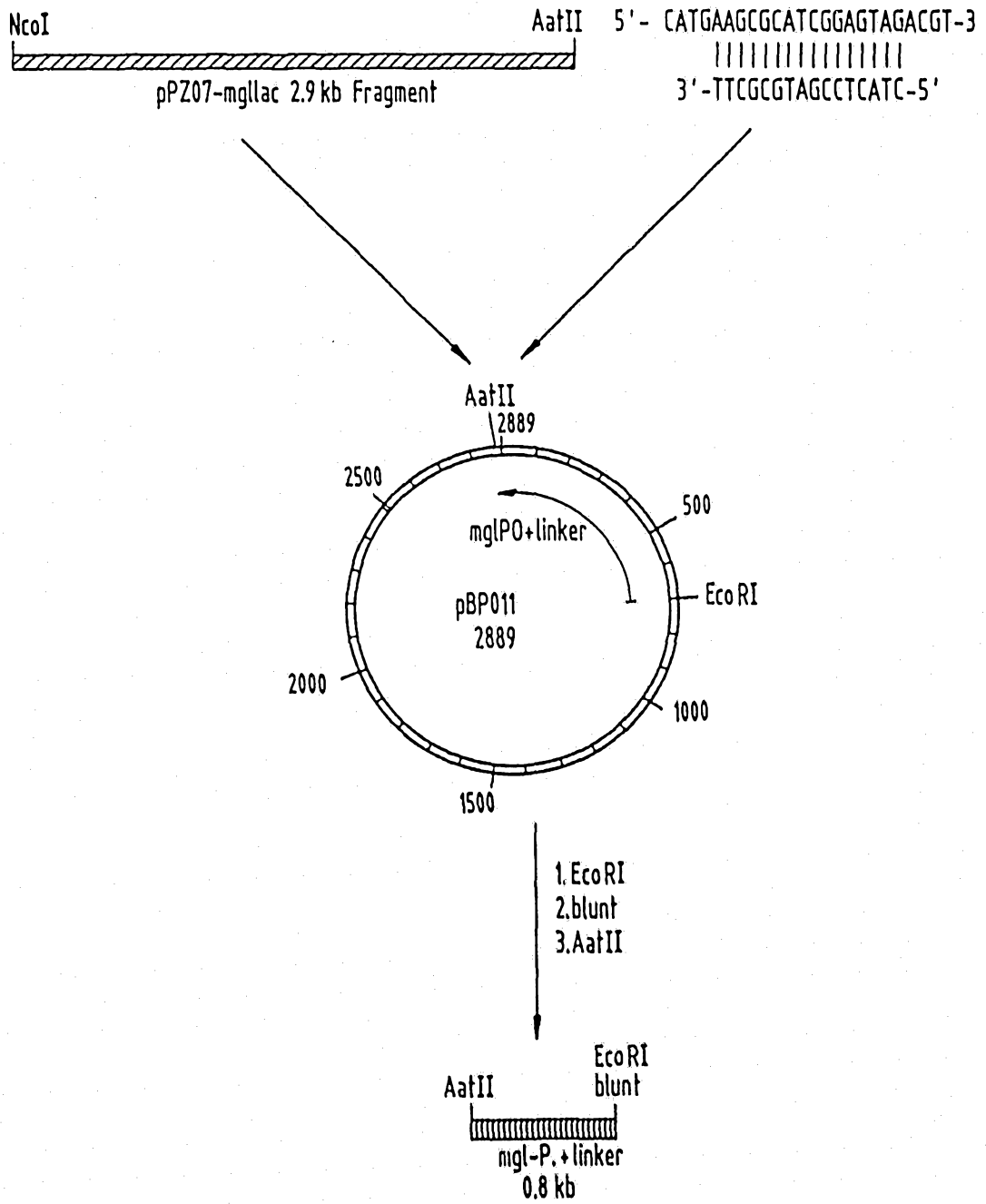
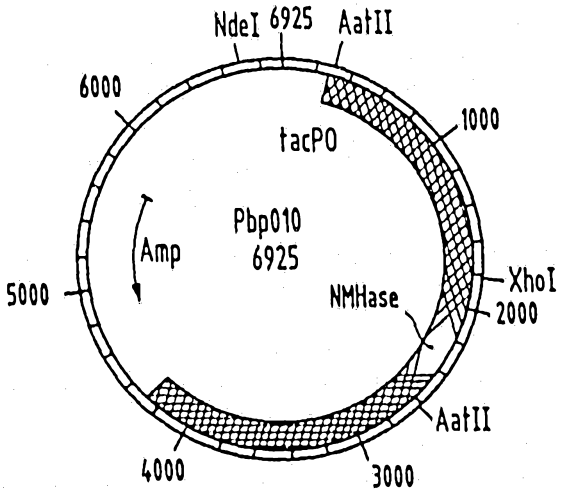

NcoI
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3'-TTCGCGTAGCCTCATC-5'
pPZ07-mgllac 2.9 kb Fragment
AatII
2889
2500
500
mglPO+linker
EcoRI
pBP011
2889
2000
1000
1500
1. EcoRI
2. blunt
3. AatII
EcoRI
blunt
AatII
mgl-P. + linker
0.8 kb

Fig. 9



1. XhoI 2. AatII

1. NdeI 2. blunt 3. XhoI

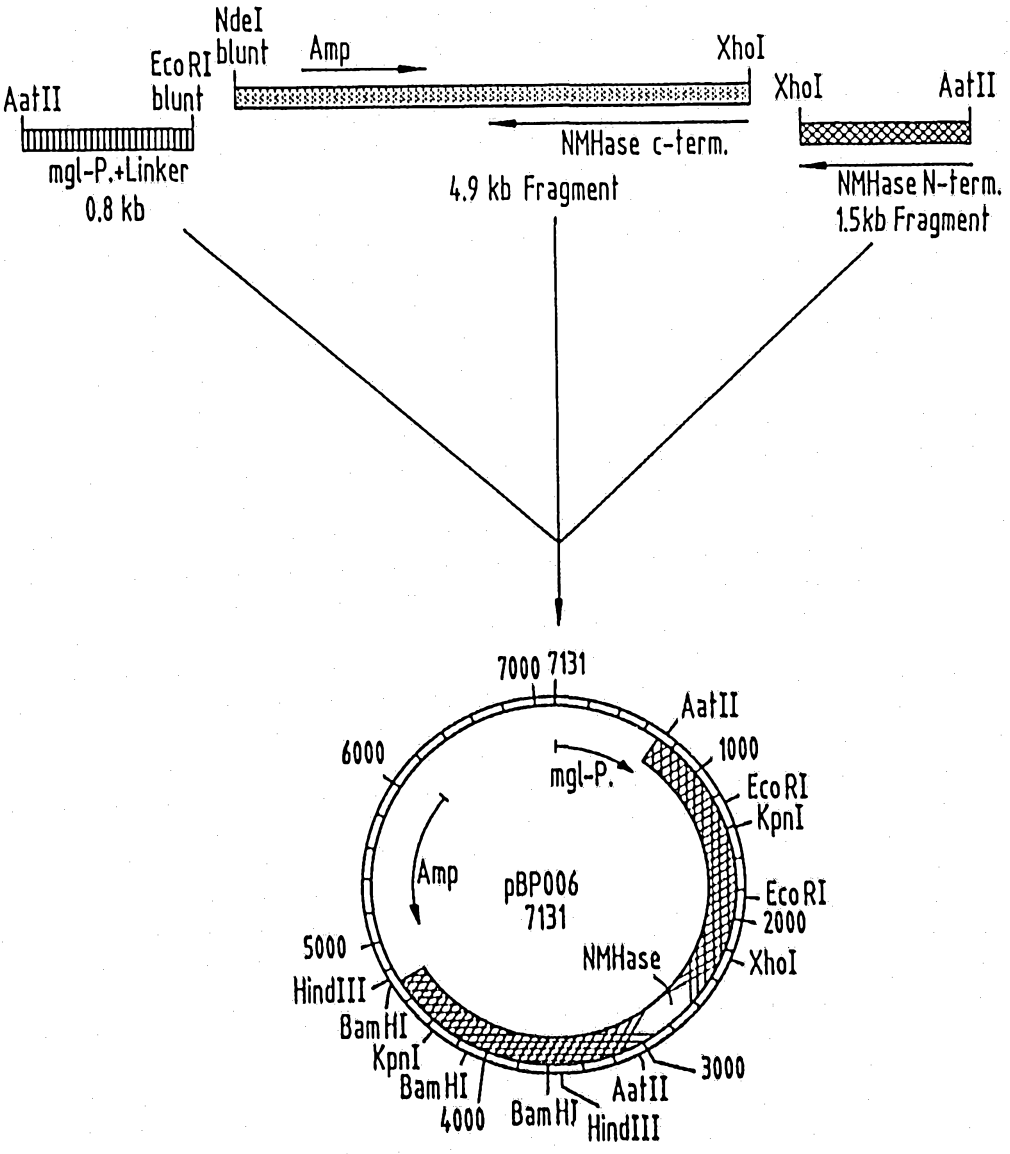


NMHase N-term.
1.5 kb Fragment



NMHase C-term.
4.9 kb Fragment

Fig.10


NdeI
blunt
Amp
XhoI
EcoRI
blunt
AatII
XhoI
AatII
NMHase c-term.
mgl-P.+Linker
0.8 kb
4.9 kb Fragment
NMHase N-term.
1.5kb Fragment
7000 7131
AatII
6000
1000
mgl-P.
EcoRI
KpnI
Amp
pBP006
7131
EcoRI
2000
5000
NMHase
XhoI
HindIII
BamHI
KpnI
BamHI
3000
AatII
4000
BamHI
HindIII