APPLICATION ACCEPTED AND AMENDMENTS ALLOWED 1-11-89

592542

SPRUSON & FERGUSON



COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

CONVENTION APPLICATION FOR A STANDARD PATENT

We, NOVO INDUSTRI A/S,

of Novo Alle, 2880 Bagsvaerd, Denmark

hereby apply for the grant of a standard patent for an invention entitled:

"METHOD FOR STABILIZING EXTRA-CHROMOSOMAL ELEMENTS IN BACTERIA DURING CULTIVATION"

which is described in the accompanying complete specification.



DETAILS OF BASIC APPLICATION

Number of Basic Application:-
5940/84

Name of Convention Country in which Basic Application was filed:-
DENMARK

Date of Basic application:-
12 December 1984

Our address for service is:-

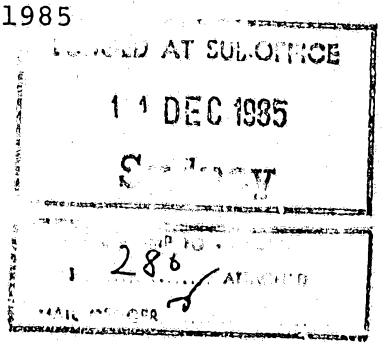
C/- Spruson & Ferguson
Patent Attorneys
Level 33 St Martins Tower
31 Market Street
Sydney New South Wales Australia

DATED this ELEVENTH day of DECEMBER 1985

NOVO INDUSTRI A/S

By:

Registered Patent Attorney.



TO: THE COMMISSIONER OF PATENTS
AUSTRALIA

SBR:ALB:7W

-2682-200=AU

SPRUSON & FERGUSON

COMMONWEALTH OF AUSTRALIA
THE PATENTS ACT 1952

AUSTRALIA CONVENTION STANDARD & PETTY PATENT DECLARATION

DECLARATION IN SUPPORT OF A CONVENTION APPLICATION FOR A PATENT

In support of the Convention Application made for a patent for an invention entitled: "METHOD FOR STABILIZING EXTRA-CHROMOSOMAL ELEMENTS IN BACTERIA DURING CULTIVATION"

Title of Invention

I/~~We~~ William Andersen

Full name(s) and address(es) of Declarant(s)

of 58, Nybro Vænge, DK-2800 Lyngby, Denmark

do solemnly and sincerely declare as follows:-

Full name(s) of Applicant(s)

1. ~~I am/We are the applicant(s) for the patent~~

(or, in the case of an application by a body corporate)

1. I am/~~We are~~ authorised by NOVO INDUSTRI A/S the applicant(~~s~~) for the patent to make this declaration on its/~~their~~ behalf.

2. The basic application(~~s~~) as defined by Section 141 of the Act was/~~were~~ made

Basic Country(ies) in Denmark

Priority Date(s) on 12 December 1984

Basic Applicant(s) by NOVO INDUSTRI A/S

3. ~~I am/We are the actual inventor(s) of the invention referred to in the basic application(s)~~

Full name(s) and address(es) of inventor(s)

(or where a person other than the inventor is the applicant)

3. Børge Krag Diderichsen
32, Estersvej, DK-2900 Hellerup
of Denmark

~~(respectively)~~

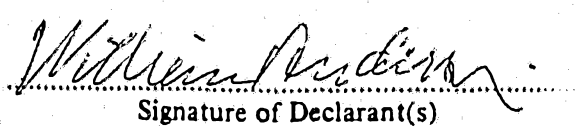
is/~~are~~ the actual inventor(~~s~~) of the invention and the facts upon which the applicant(~~s~~) is/~~are~~ entitled to make the application are as follows: NOVO INDUSTRI A/S is entitled by Contract of Employment between the inventor as employee and NOVO INDUSTRI A/S as employer, as a person who would be entitled to have the patent assigned t a patent were granted upon an application made the inve

Set out how Applicant(s) derive title from actual inventor(s) e.g. The Applicant(s) is/are the assignee(s) of the invention from the inventor(s)

4. The basic application(s) referred to in paragraph 2 of this Declaration was/~~were~~ the first application(s) made in a Convention country in respect of the invention the subject of the application.

Declared at Bagsvaerd this 24th day of January 1986

NOVO INDUSTRI A/S



Signature of Declarant(s)

William Andersen, Manager, Patents and Trade Marks

To: The Commissioner of Patents

SFP4

(12) PATENT ABRIDGMENT (11) Document No. AU-B-51081/85
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 592542

(54) Title
METHOD FOR STABILISING EXTRA-CHROMOSOMAL ELEMENTS IN BACTERIA DURING CULTIVATION

International Patent Classification(s)
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(21) Application No. : 51081/85 (22) Application Date : 11.12.85

(30) Priority Data

(31) Number (32) Date (33) Country
5940/84 12.12.84 DK DENMARK

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(71) Applicant(s)
NOVO INDUSTRI A/S

(72) Inventor(s)
BORGE KRAG DIDERICHSEN

(74) Attorney or Agent
SPRUSON & FERGUSON

(57) The invention resides in the discovery that extra-chromosomal elements can be maintained in host bacteria during cultivation in ordinary media if the extra-chromosomal elements harbour a certain function which under the given conditions is required for normal growth of the host.

When transforming a host bacterium having a mutation, a deletion or another defect in a gene encoding a function which under the given conditions is required for normal growth of the host with for instance a plasmid harbouring a gene encoding this function, only cells transformed with and maintaining the plasmid will survive because only in such cells the requirement of the host cells is supplemented by the plasmid. However, permanent plasmid maintenance is only assured if transfer of genetic information from plasmid to chromosome cannot take place and the rate of spontaneous mutation of the host bacteria into a mutant with no such requirement is insignificant.

CLAIM

1. A method to obtain stable maintenance of a plasmid in bacteria during cultivation, which method comprises inserting a functional dal gene in a plasmid which will not undergo homologous recombination with the host chromosome, and transforming a bacterial host cell having a defect in the

chromosomal dal gene for D,L-alanine racemase and not spontaneously reverting to a Dal^+ phenotype, whereby the D-alanine requirement of the host bacterium caused by its chromosomal gene defect is complemented by the dal gene contained in the plasmid and loss or modification of the plasmid during cultivation of the transformed host cell is insignificant.

7. A method according to Claim 6, wherein the mutation is a deletion of both a part of the dal gene necessary for expression of the Dal^+ phenotype and of a part of directly flanking DNA unnecessary for expression of the Dal^+ phenotype whether it be part of the dal gene or not.

12. A method for the production of a desired product in transformed bacteria which comprises providing a plasmid containing (i) a functional dal gene that will not undergo homologous recombination with the host chromosome and (ii) a gene encoding said desired product; transforming a suitable host bacterium having a defect in the chromosomal dal gene for D,L-alanine racemase and not spontaneously reverting to a Dal^+ phenotype cultivating the transformed bacterium in a suitable nutrient medium; and recovering the desired product.

592542

FORM 10 SPRUSON & FERGUSON

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

COMPLETE SPECIFICATION

(ORIGINAL)

FOR OFFICE USE:

51081/85

This document contains the amendments made under Section 49 and is correct for printing.

Class Int. Class

Complete Specification Lodged:

Accepted:

Published:

LODGED AT SUB-OFFICE
11 DEC 1985
Sydney

Priority:

Related Art:

Name of Applicant: NOVO INDUSTRI A/S

Address of Applicant: Novo Alle, 2880 Bagsvaerd, Denmark

Actual Inventor: BORGE KRAG

Address for Service: Spruson & Ferguson, Patent Attorneys,
Level 33 St Martins Tower,
31 Market Street, Sydney,
New South Wales, 2000, Australia

Complete Specification for the invention entitled:

"METHOD FOR STABILIZING EXTRA-CHROMOSOMAL ELEMENTS IN BACTERIA DURING CULTIVATION"

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

SBR:ALB:7W

A B S T R A C T

A novel method for stabilization of extra-chromosomal elements in bacteria during cultivation without use of antibiotics in the medium has been developed. The method comprises transformation of a host bacterium having a defect in a chromosomal gene needed for the synthesis or maintenance of the cell envelope with an extra-chromosomal element capable of suppressing the requirement caused by the chromosomal gene defect of the host bacterium. The invention furthermore provides for such transformed bacteria and stabilized extra-chromosomal elements and for a method for producing desirable products by inserting a gene for the desirable product into a stabilized extra-chromosomal element and cultivation of suitable host bacteria containing the stabilized extra-chromosomal element comprising the gene for the desirable product. It was demonstrated that according to the principles of this method, a plasmid including the dal gene of Bacillus subtilis and a gene encoding a desirable product can be maintained in an appropriate dal$^-$ B. subtilis host in conventional media. The method thus allows for improved product yield and quality.

According to a first aspect of the present invention there is provided a method for stabilizing extra-chromosomal elements in bacteria during cultivation, said method comprising:

providing of an extra-chromosomal element containing a DNA-sequence encoding a structural or functional component being needed for the synthesis or maintenance of the cell envelope;

and transforming a host bacterium having a defect in the chromosomal gene for such structural or functional component with the extra-chromosomal element containing said DNA-sequence;

whereby the requirement caused by the chromosomal gene defect of the host bacterium is suppressed by the extra-chromosomal element and loss of the extra-chromosomal element during cultivation is insignificant.

The method furthermore includes construction of a host bacterium from which suppression of the chromosomal defect by spontaneous mutation is insignificant and construction of an extra-chromosomal element from which the DNA-sequence supplementing the chromosomal defect of the host bacterium cannot be transferred to the chromosome of the host separately from the rest of the extra-chromosomal element.

As used herein the term "extra-chromosomal elements" means plasmids, bacteriophages or any genetic material which is not normally present in the host bacteria either as independent molecules or integrated into the chromosome. Preferred extra-chromosomal elements are plasmids although use of bacteriophages and other vector systems or integration of DNA into the chromosome may illustrate the present invention to those skilled in the art.

According to a further aspect of the present invention there is provided a transformed bacterium having a defect in a chromosomal gene needed for the synthesis or maintenance of the cell envelope and harbouring an extra-chromosomal element capable of suppressing the requirement caused by the chromosomal defect of the host bacterium.

The present invention furthermore provides a method for producing desired products (i.e. DNA, RNA, peptides, and proteins) in transformed bacteria which comprises:

providing a combined extra-chromosomal element containing i) a DNA-sequence encoding a structural or functional component being needed for the synthesis or maintenance of the cell envelope and ii) a gene encoding said desired product;

transforming a suitable host bacterium having a defect in the chromosomal gene for said structural or functional component with said combined extra-chromosomal element;

cultivating the transformed bacterium in a suitable nutrient medium; and recovering of the desired product from the culture medium.

The present invention also comprises a method for the production of a desired product in transformed bacteria which method comprises:

cultivating in a suitable nutrient medium bacteria having a defect in a chromosomal gene needed for the synthesis or maintenance of the cell envelope, said bacteria containing a combined extra-chromosomal element being capable of suppressing the requirement caused by the chromosomal defect of the host bacterium, said combined extra-chromosomal element also containing a DNA-sequence encoding said desired product; and

recovering the desired product from the culture medium.

Suitable host bacteria are bacteria belonging to the Bacillus or Enterobacteriaceae species, e.g. Bacillus subtilis and Escherichia coli although use of other suitable bacteria may be evident to those skilled in the art.

DETAILED EXPLANATION OF THE INVENTION

In the description the following terms are employed:

dal gene:	the gene for D,L-alanine racemase.
dal^+ gene:	a functional gene (wild type) for D,L-alanine racemase.
dal-1 gene:	a gene with a mutation in the D,L-alanine racemase gene normally causing requirement for external D-alanine.
dal^- host:	a host having a mutation in the dal gene (herein normally requiring external supply of D-alanine for growth).
dal^+ host:	a host with a wild type dal gene.
Dal^+ host:	a host without requirement for external D-alanine.
Dal^- host:	a host with requirement for external D-alanine.

~~CLAIMS~~

The claims defining the invention are as follows:

1. A method for stabilizing extra-chromosomal elements in bacteria during cultivation, said method comprising:

providing an extra-chromosomal element containing a DNA-sequence encoding a structural or functional component being needed for the synthesis or maintenance of the cell envelope, and

transforming a host bacterium having a defect in the chromosomal gene for said structural or functional component with the extra-chromosomal element containing said DNA-sequence,

whereby the requirement caused by the chromosomal gene defect of the host bacterium is suppressed by the extra-chromosomal element and loss or modification of the extra-chromosomal element during cultivation is insignificant.

2. A method according to claim 1, wherein the bacterium is a Bacillus species.

3. A method according to claim 2, wherein the Bacillus species is a Bacillus subtilis.

4. A method according to claim 1, wherein the bacterium is an Enterobacteriaceae species.

5. A method according to claim 4, wherein the Enterobacteriaceae species is an Escherichia coli.

6. A method according to one of the preceeding claims, wherein the structural or functional component causes the formation of D-alanine or suppresses a D-alanine requirement.

7. A method according to claim 6, wherein the structural or functional component is D,L-alanine racemase.

8. A method according to claim 1, wherein the host bacterium harbours a mutation in dal, the gene for D,L-alanine racemase, or a deletion comprising at least part of the dal gene.

9. A method according to claim 8, wherein the mutation is a deletion of both a part of the dal gene necessary for expression of the Dal^+ phenotype and of a part of directly flanking DNA unnecessary for expression of the Dal^+ phenotype whether it be part of the dal gene or not.

10. A method according to claim 1, wherein he extra chromosomal element is a plasmid or bacteriophage harbouring a functional part of the dal gene from B. subtilis.

11. An extra-chromosomal element comprising a DNA-seque ce encoding a structural or functional component causing the formation of D-alanine.

12. A transformed bacterium derived from a host bacterium having a defect in a chromosomal gene needed for the synthesis or maintenance of the cell envelope, said transformed bacterium containing an extra-chromosomal element capable of suppressing the requirement caused by the chromosomal defect of the host bacterium.

13. A transformed bacterium according to claim 12, wherein the bacterium harbours a mutation in dal, the gene for D,L-alanine racemase, or a deletion comprising at least part of the dal gene and the extra chromosomal element comprises a DNA-sequence encoding a structural or functional component causing the formation of D-alanine.

14. A transformed bacterium according to claim 13, wherein transfer of the dal^+ allele from plasmid to chromosome is prevented by insufficient DNA homology.

15. A transformed bacterium according to claim 13, wherein transfer of the *dal*$^+$ allele from plasmid to chromosome is prevented by a recombination deficiency.

16. A transformed bacterium according to claim 12, wherein the defect in the chromosomal gene is a deletion of both a part of the *dal* gene necessary for expression of the Dal$^+$ phenotype and of a part of directly flanking DNA unnecessary for expression of the Dal$^+$ phenotype whether it be part of the *dal* gene or not.

17. A transformed bacterium according to claim 16, wherein the extra-chromosomal element harbours a part of the *dal* gene which is sufficient for expression of the Dal$^+$ phenotype but does not harbour segments homologous to the DNA that flanks at least one side of the deletion on the chromosome.

18. A method for the production of a desired product in transformed bacteria which comprises:

providing a combined extra-chromosomal element containing i) a DNA-sequence encoding a structural or functional component being needed for the synthesis or maintenance of the cell envelope and ii) a gene encoding said desired product;

transforming a suitable host bacterium having a defect in the chromosomal gene for said structural or functional component with said combined extra-chromosomal element;

cultivating the transformed bacterium in a suitable nutrient medium; and

recovering the desired product from the culture medium.

19. A method for the production of a desired product in transformed bacteria which method comprises:

cultivating in a suitable nutrient medium bacteria having a defect in a chromosomal gene needed for the synthesis or maintenance of the cell envelope, said bacteria containing a combined extra-chromosomal element being capable of suppressing the requirement caused by the chromosomal defect of the host bacterium, said combined extra-chromosomal element also containing a DNA-sequence encoding said desired product; and

recovering the desired product from the culture medium.

DATED this ELEVENTH day of DECEMBER 1985

NOVO INDUSTRI A/S

Patent Attorneys for the Applicant
SPRUSON & FERGUSON

SBR:ALB:7W

Fig.1

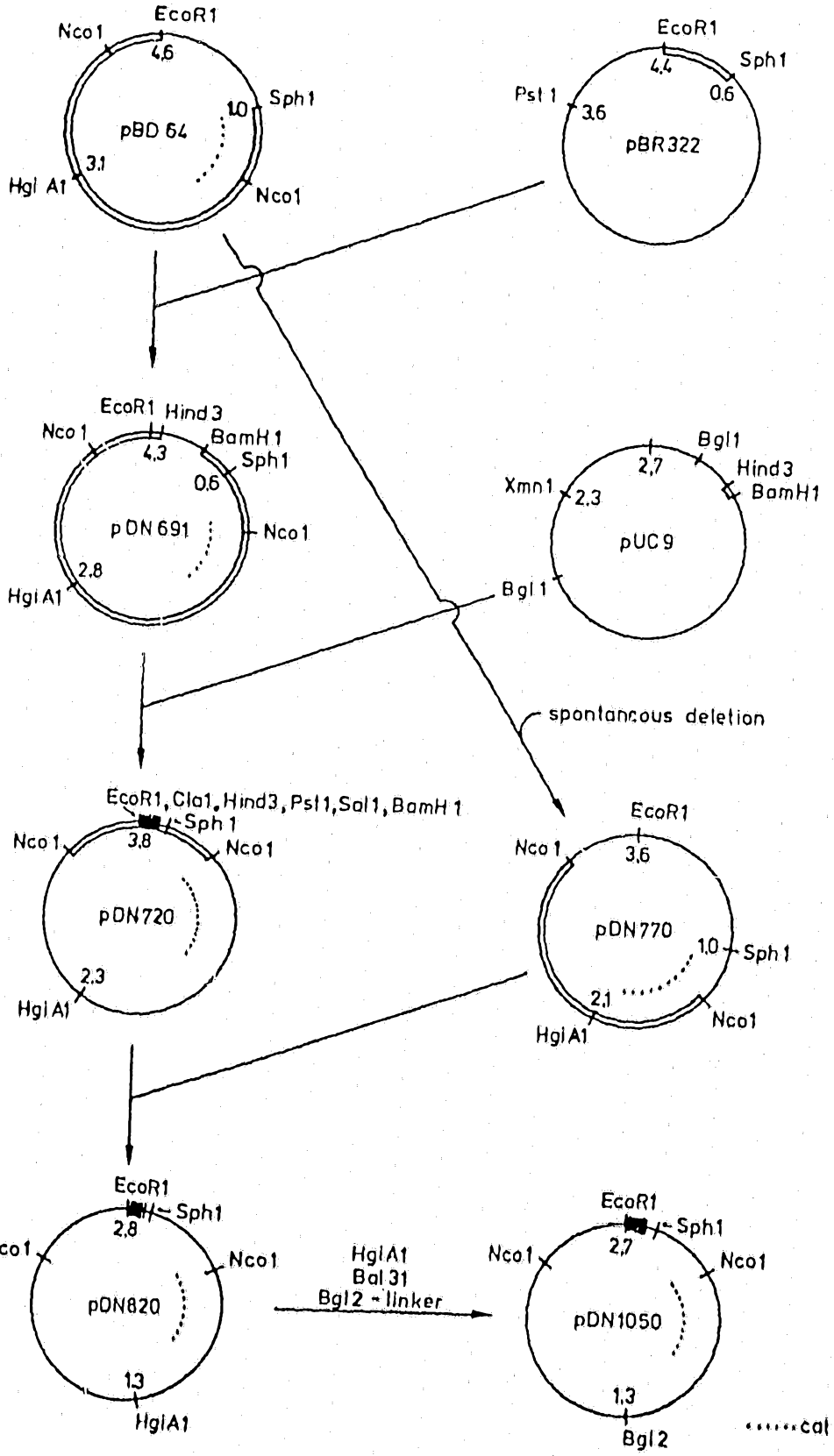

EcoR1
Nco1
4,6
Sph1
1,0
pBD 64
3,1
Hgl A1
Nco1
EcoR1
4,4
Sph1
0,6
Pst 1
3,6
pBR322
EcoR1 Hind 3
Nco 1
BamH 1
4,3
Sph1
0,6
pDN 691
Nco1
2,8
HglA1
Bgl1
2,7
Hind3
BamH1
Xmn1
2,3
pUC 9
Bgl 1
spontaneous deletion
EcoR1, Cla1, Hind3, Pst1, Sal1, BamH 1
Sph 1
Nco 1
3,8
Nco1
pDN720
2,3
HglA1
EcoR1
Nco 1
3,6
pDN770
1,0
Sph1
2,1
HglA1
Nco1
EcoR1
Sph1
2,8
Nco1
Nco1
pDN820
1,3
HglA1
HglA1
Bal 31
Bgl2 - linker
EcoR1
Sph1
2,7
Nco1
Nco1
pDN1050
1,3
Bgl2
.....cat

Fig. 2

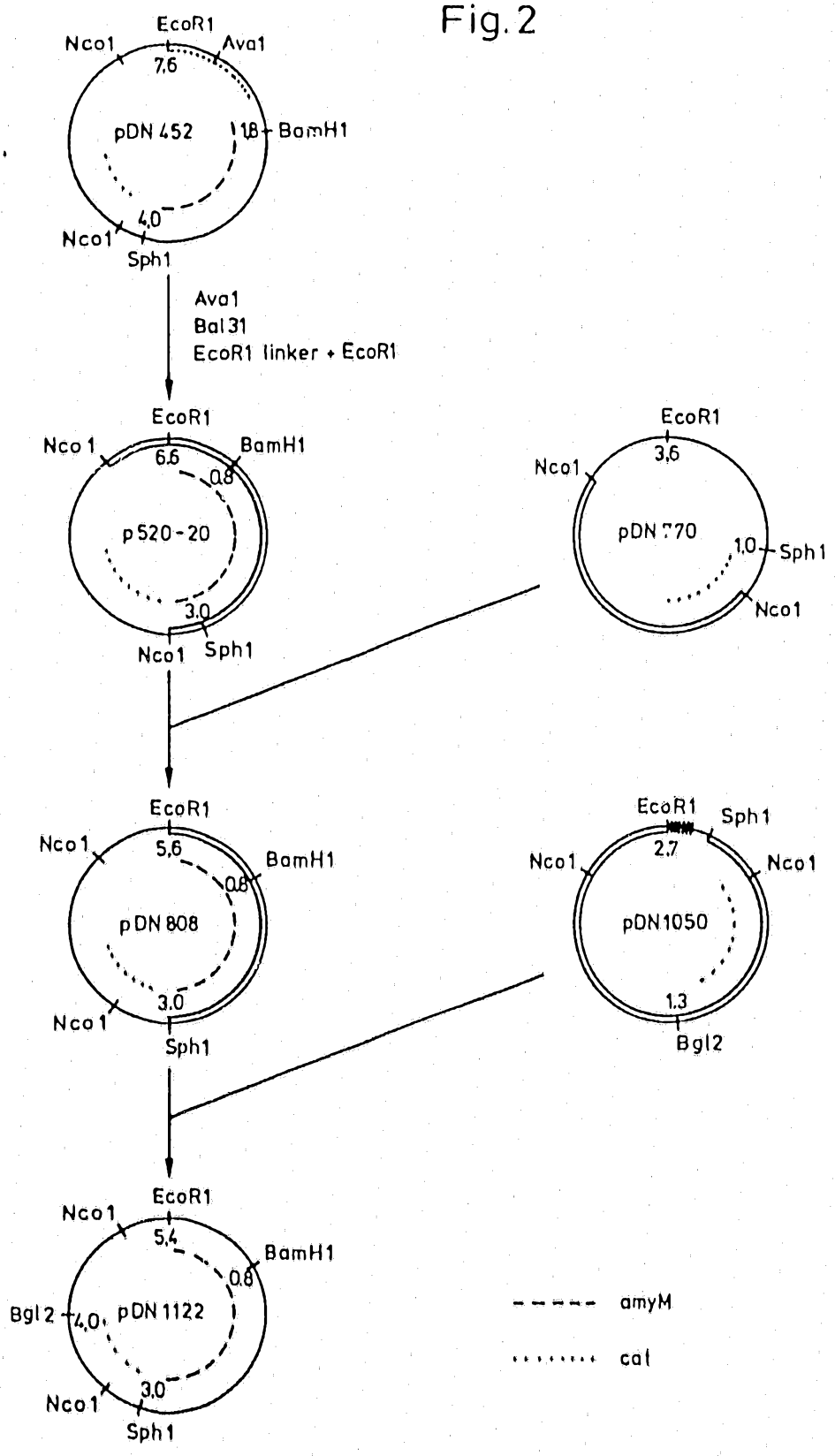

EcoR1
Nco1
Ava1
7.6
pDN 452
1.8
BamH1
4.0
Nco1
Sph1
Ava1
Bal 31
EcoR1 linker + EcoR1
EcoR1
Nco 1
BamH1
6.6
0.8
p 520-20
3.0
Nco1
Sph1
EcoR1
3.6
Nco1
pDN 770
1.0
Sph1
Nco1
EcoR1
Nco 1
5.6
BamH1
0.8
pDN 808
3.0
Nco 1
Sph1
EcoR1
Sph1
2.7
Nco1
Nco1
pDN1050
1.3
Bgl2
EcoR1
Nco 1
5.4
BamH1
0.8
Bgl 2
4.0
pDN 1122
3.0
Nco 1
Sph1
amyM
cat

Fig.3

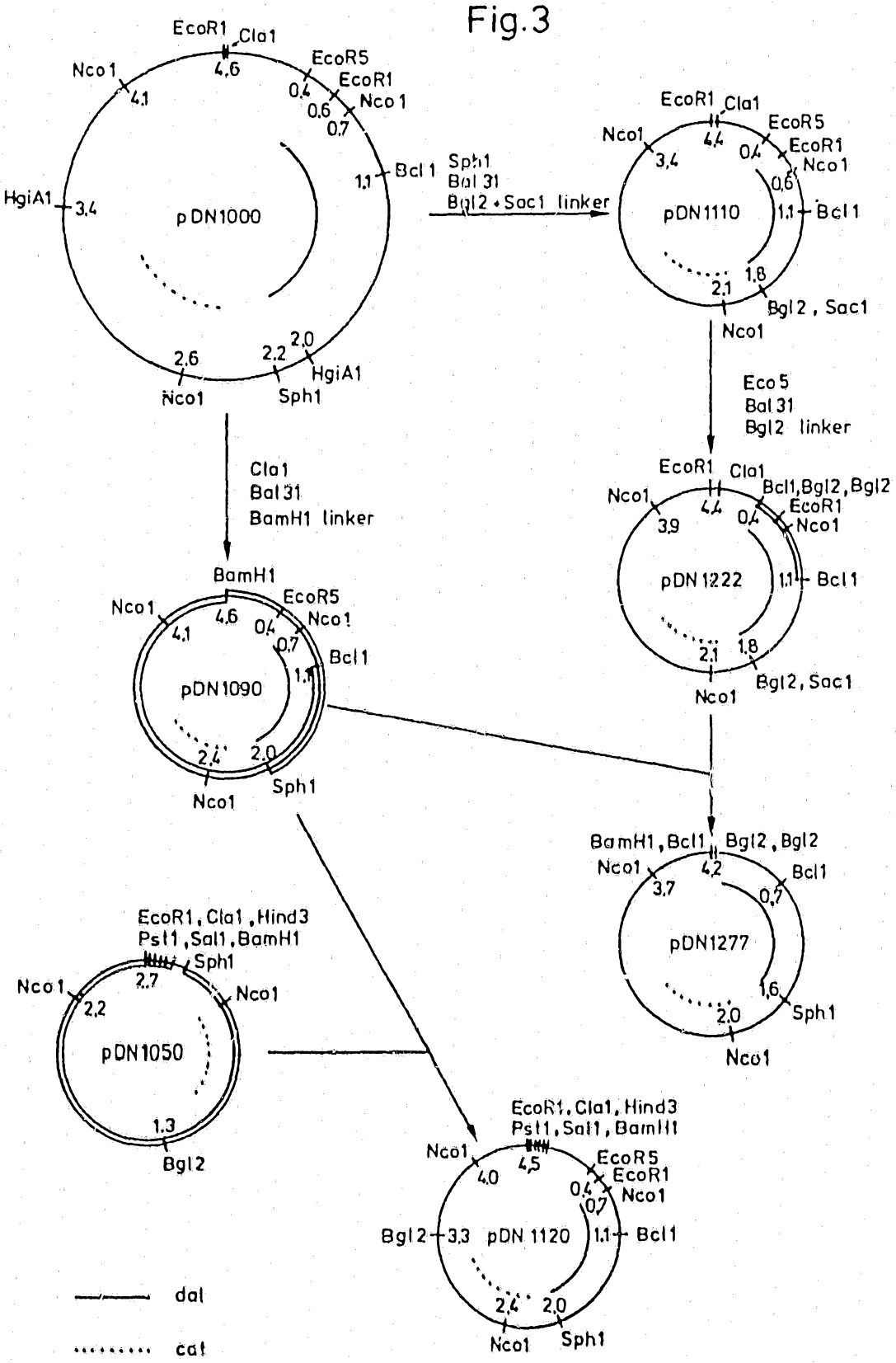

EcoR1
Cla1
EcoR5
EcoR1
Nco1
Nco1
4.1
4.6
0.4
0.6
0.7
Bcl1
1.1
HgiA1
3.4
pDN1000
2.6
2.2
2.0
HgiA1
Nco1
Sph1
Sph1
Bal 31
Bgl2 + Sac1 linker
pDN1110
Bgl2 , Sac1
Nco1
Eco5
Bal31
Bgl2 linker
Cla1
Bal31
BamH1 linker
BamH1
pDN1090
Sph1
Nco1
pDN1222
Bcl1,Bgl2,Bgl2
Bgl2, Sac1
BamH1, Bcl1
Bgl2 , Bgl2
pDN1277
Sph1
EcoR1, Cla1 , Hind3
Pst1 , Sal1 , BamH1
Sph1
pDN1050
Bgl2
EcoR1, Cla1, Hind3
Pst1, Sal1, BamH1
pDN 1120
dal
cat

Fig. 4

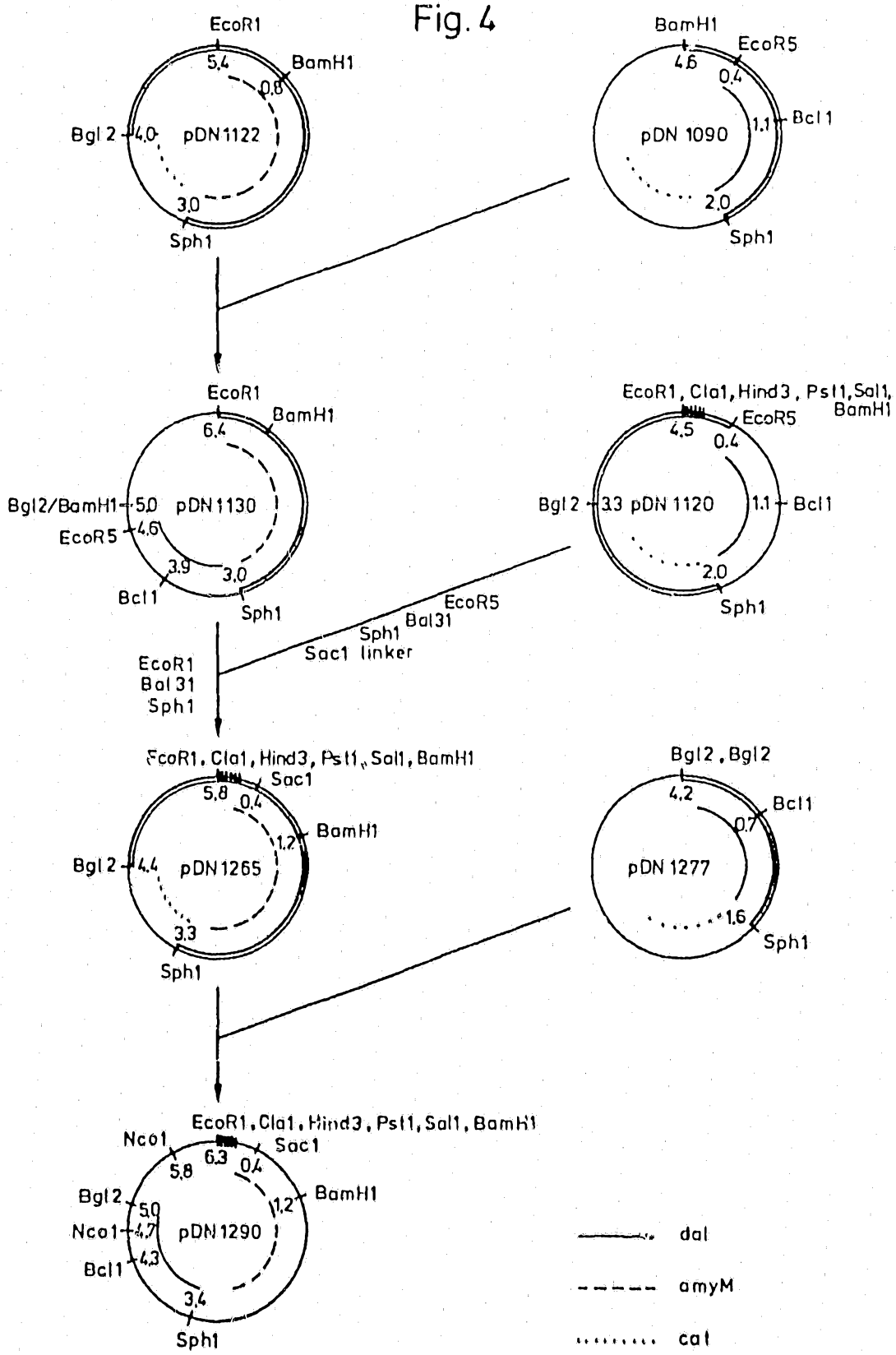
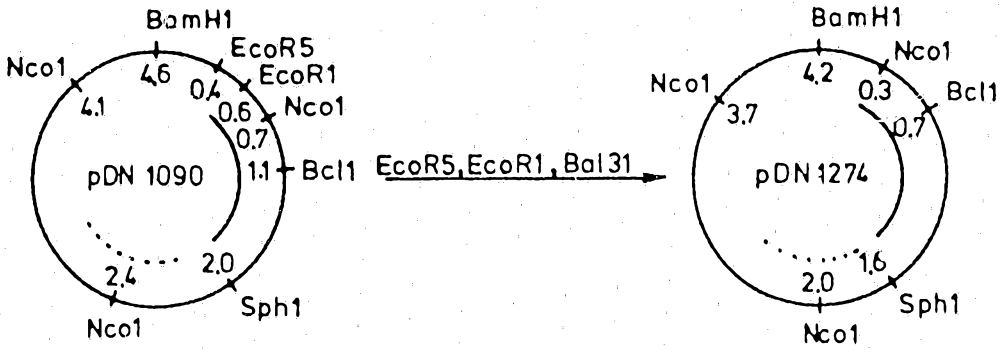

EcoR1
BamH1
5.4
0.8
Bgl 2
4.0
pDN1122
3.0
Sph1
BamH1
EcoR5
4.6
0.4
pDN 1090
1.1
Bcl 1
2.0
Sph1
EcoR1
BamH1
6.4
Bgl2/BamH1
5.0
pDN1130
EcoR5
4.6
3.9
3.0
Bcl1
Sph1
EcoR1, Cla1, Hind3, Pst1, Sal1, BamH1
EcoR5
4.5
0.4
Bgl 2
3.3
pDN 1120
1.1
Bcl1
2.0
Sph1
EcoR5
Sph1
Bal31
Sac1 linker
EcoR1
Bal 31
Sph1
EcoR1, Cla1, Hind3, Pst1, Sal1, BamH1
Sac1
5.8
0.4
BamH1
Bgl 2
4.4
pDN1265
1.2
3.3
Sph1
Bgl2, Bgl2
4.2
Bcl1
0.7
pDN 1277
1.6
Sph1
EcoR1, Cla1, Hind3, Pst1, Sal1, BamH1
Nco1
Sac1
6.3
5.8
0.4
Bgl2
5.0
BamH1
1.2
Nco1
4.7
pDN1290
Bcl1
4.3
3.4
Sph1
dal
amyM
cat

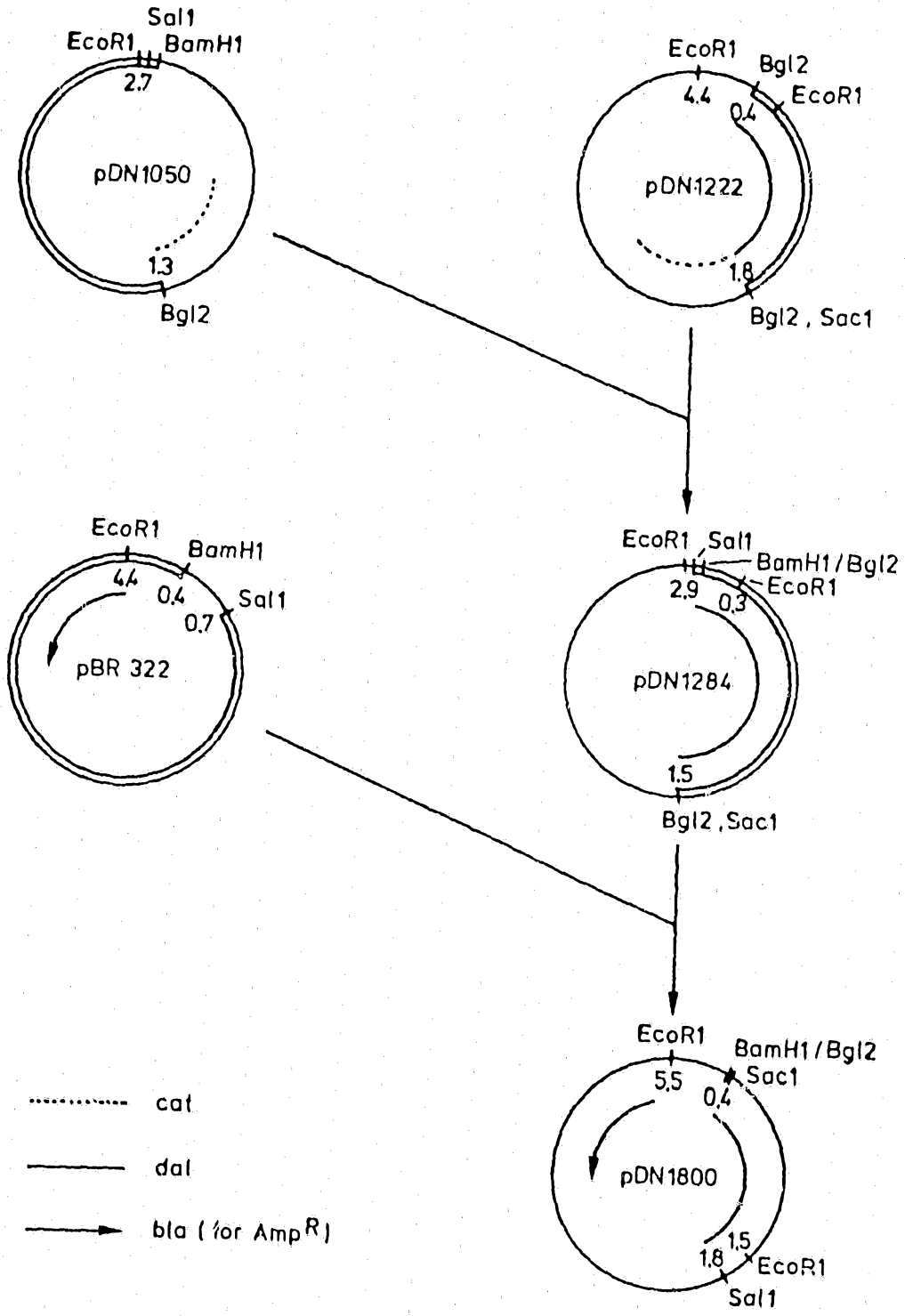
Fig.5


BamH1
EcoR5
EcoR1
Nco1
Nco1
4.6
0.4
0.6
0.7
1.1
Bcl1
4.1
pDN 1090
2.4
2.0
Nco1
Sph1
EcoR5, EcoR1, Bal31
BamH1
Nco1
Nco1
4.2
0.3
Bcl1
3.7
0.7
pDN 1274
1.6
2.0
Sph1
Nco1

——— dal

..... cat

Fig.6


Sal1
EcoR1
BamH1
2.7
pDN1050
1.3
Bgl2
EcoR1
Bgl2
4.4
0.4
EcoR1
pDN1222
1.8
Bgl2 , Sac1
EcoR1
BamH1
4.4
0.4
Sal1
0.7
pBR 322
EcoR1
Sal1
BamH1/Bgl2
EcoR1
2.9
0.3
pDN1284
1.5
Bgl2 , Sac1
EcoR1
BamH1/Bgl2
Sac1
5.5
0.4
pDN1800
1.8
1.5
EcoR1
Sal1
cat
dal
bla (for Amp^R)