

Form 1

616637

COMMONWEALTH OF AUSTRALIA

Patents Act 1952

15435-
LODGED AT SUB-OFFICE
5 AUG 1987
Melbourne

APPLICATION FOR A STANDARD PATENT

(Combined Form - Convention and Non-Convention)

+We TRANSGENE S.A., a French Body Corporate, of
16 rue Henri Regnaud, 92400 COURBEVOIE FRANCE,

hereby apply for the grant of a Standard Patent for an invention entitled
METHOD FOR THE STABILIZATION OF A PLASMID CONTAINED
IN A BACTERIAL STRAIN AND STRAIN OBTAINED
which is described in the accompanying ^{Provisional} Complete Specification.

2. This application is a convention application and is based on the application(s) for a
patent or similar protection made -
in France
on 5th August, 1986, numbered 86.11311, and
on 15th July, 1987, numbered 87.09935, and
on _____, numbered _____

3. My/Our address for service is: Care of JOWIE, THOMSON & CARTER, Patent
Attorneys, of 71 Queens Road, Melbourne, Victoria 3004, Australia.

DATED this 31st day of July, 1987.

To The Commissioner of Patents
COMMONWEALTH OF AUSTRALIA

COWIE, THOMSON & CARTER

By:

Patent Attorneys for
TRANSGENE S.A.

COWIE, THOMSON & CARTER

Patent Attorneys
71 Queens Road, Melbourne,
Victoria, 3004, Australia

PATENT DECLARATION FORM
(CONVENTION OR NON-CONVENTION)

DECLARATION IN SUPPORT OF APPLICATION FOR A PATENT

Insert name of applicant.
Insert title of invention.
Insert full name(s) and address(es) of person(s) making declaration. If applicant a company, person must be authorised to make declaration.

In support of the application made by TRANSGENE S.A.

for a patent for an invention entitled: METHOD FOR THE STABILIZATION OF PLASMID CONTAINED IN A BACTERIAL STRAIN AND STRAIN OBTAINED

I/We Etienne EISENMANN
c/o TRANSGENE S.A. - 16 rue Henri Regnault
92400 COURBEVOIE, FRANCE

Delete alternatives which do not apply

do solemnly and sincerely declare as follows:

- 1. ~~(a) I am/We are the applicant(s) for the patent.~~
- OR (b) I am authorized by the abovementioned applicant to make this declaration on its behalf.
- 2. ~~(a) I am/We are the actual inventor(s) of the invention.~~
- OR (b) Eric DEGRYSE, 6 rue d'Oslo, 67000 STRASBOURG, FRANCE

Insert name(s) and address(es) of actual inventor(s).

~~I/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:

THE SAID COMPANY IS THE ASSIGNEE OF THE INVENTION FROM THE SAID ACTUAL INVENTOR.

Insert details of entitlement to apply, e.g. Applicant is assignee of inventor(s)

3. The basic application(s) as defined by Section 141 of the Act ~~was/were~~ made in the following country of countries on the following date(s) by the following applicant(s)
- | | | | | |
|----|---|----|------------------|-------------|
| in | <u>FRANCE</u> | on | <u>AUGUST 5,</u> | <u>1986</u> |
| by | <u>TRANSGENE S.A. under no.86 11311</u> | | | |
| in | <u>FRANCE</u> | on | <u>JULY 15,</u> | <u>1987</u> |
| by | <u>TRANSGENE S.A. under n0.87 09935</u> | | | |
| in | _____ | on | _____ | 19____ |
| by | _____ | | | |
| in | _____ | on | _____ | 19____ |
| by | _____ | | | |

Delete 3 and 4 if application non-convention. Otherwise insert details of basic application(s).

4. The basic application(s) referred to in paragraph 3 of this Declaration ~~was/were~~ the first application(s) made in a Convention country in respect of the invention the subject of the application.

Place and date of Signature.

Declared at PARIS this 23th day of JULY 1987

NO ATTESTATION
OR SEAL

(MANAGER) Etienne EISENMANN

Etienne Eisenmann
Signature(s) of declarant(s).

To: The Commissioner of Patents,
Australia

(12) PATENT ABRIDGMENT (11) Document No. AU-B-76589/87
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- (56) Prior Art Documents
FR 2511032
- (57) Claim

1. A bacterium, the genome of which comprises a *dap* gene inactivated by mutation and which is transformed by a plasmid comprising a functional *dap* gene.

2. A bacterium according to claim 1, the genome of which comprises a *dapD* gene inactivated by mutation and which is transformed by a plasmid comprising a functional *dapD* gene.

7. A bacterium according to any one of claims 1 to 6 in which the plasmid comprising a functional *dap* gene also comprises a gene which encodes a protein of industrial interest and the elements ensuring its expression.

8. A bacterium according to claim 7 in which the plasmid comprising a functional *dap* gene also comprises a gene which encodes a protein of industrial interest selected from hirudin, gamma-interferon, catechol 2, 3 oxygenase and alpha-antitrypsin.

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9. A process for preparing a homogenous culture of bacterium according to any one of claims 1 to 8 which comprises culturing said bacterium in a DAP-free medium in the presence of a substantial amount of lysine.



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Form 10

PATENTS ACT 1952-1973

COMPLETE SPECIFICATION

(ORIGINAL)

FOR OFFICE USE

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Related Art:

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TO BE COMPLETED BY APPLICANT

•• Name of Applicant: TRANSGENE S.A., a French Body Corporate, of
16 rue Henri Renault 92400 COURBEVOIE FRANCE.

•• Address of Applicant:

Actual Inventor: ERIC DEGRYSE

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Complete Specification for the invention entitled:
METHOD FOR THE STABILIZATION OF A PLASMID CONTAINED IN A BACTERIAL
STRAIN AND STRAIN OBTAINED

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

1 -

*Note: The description is to be typed in double spacing, pica type face, in an area not exceeding 250 mm in depth and 180 mm in width, on tough white paper of good quality and it is to be inserted inside this form.

The practical application of recombinant DNA technology to the production of molecules of interest by microorganisms generally runs into the problem of the instability of the recombinant plasmids.

5 Cloning and expression vectors commonly used in the laboratory are generally multicopy plasmids, and their stable transmission to the progeny is assured by the large number of plasmids per cell genome (Jones et al. 1980). However, the introduction of foreign genes into these plasmids gives rise to various degrees of instability during the multiplication cycles of the bacteria. Thus, industrial production operations may require 1,000-litre cultures, resulting in more than 10^{16} cells, after more than 50 generations. Therefore, it is essential to stabilize the plasmids in the bacteria in order to ensure their presence, and therefore the expression of the foreign molecule, until the end of the culture in the fermenter.

10 The stabilization of plasmids by integrating a gene coding for resistance to an antibiotic is a conventional laboratory practice which cannot, however, be applied to an industrial scale use for several reasons:

- the use of an antibiotic-resistant bacterial strain may represent a risk to the environment;
- the quantity of antibiotic required during the culture significantly increases the cost of production;
- and
- the use of an antibiotic cannot be envisaged in the production of substances to be used in human or veterinary therapy.

15 Therefore, it is essential to develop other methods for the selection of bacteria carrying a recombinant plasmid. The few models which have already been applied are based on the same principle: the host cell is made to undergo mutation (auxotrophic mutation or introduction of a gene which is lethal to the bacterium) so as to prevent it from multiplying in the absence of a plasmid coding for a character which makes up for the deficiency of the host.

20 For example, Skogman and Nilsson (1984 and 1985) have used the complementation of a heat-sensitive val S mutation

by the *val S* gene carried by a plasmid; in this model the stability of the plasmid, which carries the tryptophan operon, is total after 200 generations at non-permissive temperature, whereas under non-selective conditions (30°C), a plasmid loss of 1.2% is observed in each generation.

Miwa et al. (1984) have used, as the host strain, a streptomycin-dependent (Sm^d) *E. coli* mutant and a plasmid carrying a gene (*rpsL* of an Sm^R strain) which masks the Sm^d phenotype and renders the strain independent of streptomycin, thereby ensuring a plasmid stability greater than 99%.

Herthberger and Rosteck (1984) have rendered a bacterium lysogenic for a prophage lambda whose repressor has been deleted; the cell can escape lysis only in the presence of a plasmid carrying the lambda *cI* repressor.

A new selection model has been developed, based on the complementation of a dap^- chromosomal mutation with a dap^+ plasmid gene, which ensures the survival of only the plasmid-carrying cells.

Diaminopimelic acid (DAP) is a component of bacterial cell walls; it is also an intermediate in the biosynthesis of lysine from aspartate. A strain which is deficient in an enzyme for DAP biosynthesis will not be able to multiply in a minimal medium; the addition of lysine to the medium will permit growth, but will soon bring about the lysis of the cell which does not incorporate DAP in its membrane (Work 1950 - Davis et al., 1973).

The deficiency of an enzyme for DAP biosynthesis may be compensated for by the introduction of the corresponding gene on a plasmid and, in particular, on the expression vector of the foreign protein required to be produced. If the bacterium loses the plasmid, it becomes dap^- and can no longer multiply. This model has the advantage that it can be applied to any DAP-free culture medium, i.e. any medium for industrial-scale production and any medium, rich or minimal, to which lysine has been added.

The system also has the advantage of not interfering with the synthesis of DNA, RNA or proteins.

5 A $dapD^-$ strain (with one of the 9 genes of the bio-synthetic pathway of lysine, the gene D, corresponding to tetrahydropicolinate-N-succinyl transferase, deleted) is constructed and the $dapD$ gene on a commonly used expression plasmid (carrying the gene to be expressed under the control of the promoter P_T) is introduced. Under selection conditions, it can be shown that the plasmid is stable for at least 150 generations. The cloning of another gene ($dapA$), coding for an enzyme for the biosynthesis of lysine, on a plasmid, has already been carried out, with a view to increasing the production of lysine (Dauce-Le Reverend et al. 1982); however, these authors have not been able to control the stability of their plasmid.

10 The $dapD$ gene of *E. coli* has already been cloned into the plasmid pBR322 and its nucleotide sequence has been published by Richaud et al. (1984); however, the object of this work is only to study the regulation of this gene.

15 For this reason, the present invention relates to a method for stabilizing a plasmid vector contained in a bacterium, wherein the bacterium comprises a dap^- chromosomal mutation and wherein the plasmid vector carries a dap^+ gene.

20 Accordingly the invention provides in one broad aspect a bacterium, the genome of which comprises a dap gene inactivated by mutation and which is transformed by a plasmid comprising a functional dap gene.

In a further aspect the invention provides a process for preparing a homogeneous culture of bacterium comprising culturing said bacterium in a dap^- free medium in the presence of a substantial amount of lysine.

Among dap^- chromosomal mutations, $dapD^-$ will preferably be



employed; however, other genes coding for an enzyme for DAP biosynthesis could also be employed, for example *dapA*, on condition that a vector plasmid carrying the corresponding gene is used; in the case of a *dapD*⁻ strain, the gene to be inserted into the plasmid will be *dapD*.

5 The *dap* stabilization system does not interfere with the expression of the protein, it may therefore be used with any expression vector.

10 In order to avoid possible homologous recombinations between the *dapD*⁺ plasmid and the mutated *dapD* gene of the chromosome, it is preferable to provide for a substantial deletion of the *dapD* gene from the chromosome.

 For this reason, the present invention relates more particularly to a method for the stabilization of a plasmid vector contained in a bacterium as described above, wherein



the dap chromosomal mutation is a deletion of at least a portion of the dapD gene and wherein the plasmid vector carries an intact dapD gene.

5 It is clear that in order to avoid any recombination, a total deletion of the dapD gene will be the most satisfactory solution.

10 However, a selection system, however strong it may be, cannot overcome the inherent instability of a plasmid. Because of this, in order to increase the stability of the expression vectors in question by genetic means it is possible to introduce into said vectors a sequence which maintains them in the monomeric state, especially the "cer" sequence.

15 The "cer" (Summers and Sherratt, 1984) is an element which does not code for any protein and the presence of which promotes the maintenance of a plasmid in the monomeric state. If the plasmid forms multimers, the number of units which can be distributed to daughter cells drops and plasmid-free cells are obtained more easily. Therefore, cer stabilizes the plasmid indirectly by maintaining it in the monomeric state.

20 For this reason, the present invention also relates to plasmid vectors which additionally comprise the gene coding for a protein of industrial value and to the elements which ensure its expression in the host bacterium and, in particular, to the gene coding for a hirudin or one of the natural or synthetic variants thereof, for example, C_{2,3}O, interferon-gamma or alpha-antitrypsin, and to strains transformed by these different vectors and to the methods for the preparation of industrial proteins by culturing said transformed strains in a complete medium and recovering the protein after culture.

25 In the following text, the expression system comprises the leftward promoter, P_L, of phage lambda; however, it could be another promoter which is active in the bacterium in question. This explains the reason for not giving a complete description of the expression elements of the heterologous protein, this type of plasmid now being widely known. It will be advisable simply to insert dapD or

30

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other gene into a non-essential site of the plasmid vector.

The plasmids in question may be employed to transform any *E. coli* strain which has been rendered $dapD^-$.

By virtue of the method according to the invention, bacterial strains in which the expression plasmids are stable in a complete medium without the need, for example, for a selection pressure by an antibiotic or for a specific amino acid-free medium, are obtained. Additionally, the method according to the invention exerts a counter selection against bacteria which have lost the plasmid.

Therefore, the invention also relates to bacterial strains, in particular dap^- *E. coli*, transformed by a plasmid vector carrying a dap^+ gene and to elements which ensure the expression of a protein of industrial value, carried by this plasmid.

The invention also relates to a method for the preparation of a protein of industrial value from a bacterium, wherein a bacterium transformed by a plasmid according to the invention is cultured in a complete medium, said plasmid additionally comprising the gene of said protein and control signals for the expression of this protein in the host bacterium.

The object of the examples below is to demonstrate the stability of plasmids carrying the $dapD$ gene in the dap^- bacteria, in comparison with plasmids carrying the Amp^r gene.

Additionally, these plasmids carrying the $dapD$ gene have been used to express the genes coding for hirudin and interferon-gamma.

These two genes are placed under the control of the phage lambda promoter P_L and the *E. coli* host strains contain the heat-sensitive repressor CI857; this system makes it possible to induce the expression of the gene controlled by P_L by increasing the temperature.

The *E. coli* strain TGE 900 which contains the repressor CI857 was modified to become $dapD^-$ by giving the strain TGE 7615 or TGE7214 and strain \checkmark 5969 was modified to become TGE7303.

The following plasmids were then prepared according to the diagrams attached hereto:

	<u>Amp^r</u>	<u>dapD</u>	<u>Foreign protein</u>
Figure 1 - pTG764	+	+	0
Figure 2 - pTG720	+	0	Hirudin
Figure 2 - pTG771	+	+	Hirudin
Figure 3 - pTG775	0	+	Hirudin
Figure 3 - pTG776	0	+	Hirudin
Figure 4 - pTG766	0	+	0
Figure 4 - pTG767	0	+	0
Figure 5 - pTG7671	0	+	IFN-gamma

Figure 6: Restriction map of the HindIII-BamHI fragment of pDB6.

Figure 7: Diagram of PstI inserts of pDB6 in M13mp8 and position of EcoRI sites introduced therein.

Figure 8: Restriction map for the KpnI-BglII fragment of pTG47.

Figure 9: Demonstration of the deletion of the dap gene in chromosomal DNAs of different strains, by "Southern blot" autoradiography.

Figure 9A: probe = kan^R gene carried by the EcoRI fragment of pTG47

bands 6 to 9	DNA cut with PstI
10 to 14	DNA cut with BamHI and HindIII
bands 6 and 10	= strains GC4540
7 and 11	TGE7213
8 and 12	TGE7214
9 and 13	TGE901
14	pDB6
15	molecular weight markers

Figure 9B: probe = 5' side of the dapD gene carried by the EcoRI fragment of M13TG620

bands 4 to 7	DNA cut with PstI
8 to 11	DNA cut with BamHI and HindIII
bands 4 and 8	= strains GC4540
5 and 9	TGE7213
6 and 10	TGE7214

7 and 11 TGE901
bands 13 molecular weight
markers
1 and 12 pDB6 cut with PstI or
BamHI and HindIII
2 M13TG620 cut with PstI
3 M13TG597 cut with PstI

Figure 9C: probe = flanking regions of the dapD gene carried
by the KpnI-BglII fragment of pTG47.

Chromosomal DNAs cut with PstI.

bands 4 = strains GC4540

5 TGE7213

6 TGE7214

7 TGE901

bands 8 = molecular weight markers

1 BamHI-HindIII fragment of pDB6 cut with
PstI

2 M13TG620 cut with PstI

3 M13TG597 cut with PstI

Figure 9D: probe = flanking regions of dapD gene carried
by the KpnI-BglII fragment of pTG47.

Chromosomal DNAs cut with PstI.

bands 4 = strains RH5345

5 RL58

6 TGE7615

7, 2 and 3 (as in 9C)
= molecular weight markers

Figure 10: Diagram and restriction map for pTG790.

Figure 11: Diagram and restriction map for pTG792.

Figure 12: Diagram and restriction map for pTG7922.

Figure 13: Diagram and restriction map for pTG769.

Figure 14: Diagram and restriction map for pTG7406.

Figure 15: SDS polyacrylamide gel electrophoresis, visuali-
zed with Coomassie blue, of proteins synthesized
by TGE7213/pTG7407.

bands 1 and 8 = molecular weight markers

bands 2 to 5 = culture at 30°C for 4 hours
(2 and 3) or 7 hours (4 and 5)

bands 6 to 12: culture initiated at 42°C for
4 hours (6, 11 and 12) or 7 hours

(7, 9 and 10)

C = insoluble fractions; 20 μ l

S = soluble fraction; 40 μ l

Figure 16: Diagram and restriction map for pTG7675.

Figure 17: Diagram and restriction map for pTG7914.

METHODS

Unless otherwise mentioned, the different enzymes are employed according to known techniques.

Transformation

Competent cells were prepared according to the method of Hanahan (1983). Their competence ranges up to 10^4 to 10^5 transformants/ μ g DN for the RL58 strain (Bollen et al. 1979) and 10^5 to 10^6 transformants/ μ g DN for the TGE 7615, TGE7214 or TGE 7303 strain.

In general, 1 to 10 μ l of a solution, at the appropriate dilution, of a DNA preparation containing the plasmid which is desired to be introduced into the strain, is added to 0.2 ml of a stock of competent cells. The cells are allowed to react with the DNA for 15 minutes at 0°C and they are then incubated at 37°C for 90 seconds. They are returned to 0°C for 5 minutes and then 0.8 ml of LB medium is added thereto. For the strain RL58 or for the strain TGE 7615, this medium must contain DAP at a rate of 8 gamma/ml. This is necessary for the growth of $dapD^-$ cells, even for those which contain the plasmid carrying the $dapD$ gene. The cells are generally incubated at 30°C, with vigorous stirring (however, they may be placed at 37°C if the plasmid does not contain the promoter P_L). The incubation period is 60 minutes, but it can be extended to 90 minutes for growth at 30°C for the strain TGE 7615.

Subsequently, defined volumes (0.1 ml) are spread on solid LB medium. In the case of plasmids containing the gene for ampicillin resistance, the clones are selected by adding 100 gamma/ml of ampicillin; the clones containing the $dapD$ gene are selected from a culture which is $dapD^-$ on the whole, on the LB medium without other additions. The dishes are incubated for 24 hours at 30°C. The colonies are then transferred with toothpicks into 3 ml of LB medium

and, after growth at 30°C overnight, the plasmid is isolated and its structure confirmed on agarose gel, after digestion with appropriate restriction enzymes.

Plasmids containing the *dapD* gene may be transformed in any *E. coli* which carries the *dapD*⁻ mutation (for example RL58, TGE75150, TGE7214 or TGE7303); if they additionally contain the bacteriophage lambda promoter P_L, they must necessarily be transformed in *dapD*⁻ *E. coli* strains which express the bacteriophage lambda repressor cI857 either on a plasmid or in the chromosome, or cI and Rec mutation (for example RecA441).

EXAMPLE 1

Introduction of a mutated *dapD*⁻ gene in *E. coli* strain TGE900

The *E. coli* host strain TGE900 was rendered *dapD*⁻ by conjugation with a known *dapD*⁻ mutant, RL58.

The strain TGE900 is a derivative of strain N4830 described by Gottesman et al. (1980), the characteristics of which are as follows: Su⁻ F⁻ his ilv bio (λ cI857 Δ Bam Δ HI) Sm^r. It is commonly used as the host for expression vectors in which the foreign gene is placed under the control of the lambda promoter P_L, as it enables the expression to be induced at will by increasing the temperature (above 37°C), which inactivates the heat-sensitive repressor (lambda cI857) carried by the bacterium.

The *dapD*⁻ RL58 (met B *dapD*₂ met D279 Hfr P₄ X) strain has been described by Bollen et al. (1979).

Starting with this strain, a trimethoprim-resistant spontaneous mutant was selected (after spreading the strain on a dish containing 2 gamma/ml of trimethoprim and confirming the resistance of a colony chosen from a medium containing 4 gamma/ml trimethoprim). In fact, the gene coding for this resistance is sufficiently close to the *dapD*⁻ mutation so that if the recipient strain, after conjugation, becomes resistant to trimethoprim, it also becomes *dapD*⁻.

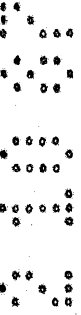
The resistant strain has been called TGE755; the useful characteristics thereof are: Hfr *dapD*⁻

Tmp^r.

After conjugation of the strains TGE900 (F⁻ Sm^{res} dap⁺), and TGE755, stopped after 15 minutes, by spreading

5

10



on a minimal medium containing DAP, streptomycin and trimethoprim, Sm^r Tmp^r colonies are selected; the dap^- nature of the strains selected and the presence of auxotrophic mutations in the parent strain ($\text{his}^- \text{ilv}^-$) in the minimal medium containing DAP, are verified on an LB medium.

One strain ($\text{his}^- \text{ilv}^- \text{dap}^-$) was chosen: TGE7615.

EXAMPLE 2

Construction of a vector plasmid carrying the E. coli dapD gene (Figure 1)

a) Construction of a multiple-use cloning plasmid comprising a polylinker with 12 restriction sites

The construction was initiated starting with a pML_2 plasmid (Lusky and Botchan, 1981), derived from pBR322 by deleting the nucleotides 1089 to 2491. This plasmid retained the origin of replication of pBR322 and the beta-lactamase gene (ampicillin resistance).

The PstI enzyme recognition sequence was removed by inserting an AhaIII - AhaII fragment of pUC8 which carries a PstI^0 mutation induced with ethanesulfonate (Vieira and Messing, 1982), between the two AhaIII sites of pML_2 , which deletes 19 base pairs from pML_2 . The resulting plasmid, pTG190 , carries the PstI^0 mutation of pUC8 .

A BglII linker (5'- dCAGATCTG -3'; Collaborative Research) was inserted into the only NruI site, by the "linker tailing" technique described by Lathe et al., 1984. The resulting construction is pTG191 .

The pTG191 was opened with EcoRI and BglII (which deletes the tetracycline-resistance gene) and religated with the EcoRI - BglII segment of phage M13TG131 (Kieny et al., 1983) which comprises a polylinker containing 12 restriction enzyme recognition sequences.

The resulting plasmid is pTG192 .

b) Cloning of a dapD gene in the pTG192 plasmid

The dapD chromosomal gene of E. coli was inserted into the plasmid pACYC184 to give pDB6 (Bendiak and Friesen, 1981). This dapD gene was recovered from pDB6 in the form of a 1.3-kb AluI fragment and inserted into the EcoRI site of pTG192 (the EcoRI ends having been repaired beforehand by treating with the Klenow fragment of DNA polymerase I).

From the resulting plasmids, pTG764, in which a single EcoRI site is reconstituted, is chosen. Thus, the pTG764 carries the dapD gene and the ampicillin-resistance gene of pTG192 (originally of pUC8).

5 EXAMPLE 3

Expression plasmid of hirudin containing dapD and Amp^r genes (figure 2)

The pTG720 plasmid described in French Patent No. 84/04,755 essentially comprises the hirudin gene under the control of the lambda promoter P_L.

10 The pTG720 plasmid, digested with BglII and BglI, gives a 2.74-kb fragment which carries the hirudin gene and a portion of the amp^r gene. This fragment, treated with phosphatase, is religated to the BglII-BglI fragment of the pTG764 plasmid which carries the dapD gene and another fragment of the amp^r gene. 15 The resultant plasmid, pTG771, carries the reconstituted while amp^r gene, the dapD gene and the gene coding for hirudin.

20 The colonies of TGE7615 transformed by this plasmid may be selected either on an LB medium (selection for the dap⁺ nature) or on an LB + ampicillin medium.

After induction, the transformed strain produces hirudin.

25 EXAMPLE 4

Expression plasmid of hirudin containing the dapD gene and no longer containing the Amp^r gene (Figure 3)

The starting plasmid is pTG771.

30 A digestion is carried out with AhaIII in order to remove the small fragment comprising the 3' end of the gene coding for Amp^r and the plasmid is closed again by ligation in the presence of an EcoRI "linker":

CCGAATTCCG

The pTG775 plasmid is obtained.

35 An EcoRI digestion followed by ligation enables the gene coding for Amp^r to be removed completely.

The pTG776 plasmid is obtained.

40 The TGE7615 bacteria transformed by this plasmid may be selected on an LB medium, without addition (selection for the dap⁺ character).

After induction, the transformed strain produces hirudin.

EXAMPLE 5

Construction of plasmids carrying the dapD gene and not containing the Amp^r gene (Figure 4)

The starting plasmid is the plasmid pTG192.

After digesting with SmaI and AhaIII and then treating with phosphatase, the 0.95-kb vector fragment is isolated and ligated with the 1.3-kb AluI fragment of pD86 which carries the dapD gene. The amp^r gene has therefore been completely deleted. The TGE8615 strain is transformed with this new plasmid and selection is carried out on an LB medium in order to obtain clones which carry the dapD gene.

Plasmids which carry the dap gene in the two orientations are obtained; the orientation of the insert may be determined by digesting with PstI. For two candidates chosen:

pTG766 releases 1.34-kb and 0.9-kb fragments, and pTG767 releases 1.85-kb and 0.4-kb fragments.

EXAMPLE 6

Expression plasmid of interferon-gamma, carrying the dapD gene (Figure 5)

The pTG40 (PED) (EP-A-0146462) plasmid carries, on a 1.2-kb HindIII fragment, the interferon-gamma gene under the control of the promoter P_L. After digesting with HindIII, this fragment is recovered and inserted into the HindIII site of pTG767 which has previously been treated with phosphatase. After ligation, TGE7615 is transformed and selection for the presence of the dapD gene is carried out on an LB medium. Two plasmids carrying the insert in opposite orientations are chosen:

pTG7671 (which has the P_L proximal of the replication origin and in the same orientation, and pTG7672.

The TGE7615 bacteria transformed by these plasmids may be selected on an LB medium, without addition (selection for the dap⁺ character).

After induction, the transformed strain produces

interferon-gamma.

The following examples are intended to demonstrate the characteristics of the strains transformed according to the present invention.

5 All the plasmids carrying the *dapD* gene are in the TGE7615 strain and the others are in the TGE900 strain.

The stability of the different plasmids was tested in suitable media:

10 "selective medium" will refer to the LB medium in the case of the plasmids according to the invention and to the LB + ampicillin medium in the case of plasmids pTG720 and pTG40; and

15 "non-selective medium" will refer to the LB medium + DAP in the case of the plasmids according to the invention and to the LB medium in the case of other plasmids.

20 A study of stability over 110 generations is carried out by several successive cycles of dilution and multiplication of the bacteria. At the end of this multiplication phase, the stability of the plasmid is determined as follows:

25 the number of viable cells in the culture is determined by suitable dilution and counting the cells in a non-selective agar medium; and

30 after a growth, a significant sample of these colonies is transferred into selective and non-selective agar media, and the percentage of colonies which have lost the plasmid, and therefore the capacity to grow in a selective medium, is determined.

The results obtained with the plasmids carrying the interferon gene are given in Table I:

TABLE 1

Determination of plasmid loss by bacteria
after 110 generations

5	Strain	TGE 900	TGE7615	TGE7615	TGE7615
	Plasmid	pTG40	pTG766	pTG767	pTG7671
	Selection	Amp	dapD	dapD	dapD
	LB medium				
	p-/c/generation*	2×10^{-3}	$< 5 \times 10^{-5}$	$< 5 \times 10^{-5}$	3×10^{-5}
10	LB medium +				
	DAP	2×10^{-3}	$< 5 \times 10^{-5}$	$< 5 \times 10^{-5}$	3×10^{-5}

* c = number of viable cells

p- = cells which have lost the plasmid

p-/c/generation = (number of p-/number of c tested)
x number of generations

15 Similar tests carried out with plasmids carrying the hirudin gene and with the cloning vector pTG766, after the multiplication of the bacteria over 170 generations in an LB medium, give the following results:

TABLE 2

Determination of plasmid loss by bacteria
after 170 generations in an LB medium

	Strain	TGE900	TGE7615	TGE7615	TGE7615
	Plasmid	pTG720	pTG771	pTG776	pTG766
	p+ (%)	96	98	100	100
	p-/c/generation	2×10^{-4}	1.5×10^{-4}	$< 1.1 \times 10^{-4}$	$< 1.1 \times 10^{-4}$

20 The results presented in Tables 1 and 2 show that:

25 1) the stability of dap vectors is increased by a factor of 10 relative to the plasmid carrying the amp^r gene;

2) the difference between the selective and non-selective media is minimal;

3) the cloning vectors pTG766 and pTG767 have a loss of less than 5×10^{-5} p-/c/generation;

35 4) the stability of pTG40 is significantly less than that of the dap⁺ vectors; however, it is worth pointing out that the phenomenon becomes particularly marked after 50 generations; at the early stages of culture, the

Loss is only 2.5×10^{-4} p-/c/generation; and

5) the loss of amp^r control plasmids pTG40 and pTG720, carrying the interferon and the hirudin genes respectively, is of the same order of magnitude; this plasmid loss with time is reduced when they carry the dapD gene (pTG766, 767, 7671 and 776).

However, it should be pointed out that the stability of the plasmid carrying the dapD gene and the gene for ampicillin resistance (pTG771) is lower and remains comparable to that of pTG720. This result is explained by an analysis of the plasmid content in the agarose gel; the latter shows that pTG771 forms tetramers, which phenomenon has repercussions on the partition of plasmids and which increases their loss (Summers and Sherratt, 1984). It should be added that over a smaller number of generations (30), this multimerization phenomenon does not take place, and that over a total of 70 generations, the loss of pTG771 remains less than 2.8×10^{-4} p-/c/generation.

Therefore, the stability of the plasmids which contain the dapD gene is increased relative to the plasmids carrying the amp^r gene, and their loss becomes minimal.

EXAMPLE 7

Stability of plasmids at 37 or 42°C

The effect of temperature on the stability of plasmids cannot be studied in the case of those which contain a gene coding for a foreign protein placed under the control of the promoter P_L without inducing the expression of this foreign protein. Nevertheless, it should be pointed out that, under the operating conditions, the induction is followed by an increase in O.D. from 0.3 to 3.6, which corresponds to 3.6 generations. Therefore, in fact, the bacteria form the largest number of generations at 30°C, before induction, with a loss which has already been determined in the preceding example.

When the expression of a foreign protein is induced (for example hirudin for pTG720 and interferon-gamma for pTG40), a mortality of the E. coli culture, at a rate of 50% to 95%, is observed after a certain period

of time (2 to 4 hours). These are cells containing the plasmid (because the p- host cell is capable of growing at 37 to 42°C). Inevitably, this mortality increases the proportion of p- cells relative to the living cells by a factor of 10 to 20. When the total number of living cells is significantly decreased, and the p- cells form a significant fraction of the population, the multiplication of the p- cells is determined, which decreases significantly the p+/p- ratio (see for example Table 3, results obtained after 5 h 30 min and 7 hours).

It is obvious that the parameter p-/cell/generation has lost its significance under such conditions because the p+ cells no longer multiply. The following parameter is proposed: p-/ml/optical density unit, which measures only the p-cells at each culture period. Additionally, it enables two different cultures to be compared for their plasmid loss. This parameter is calculated according to the following formula:

$$p-/ml/OD = [1-(\%p+/100)] \times \text{viable cells/ml/OD}.$$

Finally, if this parameter is expressed on the basis of the total number of cells (which is 4×10^8 under the present conditions), it is possible to obtain the percentage of (Fp^-) cells in the culture and to determine the degree of contamination of the culture with p- cells at each period. Fp^- is calculated as follows:

$$Fp^- = 4 \times 10^8 \text{ c/ml/OD} \times 100(p-/ml/OD).$$

Fp^- is an objective parameter which makes it possible to decide whether a culture intended for the production of a molecule is sufficiently pure to make it worthwhile to develop it further and which makes it possible to compare different plasmids with one another under the conditions of induction.

In the inductions described in the following examples, the number of viable cells and the percentage of p+ will be determined and then p/ml/OD and Fp will be calculated. These data will be presented in the form of tables.

EXAMPLE 8

Induction of hirudin

The results for hirudin induction will be presented, first with a vector containing the ampicillin resistance gene and then with the vector which also contains the dapD gene.

1) Stability of plasmids

a) Induction of hirudin expression in a vector which contains the ampicillin resistance gene (TGE900/pTG720) in an LB medium at 37°C

Typical results for TGE900/pTG720 are given in Table 3.

TABLE 3

Stability parameters for the plasmids during hirudin expression at 42°C in TGE900/pTG720

<u>Time in hours</u>	<u>c/ml/OD</u>	<u>% p+</u>	<u>p-/ml/OD</u>	<u>Fp-(%)</u>
0 h	8.3×10^7	100	$<1.7 \times 10^6$	< 0.4
1 h 30	2.4×10^8	100	$<4.8 \times 10^6$	< 1.2
3 h	2.1×10^8	96	8.4×10^6	2.1
4 h 30	4.7×10^7	82	8.5×10^6	2.1
5 h 30	1.8×10^7	48	9.4×10^6	2.35
7 h	2.8×10^7	20	2.2×10^7	5.5

It is seen that after 3 hours, the number of viable cells per OD unit decreases concurrently with the proportion of p+ cells. After 5 h 30 min, fp- (% of p- cells relative to the total number of cells) is of the order of 2.35%. After 7 hours, this number will have doubled and it is probable that this is due to the growth of the p- cells only.

b) Induction of hirudin expression in a vector which contains the dapD gene (TGE7615/pTG771) in an LB medium at 37°C

The results given in Table 4 are comparable to those obtained for pTG720.

TABLE 4

Stability parameters for the plasmids during hirudin expression at 42°C in TGE7615/pTG771

<u>Time in hours</u>	<u>c/ml/OD</u>	<u>% p+</u>	<u>p-/ml/OD</u>	<u>Fp- (%)</u>
0 h	3.4×10^6	99.8	7.7×10^5	0.2
1 h 30	2.1×10^7	99.2	1.7×10^5	0.04
3 h	9.7×10^6	100	$<1.0 \times 10^5$	<0.025
4 h	9.3×10^6	100	$<8.0 \times 10^4$	<0.005
5 h	7.5×10^6	98	1.5×10^5	0.04

It is seen from Table 4 that despite a mortality comparable to that recorded for TGE900/pTG771, the loss of plasmid (%p+) is low (98% p+ after 5 hours), which is also the case for the absolute quantity of p- cells (p-/ml/OD). The quantity of Fp- after 5 hours is 0.04%, which is 50 times less than that in the case of pTG720 at the same period. This demonstrates the greater stability of pTG771 compared with pTG720. It should be pointed out that in an induction experiment pTG771 does not form tetramers and its loss is supposed to be less than that determined over 170 generations at 30°C (see Table 2).

The results show a greater stability of the plasmid pTG771 compared with pTG720.

2) Plasmid content

Plasmids pTG720 and pTG771 were isolated at different times during the induction. In general, in the initial stages of induction, during the exponential phase, a low plasmid content per cell is observed, which increases substantially with time. Hirudin is produced during this period.

It is interesting to note that this increase in plasmid content occurs in a population in which more than 95% of cells are dead.

3) Activity induced

The hirudin activities produced from pTG720 and pTG771 are not significantly different. The values (in antithrombin units, ATU) are 2720 ATU/L/OD for pTG720 and 2380 ATU/L/OD for pTG771, after 5 hours of induction.

In conclusion, it may be stated that pTG771 shows

an increased stability relative to pTG720, while maintaining the same production capacity and the same properties with respect to increasing its copy number as pTG720, at the end of the exponential phase.

5 EXAMPLE 9

Induction of interferon-gamma

The data for interferon-gamma induction are presented in the same way as those of hirudin.

1) Stability of plasmids

10 a) Induction of interferon-gamma expression in a vector containing ampicillin resistance gene (TGE900/pTG40) in an LB medium at 42°C

15 The results given in Table 5 show that the loss of plasmids is comparable to that for pTG720, and that Fp- rises to 6% after 5 hours. However, unlike pTG720, the loss of viability is quicker and a minimum value for the number of viable cells is already reached after 3 hours (as compared with 5 hours observed in the case of pTG720); additionally, the p- cells present in the culture remain viable and are already dividing significantly after 3 hours, and contribute 6% to this Fp- after 5 hours.

TABLE 5

Stability parameters for the plasmids during interferon expression at 42°C in TGE900/pTG40

<u>Time in hours</u>	<u>c/ml/OD</u>	<u>% p+</u> (No. of colonies tested)	<u>p-/ml/OD</u>	<u>Fp-(%)</u>
0 h				
1 h 30	1.8×10^6	100 (202/202)	$< 10^6$	< 1
3 h	6.6×10^6	10 (2/20)	6.0×10^6	1.5
5 h	2.5×10^7	2.7 (10/366)	2.4×10^7	6
6 h	4.3×10^7	1.7 (11/645)	7.3×10^7	18
8 h	1.3×10^8	0.9 (4/465)	1.3×10^8	32

b) Induction of interferon expression in a vector containing the dapD gene (TGE7615/pTG7671) in LB at 42°C

The data for a TGE7615/pTG7671 induction at 42°C in LB appear in Table 6. It is observed that maximum mortality is not reached until 5 hours have elapsed and that at this period, although %p+ is comparable to that in the case of pTG40 (10%), the number of p- cells (p-/ml/OD) is one fifth that obtained after 3 hours in the case of pTG40. In fact, after 5 hours, Fp- is 0.3% whereas Fp- for pTG40 is already 6% after this period. Therefore, there is a difference of a factor of 20, which is a reflection of the increased stability of pTG7671, which observation confirms the stability data at 30°C (see Table 1).

TABLE 6

Stability parameters for the plasmids during interferon expression at 42°C in TGE7615/pTG7671

<u>Time in hours</u>	<u>c/ml/OD</u>	<u>% p+</u> (No. of colonies tested)	<u>p-/ml/OD</u>	<u>Fp-(%)</u>
0 h	1.7×10^8	100 (50/50)	$< 3.8 \times 10^6$	< 1
1 h 30	1.35×10^8	100 (50/50)	$< 2.7 \times 10^6$	< 1.5
3 h	4.2×10^7	100 (50/50)	$< 8.4 \times 10^5$	< 0.2
5 h	1.3×10^6	12 (6/50)	1.1×10^6	0.3
6 h	2.2×10^6	5 (5/100)	2.1×10^6	0.5

Under conditions of TGE7615/pTG7671 induction at 42°C, the stability of the plasmid is practically identical in the selective and the non-selective media, the stability of pTG7671 being very high even in the absence of selection. Plasmid loss is therefore reduced to a level which is very satisfactory and compatible with production on an industrial scale.

2) Plasmid content

Analysis of plasmid content on agarose gel shows that the strains transformed by the plasmids pTG40 and pTG7671 contain equal quantities of material and that this plasmid content per cell increases with time during the induction pTG40 is present in the dimer form (as shown on agarose gel).

3) Selection of p+ cells containing the dapD gene

In order to demonstrate the selective capacity of the dapD system taking into account the growth of p-cells, which will no longer be generated as soon as the major part of cells have lost their viability, an induction is carried out, during which the culture is diluted by a factor of 5, three times, at 2-hourly intervals, after which this culture is allowed to reach a stationary phase. The results for TGE7615/pTG7671 induction at 42°C are presented in Table 7 for growth in a selective medium and in Table 8 for growth in a non-selective LB + DAP medium.

TABLE 7
Stability parameters for the plasmids during interferon expression at 42°C in TGE7615/pTG7671 in a selective

<u>Time in</u> <u>hours</u>	<u>c/ml/OD</u>	<u>LB medium</u>		
		<u>% p+</u> (No. of colonies tested)	<u>p-/ml/OD</u>	<u>Fp- (%)</u>
0 h	3.8×10^7	100 (50/50)	$< 6 \times 10^5$	< 0.2
2 h	8.3×10^7	97 (74/76)	1.4×10^6	0.4
4 h	$< 10^6$			
6 h	$< 10^6$			
9 h	3.7×10^6	100 (60/60)	$< 3.7 \times 10^4$	< 0.01

TABLE 8

Stability parameters for the plasmids during interferon expression at 42°C in TGE7615/pTG7671 in an

Time in hours	c/ml/OD	LB + DAP medium		
		% p+ (No. of colonies tested)	p-/ml/OD	Fp- (%)
0 h	5.5×10^7	100 (50/50)	$< 1,2 \times 10^6$	< 0.3
2 h	9.9×10^7	99 (74/75)	1.3×10^6	0.3
4 h	$< 10^6$			
6 h	$< 10^6$			
9 h	1.7×10^7	5 (5/100)	1.6×10^7	4

It is observed in Table 8 that, after 9 hours of induction, in the selective LB medium all the viable cells contain the plasmid (the presence of the plasmid is confirmed by mini preparation from 30 colonies out of a total of 60 positive colonies in an LB medium and identification of the plasmid by agarose gel electrophoresis). In contrast, in the non-selective medium, the viability is 5 times as great, but the culture contains only 5% of the cells containing the plasmid. This greater viability in LB + DAP medium than in LB medium shows the growth of p- cells in the medium to which DAP has been added and a fortiori the mortality of the p- cells in the selective medium. Therefore, the LB medium enables an effective counter-selection of p- cells to be carried out.

4) Interferon production

pTG7671 retains the same interferon-gamma production capacity as the original plasmid pTG40.

EXAMPLE 10: Deletion of the dapD gene from the chromosome of the bacterium

Sub-cloning of the dapD gene on 2 PstI fragments

Plasmid p086 and M13mp8 are cut with PstI and ligated. The M13s containing the 5' end and the 3' end of the gene are screened with oligonucleotides specific to these regions, TG596 (GCGCTTAATAACGAGTTG) and TG598

(TGTGCATACTTTAGTC) respectively. The candidates SB96 and SB98 are chosen, and the insertion of the desired fragments confirmed by sequencing. (See diagram in Figure 6).

5 Introduction of an EcoRI site into the 5' and 3' ends of the *dapD* gene

The EcoRI sites are introduced into SB96 and SB98 by point mutation:

into SB98 with the oligonucleotide TG597:

10 GTACGCAGGAATTCCTTAATGCCG

which pairs with the 3' region of the end of the gene, but before an assumed transcription terminator; and

into SB96 with the oligonucleotide TG620:

15 AGAGGCCCGAATTCCAAACG

which pairs with the 5' region upstream of the assumed promoter for the *dapD* gene.

The transformants are analysed with the same probes as those used for introducing the EcoRI sites. The presence of this EcoRI site is confirmed by a DNA minipreparation and then by sequencing the M13 candidates chosen (see drawing in Figure 7).

Construction of a deletion vector

25 The kanamycin resistance gene of plasmid pUC-4K (sold by Pharmacia) is recovered in the form of an EcoRI fragment. The M13TG597 and 620 are cut with EcoRI and PstI, which releases the 3' end of the *dapD* gene (without the assumed terminator) and the 5' end of the gene (with its assumed promoter) respectively.

30 The cloning vector pTG192 (described in Example 2a) is cut with PstI.

35 All these fragments are ligated. After the transformation of 5K cells and spreading on an LB medium + ampicillin 0.1 μ g/ml + kanamycin 0.02 μ g/ml, the colonies are screened with oligonucleotide TG596.

One construction was selected: pTG47. Its structure is analysed by a DNA minipreparation and the orientation of the kanamycin resistance gene is determined by digesting the plasmid with HindIII, which releases 2

bands of sizes 5.3 kb and 4.0 kb. The orientation of kanamycin resistance gene in pTG47 is the same as that of the *dapD* gene in pDB6 (in the other orientation, bands of sizes 4.9 kb and 4.4 kb would have been obtained).

The diagram for pTG47 is shown in Figure 8.

Deletion of the *dapD* gene from the chromosome

Deletion of the *dapD* gene of the chromosome from an intermediate strain.

RH5345 cells, the competence of which amounts to 2.5×10^{-7} transformants/ μ g of DNA of pTG47, are transformed by the plasmid pTG47 previously cut with KpnI and SgiII (see Figure 8).

This digestion releases a fragment which contains the flanking regions of the *dapD* gene, the gene itself being replaced by the kanamycin resistance gene.

After spreading on an LB medium + DAP + kanamycin 0.01 μ g/ml, 9 candidates are selected: TGE721 to TGE729, the *dap*⁻, *kan*^R phenotype of which is confirmed.

The absence of the *dapD* gene is confirmed after transformation of these strains by pTG764, by their capacity to multiply in the LB medium.

Deletion of the *dapD* gene from the strains TGE901 and N5969

The *dapD* deletion of the strain TGE721 is then transduced into strains TGE901 and N5969 with the phage transducer P1vir/TGE721, and the *dap*⁻ recombinants selected by their resistance to 0.01 μ g/ml of kanamycin.

The candidates chosen are TGE7213, 7214 and TGE-7303 respectively. For one candidate, TGE7214, the *ile*, *val*, his requirements of the parent strain, TGE901, in addition to the *dap*⁻ *kan*^R character, were confirmed on suitable media.

EXAMPLE 11: Confirmation of the deletion of the *dap* gene in different strains by chromosome blotting.

The various strains below were analysed:

the parent strains TGE901 and RH5345, *dap*⁺,

the deleted strains TGE7213 and 7214,

the parent strain RL58 which was used to introduce the *dapD*⁻ mutation into TGE901, and the *dapD*⁻ mutant, TGE7615, derived therefrom, and

a GC4540 strain which is resistant to kanamycin by the integration of Tn5, whereas in TG...strains, the kanamycin resistance gene is derived from Tn903 (the 2 genes should not give cross hybridization owing to lack of homology: Beck et al. 1982).

The choice of restriction enzymes is based on the sequence of pDBJ; a cut site:

BamHI and HindIII releases a 9-kb chromosomal fragment containing the dapD gene and its flanking regions, in the case of wild strains; in the case of deleted strains, the resistance gene must be released by digestion with BamHI and cut into 2 fragments by digestion with HindIII; and PstI releases 2 fragments, each containing a portion of the dapD gene (2.8 kb from the 5' region and 3.4 kb from the 3' region) for the wild type strains; in the case of the deleted strains, digestion with PstI releases the kanamycin resistance gene and the 2 flanking regions (a 2.4-kb fragment on the 5' side and a 2.8-kb fragment on the 3' side) (see Figures 6 and 8).

Probes were chosen in order to reveal the dap gene or its flanking regions or the kanr^R gene:

in order to probe the kanamycin resistance gene, an EcoRI fragment of pTG47 which contains only this resistance gene (Figure 8) is isolated; in order to probe the dapD gene, a 2.4-kb EcoRI fragment of M13TG620 which contains only the 5' region of the dapD gene and which must be completely deleted in TGE721, 7213 and 7214 (Figure 7) is employed; and

in order to probe the chromosomal gap D gene and its flanking regions, a KpnI-BglII fragment of pTG47 which contains the kanamycin resistance gene and the 2 regions (the 5' and the 3' sides) of the chromosome which flanks the dapD gene (Figure 8) is employed.

Therefore, revelation of the following is expected: in the wild strains, a 2.8-kb band corresponding

to the 5' region of the *dapD* gene and a 3.4-kb band corresponding to the 3' region of this gene (Figure 6); and

in the deleted strains, the kanamycin resistance gene, the 5' region flanking the *dapD* gene which still exists (2.4 kb) and the 3' region flanking the *dapD* gene which still exists (2.8 kb) (Figure 8).

Demonstration of the incorporation of the kanamycin resistance gene

The chromosomal DNAs of GC4540, TGE7213, TGE7214 and TGE901, cut with *Pst*I or *Bam*HI and *Hind*III were compared and probed with the *Eco*RI fragment of pTG47.

*Pst*I releases the 1.3-kb band; a *Bam*HI and *Hind*III restriction gives 2 fragments of 0.7 kb and 0.6 kb for the deleted strains only. No band is revealed in TGE901 or in GC4540 (Figure 9A).

These results prove that the kanamycin resistance gene is incorporated into the chromosome of the deleted strains and that this gene originates from pUC-4K.

Demonstration of the deletion of the *dapD* gene from the chromosome

The chromosomal DNAs of GC4540, TGE7213, 7214 and TGE901 are compared with, as controls, M13TG620, M13TG597, the *Bam*HI-*Hind*III fragment of pDB6 and the same fragment cut with *Pst*I (Figure 6).

The chromosomal DNAs are cut with *Pst*I or *Bam*HI and *Hind*III.

The M13TG620 cut with *Eco*RI is isolated from the band specifically containing the 5' side of the *dapD* gene in order to use it as probe (Figure 9B).

After digestion with *Pst*I, it is seen that in the wild strains, a 2.8-kb band corresponding to the 5' region of the *dapD* gene and also an unexpected 1.7-kb band are revealed.

After digestion with *Bam*HI and *Hind*III, an approximately 12-kb band which is larger than the band (9 kb) originating from pDB6 is revealed. Furthermore, an additional 2.5-kb band is also present in the wild strains (Figure 9B).

For the deleted strains, no significant homology is apparent in any digestion fragment.

These observations show that:

5 a probe which covers the 5' portion of the *dapD* gene does not reveal any band in the chromosome of strains TGE7213 and 7214, which demonstrates the deletion of the 5' side of this gene;

and

10 in the wild strains, as a second band specific for the *dapD* gene is revealed in addition to the expected band it seems that this gene is duplicated in these strains.

15 As a duplication of the *dapD* gene was unexpected, we have confirmed this duplication in some wild strains and confirmed the deletion of the *dapD* gene from the 3' side in addition to the 5' side.

The same chromosomal and control DNAs are employed after digestion with *Pst*I (Figure 8).

20 The probe is pTG47 cut with *Kpn*I and *Bgl*II. In addition to the bands predicted above, this probe must reveal at least a 1.7-kb band. In fact, pTG47 contains 300 bp and 100 bp homologous to the *dapD* gene, from the 5' side and the 3' side respectively, which must be revealed in the deleted strains.

25 Figure 9C shows that:

2.8-kb and 2.4-kb bands are revealed in the deleted strains and 3.4-kb and 2.8-kb bands in the wild strains; additionally, a 1.3-kb band corresponding to the kanamycin resistance gene is revealed. This proves the deletion in strains TGE7213 and 7214; and two

30 less intense bands, due to the duplicated *dapD* gene, are also revealed in the wild strains only.

35 As it is known that the 1.7-kb band is revealed with the 5' portion, the 2.1-kb band must originate from the 3' side. This proves that these strains indeed contain a duplication which has disappeared in the deleted strains.

The chromosomal DNA cut with PstI of strains RH5345 and RL58, and the *dapD*⁻ mutant obtained by conjugation of RL58 with TGE901 were compared. These DNAs were probed with the KpnI, BglII fragment isolated from pTG47 which contains the kanamycin resistance gene (which should not reveal anything) and the regions flanking the *dapD* gene. Figure 9D shows that in RH5345, only the 3.4 and 2.8-kb bands are revealed and not the bands due to the duplication of the gene, which are present in GC4540 or TGE901. Additionally, only a 7-kb band is observed for RL58 and TGE7615, which indicates loss of a PstI site; this proves that these 2 mutants are identical and are affected at least in the PstI site of the *dapD* gene.

In conclusion, these experiments show that: strains TGE7213 and 7214 are deleted for the *dapD* gene and they contain the kanamycin resistance gene;

the *dapD*⁻ mutation of RL58 and TGE7615 is present at least at the PstI site of the *dapD* gene; and some strains of *E. coli* have a duplication of the *dapD* gene and this duplication is not present in the deleted strains.

As the 2 *dapD* genes of the recipient strain are successfully deleted by transduction, it may be concluded that the duplicated gene must be close (at less than 2' on the chromosomal map of *E. coli*) to the first *dapD* gene.

EXAMPLE 12: Cloning of the *cer* gene.

The *cer* gene is recovered from the Col E1 plasmid, the sequence for which has been published by Chan et al. (1985); in the form of a 1.85-kb HaeII fragment.

The HaeII fragment is then cut with HpaII, treated with Klenow and a 0.4-kb band is recovered. The M13mp130 is cut with EcoRV and treated with phosphatase. The 0.4-kb fragment of Col E1 is ligated to M13mp130 and it is introduced into the strain JM103. The presence of the Col E1 *cer* fragment was confirmed by sequencing the 0.4-kb band released by cutting with SmaI and HindIII.

The *cer* gene inserted into the polylinker of M13mp131 is then isolated after digestion with SmaI and

HindIII and ligated to the pTG720 vector (carrying the hirudin gene, Figure 2) cut with BglII and treated with Klenow. The resulting plasmid is pTG720cer.

EXAMPLE 13: Construction of cloning vectors containing the cer gene and the dapD gene.

Inversion of the orientation of the M13mp131 polylinker in a vector coding for ampicillin resistance

The pTG192 (Figure 1) is cut with EcoRI and BglII in order to release the M13mp131 polylinker and is shortened by HaeIII digestion. A plasmid carrying the ampicillin resistance gene, for example pTG730 (expression vector of hirudin, described in French Patent 86/16,723) is employed; this plasmid is cut with BglII and EcoRI and ligated to the EcoRI-BglII fragment of pTG192. In this way, the expression block comprising the P_L and the hirudin structural gene of pTG730 are lost, being replaced with the M13mp131 polylinker. This new plasmid is called pTG790 (Figure 10).

Introduction of the cer fragment into the cloning vector

pTG790 is cut with SstI and KpnI and treated with phosphatase. The fragment resulting from this digestion is ligated to pTG720cer, cut with SstI and KpnI (which releases the cer fragment) and shortened by BglII digestion. The resulting vector, pTG792, contains the cer fragment (Figure 11).

Introduction of the dapD gene into a vector containing the cer fragment

pTG792 is cut with EcoRI, treated with Klenow and phosphatase. The resulting fragment is ligated with the 1.3-kb AluI fragment resulting from pDB6 which contains the dapD gene (Figure 6). 2 plasmids, pTG7922 and pTG7923, result therefrom, and differ only by the orientation of the dapD gene located between 2 EcoRI sites. For pTG7922, the promoters for the 3 genes, replication origin, ampicillin resistance and dapD are oriented in the same way (Figure 12).

Deletion of the ampicillin resistance gene

The following constructions have a multiple aim: to delete the ampicillin resistance gene from

vectors containing the *dapD* gene;
to obtain a *dapD* cloning vector containing the
cer gene;
to obtain a *dapD* cloning vector which contains
the M13mp131 polylinker (minus the *EcoRV* site,
used for the cloning of *cer*); and
to obtain a *dapD* vector with a single *EcoRI* site.

A *PstI* fragment is recovered from pTG7922 and
pTG7923 containing, respectively, the 3' and 5' por-
tions of the *dapD* gene as well as the *cer* gene in order to
introduce it into a *dap* vector containing an analogous
fragment but without any *EcoRI* or *AvaI* site, or *cer*. The
analogous vectors are respectively pTG767 and pTG766 de-
scribed above.

The pTG7922 and pTG7923 are cut with *PstI*, shor-
tened by *BglII* digestion and ligated respectively to pTG767
and pTG766 cut with *PstI* and treated with phosphatase.
The cloning vectors which result therefrom are pTG769 and
pTG768 respectively (pTG769 is shown in Figure 13).

EXAMPLE 14: Application of the *dap* model to the con-
struction of expression vectors for catechol
2,3-oxygenase ($C_{2,30}$).

Vector without *BamHI* site upstream of $C_{2,30}$

The structural gene for $C_{2,30}$ is recovered from
pTG444, to be introduced into the *dap* vector pTG7671
(described above).

pTG444 is identical to pTG445 described by
Zukowski et al. (1984) except for a non-regenerated *XmaIII*
site. The pTG444 is cut with *BamHI* and *HindIII* and ligan-
ted to pTG769 cut with *BamHI* and *HindIII* and treated with
phosphatase. The resulting plasmid, called pTG7401, does
not contain the structural gene for $C_{2,30}$.

pTG7671 contains 2 *BglII* sites: a site upstream
of P_L forming part of the polylinker and a site down-
stream of P_L located in the ribosome binding site,
upstream of the structural gene for interferon-gamma.
The pTG7671 is cut with *BglII* and shortened by *KpnI* diges-
tion. The resulting mixture is ligated to pTG7401, cut
at its polylinker, with *BglII* and *BamHI* and treated with

phosphatase. The BglII site is reconstituted by this ligation, but the BamHI site ligated to the BglII site is lost. Two orientations of P_L with respect to the structural gene for $C_{2,30}$ are possible. In order to distinguish the construction in which $C_{2,30}$ is under the control of P_L , cutting is carried out with BamHI and BglII (in fact, for the orientation desired, a BamHI site is present near the BglII site and in practice, the digestion will give only a 4.3-kb band; in the other orientation, the digestion releases a 3.9-kb band and a 0.4-kb band.

The plasmid chosen, pTG7407, having $C_{2,30}$ under the control of P_L , has a structure close to pTG7406 described later (compare with Figure 14), however, it has lost its BamHI site upstream of $C_{2,30}$.

Vector with a BamHI site upstream of $C_{2,30}$

pTG769 is cut with BglII and BamHI, treated with phosphatase and ligated with a BamHI-BglII fragment of any expression plasmid (pTG907) which contains P_L and the complete N gene of λ . A construction, pTG7400, results therefrom, which may be identified by a BamHI and BglII cut which releases 2 bands, of 2.6 kb and 1.3 kb. This construction contains the P_L and the complete N gene.

The pTG7400 is then cut with HpaI and a BamHI linker, CCGGATCCGG (sold by BRL), which has been phosphorylated and hybridized, is inserted therein. This gives pTG7402 which has lost its HpaI site but which contains 2 BamHI sites.

The pTG7402 is cut with BamHI and religated to give pTG7404. By this procedure, a BamHI site is removed and the N gene is truncated.

Introduction of the $C_{2,30}$ gene into a dap-cer vector

The pTG7402 is cut with BamHI and HindIII, treated with phosphatase, and the BamHI-HindIII fragment of pTG444 is introduced therein to give pTG7406 (see Figure 14). It differs from pTG7407 in that the $C_{2,30}$ gene may be removed by a BamHI-HindIII cut so as to recover the fragment introduced from pTG444.

Expression of C_{2,30} in dap⁻ E. coli strains transformed by the plasmid pTG7407

5 The expression of the C_{2,30} gene in the bacteria TGE7213/pTG7407 was determined at 30°C after 4 h and 7 h of culture and during induction at 42°C after 4 h and 7 h. For each determination, a sample is harvested from the culture and centrifuged; the pellet is washed and taken up with phosphate buffer (as described by Zukowski et al., 1983) and then treated with ultrasound 3 times for 20 seconds each.

10 After centrifuging for 10 min at 10,000 g, the pellet is considered as the insoluble fraction (P) and the supernatant as the soluble fraction (S).

15 The proteins present in each fraction are analysed by electrophoresis on SDS polyacrylamide gel. The bands are visualized by staining with Coomassie blue. The results are presented in Figure 15. The intensity of the band for MW 35,000 is noted, especially after 7 h of induction at 42°C. The "scanning" of the gel gives approximately 64% and 75% of C_{2,30} in fractions S and P respectively.

20 In the richer sample (S, 7 h at 42°C), the specific activity of C_{2,30} is determined (according to the method described by Zukowski et al., 1983), by adding catechol as substrate. A specific activity of 28 to 35 U/mg is measured.

25 The specific activity of a pure enzyme preparation being 280 U/mg, the extracts analyzed contain approximately 12% of active C_{2,30}.

30 EXAMPLE 15: Introduction of the cer fragment into an expression vector of interferon-gamma.

35 The pTG7671 described above is cut at its poly-linker with SstI (identical to SacI) and KpnI and then treated with phosphatase and ligated to fragments pTG720cer cut with SstI and KpnI. After transformation in TGE7615, one candidate, pTG7675, which releases 2 fragments of 400 bp and 3.4 kb after digestion with SstI and KpnI (Figure 16) is chosen.

EXAMPLE 16: Demonstration of the stability of the plasmids during the induction of interferon-gamma expression.

5 a) Induction of interferon-gamma expression in LB medium at 42°C for a vector carrying the ampicillin resistance gene: TGE901/pTG40.

10 The results are given in Table 9. The total number of cells/ml/OD unit is determined in order to ensure that the loss of viability is a true phenomenon and not, for example, a change in cell volume. These data are also given in Table 9. F_p^- was defined in example 7 and relates the number of p^- cells to the total number of cells present at a given time.

15 It will be noted that F_p^- after 7 h 30 min of induction reaches a value of approximately 2%. Therefore, it is less than in the previous experiment (see Example 9), which can be attributed only to a difference in the structure of the plasmid pTG40 in these 2 experiments: in fact, although the quantity of plasmid is the same at different times of induction, in the first experiment the plasmid was in the dimeric form, whereas in the experiment described here it is mainly in the monomeric form (as shown by analysis on gel). The condition of the plasmid does not affect interferon-gamma production, but illustrates conclusively the loss of stability if the monomeric form of a plasmid is not maintained.

20
25
30 b) Induction of interferon-gamma expression in a strain mutated for the *dap* gene and transformed by a vector containing the *dapD* gene and the *cer* gene, TGE7615/pTG7675

35 The results are given in Table 10. The total number of cells/OD unit/ml are determined; there is no marked difference with TGE901/pTG40. A real loss of viability is thus confirmed: after 7 h 30 min of induction, only 0.02% of the culture remains viable. This should be compared with TGE901/pTG40 in which case the value obtained is 2.4%. This manifests itself in F_p^- , which is decreased by a factor of 1000 in the case of TGE7615/pTG-7675 compared with TGE901/pTG40. Nevertheless, some p^-

cells still remain, which appear at the end of induction. The plasmid content has the same features as before, i.e. increase in the copy number at the end of growth, but the multimeric forms, which are present in variable numbers with the plasmids without cer, are almost absent in this case. The interferon-gamma production is slightly greater than that obtained with pTG40.

c) Induction of interferon-gamma expression in a host cell deleted for the *dapD* gene and transformed by a vector containing the *dapD* gene and cer, TGE7213/pTG7675

The results are given in Table 11. The conclusions are identical to those drawn from the comparison between TGE901/pTG40 and TGE7615/pTG7675: the mortality reaches a factor of 3.5×10^{-4} , and the value for total number of cells/ml/OD unit does not vary significantly during the induction. In contrast, even after 7 h 30 min of induction, p^- cells do not appear (after 24 h, the entire culture became p^- in the case of TGE901/pTG40 and remained 100% p^+ in the case of TGE7213/pTG7675). The plasmid content is comparable to that of TGE7615/pTG7675 in the absence of multimers. Interferon-gamma production is slightly greater than that obtained with TGE7615/pTG7675.

Table 9

Induction of TGE901/pIG40 at 42°C in LB medium

	O.D. 600 nm	c/ml/O.D.	total number of cells/ml/O.D.	Fp-
0 h 00 min	0.370	3.9×10^8		< 1.4%
1 h 00 min	1.29	3.8×10^8	6.5×10^8	
2 h 00 min	1.67	3.0×10^7	7.2×10^8	0.03%
3 h 00 min	2.13	2.7×10^6		0.11%
4 h 00 min	2.42	2.8×10^6	6.7×10^8	0.23%
6 h 15 min	2.89	7.0×10^6	6.5×10^8	0.70%
7 h 30 min	3.08	9.5×10^6		1.73%

NOTE: Fp- is the percentage of p- cells over the total number of cells present at a given time.

Table 10

7GE7615/pTG7675 at 42°C in LB medium

	O.D. 600 nm	c/ml/O.D.	total number of cells/ml/O.D.	Fp-
0 h 00 min	0.310	3.5×10^8		
1 h 00 min	1.07	3.3×10^8	4.7×10^8	
2 h 00 min	1.64	1.5×10^8	5.1×10^8	
3 h 00 min	2.31	1.44×10^7		
4 h 00 min	2.67	7.1×10^6	7.9×10^8	
6 h 15 min	3.26	6.1×10^4	6.1×10^8	0.0017%
7 h 30 min	3.44	6.4×10^4		0.0018%

Table 11

Induction of TGE7214/pTG7675 at 42°C in LB medium

	O.D. 600 nm	c/ml/O.D.	total number of cells/ml/O.D.	Fp-
0 h 00 min	0.400	3.4×10^8		< 1.1%
1 h 00 min	1.25	3.7×10^8	5.8×10^8	
2 h 00 min	1.71	3.8×10^7	7.1×10^8	
3 h 00 min	2.45	3.0×10^5		
4 h 00 min	3.32	6.6×10^4	12.8×10^8	
6 h 15 min	4.25	7.3×10^4	5.8×10^8	
7 h 30 min	4.21	1.2×10^5		< $6 \times 10^{-4}\%$

EXAMPLE 17: Application of the dap model to the construction of an expression vector for alpha-1 antitrypsin.

5 The PstI fragment containing the alpha-1 antitrypsin expression block, i.e. the phage lambda promoter P_L, the truncated N gene, a ribosome binding site and the structural gene for alpha-1 antitrypsin (Arg³⁵⁶), originating from pTG2901 (truncated derivative of pTG983, described in French Patent 85/07,393) is introduced into pTG792 (described above), cut with PstI and treated with phosphatase.

10 The resulting expression vector, pTG7913, is then cut with BglII and SstI and the expression block containing the alpha 1 antitrypsin and the cer gene is introduced into pTG767, cut with BglII and SstI and treated with phosphatase.

15 The resulting plasmid, pTG7914, contains the dapD gene, the cer gene and the alpha-1 antitrypsin expression block (arg³⁵⁸) (figure 17).

20 Deposition of representative strains of the invention

The following strains are deposited at the Collection Nationale de Cultures des Microorganismes (National Collection of Microbial Cultures) (25 Rue du Dr. Roux, Paris):

- 25 TGE7615/pTG7671 under the number 1-586) on
TGE7615/pTG771 under the number 1-585) 25.07.86
TGE7214, dapD gene-deleted coli strain, under
the number I-652;
TGE7303, dapD gene-deleted coli strain, under
30 the number I-653;
(the 2 strains are transformed by the plasmid
pTG768 which carries the dapD and cer gene); and
TGE7214/pTG7407, dapD⁻ strain transformed by the
expression plasmid for C_{2,3}O, under the number
35 I-655 on 10.03.87.

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The claims defining the invention are as follows:

1. A bacterium, the genome of which comprises a dap gene inactivated by mutation and which is transformed by a plasmid comprising a functional dap gene.

5 2. A bacterium according to claim 1, the genome of which comprises a dapD gene inactivated by mutation and which is transformed by a plasmid comprising a functional dapD gene.

3. A bacterium according to claim 2, the genome of which comprises a dapD gene which is inactivated by deletion of at least part of the gene.

10 4. A bacterium according to any one of claims 1 to 3, in which the plasmid comprising a functional dap gene also comprises a sequence which maintains said plasmid in a monomeric state.

5. A bacterium according to claim 4, in which said sequence is a "cer" sequence.

15 6. A bacterium according to any one of claims 1 to 5, which is an E. coli bacterium.

7. A bacterium according to any one of claims 1 to 6 in which the plasmid comprising a functional dap gene also comprises a gene which encodes a protein of industrial interest and the elements ensuring its
20 expression.

8. A bacterium according to claim 7 in which the plasmid comprising a functional dap gene also comprises a gene which encodes a protein of industrial interest selected from hirudin, gamma-interferon, catechol 2, 3 oxygenase and alpha-antitrypsin.

9. A process for preparing a homogenous culture of bacterium according



to any one of claims 1 to 8 which comprises culturing said bacterium in a DAP-free medium in the presence of a substantial amount of lysine.

10. A process for preparing a protein of industrial interest which comprises culturing in a DAP-free medium containing a substantial amount of lysine, a bacterium according to claim 7 or 8 and recovering said protein from the culture.

11. A process according to claim 8, which comprises culturing in a rich medium, a bacterium according to claim 7 or 8, and recovering said protein from the culture.

12. A bacterium according to any one of claims 1 to 8 substantially as hereinbefore described.

13. A process according to any one of claims 9 to 11 substantially as hereinbefore described.

DATED this July 31, 1991

CARTER SMITH & BEADLE

Fellows Institute of Patent Attorneys of Australia

Patent Attorneys for the Applicant:

TRANSGENE S.A.



FIG. 1

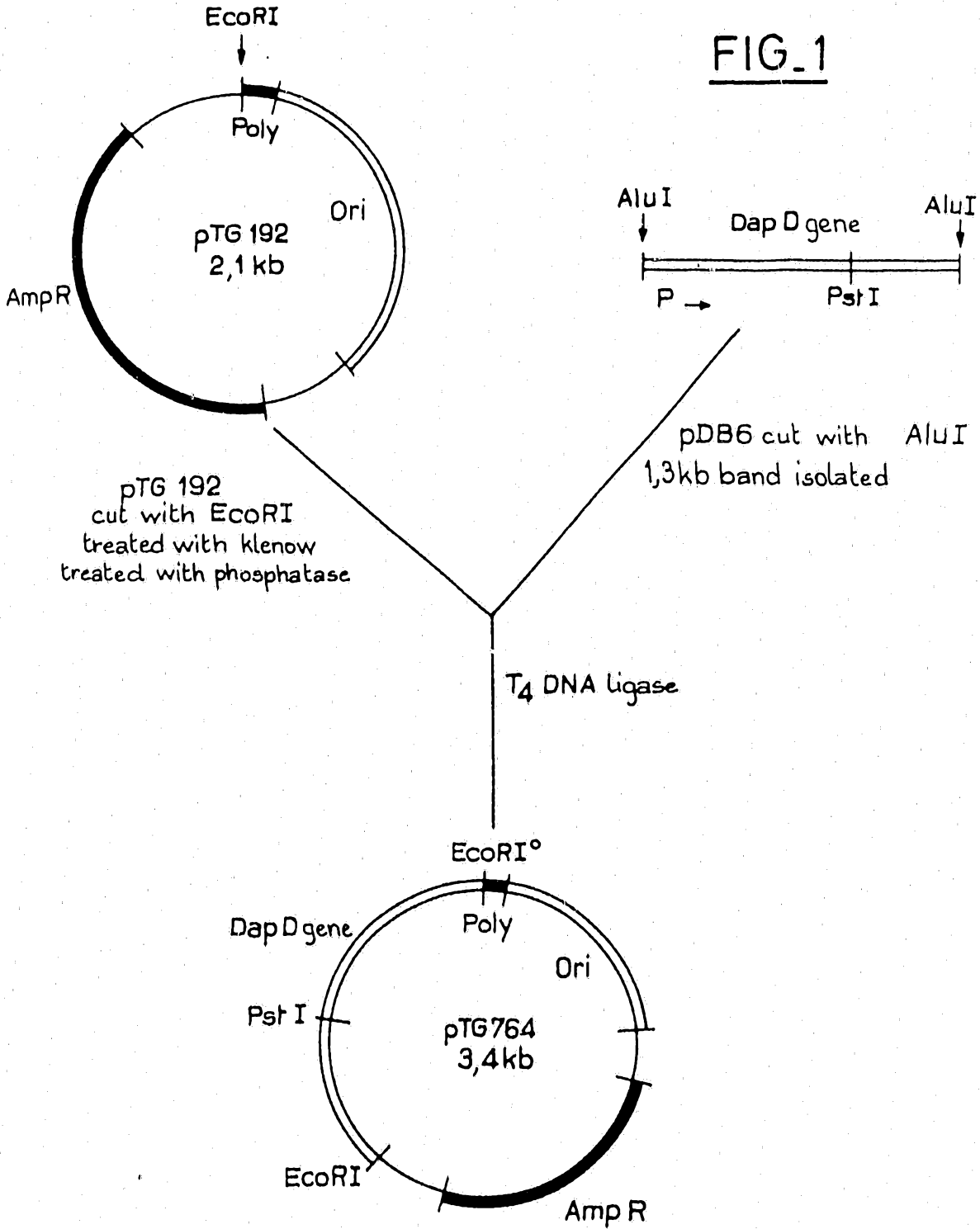


FIG. 2

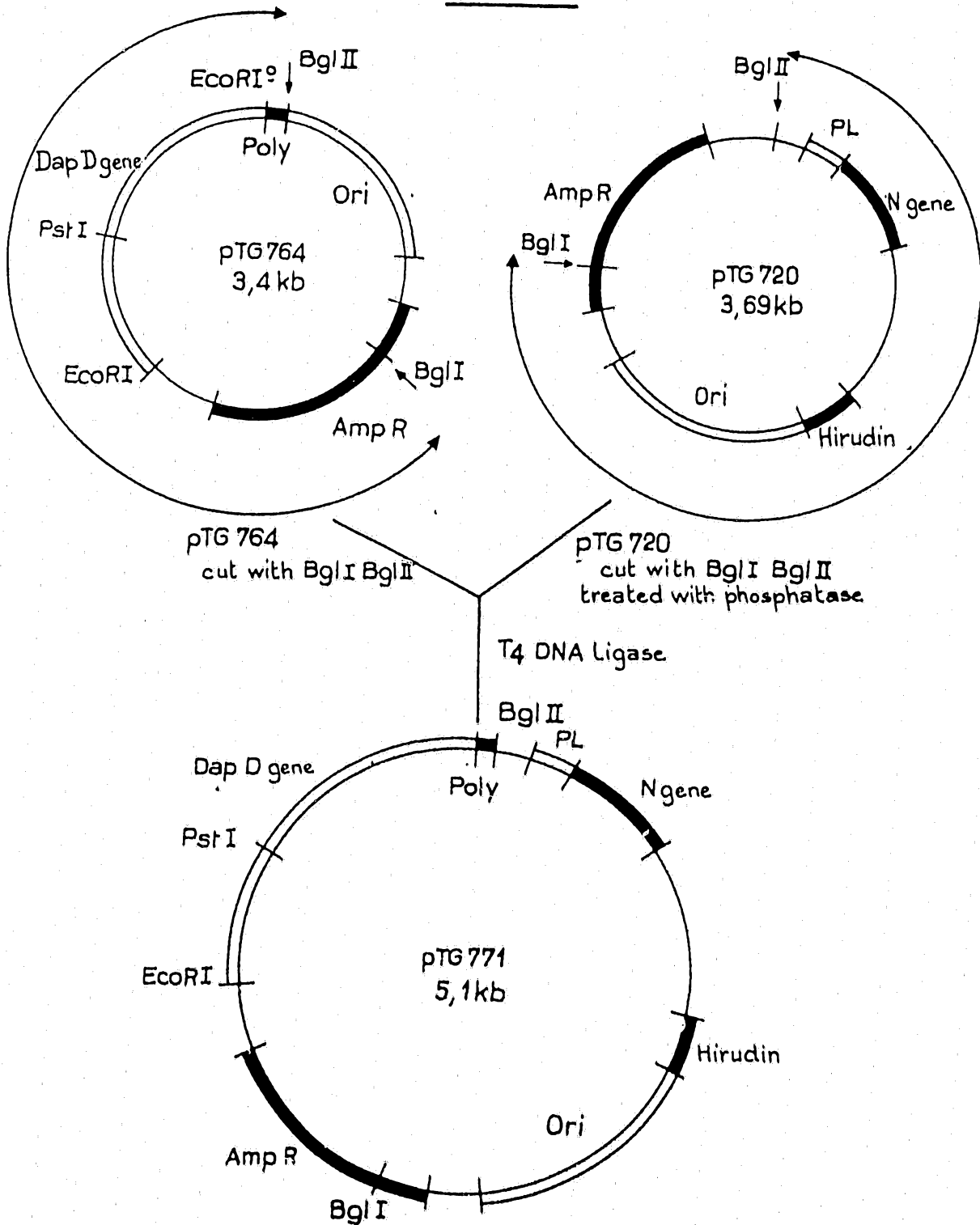
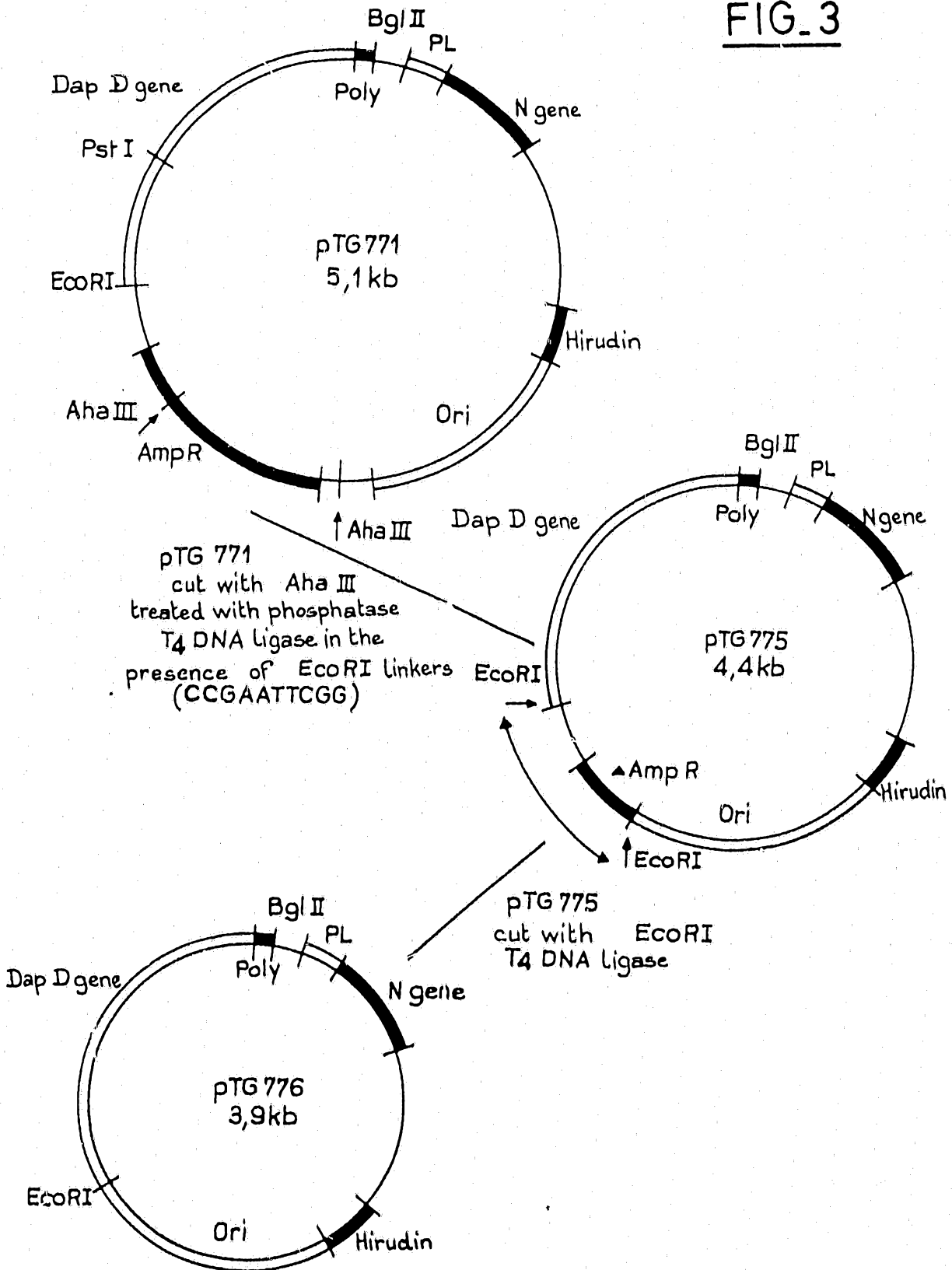


FIG. 3



FIG_4

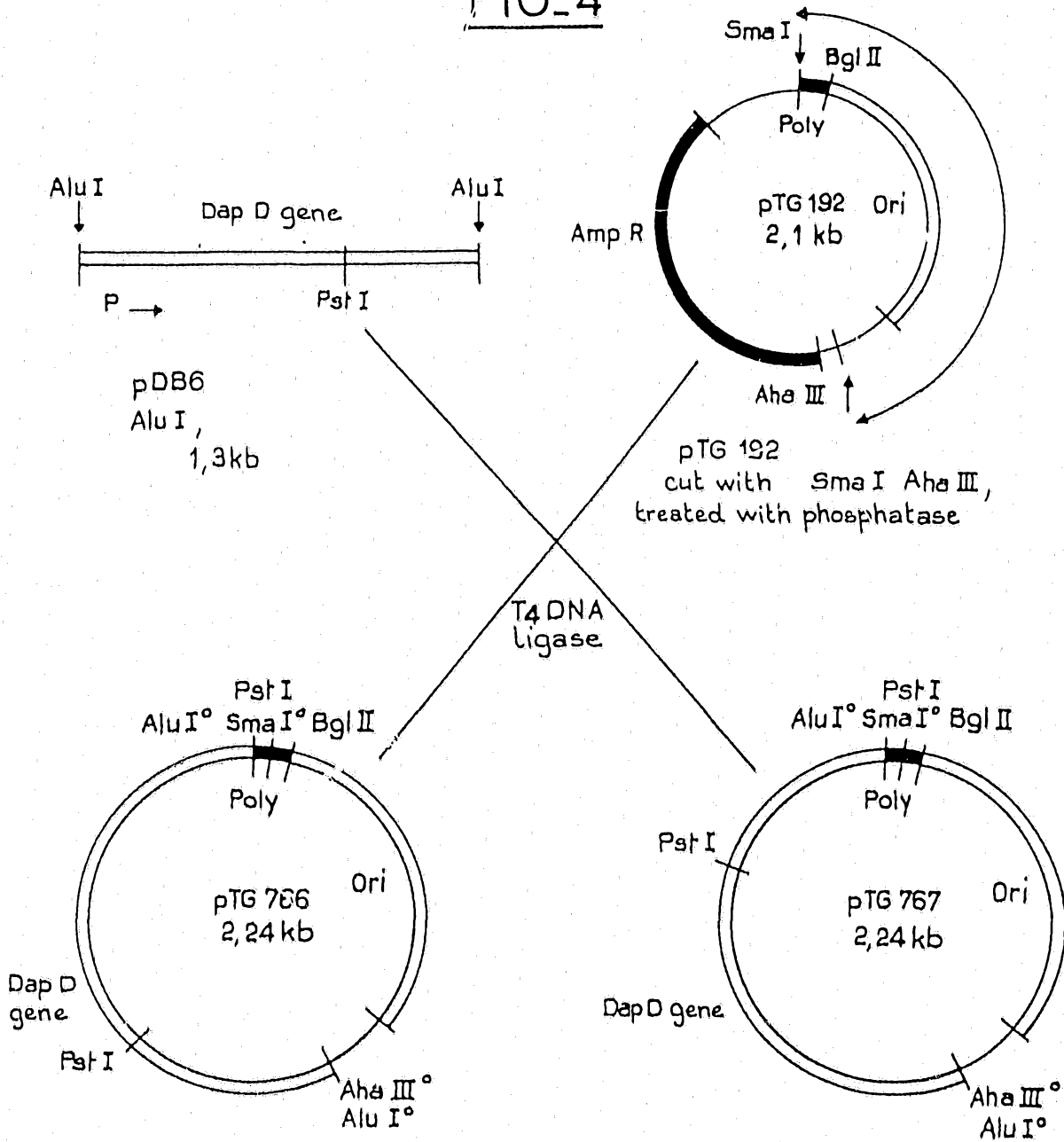
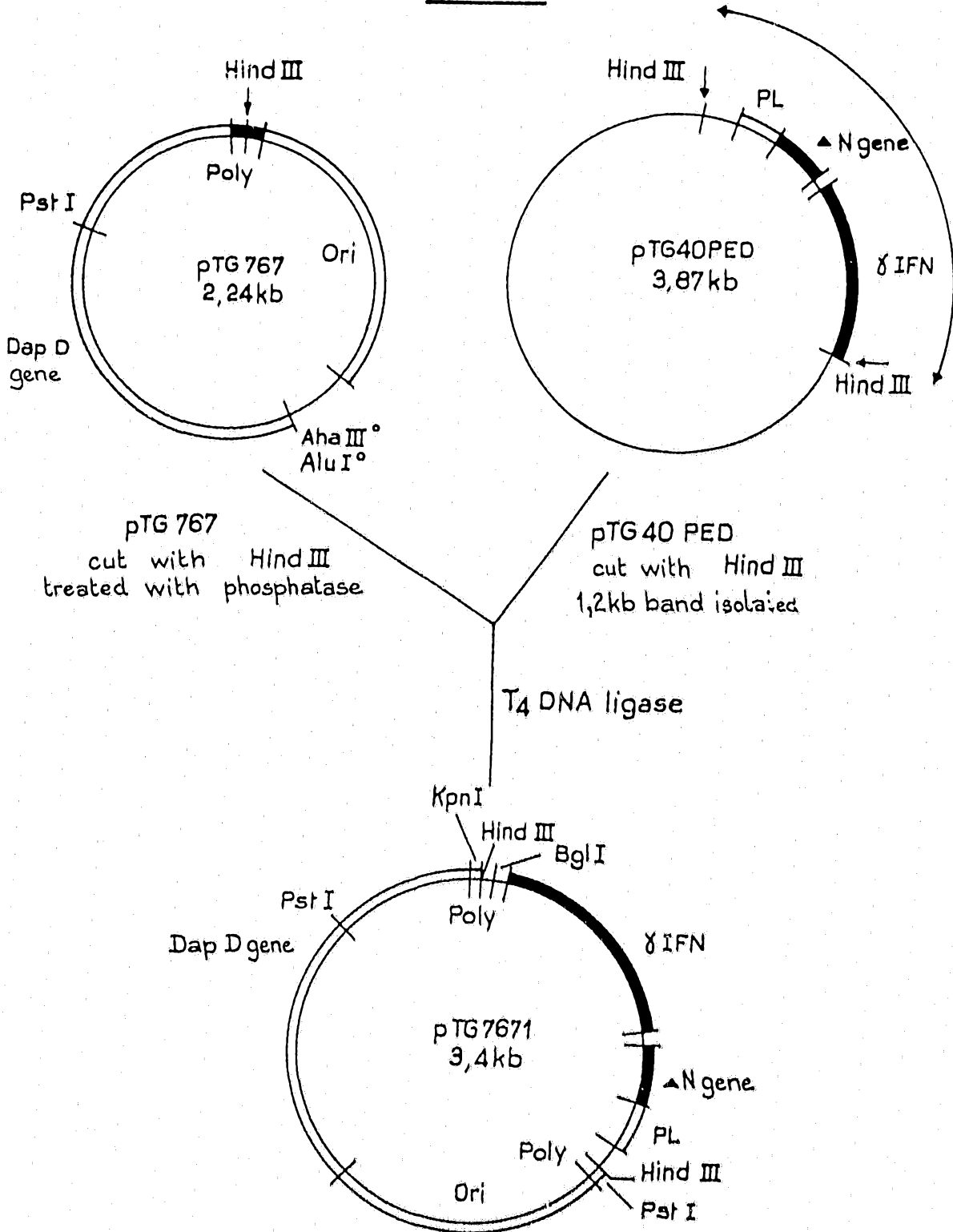


FIG. 5



pDB6 :

Hind III - BamH I fragment

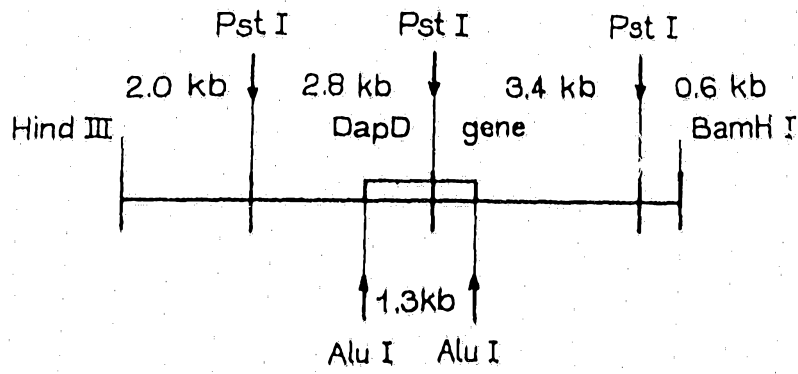


FIG-6

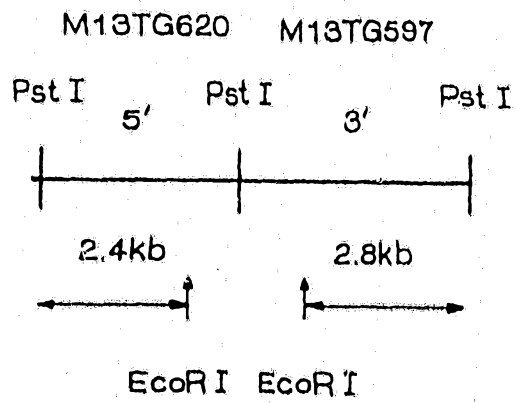


FIG-7

pTG47 :

KpnI - BglII fragment

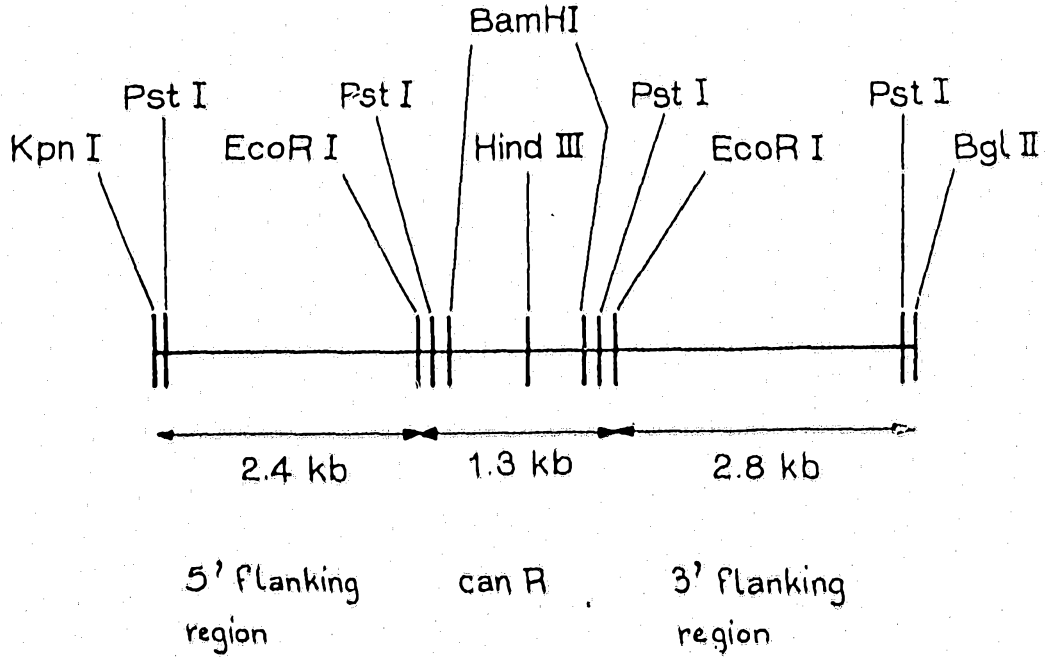


FIG-8

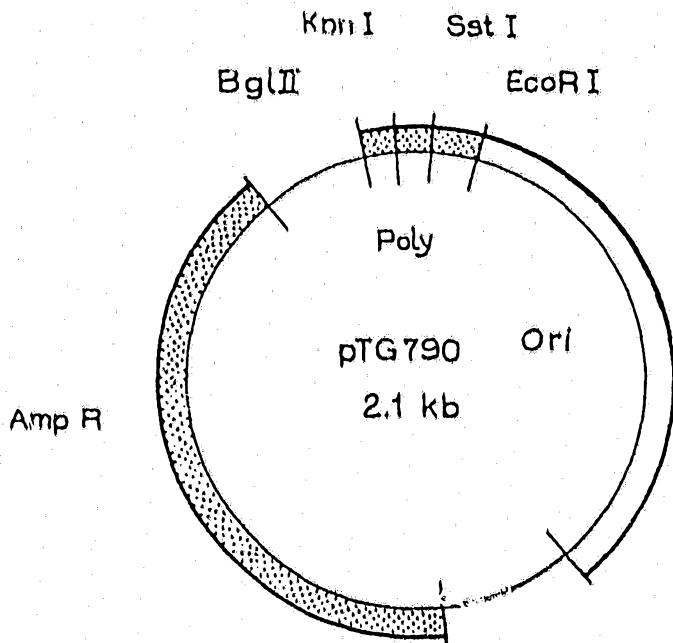


FIG-10

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16



FIG-9A

2 3 4 5 6 7 8 9 10 11 12 13

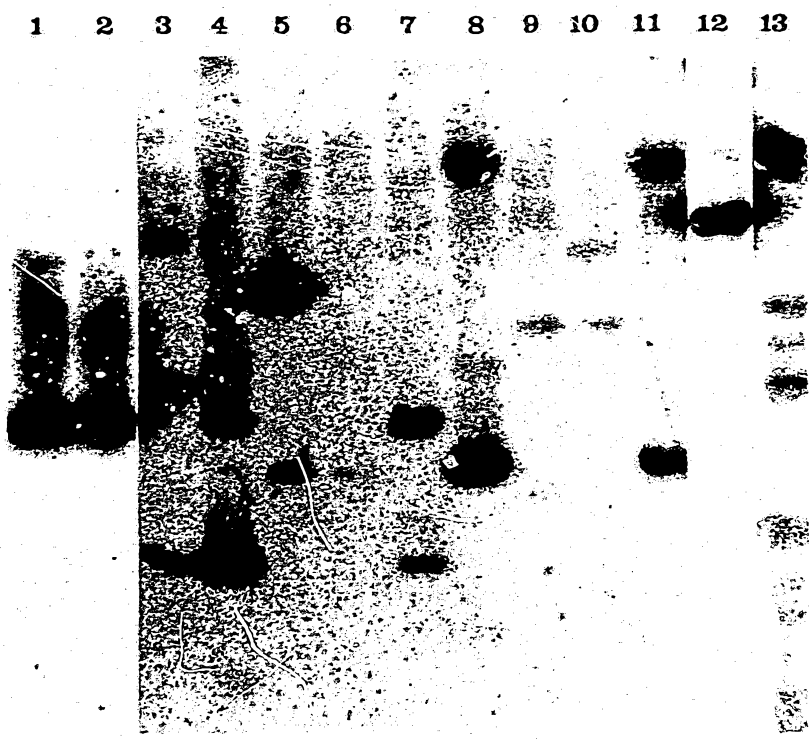


FIG-9B

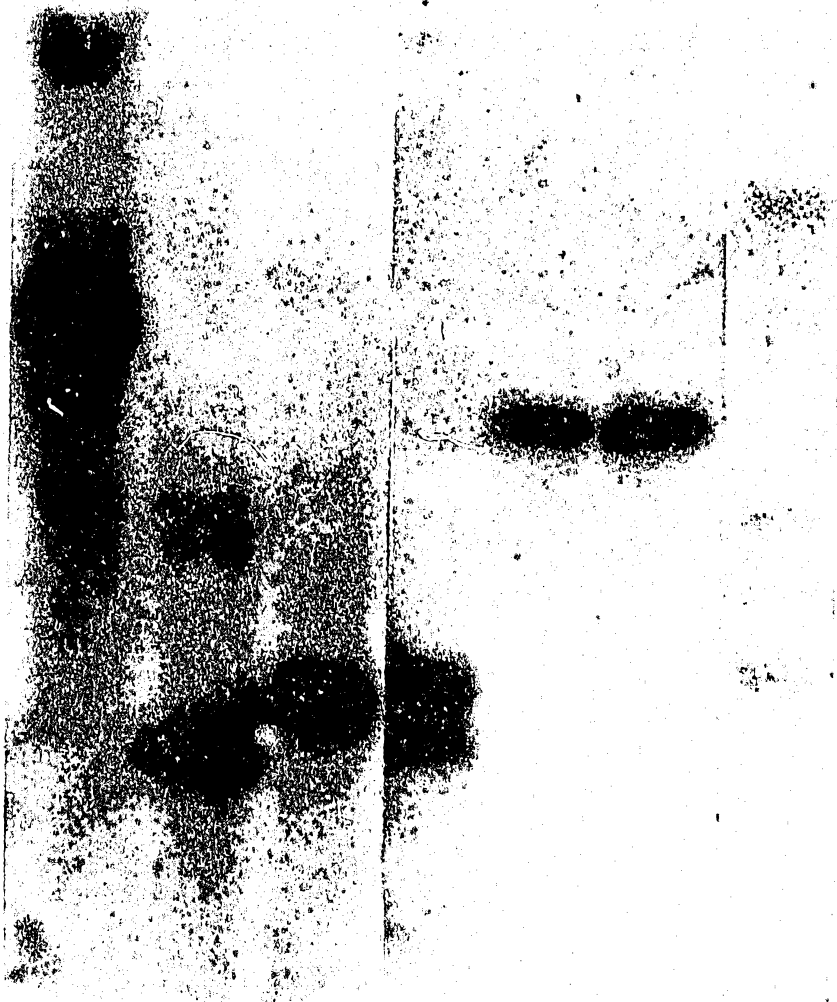
9 / 15

10/15



FIG-9C

1 2 3 4 5 6 7



FIG_9D

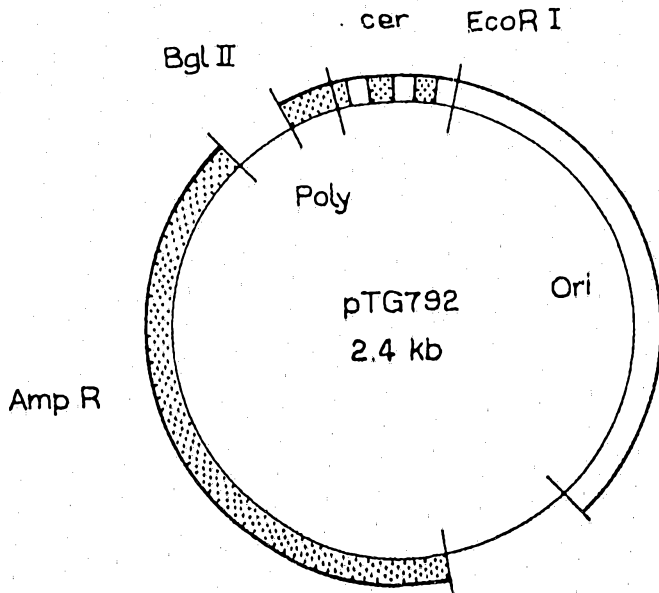


FIG-11

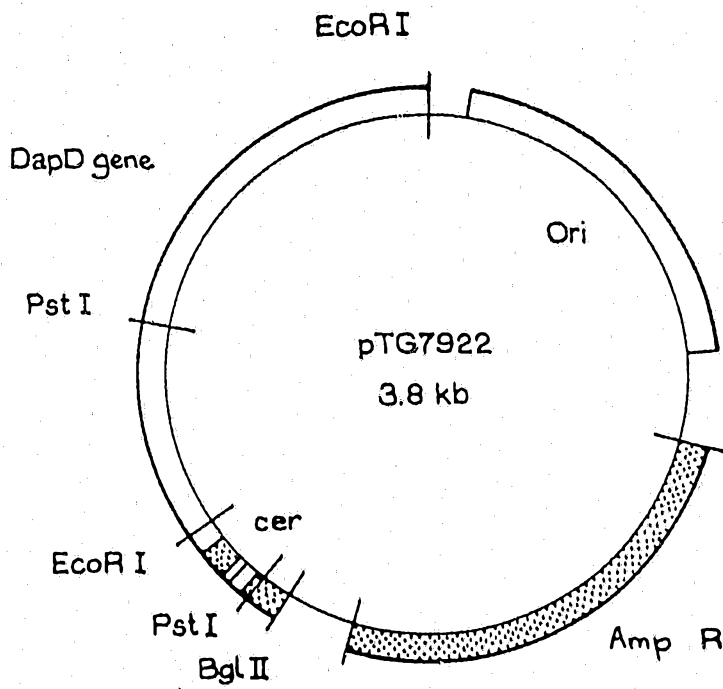


FIG-12

13 / 15

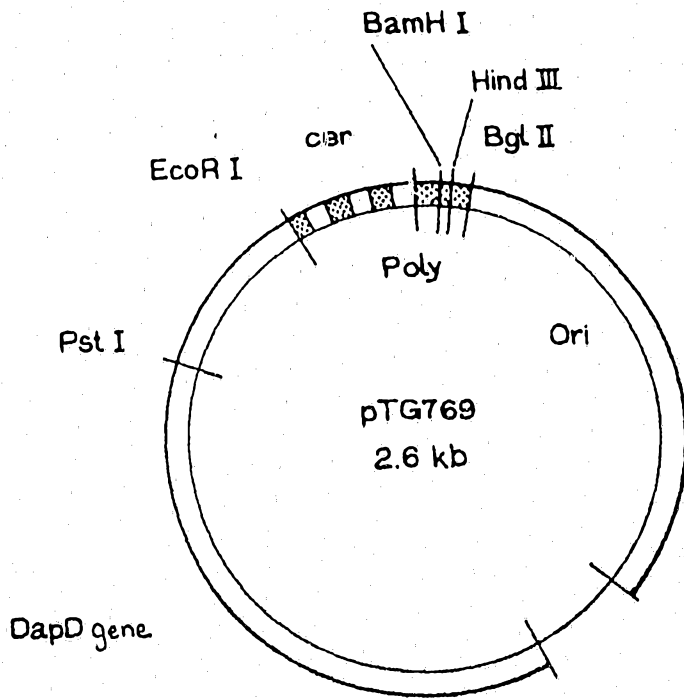


FIG-13

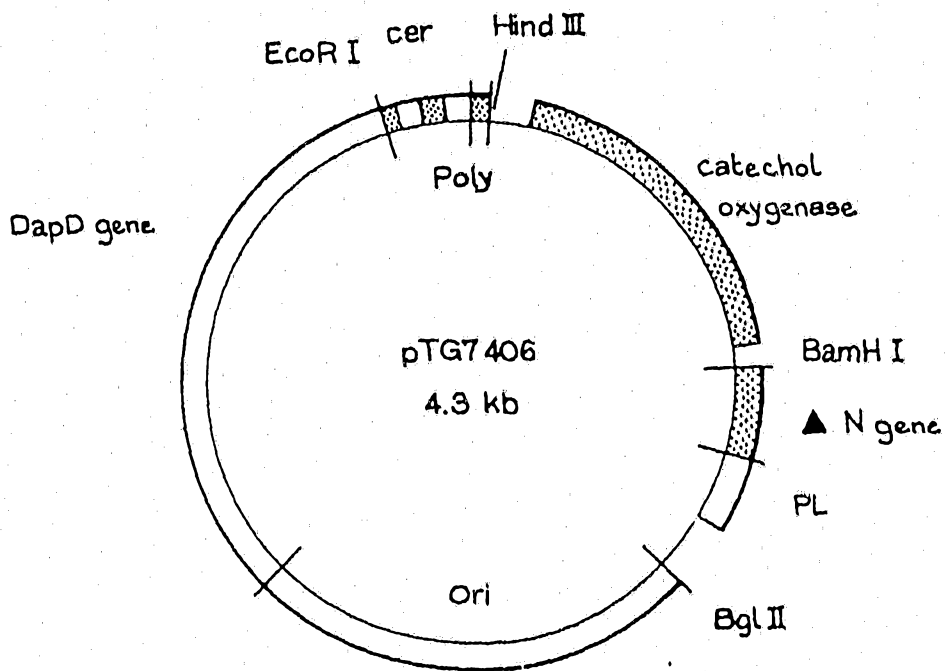


FIG-14

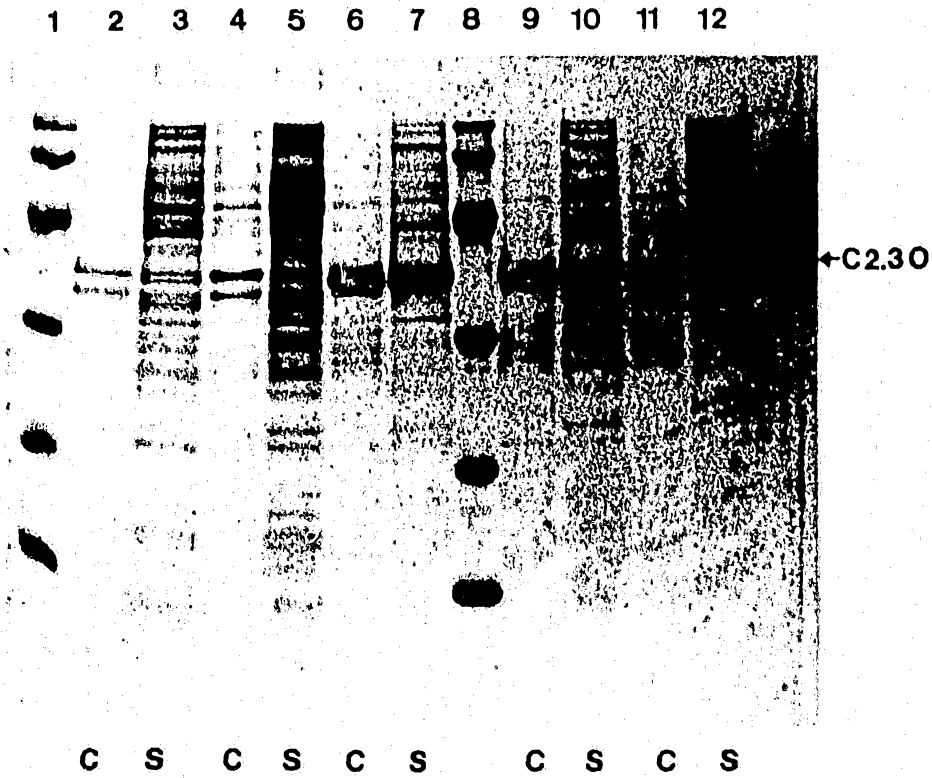


FIG-15

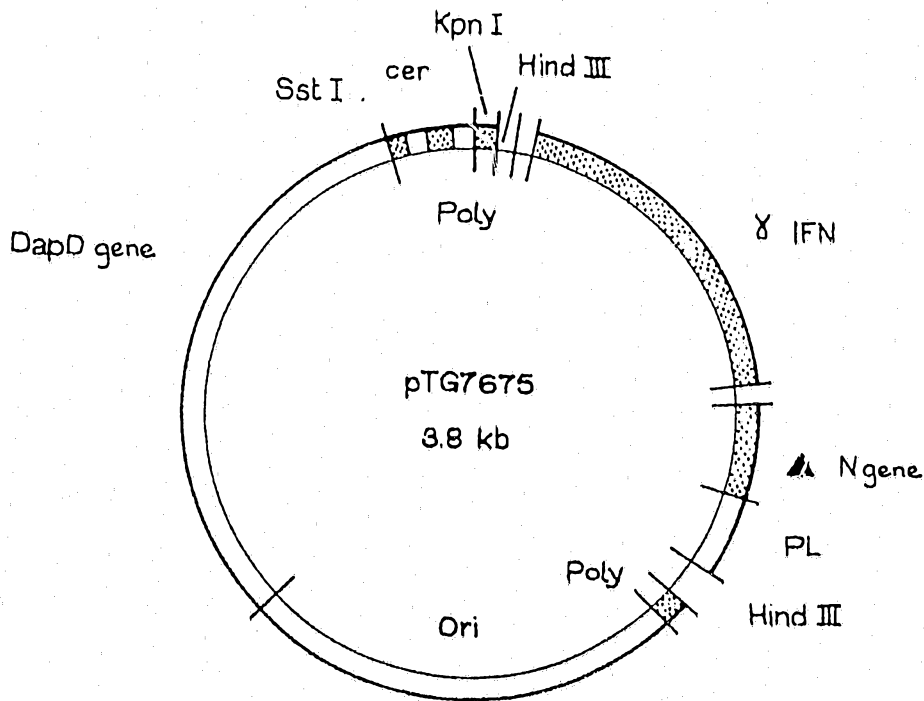


FIG-16

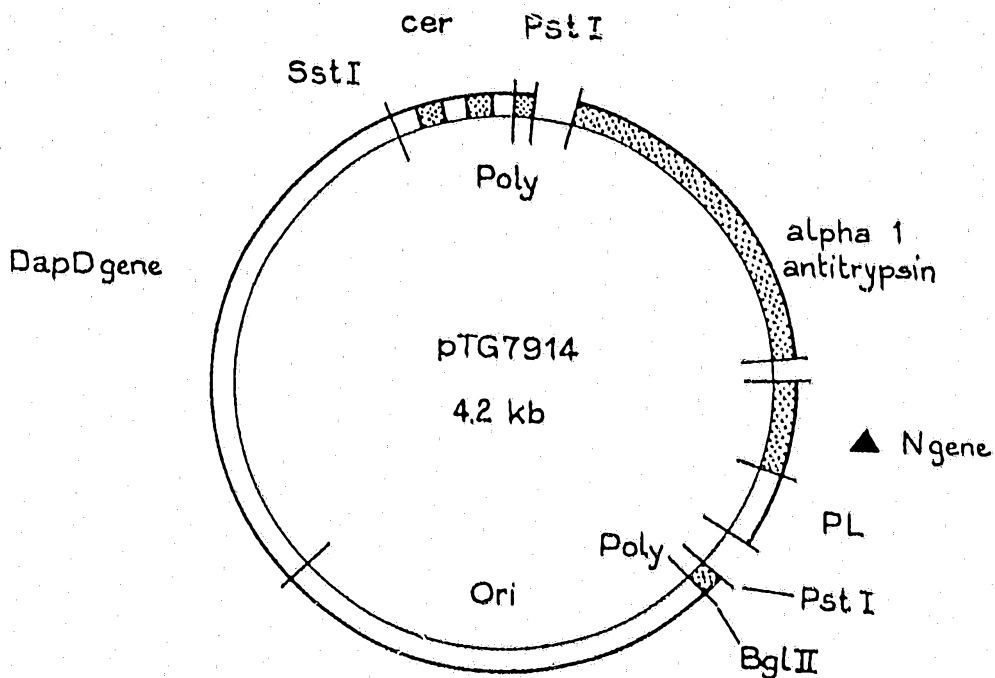


FIG-17