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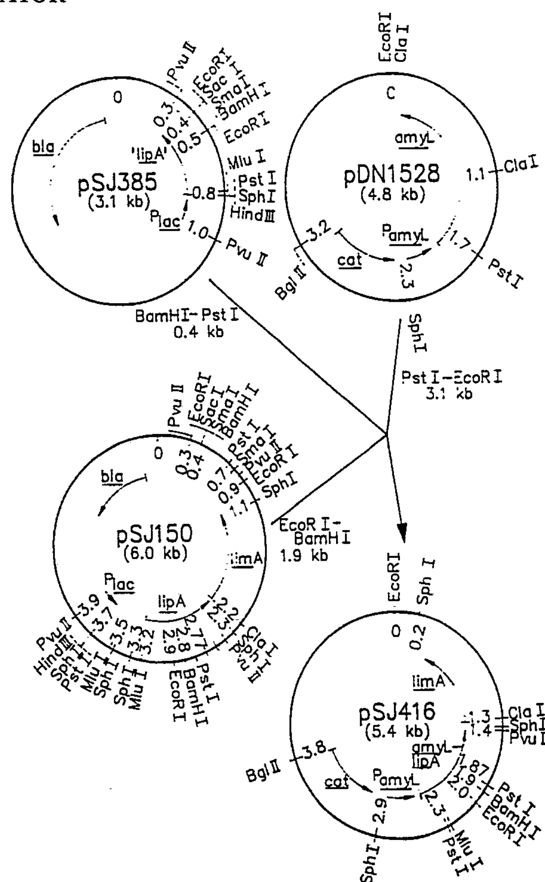
(54) Title: DNA ENCODING A LIPASE AND A LIPASE MODULATOR

(57) Abstract

A DNA sequence encoding a factor which acts *in trans* as a modulator of the production of a *Pseudomonas cepacia* lipase, or encoding an analogue of the lipase modulating factor, will, when present in a host cell which also contains a DNA sequence encoding said lipase, exert a beneficial effect on lipase production. The lipase modulating factor has this effect even when it or the lipase are expressed from heterologous expression signals or in heterologous cells.



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DNA ENCODING A LIPASE AND A LIPASE MODULATOR

FIELD OF INVENTION

The present invention relates to a DNA sequence encoding a lipase, a DNA sequence the product of which affects the pro-
5 duction of the lipase, expression vectors and host cells comprising these sequences, and a method of producing lipase by cultivating the host cells.

BACKGROUND OF THE INVENTION

Lipases are enzymes which catalyze the hydrolysis of ester
10 bonds in triglycerides resulting in the formation of diglycerides, monoglycerides, glycerine and free fatty acids. Some lipases also catalyze other reactions involving ester bonds such as synthesis of ester bonds or transesterification reactions. Lipases are produced by a wide variety of different
15 organisms. Microbial lipases in particular are of considerable practical utility for a variety of purposes where lipolysis of fats is desired, e.g. in the food industry and in detergents.

One particular lipase which has been found to be particularly
20 advantageous for inclusion in a detergent for the removal of fatty stains or soiling from fabrics is a lipase produced by strains of Pseudomonas cepacia. In EP 214 761 (to Novo Industri A/S), this lipase is disclosed as a lipase which is active at a temperature below 60°C, which is important as most
25 present-day fabrics are washed at temperatures below 60°C.

Another important Pseudomonas cepacia lipase for use as a detergent additive is the one disclosed in WO 89/01032 (to Novo Industri A/S) as a positionally non-specific lipase, i.e. one which is able to react with all three fatty acyl
30 groups of a triglyceride.

In order to facilitate Pseudomonas cepacia lipase production, it may be advantageous to employ recombinant DNA techniques, for instance in order to optimize lipase expression by introducing a stronger promoter from which the DNA sequence en-
5 coding the enzyme is expressed or by introducing more efficient ribosome binding sites or signal peptide coding sequences, or in order to select a host organism for the production of the enzyme which is easier to cultivate (e.g. in terms of its being a standard production organism such as
10 E.coli, B. subtilis or the like) or which results in higher lipase yields. As described below, such approaches will sometimes fail to yield the expected results, e.g. in cases where one or more genes in addition to the structural gene coding for the protein in question, play some part in the production
15 of the gene product (examples of such genes are the Bacillus sac (Honjo, M., et al. (1987), Journal of Biotechnology, 6:191-204) and iep (Tanaka, T., Kawata, M. (1988), Journal of Bacteriology, 170:3593-3600) genes, and genes required for the production of Klebsiella pullulanase and E.coli hemoly-
20 sin).

The cloning of a lipase gene from another Pseudomonas species, Pseudomonas fragi, is known from, e.g., S. Aoyama et al., FEBS Letters 242(1), December 1988, pp. 36-40, and W. Kugimiya et al., Biochem. Biophys. Res. Comm. 141(1), Novem-
25 ber 26, 1986, pp. 185-190. However, the lipase produced by P.fragi differs from that of P.cepacia in its amino acid sequence, and in these publications, there is no indication that one or more additional genes may be required in order to achieve a significant lipase production in a host organism.

30 EP 331 376 discloses a recombinant DNA encoding a Pseudomonas cepacia lipase as well as a protein participating in the production of the lipase. There is, however, no indication that the gene encoding this protein may also be functional in a heterologous host organism.

SUMMARY OF THE INVENTION

The present inventor has isolated and cloned a gene encoding a *Pseudomonas cepacia* lipase and identified a gene the expression of which is important to achieve production of the lipase in significant yields.

Accordingly, in a first aspect, the present invention relates to a DNA construct which comprises a DNA sequence encoding a factor which acts *in trans* as a modulator of the production of a *Pseudomonas cepacia* lipase, or encoding a functional analogue of the *P. cepacia* lipase modulating factor, the *P. cepacia* lipase modulating factor or functional analogue thereof being capable of modulating lipase production when the *P. cepacia* lipase is expressed using expression signals which are heterologous to *Pseudomonas* and/or when the *P. cepacia* lipase is expressed in a heterologous host cell.

In the course of the research leading to the present invention, it was surprisingly found that a region downstream of the DNA sequence coding for the lipase (as present on the chromosome of *P. cepacia*) has a pronounced beneficial effect on the production of the lipase. It was experimentally established (among other things, by inserting this region on a another plasmid than that carrying the DNA sequence encoding the lipase) that the region does not merely provide a site on the DNA sequence (such as a promoter, terminator, enhancer, or the like) which is required for lipase expression, but that the region includes a gene encoding a factor which is able to affect the production of the lipase even when the DNA sequences coding for the two products are not located on the same vector. The lipase modulating factor may be an RNA molecule or a polypeptide, but is most likely a polypeptide (cf. Example 3 below).

In the following, the DNA sequence encoding the lipase modulating factor will usually be referred to as the "*lim*" gene,

while the modulating factor will usually be referred to as the "Lim" factor.

It has surprisingly been found that expression of the lim gene exerts a beneficial effect on lipase production even 5 when the lipase is expressed using expression signals which are heterologous to those of Pseudomonas (e.g. a promoter, ribosome binding site and signal peptide-coding region from a Bacillus α-amylase gene) or when the host organism is not Pseudomonas. Therefore, the Lim factor does not act on the 10 initiation of either transcription or translation. However, the precise function of the Lim factor has yet to be elucidated.

The term "functional analogue" is understood to indicate a factor which has a similar lipase-production modulating func-15 tion to that of the P.cepacia Lim factor and which is derived from another organism than P.cepacia, or which is a derivative of the P.cepacia Lim factor produced by modifying the DNA sequence in a manner resulting in addition, substitution, insertion or deletion of one or more amino acids in the na-20 tive sequence. There is reason to believe, however, that such modifications of the native sequence should not be too extensive as the modulating function of the Lim factor may otherwise be lost. Apart from this, a functional analogue may be a homologous polypeptide (i.e. one encoded by DNA which hybri-25 dizes to the same probe as the DNA coding for the Lim factor under certain specified conditions [e.g. presoaking in 5xSSC and prehybridizing for 1h at ~40°C in a solution of 20% formamide, 5xDenhardt's solution, 50mM sodium phosphate, pH 6.8, and 50μg of denatured sonicated calf thymus DNA, followed by 30 hybridization in the same solution supplemented with 100μM ATP for 18h at ~40°C]), or which is reactive with antibodies raised or directed against the Lim factor. When the term "Lim factor" is used in the following description, this is intended implicitly to include such functional analogues as 35 well as the native P.cepacia lipase modulating factor.

DETAILED DISCLOSURE OF THE INVENTION

The DNA construct of the invention comprising the DNA sequence encoding the Lim factor may be of genomic or cDNA origin, for instance obtained by preparing a genomic or cDNA
5 library of P.cepacia and screening for DNA sequences coding for all or part of the Lim factor by hybridization using synthetic oligonucleotide probes in accordance with standard techniques (cf. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1982).

10 The DNA sequence encoding the Lim factor may be any one obtained from a lipase-producing P.cepacia strain. Examples of such strains are the ones deposited in the Deutsche Sammlung von Mikroorganismen in connection with the invention disclosed in EP 214 761, with the deposit numbers DSM 3333-3337
15 and DSM 3401, as well as the strain deposited in the Deutsche Sammlung von Mikroorganismen in connection with the invention disclosed in WO 89/01032, with the deposit number DSM 3959. It is, however, envisaged that a DNA sequence equivalent to the lim gene may also be derived from another lipase-pro-
20 ducing organism. Such a gene may be screened for by hybridization using probes that hybridize to DNA encoding the Lim factor as described above or selected by the reactivity of its gene product with the same antibodies as the Lim factor or identified by screening for the ability to confer lipase
25 production to strains containing the lipase gene but not the lim gene.

The DNA construct of the invention encoding the Lim factor may also be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by S.L.
30 Beaucage and M.H. Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the method described by Matthes et al., EMBO Journal 3, 1984, pp. 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an

automatic DNA synthesizer, purified, ligated, and cloned in an appropriate vector.

Finally, the DNA construct may be of mixed synthetic and genomic, mixed synthetic and cDNA or mixed genomic and cDNA
5 origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire DNA construct, in accordance with standard techniques.

Preferred DNA constructs encoding the Lim factor are those
10 comprising a DNA sequence derived from the chromosome of P.cepacia, in particular those wherein the DNA sequence is located on the P. cepacia chromosome downstream of the sequence encoding a ~33 kD lipase. A particularly preferred DNA construct encoding the Lim factor is one which has the DNA
15 sequence shown in Fig. 1 A-C appended hereto or a modification thereof encoding a functional analogue of the P. cepacia Lim factor as defined above.

Examples of suitable modifications of the DNA sequence are nucleotide substitutions which do not give rise to another
20 sequence of the Lim factor, but which may correspond to the codon usage of the host organism into which the DNA construct is introduced or nucleotide substitutions which do give rise to a different sequence and therefore, possibly, a different structure without, however, impairing the properties of
25 either the lipase or the Lim factor. Other examples of possible modifications are insertion of one or more nucleotides into the sequence, addition of one or more nucleotides at either end of the sequence and deletion of one or more nucleotides at either end of or within the sequence.

30 For some purposes, it may be convenient to provide a DNA sequence encoding a Pseudomonas cepacia lipase on the same DNA construct as the DNA sequence encoding the Lim factor. Thus, the present invention further relates to a DNA construct

which comprises a first DNA sequence encoding a *P. cepacia* lipase or a derivative thereof and a second DNA sequence encoding the *P. cepacia* lipase modulating factor.

In the present context, the term "derivative" is intended to indicate a protein with lipolytic activity which is derived from the native lipase by suitably modifying the DNA sequence coding for the native lipase, resulting in the addition of one or more amino acids to either or both the C- and N-terminal end of the the native protein, substitution of one or more amino acids at one or a number of different sites in the native amino acid sequence, deletion of one or more amino acids at either or both ends of the native protein or at one or more sites in the amino acid sequence, or insertion of one or more amino acids at one or more sites in the native amino acid sequence. Such modifications of DNA coding for native proteins are well known and widely practised in the art.

Preferred DNA constructs encoding the *P. cepacia* lipase are those comprising a DNA sequence derived from *P. cepacia*, DSM 3959, encoding a lipase of ~33 kD which in nature is present on the chromosome of *P. cepacia*. A particularly preferred DNA construct encoding the lipase is one wherein the DNA sequence encoding the lipase is the one shown in Fig. 2 A-C appended hereto or a modification thereof encoding a derivative of the *P. cepacia* lipase as defined above.

In one embodiment of the DNA construct of the invention, the lipase DNA sequence may be located upstream of the sequence encoding the Lim factor, and in another embodiment, the DNA sequence encoding the lipase may be located downstream of the sequence encoding the Lim factor. In either case, the DNA sequences may be located relative to each other in such a way that they are transcribed from the same promoter under conditions permitting expression of the lipase and Lim factor when the DNA construct is present in a host cell. In a particular embodiment, the first DNA sequence encoding the lipase and/or

the second DNA sequence encoding the Lim factor may further comprise expression signals (e.g. a promoter, ribosome binding site and/or signal peptide-coding sequence) heterologous to those of Pseudomonas.

5 In a further aspect, the present invention relates to a replicable expression vector which carries an inserted DNA sequence encoding the Lim factor or a functional analogue thereof, as described above.

In a still further embodiment, the invention relates to a 10 replicable expression vector which carries a first inserted DNA sequence encoding the P. cepacia lipase or a derivative thereof (as described above) and a second DNA sequence encoding the Lim factor (as described above). In this case, the first and second DNA sequences may be expressed from the same 15 promoter although this need not be required. When the first and second DNA sequences are expressed from the same promoter, the lipase-encoding sequence may be located downstream or upstream of the Lim-encoding sequence without adversely affecting the modulation of lipase production effected by the 20 Lim factor.

Alternatively, the DNA sequences encoding the lipase and the Lim-polypeptide, respectively, may each be expressed from a separate promoter, even while carried on the same vector.

In a particular embodiment, the first DNA sequence encoding 25 the lipase and/or the second DNA sequence encoding the Lim factor may further comprise expression signals (e.g. a promoter, ribosome binding site and/or signal peptide-coding sequence) heterologous to those of Pseudomonas.

The replicable expression vector carrying a DNA sequence en-30 coding the lipase and/or Lim factor may be any vector which is capable of replicating autonomously in a given host organism, typically a plasmid or bacteriophage. In the vector,

the DNA sequence encoding the lipase and/or Lim factor should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell and may be derived from genes encoding proteins either homologous or heterologous to the host organism, e.g. a promoter which is heterologous to Pseudomonas. The promoter is preferably derived from a gene encoding a protein homologous to the host organism. Examples of suitable promoters are lac of E.coli, dagA of Streptomyces coelicolor and amyL of Bacillus licheniformis.

The replicable expression vector of the invention further comprises a DNA sequence enabling the vector to replicate in the host cell. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene whose product confers antibiotic resistance, such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance, or the dal genes from B.subtilis or B.licheniformis.

The procedures used to ligate the DNA sequences coding for the lipase and/or the Lim factor and the promoter, respectively, and to insert them into suitable vectors containing the information necessary for replication in the host cell, are well known to persons skilled in the art (cf., for instance, Sambrook et al., op.cit.). It will be understood that the vector may be constructed either by first preparing a DNA construct containing the entire DNA sequence coding for the lipase and for the Lim factor and subsequently inserting this construct into a suitable expression vector, or by preparing separate DNA constructs comprising DNA sequences encoding the lipase or the Lim factor, respectively, or by sequentially inserting DNA fragments containing genetic information for the individual elements (such as the lipase or Lim factor) followed by ligation after each step. It should further be understood that, in this connection, the DNA sequence en-

coding the lipase may be of genomic origin, while the sequence encoding the Lim factor may be prepared synthetically or _vice versa_, as described above.

In a still further aspect, the present invention relates to a
5 host cell which contains either two separate DNA constructs comprising a DNA sequence encoding the _P. cepacia_ lipase and a DNA sequence encoding the Lim factor as defined above, respectively, or a DNA construct comprising both DNA sequences on the same fragment; in either case, the DNA constructs may
10 be integrated in the host chromosome which may be an advantage as the DNA sequences are more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous recombination. The two
15 DNA sequences may be integrated in the host chromosome in such a way that they are each expressed from a separate promoter. In a particular embodiment, the DNA sequence encoding the lipase and/or the DNA sequence encoding the Lim factor may further comprise expression signals (e.g. a promoter,
20 ribosome binding site and/or signal peptide-coding sequence) heterologous to those of _Pseudomonas_.

Alternatively, the host cell may be transformed with a replicable expression vector which contains both DNA sequences, or with two vectors containing the DNA sequence encoding the
25 lipase and the DNA sequence encoding the Lim factor, respectively.

As a further alternative, the DNA sequence encoding the lipase may be integrated in the host chromosome, and the DNA sequence encoding the Lim factor may be carried on a replic-
30 able expression vector (as described above), or _vice versa_.

The host cell used in the process of the invention may be any suitable bacterium, yeast or filamentous fungus which, on cultivation, produces large amounts of the _P. cepacia_ lipase.

Examples of suitable bacteria may be grampositive bacteria such as Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagu-
5 lans, Bacillus circulans, or Streptomyces lividans or Streptomyces murinus, or gramnegative bacteria such as E.coli. The transformation of the bacteria may for instance be effected by protoplast transformation or by using competent cells in a manner known per se.

10 The yeast organism may favourably be selected from a species of Saccharomyces, e.g. Saccharomyces cerevisiae. The filamentous fungus may advantageously belong to a species of Aspergillus, e.g. Aspergillus oryzae or Aspergillus niger. Fungal cells may be transformed by a process involving protoplast
15 formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. The use of Aspergillus as a host organism is described in, e.g., EP 238 023.

In a yet further aspect, the present invention relates to a
20 method of producing the P. cepacia lipase or a derivative thereof, which method comprises cultivating a host cell as described above under conditions conducive to the production of the lipase or analogue thereof (including conditions ensuring the expression of the Lim factor), and recovering the
25 lipase or analogue from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing bacteria. The lipase may be recovered from the medium by conventional procedures including separating the cells from the medium by centrifugation or
30 filtration, if necessary after disruption of the cells, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, followed by purification by a variety of chromatographic procedures,

e.g. ion exchange chromatography, affinity chromatography, or the like.

The invention is further illustrated by the following examples which are not in any way intended to limit the scope 5 of the invention, with reference to the appended drawings, wherein

Fig. 1 A-C shows the DNA sequence of the P. cepacia lim gene, the derived amino acid sequence being shown below in the conventional three-letter code,

10 Fig. 2 A-C shows the DNA sequence encoding the ~33kD P. cepacia lipase, the derived amino acid sequence being shown below in the conventional three-letter code,

Fig. 3 schematically shows the construction of plasmid pSJ518,

15 Fig. 4 schematically shows the construction of plasmid pSJ910,

Fig. 5 schematically shows the construction of plasmid pSJ485,

Fig. 6 A-B schematically shows the construction of plasmids 20 pSJ622 and pSJ624,

Fig. 7 schematically shows the construction of plasmid pSJ424,

Fig. 8 schematically shows the construction of plasmids pSJ494 and 909,

25 Fig. 9 schematically shows the construction of plasmid pSJ729,

Fig. 10 schematically shows the construction of plasmid pSJ416,

Fig. 11 schematically shows the construction of plasmid pSJ600,

Fig. 12 shematically shows the construction of plasmid pSJ671, and

Fig. 13 A-B schematically shows the construction of plasmid pSJ669.

MATERIALS AND METHODS

Bacterial strains:

E. coli HW1 is a *lac*I^q, z M15 derivative of strain 803 described in Wood, 1966.

E. coli NM539 is described in Frischauf et al., 1983, and was obtained from Promega.

Pseudomonas cepacia SB10, DSM 3959, is described in WO 89/01032.

Bacillus subtilis DN1885 is a *amy*E, *amy*R2, Spo$^+$, Pro$^+$ derivative of *B. subtilis* 168.

Bacillus licheniformis ATCC 9789.

Streptomyces lividans TK24 is described in Hopwood et al., 1983.

Phages:

Lambda EMBL4 is described in Frischauf et al., 1983. It was obtained from Promega.

Plasmids:

5 pUC18 and pUC19 are described in Yanisch-Perron et al., 1985.

pACYC177 is described in Chang and Cohen 1978.

pIJ702 is described in Katz et al., 1983.

pIJ4642 is an E.coli cloning vector allowing positive selection for inserts. It resembles pIJ666 (Kieser and Melton,
10 Gene 65, 1988, pp. 83-91) but has more useful sites for cloning. It was obtained from T. Kieser.

pDN1528 is a derivative of the B. subtilis plasmid pUB110 (Gryczan et al.,1978), carrying a 2.3 kb HindIII - SphI fragment containing the alpha-amylase gene from B. licheniformis
15 strain DN52, an amylase-overproducing derivative of ATCC9789 produced by conventional mutagenesis procedures.

pPL1131 is a pUB110-derived plasmid containing the amylase promoter and signal peptide-coding region of pDN1528 followed by a synthetic linker.

20 pIJ2002 is a pUC18 derivative containing the dagA (agarase) gene of S. coelicolor A3(2). It is described in Buttner et al., 1987, and was obtained from M. Bibb.

General Methods:

Standard DNA manipulations were performed essentially as de-
25 scribed in Maniatis et al., 1982.

Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, DNA Polymerase I (Klenow Fragment), and Exonuclease III were obtained from New England Biolabs and used as recommended by the supplier. Nuclease S1 and the restriction enzyme HindII were obtained from Boehringer Mannheim and used as recommended by the supplier.
Chicken egg white lysozyme was obtained from Sigma.

Preparation of plasmids from all strains was conducted by the method described by Kieser (1984).

Lambda DNA was packaged in vitro using the Packagene extract from Promega.

DNA sequencing was performed by the dideoxy chain termination method (Sanger et al., 1977) using ^{35}SdATP (DuPont NEN NEG034H, >1000 Ci/mmol) as the radioactive label and using denatured plasmid DNAs as templates. Sequencing was either carried out as described in Hattori and Sakaki, 1986, using the Klenow fragment, or by means of Sequenase™ as described in the booklet from the supplier (United States Biochemical Corporation). Primers were either the M13 sequencing and reverse sequencing primers from New England Biolabs or oligonucleotides (17-21 mers) complementary to regions of the cloned Pseudomonas DNA prepared by the present inventor.

Transformation of E. coli:

Cells of E. coli were made competent and transformed as described by Mandel and Higa, 1970.

Transformation of B. subtilis:

Competent cells were prepared and transformed as described by Yasbin et al., 1975.

Transformation of B. licheniformis:

Plasmids were introduced into B.licheniformis by polyethylene glycol-mediated protoplast transformation as described by Akamatzu (1984).

Transformation of S. lividans:

Transformation and other procedures for Streptomyces were as described in Hopwood et al., 1985.

Oligonucleotide synthesis:

5 Synthesis was performed on an automatic DNA synthesizer using the phosphoamidite method described by Beaucage and Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869. Crude oligonucleotides were purified by polyacrylamide gel electrophoresis.

10 Preparation of phage DNA:

Phage lambda DNA was prepared from small-scale liquid cultures as described in Maniatis et al., 1982.

Exo III deletions:

15 Unidirectional deletions were made using Exonuclease III as described by Henikoff, 1984.

Lipase analysis

Lipase was measured by a pH-stat method using tributyrine as substrate. 1 LU (Lipase Unit) is the amount of enzyme which 20 liberates 1 umole titratable butyric acid per minute under the following conditions:

Temperature	30.0 °C
pH	7.0
Emulsifier	Gum Arabic, 1 g/l
25 Substrate	Tributyrine, 50 ml/l

Media:

TY:	Trypticase	20	g/l
	Yeast extract	5	g/l
	$FeCl_2.4H_2O$	6	mg/l
30	$MnCl_2.4H_2O$	1	mg/l
	$MgSO_4.7H_2O$	15	mg/l
	pH		7.3

BPX:

Potato starch	100	g/l
Barley flour	50	g/l
BAN 5000 SKB	0.1	g/l
Sodium caseinate	10	g/l
Soy Bean Meal	20	g/l
Na_2HPO_4, 12 H_2O	9	g/l
Pluronic	0.1	g/l

LB agar:

Bacto-tryptone	10 g/l
Bacto yeast extract	5 g/l
NaCl	10 g/l
Bacto agar	15 g/l

Adjusted to pH 7.5 with NaOH

LB agar with tributyrine:

To 350 ml of melted LB agar is added 3.5 ml tributyrine and 0.35 g Gum Arabic, the mixture is emulsified using an Ultra Turrax emulsifier and then autoclaved.

Nile Blue indicator plates:

The bottom layer consisted of 15 ml LB agar.
The top layer contained a mixture of 3 ml soft agar (LB with 0.6 % agar), 300 µl olive oil emulsion and 20 µl of a 5% Nile Blue solution (made up in water and sterile filtered).

Olive Oil emulsion:

Olive oil	10 ml
Gum Arabic	1 g
Deionised water	90 ml

mixed using an Ultra Turrax emulsifier.

Buffers:

TE buffer: 10 mM Tris, pH 8, 1 mM EDTA, pH 8.

Phage buffer: 0.01 M NaCl
0.01 M $MgCl_2$
0.01 M Tris.HCl, pH 7.0

Ligation buffer: 0.066 M Tris.HCl pH 7.5, 0.01 M $MgCl_2$, 25
5 µg/ml gelatine, 0.001 M ATP, 0.01 M DTT.

EXAMPLE 1

Cloning of the Pseudomonas cepacia lipase gene

A) Preparation of chromosomal DNA from Pseudomonas cepacia SB10.

10 A pellet of frozen cells from a 3 ml TY culture was resuspended in 1 ml TE buffer containing 1 mg/ml lysozyme. 0.1 ml EDTA (0.5 M, pH 8.0) was added and the mixture was incubated at 37 °C for 15 min. with gentle shaking. 5 µl 20% SDS was added, and the solution was repeatedly extracted with phenol 15 (300 µl portions) until no interface was present. The supernatant was then extracted once with $CHCl_3$ (300 µl). To 900 µl supernatant, 90 µl 3 M Na-acetate and 500 µl isopropanol were added. After 5-10 minutes, the precipitated DNA was recovered on a plastic inoculating loop, washed in 70 and 96 % EtOH and 20 resuspended in 500 µl TE. The solution of chromosomal DNA (chrDNA) was kept at -20 °C.

B) Partial cutting and fractionation of chrDNA from P. cepacia.

80 µg chrDNA from P.cepacia SB10 (prepared as described in 25 section A above) was digested with 5 units Sau3A at 37 °C for 5 minutes in 400 µl of the buffer recommended by the supplier, and heated at 70 °C for 10 min, and the total sample was then loaded on a preparative agarose gel. After elec-

trophoresis, gel segments containing DNA fragments of 9-23 kb were cut out. The chromosomal DNA fragments were recovered in the liquid formed after freezing/thawing the gel segments, extracted twice with phenol, once with $CHCl_3$, ethanol preci-
5 pitated and dissolved in TE buffer.

C) Construction of a P. cepacia DNA library in phage lambda.

2 µg lambda EMBL4 DNA was digested completely with restriction enzymes BamHI and SalI, extracted twice with phenol, once with $CHCl_3$, ethanol precipitated and resuspended in 10
10 µl TE. The suspension was mixed with 2.5 µg size-fractionated chrDNA (in 50 µl TE), ethanol precipitated, resuspended in 10 µl ligation buffer, 100 units of T4 DNA ligase were added and ligation was carried out for 2 h at room temperature followed by 16 hours at 4°C. The ligated DNA was then packaged into
15 lambda phage particles using the Packagene™ extract from Promega, diluted in 500 µl phage buffer and 50 µl $CHCl_3$ were added. This was used for making an amplified phage stock as follows: Packaged phage particles were mixed with an equal volume of a fresh overnight culture of E. coli NM539 grown in
20 TY + 0.4 % maltose + 20 mM $MgSO_4$, allowed to adsorb for 20 minutes at 37°C and plated on fresh LB plates in 4 ml soft LB agar (0.6 %) containing 20 mM $MgSO_4$. After incubation at 37°C for 16 hours, the partially lysed top layer containing about 2500 plaques was scraped into a 40 ml centrifuge tube con-
25 taining 5 ml phage buffer, and phage eluted for 2 hours. The supernatant after centrifugation at 5000 rpm for 5 min. in a Sorvall SS34 rotor was kept as an amplified stock and used for screening.

D) Isolation of a recombinant lambda phage carrying a lipase
30 gene.

About 200000 phages from the amplified library were adsorbed to NM539 and plated on 20 LB plates in soft agar containing 1 % glycerol tributyrate emulsion, resulting in total lysis on

each plate. 170 clear plaques (hydrolysing the tributyrine) were identified in the turbid tributyrine emulsion, and a number reisolated. 35 plaques were streaked on Nile Blue indicator plates and 10 of these gave a positive reaction, i.e. a strong blue colouring. Phage DNA was isolated from three of these phages, which were found to be identical, based on restriction enzyme digests.

E) Subcloning into pUC19

1 μg of the phage DNA prepared in section D above was partially cut with SalI, mixed with 0.3 μg SalI digested pUC19 DNA, ligated, transformed into competent E. coli HW1 and plated on LB plates containing 200 μg/ml ampicillin. These plates were replicated onto LB plates with 200 μg/ml ampicillin and 1 % glycerol tributyrate emulsion, and colonies surrounded by clear halos were isolated. One of these, SJ150, was shown to harbour a plasmid, designated pSJ150, of approximately 6 kb, including the lipase-coding gene and the lipase modulating (lim) gene.

F) DNA sequencing

The sequence of most of the cloned DNA contained on pSJ150 was determined by the dideoxy chain terminating method directly on double-stranded templates, as described in Materials and Methods above. The sequences of both strands were determined, using a combination of subcloning of restriction fragments, deletions from either end of the cloned DNA using Exonuclease III, and synthetic oligonucleotide primers. This allowed identification of the lipase coding sequence shown in Fig. 2A-C, and the lipase modulator (lim) coding sequence shown in Fig. 1A-C. On pSJ150, the sequence TCG separates the lipase modulator start codon (ATG) from the lipase stop codon (TAA).

EXAMPLE 2

Expression of the Pseudomonas cepacia lipase

A. Expression of the unmodified lipase in E.coli

(1) lip without lim

5 Plasmid pSJ518 was constructed by subcloning the 1.5 kb HindIII-ClaI fragment containing the lipase (lip) gene from pSJ150 into pUC19, as shown in Fig. 3. Strain SJ518 (E. coli HW1 containing pSJ518) does not produce any lipase as seen on plates containing 1 % glycerol tributyrate (i.e. no halos 10 surround the colonies).

(2) lip + lim on the same plasmid

On pSJ150, the lipase is expressed from its own as well as from the pUC19 lac promoter. The plasmid pSJ910, containing the same fragment of cloned DNA but in the reverse orienta-15 tion with respect to the lac promoter, was constructed as outlined in Fig. 4 and introduced into E. coli HW1 to form strain SJ910. When plated on plates containing 1 % glycerol tributyrate, SJ910 colonies were surrounded by smaller halos than colonies of SJ150. This indicates the presence of a pro-20 moter, active in E. coli, for the lip and lim genes.

The region between the lip and lim genes can be changed without negatively affecting lipase expression. pSJ150 contains a unique ClaI site one basepair after the lipase stop codon. Plasmid pSJ485 was constructed by insertion of an 8 basepair 25 BglII linker (New England Biolabs) into this ClaI site which had been filled-in using the large fragment of DNA polymerase I (Klenow fragment) and dNTP's, thus giving a 10 basepair insertion (Fig. 5). Strain SJ485 (E. coli HW1 containing pSJ485) produced the same amounts of lipase as SJ150.

(3) lip and lim on separate plasmids

The lim gene was excised on a 1.2 kb SphI fragment from pSJ150 and inserted into the SphI site of pUC19. This resulted in plasmids pSJ377, with the lac promoter reading correctly into the lim gene, and pSJ378, with the lac promoter reading backwards into the lim gene. From pSJ377 and pSJ378, the 1.2 kb insert + the lac promoter could be excised as a 1.5 kb PvuII fragment. This was inserted into the HindII site of pACYC177 to give pSJ622 (insert from pSJ377) and pSJ624 (insert from pSJ378). The construction of pSJ622 and pSJ624 is outlined in Fig. 6.

Plasmids pSJ622 and pSJ624 were introduced into competent SJ518 (the strain containing pSJ518 with the lipase gene alone), selecting for ampicillin and kanamycin resistance, and the transformants were replicated onto plates containing glycerol tributyrate. Large halos were formed around transformants containing pSJ622, but none around transformants containing pSJ624, indicating lipase production from the former but not from the latter.

B. Expression of a modified lipase gene in E.coli

(1) lip without lim

pSJ494 is an expression plasmid for the P.cepacia lipase in Bacillus. It has the promoter, ribosome binding site and signal peptide coding region from the B. licheniformis alpha-amylase gene fused in-frame to DNA coding for the mature lipase, but does not contain the lim gene. The construction of pSJ494 is outlined in Figs. 7 and 8. The sequence of the fusion region is as follows:

ATG AAA CAA CAA AAA CGG CTT TAC GCC CGA TTG CTG ACG CTG
Met Lys Gln Gln Lys Arg Leu Tyr Ala Arg Leu Leu Thr Leu
alpha-amylase signal peptide

PstI
TTA TTT GCG CTC ATC TTC TTG CTG CCT CAT TCT GCA GCA GCG GCC
Leu Phe Ala Leu Ile Phe Leu Leu Pro His Ser Ala Ala Ala Ala

MluI
5 GCA GCT GGC TAC GCG GCG ACG CGT TAC CCG ATC - -
Ala Ala Gly Tyr Ala Ala Thr Arg Tyr Pro Ile - -
| mature lipase sequence

In order to introduce this alpha-amylase/lipase fusion gene into E. coli, plasmid pSJ909 was constructed (Fig. 8). Strain 10 SJ909 (E. coli HW1 containing pSJ909) does not produce any halos on plates containing glycerol tributyrate.

(2) lip and lim on separate plasmids

Strain SJ909 was made competent and transformed with either pSJ377 (constructed as shown in Fig. 6) or pSJ729, which was 15 derived from pSJ377 by deleting about 600 basepairs from the 5' terminal of the lim gene (Fig. 9). When the double transformants were streaked on plates containing glycerol tributyrate, those containing pSJ377 (the lim^+ plasmid) formed clear halos around the colonies, indicating lipase produc- 20 tion, whereas those containing pSJ729 (lim^-) did not form any halos.

C. Expression of a modified lipase gene in B.subtilis

(1) lip without lim

Plasmids pSJ493 and pSJ495 are identical to pSJ494, described 25 in section B.1), carrying the amylase-lipase fusion but no lim gene. They were introduced into B. subtilis strain DN1885 and transformants were grown for 4 days at 37 °C in shake flasks containing BPX growth medium, with the addition of oleyl alcohol to 30 g/l and chloramphenicol to 12 µg/ml.

Lipase activity in the culture broth was measured by the LU-titrimetric method (described in Materials and Methods) and was 1-2 LU/ml. This is not above the background level found for strain DN1885, which varies between 0-5 LU/ml.

5 (2) lip and lim on the same plasmid

Plasmid pSJ416 contains the same amylase-lipase fusion as pSJ493-495, but the lipase gene is followed immediately downstream by the lim gene in exactly the same way as on pSJ150 (the construction of pSJ416 is outlined in Fig. 10).

10 When this plasmid was introduced into B. subtilis DN1885 and transformants grown in shake flasks as described above, yields reached 40 LU/ml in 4 days.

D. Expression of a modified lipase gene in B.licheniformis

(1) lip without lim

15 Plasmid pSJ488 contains the same amylase-lipase fusion as pSJ493, but on the Bacillus vector pPL1131 conferring kanamycin resistance to its host strain (Fig. 11). This plasmid was introduced into B. licheniformis strain ATCC 9789 by protoplast transformation, and the transformants were streaked 20 on plates containing glycerol tributyrate. The halos formed around the transformants were very small, of the same size as those formed around the untransformed strain.

(2) lip and lim on the same plasmid

Plasmid pSJ600 contains the same amylase-lipase fusion as 25 pSJ488, but the lipase gene is followed immediately downstream by the lim gene in exactly the same way as on pSJ150 (the construction of pSJ600 is outlined in Fig. 11). When this plasmid was introduced into B. licheniformis ATCC 9789 by protoplast transformation, and the transformants were

streaked on plates containing glycerol tributyrate, pronounced halos were formed around the colonies indicating lipase production.

E. Expression of a modified lipase gene in S.lividans

5 (1) lip without lim-expression

Plasmid pSJ604 was constructed by isolating a 880 bp HindIII-AvaII fragment from plasmid pIJ2002, carrying the dagA promoters and part of the signal peptide-encoding sequence (cf. Fig. 2 in Buttner et al., 1987). To the fragment were added 10 two complementary, phosphorylated and annealed oligonucleotides of the following sequence:

AvaII MluI XhoI

GTCCCGCACCCGCCGCTCATGCCGCAGCTGGCTACGCGGCGACGCGTC

GGCGTGGGCGGCGAGTACGGCGTCGACCGATGCGCCGCTGCGCAGAGCT

15 This mixture was ligated to pUC19 which had previously been digested with HindIII and SalI to give plasmid pSJ604.

Plasmid pSJ671 is an E. coli-S. lividans shuttle plasmid carrying an in-frame fusion between the promoter, ribosome binding site and signal peptide coding region of the S. 20 coelicolor agarase gene (dagA, Buttner et al., 1987) and the DNA coding for the mature lipase. The lim gene is present on the plasmid, but in such a position that it is not transcribed by any known promoters. The construction of pSJ671 is outlined in Fig. 12, and the sequence of the fusion region is 25 as follows:

GTG GTC AAC CGA CGT GAT CTC ATC AAG TGG AGT GCC GTC GCA
Met Val Asn Arg Arg Asp Leu Ile Lys Trp Ser Ala Val Ala
agarase signal peptide

AvaII
CTC GGA GCG GGT GCG GGG CTC GCG GGT CCC GCA CCC GCC GCT CAT
Leu Gly Ala Gly Ala Gly Leu Ala Gly Pro Ala Pro Ala Ala His

5

MluI
GCC GCA GCT GGC TAC GCG GCG ACG CGT TAC CCG ATC -
Ala Ala Ala Gly Tyr Ala Ala Thr Arg Tyr Pro Ile
| mature lipA sequence

10 When pSJ671 was introduced into S. lividans TK24 by protoplast transformation and the resulting transformants were streaked on plates containing glycerol tributyrate, very small halos were formed around the colonies, indicating little or no lipase production.

15 (2) lip and lim on the same plasmid

pSJ669 is similar to pSJ671 with the exception that pSJ669 contains the lim gene immediately downstream from the lipase gene in exactly the same way as on pSJ150 (the construction of pSJ669 is outlined in Fig. 13).

20 When pSJ669 was introduced as described above into S. lividans TK24 and transformants were streaked on plates with glycerol tributyrate, very large halos were formed around the colonies, compared to those around pSJ671.

F. Effect of a modified lim gene

25 pSJ377, shown in Fig. 6, contains the lim gene expressed from a lacZ promoter. This plasmid was digested with ClaI, and deletions around the ClaI site were generated using ExoIII. One such deletion plasmid is pSJ721, in which 15 base pairs were deleted, which created a fusion protein derived from the

9 N-terminal codons from the pUC19 lacZ gene plus 8 C-terminal codons from the lip gene fused to a lim gene lacking only the first two codons. The sequence of the fusion region is as follows:

5 LacZ start SphI lip C-terminal part
ATG ACC ATG ATT ACG CCA AGC TTG CAT GCG AAC CGG CTG AAG CTG
Met Thr Met Ile Thr Pro Ser Leu His Ala Asn Arg Leu Lys Leu

: lim from 3. codon
GCG GGG:GCA CGA GGA GGA CGC GCG CCG CTG GCG CGC CGC GCC GTG
10 Ala Gly Ala Arg Gly Gly Arg Ala Pro Leu Ala Arg Arg Ala Val

GTC
Val

The fusion construct plus the lac promoter was excised from pSJ721 as a 1.5 kb PvuII fragment and inserted into the 15 HindII site of pACYC177 to give pSJ812, in the same manner as in the construction of pSJ622 shown in Fig. 6.

pSJ812 was introduced into competent SJ518 (the strain containing pSJ518 with the lipase gene alone), selecting for ampicillin and kanamycin resistance, and the transformants 20 were replicated onto plates containing glycerol tributyrate. The halos formed around the transformants were of approximately the same size as those formed around transformants of SJ518 with pSJ622, expressing the non-modified lim gene.

EXAMPLE 3

25 **Expression and identification of the lim encoded polypeptide**

The lim gene was excised from pSJ150 as a 1.17 kb ClaI - SphI fragment and inserted into the NdeI site of plasmid pJW2

(Wang et al., 1990) after creating blunt ends with the Klenow fragment of DNA polymerase I. Recombinant plasmids were obtained with the lim gene inserted in either of the two possible orientations. pCBE3 and pCBE4 both contain the lim gene
5 in the correct orientation for transcription from the temperature-inducible lambda pR and pL promoters on pJW2, whereas pCBE2 contain the lim gene in the opposite orientation. The plasmids were introduced into E. coli JA221 (Clarke and Carbon, 1978), and transcription from the pL and pR promoters
10 was induced by increasing the temperature of the cultures to 42 °C. Proteins produced upon induction were identified by polyacrylamide gel electrophoresis of cells harvested from the induced cultures. This allowed identification of a protein of an apparent molecular weight of 34000 which was found
15 in induced cultures of transformants carrying pCBE3 and pCBE4, but not in the induced culture of the transformant carrying pCBE2, or in uninduced cultures.

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CLAIMS

1. A DNA construct which comprises a DNA sequence encoding a factor which acts in trans as a modulator of the production of a Pseudomonas cepacia lipase, or encoding a functional
5 analogue of the P. cepacia lipase modulating factor, the P. cepacia lipase modulating factor or functional analogue thereof being capable of modulating lipase production when the P. cepacia lipase is expressed using expression signals which are heterologous to Pseudomonas and/or when the P. ce-
10 pacia lipase is expressed in a heterologous host cell.

2. A DNA construct according to claim 1, wherein the DNA sequence is derived from the chromosome of P. cepacia.

3. A DNA construct according to claim 2, wherein the DNA sequence is located on the P. cepacia chromosome downstream of
15 a DNA sequence encoding a ~33 kD lipase.

4. A DNA construct according to claim 1, wherein the DNA sequence is the one shown in the appended Fig. 1 A-C or a modification thereof encoding a functional analogue of the P. cepacia lipase modulating factor.

20 5. A DNA construct which comprises a first DNA sequence encoding a P. cepacia lipase or a derivative thereof, and a second DNA sequence encoding a P. cepacia lipase modulating factor as defined in any of claims 1-4.

6. A DNA construct according to claim 5, wherein the first
25 DNA sequence encodes a ~33 kD lipase.

7. A DNA construct according to claim 5, wherein the DNA sequence is the one shown in the appended Fig. 2 A-C or a modification thereof encoding a derivative of the P. cepacia lipase.

8. A DNA construct according to claim 5, wherein the DNA sequence encoding the P. cepacia lipase is located upstream of the DNA sequence encoding P. cepacia lipase modulating factor.

5 9. A DNA construct according to claim 5, wherein the DNA sequence encoding the P. cepacia lipase is located downstream of the DNA sequence encoding the P. cepacia lipase modulating factor.

10. A DNA construct according to any of claims 5-9, wherein
10 the first and/or second DNA sequence further comprises expression signals heterologous to those of Pseudomonas.

11. A replicable expression vector which carries an inserted DNA sequence encoding a factor which acts in trans as a modulator of the production of a P. cepacia lipase, or encoding a
15 functional analogue of the P. cepacia lipase modulating factor, the P. cepacia lipase modulating factor or functional analogue thereof being capable of modulating lipase production when the P. cepacia lipase is expressed using expression signals which are heterologous to Pseudomonas and/or when the
20 P. cepacia lipase is expressed in a heterologous host cell.

12. A vector according to claim 11, wherein the DNA sequence is as defined in any of claims 2-4.

13. A replicable expression vector which carries a first inserted DNA sequence encoding a P. cepacia lipase or a deriva-
25 tive thereof, and a second inserted DNA sequence encoding a P. cepacia lipase modulating factor as defined in any of claims 1-4.

14. A vector according to claim 13, wherein the first DNA sequence encodes a ~33 kD lipase.

15. A vector according to claim 14, wherein the DNA sequence is the one shown in the appended Fig. 2 A-B or a modification thereof encoding a derivative of the P. cepacia lipase.

16. A vector according to claim 13, wherein the first and
5 second DNA sequence are expressed from the same promoter.

17. A vector according to claim 16, wherein the first DNA sequence encoding the P. cepacia lipase is located upstream of the second DNA sequence encoding the P. cepacia lipase modulating factor.

10 18. A vector according to claim 16, wherein the first DNA sequence encoding the P. cepacia lipase is located downstream of the second DNA sequence encoding the P. cepacia lipase modulating factor.

19. A vector according to claim 13, wherein the first DNA
15 sequence encoding the P.cepacia lipase and the second DNA sequence encoding the P. cepacia lipase modulating factor are each expressed from a separate promoter.

20. A vector according to any of claims 13-19, wherein the first and/or second DNA sequence further comprises expression
20 signals heterologous to those of Pseudomonas.

21. A host cell containing a first DNA construct comprising a DNA sequence encoding a P. cepacia lipase or a derivative thereof, and a second DNA construct according to any of claims 1-4.

25 22. A host cell according to claim 21, wherein the first DNA construct encodes a ~33 kD lipase.

23. A host cell according to claim 22, wherein the DNA sequence of the DNA construct is the one shown in the appended

Fig. 2 A-B or a modification thereof encoding a derivative of the P. cepacia lipase.

24. A host cell according to any of claims 21-23, wherein the first and/or second DNA construct further comprises expres-
5 sion signals heterologous to those of Pseudomonas.

25. A host cell containing a DNA construct according to any of claims 5-10.

26. A host cell according to claim 21, which is transformed with a replicable expression vector carrying the first DNA
10 construct, and with a replicable expression vector according to claim 11.

27. A host cell according to claim 26, wherein the DNA sequence encoding the P. cepacia lipase further comprises expression signals heterologous to those of Pseudomonas.

15 28. A host cell according to claim 21 which is transformed with a replicable expression vector according to any of claims 13-20.

29. A host cell according to claim 21, which carries the first DNA construct integrated in its chromosome, and which
20 contains a replicable expression vector according to claim 11.

30. A host cell according to claim 21, which carries the second DNA construct integrated in its chromosome, and which contains a replicable expression vector containing the first
25 DNA construct.

31. A host cell according to claim 21, which carries the first and second DNA construct integrated in its chromosome in such a way that the DNA sequence coding for the P. cepacia

lipase and the DNA sequence coding for the lipase modulating factor are each expressed from a separate promoter.

32. A host cell according to any of claims 21-31 which is a bacterium, yeast or filamentous fungus.

5 33. A host cell according to claim 32 which is a grampositive bacterium.

34. A host cell according to claim 33, wherein the grampositive bacterium is selected from the group consisting of Bacillus subtilis, Bacillus licheniformis, Bacillus lentus,
10 Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans, or Streptomyces lividans or Streptomyces murinus.

35. A host cell according to claim 32 which is a gramnega-
15 tive bacterium, e.g. Escherichia coli.

36. A method of producing a P. cepacia lipase or a derivative thereof, which method comprises cultivating a host cell according to any of claims 21-35 under conditions conducive to the production of the lipase or analogue, and recovering the
20 lipase or analogue from the culture medium.

```
                 10                  20                  30                  40
                  |                   |                   |                   |
ATG ACG GCA CGA GGA GGA CGC GCG CCG CTG GCG CGC CGC GCC GTG
MET Thr Ala Arg Gly Gly Arg Ala Pro Leu Ala Arg Arg Ala Val

         50                  60                  70                  80                  90
          |                   |                   |                   |                   |
GTC TAT GGT GCC GTG GGG CTG GCG GCG ATT GCC GGC GTG GCG ATG
Val Tyr Gly Ala Val Gly Leu Ala Ala Ile Ala Gly Val Ala MET

                100                 110                 120                 130
                  |                   |                   |                   |
TGG AGC GGC GCG GGC CGG CAT GGC GGG ACG GGC GCA TCC GGC GAG
Trp Ser Gly Ala Gly Arg His Gly Gly Thr Gly Ala Ser Gly Glu

        140                 150                 160                 170                 180
          |                   |                   |                   |                   |
CCG CCG GAT GCG TCG GCG GCA CGC GGA CCG GCT GCC GCA CCG CCG
Pro Pro Asp Ala Ser Ala Ala Arg Gly Pro Ala Ala Ala Pro Pro

                190                 200                 210                 220
                  |                   |                   |                   |
CAG GCC GCC GTG CCG GCA AGC ACG AGC CTG CCG CCG TCG CTC GCC
Gln Ala Ala Val Pro Ala Ser Thr Ser Leu Pro Pro Ser Leu Ala

        230                 240                 250                 260                 270
          |                   |                   |                   |                   |
GGC TCC AGC GCG CCC CGC TTG CCG CTC GAT GCC GGC GGC CAT CTC
Gly Ser Ser Ala Pro Arg Leu Pro Leu Asp Ala Gly Gly His Leu

                280                 290                 300                 310
                  |                   |                   |                   |
GCG AAG GCG CGC GCG GTG CGG GAT TTC TTC GAC TAC TGC CTG ACC
Ala Lys Ala Arg Ala Val Arg Asp Phe Phe Asp Tyr Cys Leu Thr

        320                 330                 340                 350                 360
          |                   |                   |                   |                   |
GCG CAG AGC GAC CTG AGT GCG GCC GGG CTC GAT GCG TTC GTC ATG
Ala Gln Ser Asp Leu Ser Ala Ala Gly Leu Asp Ala Phe Val MET

                370                 380                 390                 400
                  |                   |                   |                   |
CGC GAG ATT GCC GCA CAG CTC GAC GGG ACC GTT GCG CAG GCC GAG
Arg Glu Ile Ala Ala Gln Leu Asp Gly Thr Val Ala Gln Ala Glu

        410                 420                 430                 440                 450
          |                   |                   |                   |                   |
GCG CTC GAC GTG TGG CAC CGG TAT CGC GCG TAT CTC GAC GCA CTC
Ala Leu Asp Val Trp His Arg Tyr Arg Ala Tyr Leu Asp Ala Leu

                460                 470                 480                 490
                  |                   |                   |                   |
GCG AAA TTG CGC GAT GCC GGC GCG GTC GAC AAG TCG GAC CTG GGT
Ala Lys Leu Arg Asp Ala Gly Ala Val Asp Lys Ser Asp Leu Gly
```

Fig. 1a

```
 500          510           520          530          540
  |            |             |            |            |
GCA TTG CAG CTC GCG CTC GAC CAG CGC GCG TCG ATC GCG TAC CGG
Ala Leu Gln Leu Ala Leu Asp Gln Arg Ala Ser Ile Ala Tyr Arg

              550          560          570          580
               |            |            |            |
TGG CTC GGC GAC TGG AGC CAG CCG TTC TTC GGT GCG GAG CAA TGG
Trp Leu Gly Asp Trp Ser Gln Pro Phe Phe Gly Ala Glu Gln Trp

 590          600           610          620          630
  |            |             |            |            |
CGG CAG CGC TAC GAC CTC GCG CGG CTG AAG ATC GCG CAG GAC CCC
Arg Gln Arg Tyr Asp Leu Ala Arg Leu Lys Ile Ala Gln Asp Pro

              640          650          660          670
               |            |            |            |
GCG CTG ACG GAT GCG CAG AAG GCC GAA CGG CTC GCG GCG CTC GAA
Ala Leu Thr Asp Ala Gln Lys Ala Glu Arg Leu Ala Ala Leu Glu

 680          690           700          710          720
  |            |             |            |            |
CAG CAG ATG CCG GCC GAC GAA CGC GCC GCG CAG CAG CGC GTC GAC
Gln Gln MET Pro Ala Asp Glu Arg Ala Ala Gln Gln Arg Val Asp

              730          740          750          760
               |            |            |            |
CGG CAG CGC GCG GCG ATC GAC CAG ATC GCG CAA TTG CAG AAG AGC
Arg Gln Arg Ala Ala Ile Asp Gln Ile Ala Gln Leu Gln Lys Ser

 770          780           790          800          810
  |            |             |            |            |
GGG GCG ACG CCC GAT GCG ATG CGC GCA CAA CTG ACG CAG ACG CTC
Gly Ala Thr Pro Asp Ala MET Arg Ala Gln Leu Thr Gln Thr Leu

              820          830          840          850
               |            |            |            |
GGC CCC GAA GCG GCC GCG CGC GTC GCG CAG ATG CAG CAG GAC GAC
Gly Pro Glu Ala Ala Ala Arg Val Ala Gln MET Gln Gln Asp Asp

 860          870           880          890          900
  |            |             |            |            |
GCA TCG TGG CAG AGG CGC TAC GCG GAC TAC GCG GCG CAG CGT GCG
Ala Ser Trp Gln Arg Arg Tyr Ala Asp Tyr Ala Ala Gln Arg Ala

              910          920          930          940
               |            |            |            |
CAG ATC GAG TCG GCC GGC CTG TCG CCG CAG GAT CGC GAC GCG CAG
Gln Ile Glu Ser Ala Gly Leu Ser Pro Gln Asp Arg Asp Ala Gln

 950          960           970          980          990
  |            |             |            |            |
ATC GCC GCG CTG CGG CAG CGC GTG TTT ACG AAG CCC GGC GAA GCC
Ile Ala Ala Leu Arg Gln Arg Val Phe Thr Lys Pro Gly Glu Ala
```

Fig. 1b

```
        1000            1010            1020             1030
          |               |               |                |
GTG CGC GCG GCA TCG CTC GAT CGC GGG GCG GGC AGC GCG CGG
Val Arg Ala Ala Ser Leu Asp Arg Gly Ala Gly Ser Ala Arg
```

Fig. 1c

```
                  10                  20                  30                  40
                   |                   |                   |                   |
ATG GCC AGG ACG ATG CGT TCC AGG GTG GTG GCA GGG GCA GTG GCA
MET Ala Arg Thr MET Arg Ser Arg Val Val Ala Gly Ala Val Ala

       50                  60                  70                  80                  90
        |                   |                   |                   |                   |
TGC GCG ATG AGC ATC GCG CCG TTC GCG GGG ACG ACC GCG GTG ATG
Cys Ala MET Ser Ile Ala Pro Phe Ala Gly Thr Thr Ala Val MET

                 100                 110                 120                 130
                   |                   |                   |                   |
ACG CTC GCG ACG ACG CAC GCG GCA ATG GCG GCC ACC GCG CCC GCC
Thr Leu Ala Thr Thr His Ala Ala MET Ala Ala Thr Ala Pro Ala

     140                 150                 160                 170                 180
       |                   |                   |                   |                   |
GCT GGC TAC GCG GCG ACG CGT TAC CCG ATC ATC CTC GTG CAC GGG
Ala Gly Tyr Ala Ala Thr Arg Tyr Pro Ile Ile Leu Val His Gly

                 190                 200                 210                 220
                   |                   |                   |                   |
CTC TCG GGT ACC GAC AAG TAC GCC GGC GTG CTC GAG TAT TGG TAC
Leu Ser Gly Thr Asp Lys Tyr Ala Gly Val Leu Glu Tyr Trp Tyr

       230                 240                 250                 260                 270
        |                   |                   |                   |                   |
GGC ATC CAG GAG GAC CTG CAA CAG AAC GGT GCG ACC GTC TAC GTC
Gly Ile Gln Glu Asp Leu Gln Gln Asn Gly Ala Thr Val Tyr Val

                 280                 290                 300                 310
                   |                   |                   |                   |
GCG AAC CTG TCG GGT TTC CAG AGC GAC GAC GGC CCG AAC GGG CGC
Ala Asn Leu Ser Gly Phe Gln Ser Asp Asp Gly Pro Asn Gly Arg

       320                 330                 340                 350                 360
        |                   |                   |                   |                   |
GGC GAA CAG TTG CTC GCT TAC GTG AAG ACG GTG CTC GCG GCG ACG
Gly Glu Gln Leu Leu Ala Tyr Val Lys Thr Val Leu Ala Ala Thr

                 370                 380                 390                 400
                   |                   |                   |                   |
GGG GCG ACC AAG GTC AAT CTC GTC GGT CAC AGC CAG GGC GGC CTC
Gly Ala Thr Lys Val Asn Leu Val Gly His Ser Gln Gly Gly Leu

       410                 420                 430                 440                 450
        |                   |                   |                   |                   |
TCG TCG CGC TAT GTT GCT GCC GTC GCG CCC GAT CTC GTT GCG TCG
Ser Ser Arg Tyr Val Ala Ala Val Ala Pro Asp Leu Val Ala Ser

                 460                 470                 480                 490
                   |                   |                   |                   |
GTG ACG ACG ATC GGC CCA GCC GAT CGC GGC TCC GAA TTC GCC GAC
Val Thr Thr Ile Gly Pro Ala Asp Arg Gly Ser Glu Phe Ala Asp
```

Fig. 2 a

```
    500         510         520         530         540
     |           |           |           |           |
TTC GTG CAG GAC GTG CTC GCG TAC GAT CCG ACC GGG CTT TCG TCA
Phe Val Gln Asp Val Leu Ala Tyr Asp Pro Thr Gly Leu Ser Ser

            550         560         570         580
             |           |           |           |
TCG GTG ATC GCC GCG TTC GTC AAT GTG TTC GGG ATC CTG ACG AGC
Ser Val Ile Ala Ala Phe Val Asn Val Phe Gly Ile Leu Thr Ser

    590         600         610         620         630
     |           |           |           |           |
AGC AGC CAC AAC ACC AAC CAG GAC GCG CTC GCC GCA CTG CAG ACG
Ser Ser His Asn Thr Asn Gln Asp Ala Leu Ala Ala Leu Gln Thr

            640         650         660         670
             |           |           |           |
CTG ACC ACC GCA CGG GCC GCC ACG TAC AAC CAG AAC TAT CCG AGC
Leu Thr Thr Ala Arg Ala Ala Thr Tyr Asn Gln Asn Tyr Pro Ser

    680         690         700         710         720
     |           |           |           |           |
GCG GGC CTG GGT GCG CCG GGC AGT TGC CAG ACC GGT GCG CCG ACC
Ala Gly Leu Gly Ala Pro Gly Ser Cys Gln Thr Gly Ala Pro Thr

            730         740         750         760
             |           |           |           |
GAA ACC GTC GGC GGC AAC ACG CAC CTG CTG TAT TCG TGG GCC GGC
Glu Thr Val Gly Gly Asn Thr His Leu Leu Tyr Ser Trp Ala Gly

    770         780         790         800         810
     |           |           |           |           |
ACG GCG ATC CAG CCG ACG CTC TCC GTG TTC GGC GTC ACG GGC GCG
Thr Ala Ile Gln Pro Thr Leu Ser Val Phe Gly Val Thr Gly Ala

            820         830         840         850
             |           |           |           |
ACG GAC ACG AGC ACC CTT CCG CTC GTC GAT CCG GCG AAC GTG CTC
Thr Asp Thr Ser Thr Leu Pro Leu Val Asp Pro Ala Asn Val Leu

    860         870         880         890         900
     |           |           |           |           |
GAC CTG TCG ACG CTC GCG CTG TTC GGC ACC GGC ACG GTG ATG ATC
Asp Leu Ser Thr Leu Ala Leu Phe Gly Thr Gly Thr Val MET Ile

            910         920         930         940
             |           |           |           |
AAC CGC GGC TCC GGG CAG AAC GAC GGG CTC GTG TCG AAG TGC AGT
Asn Arg Gly Ser Gly Gln Asn Asp Gly Leu Val Ser Lys Cys Ser

    950         960         970         980         990
     |           |           |           |           |
GCG CTG TAC GGC AAG GTG CTG AGC ACG AGC TAC AAG TGG AAC CAC
Ala Leu Tyr Gly Lys Val Leu Ser Thr Ser Tyr Lys Trp Asn His
```

Fig. 2 b

```
                1000        1010        1020            1030
                   |           |           |               |
CTC GAC GAG ATC AAC CAG CTG CTC GGC GTG CGC GGC GCG TAT GCG
Leu Asp Glu Ile Asn Gln Leu Leu Gly Val Arg Gly Ala Tyr Ala

   1040        1050            1060        1070        1080
      |           |               |           |           |
GAA GAT CCC GTC GCG GTG ATC CGC ACG CAT GCG AAC CGG CTG AAG
Glu Asp Pro Val Ala Val Ile Arg Thr His Ala Asn Arg Leu Lys

         1090
            |
CTG GCG GGC GTG
Leu Ala Gly Val
```

Fig. 2 c

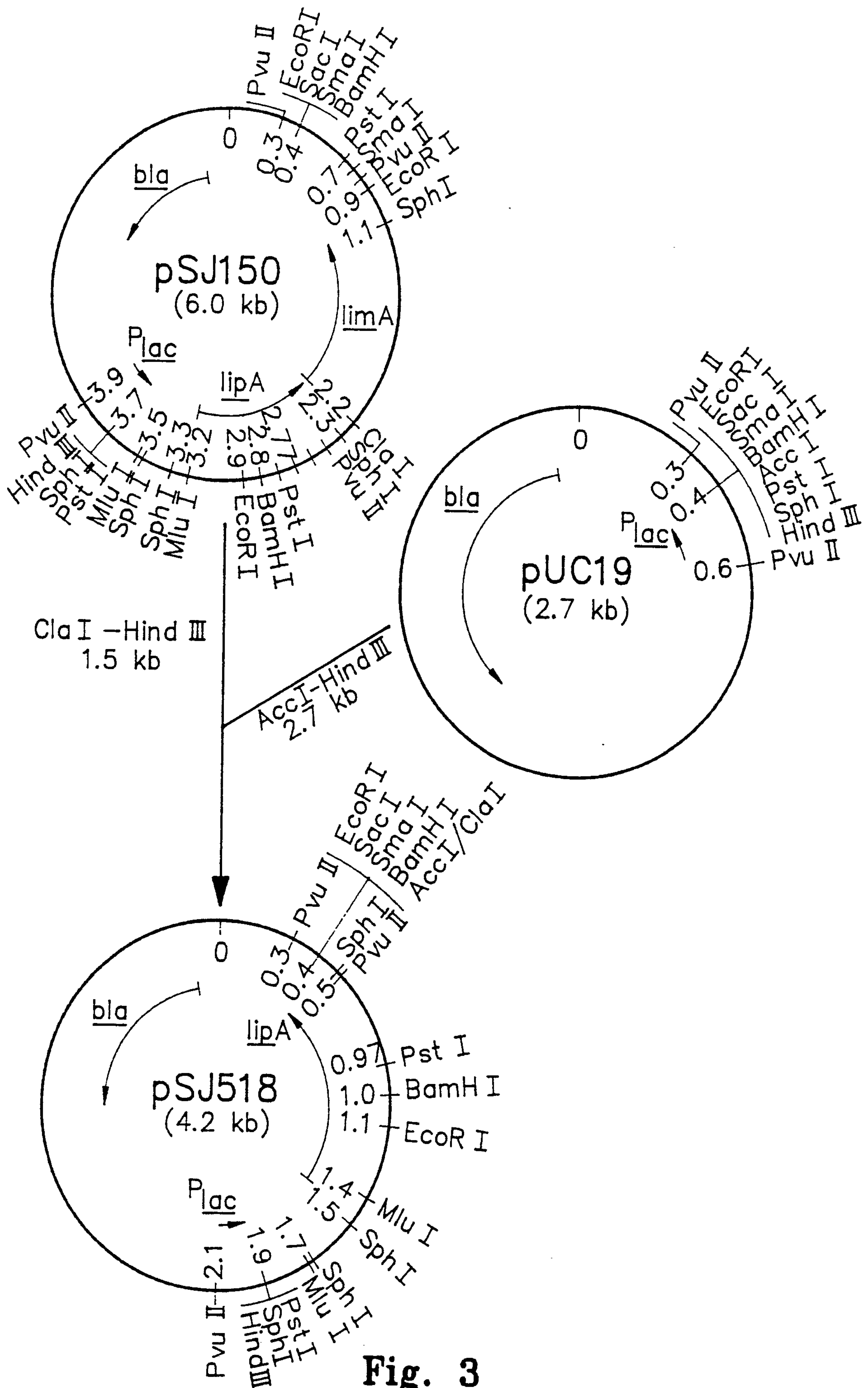

Fig. 3

Fig. 4

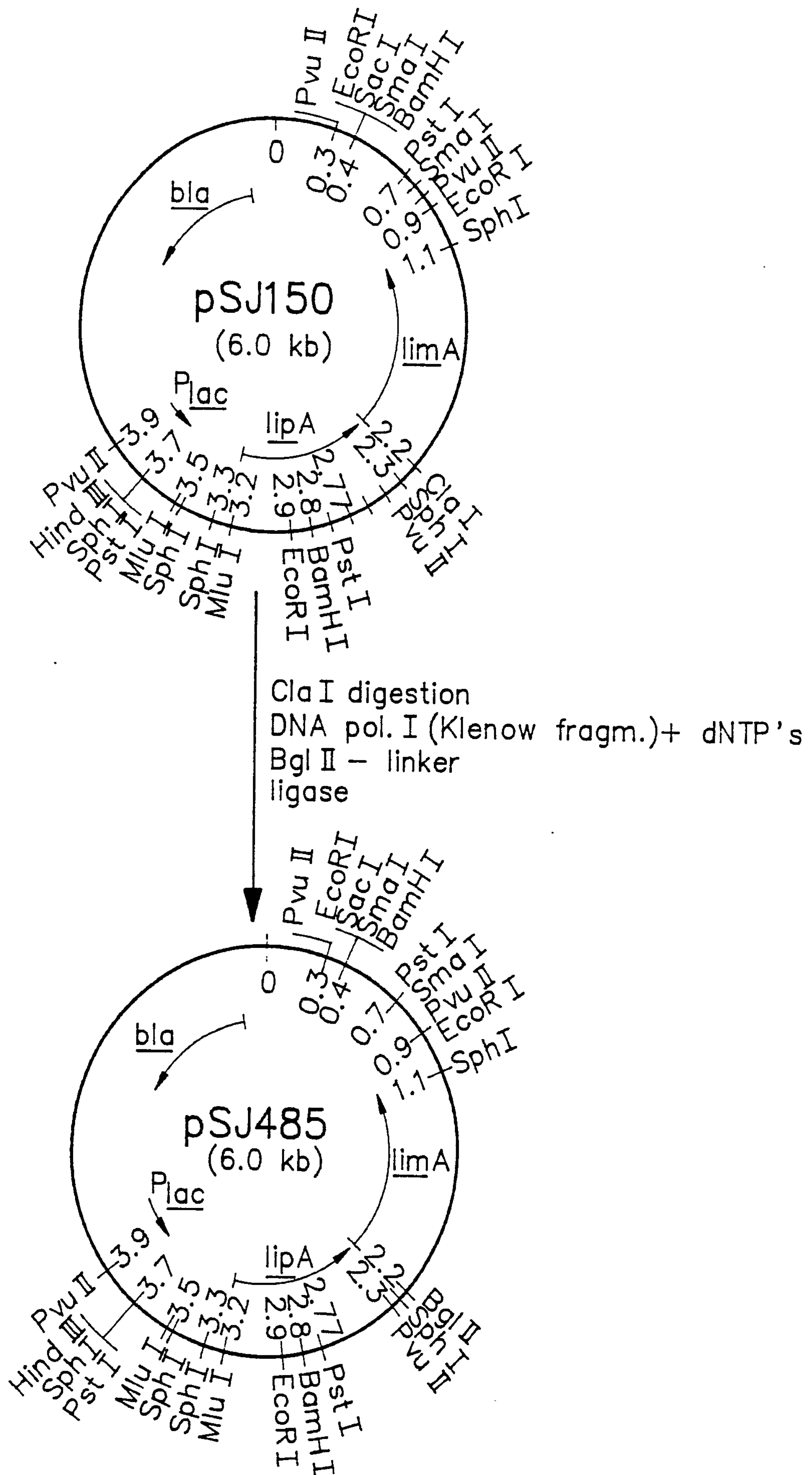

Fig. 5

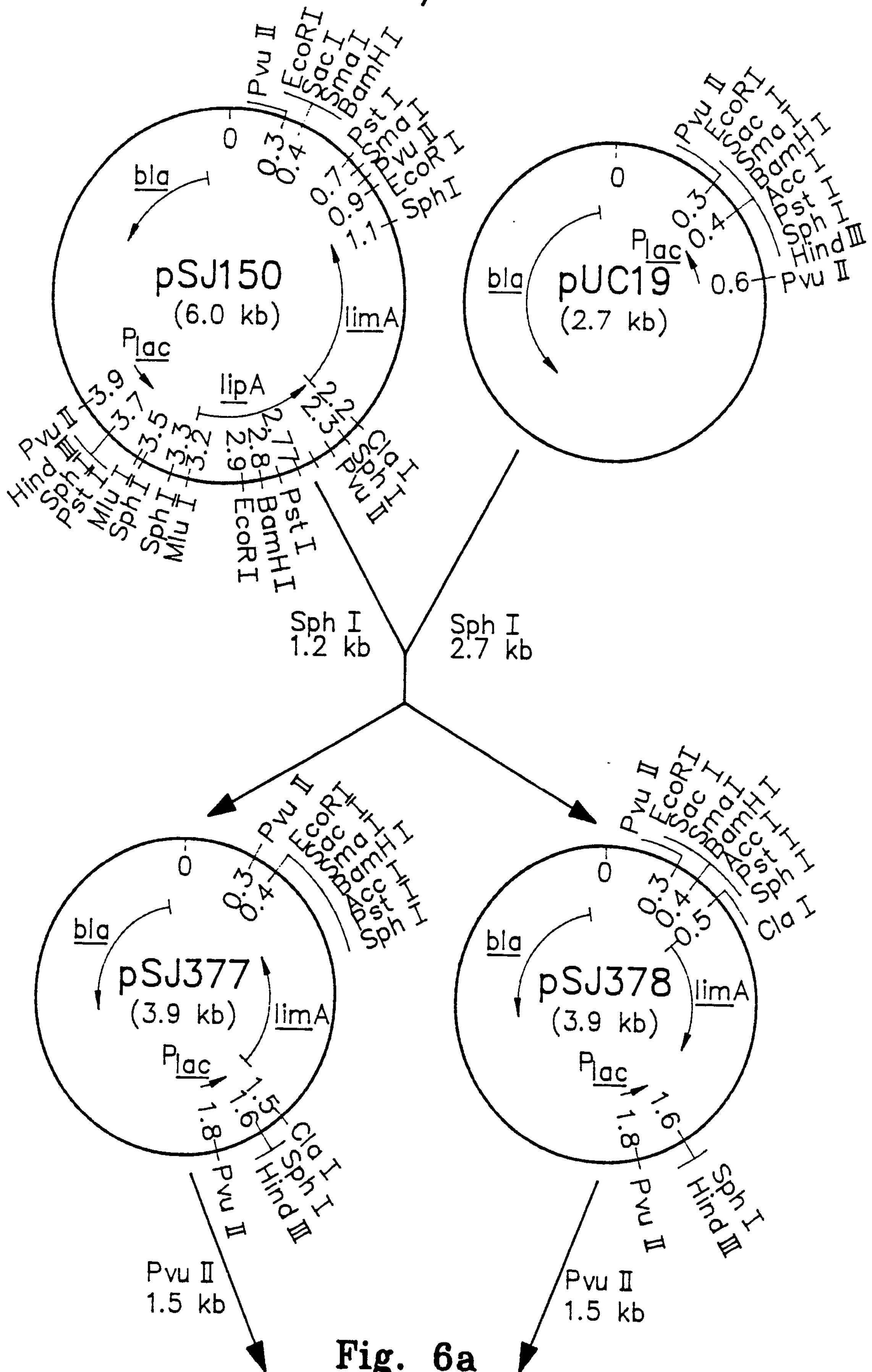

Fig. 6a

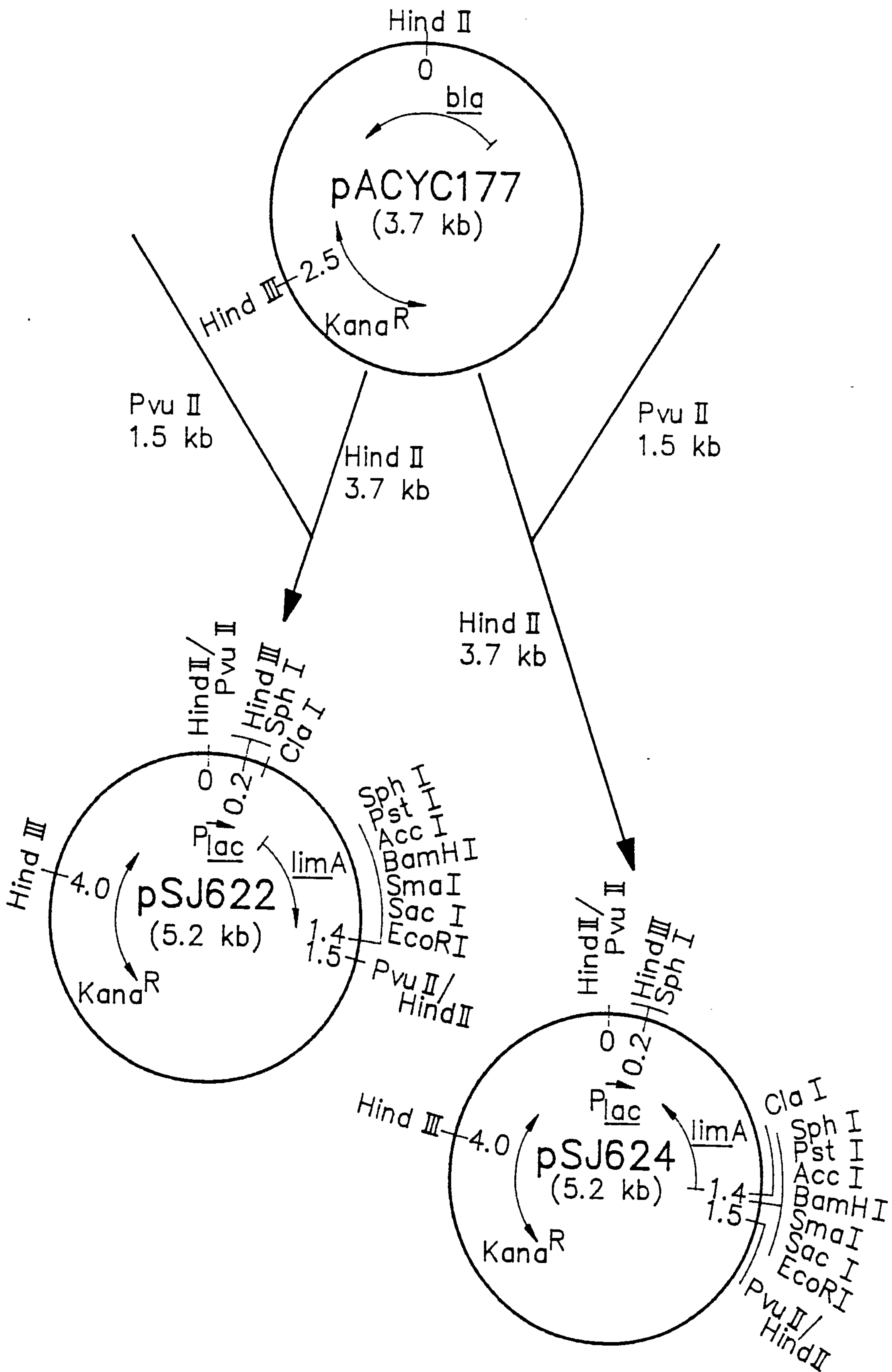

Fig. 6b

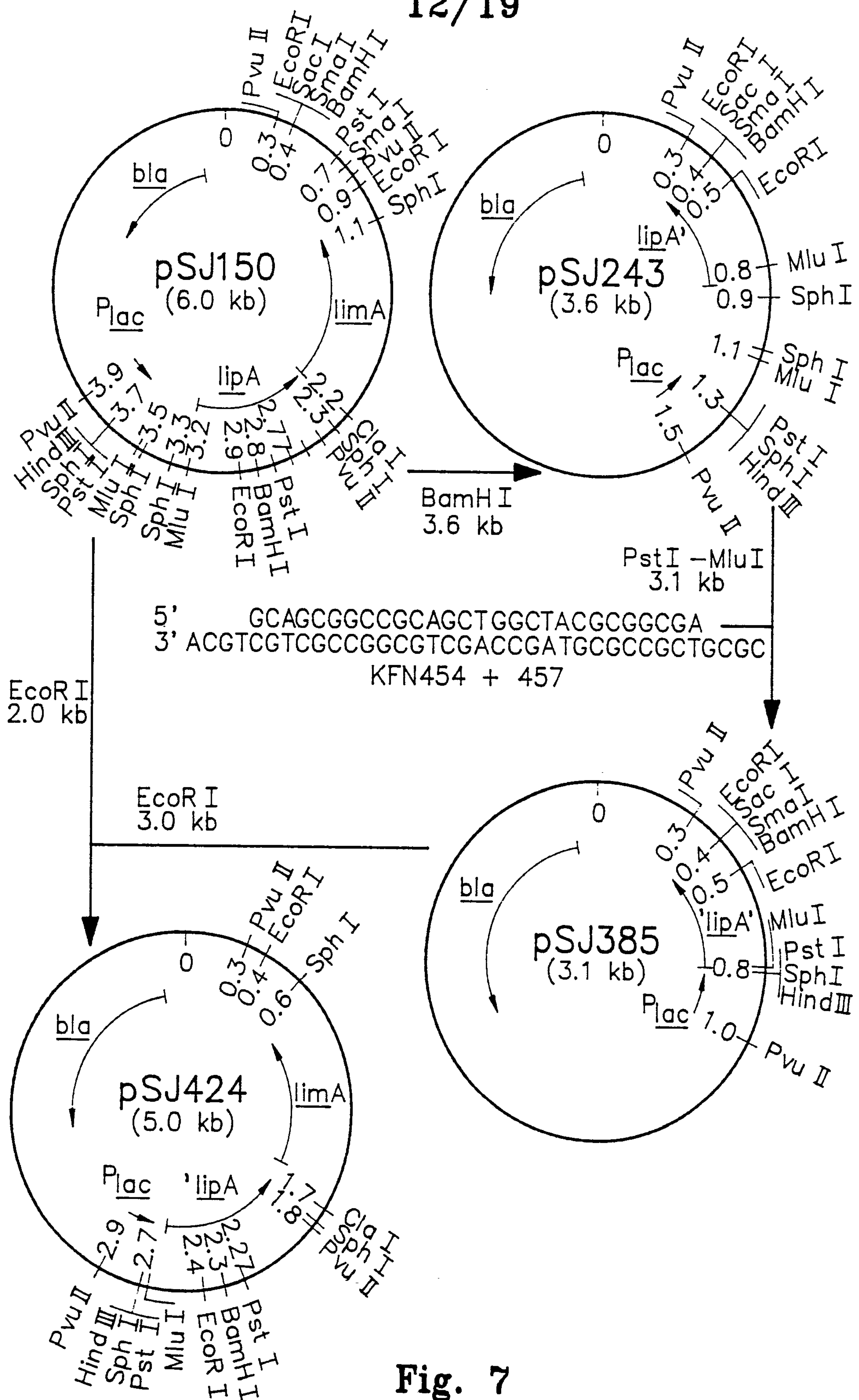

Fig. 7

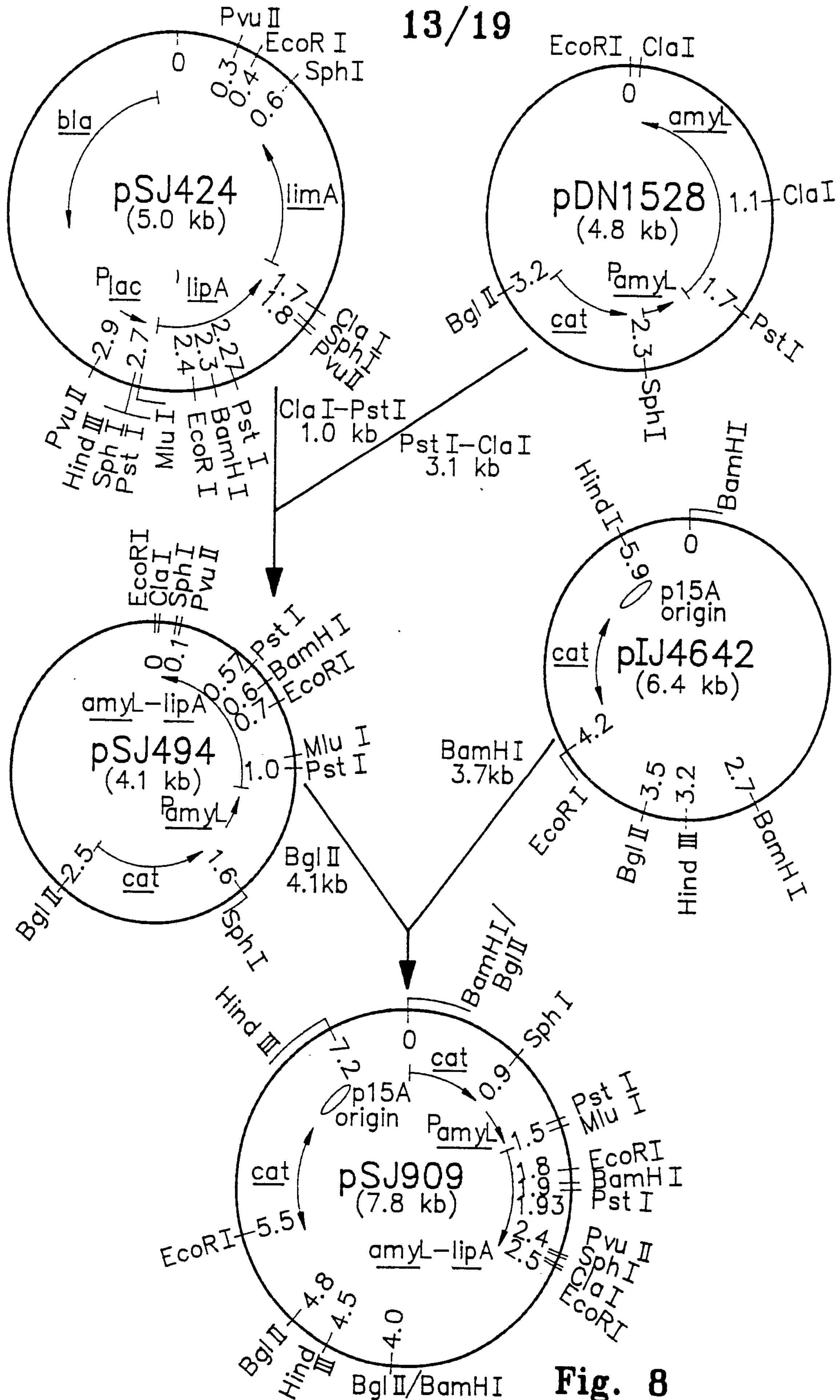

Fig. 8

FIG. 9

pSJ385 (3.1 kb)

pDN1528 (4.8 kb)

BamHI- PstI 0.4 kb

PstI-EcoRI 3.1 kb

EcoRI-BamHI 1.9 kb

pSJ150 (6.0 kb)

pSJ416 (5.4 kb)

Fig. 10

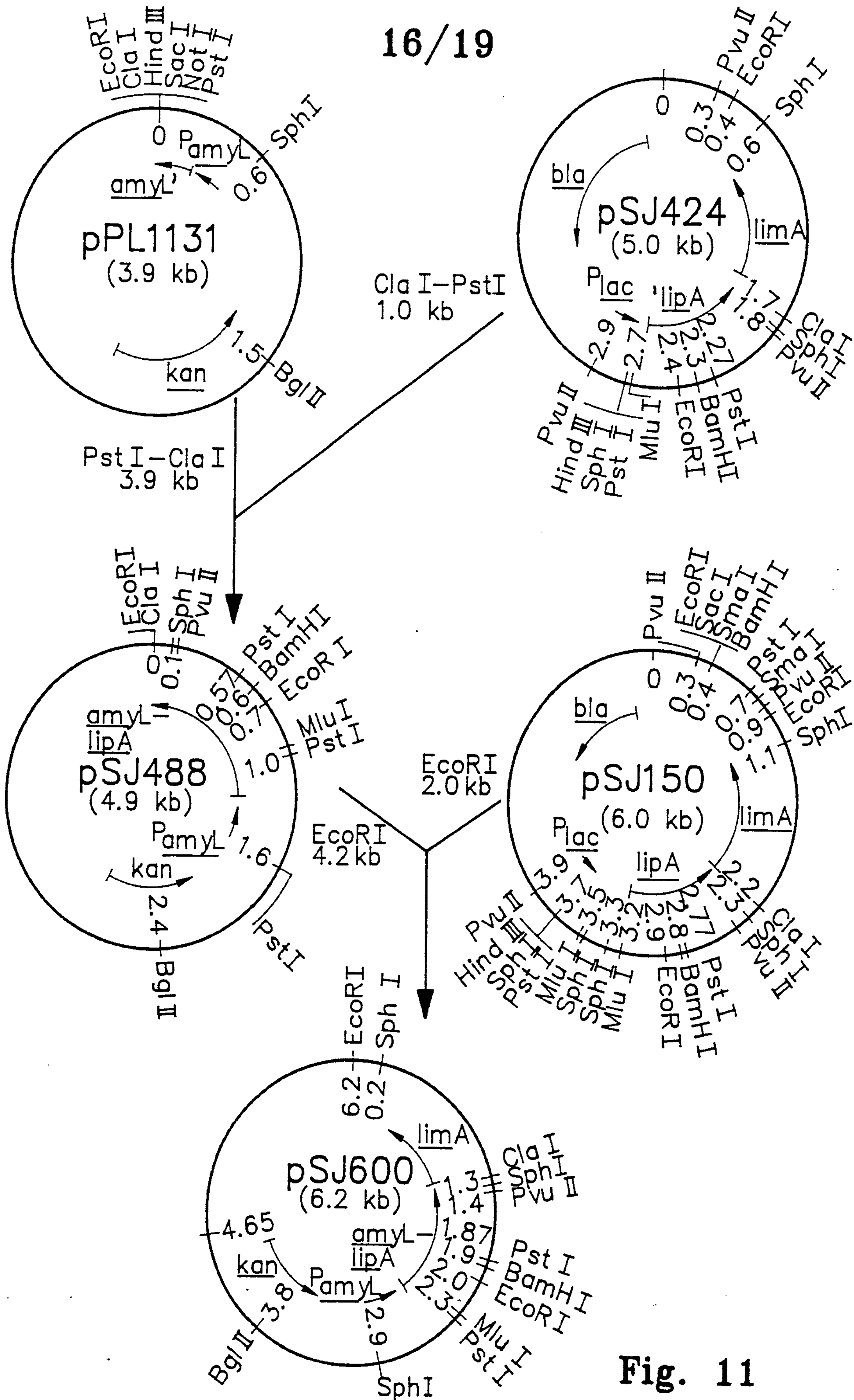

Fig. 11

Fig. 12

SacI+SmaI
Exo III
S1 nuclease
Klenow
Bgl II -linker
ligase

Hind III-Mlu I
4.7 kb

Mlu I-
Hind III
0.9 kb

Bgl II
5.6kb

Fig. 13a

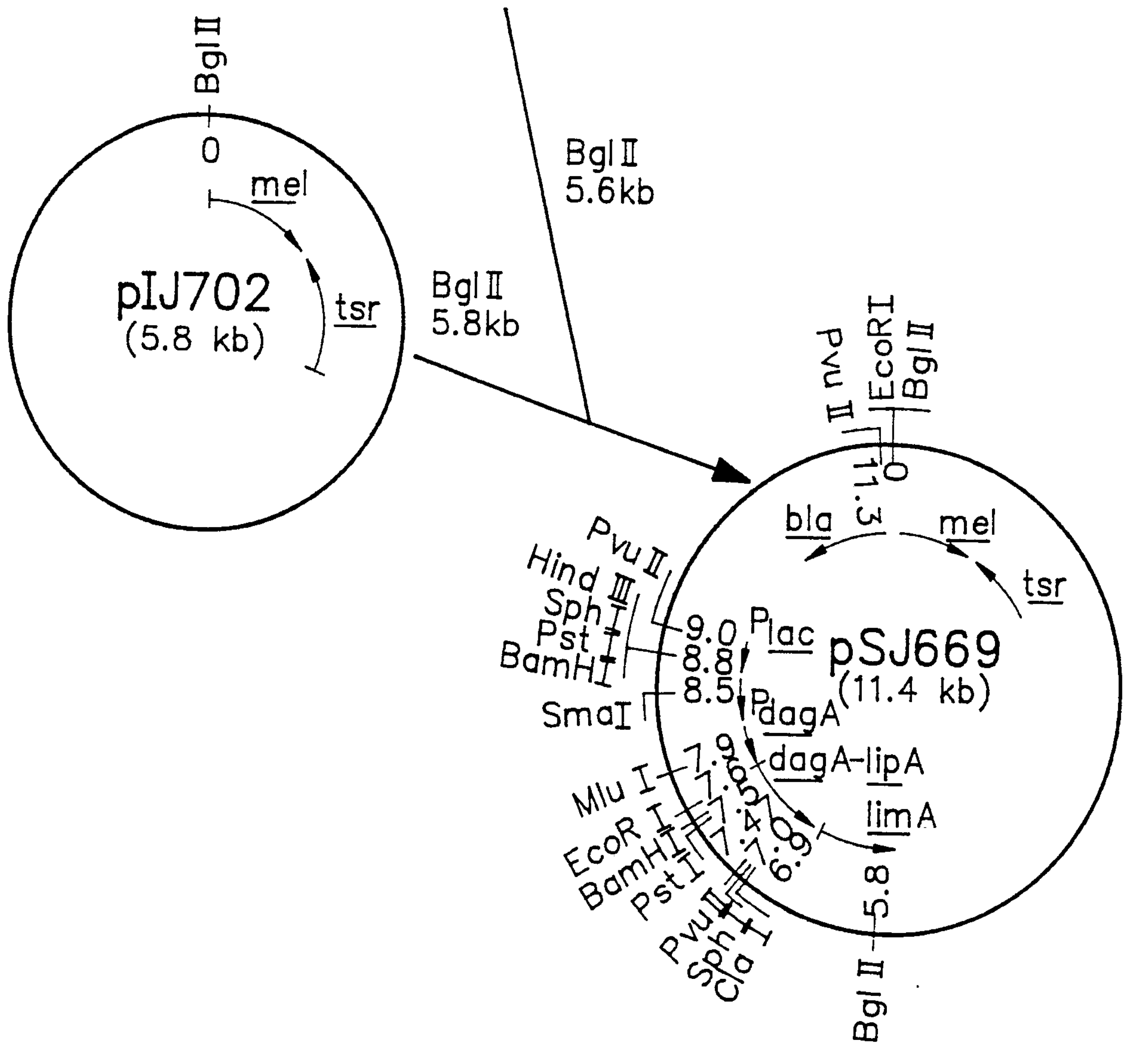

Fig. 13b

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 90/00170

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 N 15/31, 15/55, 9/20

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	EP, A2, 0331376 (AMANO PHARMACEUTICAL CO., LTD) 6 September 1989, see the whole document --	1-36
A	EP, A2, 0318775 (CHISSO CORPORATION) 7 June 1989, see the whole document --	1-36
A	WO, A1, 8901032 (NOVO INDUSTRI A/S) 9 February 1989, see the whole document --	1-36
A	EP, A2, 0214761 (NOVO INDUSTRI A/S) 18 March 1987, see the whole document --	1-36

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* Special categories of cited documents: [10]

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"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
8th October 1990	1990 -10- 12
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Mikael G:son Bergstrand

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 90/00170

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The members are as contained in the Swedish Patent Office EDP file on 90-08-28
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A2- 0331376	89-09-06	NONE		
EP-A2- 0318775	89-06-07	JP-A-	1291789	89-11-24
		JP-A-	2039890	90-02-08
WO-A1- 8901032	89-02-09	EP-A-	0382738	90-08-22
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		US-A-	4876024	89-10-24