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(54) **PREPARATION D'ACIDES AMINES D PAR DES MATERIAUX ET DES PROCEDES DE FERMENTATION DIRECTE**

(54) **PREPARATION OF D-AMINO ACIDS BY DIRECT FERMENTATIVE MEANS**

(57) L'invention concerne des matériaux et des procédés, qui sont destinés à produire des acides aminés D naturels et artificiels. L'invention concerne en particulier un procédé de fermentation pour la production d'acides aminés D au moyen de cellules hôtes de recombinaison. L'invention se rapporte spécifiquement à un procédé de production d'acide aminé D dans une cellule, qui consiste à: (a) incorporer à la cellule un gène d'aminotransférase D et un gène d'aminotransférase L; (b) cultiver ladite cellule dans un milieu de culture cellulaire; et (c) isoler l'acide aminé D dudit milieu de culture cellulaire. L'invention a également pour objet un procédé de production de phénylalanine D dans une cellule, qui consiste à: (a) incorporer à la cellule un gène d'aminotransférase D, un gène d'aminotransférase L et un moyen servant à accroître la production de phénylpyruvate; (b) cultiver ladite cellule dans un milieu de culture cellulaire; et (c) isoler la phénylalanine D dudit milieu de culture cellulaire. L'invention concerne enfin la préparation de cellules de recombinaison utilisées dans la production d'acides aminés D énantiomorphiquement purs.

(57) The present invention relates to materials and methods for production of natural and unnatural D-amino acids. In particular, the present invention relates to a fermentation method for the production of D-amino acids using recombinant host cells. Specifically, the invention relates to a method for producing a D-amino acid in a cell, comprising: (a) incorporating into the cell a D-aminotransferase gene and an L-aminodeaminase gene; (b) culturing the cell in a cell culture medium; and (c) isolating the D-amino acid from the cell culture medium.

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(57) Abstract

The present invention relates to materials and methods for production of natural and unnatural D-amino acids. In particular, the present invention relates to a fermentation method for the production of D-amino acids using recombinant host cells. Specifically, the invention relates to a method for producing a D-amino acid in a cell, comprising: (a) incorporating into the cell a D-aminotransferase gene and an L-aminodeaminase gene; (b) culturing the cell in a cell culture medium; and (c) isolating the D-amino acid from the cell culture medium.

TITLE

PREPARATION OF D-AMINO ACIDS BY DIRECT FERMENTATIVE MEANS

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates to materials and methods for the production of D-amino acids. In particular, the present invention relates to the preparation of both natural and unnatural D-amino acids using recombinant host cells. Specifically, the invention relates to a fermentation process using recombinant cells to produce enantiomerically pure D-amino acids.

BACKGROUND OF THE INVENTION

With the exceptions of glycine, threonine, and isoleucine, each of the common, naturally-occurring amino acids exist as one of two optical isomers, termed levorotatory or dextrorotatory, depending upon the direction in which they cause a plane of polarized light to rotate. Glycine, having no asymmetric carbon, has no optical isomers. Threonine and isoleucine, each having two asymmetric carbons, have four optical

isomers each. Some amino acids, such as alanine and glutamine are dextrorotatory, producing a positive (right-handed) rotation. Others, such as phenylalanine and tryptophan, are levorotatory, producing a negative (left-handed) rotation. Thus, amino acids may be referred to as l- or d-amino acids in order to reflect their chirality in isolation. Specific rotation produced by a given amino acid varies with temperature and pH.

By convention, amino acids are also referred to as D or L (as opposed to the d or l designations referred to above) based upon whether the configuration about the α-carbon of the amino acid corresponds to the D or L stereoisomer (enantiomer) of glyceraldehyde, the arbitrary standard. Based upon that standard, most naturally-occurring amino acids are L-amino acids, despite the fact that some of them are dextrorotatory (d) when placed in aqueous solution at neutral pH. Most enzymes which act upon amino acids have asymmetric binding domains which recognize only the L-form of the amino acid. Accordingly, most naturally-occurring proteins comprise L-amino acids.

There are, however, exceptions wherein D-amino acids are produced and utilized by cells. Principal among these is the production of D-glutamate and D-alanine by certain microorganisms. D-glutamate and D-alanine are primarily produced in bacterial cells and are utilized in murein synthesis. In the absence of D-glutamate and D-alanine, a defective bacterial cell wall is produced, resulting in cell lysis. Most bacteria produce D-amino acids not by direct synthesis, but through conversion of the corresponding L-amino acid by an amino acid-specific racemase. For example, many bacterial cells possess an alanine racemase which catalyzes bidirectional conversion between L-alanine and D-

alanine, resulting in a racemic (50:50) mixture of L- and D-alanine. Similarly, a glutamate racemase produces a racemic mixture of D-glutamate and L-glutamate, the former for incorporation into the cell wall and the latter for, *inter alia*, formation of protein. The specificity of those two enzymes is demonstrated by the fact that the lack of either one results in cell lysis due to defective cell wall formation.

Certain bacteria, such as members of the genus *Bacillus*, possess an alternative to racemases for making D-amino acids in the form of an enzyme known as D-aminotransferase. Such an enzyme reversibly catalyzes the transamination of various D-amino acids and corresponding α-keto acids. In PCT Publication WO 91/05870, Manning reports a method for microbial synthesis of D-alanine and D-glutamate via catalysis by an aminotransferase. While Manning reports, at page 2, the use of a *Bacillus sphaericus* D-aminotransferase, that publication actually only reports the cloning, isolation, and use of a thermophilic species of D-aminotransferase which is not capable of effectively catalyzing synthesis of more than trace amounts of the D-amino acid. Moreover, Manning fails to report any means for isolating or using a *Bacillus sphaericus* D-aminotransferase or any other D-aminotransferase which catalyzes the synthesis of enantiomerically pure D-amino acids.

Evidence that Manning's reference to a *Bacillus sphaericus* D-aminotransferase is an error is found at page 2 of the Manning publication, wherein Manning states that the D-aminotransferase DNA was cloned onto plasmid pICT113. As reported in Stoddard, et al., *J. Mol. Biol.*, *196*: 441-442 (1987), plasmid pICT113 carries the thermophilic species of D-aminotransferase

and not the *Bacillus sphaericus* species. The significance of that fact is that the thermophilic species cannot effectively catalyze significant production of a D-phenylalanine and, therefore, is useless in recombinant methods for production of a D-phenylalanine acid.

Prior to the present application, the only report of a *Bacillus sphaericus* D-aminotransferase is a partial C-terminal sequence found in *Transaminases*, Christen, et al., (eds.), 464 (1985). However, as will be apparent from the present invention that partial sequence is wrong and is not useful in isolating the *Bacillus sphaericus* D-aminotransferase. Accordingly, no prior reference reports a *Bacillus sphaericus* D-aminotransferase in the production, by recombinant means or otherwise, of a D-amino acid. Other D-aminotransferases have been isolated but, unlike that produced by the *Bacillus sphaericus* species, D-phenylalanine is a relatively poor substrate for those enzymes. Tanizawa et al., *J. Biol. Chem.*, 264: 2445-2449 (1989).

This invention provides recombinant materials and methods for producing enantiomerically-pure natural and unnatural D-amino acids.

SUMMARY OF THE INVENTION

The present invention relates to materials and methods for production of natural and unnatural D-amino acids. In particular, the present invention relates to a fermentation method for the production of D-amino acids using recombinant host cells.

Specifically, the invention relates to a method for producing a D-amino acid in a cell, comprising:

(a) incorporating into the cell a D-aminotransferase gene and a L-aminodeaminase gene;

(b) culturing the cell in a cell culture medium; and

(c) isolating the D-amino acid from the cell culture medium.

The invention also relates to a method for producing D-phenylalanine in a cell, comprising:

(a) incorporating into the cell a D-aminotransferase gene, a L-aminodeaminase gene and means for increasing production of phenylpyruvate;

(b) culturing the cell in a cell culture medium; and

(c) isolating the D-phenylalanine from the cell culture medium.

The methods of the present invention may further comprise the step of introducing a D-aminodeaminase gene mutation into the cell such that the D-aminodeaminase gene is nonfunctional.

The invention also relates to the preparation of recombinant cells for use in the production of enantiomerically pure D-amino acids.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. is a general scheme illustrating the method of the present invention for the production of D-amino acids.

Figure 2. is a scheme illustrating the production of D-phenylalanine using the method of the present

invention. The following abbreviations are used in Figure 2, E4P is erythrose-4-phosphate, PEP is phosphoenolpyruvate, and DAHP is 3-deoxy-D-arabino-heptulosonate-7-phosphate.

Figure 3. is a schematic diagram showing construction of plasmid pIF1002.

Figure 4. is a schematic diagram showing construction of plasmid pIF1003.

Figure 5. is a schematic diagram showing construction of plasmid pIF318.

Figure 6. is a schematic diagram showing construction of plasmid pJN326.

Figure 7. is a schematic diagram showing construction of plasmid pIF319.

Figure 8. is a schematic diagram showing construction of plasmid pIF320.

Figure 9. is a schematic diagram showing construction of plasmid pIF321.

Figure 10. is a schematic diagram showing construction of plasmid pIF333.

Figure 11. is a schematic diagram showing construction of plasmid pALR18.

Figure 12. is a schematic diagram showing construction of plasmid pPT362.

Figure 13. is a schematic diagram showing construction of plasmid pPT363.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to materials and methods for the production of D-amino acids. The general method of the present invention is illustrated in Figure 1. The invention relates to a method in which a D-aminotransferase gene (*dat*) and a L-aminodeaminase gene (*lad*) are introduced into a bacterial cell. The D-aminotransferase gene product, i.e., the D-aminotransferase enzyme (Dat), catalyzes a transamination reaction between a D-amino acid substrate and a keto acid precursor. In the transamination reaction the keto acid precursor is converted to its corresponding D-amino acid and the D-amino acid substrate is converted to its keto acid form. Thus, the D-amino acid substrate serves the function of being an amino donor in the transamination reaction.

A L-aminotransferase gene product, i.e., a L-aminotransferase enzyme (Lat) is naturally present in cells. The D-aminotransferase gene product competes in the cell with the L-aminotransferases gene product for the keto acid precursor as a substrate. The L-aminotransferase enzyme catalyzes the transamination reaction between an L-amino acid substrate and the keto acid precursor to form the L-amino acid of the form of the keto acid precursor. However, if a L-aminodeaminase gene is introduced into the cell, its gene product catalyzes the deamination of any L-amino acid present in the cell to its corresponding keto acid form. The keto acid formed due to deamination of the L-amino acid provides further keto acid precursor for use as a substrate by the D-aminotransferase enzyme. Conversion of the keto acid precursor to its corresponding D-amino acid form by D-aminotransferase is irreversible as there is no D-aminodeaminase gene

present in the cell to produce a D-aminodeaminase enzyme to deaminate the D-amino acid product.

In one preferred embodiment of the present invention, genes encoding enzymes for the production of amino acid substrates and keto acid precursors may also be incorporated into the cell in order to overproduce the desired substrates that are available to the D-aminotransferase and L-aminotransferase enzymes. The genes incorporated may be racemase genes or genes that encode rate limiting enzymes involved in the biosynthesis of amino acid substrates or keto acid precursors. Alternatively, the amino acid substrates and/or the keto acid precursors may be provided as part of the culture medium for the cells during the production of the D-amino acids. In the case of the cell culture medium containing L-amino acids or racemic amino acids as the substrate, a racemase gene is preferably incorporated into the cell in order to provide an overproduction of a racemase enzyme to convert the L-amino acid added as part of the cell culture medium to D-amino acid. In addition, the presence of the L-aminodeaminase gene product will deaminate the L-amino acid present in the cell to produce its corresponding keto-acid precursor for use as a substrate by D-aminotransferase enzyme.

Cells which are suitable for use in the method of the present invention include, but are not limited to the following bacterial cells, such as *Bacillus subtilis*, *Bacillus sphaericus*, *Bacillus stearothermophilus*, *Pseudomonas*, *Klebsiella*, *Salmonella*, *Brevibacterium*, *Micrococcus*, *Corynebacterium* and *Escherichia coli*. In another preferred embodiment of the method of the present invention the cell is *Escherichia coli*.

In another preferred embodiment of the present invention, the use of *Bacillus stearothermophilus* cells have the additional advantage of being moderate thermophiles thereby allowing the preparation of D-amino acids to be performed at elevated temperatures where reaction rates are faster. Accordingly, production times for the preparation of D-amino acids may be reduced.

In one preferred embodiment an L-aminodeaminase gene from *Proteus myxofaciens* and a D-aminotransferase gene from *Bacillus sphaericus* are introduced into a cell. Both of these genes encode enzymes that have very broad substrate ranges as shown in the following Table 1. The substrates include both natural and unnatural D- and L-amino acids. In addition, the substrate range for these enzymes may be increased by mutation of the respective genes using standard mutation procedures.

Lad Substrate	**Dat Substrate**
Alanine	Pyruvic Acid
Phenylalanine	Phenylpyruvic acid
Isoleucine	alpha-ketoisocaproate
Leucine	alpha-ketoisovaleric acid
Tryptophan	Indole-3-Pyruvic acid
Tyrosine	Hydroxy phenylpyruvic acid
Valine	alpha-ketoisovaleric acid
Arginine	5-Guanidino-2-Oxovaleric Acid
Asparagine	2-Oxosuccinamic Acid
Glutamine	Not tested
Methionine	2-Oxo-4-(methylthio)butyric acid
Ornithine	5-Amino-2-Oxopentanoic acid
Serine	3-hydroxypyruvic acid
Norleucine	2-Oxohexanoic acid
Norvaline	2-Oxopentanoic acid
Dihydroxyphenyl alanine	Dihydroxyphenylpyruvic acid
Citrulline	alpha-Oxo-gamma-ureidonovaleric acid
Cysteine	Not tested
Histidine	2-Oxo-4-Imidazolepropionic acid
Lysine	6-Amino-2-Oxohexanoic acid

Table 1. Natural and unnatural amino acid substrates for Lad and Dat enzymes.

In another preferred embodiment an L-aminodeaminase gene from *Proteus mirabilis* and a D-aminotransferase gene from *Bacillus sphaericus* are introduced into a cell.

In one preferred embodiment of the present invention, the preferred host cell is an *Escherichia coli* strain pIF3. The *Escherichia coli* strain pIF3 is derived from a RY347 strain which may be obtained from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A.(ATCC Accession Number 69766). The pIF3 strain differs from RY347 in that wild copies of the L-aminotransaminase genes *typ*B+ and *ilv*E have been introduced to the chromosome cell by transduction with a bacteriophage P1 as described in Miller et al., *A Short Course in Bacterial Genetics*, Cold Spring Harbor Laboratory Press (1992), incorporated by reference herein. The *tyr*B+ and *ilv*E genes encode L-aminotransaminase enzymes that convert keto acid precursors to their corresponding L-amino acid form.

The reintroduction of the wild type aminotransaminase genes *tyr*B+ and *ilv*E into pIF3 cells has the added benefit of improving cell growth over that of RY347, presumably due to some undefined additional function of the L-aminotransaminase gene products. In particular, preferred L-aminotransaminase genes, include but are not limited to *asp*C, *tyr*B and *ilv*E.

The chromosomes of the cells used in the production of D-amino acids of the present invention may be mutated using standard techniques, as described in Miller et al., *A Short Course in Bacterial Genetics*, Cold Spring Harbor Laboratory Press (1992), incorporated by

reference herein. In one particular embodiment, a *dadA* gene mutation is introduced into the *Escherichia coli* cells such that the *dadA* gene is nonfunctional. *Escherichia coli* cells have a *dad* operon which comprises the genes *dadA* and *dadX*. The *dadX* gene encodes alanine racemase enzyme which is involved in racemizing amino acids between its D- and L-forms. The *dadA* gene encodes a D-aminodeaminase which carries out the oxidative deamination of a range of D-amino acids. The *dad* operon is induced in the presence of D-alanine and produces the D-aminodeaminase and D-alanine racemase enzymes. The DadX and DadA enzymes form a membrane complex which is involved in the uptake and catabolism of D-alanine to pyruvate. The DadA enzyme can also deaminate other D-amino acids such as D-phenylalanine. Accordingly, in *Escherichia coli* cells that are involved in the overproduction of D-amino acids, it is advantageous to mutate the *dadA* gene in order to prevent production of the DadA enzyme.

Additionally, *Escherichia coli* strains bearing mutations in L-aminotransaminase genes *aspC*, *ilvE*, *tyrB* or in the D-aminodeaminase *dadA* gene may be obtained from the *coli* Genetic Stock Center (Yale University, New Haven, CT). For example, the following *Escherichia coli* strains, DG30, DG31, DG34, and DG, having mutations in L-aminotransaminase genes *aspC*, *ilvE*, and *tyrB* and the *Escherichia coli* strain, EB105 having a mutation in the D-aminodeaminase *dadA* gene may be obtained the *coli* Genetic Stock Center.

Mutations including deletions may be introduced to the chromosome of the cell in a site directed fashion using temperature sensitive recombinant plasmids, which carry *in vitro* generated fragments of the target gene into the host cell chromosome. For example, plasmid pHSG415 disclosed in U.S. Patent No. 5,354,672 in which the

temperature sensitive nature of the plasmid replication control region can be used to identify recombinant events between the plasmid and the host cell chromosome. The deleted copy of a target gene on the plasmid may be exchanged for the wild type copy of the same gene on the cell chromosome using pHSG415. Subsequent loss of the plasmid from the host cell renders the cell mutated in the target gene. Accordingly, pHSG415 provides an effective means in which to either mutate a host cell chromosome or to reintroduce a wild type gene back into a host cell chromosome that had been mutated.

In one preferred embodiment of the present invention, a method for producing D-phenylalanine in a cell comprises, incorporating into the cell a D-aminotransferase gene and a L-aminodeaminase gene. The D-aminotransferase gene product catalyzes a transamination reaction between a D-alanine substrate and a keto acid precursor, phenylpyruvate, to produce D-phenylalanine and pyruvate. The substrates D-alanine and phenylpyruvate are normally present in the cell, the former for incorporation into the cell wall, the latter as the last precursor in the pathway leading to L-phenylalanine biosynthesis. In addition, the naturally present L-aminotransferase gene product catalyzes the transamination reaction between L-alanine and phenylpyruvate to produce L-phenylalanine and pyruvate. However, introduction of the L-aminodeaminase gene into the cell results in production of L-aminodeaminase enzyme which deaminates most of the L-phenylalanine synthesized back to phenylpyruvate while the rest of the L-phenylalanine present is used in the production of protein . The phenylpyruvate produced as a result of the deamination reaction can be utilized by the D-aminotransferase enzyme as a substrate to produce more D-phenylalanine. Production of D-phenylalanine in

the cell is irreversible because there is no D-aminodeaminase gene product present in the cell to deaminate the D-phenylalanine.

In the production of D-amino acids using the method of the present invention it is desirable to have increased levels of D-amino acid substrate for use as an amino donor in the transamination reaction. For example, in the preparation of D-phenylalanine addition of D-alanine to the cell assures sufficiently high levels of D-alanine substrate for the transamination reaction.

In a preferred embodiment of the present invention a racemic mixture of alanine is added to the cells as part of the cell culture medium during fermentation. Additionally, a cyctoplasmic alanine racemase gene (*alr*) encoding an alanine racemase enzyme is introduced into the cell. The alanine racemase enzyme maintains the 50/50 D-, L-alanine equilibrium in the cell. As the amount of D-alanine in the cell is being consumed due to the action of the D-aminotransaminase enzyme, the alanine racemase enzyme converts L-alanine to D-alanine. In this manner, all of the D-, L-alanine mixture is made available to the D-aminotransferase enzyme as D-alanine substrate for use as an amino donor in the transamination reaction, other than the small amount incorporated into the cell wall. In one preferred embodiment, the *alr* gene incorporated into the cell is cloned from *Salmonella typhimurium*.

Other suitable amino donors that may be added to cell cultures during the production of D-amino acids include L-alanine, L-glutamate, L-phenylalanine, L-aspartate or a racemic mixture one of the aforementioned L-amino acids. Preferably, a racemase gene is also incorporated into the cell, such as glutamate racemase, aspartate racemase or phenylalanine racemase depending

on the amino donor present. Accordingly, D-aminotransaminase enzyme has increased amounts of D-amino donor substrate available for use in the transamination reaction.

In order to increase the production of D-phenylalanine in the cell, the amount of the keto acid precursor, i.e., phenylpyruvate, may be increased in the cell by introducing genes that encode the rate limiting enzymes that produce phenylpyruvate. Phenylpyruvate production from the cellular aromatic amino acid biosynthetic pathway is regulated by two rate limiting enzymes, PheA and AroH. Introduction of the genes that encode PheA and AroH into the cell results in an overproduction of phenylpyruvate. Accordingly, increasing the amount of phenylpyruvate provides more substrate for the D-aminotransferase gene product to convert to D-phenylpyruvate.

The amount of the keto acid precursor in the cell may also be increased by addition of the corresponding L-amino acid to the cell. In the case of the addition, of a L-amino acid, the L-aminodeaminase enzyme deaminates the L-amino acid to form the corresponding keto acid precursor. The keto acid precursor can then be used as a substrate by the D-aminotransferase enzyme to be converted to its corresponding D-amino acid.

The present invention also relates to a recombinant cell, comprising an exogenous D-aminotransferase gene and an exogenous L-aminodeaminase gene. The recombinant cell of the present invention may further comprise a D-aminodeaminase gene mutation in the cell such that the D-aminodeaminase gene is nonfunctional. The recombinant cell of the present invention may further comprise an exogenous alanine racemase gene, an exogenous *aroH* gene and an exogenous *pheA* gene. The

exogenous D-aminotransferase gene may be a *Bacillus sphaericus* D-aminotransferase gene, the exogenous L-aminodeaminase gene may be a *Proteus myxofaciens* L-aminodeaminase gene or a *Proteus mirabilis* L-aminodeaminase gene and the exogenous racemase gene may be a *Salmonella typhimurium* racemase gene.

Cultures of recombinant cells of the present invention are used to produce enantiomerically pure D-amino acids. The percentage enantiomeric excess (ee) of a D-amino acid over an L-amino acid produced using the disclosed method may be determined by subtracting the amount of L-amino acid present from that of the D-amino acid present, dividing by the total amount of D-, and L- amino acid and multiplying by 100. In a preferred embodiment, D-phenylalanine is produced in substantially pure form and in high yields. The method of production of D-phenylalanine is illustrated in Figure 2.

Using cultures of recombinant cells of the present invention with the addition of D-, L-alanine and L-phenylalanine as additional sources of D-alanine and phenylpyruvate substrates for the D-aminotransferase gene product resulted in the production of 13.66 g/l of D-phenylalanine and 0.47 g/l of L-phenylalanine, a 94% enantiomeric excess. In the case where only D-, L-alanine was added to the cultures during the fermentation process resulted in the production of 4.15 g/l of D-phenylalanine and no L-phenylalanine, a 100% enantiomeric excess. In contrast, when no D-, L-alanine or L-phenylalanine was added to the cell cultures during the fermentation process, 1.12 g/l of D-phenylalanine and 0.47 g/l of L-phenylalanine is produced, a 41% enantiomeric excess.

The D-amino acids produced according to the method of the present invention may be isolated using procedures well-known to those skilled in the art. For example, one method of isolating the D-amino acids prepared using the disclosed method is as follows. On completion of fermentation, the fermentation broth is decanted from the cells. The broth may be reduced in volume to increase the concentration of the D-amino acid product. The reduction of the broth is typically carried out by heating the broth to temperatures of between 30°C to 100°C under a vacuum. The D-amino acid is then precipitated by adjusting the pH of the broth to a range of +/-1°C from the isoelectric point of the amino acid product. During the pH adjustment the D-amino acid product will precipitate. Following, precipitation the D-amino acid is separated from the broth by standard methods, which may include filtration, centrifugation or decanting. The isolated D-amino acid product is then washed and dried.

In *Escherichia coli*, the amino acids alanine, aspartic acid, glutamic acid, phenylalanine, tyrosine, valine, leucine and isoleucine are synthesized directly from their keto acid precursors. In addition to adding either L-amino acids or racemic mixtures to the recombinant cells during fermentation, the keto acid precursor of a desired amino acid may be overproduced by the introduction of genes that produce the rate limiting enzymes for a particular keto acid.

The following examples are provided to more specifically set forth and detail particular embodiments of practicing the present invention. They are for illustrative purposes only and it is recognized that minor changes and alterations can be made to the starting materials and/or the process parameters. To the extent that any such changes do not materially

alter the process or final end product they are deemed as falling within the spirit and scope of the present invention as recited by the claims that follow.

Example 1
ISOLATION OF D-AMINOTRANSFERASE DNA

Cultures of *Bacillus sphaericus*, were obtained from the American Type Culture Collection, ATCC, (ATCC Accession No. 10208), as a source of D-aminotransferase DNA. Cultures were streaked on unsupplemented LB medium and allowed to grow overnight at 37°C. In order to prepare chromosomal DNA, a single colony was used to inoculate 50 ml Luria Broth in a 1 L flask which was shaken overnight at 300 rpm and 37°C. Cells were then harvested by centrifugation at 10,000 G for 5 minutes, washed in 0.85% saline and centrifuged again at 10,000 G for 5 minutes. The resulting pellet was re-suspended in 5 ml of 10 mM glucose, 25 mM Tris HCl, pH 8.0, and 10 mM ethylenediamine tetraacetic acid (EDTA). An aliquot of 50 µl RNase A was added and the solution was mixed gently. Subsequently, 10 ml of 0.4% sodium dodecyl sulphate (SDS) and 100 µg/ml protease K were added to the mixed solution which was then incubated at 37°C until clear. Sodium acetate, pH 5.2, was then added to a final concentration of 300 mM. Gentle phenol extractions were carried out using a volume of phenol approximately equal to the aqueous phase until no white precipitate was visible at the phase interface. The aqueous phase was then removed and the chromosomal DNA was precipitated using 2.5 volumes of ethanol. The DNA pellet was removed and re-solubilized in 300 mM sodium acetate, pH 5.2. Ethanol precipitation was carried out and the DNA pellet was removed, dried and dissolved in 2 ml distilled water. The DNA concentration was determined to be 150 µg/ml. In addition to the procedure described above, standard

procedures are known for the isolation of bacterial DNA and are reported, for example, in *Current Protocols in Molecular Biology*, 2.4.1-2.4.5 (Ausubel, et al., eds., 1994), incorporated by reference herein.

The chromosomal DNA obtained as described above was then partially digested with *MboI*. Ideal digestion, yielding fragments in the range of 2-10 kb, was obtained using 13 μg chromosomal DNA and digesting for 40 minutes with 2.5 *MboI* (New England Biolabs, Beverly, MA). Approximately 13 μg chromosomal DNA prepared as indicated above was partially digested with 2.5 U of *MboI* in a total volume of 100 μl at 37°C in Biolabs *MboI* buffer. Samples of 17 μl were taken at 5, 10, 20, 30, 40 minutes and a sample of 15 μl was taken at 50 minutes. All samples were heated to 65°C in order to destroy any restriction enzyme present in the sample which was then placed on ice. A 5 μl aliquot of each sample was electrophoresed on a 0.8% agarose gel using TBE buffer as described in *Sambrook, et al. (eds.), Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press): 6.3-6.32 (1989), incorporated by reference herein. From the electrophoresis data, it was determined that the sample taken at 40 minutes contained the majority of the DNA in the 2-10 kb size range and it was those fragments which were used to construct a library in plasmid pIF306 for expression of the D-aminotransferase.

Plasmid pIF306 was derived from pBR322 (New England Biolabs, Beverly, MA). In order to construct pIF306, a modified *phe*A promoter was inserted between unique *Hind*III and *Sph*I sites on pBR322. Within the *Hind*III to *Sph*I insert there exists unique *BamHI* and *Bg1II* sites. The modified *phe*A promoter was derived from that characterized in co-owned U.S. Patent No. 5,120,837 to Fotheringham et al. which is incorporated

by reference herein, such that the sequence was as follows:

HindIII -35 -10

AAGCTTTTTTGTTGACAGCGTGAAAACAGTACGGGTATAATACT

BamHI Start

AAAGTCACAAGGAGGATCCACTATGACATCGGAAAACCCGTTACT

HaeII

GGCGCT (SEQ ID NO: 1).

Vector DNA was prepared by digesting pIF306 to completion with *BamHI* and *Bg1II*, each of which produces ends compatible with those produced by *MboI*. The digest was carried out at 37°C in a total volume of 20 µl for 2 hours using 0.5 µg of plasmid DNA and 2 units of each enzyme. Fragments of 4.25 kb and 1.25 kb were produced and separated by electrophoresis on a 1% agarose TBE gel. The desired 4.25 kb fragment was excised from the gel and recovered using a Gel Extraction Kit (Qiagen Inc., Chatsworth, CA). That fragment was then treated with calf intestinal phosphatase (New England Biolabs, Beverly, MA) at 37°C for 1 hour in a volume of 20 µl with 1 unit of enzyme in Biolabs buffer #2 according to the manufacturer's instructions in order to dephosphorylate the ends of the DNA and to prevent re-circularization. The mixture was then treated with a PCR purification kit (Qiagen) in order to isolate the DNA fragment free of enzyme.

The pIF306 vector fragment was ligated to the fragments from the 40 minute partial digest (see above) of ATCC 10208 chromosomal DNA by combining approximately 20 ng of vector fragment with the remaining approximately 12µl of the 40 minute partial digest. Ligation was accomplished using a Takara Ligation Kit (Takara Biochemicals, PanVera Corporation, Madison, WI) according to the manufacturer's instructions. The ligation was carried out at 17°C for 2 hours, at which

time the DNA was recovered using a PCR purification kit (Qiagen) in a final volume of 50 μl. The resulting plasmids were introduced into *Escherichia coli*, XL1-Blue (Stratagene, La Jolla, CA) by electroporation using a Bio-Rad Gene Pulser™ set to 2.5 kv with 25 μF capacitance and a Bio-Rad pulse controller set to 200 ohms resistance.

Transformants were plated on LB medium supplemented with 50 μg/ml ampicillin. Approximately 20,000 transformants were produced and pooled. Plasmid DNA was then isolated as reported in *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., eds. 2d ed. 1989), incorporated by reference herein. The resulting plasmid DNA was incorporated into *Escherichia coli*, strain WM335 by electroporation using a Bio-Rad Gene Pulser™ set to 2.5 kv with 25 μF capacitance and a Bio-Rad pulse controller set to 200 ohms resistance. Strain WM335 may be obtained from the Phabagen Collection, Department of Molecular Cell Biology, State University of Utrecht, The Netherlands and was reported in Lugtenberg, et al., *J. Bacteriol.*, 114: 499-506 (1973), incorporated by reference herein. Cells were pulsed in BioRad Gene Pulser™ cuvettes with a 0.2 cm gap. *Escherichia coli* cells to be transformed were grown (50 ml cultures) to an optical density of 0.7 at 600 nm. The cells were then recovered by centrifugation at 10,000 G for 5 minutes and washed in 30 ml deionized distilled water. The cells were re-spun and re-suspended in 200 μl deionized distilled water and 40 μl of cells were combined with 10 μl of the recovered ligation mix and placed in an electroporation cuvette. A single pulse was applied to the cuvette and 500 μl SOC medium (GIBCO/BRL, Gaithersburg, MD) was added and mixed with the cell suspension. The contents of the cuvette were then transferred to a 20 ml pvc tube and incubated for 30

minutes at 37°C. Cells were then plated on appropriate media and selected as described below. Numerous medium for transforming/transfecting DNA into microorganisms are known and may be used in methods according to the invention. *See, e.g., Chang, et al. (eds.), Guide to Electroporation and Electrofusion* (Academic Press, 1992).

Transformants were plated on LB medium supplemented with 50 μg/ml thymine and 60 μg/ml ampicillin but lacking D-glutamate. Only those transformants able to make D-glutamate survive on that medium. According to reports in the literature, all such cells should have necessarily been transformants carrying the *dat* gene of *Bacillus sphaericus* because *Bacillus sphaericus* was thought to lack a glutamate racemase. However, two different classes of transformants were isolated by the procedure described above, one carrying the *dat* gene and the other carrying a glutamate racemase. The racemase-containing clone was designated pIF1001 and the *dat*-containing clone was designated pIF1002. Figure 3 is a schematic diagram showing construction of pIF1002.

In each case, the clones were mapped by restriction endonuclease digestion and the genes were sequenced. The sequence of the *dat* gene and the deduced amino acid sequence of the encoded protein are shown in SEQ ID NOS: 2 and 3. It was found that the *dat* gene had a high degree of sequence homology with the only other known *dat* gene sequence. *See Tanizawa, et al., J. Biol. Chem.*, 264: 2450-2454 (1989). However, the C-terminal amino acid sequence of the D-aminotransferase encoded by the *Bacillus sphaericus dat* gene in pIF1002 did not agree with that of the only other published report of a *Bacillus sphaericus* D-aminotransferase in which only a C-terminal sequence was published. That

sequence, reported in *Transaminases*, Christen, *et al.* (eds.), 464 (1995) was Val-Ile-(Phe-Tyr)-Leu-Ala-Leu (SEQ ID NO: 4). In contrast, the correct C-terminal sequence as provided in the present invention is Leu-Pro-Ile-Ser-Ile-Asn-Ala (SEQ ID NO: 5). It was attempted, without success, to use the sequence reported in Christen in order to isolate a *Bacillus sphaericus* D-aminotransferase-encoding gene.

Both clones were then subjected to a biological assay for the presence of the *dat* gene. That assay was reported in *Methods in Enzymology*, 113: 108-113 (19), incorporated by reference herein. Briefly, cultures of pIF1001 or pIF1002 in WM335 cells were set up in 50 ml of LB medium supplemented with 50 μg/ml thymine and 200 μg/ml ampicillin. The cultures were grown overnight in 500 ml flasks in a shaking incubator at 37°C. Cells were harvested by centrifugation at 10,000 G for 5 minutes and washed in 50 mM potassium phosphate at pH 8.5. The cells were re-spun and taken up in 1 ml 50 mM potassium phosphate at pH 8.5. The cells were then lysed using a French Pressure Cell at 1000 lbs/in^2 and the lysates were centrifuged at 14,000 G in a microfuge for 30 minutes, at which time supernatant was extracted by micropipette. The resulting cell extracts were assayed using the lactatedehydrogenase-coupled assay as reported in *Methods in Enzymology*, 113: 108-113 (19), incorporated by reference herein. The assay mixture contained 0.3 M potassium phosphate, pH 8.5, 25 mM D-alanine, 25 mM α-keto-glutarate, 0.1 mM NADH, 70 μg/ml lactate dehydrogenase and 50 μl cell extract. The reaction was started by addition of the NADH and lactate dehydrogenase to the other components in a 1 ml cuvette at 25°C. The reaction produced a change in absorbance at 338 nm as evidence of oxidation of NADH. To correct for non-specific oxidation, control assays were run using an assay mixture lacking cell extract.

As an additional control, assays were run using an assay mixture lacking D-alanine. Extracts of untransformed WM335 cells and controls produced essentially identical changes in absorbance; whereas WM335 cells bearing pIF1002 showed changes in absorbance in excess of 30-fold greater than controls. The *dat*-containing clone had levels of activity about 100-fold greater than extracts of *Bacillus sphaericus*, a consequence of overexpression on the high copy number plasmid, pIF306. Plasmid pIF1001 had activity identical to that of the controls.

Example 2
CONSTRUCTION OF PLASMID pIF1003

Plasmid pIF1003 was a derivative of pIF1002 which carries the partition (Par) locus of plasmid pLG338 (Stoker et al., Gene 18:355-341 (1982)). The partition locus of plasmid pLG338 (Stoker et al., Gene 18:355-341 (1982). The partition locus controls plasmid partitioning during cell division and in doing so confers increased segregational stability on plasmid vectors. It is useful in reducing or eliminating the need for antibiotic selection in plasmid maintenance. The partition locus can be isolated from pLG338 using PCR with the oligonucleotide primers:

5'GCCATCTCCTTGCATGCACCATTCC 3' (SEQ ID NO: 6)

5'CCCTCGCAAGCTCGTCCGGAGGCAAATCGCTGAATATTCC 3' (SEQ ID NO: 7)

The resulting 992bp fragment was then digested with the restriction enzymes *Sph*I and *BspE*I (New England Biolabs, Beverly, MA) and the resulting 965bp, *Sph*I to BspEI fragment was isolated using a QIAquick gel extraction kit (QIAGEN) following electrophoresis on a

1% agarose TBE gel. This fragment was then ligated to the 5.8kB DNA fragment produced by BspEI cleavage and partial *Sph*I cleavage of pIF1002 to generate pIF1003. Figure 4 is a schematic diagram showing construction of pIF1003.

Example 3
CONSTRUCTION OF PLASMID pIF321

In order to construct a vector which enables production of D-phenylalanine in a host cell, the *dat* gene was isolated from pIF1002 using PCR. Amplification of the *dat*-encoding region was accomplished using an Amplitaq™ PCR Kit (Perkin-Elmer, Norwalk, CT) in a 0.2 ml MicroAmp™ reaction tube (Perkin-Elmer, Norwalk, CT) to which was added 100 ng pIF1002 DNA (1 μl); 5 μl each of primers,

MB1809 5' CGCAGATCTACTATGGCATACTCATTATGG 3' (SEQ ID NO: 8); and

MB1810 5' CATGCCATGGATCCTCCTTTTAGGTAGCTCTTTTTAATC 3' (SEQ ID NO: 9)

at a concentration of 10 nanomoles/ml each; 2 μl each of dATP, dCTP, dTTP, and dGTP (10 mM each); 10 μl buffer comprising 15 mM $MgCl_2$, 500 mM KCl_2, 100 mM Tris (pH 8.3), and 0.01% gelatin; a Taq DNA polymerase (0.5 μl at 5 u/μl, Amplitaq™); and distilled water to a total volume of 100 μl. The tube was capped and placed in a Perkin Elmer 9600 Thermal Cycler. Amplification was carried out by pre-heating at 94°C for 3 minutes, followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 90 seconds. The reaction mixture was stored at 4°C.

The resulting approximately 914 bp PCR product was digested with *Bgl*II and *Nco*I and the product was then ligated into the 4.5 kb *BamH*I to *Nco*I fragment of pIF306 using a Ligation Kit (Takara Biochemicals) according to the manufacturer's instructions. The resulting plasmid was designated pIF318. Construction of pIF318 is shown in Figure 5.

The pIF319 plasmid was based upon the pLG338 plasmid disclosed in co-owned U.S. Patent No. 5,354,672, incorporated by reference herein, with the kanamycin resistance marker replaced by a chloramphenicol resistance marker to avoid conflict with a potential host strain, *Escherichia coli* HW857, which carries a kanamycin resistance gene. Plasmid pIF319 contains the *phe*A34 gene, as disclosed in co-owned U.S. Patent 5,120,837, incorporated by reference herein, and the *aro*H gene in a synthetic operon between unique *Eco*RI and *Sal*I sites in pLG338. The *phe*A34 allele contains a modification in the *phe*A coding sequence which substantially reduces phenylalanine-mediated feedback inhibition of the enzyme. It also contains a deregulated version of the *phe*A promoter region which lacks the attenuator sequence and allows increased expression of associated genes. The presence of *phe*A34 and *aro*H effectively deregulate pathways to phenylpyruvate in *Escherichia coli* W3110 and in any *Escherichia coli*, K12 strain. Plasmid pIF319 may also be derived from pJN307, disclosed in U.S. Patent No. 5,120,837, by introduction of the *Escherichia coli aro*H gene between unique *Bam*HI and *Sal*I sites in pJN307 followed by introduction of the *Escherichia coli asp*C promoter into the *Bam*HI site. The *aro*H gene was isolated from the *Escherichia coli* W3110 by PCR using primers 5'CGCGGATCCTCGTCATGAACAGAACTGACGAACTCCG 3' (SEQ ID NO: 10) and 5' ACGCGTCGACTCAGAAGCGGGTATCTACCGCAGAGG 3' (SEQ ID NO: 11). The resulting PCR fragment was

cleaved with *Bam*HI and *Sal*I and ligated to the 8 kb fragment generated by similar cleavage of pJN307. The *asp*C promoter region was then inserted at the unique *Bam*HI site in the resulting intermediate plasmid. The *asp*C promoter region was isolated from *Escherichia coli* W3110 by PCR using primers 5' GGAAGATCTTACATCATCAACCAGATCGATTCTG 3' (SEQ ID NO: 12) and 5' CGCGGATCCATTATGGTTACAGAAGGGAAGTCC 3' (SEQ ID NO: 13). The resulting approximately 278 bp fragment was then cleaved with *Bgl*II and *Bam*HI and ligated to the vector cleaved at a unique *Bam*HI site. The resulting ligation results in a DNA sequence that cannot be cleaved with *Bgl*II and only singly with *Bam*HI and, therefore, provides a simple means for verification of the orientation of the *asp*C promoter. The resulting construction is pJN326. Construction of pJN326 is shown in Figure 6. Plasmid pJN319 was generated from pJN326 by deletion of most (520 bp) of the kanamycin resistance gene by cleavage with *Hind*III and *Xho*I and insertion of a DNA fragment encoding the chloramphenicol resistance gene of pHSG415. The chloramphenicol resistance gene of pHSG415 was isolated by PCR using the primers 5'CCGCTCGAGCCCGACGCACTTTGCGCCGA 3' (SEQ ID NO: 14) and 5' CCCAAGCTTATCAGGCTCTGGGAGGCAG 3' (SEQ ID NO: 15). The resulting approximately 1191 bp fragment was cleaved with *Hind*III and *Xho*I and ligated to the 8.87 kb fragment generated by similar cleavage of pJN326. The resulting plasmid is pIF319. Construction of pJN319 is shown in Figure 7.

The pIF318 plasmid was cleaved with *Bam*HI and *Sph*I for the insertion of a *dadX* gene in order to construct the pIF320 plasmid. The MB1810 primer referred to above contains a *Bam*HI site (GGATCC) which overlaps the *Nco*I site in that primer. It is the *Bam*HI site (and the downstream *Sph*I site) that was used for introduction of

dadX to form a synthetic operon comprising *dat* and *dadX*. The *dadX* gene sequence was obtained from the Genbank database, reference code ECODADAX. From that sequence, PCR primers

MB1811, 5'CGCGGATCCACTATGACCCGTCCGATACAGGCC 3' (SEQ ID NO: 16) and
MB1816, 5' TGCCATGCATGCCTACAGTTGCTGACCAGCCGG 3' (SEQ ID NO: 17)

were designed and used to isolate the *dadX* gene from *Escherichia coli*, strain W3110 (ATCC Accession Number 27325). Amplification conditions were exactly as described above. The gene was isolated without its native promoter and ligated immediately downstream of the *dat* gene insert. Amplification results in an approximately 1171 bp fragment which was cleaved with *BamHI* and *SphI* and ligated to pIF318 which was similarly digested to form an approximately 4.8 kb fragment. The resulting plasmid was designated pIF320 and carries the *dat* and *dadX* genes in a synthetic operon. Construction of pIF320 is shown in Figure 8.

An additional plasmid, designated pIF321 was then constructed. Plasmid pIF321 was generated by cleaving pIF320 with *Hind*III and *Sph*I and isolating the 2.1 kb fragment carrying the *dat* and *dadX* genes which was then ligated to the 9.2 kb fragment produced by similar cleavage of pIF319. Construction of pIF321 is shown in Figure 9. The pIF321 plasmid contained *dat* and *dadX* genes of pIF320 isolated on a *Hind*III-to-*Sph*I fragment (*Hind*III - promoter - *dat* - *dadX* - *Sph*I) and ligated into pIF319, which contains the above-described *phe*A34 allele along with the *aro*H gene which encodes the tryptophan-dependent DAHP synthase of *Escherichia coli*.

Example 4
CONSTRUCTION OF PLASMID pIF333

In order to generate plasmid pIF333, plasmid pIF321 was first cleaved using the enzymes *SphI* and *SalI* to yield fragments of 6.9 kB and 4.5 kB. The 6.9 kB fragment can be isolated using a QIAquick gel extraction kit (QIAGEN) following electrophoresis on a 1% agarose TBE gel. This fragment was then ligated to the 89 bp fragment generated from *SphI* and *SalI* cleavage of pBR322 (New England Biolabs, Beverly, MA) and similarly isolated from a 2% agarose TBE gel. The resulting plasmid is pIF333. Construction of pIF333 is shown in Figure 10.

Example 5
CONSTRUCTION OF pALR18

The *alr* gene encoding alanine racemase was isolated from *Salmonella typhimurium* strain ATCC Accession Number 19585 obtained from the ATCC. The *alr* gene was isolated by PCR using the oligonucleotide primers:

5' CGCGGATCCACTATGCAAGCGGCAACAGTCGTC 3' (SEQ ID NO: 18)
5' GGAGCATGCTTATTCAATATACTTCATCGCCAC 3' (SEQ ID NO: 19)

The 1098bp PCR product was cleaved with *BamHI* and *SphI* yielding a 1082 *BamHI* to *SphI* fragment which was isolated using a QIAquick gel extraction kit (QIAGEN) following electrophoresis on a 1% agarose TBE gel. This fragment was then ligated to the 5.7kB fragment of pIF333 to generate pALR18. Construction of pALR18 is shown in Figure 11.

Example 6

ISOLATION OF THE L-AMINODEAMINASE GENE AND CONSTRUCTION OF THE pPT363 PLASMID

The L-aminodeaminase gene (*lad*) was isolated from the chromosome of a *Proteus myxofaciens* strain ATCC acession number 19692 using a PCR reaction carried out under standard conditions using an extension time of 2 minutes and the following oligonucleotides:

MB 2198:
5'TTTAGCGCATGCAAGGAGGATCAACTATGAACATTTCAAGGAGAAAG 3' (SEQ ID NO:20)

MB2201:
5'AGCTTTGTCGACGGGCCCTTACTTAAAACGATCCAAAC 3' (SEQ ID NO:21)

The fragment was cleaved by the enzymes *Sph*I and *Sal*I and ligated to the 6.84kb fragment of pALR18 produced form similar cleavage. The resulting plasmid was named pPT362. Construction of pPT362 is shown in Figure 12.

Plasmid pPT363 was generated from pPT362 and plasmid pIF321. Both pPT362 and pIF321 were cleaved with XhoI and ApaI. The 4.67kB fragment of pPT362 and the 7.49kB fragment of pIF321 were isolated and ligated to generate pPT363. Construction of pPT363 is shown in Figure 13.

Example 7

CONSTRUCTION OF THE STRAIN IF3

The *Escherichia coli* strain pIF3 was derived from RY347 (ATCC Accession Number 69766). RY347 was transduced to *tyr*B+ using standard P1 transducing methodology as described in Miller et al., *A Short Course in Bacterial*

Genetics, incorporated by reference herein. The selection for *tyr*B+ transductants was the loss of tyrosine auxotrophy, similarly the strain was transduced to *ilv*E+ selecting for loss of isoleucine auxotrophy. The resulting isolate was designated pIF3.

Example 8
FERMENTATION PROCESS FOR THE PRODUCTION OF D-PHENYLALANINE WITHOUT THE ADDITION OF AN EXTERNAL AMINO DONOR

The strain IF3 was transformed with plasmids pPT363 and pIF1003. The transformed IF3 strain was used to inoculate a 2800 ml Fernbach flask containing 1L of the following growth medium:

Potassium Phosphate (dibasic)	13g
Potassium Phosphate (monobasic)	2g
Ammonium Phosphate	4g
Ferric Ammonium Citrate	0.24g
Yeast Extract	2g
Magnesium Sulphate ($7*H_2O$)	1g
Water	930 mls

The strain was grown to 800-900 Klett Units and used to inoculate the fermentor. The fermentor was a Biolaffite 78-100 (St Germain-en Laye, France) 20L. The following are the conditions under which the fermentor was operated.

Agitation	500rpm
Temperature	32°C
Backpressure	0.7 Bar
pH	7.2 with 50% KOH
Aeration	1 vvm
Set Volume	10 L
Inoculation	1 L
Run Time	67 hrs

The fermentation medium used is listed in the following table.

Magnesium Sulphate (7*H_2O)	5.35 g/l
Ferric Ammonium Citrate	0.3 g/l
Potassium Phosphate (Dibasic)	4.6 g/l
Manganese Sulphate	0.023 g/l
Antifoam (Mazur Mazu) DF204	0.4 ml
$(NH_4)_2HPO4_4$	21 g/l
Yeast Extract	5 g/l
L-alanine	1 g/l

During the fermentation process glucose was fed at a variable rate to achieve a concentration of 10-15 g/l for the first 12 hrs then less than 1 g/l for the remaining time for a total of 1204 g in 48 hours. The fermentation resulted in 1.12 g/l of D-phenylalanine and 0.47 g/l of L-phenylalanine being produced.

Example 9
FERMENTATION PROCESS FOR THE PRODUCTION OF D-PHENYLALANINE WITH THE ADDITION OF D-, L-ALANINE FEED AS AN AMINO DONOR

The fermentation process for Example 9 was identical to the fermentation process in Example 8, except for the following aspects. The total glucose fed was 1976 g over 48 hours. The yeast extract was used at 2 g/l. The fermentation medium included a D-, L-alanine feed whereby a total of 1400 mls of 167 g/l D-, L-alanine was fed at a rate of 1.9 ml/min starting 12 hrs from the beginning of the fermentation. The fermentation resulted in 4.15 g/l of D-phenylalanine and 0 g/l of L-phenylalanine being produced.

Example 10
FERMENTATION PROCESS FOR THE PRODUCTION OF D-PHENYLALANINE WITH THE ADDITION OF D-,L-ALANINE AS AN AMINO DONOR AND

L-PHENYLALANINE AS A KETO ACID PRECURSOR

The fermentation process for Example 10 was identical to Example 8 except for the following aspects. The growth medium used in the fermentation is listed in the following table:

Magnesium Sulphate (7*H_2O)	8.03 g/l
Ferric Ammonium Citrate	0.195 g/l
Potassium Phosphate (Dibasic)	6.9 g/l
Manganese Sulphate	0.0345 g/l
Antifoam (Mazur Mazu) DF204	0.6 ml
$(NH_4)_2HPO_4$	31.5 g/l
Yeast Extract	7.5 g/l
L-alanine	1.5 g/l

The amount of glucose fed was 2021 g over 52 hours. The fermentation medium included a D-, L-alanine feed whereby a total of 1400 mls of 167 g/l D-, L-alanine was fed at a rate of 1.9 ml/min starting 12 hrs from the beginning of the fermentation. In addition, L-phenylalanine was fed at the same concentration and rate as the D-, L-alanine. The fermentation resulted in 13.66 g/l of D-phenylalanine and 0.87 g/l L-phenylalanine being produced.

Example 11

CONSTRUCTION OF PLASMID pPT361

Plasmid pPT361 was derived from pIF306 as follows. pIF306 was cleaved with the enzymes *BamHI* and *Sph*I. The 3.9 kb fragment was isolated and ligated to a similarly cleaved fragment containing the *Escherichia coli* K12 *ilv*E gene which was generated by PCR from W3110 chromosome using the following oligonucleotide primers:

5' CGC GGA TCC ACT ATG ACC ACG AAG AAA GCT GAT TAC ATT TGG 3' (SEQ ID NO:22)

5' CAG CGT GCA TGC TTA TTG ATT AAC TTG ATC TAA CCA GC 3'
(SEQ ID NO:23)

The resulting vector was named pIF307. Plasmid pIF307 was cleaved with enzymes *EcoRI* and *PstI* and the 4.1 kB fragment isolated. This was ligated to a similarly cleaved and purified 982 bp DNA fragment containing the kanamycin resistance gene from pLG338. This was generated using PCR with the following oligonucleotide primers:

5' CCG GAA TTC ACG TTG TGT CTC AAA ATC TCT GAT 3'
(SEQ ID NO:24)

5' CCG CTG CAG GCC GTC CCG TCA AGT CAG CGT AAT G 3'
(SEQ ID NO:25)

The resulting plasmid cleaved was named pIF312. Plasmid pIF12 was cleaved by *EcoRI* and *BamHI* and ligated to the phage lambda C1857 gene which was similarly cleaved following isolation by PCR using the Lambda ZapII vector (Stragene, La Jolla, CA) as template and the following oligonucleotide primers:

5' TTTGGATCCTCCTTAGTACATGCAACC 3'
(SEQ ID NO:26)

5' TTTGAATTCGGATGAAGATTCTTGCTCGATTGT 3'
(SEQ ID NO:27)

The resulting plasmid was named pPT353. This plasmid was then cleaved with *PstI* and *EagI* and the 3.17 kb fragment was isolated. This was ligated to the similarly cleaved 2.5 kb fragment generated by similar cleavage of pIF1003. The resulting vector was named

4.7 kb fragment isolated. this was ligated to the following oligonucleotide linker

5'
GATCCTAGGTACCGGTGCGGCCGCATGCTGACTGACTGAAGATCCCGGGCGATTC
TACGCCCGGGTTTTTTATG 3' (SEQ ID NO:28)

5'
TCGACATAAAAAACCCGGGCGTAGAATCGCCCGGGATCTTCAGTCAGTCAGCATG
CGGCCGCACCGGTACCTAG 3' (SEQ ID NO:29)

The resulting plasmid was named pPOT2. This plasmid was cleaved with *Xho*I and *Pst*I and the 3.9 kb fragment isolated. This was ligated to a fragment containing the chloramphenicol resistance gene which was isolated bay PCR using pIF319 plasmid DNA as template and the following oligonucleotide primers.

5' GAC CTC GAG GCA CTT TGC GCC GAA TAA ATA CCT GTG 3'
(SEQ ID NO:30)

5' GAC CTG CAG CAC CAG GCG TTT AAG GGC ACC AAT AAC 3'
(SEQ ID NO:31)

The resulting plasmid was named pPOT3. This was cleaved with *BamH*I and *Sph*I. The 4.8 bp fragment was isolated and ligated to similarly cleaved fragment containing the *Proteus myxofaciens Lad* gene. This was isolated by PCR from the chromosome from ATCC 19692 using the following oligonucleotide primers:

5' TTTGGATCCAAGATGAACATTTCAAGGAGAAAG 3'
(SEQ ID NO:32)
5' AGCTTTGTCGACGCATGCTTACTTCTTAAAACGATCCAAAC 3'
(SEQ ID NO:33)

Example 12

DETERMINATION OF Lad AMINO ACID SUBSTRATES

Each of the amino acid substrates listed in Table 1 were determined to be a suitable substrate for the Lad enzyme using the following thin layer chromatography (TLC) Lad assay. All of the chemicals used were obtained from Sigma Chemical Company, St. Louis, MO.

The assay mix contained 10mg/ml of one of the amino acid substrates listed in Table 1 and 100mM Tris HCl with a pH of 7.5. The assay mix (2 mls) was added to 100 mg of cell pellet from Strain W3110 containing plasmid pPT361 which contained the Lad gene.

Cells were prepared from overnight culture of 200mls of LB medium (Difco, Detroit, Michigan) at 37°C in 1L shake flasks. Cells were washed once in 100mM tris HCL pH 7.5 and pelleted by centrifugation. The reaction was carried out for 16 hours at 37°C 0.005ml of reaction mix was spotted on Silica TLC plates #60 F-254 (EM Science Cincinnati OH).

The chromatography was carried out using the following solvent: water (40%); methanol (40%); and acetonitrile (20%). The TLC plates were air dried and sprayed with 2% Ninhydrin in ethanol and then baked for 10 minutes.

The conversion of each of the amino acids listed in Table 1 to their corresponding keto acids was determined by the absence of the amino acid derived spots against co-chromatographed known standards. Each of the amino acid substrates listed in Table 1 were found to be suitable substrates for the Lad enzyme.

Example 13

DETERMINATION OF Dat KETO ACID SUBSTRATES

The Dat enzyme was assayed with each keto acid substrate listed in Table 1 in a coupled enzyme assay under the following conditions. All of the chemicals used were obtained from Sigma Chemical Company, St. Louis, MO.

The assay mix contained 500 u/ml Dat; 30 mM D-Alanine; 30 mM Keto Acid Substrate; 0.2mMNADH; and 100mM Tris-HCl. The pH of the assay mixture was 8.3. The assay was carried out using 1 ml of solution containing 0.85 ml of assay mix, 0.05 ml of D-Lactate and 0.1ml of W3110 cells (ATCC27325) containing plasmid pIF1003 at an $O.D._{650}$ of 0.5-1.0.

Cells were prepared from overnight culture in 200mls of LB medium (Difco, Detroit, Michigan) at 37°C in 1L shake flasks. Cells were washed once in 100mM Tris HCl pH 7.5, centrifuged and taken up in water. The reaction for each of the keto acid substrates in Table 1 was monitored by measuring ΔA_{340} at 37°C. Each of the keto acid substrates assay in Table 1 were found to be suitable substrates for the Dat enzyme.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Taylor, Paul P.
Ton, Jennifer L.

(ii) TITLE OF INVENTION: Preparation of D-Amino Acids By Direct Fermentative Means

(iii) NUMBER OF SEQUENCES: 33

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(ix) TELECOMMUNICATION INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 95 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAGCTTTTTT GTTGACAGCG TGAAAACAGT ACGGGTATAA TACTAAAGTC ACAAGGAGGA 60

TCCACTATGA CATCGGAAAA CCCGTTACTG GCGCT 95

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1424 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS

(B) LOCATION: 427..1275

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
ACAAGGAGGA TCCGTTAATC CAAACGTTAG CTGGTGTTTA TCGCCGACAA ACGGGCGATA      60

ACGAAACACC TTTACTTTCA ACAGGCGGTG GAACGTATGC ACGCGTCTTG AAAAAAGGTG     120

TGGCATTCGG CATGCTTTTC CCTGGTGATC CAGATGTCAT GCATCGTGCG GATGAATATG     180

TAATTGTTGA TAAATTAGTA CAAGCTGCTG CTATTTATGC AGAAGCCATT GCAGAACTGG     240

CTGGGAAGTA AGTGTCATTA AGAGCGTAAT GTTTTCTTGC CAAAGAGATC ACGAAGCTTC     300

ACACGCCAAG CACTTCACTG AAAAATCTAC TTTGATTTAC TGCATCTGGT CTTACTTGAT     360

CGTCTAGTGG GAATCATTGT ACTTAAAAAT GTGAAAATAA CTTAAAAATG AAAAGGATGT     420

ATAAAC ATG GCA TAC TCA TTA TGG AAT GAC CAA ATC GTT GAA GAA GGA          468
       Met Ala Tyr Ser Leu Trp Asn Asp Gln Ile Val Glu Glu Gly
         1               5                  10

TCT ATT ACA ATT TCA CCA GAA GAC CGT GGT TAT CAA TTT GGT GAT GGT        516
Ser Ile Thr Ile Ser Pro Glu Asp Arg Gly Tyr Gln Phe Gly Asp Gly
 15                  20                  25                  30

ATT TAC GAA GTA ATC AAA GTA TAT AAC GGG CAT ATG TTT ACA GCA CAA        564
Ile Tyr Glu Val Ile Lys Val Tyr Asn Gly His Met Phe Thr Ala Gln
                 35                  40                  45

GAG CAC ATC GAT GCT TTC TAT GCT AGT GCC GAA AAA ATT CGC CTT GTT        612
Glu His Ile Asp Ala Phe Tyr Ala Ser Ala Glu Lys Ile Arg Leu Val
             50                  55                  60

ATT CCT TAT ACA AAA GAT GTA TTA CAC AAA TTA TTG CAT GAT TTA ATC        660
Ile Pro Tyr Thr Lys Asp Val Leu His Lys Leu Leu His Asp Leu Ile
         65                  70                  75

GAA AAA AAT AAT TTA AAT ACA GGT CAT GTT TAC TTC CAA ATT ACA CGT        708
Glu Lys Asn Asn Leu Asn Thr Gly His Val Tyr Phe Gln Ile Thr Arg
     80                  85                  90

GGA ACA ACT TCT CGT AAC CAC ATT TTC CCG GAT GCA AGC GTA CCA GCA        756
Gly Thr Thr Ser Arg Asn His Ile Phe Pro Asp Ala Ser Val Pro Ala
 95                 100                 105                 110

GTG CTA ACA GGT AAT GTT AAA ACT GGT GAA CGT TCA ATT GAA AAT TTC        804
Val Leu Thr Gly Asn Val Lys Thr Gly Glu Arg Ser Ile Glu Asn Phe
                115                 120                 125

GAA AAA GGC GTA AAA GCG ACA TTG GTT GAA GAT GTT CGT TGG TTA CGT        852
Glu Lys Gly Val Lys Ala Thr Leu Val Glu Asp Val Arg Trp Leu Arg
            130                 135                 140

TGT GAT ATT AAA TCT TTA AAT TTA CTT GGC GCG GTA CTT GCG AAA CAA        900
Cys Asp Ile Lys Ser Leu Asn Leu Leu Gly Ala Val Leu Ala Lys Gln
        145                 150                 155

GAA GCA TCT GAA AAA GGT TGT TAC GAA GCC ATT TTA CAC CGT GGA GAT        948
Glu Ala Ser Glu Lys Gly Cys Tyr Glu Ala Ile Leu His Arg Gly Asp
    160                 165                 170

ATT ATC ACA GAA TGT TCT TCT GCT AAT GTC TAT GGT ATT AAA GAT GGT        996
Ile Ile Thr Glu Cys Ser Ser Ala Asn Val Tyr Gly Ile Lys Asp Gly
175                 180                 185                 190
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AAA CTT TAT ACG CAC CCA GCA AAT AAC TAC ATC TTA AAT GGT ATT ACA      1044
Lys Leu Tyr Thr His Pro Ala Asn Asn Tyr Ile Leu Asn Gly Ile Thr
                195                 200                 205

CGC CAA GTT ATA TTA AAA TGT GCC GCT GAA ATA AAT TTA CCA GTG ATT      1092
Arg Gln Val Ile Leu Lys Cys Ala Ala Glu Ile Asn Leu Pro Val Ile
            210                 215                 220

GAA GAG CCG ATG ACA AAA GGC GAT TTA TTA ACA ATG GAT GAA ATT ATT      1140
Glu Glu Pro Met Thr Lys Gly Asp Leu Leu Thr Met Asp Glu Ile Ile
        225                 230                 235

GTG TCT TCT GTT TCA TCT GAA GTG ACA CCG GTT ATC GAT GTG GAT GGT      1188
Val Ser Ser Val Ser Ser Glu Val Thr Pro Val Ile Asp Val Asp Gly
    240                 245                 250

CAG CAA ATT GGT GCA GGT GTT CCT GGT GAA TGG ACT CGT AAA TTG CAA      1236
Gln Gln Ile Gly Ala Gly Val Pro Gly Glu Trp Thr Arg Lys Leu Gln
255                 260                 265                 270

AAA GCA TTT GAG GCA AAA TTA CCA ATT TCA ATT AAT GCC TAATCTGTAT      1285
Lys Ala Phe Glu Ala Lys Leu Pro Ile Ser Ile Asn Ala
                275                 280

AAATGATTAA AAAGAGCTAC CTAAAACTTG GTTATTCGCC AAGTTAGGAG GGTAGCTCTT   1345

TTTTATAGAA TAAAATATGC ATGTATTCTC CTGAAACGTC ATGTAAAATA AAAAAGATAG   1405

CGCCTTTAGT CGATATCAC                                               1424
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 283 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Met Ala Tyr Ser Leu Trp Asn Asp Gln Ile Val Glu Glu Gly Ser Ile
  1               5                  10                  15

Thr Ile Ser Pro Glu Asp Arg Gly Tyr Gln Phe Gly Asp Gly Ile Tyr
            20                  25                  30

Glu Val Ile Lys Val Tyr Asn Gly His Met Phe Thr Ala Gln Glu His
        35                  40                  45

Ile Asp Ala Phe Tyr Ala Ser Ala Glu Lys Ile Arg Leu Val Ile Pro
    50                  55                  60

Tyr Thr Lys Asp Val Leu His Lys Leu Leu His Asp Leu Ile Glu Lys
65                  70                  75                  80

Asn Asn Leu Asn Thr Gly His Val Tyr Phe Gln Ile Thr Arg Gly Thr
                85                  90                  95

Thr Ser Arg Asn His Ile Phe Pro Asp Ala Ser Val Pro Ala Val Leu
            100                 105                 110

Thr Gly Asn Val Lys Thr Gly Glu Arg Ser Ile Glu Asn Phe Glu Lys
        115                 120                 125

Gly Val Lys Ala Thr Leu Val Glu Asp Val Arg Trp Leu Arg Cys Asp
```

130 135 140

Ile Lys Ser Leu Asn Leu Leu Gly Ala Val Leu Ala Lys Gln Glu Ala
145 150 155 160

Ser Glu Lys Gly Cys Tyr Glu Ala Ile Leu His Arg Gly Asp Ile Ile
165 170 175

Thr Glu Cys Ser Ser Ala Asn Val Tyr Gly Ile Lys Asp Gly Lys Leu
180 185 190

Tyr Thr His Pro Ala Asn Asn Tyr Ile Leu Asn Gly Ile Thr Arg Gln
195 200 205

Val Ile Leu Lys Cys Ala Ala Glu Ile Asn Leu Pro Val Ile Glu Glu
210 215 220

Pro Met Thr Lys Gly Asp Leu Leu Thr Met Asp Glu Ile Ile Val Ser
225 230 235 240

Ser Val Ser Ser Glu Val Thr Pro Val Ile Asp Val Asp Gly Gln Gln
245 250 255

Ile Gly Ala Gly Val Pro Gly Glu Trp Thr Arg Lys Leu Gln Lys Ala
260 265 270

Phe Glu Ala Lys Leu Pro Ile Ser Ile Asn Ala
275 280

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Val Ile Phe Tyr Leu Ala Leu
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Leu Pro Ile Ser Ile Asn Ala
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCCATCTCCT TGCATGCACC ATTCC 25

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCCTCGCAAG CTCGTCCGGA GGCAAATCGC TGAATATTCC 40

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGCAGATCTA CTATGGCATA CTCATTATGG 30

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CATGCCATGG ATCCTCCTTT TAGGTAGCTC TTTTTAATC 39

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGCGGATCCT CGTCATGAAC AGAACTGACG AACTCCG 37

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ACGCGTCGAC TCAGAAGCGG GTATCTACCG CAGAGG 36

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGAAGATCTT ACATCATCAA CCAGATCGAT TCTG 34

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CGCGGATCCA TTATGGTTAC AGAAGGGAAG TCC 33

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CCGCTCGAGC CCGACGCACT TTGCGCCGA 29

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CCCAAGCTTA TCAGGCTCTG GGAGGCAG 28

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGCGGATCCA CTATGACCCG TCCGATACAG GCC 33

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TGCCATGCAT GCCTACAGTT GCTGACCAGC CGG 33

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CGCGGATCCA CTATGCAAGC GGCAACAGTC GTC 33

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGAGCATGCT TATTCAATAT ACTTCATCGC CAC 33

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TTTAGCGCAT GCAAGGAGGA TCAACTATGA ACATTTCAAG GAGAAAG 47

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGCTTTGTCG ACGGGCCCTT ACTTAAAACG ATCCAAAC 38

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CGCGGATCCA CTATGACCAC GAAGAAAGCT GATTACATTT GG 42

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CAGCGTGCAT GCTTATTGAT TAACTTGATC TAACCAGC 38

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CCGGAATTCA CGTTGTGTCT CAAAATCTCT GAT 33

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CCGCTGCAGG CCGTCCCGTC AAGTCAGCGT AATG 34

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TTTGGATCCT CCTTAGTACA TGCAACC 27

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TTTGAATTCG GATGAAGATT CTTGCTCGAT TGT 33

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 74 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GATCCTAGGT ACCGGTGCGG CCGCATGCTG ACTGACTGAA GATCCCGGGC GATTCTACGC 60

CCGGGTTTTT TATG 74

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 74 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TCGACATAAA AAACCCGGGC GTAGAATCGC CCGGGATCTT CAGTCAGTCA GCATGCGGCC 60

GCACCGGTAC CTAG 74

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GACCTCGAGG CACTTTGCGC CGAATAAATA CCTGTG 36

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GACCTGCAGC ACCAGGCGTT TAAGGGCACC AATAAC 36

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TTTGGATCCA AGATGAACAT TTCAAGGAGA AAG 33

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

AGCTTTGTCG ACGCATGCTT ACTTCTTAAA ACGATCCAAA C 41

What is claimed is:

1. A method for producing a D-amino acid in a cell, comprising:
   (a) incorporating into the cell a D-aminotransferase gene and a L-aminodeaminase gene;
   (b) culturing the cell in a cell culture medium;
   (c) and isolating the D-amino acid from the cell culture medium.

2. The method of claim 1, further comprising the step of introducing a D-aminodeaminase gene mutation into the cell such that the D-aminodeaminase gene is nonfunctional.

3. The method of claim 1, wherein the cell is a bacterial cell.

4. The method of claim 2, wherein the bacterial cell is selected from the group consisting of *Bacillus subtilis*, *Bacillus sphaericus*, *Bacillus stearothermophilus*, *Pseudomonas*, *Klebsiella*, *Salmonella*, *Brevibacterium*, *Micrococcus*, *Corynebacterium* and *Escherichia coli*.

5. The method of claim 4, wherein the cell is a *Escherichia coli*.

6. The method of claim 5, further comprising the step of introducing a *dadA* gene mutation into the *Escherichia coli* cell such that the *dadA* gene is nonfunctional.

7. The method of claim 1, wherein the D-aminotransferase gene is a *Bacillus sphaericus* D-aminotransferase gene.

8. The method of claim 1, wherein the L-aminodeaminase gene is a *Proteus myxofaciens* L-aminodeaminase gene or a *Proteus mirabilis* L-aminodeaminase gene.

9. The method of claim 1, further comprising the step of incorporating into the cell a racemase gene.

10. The method of claim 9, wherein the racemase gene is selected from the group consisting of alanine racemase, glutamate racemase, aspartate racemase and phenylalanine racemase.

11. The method of claim 10, wherein the racemase gene is alanine racemase.

12. The method of claim 1, wherein the D-amino acid is a natural or unnatural D-amino acid.

13. The method of claim 12, wherein the natural or unnatural D-amino acid is selected from the group consisting of isoleucine, leucine, tryptophan, tyrosine, valine, arginine, asparagine, glutamine, methionine, ornithine, serine, norleucine, norvaline, phenylalanine, dihydroxyphenylalanine, citrulline, cysteine, histidine and lysine.

14. The method of claim 13, wherein the natural D-amino acid is phenylalanine.

15. The method of claim 1, wherein the culture medium contains an amino donor.

16. The method of claim 15, wherein the amino donor is selected from the group consisting of L-alanine, L-glutamate, L-phenylalanine, L-aspartate and a

racemic mixture one of the aforementioned L-amino acids.

17. The method of claim 16, wherein the amino donor racemic mixture is aspartate.

18. The method of claim 1, wherein the culture medium contains an L-amino acid substrate.

19. The method of claim 18, wherein the L-amino acid substrate is selected from the group consisting of isoleucine, leucine, tryptophan, tyrosine, valine, arginine, asparagine, glutamine, methionine, ornithine, serine, norleucine, norvaline, phenylalanine, dihydroxyphenylalanine, citrulline, cysteine, histidine and lysine.

20. A method for the preparation of a substantially pure D-amino acid using a culture of the cell of claim 1.

21. The method of claim 20, wherein the D-amino acid is produced in high yields.

22. The method of claim 1, wherein the D-aminotransferase gene and the L-aminodeaminase gene are incorporated into the cell using a plasmid.

23. A method for producing D-phenylalanine in a cell, comprising:

(a) incorporating into the cell a D-aminotransferase gene, a L-aminodeaminase gene and means for increasing production of phenylpyruvic acid;

(b) culturing the cell in a cell culture medium; and

(c) isolating the D-phenylalanine from the cell culture medium.

24. The method of claim 23, further comprising the step of introducing a D-aminodeaminase gene mutation into the cell such that the D-aminodeaminase gene is nonfunctional.

25. The method of claim 23, wherein the cell is a bacterial cell.

26. The method of claim 25, wherein the bacterial cell is selected from the group consisting of *Bacillus subtilis*, *Bacillus sphaericus*, *Bacillus stearothermophilus*, *Pseudomonas*, *Klebsiella*, *Salmonella*, *Brevibacterium*, *Micrococcus*, *Corynebacterium* and *Escherichia coli*.

27. The method of claim 26, wherein the cell is a *Escherichia coli*.

28. The method of claim 27, further comprising the step of introducing a *dadA* gene mutation into the *Escherichia coli* cell such that the *dadA* gene is nonfunctional.

29. The method of claim 23, wherein the D-aminotransferase gene is a *Bacillus sphaericus* D-aminotransferase gene.

30. The method of claim 23, wherein the L-aminodeaminase gene is a *Proteus myxofaciens* L-aminodeaminase gene or a *Proteus mirabilis* L-aminodeaminase gene.

31. The method of claim 23, further comprising the step of incorporating into the cell a racemase gene.

32. The method of claim 31, wherein the racemase gene is selected from the group consisting of alanine racemase, glutamate racemase, aspartate racemase or phenylalanine racemase.

33. The method of claim 32, wherein the racemase gene is alanine racemase.

34. The method of claim 23, wherein the culture medium contains an amino donor.

35. The method of claim 34, wherein the amino donor is selected from the group consisting of L-alanine, L-glutamate, L-phenylalanine, L-aspartate and a racemic mixture one of the aforementioned L-amino acids.

36. The method of claim 35, wherein the racemic mixture is aspartate.

37. The method of claim 23, wherein the culture medium contains L-phenylalanine as a substrate.

38. The method of claim 23, wherein means for increasing production of phenylpyruvate comprises incorporating into the cell an *aroH* gene.

39. The method of claim 23, wherein means for increasing production of phenylpyruvate comprises incorporating into the cell a *pheA* gene.

40. A method for the preparation of a substantially pure D-phenylalanine acid using a culture of the cell of claim 23.

41. The method of claim 40, wherein the D-phenylalanine is produced in high yields.

42. The method of claim 41, wherein the D-aminotransferase gene and the L-aminodeaminase gene are incorporated into the cell using a plasmid.

43. A recombinant cell, comprising an exogenous D-aminotransferase gene and an exogenous L-aminodeaminase gene.

44. The recombinant cell of claim 43, further comprising a D-aminodeaminase gene mutation in the cell such that the D-aminodeaminase gene is nonfunctional.

45. The recombinant cell of claim 43, wherein the exogenous D-aminotransferase gene is a *Bacillus sphaericus* D-aminotransferase gene.

46. The recombinant cell of claim 43, wherein the exogenous L-aminodeaminase gene is a *Proteus myxofaciens* L-aminodeaminase gene or a *Proteus mirabilis* L-aminodeaminase gene.

47. The recombinant cell of claim 43, further comprising an exogenous racemase gene.

48. The recombinant cell of claim 47, wherein the exogenous racemase gene is a *Salmonella typhimurium* gene.

49. The recombinant cell of claim 48, wherein the *Salmonella typhimurium* gene is alanine racemase.

50. The recombinant cell of claim 43, further comprising an exogenous *aroH* gene and an exogenous *pheA* gene.

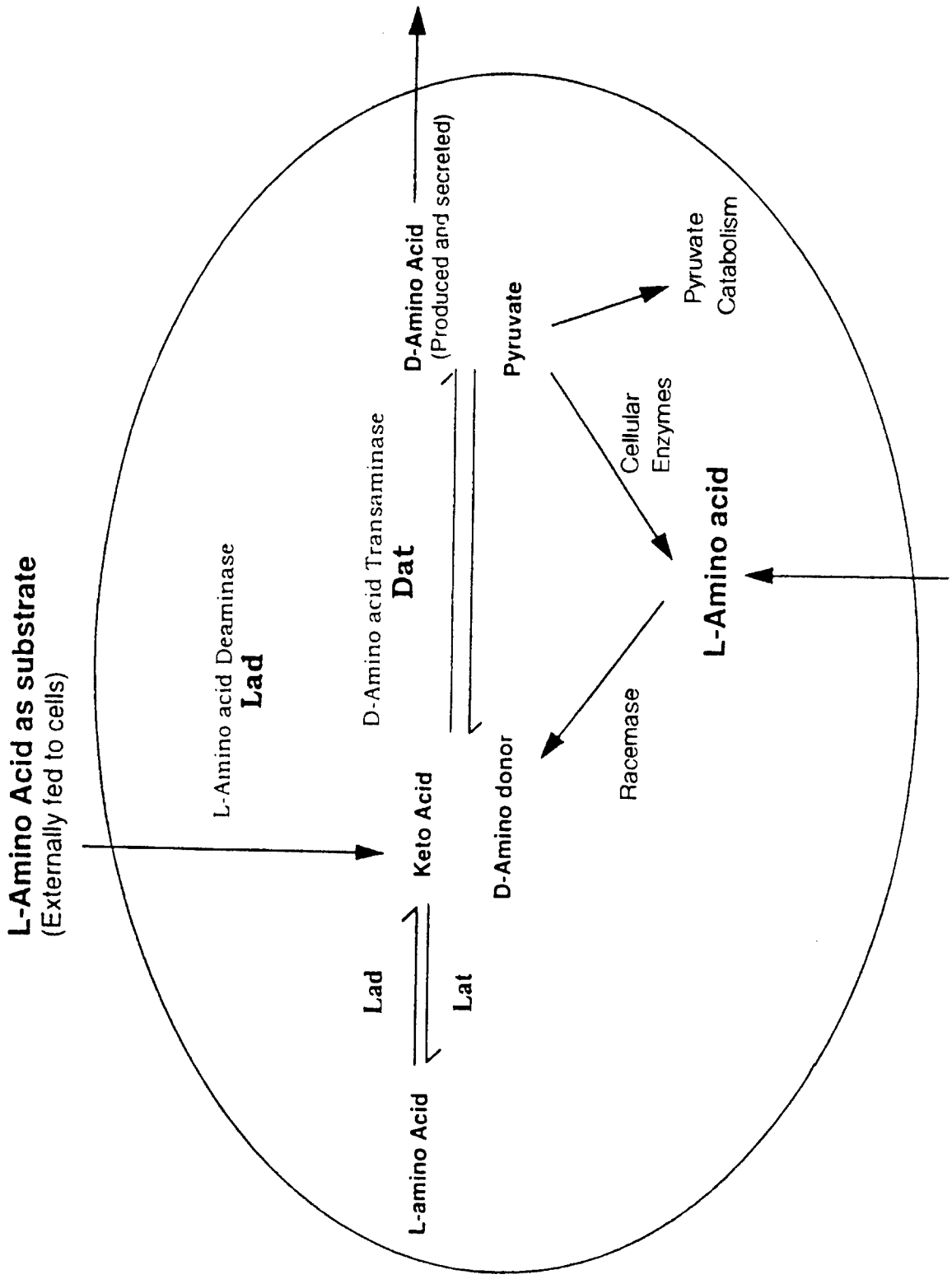
L-Amino Acid as substrate
(Externally fed to cells)
L-Amino acid Deaminase
Lad
D-Amino acid Transaminase
Dat
D-Amino Acid
(Produced and secreted)
Pyruvate
Pyruvate
Catabolism
Cellular
Enzymes
L-Amino acid
L-Amino acid as amino donor
(Externally fed to cells)
Keto Acid
D-Amino donor
Racemase
Lad
Lat
L-amino Acid

FIG. 1

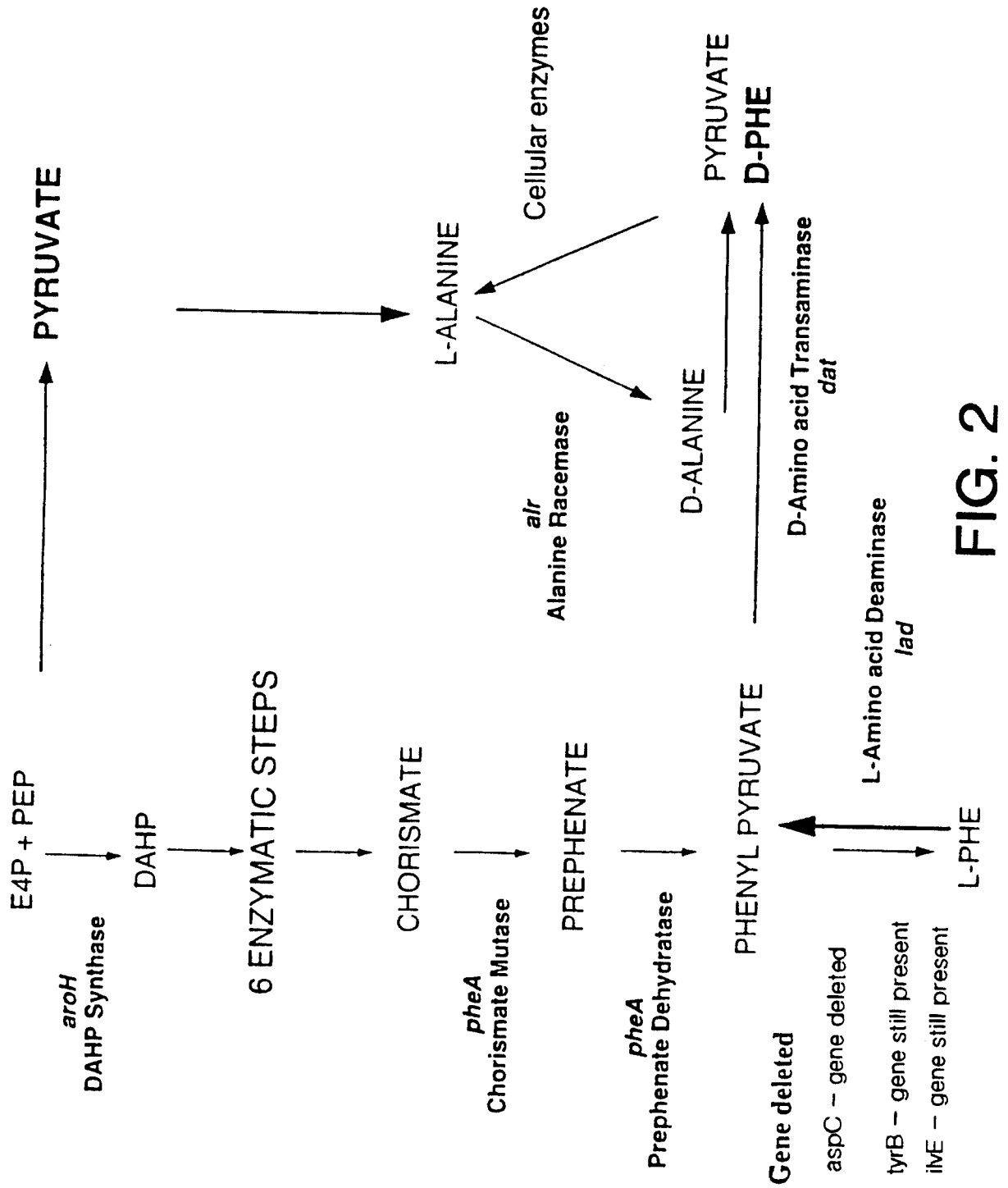
E4P + PEP
aroH
DAHP Synthase
DAHP
6 ENZYMATIC STEPS
CHORISMATE
pheA
Chorismate Mutase
PREPHENATE
pheA
Prephenate Dehydratase
PHENYL PYRUVATE
L-Amino acid Deaminase
lad
L-PHE
PYRUVATE
L-ALANINE
Cellular enzymes
alr
Alanine Racemase
D-ALANINE
D-Amino acid Transaminase
dat
PYRUVATE
D-PHE
Gene deleted
aspC – gene deleted
tyrB – gene still present
ilvE – gene still present

FIG. 2

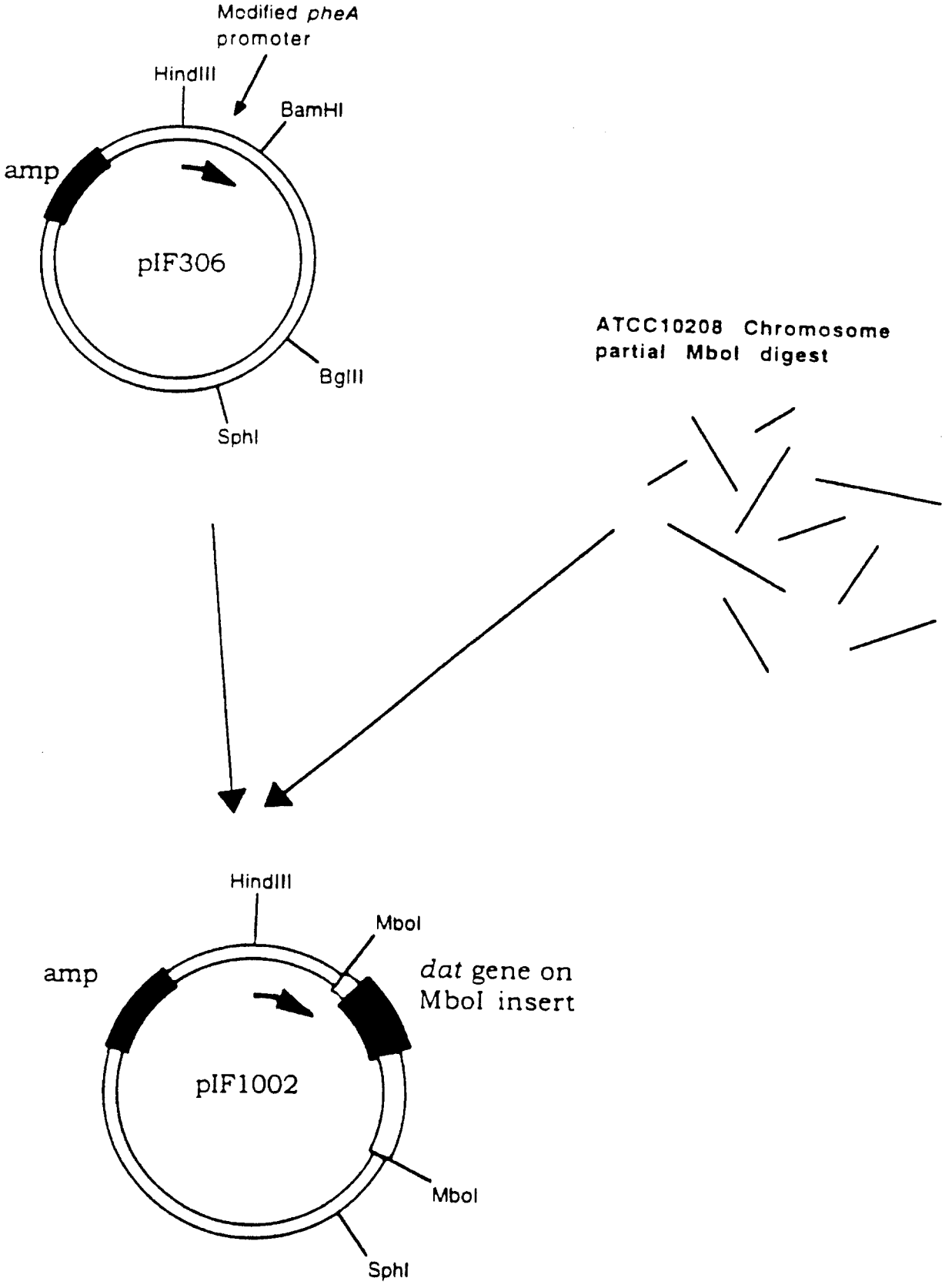
Modified *pheA* promoter
HindIII
BamHI
amp
pIF306
BglII
SphI
ATCC10208 Chromosome partial MboI digest
HindIII
MboI
amp
dat gene on MboI insert
pIF1002
MboI
SphI

FIG. 3

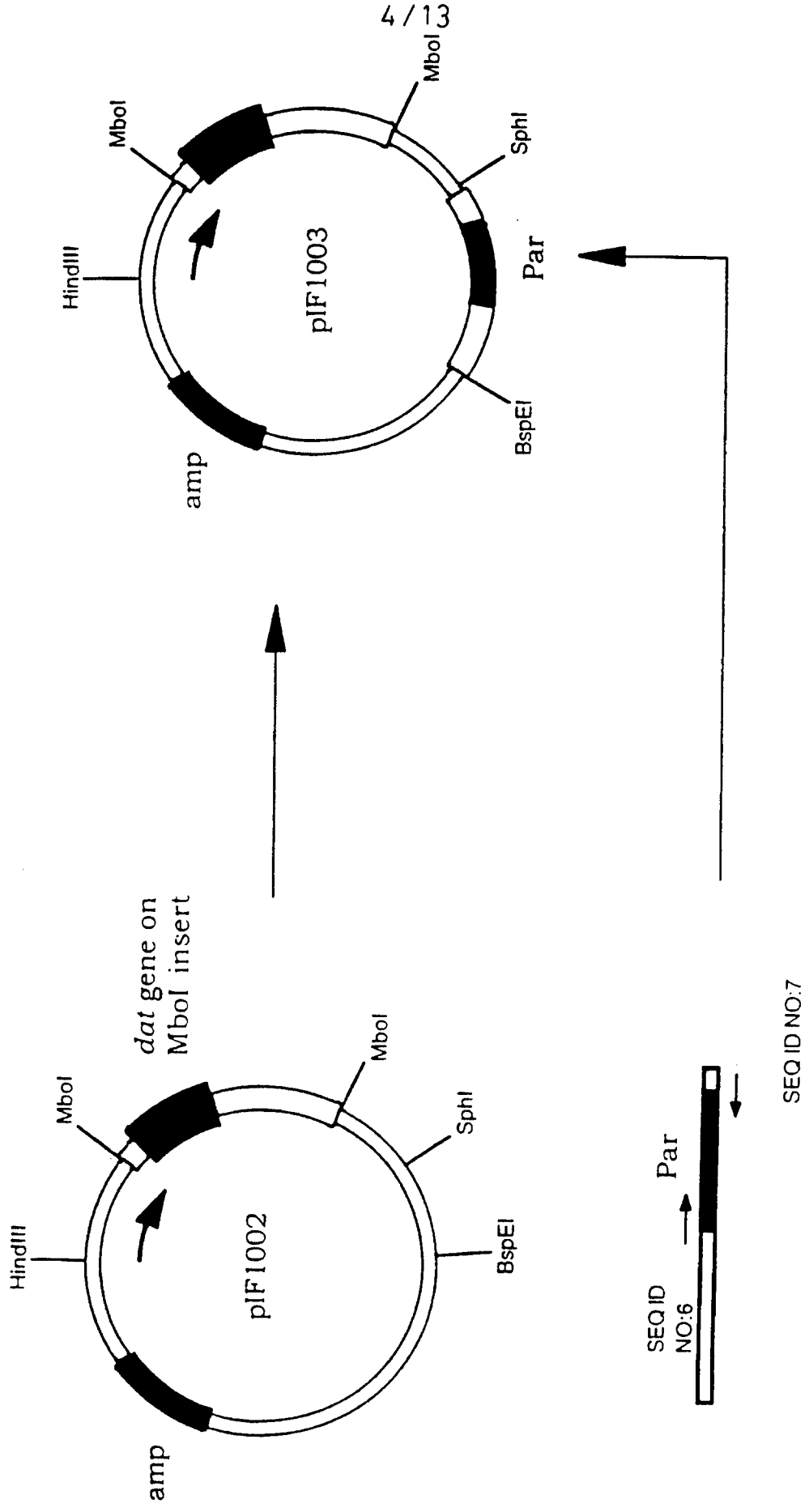
MboI
SphI
Par
BspEI
amp
HindIII
MboI
pIF1003
dat gene on MboI insert
MboI
SphI
BspEI
amp
HindIII
MboI
pIF1002
Par
SEQ ID NO:6
SEQ ID NO:7

FIG. 4

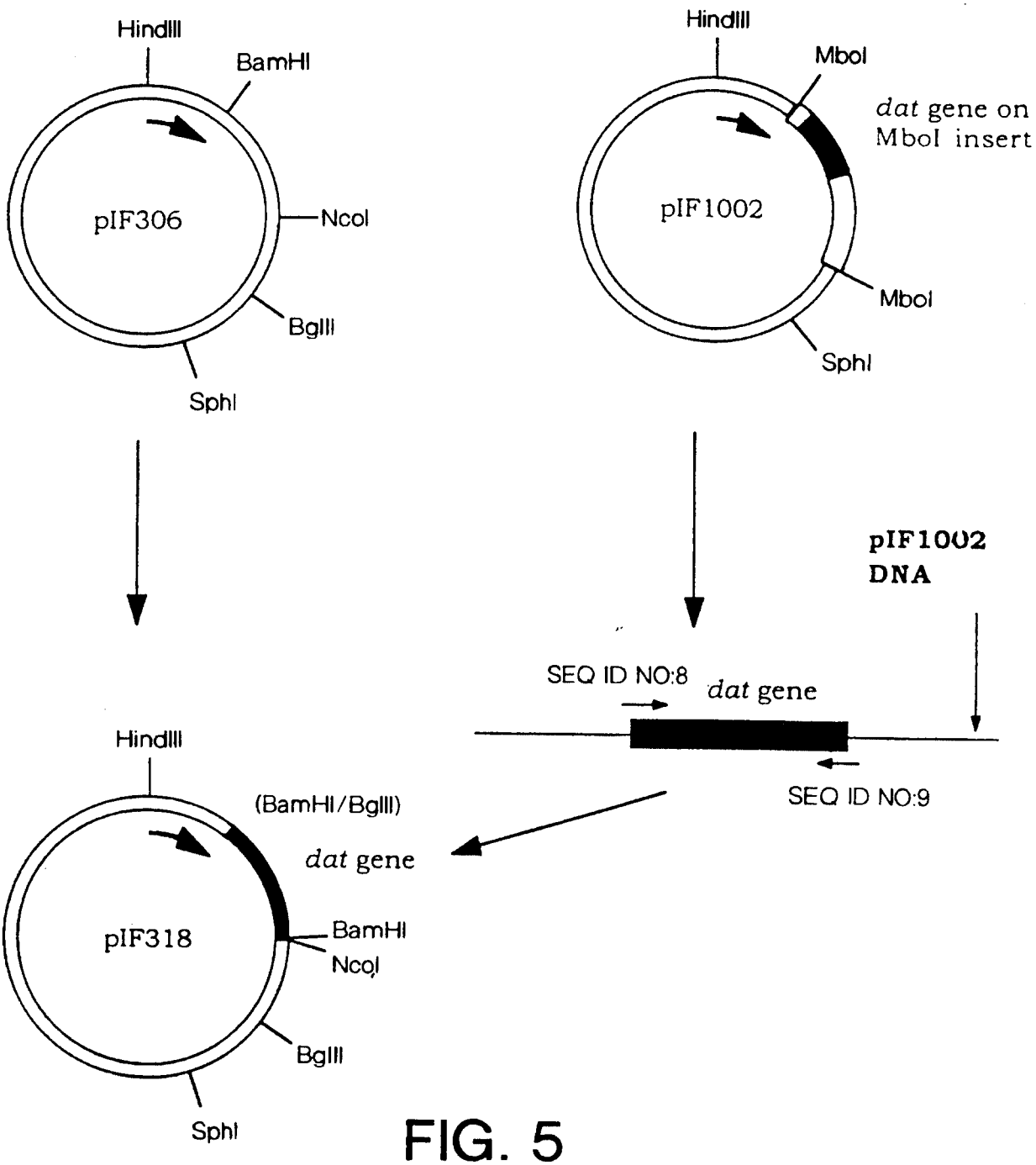
HindIII
BamHI
pIF306
NcoI
BglII
SphI
HindIII
MboI
dat gene on
MboI insert
pIF1002
MboI
SphI
pIF1002
DNA
SEQ ID NO:8
dat gene
SEQ ID NO:9
HindIII
(BamHI/BglII)
dat gene
pIF318
BamHI
NcoI
BglII
SphI

FIG. 5

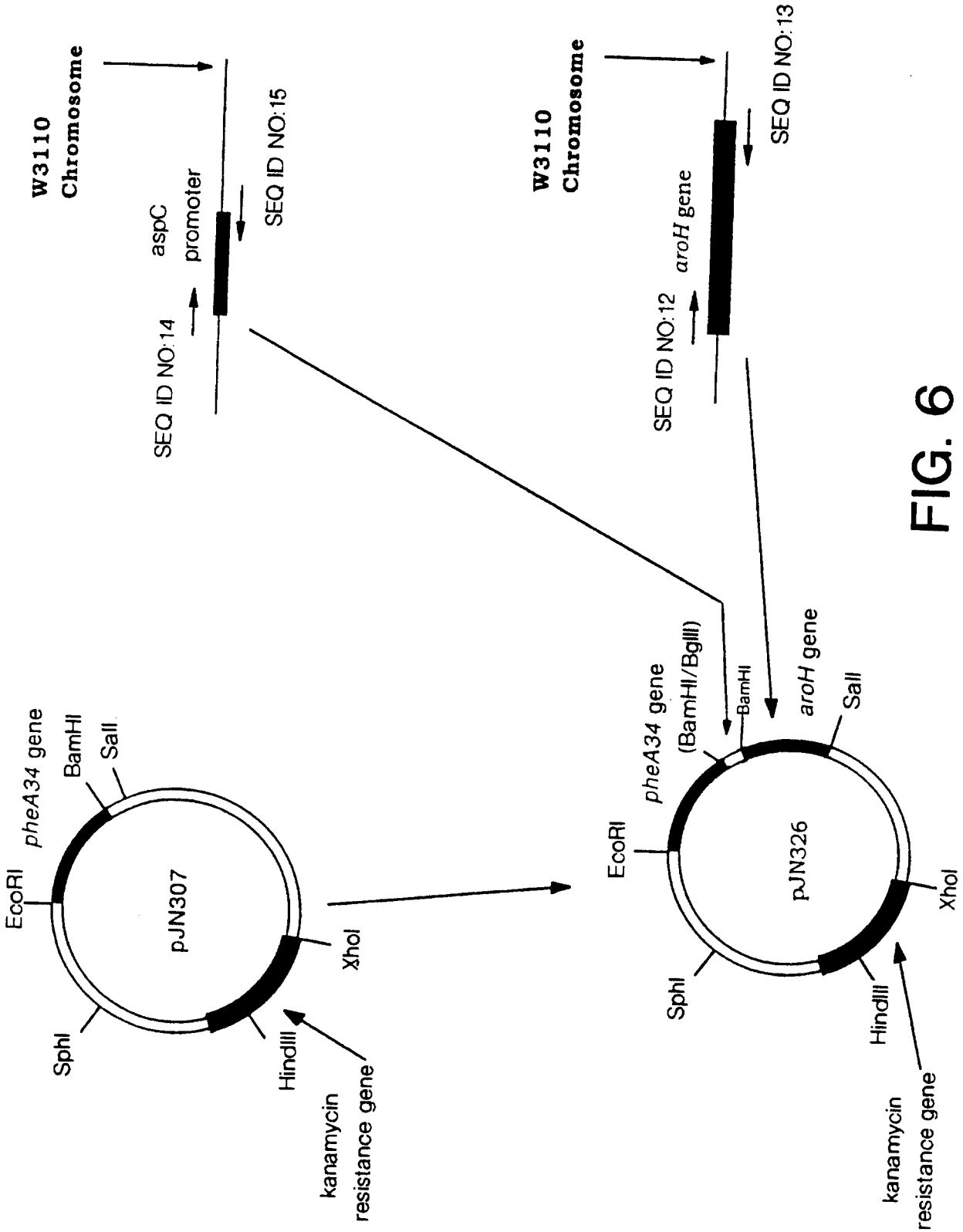
W3110 Chromosome
aspC promoter
SEQ ID NO:14
SEQ ID NO:15
W3110 Chromosome
aroH gene
SEQ ID NO:12
SEQ ID NO:13
pheA34 gene
BamHI
SalI
EcoRI
pJN307
XhoI
SphI
HindIII
kanamycin resistance gene
pheA34 gene (BamHI/BglII)
BamHI
aroH gene
SalI
EcoRI
pJN326
XhoI
SphI
HindIII
kanamycin resistance gene

FIG. 6

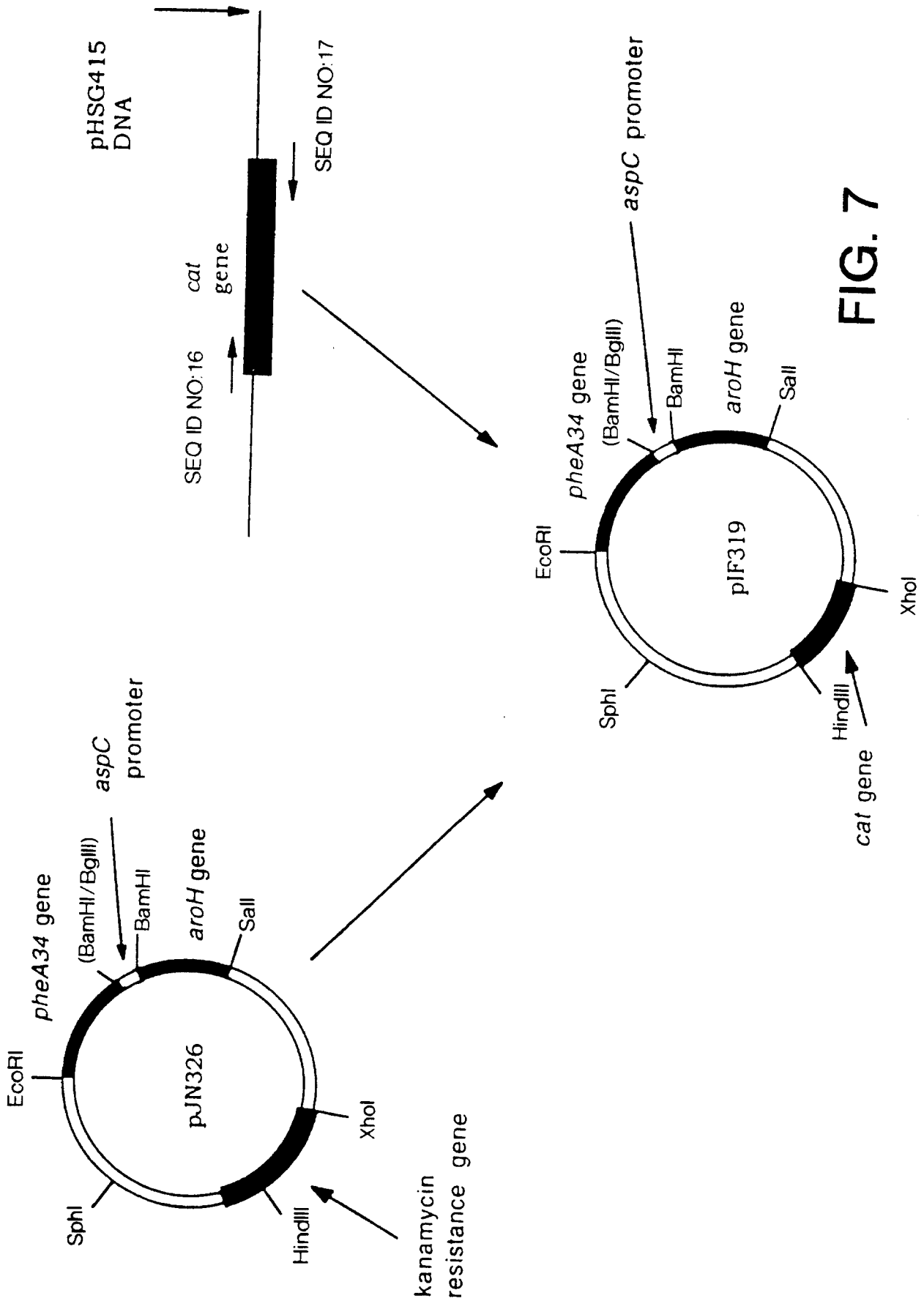
pHSG415 DNA
SEQ ID NO:17
SEQ ID NO:16
cat gene
aspC promoter
pheA34 gene
(BamHI/BglII)
BamHI
aroH gene
SalI
EcoRI
pIF319
XhoI
SphI
HindIII
cat gene
aspC promoter
pheA34 gene
(BamHI/BglII)
BamHI
aroH gene
SalI
EcoRI
pJN326
XhoI
SphI
HindIII
kanamycin resistance gene

FIG. 7

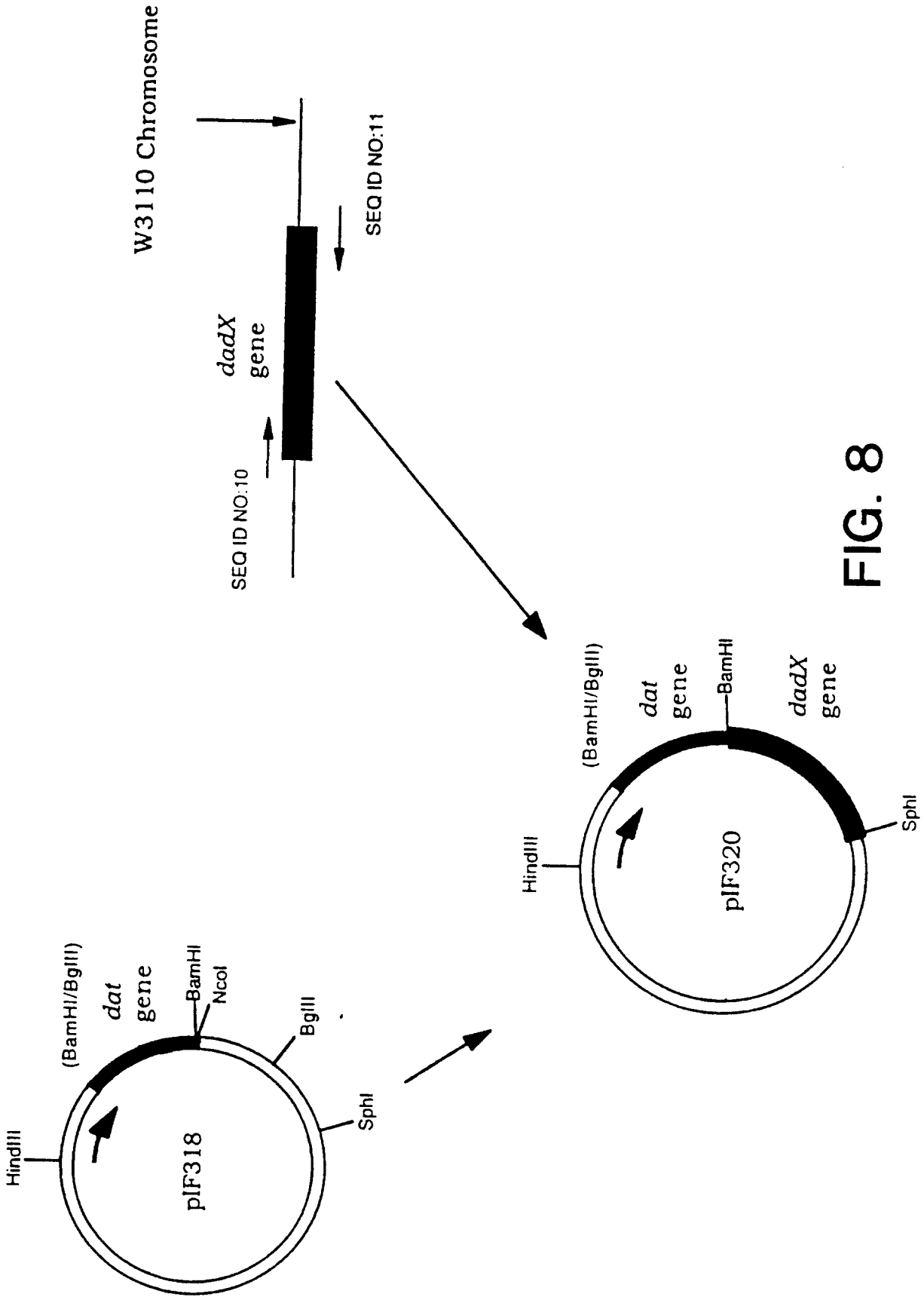
W3110 Chromosome
SEQ ID NO:11
dadX gene
SEQ ID NO:10
(BamHI/BglII)
dat gene
BamHI
dadX gene
HindIII
pIF320
SphI
(BamHI/BglII)
dat gene
BamHI
NcoI
BglII
HindIII
pIF318
SphI

FIG. 8

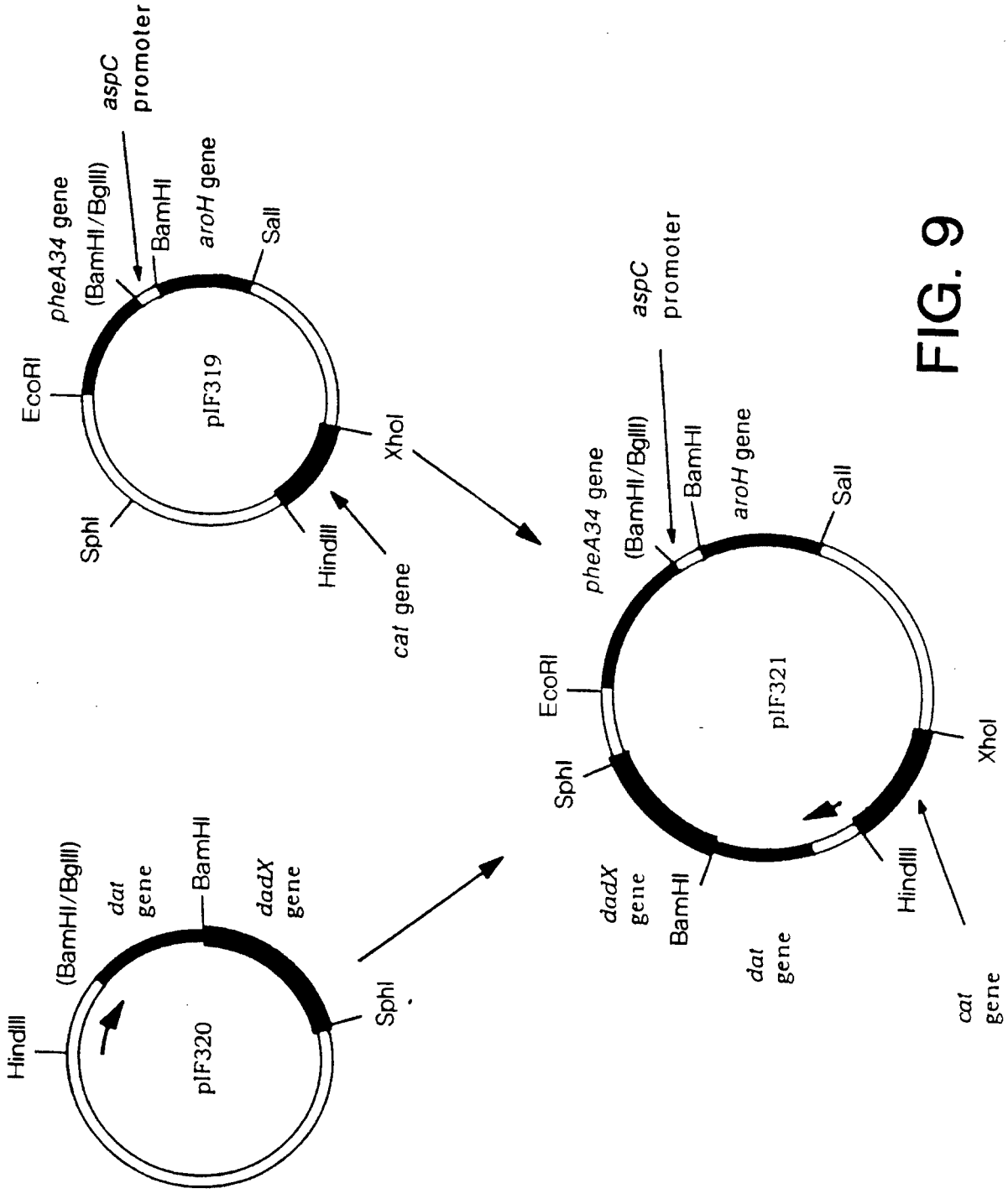
aspC promoter
pheA34 gene (BamHI/BglII)
BamHI
aroH gene
SalI
EcoRI
pIF319
XhoI
SphI
HindIII
cat gene
(BamHI/BglII)
dat gene
BamHI
dadX gene
HindIII
pIF320
SphI
aspC promoter
pheA34 gene (BamHI/BglII)
BamHI
aroH gene
SalI
EcoRI
pIF321
XhoI
SphI
dadX gene
BamHI
dat gene
HindIII
cat gene

FIG. 9

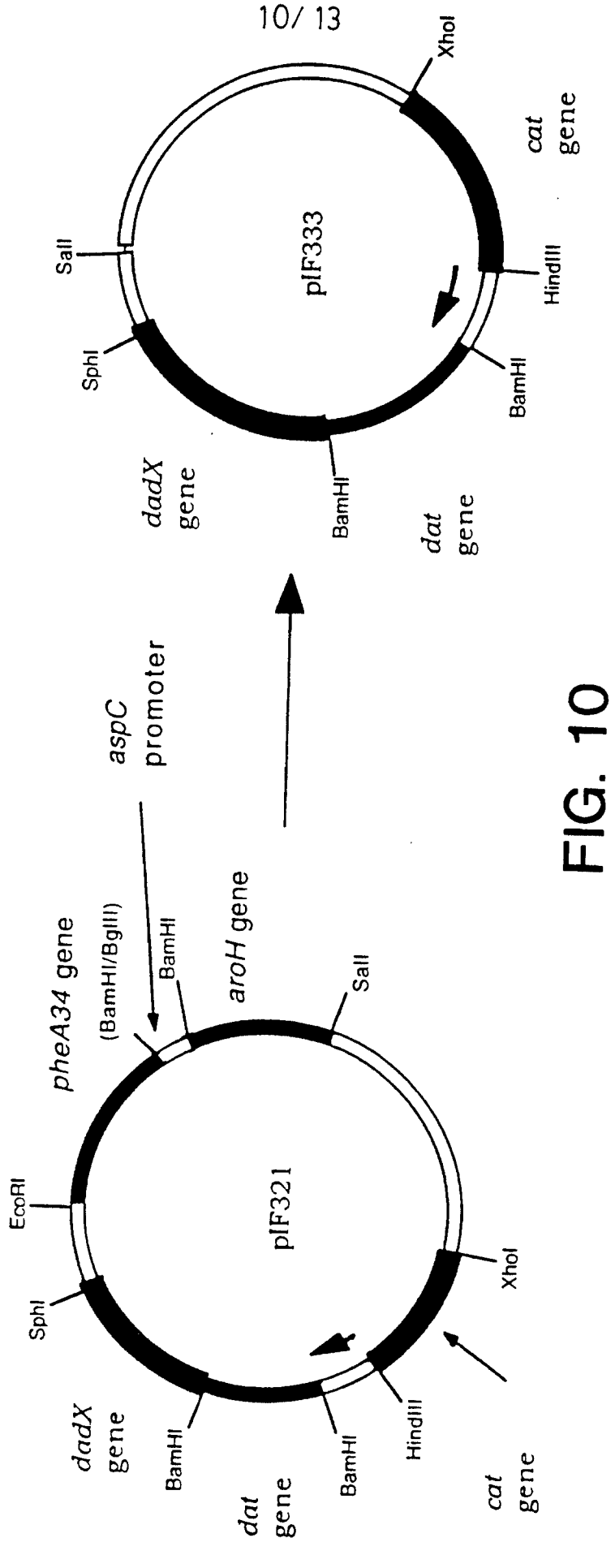
pIF333
XhoI
cat gene
HindIII
BamHI
dat gene
BamHI
dadX gene
SphI
SalI
aspC promoter
pheA34 gene
(BamHI/BglII)
BamHI
aroH gene
SalI
pIF321
XhoI
cat gene
HindIII
BamHI
dat gene
BamHI
dadX gene
SphI
EcoRI

FIG. 10

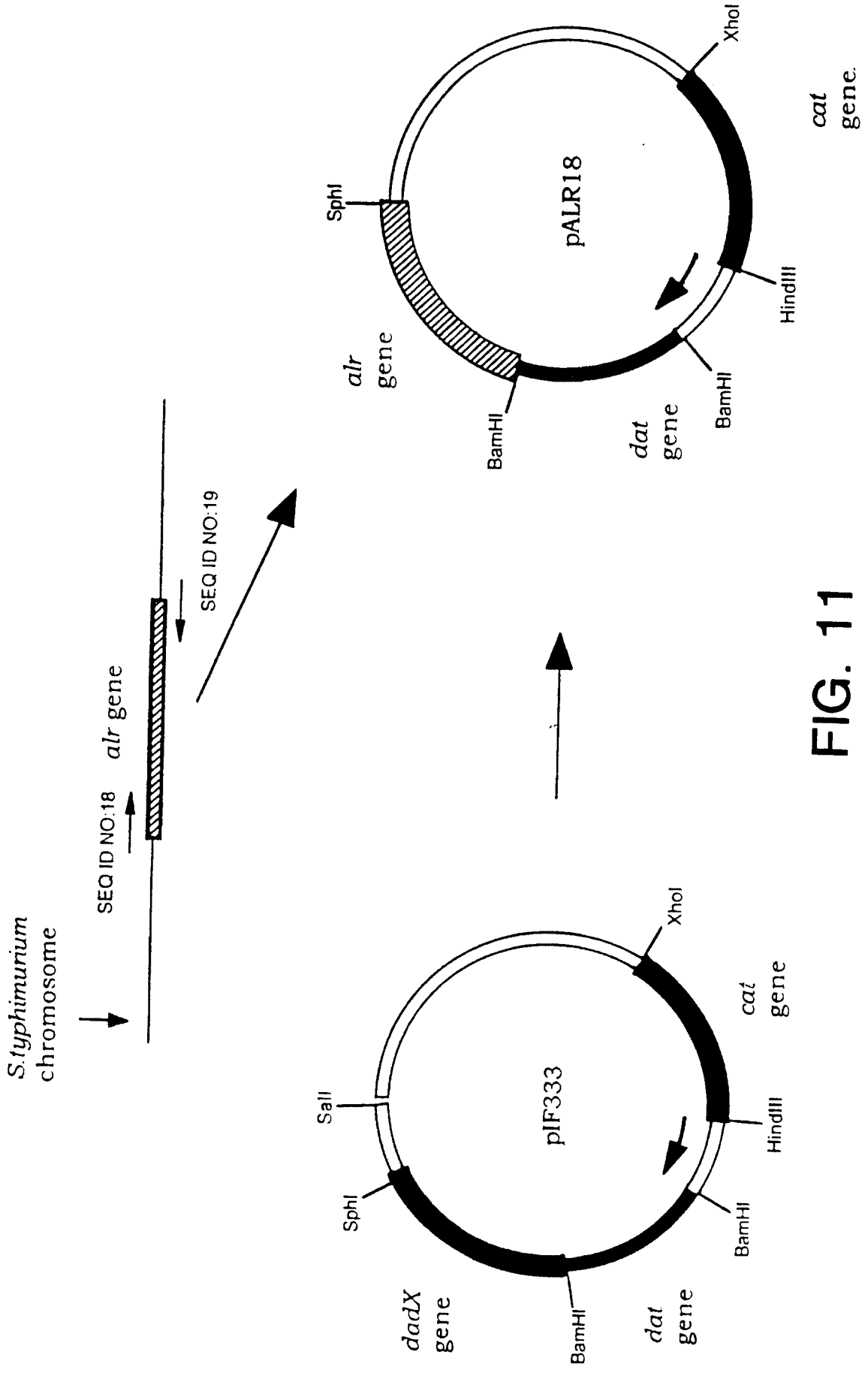
S.typhimurium chromosome
SEQ ID NO:18
alr gene
SEQ ID NO:19
pALR18
alr gene
SphI
BamHI
dat gene
BamHI
HindIII
XhoI
cat gene
pIF333
dadX gene
SphI
SalI
BamHI
dat gene
BamHI
HindIII
XhoI
cat gene

FIG. 11

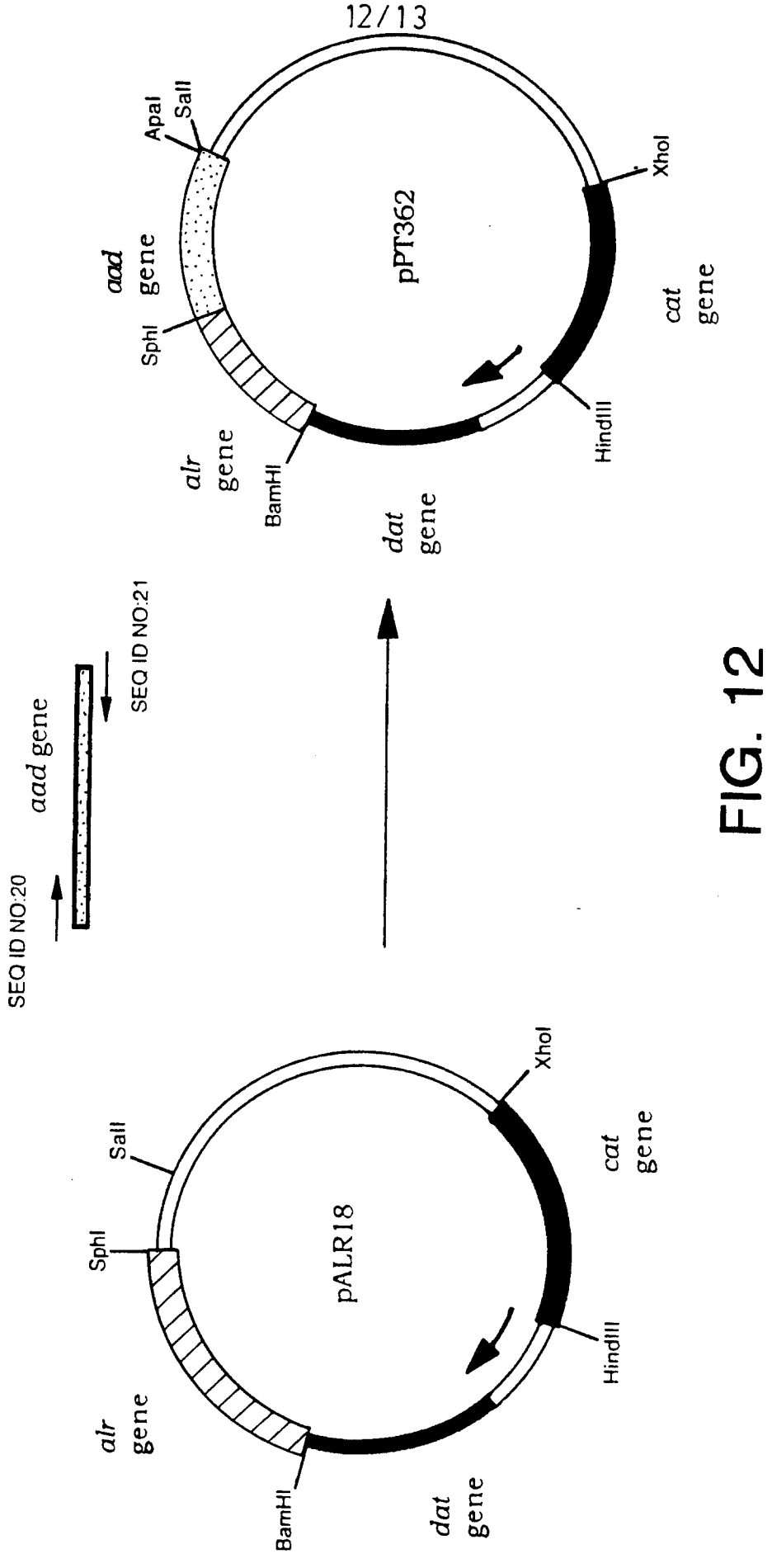
ApaI
SalI
aad gene
SphI
alr gene
BamHI
pPT362
XhoI
cat gene
HindIII
dat gene
SEQ ID NO:21
aad gene
SEQ ID NO:20
SalI
SphI
alr gene
BamHI
pALR18
XhoI
cat gene
HindIII
dat gene

FIG. 12

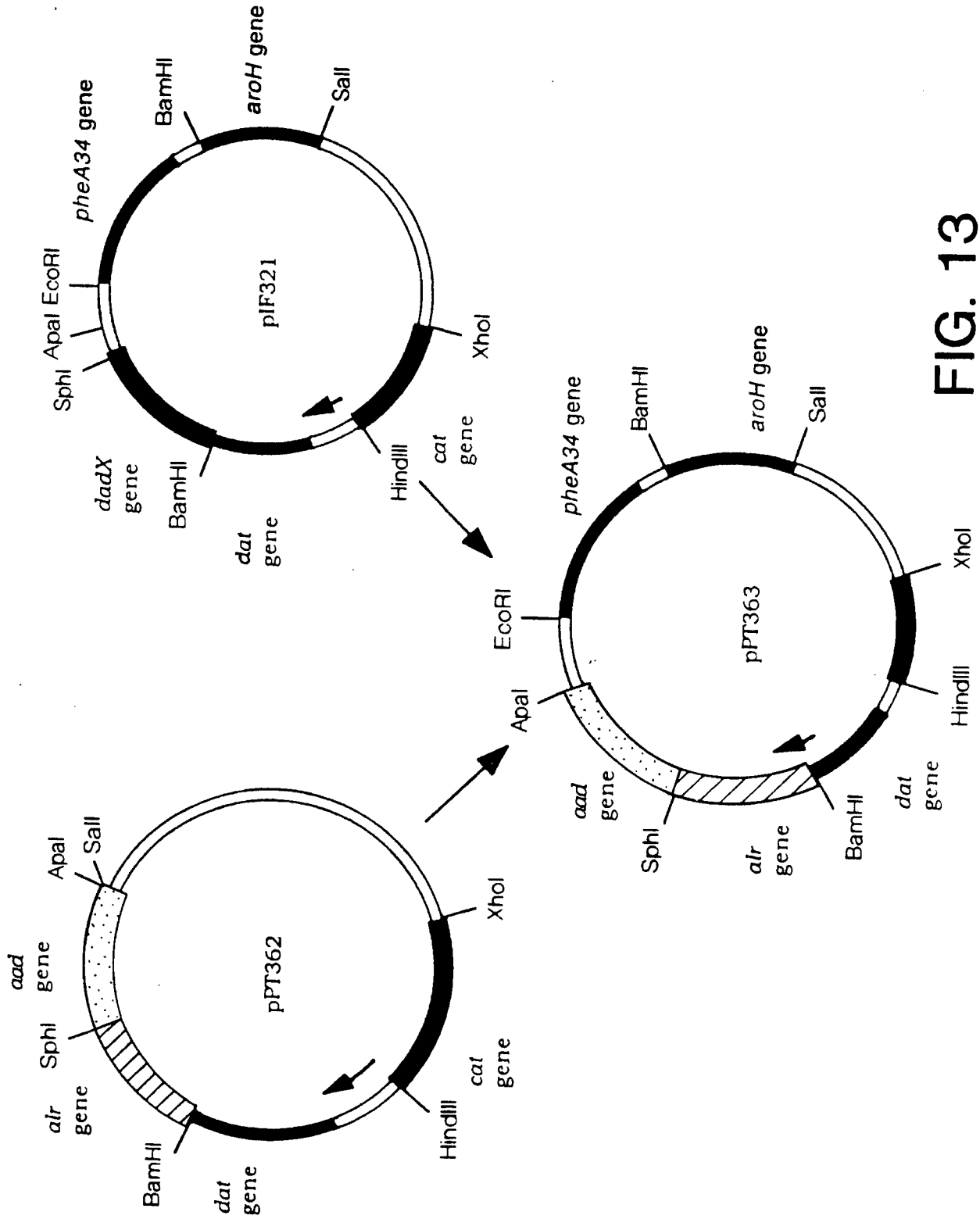
pheA34 gene
BamHI
aroH gene
SalI
ApaI EcoRI
SphI
pIF321
XhoI
dadX gene
BamHI
HindIII
cat gene
dat gene
pheA34 gene
BamHI
aroH gene
SalI
EcoRI
pPT363
XhoI
ApaI
HindIII
aad gene
SphI
alr gene
BamHI
dat gene
aad gene
ApaI
SalI
SphI
pPT362
XhoI
alr gene
HindIII
cat gene
BamHI
dat gene

FIG. 13