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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/80, 15/53, 9/08 C12N 1/15 // (C12N 1/15 C12R 1:68)

A2

(11) International Publication Number: **WO 93/18166**

(43) International Publication Date: 16 September 1993 (16.09.93)

(21) International Application Number: PCT/US93/02020

(22) International Filing Date: 4 March 1993 (04.03.93)

(30) Priority data:

07/846,181	4 March 1992 (04.03.92)	US
07/845,989	4 March 1992 (04.03.92)	US

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(81) Designated States: CA, FI, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: PRODUCTION OF *ASPERGILLUS NIGER CATALASE-R*

(57) Abstract

The invention discloses the application of genetic engineering techniques to create novel strains of *A. niger* which produce high levels of catalase *catR* gene product, catalase-R) while generating minimal sodium gluconate waste material. Use of an Aspergillus glucoamylase promoter gene.

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PRODUCTION OF *ASPERGILLUS NIGER* CATALASE-R

Field of the Invention:

The invention relates to the application of genetic engineering techniques to create novel strains of *A. niger* which produce high levels of an endogenous catalase enzyme (*catR* gene product, catalase-R) while generating minimal sodium gluconate waste material. Specifically, high levels of catalase-R are generated through replacement of the endogenous *catR* gene promoter with the *A. niger* glucoamylase (*glaA*) gene promoter which results in not only higher levels of catalase-R, but also eliminates the requirement for hydrogen peroxide to act as an inducer for catalase synthesis, and deletion of the endogenous glucose oxidase (*goxA*) gene greatly reduces the level of sodium gluconate waste product, thereby minimizing the need for expensive waste handling.

Background of the Invention:

Catalases [hydrogen peroxide: hydrogen peroxide oxidoreductases (EC 1.11.1.6)] are enzymes which catalyze the conversion of hydrogen peroxide (H_2O_2) to oxygen (O_2) and water (H_2O) according to the following formula:

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

These ubiquitous enzymes have been purified from a variety of animal tissues, plants and microorganisms (Chance and Maehly 1955 Methods Enzymol. **2**: 764-791; Jones and Wilson 1978 in H. Sigel (ed.), *Metal Ions in Biological Systems*, Vol. 7, Marcel Dekker Inc., New York). Nearly all forms of the enzyme which have been characterized consist of four polypeptide subunits, each having a molecular weight of 50,000 to 60,000 and containing one protohemin prosthetic group per subunit (Wasserman and Hultin 1981 Arch. Biochem. Biophys. **212**: 385-392; Hartig and Ruis 1986 Eur. J. Biochem. **160**: 487-490). Bovine liver catalase has been the most extensively studied variety of this enzyme [Schonbaum and Chance 1976 in *The*

Enzymes (P.D. Boyer, ed.) 3rd edn., vol. 13, pp. 363-408, Academic Press, New York]. The complete amino acid sequence and three dimensional structure of bovine liver catalase are known (Schroeder, et al., 1982 Arch. Biochem. Biophys. **214**: 397-412; Murthy, et al., 1981 J. Mol. Biol. **152**: 465-499).

Although less well-studied from a biochemical and biophysical standpoint, catalases from filamentous fungi have several characteristics that distinguish them from their mammalian counterparts. While similar in subunit number and heme content, fungal catalases are substantially larger molecules than those from other organisms, having subunit molecular weights ranging from 80,000 to 97,000 (Vainshtein, et al., 1986 J. Mol. Biol. **188**: 63-72; Jacob and Orme-Johnson 1979 Biochem. **18**: 2967-2975; Jones, et al., 1987 Biochim. Biophys. Acta **913**: 395-398). More importantly, catalases from fungi such as *Aspergillus niger* are more stable than beef liver catalase to proteolysis and to inactivation by glutaraldehyde, SDS, and have lower affinity for catalase inhibitors such as cyanide, azide and fluoride (Wasserman and Hultin 1981 Arch. Biochem. Biophys. **212**: 385-392). In addition, *A. niger* catalase is significantly more stable than beef liver catalase when subjected to extremes of pH, hydrogen peroxide, and temperature (Scott and Hammer 1960 Enzymologia **22**: 229-237). Although fungal catalases offer stability advantages, the corresponding mammalian enzymes such as beef liver catalase appear to have higher catalytic activity (Gruft, et al., 1978; Kikuchi-Torii, et al., 1982). However, since enzyme stability is an important factor in the biotechnological utilization of enzymes, there has been considerable interest in the use of fungal catalases, especially for applications involving neutralization of high concentrations of hydrogen peroxide. Vasudevan and Weiland (1990 Biotechnol. Bioeng. **36**: 783-789) observed that the rate of deactivation in H_2O_2 was at least an order of magnatude lower for *A. niger* catalase than for beef liver catalase. The differences in stability of these two enzymes can probably be attributed to differences in structural characteristics and

composition of the proteins [Vasudevan and Weiland 1990 Biotechnol. Bioeng. **36:** 783-789].

Catalase preparations from *A. niger* are sold commercially for diagnostic enzyme kits, for the enzymatic production of sodium gluconate from glucose, for the neutralization of H_2O_2 waste, and for the removal of H_2O_2 and/or generation of O_2 in foods and beverages. Traditionally, beef liver catalase has been the preferred enzyme for diagnostic purposes and for pharmaceutical-related applications (e.g., contact-lens cleaning/disinfection/H_2O_2 neutralization). However, recent outbreaks of a slow-virus disease known as BSE (bovine spongiform encephalopathy) in European cattle herds and fear that this disease might be spread to man [Dealler and Lacey 1991 Nutr. Health (Bicester) **7:** 117-134; Dealler and Lacey 1990 Food Microbiol. **7:** 253-280] have aroused interests in finding alternatives to beef liver catalase for most industrial applications.

Little information has been published regarding the regulation of catalase synthesis in *A. niger*. However, it has been observed that catalase is produced in response to the generation of H_2O_2 during growth of the organism on glucose or fatty acids. For example, during the metabolism of glucose, H_2O_2 is formed by oxidation of the sugar to give gluconate. This reaction is catalyzed by the enzyme glucose oxidase:

$$\text{Glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{Gluconate} + H_2O_2$$

Cellular metabolism of fatty acids, which occurs in specialized organelles known as peroxisomes, also yields H_2O_2 which induces the formation of catalase. However, in a distantly related fungus (yeast), *Saccharomyces cerevisiae*, a specific catalase is induced during growth on fatty acids. This catalase, termed catalase-A (atypical), is localized chiefly in peroxisomes where fatty acid oxidation occurs. A second *S. cerevisiae* enzyme, catalase-T (typical) is a soluble cytoplasmic enzyme

which is synthesized in response to a variety of other metabolic and environmental stresses. These two yeast catalases are the products of two different nuclear genes, designated *CTA1* and *CTT1*. Similarly, two catalase genes have been isolated from *A. niger* (Genencor International, Inc., unpublished). The *A. niger catA* gene, cloned by cross-hybridization to the yeast *CTA1* gene, encodes a catalase enzyme which is induced primarily during growth on fatty acids and is presumably peroxisomal. This enzyme (catalase-A) is not of commercial importance at this time, however, a second cloned *A. niger* catalase gene, designated as *catR*, encodes a soluble cytoplasmic enzyme (catalase-R) which represents the major activity in commercial catalase preparations.

Because of the obvious commercial interest in *A. niger* catalases, it would be desirable to obtain *A. niger* strains which produce increased levels of the *catR* gene product. Furthermore, it would be a significant advantage to effect high levels of catalase synthesis without the need to generate hydrogen peroxide as an inducer. Concomitant with the generation of hydrogen peroxide is the formation of sodium gluconate which represents a waste disposal problem. Thus, it is also highly desirable to minimize the production of gluconate in large scale fermentations with catalase production strains of *A. niger*. This invention discloses a solution for simultaneously accomplishing all of these objectives.

Summary of the Invention:

It has been discovered that it is possible to increase the expression of catalase-R (*catR* gene product) without the need to supply hydrogen peroxide as an inducer of catalase synthesis. Simultaneously, it was discovered that elimination of glucose oxidase gene expression (by *goxA* gene deletion) minimizes the generation of gluconate waste material, thereby circumventing the need for expensive waste treatment processes.

The invention includes a gene encoding *Aspergillus niger* catalase-R (*catR* gene) to which promoter and terminator elements of the *A. niger* glucoamylase (*glaA*) gene were functionally attached. Concomitantly, the coding region of the *A. niger* glucose oxidase (*goxA*) gene was destroyed using a targeted gene replacement strategy. The invention also includes a transformed *A. niger* organism which is capable of expressing high levels of catalase-R without hydrogen peroxide induction. This organism contains a functional expression unit comprising the *catR* gene, to which the *A. niger glaA* gene promoter and terminator sequences have been functionally attached.

The inventors also disclose a method for producing high levels of catalase-R comprising growth of transformed *A. niger* cells which contain chromosomally integrated copies of the *catR* gene under operational control of the *A. niger glaA* promoter.

Figures:

Figure 1 is a diagrammatic representation of the construction of the *catR* expression plasmid which contains the *A. niger glaA* promoter, *catR* coding region, *glaA* terminator and *A. niger pyrG* gene. A linear fragment (EC2L) containing these components was excised by digestion with *NotI* and *PmeI* and used to transform the host strain *A. niger ΔgoxA pyrG metC*.

Figure 2 shows the nucleotide sequence and deduced amino acid sequence of the *A. niger catR* gene and flanking regions. Restriction sites for enzymes recognizing hexanucleotide and octanucleotide sequences are shown. Introns are denoted by dashed lines. Deduced amino acid sequences corresponding to peptides sequenced directly from the catalase-R protein are underlined with a solid bar.

Figure 3 is the complete nucleotide sequence of the linear fragment (EC2L) used to transform *A. niger ΔgoxA pyrG metC*.

Figure 4, Panel A is a diagrammatic representation of the construction of the *A. niger* vector for deletion of the glucose oxidase (*goxA*) gene. A linear fragment comprising the *SmaI* - *ClaI* segment was excised and used to transform the host strain *A. niger pyrG*. Panel B is a schematic showing the expected integration event at the *goxA* locus which results in replacement of the *goxA* coding region with the *A. niger pyrG* gene.

Figure 5 is a graph showing catalase production among strains of *A. niger ΔgoxA pyrG metC* transformed with the *catR* expression cassette (EC2L). The original parent strain, *A. niger* FS-1, and the host strain *A. niger ΔgoxA pyrG metC* are included as controls. Each strain was grown in duplicate and the assay results from each are shown.

Description of the Preferred Embodiments.

The details of the *catR* expression vector construction and genetic modifications used to derive improved catalase production strains are described. One skilled in the art will understand that various changes in the following examples could be made. Accordingly, the examples are not intended to be limiting.

The techniques used in cloning the *A. niger catR* gene and construction of the *catR* expression cassette are conventional techniques described in Sambrook, et al., 1989 *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY.

1. Cloning and Characterization of the *A. niger catR* Gene.

Purified catalase-R was obtained from a commercial preparation of *A. niger* catalase (Fermcolase 1000, Genencor International, Inc.) and a series of proteolytic fragments were generated. These peptide fragments were subjected to amino acid sequence analysis. The amino acid sequence information was employed to design synthetic DNA probes for identification of *catR*-specific

cDNA sequences contained within a λgt11 library. Briefly, the peptide fragment Met-Phe-Trp-Asn-Ser-Leu-Ile-Pro-Ala-Glu-Gln-Gln-Met was used to design a pool of three synthetic oligonucleotides having the following sequences:

5' ATG TTC TGG AAC AGC CTG ATC CCC GCC GAG CAG CAG ATG 3'
5' ATG TTC TGG AAC TCC CTG ATC CCC GCC GAG CAG CAG ATG 3'
5' ATG TTC TGG AAC AGC TTG ATC CCC GCC GAG CAG CAG ATG 3'

This peptide was chosen because the amino acids give minimally degenerate codon choices, i.e., the differences among the three synthetic oligonucleotides represent alternate codon choices where there was no strong bias in the known codon usage pattern for *A. niger*. This position of this proteolytic fragment corresponds to peptide 3 shown in Figure 2. A clone containing a partial cDNA fragment was positively identified by hybridization with the synthetic DNA probe and nucleotide sequence analysis of this clone confirmed that it encoded catalase-R. This cloned cDNA segment was used to probe a library of *A. niger* genomic DNA. Subsequently, the entire *catR* gene, plus upstream and downstream transcriptional control elements, was assembled as a 9.0 kb *Hind*III - *Xho*I restriction fragment. The nucleotide sequence of the *catR* coding region has been determined and is given in Figure 3.

2. Construction of a Catalase Expression Vector-Cassette (EC2) Used for Transformation of *A. niger*.

The *catR* expression vector used for these studies utilizes transcriptional and translational control signals from the well-characterized *A. niger* glucoamylase *(glaA)* gene. Unlike the *catR* promoter, the strong *glaA* promoter does not require H_2O_2 for induction. Instead, the *glaA* promoter responds to the presence of starch, maltose or other malto-oligosaccharides (Nunberg, et al., 1984 Mol. Cell. Biol. **4**: 2306-2316; Barton, et al., 1972 J. Bacteriol. **111**: 771-777; Fowler, et al., 1990 Curr. Genet. **18**: 537-545). Thus, use of the *glaA* promoter allows construction of catalase production strains which are not dependent on the generation of hydrogen peroxide for induction of catalase synthesis. Construction of the

vector-cassette for expression of catalase under transcriptional control of the *glaA* promoter is outlined in Figure 1. The essential feature of this construct is that the glucoamylase-catalase expression unit (i.e., *glaA* promoter + *catR* coding region + *glaA* terminator) and the adjacent selectable marker (the *A. niger pyrG* gene) can be excised on a single *NotI* - *PmeI* restriction fragment (Figure 1).

The *catR* coding region was joined to the *glaA* promoter utilizing a synthetic oligonucleotide linker (13 base pairs) designed to couple these two DNA segments via a *BglII* site in the *glaA* promoter to a unique *SspI* site four base pairs after the *catR* start codon (introduced by site-directed mutagenesis). Insertion of this linker restores the nucleotide sequence of *catR* to that which existed prior to the site-directed mutagenesis and precisely fuses the *catR* coding region to the *glaA* promoter. In a description of the *glaA* promoter region given by Fowler, et al., (1990 Curr. Genet. **18**: 537-545) it was noted that there are DNA sequences far upstream of the start codon which are required for high level expression. These sequences, which presumably represent transcriptional enhancer elements, are included on the 1.9 kb *glaA* promoter segment included in construction of the *catR* expression cassette. Similarly, the *glaA* terminator region was linked to the 3'-end of *catR* via a naturally-occurring *ClaI* site downstream of the catalase-R gene stop codon. An *XbaI* site adjacent to *ClaI* was incorporated using a synthetic DNA linker and was then used to complete the terminator fusion. This terminator segment, which encodes information necessary for proper polyadenylation and termination of transcription, is the same segment as that which was used for Genencor's chymosin expression vector (Cullen, et al., 1987 Bio/Technol. **5**: 369-376). A restriction fragment containing the *A. niger pyrG* gene (Wilson, et al., 1988 Nucl. Acids Res. **16**: 2339) was subcloned adjacent to the *glaA* terminator such that the entire glucoamylase-catalase-selectable marker cassette was encoded on

a single restriction fragment (the nucleotide sequence of this fragment (EC2L) is given in Figure 3).

3. Development of *A. niger* Strains to be Used in the Production of Catalase.

Features of the *A. niger* strain used as a host for expression of the glucoamylase-catalase cassette include a) uridine-requiring auxotrophy, specifically a *pyrG* auxotrophic mutation, b) deletion of the gene encoding glucose oxidase, *goxA*, and (c) a methionine-requiring auxotrophy, specifically mutation which renders the cells deficient in cystathionase (*metC*) activity. While the *metC* marker is not required for high level expression of catalase-R, it was included as a feature of the host strain to satisfy limited survivability regulation of government regulatory agencies. The catalase expression cassette described above was used to transform the *A. niger* Δ*goxA pyrG metC* strain and the resulting transformants were screened in shake flask cultures for their ability to produce high levels of catalase. From these transformants, the highest catalase producers were selected for further study. Shake flask cultures were grown for two days at 33°C in 50 ml of a liquid medium that was made according to the following recipe: For each liter of medium add maltodextrin [Staley 200, A.E. Staley Co., (100 g)], ammonium sulfate (4 g), calcium chloride (0.4 g), magnesium sulfate (0.6 g), corn steep liquor [Archer Daniels Midland Co., (10 g)], and potassium phosphate (3 g); The volume is brought to 500 ml with distilled water, the pH is adjusted to 7.0, and the solution is autoclaved; Separately a 500 ml solution of 12% calcium carbonate is made in distilled water, the pH is adjusted to 7.0, and the solution autoclaved. The two sterile mixtures were combined aseptically to give one liter of catalase production medium. After two days growth, the mycelia were harvested by filtration (Miracloth, Calbiochem, Inc.), and the cells were rapidly frozen in liquid nitrogen. The cells were disrupted by grinding the frozen pellet in an electric coffee grinder for approximately 60 sec or until a fine powder was obtained. The disrupted cells were resuspended in an extraction buffer that

contained 100 mM sodium formate, pH 7, 0.01% sodium dodecylsulfate, and 1 mM each of phenylmethyl sulfonyl fluoride and pepstatin. Insoluble debris was removed by centrifugation at approximately 1500 ¥*g*, and the activity of soluble catalase in the extract was measured by previously described methods (Patti and Bonet-Maury 1953 Bull Soc. Biol. **35**: 1177; Teranishi, et al., 1974 Agric. Biol. Chem. **38**: 1213). Specific methods for generation of the catalase production organisms are outlined below. The parental strain for all studies described herein was *A. niger* FS-1 (NRRL3).

Isolation of A. niger FS-1 pyrG Strains.
5-Fluoro-orotic acid (FOA), a toxic analog of orotic acid, has been used to select uridine-requiring auxotrophs in filamentous fungi and yeasts (VanHartingveldt, et al., 1987 Mol. Gen. Genet. **206**: 71-75). Fungal strains deficient in orotidine-5'-monophosphate decarboxylase (*pyrG* gene product), are resistant to FOA and require exogenous uridine for growth. The *A. niger pyrG* gene was cloned (Wilson, et al., 1988 Nucl. Acids Res. **16**: 2339) and used as a selectable marker for the transformation of *pyrG* mutant strains. An advantage of using FOA as a positive selection for *pyrG* auxotrophs is that spontaneous mutants can be selected without need for excessive mutagenesis and screening. The method of selecting *A. niger* FS-1 *pyrG* mutants is as follows: Spores of *A. niger* FS-1 were spread onto the surface of minimal medium plates containing 2 mg/ml uridine and 1.2 mg/ml FOA. Resistant colonies (FOA^r) were evident after 2-3 days growth at 37°C. Spores from six FOA^r colonies were streaked onto fresh medium containing FOA, and isolated colonies were picked for further analysis. Three of the six FOA^r strains were shown to require uridine for growth. To determine which of the uridine-requiring strains had a non-functional *pyrG* gene, each of the strains was tested for its ability to be transformed (i.e., complemented) with a plasmid containing the *A. nidulans pyrG* gene. Only one strain, FS-1 *pyrG1*, gave transformants (an approximate frequency of 10 transformants per μg DNA) indicating that it carried a *pyrG*

mutation. This strain was used for all subsequent experimentation.

Generation of A. niger FS-1 ΔgoxA Strains.

To generate a chromosomal deletion in the *goxA* gene, a vector was constructed which contained 5'- and 3'-flanking DNA sequences from the *goxA* gene and a selectable *pyrG* gene inserted in place of a portion of the *goxA* coding region (see Figure 4). For complete information regarding the nucleotide sequence of the *goxA* gene, consult Frederick, et al., 1990 J. Biol. Chem. **265**: 3793-3802 989 and Kriechbaum, et al., 1989 FEBS Lett. **255**:63-66. Briefly, a 4.1 kb *ClaI-SmaI* fragment comprising the *A. niger* FS-1 *goxA* gene was subcloned into a pUC218-derivative (from which the *Eco*RI site had previously been removed) to give pUC218*goxA*. The *A. niger pyrG* gene was isolated from pUC4XL as an *Eco*RI fragment having 27 bp and 16 bp of pUC4XL polylinker DNA at either end. The *goxA* coding region was subsequently removed by digestion with *Eco*RI and the remaining plasmid fragment was ligated with the *Eco*RI fragment containing the *A. niger pyrG* gene to create pUC218Δ*goxA*. From this plasmid a 4.75 kb *SmaI-XbaI* restriction fragment which contains 5'- and 3'-flanking regions of the *goxA* gene with part of the *goxA* coding sequence removed and replaced with a functional *pyrG* gene was isolated. Use of this fragment to transform *A. niger* FS-1 *pyrG1* with selection for uridine prototrophy resulted in the isolation of several strains which failed to give a blue color on glucose oxidase indicator plates (Witteveen, et al., 1990 Appl. Microbiol. Biotechnol. **33**: 683-686). Southern blotting analysis of genomic DNA extracted from these *goxA*-deficient transformants indicated that the Δ*goxA::pyrG* cassette had integrated via a homologous recombination event at the *goxA* locus (as diagramed in Figure 4B). In other words, the selectable *pyrG* gene had replaced the *goxA* coding region.

As shown in Figure 5, catalase production in Δ*goxA* mutants was approximately three- to six-fold lower than the parental strain

FS-1. We interpret these data to indicate that in the absence of glucose oxidase little hydrogen peroxide is generated, and this in turn has an adverse effect on catalase induction.

Isolation of A. niger FS-1 ΔgoxA pyrG Strains.
Spontaneous uridine-requiring mutants of *A. niger FS-1 ΔgoxA* were selected using FOA as described above. This step was necessary for subsequent transformation of the strain with the *pyrG*-based EC2 cassette.

Isolation of an A. niger FS-1 ΔgoxA pyrG metC Strain.
In order to limit the survivability of a recombinant catalase production organism in the environment, a methionine-requiring auxotrophy was introduced in the following manner. Spores of *A. niger FS-1 ΔgoxA pyrG* were mutagenized with UV light (95% killing) and survivors were subjected to filtration enrichment in *Aspergillus* minimal medium. With this technique, unwanted prototrophs germinate and grow to form mycelia which can be removed by filtration. Auxotrophic cells cannot germinate or grow in minimal medium, and therefore pass through porous filters (e.g., Miracloth, Calbiochem, Inc.). After several rounds of filtration and growth, the remaining spores were plated onto complete medium. Colonies were patched from these plates onto minimal medium agar and to fresh complete medium plates. Those which grew on complete medium but not on minimal agar were auxotrophic. From the population of auxotrophs, one colony was identified which grew on minimal medium supplemented with methionine. Upon further testing, it was discovered that the strain was defective in a specific step of the methionine biosynthetic pathway. Growth was supported by the addition of either homocysteine or methionine, but not by either homoserine or cystathionine. Based on the known biosynthetic pathway for methionine, it appears that this methionine-requiring auxotroph was deficient in cystathionase activity, and thus, it was given the designation of *metC* by convention with other organisms.

4. Transformation of the *A. niger FS-1 ΔgoxA pyrG metC* Strain and Characterization of Catalase Overproducing Strains.

The catalase expression cassette (in linear form) was isolated following digestion of the pUC-EC2 plasmid with *PmeI* and *NotI* and purification of the EC2 fragment by preparative gel electrophoresis. The purified DNA fragment was then used to transform the *A. niger ΔgoxA pyrG metC* strain, and prototrophic transformants were screened in shake flask culture for their ability to produce catalase. From approximately fifty transformants screened in shake flasks, ten were identified that produced significantly higher catalase levels than control strains. These ten strains were re-evaluated in duplicate shake flask cultures, and the results of catalase activity assays are shown in Figure 5. Nine of the ten strains produced significantly higher levels of catalase-R than the parent strain FS-1. Two of the transformants (EC2L-19, EC2L-23) produced catalase yields in shake flask cultures that were roughly ten to fifteen times the level produced by *A. niger* FS-1, and these strains were chosen for testing under large scale production conditions. Fermentation experiments at the 10 liter and 50,000 liter scale have shown that catalase-R production from transformant EC2L-23 corresponds to the level of catalase-R expression seen in shake flask studies.

Furthermore, HPLC analyses of organic acids produced during fermentations of *A. niger* EC2L-23 and the parental strain FS-1 gave the following yields of sodium gluconate:

Strain	sodium gluconate (mg/L)
FS-1	>200,000
EC2L-23 (run 27)	48
EC2L-23 (run 28)	123

These data show a dramatic decrease in the production of sodium gluconate waste material by transformant EC2L-23.

WHAT IS CLAIMED IS:

1. A gene sequence consisting essentially of the *Aspergillus niger catR* gene wherein the native *Aspergillus niger catR* promoter has been deleted and an *Aspergillus* glucoamylase promoter gene has been functionally attached.

2. The gene sequence according to Claim 1 wherein the *Aspergillus* glucoamylase promoter is from *A. niger*.

3. The gene sequence according to Claims 1 or 2 which has been inserted into a replicable plasmid of a vector capable of integration into an *Aspergillus niger* genome.

4. A gene sequence encoding for catalase-R from *Aspergillus Niger*.

5. A gene sequence encoding for catalase-R from *Aspergillus* comprising the sequence in SEQ ID NO:4 or single or multiple base substitutions, deletions, insertions or inversion encoding said catalase-R having catalase activity.

6. An *Aspergillus niger* which has been transformed with a gene comprising the *Aspergillus catR* gene without the *Aspergillus niger catR* promoter and wherein an *Aspergillus* glucoamylase promoter gene has been functionally attached.

7. The *Aspergillus niger* according to Claim 6 wherein the glucoamylase promoter is from *A. niger*.

8. The *Aspergillus niger* according to Claim 6 wherein the native *catR* promoter is deleted.

9. The *Aspergillus niger* according to Claim 6 wherein the native glucose oxidase gene is deleted.

10. A method of producing the gene product of the *catR* gene which comprises culturing an *Aspergillus niger* transformed with the *catR* gene to which an *Aspergillus* glucoamylase promoter is functionally attached using assimilable sources of carbon and nitrogen.

11. The method according to Claim 10 wherein the glucoamylase promoter is from *A. niger*.

12. Substantially pure catalase-R from *Aspergillus Niger*.

13. An isolated catalase-R protein from *Aspergillus* comprising the amino acid sequence in SEQ ID NO:5 or functionally equivalent species or allelic variations thereof.

14. A method of producing the gene product of the *catR* gene which comprises transforming an *Aspergillus niger* host with a recombinant DNA vector comprising the *catR* gene fragment and culturing said host under conditions wherein said gene fragment is transcribed and translated.

15. The method of claim 14 wherein said *catR* gene fragment comprises the sequence shown in SEQ ID NO:6 or a portion thereof having catalase activity.

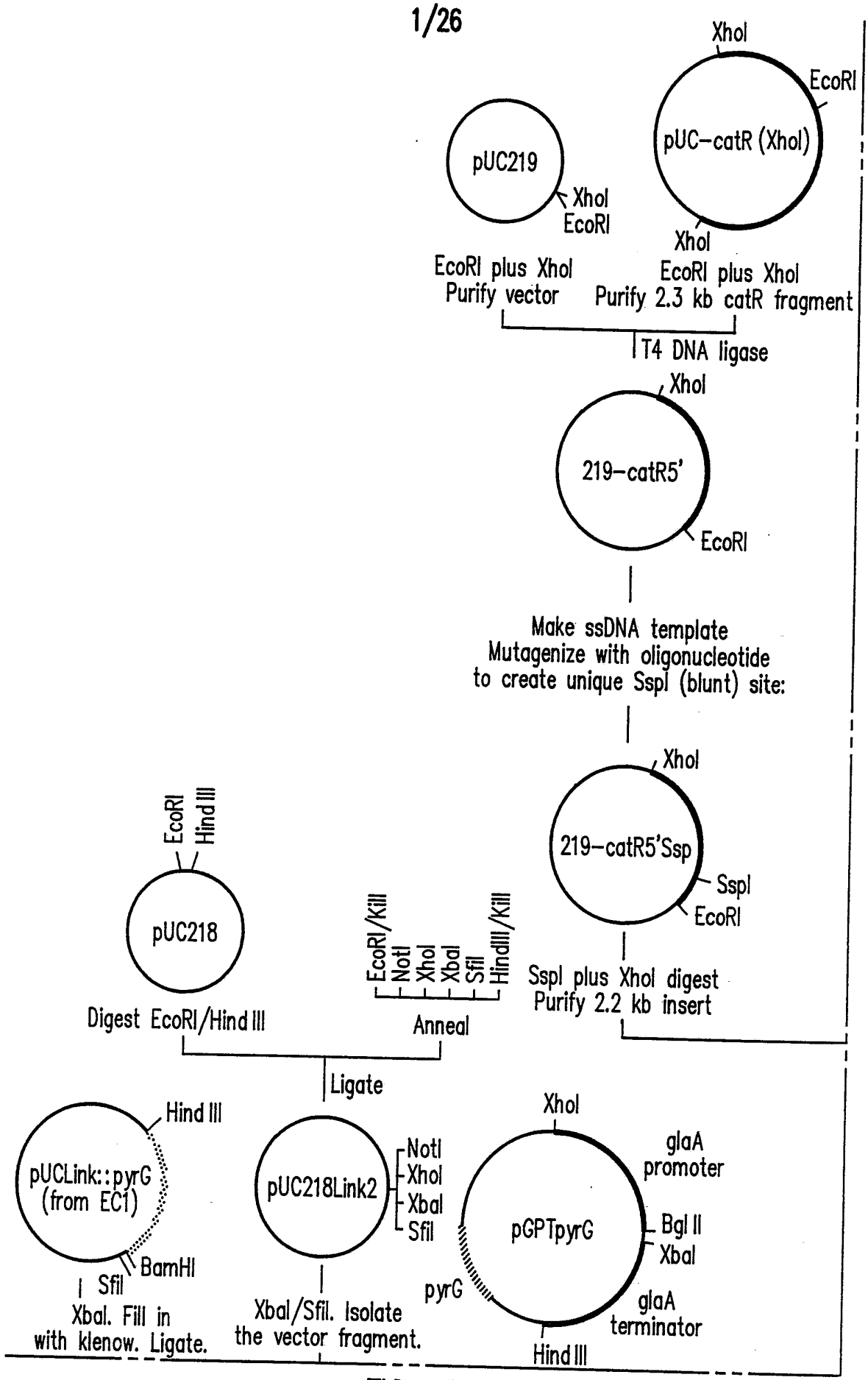

XhoI
EcoRI
pUC219
XhoI
EcoRI
pUC-catR (XhoI)
XhoI
EcoRI plus XhoI
Purify vector
EcoRI plus XhoI
Purify 2.3 kb catR fragment
T4 DNA ligase
XhoI
219-catR5'
EcoRI
Make ssDNA template
Mutagenize with oligonucleotide
to create unique SspI (blunt) site:
XhoI
219-catR5'Ssp
SspI
EcoRI
SspI plus XhoI digest
Purify 2.2 kb insert
EcoRI
Hind III
pUC218
Digest EcoRI/Hind III
EcoRI/KIII
NotI
XhoI
XbaI
SfiI
HindIII/KIII
Anneal
Ligate
Hind III
pUCLink::pyrG
(from EC1)
BamHI
SfiI
XbaI. Fill in
with klenow. Ligate.
pUC218Link2
NotI
XhoI
XbaI
SfiI
XbaI/SfiI. Isolate
the vector fragment.
XhoI
glaA
promoter
pGPTpyrG
BglII
XbaI
pyrG
glaA
terminator
Hind III

FIG. 1A

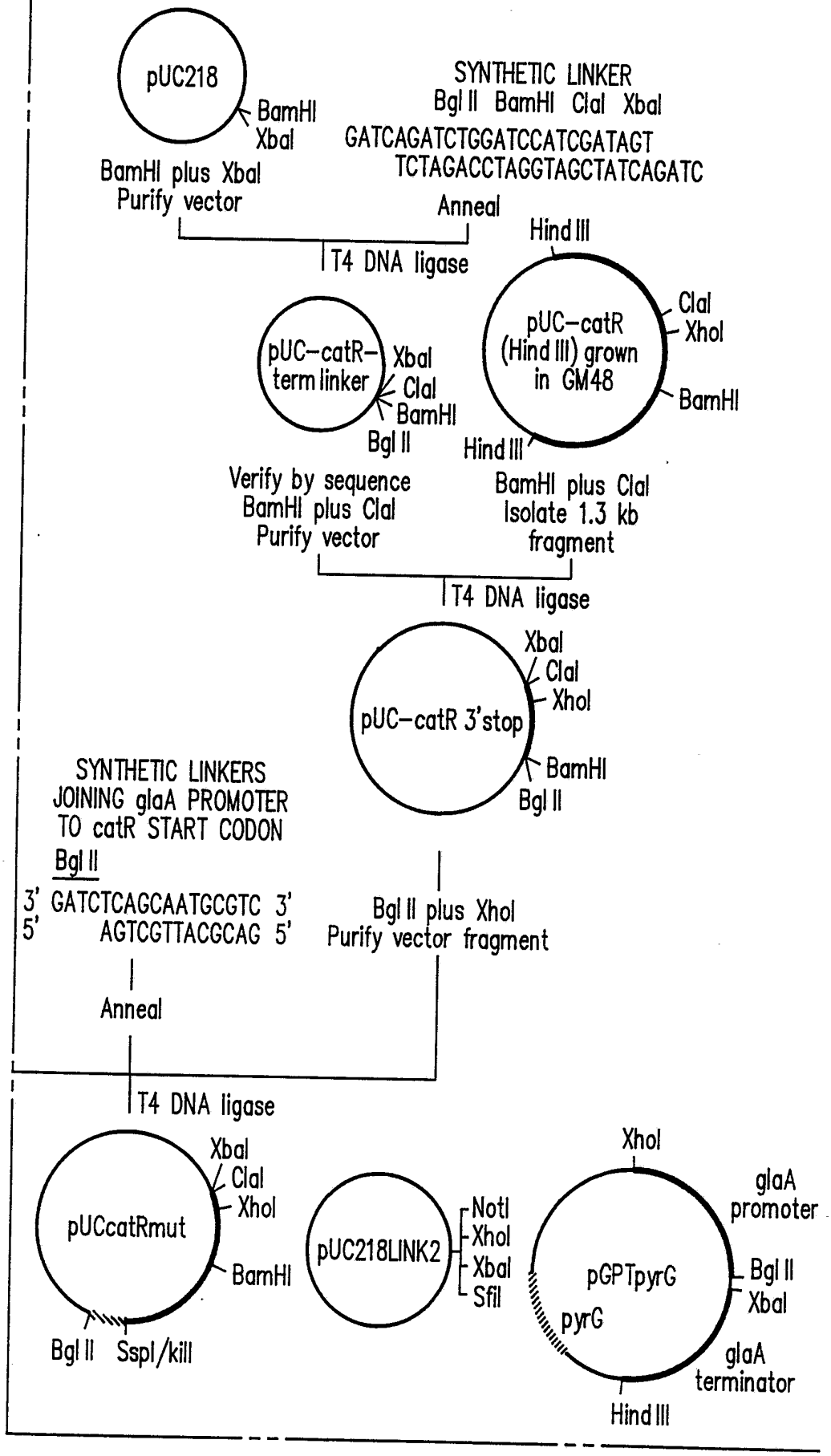

pUC218
BamHI
XbaI
BamHI plus XbaI
Purify vector
SYNTHETIC LINKER
Bgl II BamHI ClaI XbaI
GATCAGATCTGGATCCATCGATAGT
TCTAGACCTAGGTAGCTATCAGATC
Anneal
T4 DNA ligase
Hind III
pUC-catR
(Hind III) grown
in GM48
ClaI
XhoI
BamHI
Hind III
pUC-catR-
term linker
XbaI
ClaI
BamHI
Bgl II
Verify by sequence
BamHI plus ClaI
Purify vector
BamHI plus ClaI
Isolate 1.3 kb
fragment
T4 DNA ligase
pUC-catR 3'stop
XbaI
ClaI
XhoI
BamHI
Bgl II
SYNTHETIC LINKERS
JOINING glaA PROMOTER
TO catR START CODON
Bgl II
3' GATCTCAGCAATGCGTC 3'
5' AGTCGTTACGCAG 5'
Anneal
Bgl II plus XhoI
Purify vector fragment
T4 DNA ligase
pUCcatRmut
XbaI
ClaI
XhoI
BamHI
Bgl II
SspI/kill
pUC218LINK2
NotI
XhoI
XbaI
SfiI
XhoI
glaA
promoter
pGPTpyrG
Bgl II
XbaI
pyrG
glaA
terminator
Hind III

FIG. 1B

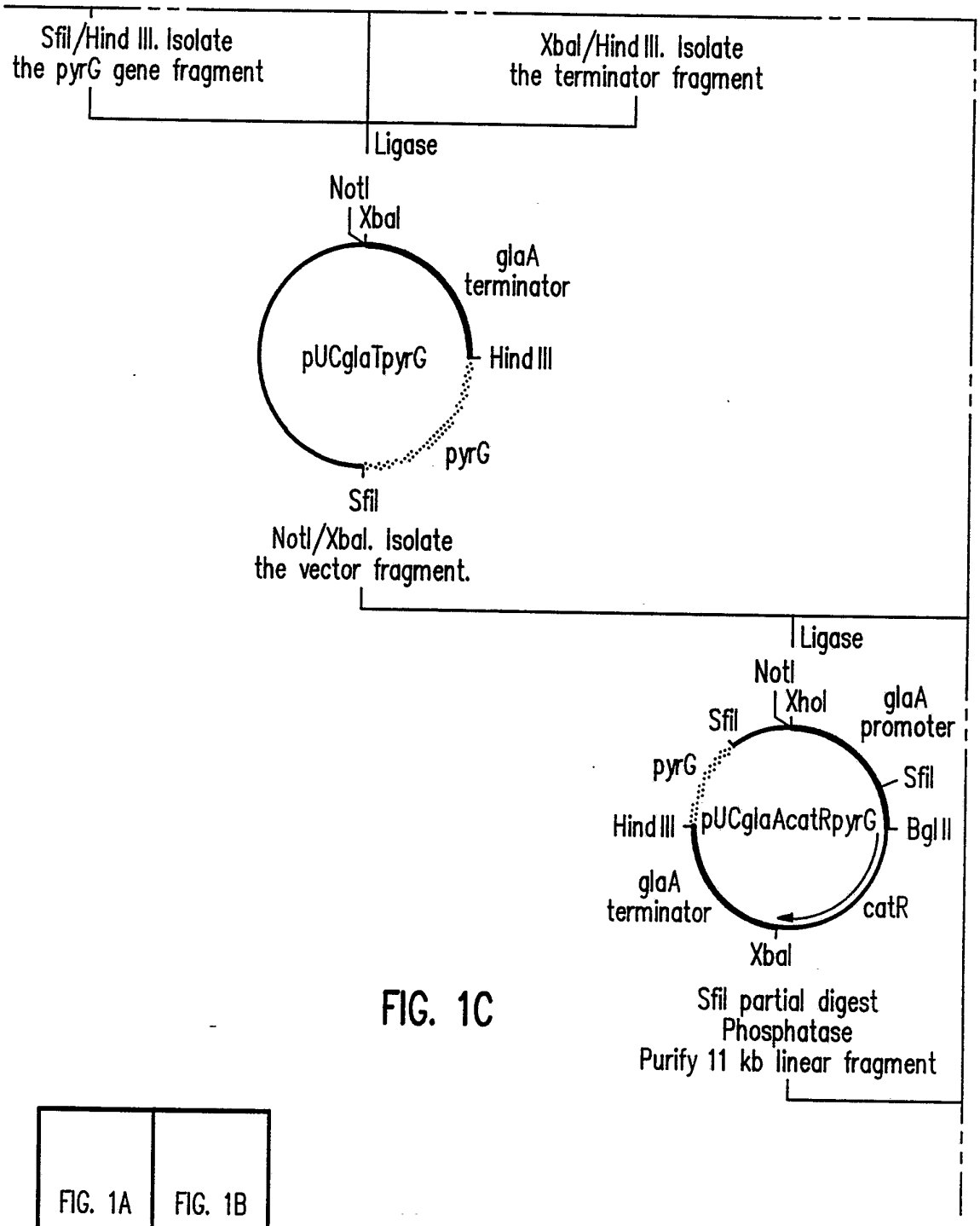


FIG. 1C

FIG. 1A	FIG. 1B
FIG. 1C	FIG. 1D

KEY TO FIG. 1

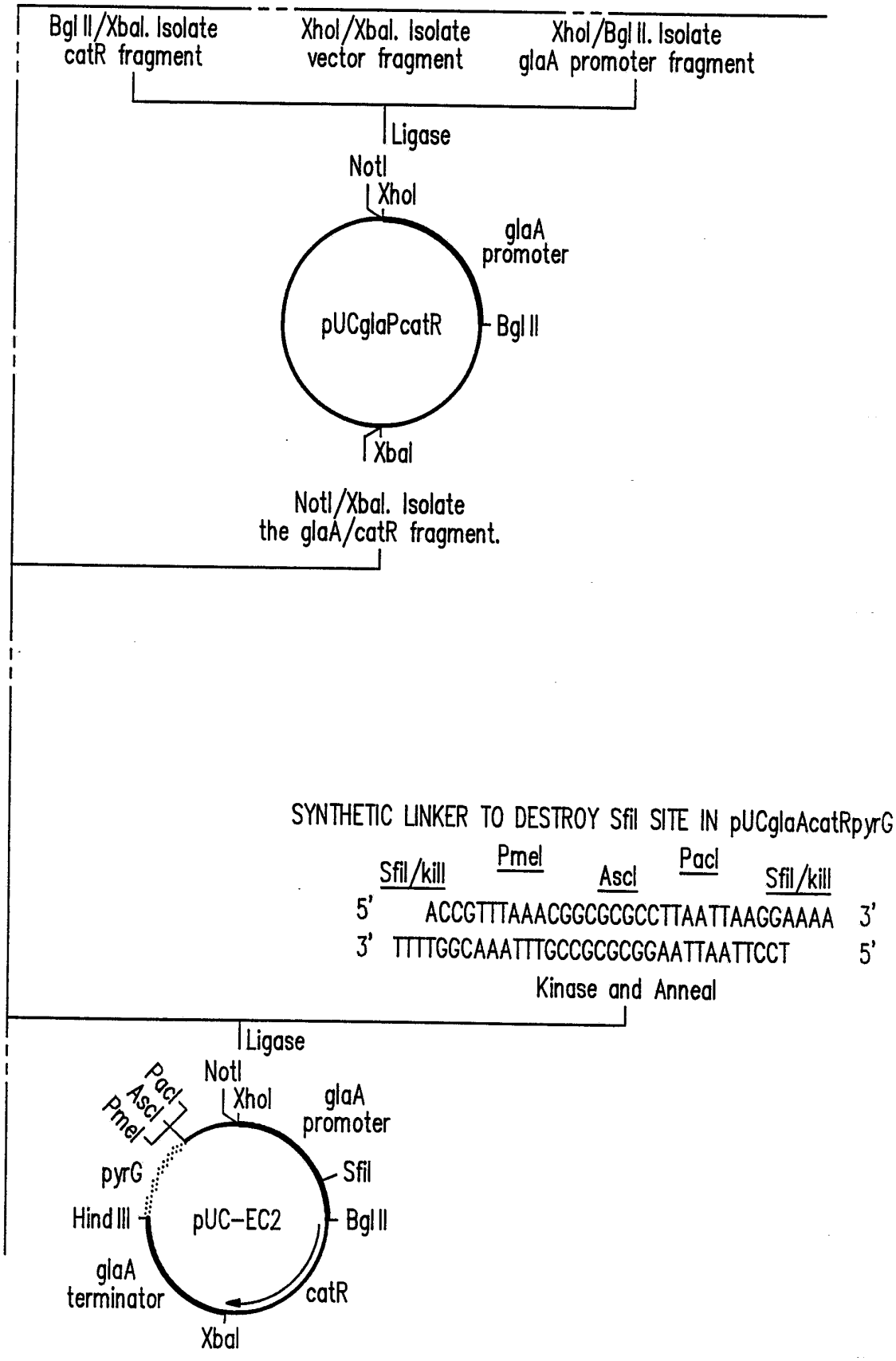

BglII/XbaI. Isolate catR fragment
XhoI/XbaI. Isolate vector fragment
XhoI/BglII. Isolate glaA promoter fragment
Ligase
NotI
XhoI
glaA promoter
pUCglaPcatR
BglII
XbaI
NotI/XbaI. Isolate the glaA/catR fragment.
SYNTHETIC LINKER TO DESTROY SfiI SITE IN pUCglaAcatRpyrG
SfiI/kill
PmeI
AscI
PacI
SfiI/kill
5' ACCGTTTAAACGGCGCGCCTTAATTAAGGAAAA 3'
3' TTTTGGCAAATTTGCCGCGCGGAATTAATTCCT 5'
Kinase and Anneal
Ligase
PacI
AscI
PmeI
NotI
XhoI
glaA promoter
pyrG
SfiI
HindIII
pUC-EC2
BglII
glaA terminator
catR
XbaI

FIG. 1D

```
 Dra III                                                           Esp3 I       Bcl I
CTTGTCACCGAGTGCCCGTTTGTCACTTGTTGTGGTGATCTTGAGCACATCGCGTTCCTCTCGTCTCATCACATCGAGTGATCAACATTG   90
                              Esp3 I      Acc III
CATGACCCTAGTGGAGCCCCTTCGTCTCCCAACAGGAGGGTCCGGATTACCAAGTCCCGACACCGTTTGGCTGTAATTCGACTCAAATTC  180
                        Mlu I
                        Eam1105 I  Hpa I   Nco I               Bln I
TGGATTCGTAGCTTAACTAAGACGCGTGGTCTGTTAACCGGCCTCGCCATGGATGCCGATATAAGGACCCTAGGGGACTCCCCCCTGGTG  270
                              EcoR I
  PshA I                      Bbv II                                             Pvu II
ACTCTCGTCGGAAGATCGCAGCACTCTGAATTCTCCTAGTCTTCGTTTACTCCGCCATGCGTCATTTCTGCCTTTTGCCAGCTGTTGCTG  360
                                                        Met Arg His Phe Trp Leu Leu Pro Ala Val Ala
                                                                Taq II-2
GTATCGCTGGGGCTCAATGCCCCTACCTGTCGGGTGAAATGAGTTTCACCCAGGAGCAGGACAATGCTGGCGATACCATTGAGGTCACGG  450
Gly Ile Ala Gly Ala Gln Cys Pro Tyr Leu Ser Gly Glu Met Ser Phe Thr Gln Glu Gln Asp Asn Ala Gly Asp Thr Ile Glu Val Thr
                                    Age I
AGCAGCCCATTGACAACACCCTGTATGTCAATGACACCGGTAGCTACATGACTACCGACTTTGGCACTCCGATCTCCGACCAGACCAGTC  540
Glu Gln Pro Ile Asp Asn Thr Leu Tyr Val Asn Asp Thr Gly Ser Tyr Met Thr Thr Asp Phe Gly Thr Pro Ile Ser Asp Gln Thr Ser
      Bsp120 I
         Apa I              EcoN I       Eco57 I     HinD III
TCAAGGCCGGGCCCCGTGGTCCTACCCTGTTGGAGGACTTTATCTTCCGTCAGAAGCTTCAGCGGTTCGACCATGAGCGTGTAAGTACAG  630
Leu Lys Ala Gly Pro Arg Gly Pro Thr Leu Leu Glu Asp Phe Ile Phe Arg Gln Lys Leu Gln Arg Phe Asp His Glu Arg-------
```

FIG. 2A'

AlwnI
BstXI
TAACTGCTGCGGTGTGTAGTAACAATAAATTGACCCAGTGGTTTTCAATTAGGTCCCCGAGCGCGTCGTCCACGCCCGTGGTGCCGGTGC 720
Val Pro Glu Arg Val Val His Ala Arg Gly Ala Gly Ala
NdeI
BsaI
ATATGGTACTTTCAAATCCTACGCCGACTGGTCGAACGTCACGGCTGCCGATTTCTTGAGTGCCAACGATAAGGAGAGCCCCTATGTTCTG 810
Tyr Gly Thr Phe Lys Ser Tyr Ala Asp Trp Ser Asn Val Thr Ala Ala Asp Phe Leu Ser Ala Asn Asp Lys Glu Thr Pro Met Phe Cys
TCGCTTCTCTACTGTGGTCGGTTTCCGTGGTAGTGTTGACACTGCGCGTGATGTTCACGGTCACGCTTGTCGGTTCTACACTGACGAGGG 900
Arg Phe Ser Thr Val Val Gly Phe Arg Gly Ser Val Asp Thr Ala Arg Asp Val His Gly His Ala Cys Arg Phe Tyr Thr Asp Glu Gly
BstE II
Taq II–2
Tth111I
TAACTATGGTATCTTGATATGGTCACCCAACAATAAATTCAATACATGCTAACAGATATGTCTCTACTAGACATCGTCGGTATCAATTTCG 990
Asn Tyr
Asp Ile Val Gly Ile Asn Phe
BsgI
CCCCCTTCTTCATCCAGGACGCCATCCAGTTCCCCGATCTTGTCCACGCCCATCAAGCCCATGCCCAACAATGAGATCCCCCAGGCCGCTA 1080
Ala Pro Phe Phe Ile Gln Asp Ala Ile Gln Phe Pro Asp Leu Val His Ala Ile Lys Pro Met Pro Asn Asn Glu Ile Pro Gln Ala Ala
Eco57I
CTGCACACACTTCCGCTTGGGACTTCTTCAGCCAGCAGAGCACTGCCCTCCACAGTGCCTTGTGGCTGATGTCTGGTAACGGTATTCCTC 1170
Thr Ala His Thr Ser Ala Trp Asp Phe Phe Ser Gln Gln Ser Thr Ala Leu His Ser Ala Leu Trp Leu Met Ser Gly Asn Gly Ile Pro
Peptide 1
GTTCTTTCCGCCACATGAACGGCTACGGAGTCCACAGCTTCCGCTTCGTCGCTGCCAATGGCACTTCCAAGGTGGTGCGAACACCTTGGA 1260
Arg Ser Phe Arg His Met Asn Gly Tyr Gly Val His Ser Phe Arg Phe Val Ala Ala Asn Gly Thr Ser Lys Val Val Arg Thr Pro Trp
Peptide 1
Peptide 5

FIG. 2A"

FIG. 2A'
FIG. 2A"
KEY TO FIG. 2A

```
                     .RieA I                              .Bpu10 I                                                              .Alwn I
AGTCCCAACAGGGTGTTGCCAGTCTGGTGTGGGATGAAGCTCAGGCCGCTGCTGGTAAGAACAGTGACTACCACCGCCAGGATCTGTACA  1350
Lys Ser Gln Gln Gly Val Ala Ser Leu Val Trp Asp Glu Ala Gln Ala Ala Ala Gly Lys Asn Ser Asp Tyr His Arg Gln Asp Leu Tyr

                                                                         .BsaB I
                   .Bal I                                                     .Cla I
ATGCGATGCCCAATGGCCACTACCCGAAATACGAGGTCAGCCAATCCCTTGATGTCTATCGATAGAGCCTTTTGCTGACAATCCCCTAGG  1440
Asn Ala Met Pro Asn Gly His Tyr Pro Lys Tyr Glu-------------------------------------------

                                                                         .BamH I
                 .PflM I                                                 .Bsu90 I        .BstX I
TCCAAGCCCAGATCATGGATGAGGCTGACATGCTTCGTTTCGGCTTCGACCTTCTGGATCCCACCAAGTTGGTCCCCGAGGAGGTTGTCC  1530
Leu Gln Ala Gln Ile Met Asp Glu Ala Asp Met Leu Arg Phe Gly Phe Asp Leu Leu Asp Pro Thr Lys Leu Val Pro Glu Glu Val Val

                                  .Ecl136 II
                                    .Sac I                                                  .Eco57 I
CTTACACTCCTCTCGGAATGATGGAGCTCAATGCCAACCCCACCAACTACTTTGCTGAAGTTGAACAGGCTGGTGTATGTATTCCCCATT  1620
Pro Tyr Thr Pro Leu Gly Met Met Glu Leu Asn Ala Asn Pro Thr Asn Tyr Phe Ala Glu Val Glu Gln Ala Gly -----------

                                         .Pst I
CATCAAATGCCAGACATAATCTAACTTCTGCAGTTCCAACCCGGTCACGTCGTTCCTGGCATTGACTTCACCGACGACCCCCTGCTGCAA  1710
--------------------------Phe Gln Pro Gly His Val Val Pro Gly Ile Asp Phe Thr Asp Asp Pro Leu Leu Gln

         .Esp3 I
           .Eam1104 I
GGCCGTCTCTTCTCCTACCTCGACACTCAGTTGACCCGTCACGGCGGTCCCAACTTCGAGCAAATCCCCGTCAACCGTCCTCGCAAGCCC  1800
Gly Arg Leu Phe Ser Tyr Leu Asp Thr Gln Leu Thr Arg His Gly Gly Pro Asn Phe Glu Gln Ile Pro Val Asn Arg Pro Arg Lys Pro

                                                               .PflM I                              .BstE II
GTTCACAACAACAACCGTGACGGCTTCGGCCAGCAGCAGATCCCCACCAACAACTGGGCCTACACCCCCAACAGCATGAGCAACGGTTAC  1890
Val His Asn Asn Asn Arg Asp Gly Phe Gly Gln Gln Gln Ile Pro Thr Asn Asn Trp Ala Tyr Thr Pro Asn Ser Met Ser Asn Gly Tyr
```

FIG. 2B'

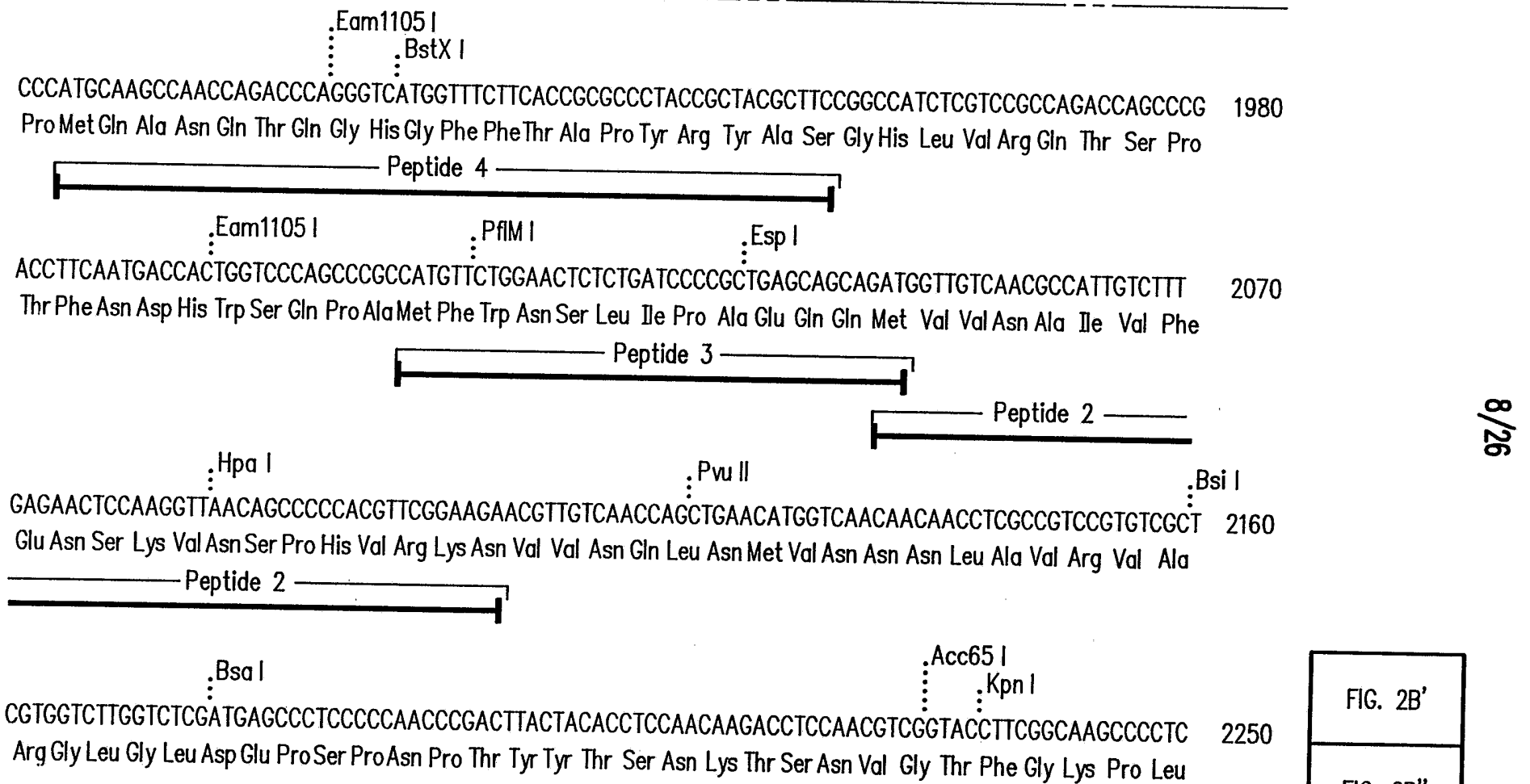


FIG. 2B"

KEY TO FIG. 2B

```
                                                    .Nco I
                                                    .PflM I
.Bpu10 I  .BspM I      .Pst I                       .
CTCAGCATCGAGGGTCTGCAGGTCGGCTTCCTGGCCTCGAACTCCCACCCCGAATCCATCAAGCAGGGCCAGGCCATGGCCGCCGCCAGTTC  2340
 Leu Ser  Ile Glu Gly Leu Gln Val Gly Phe Leu Ala Ser Asn Ser His Pro Glu Ser  Ile Lys Gln Gly Gln Ala Met Ala Ala Gln Phe
                    .Sal I                  .Stu I
                                            .
TCTGCCGCTGGCGTCGACCTGAACATTGTCACCGAGGCCTACGCCGATGGTGTCAACACCACCTACGCCCTGTCTGATGCCATCGACTTT  2430
 Ser Ala  Ala Gly  Val Asp Leu Asn Ile Val  Thr Glu  Ala Tyr Ala Asp Gly Val Asn Thr Tyr Ala Leu Ser Asp  Ala  Ile  Asp Phe
                                        .Eam1104 I
                                        .
                                .Bsg I  .
                                .       .
GACGCCCTCATCATCGCCGATGGTGTGCAGAGCCTCTTCGCCTCCCCCGCTCTCGCTAACCAGATGAACTCTACCGCCACCTCTACTCTC  2520
 Asp Ala  Leu Ile  Ile Ala  Asp Gly Val Gln Ser Leu Phe Ala Ser Pro Ala Leu Ala Asn Gln Met Asn Ser Thr  Ala  Thr  Ser  Thr  Leu
                                AlwN I
                                .PflM I
                                .
TACCCTCCTGCCAGACCTTTCCAGATCCTGGTCGATTCTTTCAGGTACGGTAAGCCCCGTGGCTGCTGTCGGCAGTGGCAGTGTTGCGCTC  2610
 Tyr Pro Pro Ala Arg Pro Phe Gln Ile Leu Val  Asp Ser Phe Arg Tyr Gly Lys Pro Val Ala Ala Val Gly Ser Gly Ser  Val  Ala  Leu
                                        .Xho I
                                        .Esp3 I
                                        .MlaA I
                                        .Sci I
                                        .                   .Xcm I
                                        .                   .
AAGAACGCTGGTATTGATTCCTCCCGCTCTGGTGTGTACACTGGCTCGAGCGACGGAGAAGATCGCCCAAGGAGGTCTTGGAGGGA  2700
 Lys Asn Ala Gly Ile Asp Ser Ser Arg Ser Gly Val  Tyr  Thr Gly Ser Ser Glu Thr Thr Glu Lys  Ile  Ala Lys Glu Val  Leu Glu  Gly
```

FIG. 2C'

```
                    .Age I
                    :
CTCTACACTTTCCGTTTTGTGGACCGGTTTGCGCTGGATGAGTAAGGGTATCACGTTTGTACTTGTACTCACGTTCATCGTTTGTGATGA   2790
 Leu Tyr Thr Phe Arg Phe Val Asp Arg Phe Ala Leu Asp Glu
              .Cla I                .Bst1107 I
              :                     :
TACATTGATTGATCGATAGATATTTTGTGAGATAGATATACTAGAGWGKACATATCTCTACTGATGAGGTGTTGTGCTGCTGCAA   2880
                                .Eam1104 I .Nhe I                .Hpa I     .Eam1104 I     .BsaB I
                                :          :                     :          :              :
CACATATTTATGAATATATATTCTCTTCTTTGTGAAAGCTAGCCTTCTATATAATCAGCAATGGTTAACTCTTCCAATTCTATAGATACC   2970
AATCACCTAACCCACTCGGAATGACGACAGAAAACATCGACATGTTCGCCCAAGTAAAGCTACTTGAACTTCTACATTTATGCTATGCTG   3060
                    .Gsu I
                    :
GAGTCCTCTCATAAGTCCAGAATAAACAAAGAGATCCGATCCTGCTC   3107
```

FIG. 2C"

KEY TO FIG. 2C

GCGGCCGCCTCGAGGATTGTCTGAACATTGACATTCGGCGCCCAGCGAACCCCAACTGCGGACGCGAATGCCCGTGCTGGTCTCGGATCT
90
linker
glaA promoter
TTGGCGGAGGCTTTGAACTTGGTTCAAAGGCCATGTATGACGGCACAACGATGGTATCATCGTCGATAGACAAGAATATGCCTATCGTGT
180
glaA promoter
TTGTAGCGATGAACTATACGCGTAGGGGGCTTCGGGTTTCTGCCCGGAAAGGAGATTCTGGAGGACGGGTCCGCCAACTTAGGTCTTTGAC
270
glaA promoter
CAAGCCTTGCCCCTAGTGGGTGGCCGACACAACATCGAGGCGTTTGGTGGAGACCAGACAAGGTGACAATCTGGGGAGAATCAGCAGGGGCTA
360
glaA promoter
TTTCTGTCTTGATCAGATGATCTGTACGACGGAAACATCGCTTACAAGGACAAGCCCTTGTTTCGGGAGCCATCATGGACTCCGGTATGT
450
glaA promoter
GTTCCCGCAGACCCTGTCGACGGGGTCAAGGGATCAGCAAGTATATGATGCGGTTGTGGACTCTGCAGGCTGTTCCTCTTCCAACGACAC
540
glaA promoter
CCTGGCTTGTCTGCGTGAGCTAGACTACACCGACTATCTCAATGCGGCAAACTCGTGCCGGGGATCCTAGGTTATCACCGTGGCGCTATC
630
glaA promoter
ATATGTGCCTCGACCAGACGGGACGGCATTTGTCGGCGTCGCCAGATTTTGGGTAAAGCAGGGAAGTATGCGCGGGTCCCATTCATCGTG
720
glaA promoter
GGCGACCAAGAGGATGAGGGGACCTTGTTCGCCTTGTTTCAGTCCTTACGACGATCGACGAGGTAGTCGACTATCTGGGCACCTACTTCT
810
glaA promoter

FIG. 3A'

TCTATGACGCTAGCCAGGAGCAGCTTGAAGAATTAGTGGCCCTGTACCCAGACACCACCACATATGGGTCTCCCTTCAGGACGGGCAGGC 900

glaA promoter

CAACAACTGGTATCCGCAATTTAAGCGATTGGCCGCCATTCTCGGCGACTTGGTCTTCACCATTACCCGGCGGCATTCCTGTCATATGCA 990

glaA promoter

GAGGAGCTCTCCCCTGACCTCCCGAAATGGTCGTACCTGGCGACCTATGACTATGGCAGCCAATTCTGGGGACCTTCCATGGAAGTGACC 1080

FIG. 3A"

glaA promoter

TGCTGCAGGTGTTCTATGGGATCAAGCCGAACTATGCAGCGAGTTCCAGCCACACGTATTATCTGAGTTTTGTATACACGCTGGATCCGA 1170

glaA promoter

ACTCCAATCGGGGGGGAGTACATGGAATGGCCCCAGTGGCAGCCGACAGTTGATGAATTTCGGAGCGAACAGCAGTCTCCTTACGGATGAT 1260

glaA promoter

TTCCGCAACGGGACATATGAGTTCATCCTGCAGAATACCGCGGCGTTCCACATCTGATGCCATTCGGGAGGGGTCCGGAGGTCAGGGACT 1350

glaA promoter

AGCCTTATGAACGTAATGATGGAAGTGTCTGGCCTCGGCAAAGGATATATAGGTCATAATAAGTAGTACTAGTTATATTAATGGAAGGG 1440

glaA promoter

TATATACCACGCGTTGGACCTTGGGACCTGCATTATAGCTTCCCGTTAGGTATAATTACCGTTGTTATAGCAGCCAATCAAGCCACCACG 1530

glaA promoter

CTCGACCGCGGGGACGGCGAATCCCCCGGGAATTGAAATAAATTGCAATTCAGGTCAATGCGGCCAGCGATTGGACACATCTCCAAGGCACA 1620

glaA promoter

FIG. 3A'
FIG. 3A"

KEY TO FIG. 3A

GGGCCATTCTGCAGTGCCGGGGATTCAGTGCATTCCCCCGGGCCCGGGCCCCGACACGCGATAGGCTGGTTCTTCCACACCACCGGAGATTC
1710
glaA promoter
GTCGTTCTGAAGAGCTGAAGTGGCGAGATGGTCTCTGCAGGAATTCAAGCTAGATGCTAAGCGATATTGCATGGCAATATGTGTTGATGC
1800
glaA promoter
ATGTGCTTCTTCCTTCAGCTTCCCCTCGTGCAGATGAAGGTTTGGCTATAAAATTGAAGTGGTTGGTCGGGGGTTCCGTGAGGGGCTGAAG
1890
glaA promoter
TGCTTCCTCCCTTTTAGACGCAACTGAGAGCCTGAGCTTCATCCCCAGCATCATTAGATCTCAGCAATGCGTCATTTCTGGCTTTTGCTG
1980
glaA promoter
catR coding region
CTGTTGCTGGTATCGCTGGCTCAATGCCCCTACTGTCGGGTGAAATGAGTTTCACCCAGGAGCAGGACAAATGCTGGCGATACCATTGAGG
2070
catR coding region
TCACGGAGCAGCCCATTGACAACACCCTGTATGTCAATGACACCGGTAGCTACATGACTACCGAACTTTGGCACTCCGATCTCCGACCAGA
2160
catR coding region
CCAGTCTCAAGGCCGGGCCCCGTGGTCCTACCCTGTTGGAGGACTTTATCTTCCGTCAGAAGCTTCAGCGGTTCGACCATGAGCGTGTAA
2250
catR coding region
GTACAGTAACTGCTGCGGTGTGTAGTAACAATAAATTGACCCAGTGGTTTTCAATTAGGTCCCCGAGCGCGTCGTCCACGCCCGTGGTGC
2340
catR coding region
CGGTGCATATGGTACTTTCAAATCCTACGCCGACTGGTCGAACGTCACGGCTGCCGATTTCTTGAGTGCCAACGATAAGGAGACCCCTAT
2430
catR coding region

FIG. 3B'

FIG. 3B"

GTTCTGTCGCTTCTCTACTGTGGTCGGTTCCGTGGTAGTGTTGACACTGCGCGTGATGTTCACGGTCACGCTTGTCGGTTCTACACTGA 2520

catR coding region

CGAGGGTAACTATGGTATCTTGATATGGTCACCCAACAATAATTCAATACATGCTAACAGATATGTCTCTACTAGACATCGTCGGTATCA 2610

catR coding region

ATTTCGCCCCCCTTCTTCATCCAGGACGCCATCCAGTTCCCCGATCTTGTCCACGCCATCAAGCCCATGCCCAACAATGAGATCCCCCAGG 2700

catR coding region

CCGCTACTGCACACACTTCCGCTTGGGACTTCTTCAGCCAGCAGGCACTGCCCTCCACAGTGCCTTGTGGCTGATGTCTGGTAACGGTA 2790

catR coding region

TTCCTCGTTCTTTCCGCCACATGAACGGCTACGGAGTCCACAGCTTCCGCTTCGTCGCTGCCAATGGCACTTCCAAGGTGGTGCGAACAC 2880

catR coding region

CTTGGAAGTCCCAACAGGGTGTTGCCAGTCTGGTGTGGGATGAAGCTCAGGCCGCTGCTGGTAAGAACAGTGACTACCACCGCCAGGATC 2970

catR coding region

TGTACAATGCGATGCCCAATGGCCACTACCCGAAATACGAGGTCAGCCAATCCCTTGATGTCTATCGATAGAGCCTTTTGCTGACAATCC 3060

catR coding region

CCTAGCTCCAAGCCCAGATCATGGATGAGGCTGACATGCTTCGTTTCGGCTTCGACCTTCTGGATCCCACCAAGTTGGTCCCCGAGGAGG 3150

catR coding region

TTGTCCCTTACACTCCTCTCGGAATGATGGAGCTCAATGCCAACCCCACCAACTACTTTGCTGAAGTTGAACAGGCTGGTGTATGTATTC 3240

catR coding region

FIG. 3B'

FIG. 3B"

KEY TO FIG. 3B

CCCATTCATCAAATGCCAGACATAATCTAACTTCTGCAGTTCCAACCCGGTCACGTCGTTCCTGGCATTGACTTCACCGACGACCCCCTG
3330
catR coding region
CTGCAAGGCCGTCTCTTCTCCTACCTCGACACTCAGTTGACCCGTCACGGCGGTCCCAACTTCGAGCAAATCCCCGTCAACCGTCCTCGC
3420
catR coding region
AAGCCCGTTCACAACAACAACCGTGACGGCTTCGGCCAGCAGATCCCCACCAACACTGGGCCTACACCCCCAACAGCATGAGCAAC
3510
catR coding region
GGTTACCCCATGCAAGCCAACCAGACCCAGGGTCATGGTTTCTTCACCGCGCCCTACCGCTACGCTTCCGGCCATCTCGTCCGCCAGACC
3600
catR coding region
AGCCCGACCTTCAATGACCACTGGTCCCAGCCCGCCATGTTCTGGAACTCTCTGATCCCCGCTGAGCAGCAGATGGTTGTCAACGGCCATT
3690
catR coding region
GTCTTTGAGAACTCCAAGGTTAACAGCCCCCACGTTCGGAAGAACGTTGTCAACCAGCTGAACATGGTCAACAACACCTCGCCGTCCGT
3780
catR coding region
GTCGCTCGTGGTCTTGGTCTCGATGAGCCCTCCCCCAACCCGACTTACTACACCTCCAACAAGACCTCCAACGTCGGTACCTTCGGCAAG
3870
catR coding region
CCCCTCCTCAGCATCGAGGGTCTGCAGGTCGGCTTCCTGGCCTCGAACTCCCACCCCGAATCCATCAAGCAGGGCCAGGCCATGGCGCGC
3960
catR coding region
AGTTCTCTGCCGCTGGCGTCGACCTGAACATTGTCACCGAGGCCTACGCCGATGGTGTCAACACCACCTACGCCCTGTCTGATGCCATCG
4050
catR coding region

FIG. 3C'

FIG. 3C"

ACTTTGACGCCCTCATCATCGCCGATGGTGTGCAGAGCCTCTTCGCCTCCCCCGCTCTCGCTAACCAGATGAACTCTACCGCCACCTCTA 4140

catR coding region

CTCTCTACCCTCCTGCCAGACCTTTCCAGATCCTGGTCGATTCTTTCAGGTACGGTAAGCCCCGTGGCTGCTGTCGGCAGTGGCAGTGTTG 4230

catR coding region

CGCTCAAGAACGCCTGGTATTGATTCCTCCCGCTCTGGTGTGTACACTGGCTCGAGCGAGACGGAGAAGATCGCCCAAGGAGGTCTTGG 4320

catR coding region

AGGGACTCTACACTTTCCGTTTTGTGGACCGGTTTGCGCTGGATGAGTAAGGGTATCACGTTTGTACTTGTACTCACGTTCATCGTTTGT 4410

catR coding region — catR 3' non coding DNA

GATGATACATTGATTGATCGATAGTCTAGAGTCGACCGCGACCGGTGACCGACACCTGGCGGGTAGACTATTTATTCCTGTTGATATGAAGG 4500

-catR 3' non coding DNA — linker — glaA poly adenylation & termination signals

ATGAGCATGAGGGTAATTGCTCATATAATCATGTATGTAGTGGATGTGCATAAGAGCAACGAAATGGAAGCCTGATCATGTGATTGTATT 4590

glaA poly adenylation & termination signals

GCGACCGACGGAAATTGAGGATATGCGGAGATACGGACAGTGCCAGAGCCATTGTCTTCACGTAAAGTACCAGACGGTCCCTGATTTCTT 4680

glaA poly adenylation & termination signals

CTTGCACATAGCATTAGGCAATTGACATGTTGTCGCTCTACTGATATCACTGTCCCTCAAAGCATAGCCATGAGCTCATCTTAGATCCAA 4770

glaA poly adenylation & termination signals

GCACGTAATTCCATAGCCGAGGTCCACAGTGGAGCAACAGCAGCATCCATCATTGCTTCTCCCCCAGGGGCCTCTTAGCGACTAAACCTG 4860

glaA poly adenylation & termination signals

KEY TO FIG. 3C
FIG. 3C'
FIG. 3C"

GAGTATGTCTCAACCAGCCAATGAATCGTCTTCGCTTCAATGTCCTTGACACTTCTGAGAGGGTCCCCATCCCTCAATGCTAATTCAAAA 4950

glaA poly adenylation & termination signals

TATAGCCGAGATGCATGGTGGAGTCCAAAGTAGACAGTATTGCCGCGGAATGACGGGGCCAGTTGCGCCGAGGTCATTGGCCCGGCTGTGATG 5040

glaA poly adenylation & termination signals

CCATCTGCCACTAAATCCGATCATTGATCCACCGCCCACGAGGGCCGTCTTTGCTTTTGCTTTGCGCTGCGTCCAGGTTCACACATCTCTCTTC 5130

glaA poly adenylation & termination signals

TGCAGCTCCAGACTGACCAGACTATTCTACTTACTGGTCTGATCGGCTCCATCAGAGCTATGGCGTTATCCCGTGCCGTTGCTGCGCCAT 5220

glaA poly adenylation & termination signals

CGCTATCTTGATCGCGAGCTCGAACTCACTTCTTGTTTTAATAGTTGTTCTCGGTGACTGAGTGTCGGTGAGTGACAGACCACAACACCA 5310

glaA poly adenylation & termination signals

TTGTTGCAGGGGGTAAATTTATTCAATTCAGGAATTGGATTGTTCGTCCCGCCATGATGTTCTTGCCGGCTTTGTTGGCCCTGTTTGTCG 5400

glaA poly adenylation & termination signals

GATGCGACGCCCTCGCTGTGCAGCAGGCAGGTACTGCTGGATGATGAGCCGTCGGTCTCCGCGCCGCAAGCCTAACTTCCTCTTCATTCTT 5490

glaA poly adenylation & termination signals

ACGGATGATCAGGATCTGCAGATCGAATTCCACCGGCGTATATGCCGTATACACAGGCGAGAATCAAGGAGAAGGGTACTGAGTTTTGAA 5580

glaA poly adenylation & termination signals

TCATTTGTTACTACTGGCTCTGTGCTGTCCGTCGCGCGTGAGTCTTTGGACGGAAGACAGGCTCATAATACTAATGTGACGGATGTGAAC 5670

glaA poly adenylation & termination signals

FIG. 3D'

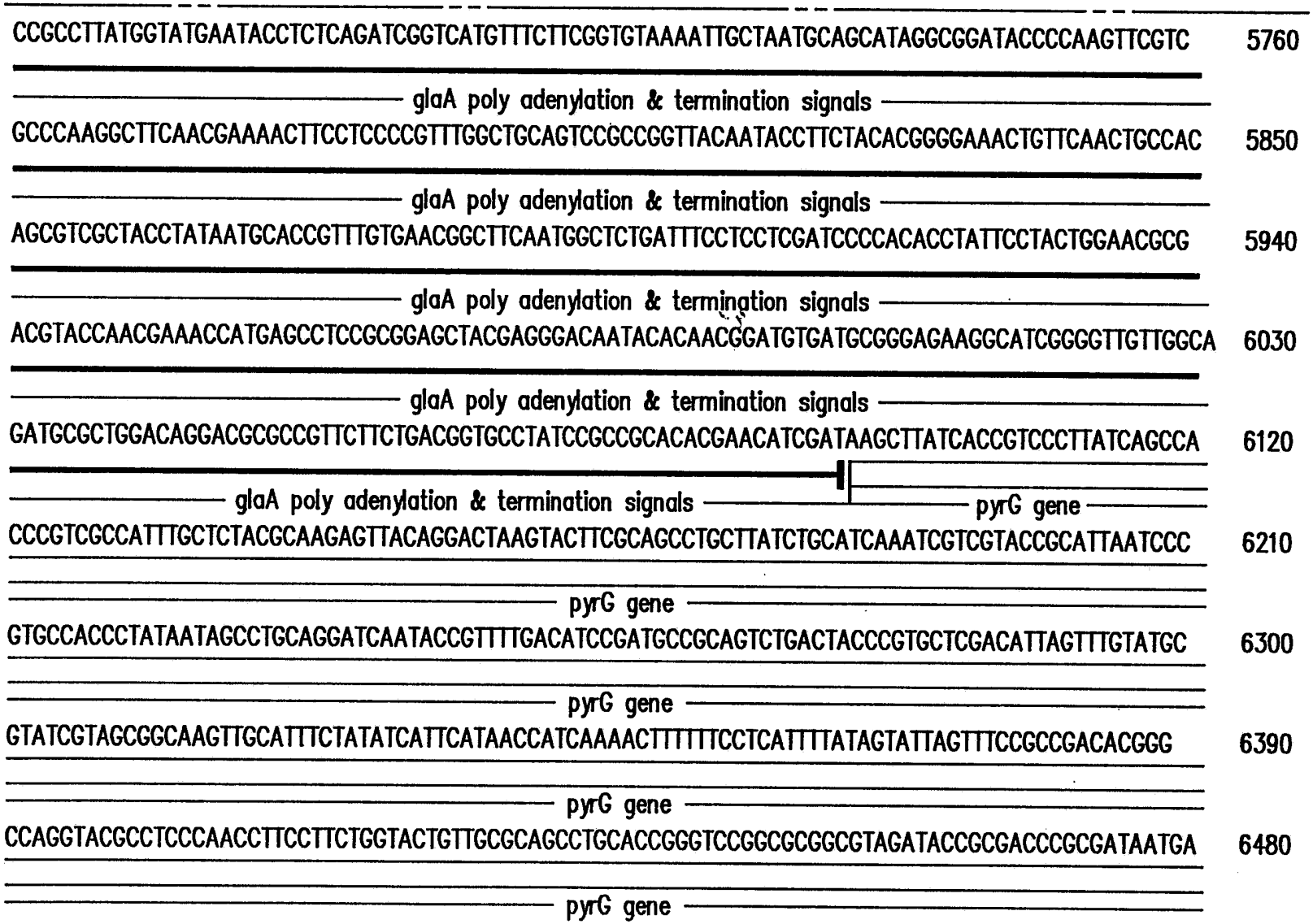
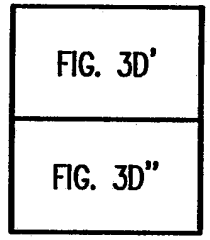


FIG. 3D"



KEY TO FIG. 3D

AGTCAGCACCCCGACCGATAGCCGATGCGGGAGTCTGGTACTGCTGACCGAGCTTATCTCCCTTGGACGAAATGTTCACACCAGTCGTGA 6570

pyrG gene

AGACCACAAAGTCCTCCTCATCCGAAGGAGAGCTGACTTCCGACTGCACCTCACCCAACGAGCGGGTCGACACAAATCCCATGACGAAGT 6660

pyrG gene

TCTTGTATTTCCGGGCATAATCAACCGAAGAAGTAGTGTACTGGCCCGGTGGCCAAGGAACCCTTAGAGGTCATTTCCGCCAAGATCAACA 6750

pyrG gene

GACCACGTTCGGGGCCCGTAGGAGAAGTCCGGTGCAGACGCCGTCTGAGCGAGAGCCTCGACGATACCCTCGCCAGGCAGGATGCTGCAGT 6840

pyrG gene

TGATGATATGGGCCCATTCTGAGATGCGGAGGGTACCACGGTGGTATTGCTTCTGGACAGTGTTGCCAATGTCGATGAATTTGCGGTCCT 6930

pyrG gene

CGAAGATGAGGAAGTTGTGCTTCTGCGCAAGAGCCTTGAGGCCCTCAATGGTCTCGTCGCTGAAGTCAGAGAGGATATCGATGTGGGTTT 7020

pyrG gene

TGATCACGGCGATGTAGGGACCGAGACCTCAGTCCGGTATCACCGTTAATAAGTTTGTATGCAGCATAAACAGGCAGAATGGCGGGTCGG 7110

pyrG gene

CCTACGGTCAGCAAGATCTAGTAGCTCCTTAGTGGTGGTAACGTCGGCAGAGACGGTCACATTGGTCTTCTTGGCCTCAGCAATTTCGAA 7200

pyrG gene

CAGCCGCTTGGCCAGAGCATTGGGGTGCTTGCTGGCACGGGCAGTGTAGGTCAATTGCGACTTGGAGGACATGGTGTCGGTGGAGGGGTT 7290

pyrG gene

FIG. 3E'

FIG. 3E"

AATGCGGGGATGAAAGAGGCTTGTGCAATATGAGTAGCTTGGAGTTTCGACTGATAGGCCCTAATTGGTAGATCCAGAGATGCGCAAATA 7380

pyrG gene

CTACCGAATAATTTAGCAGCGACTGGCCCTTATATGAGGTGAACAATGCACATTCAATGTCGAGCAAAAGAGGAGCTCAGTAAATCATCG 7470

pyrG gene

CGACCCTCCACGCACCAGCCACATCGGGTGATTTCGCCGCCTCCGACCGGAACCGTGGGGTTCAGCCACACCTGCAAAGGCAGTTCCTTT 7560

pyrG gene

CCATTGAAGTTGCCACACCCAGGTTCATTGGAGCTCGTATTTTTCCCTGCTGCACATGGGGAAATAGACCAGCTCAATCAGAAAGCCATT 7650

pyrG gene

GTCATTCCCGACCCTAGCAGTACGCATAGTAAACGCGTCGTGGAGTAGTAATATACAAGTGAGAAATTTATTACATATAGCGTGGTATAG 7740

pyrG gene

CCAACAGCGCCAATCACACCCGACGGAAGTCAATCCAAACTTTAAAAGGTAGGGAAATCAACTCCCTCGCGACTTCCAAAAGAGGTCAAT 7830

pyrG gene

CCCCAAAGAGCTCCCTGTGCAAGCAAGTAGAAGCTGCCGTACAACCGGACCCCGGCTTGCCGGAGTACACGTATCCGTAAAGGAAC 7920

pyrG gene

AGTGAGCGACCGAGAACCCAAATGCTTCCAAGGCCAGTTGCCAACTGGGGGTACTTCAATCCAGCCACCAGGATGAAGAGCATAGTTTGG 8010

pyrG gene

CTGGAGTTCTCAAGGAAGTTGGCATGAGCGTGAGCGAGTTAACTGCTCAGCCTTGGGCTGCACGATTGGAATGTATGTTAGCTCGAGGAA 8100

pyrG gene

FIG. 3E'
FIG. 3E"

KEY TO FIG. 3E

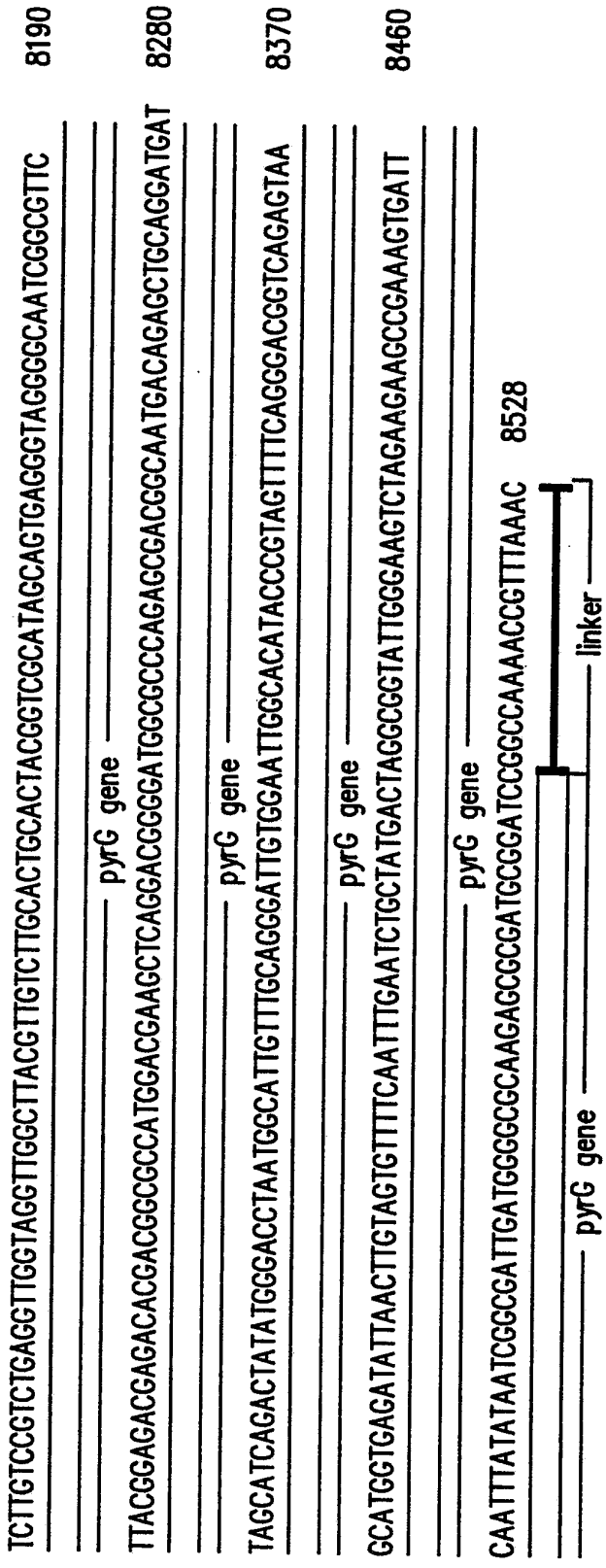

TCTTGTCCGTCTGAGGTTGGTAGGTTGGCTTACGTTGTCTTGCACTGCACTACGGTCGCATAGCAGTGAGGGTAGGGGCAATCGGCGTTC
8190
pyrG gene
TTACGGAGACGAGACACGACGGCGCCCATGGACGAAGCTCAGGACGGGGATGGCGCCCAGAGCGACGGCAATGACAGAGCTGCAGGATGAT
8280
pyrG gene
TAGCATCAGACTATATGGGACCTAATGGCATTGTTTGCAGGGATTGTGGAATTGGCACATACCCGTAGTTTTCAGGGACGGTCAGAGTAA
8370
pyrG gene
GCATGGTGAGATATTAACTTGTAGTGTTTTCAATTTGAATCTGCTATGACTAGGCGCTATTGGGAAGTCTAGAAGAAGCCGAAAGTGATT
8460
pyrG gene
CAATTTATATAATCGGCGATTGATGGGGCGCAAGAGCGCGATGCGGATCCGGCCAAAACCGTTTAAAC
8528
pyrG gene
linker

FIG. 3F

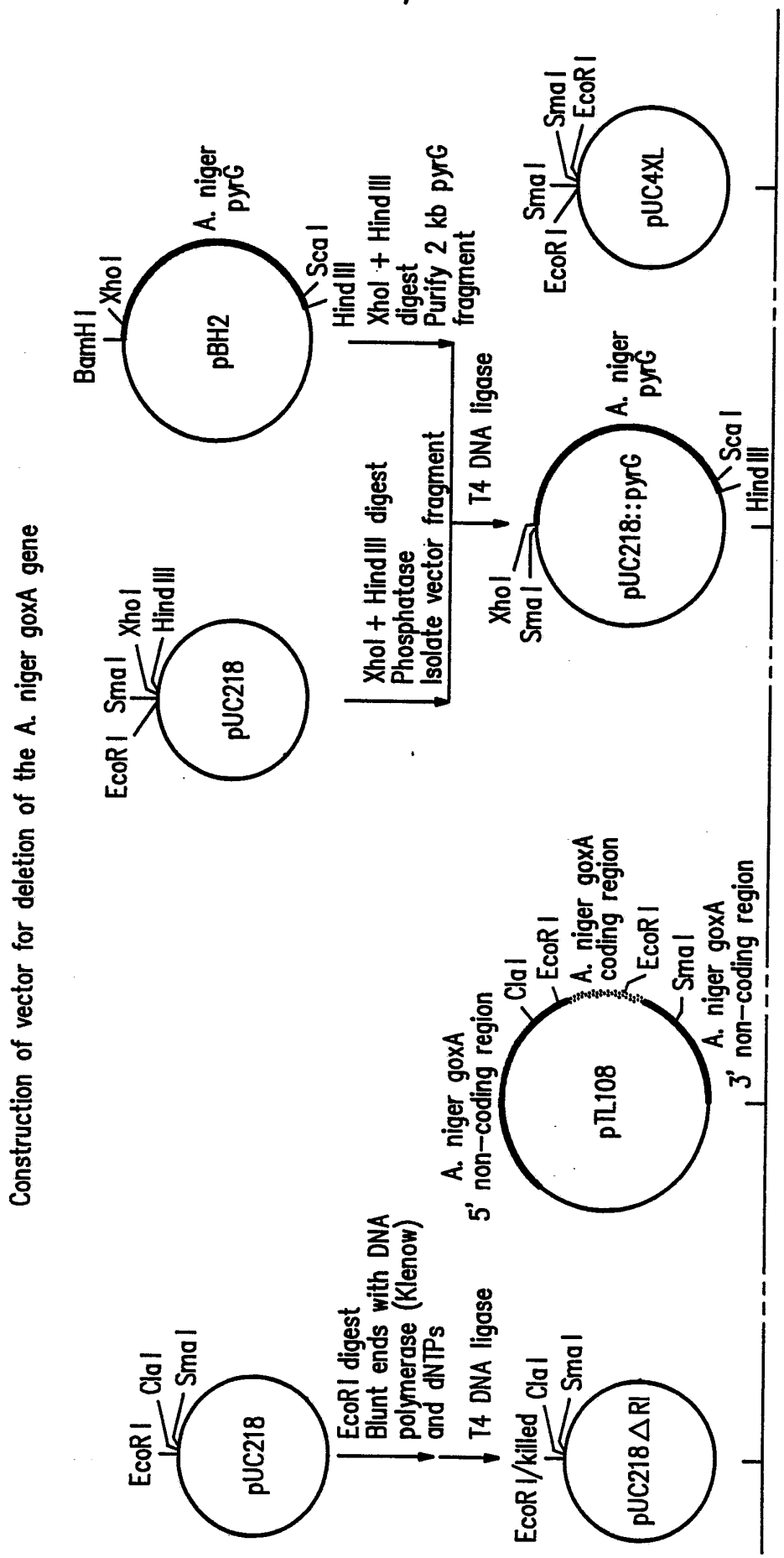

Construction of vector for deletion of the A. niger goxA gene
pUC218
EcoR I
Sma I
Cla I
EcoR I digest
Blunt ends with DNA polymerase (Klenow) and dNTPs
T4 DNA ligase
EcoR I/killed
pUC218 ΔRI
A. niger goxA 5' non-coding region
pTL108
A. niger goxA coding region
A. niger goxA 3' non-coding region
pBH2
BamH I
Xho I
A. niger pyrG
Sca I
Hind III
Xho I + Hind III digest
Phosphatase
Isolate vector fragment
Xho I + Hind III digest
Purify 2 kb pyrG fragment
T4 DNA ligase
pUC218::pyrG
pUC4XL

FIG. 4A'

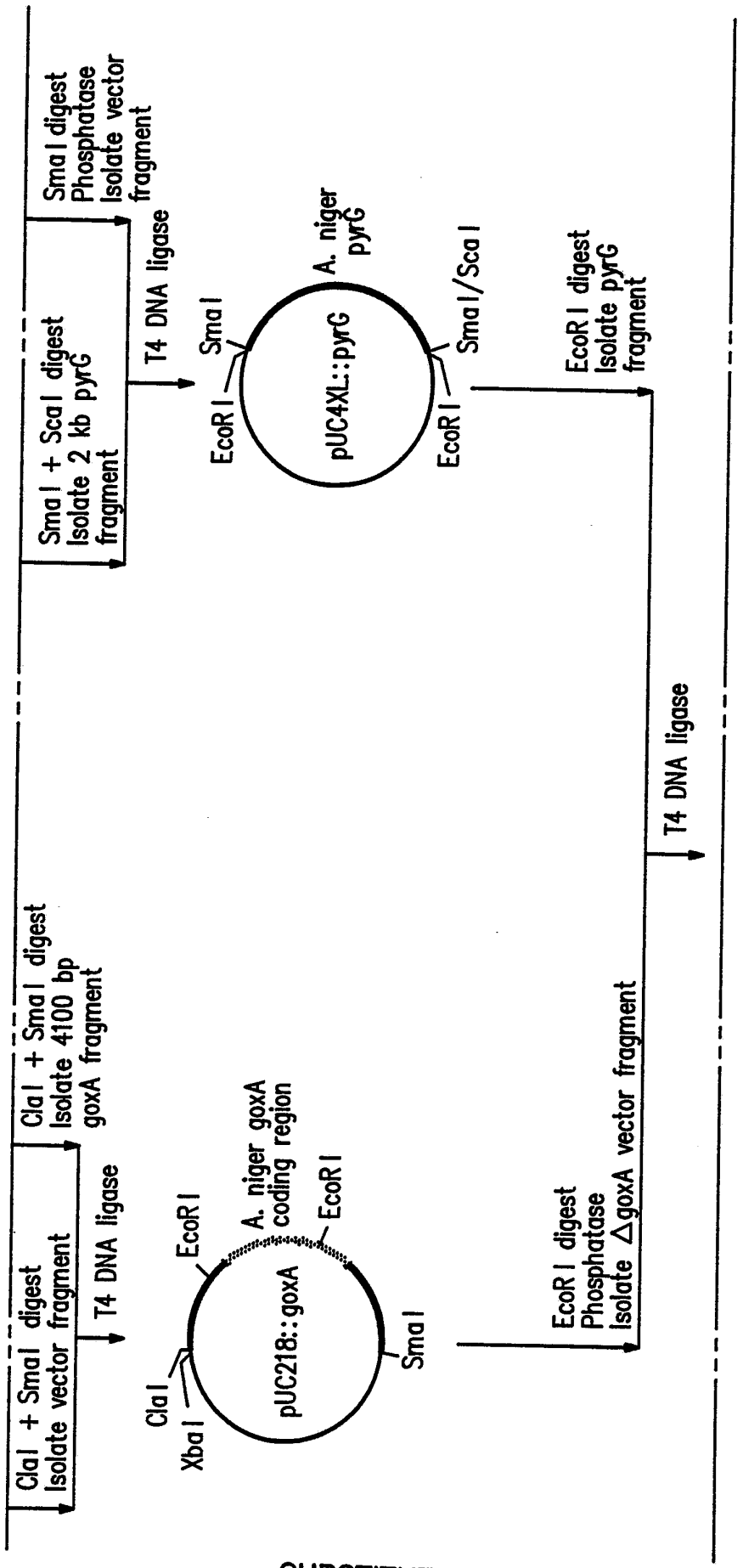

ClaI + SmaI digest
Isolate vector fragment
T4 DNA ligase
ClaI + SmaI digest
Isolate 4100 bp
goxA fragment
ClaI
XbaI
EcoR I
A. niger goxA
coding region
EcoR I
pUC218::goxA
SmaI
EcoR I digest
Phosphatase
Isolate ΔgoxA vector fragment
T4 DNA ligase
SmaI + ScaI digest
Isolate 2 kb pyrG
fragment
T4 DNA ligase
SmaI digest
Phosphatase
Isolate vector
fragment
SmaI
EcoR I
A. niger
pyrG
pUC4XL::pyrG
EcoR I
SmaI/ScaI
EcoR I digest
Isolate pyrG
fragment

FIG. 4A"

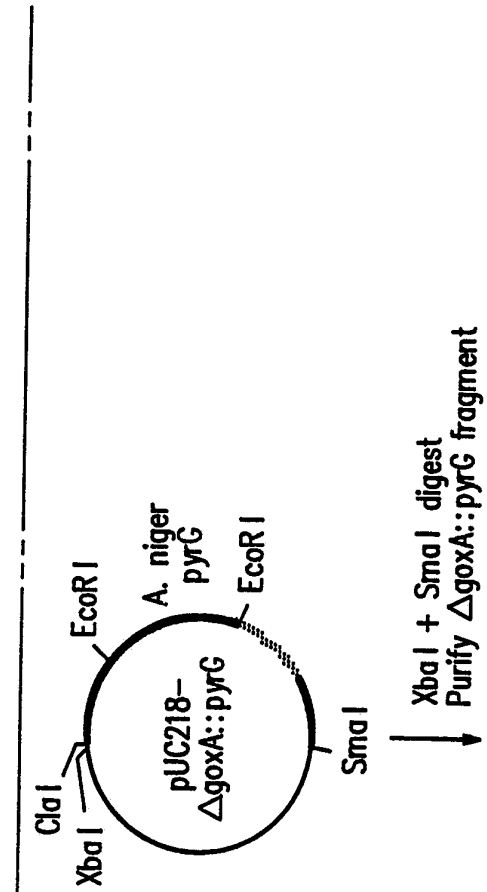

Cla I
Xba I
EcoR I
A. niger pyrG
EcoR I
pUC218-ΔgoxA::pyrG
Sma I
Xba I + Sma I digest
Purify ΔgoxA::pyrG fragment
A. niger pyrG
Use linear DNA fragment to transform A. niger FS-1 pyrG strain. Screen transformants for loss of glucose oxidase activity on indicator plates.

FIG. 4A"

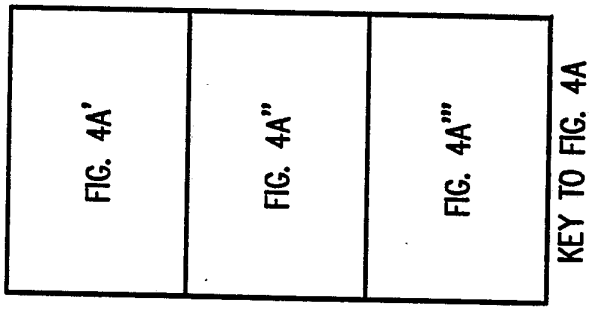


FIG. 4A'
FIG. 4A"
FIG. 4A"'
KEY TO FIG. 4A

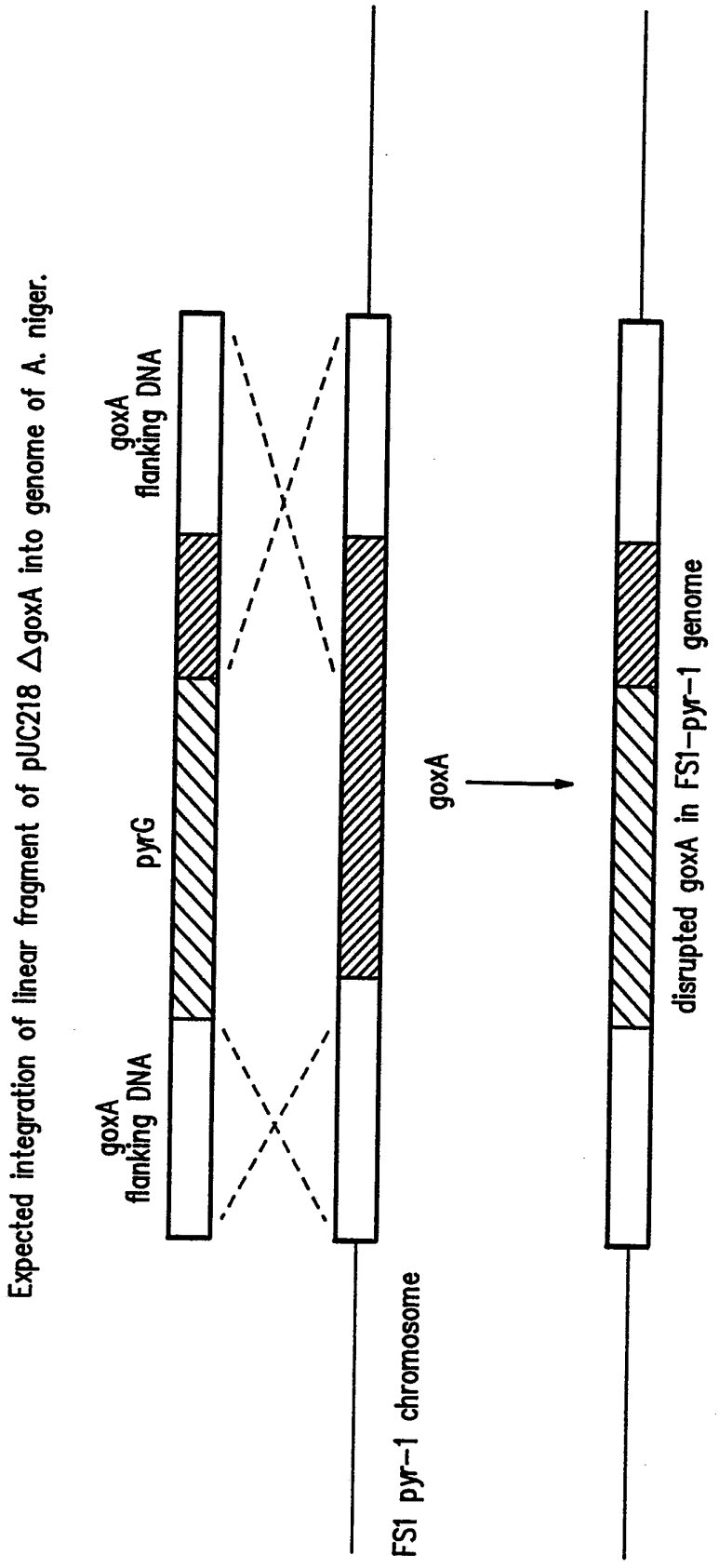
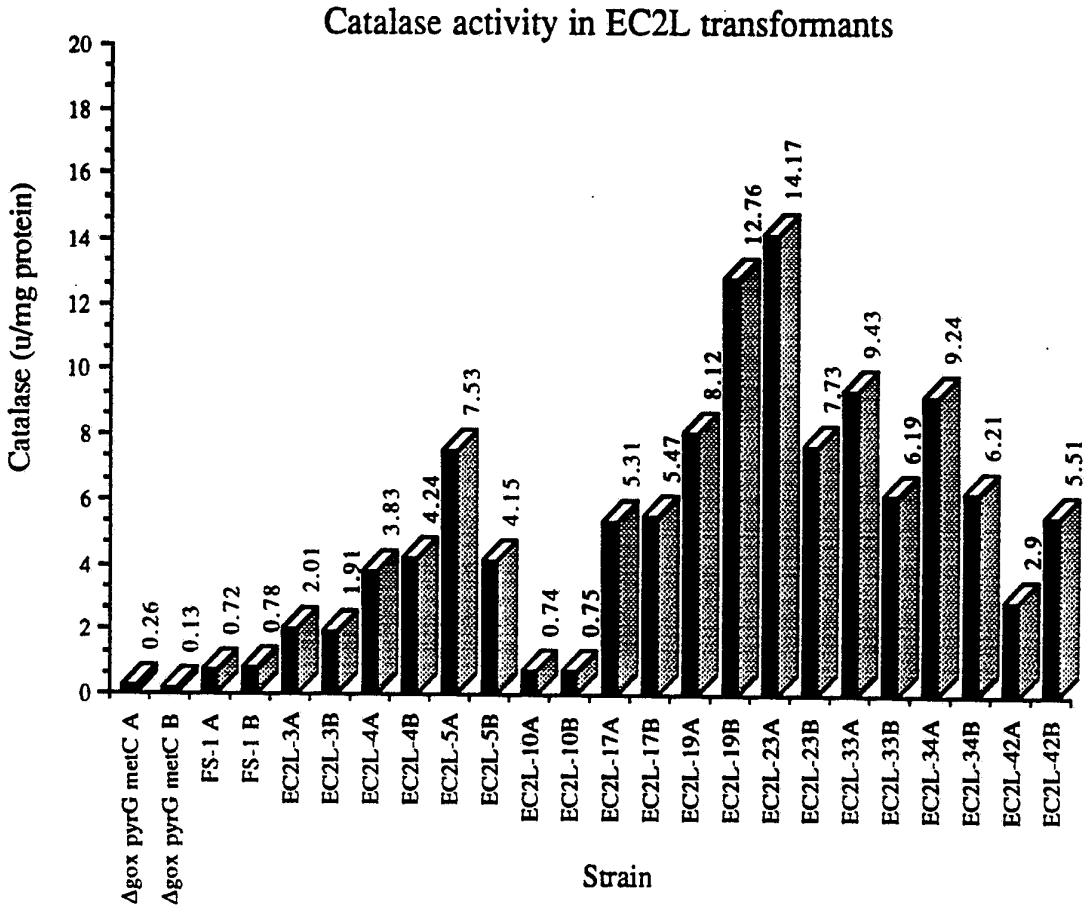

Expected integration of linear fragment of pUC218 ΔgoxA into genome of A. niger.
goxA flanking DNA
pyrG
goxA flanking DNA
FS1 pyr-1 chromosome
goxA
disrupted goxA in FS1-pyr-1 genome

FIG. 4B

FIGURE 5.


Catalase activity in EC2L transformants
Catalase (u/mg protein)
0
2
4
6
8
10
12
14
16
18
20
0.26
0.13
0.72
0.78
2.01
1.91
3.83
4.24
7.53
4.15
0.74
0.75
5.31
5.47
8.12
12.76
14.17
7.73
9.43
6.19
9.24
6.21
2.9
5.51
Δgox pyrG metC A
Δgox pyrG metC B
FS-1 A
FS-1 B
EC2L-3A
EC2L-3B
EC2L-4A
EC2L-4B
EC2L-5A
EC2L-5B
EC2L-10A
EC2L-10B
EC2L-17A
EC2L-17B
EC2L-19A
EC2L-19B
EC2L-23A
EC2L-23B
EC2L-33A
EC2L-33B
EC2L-34A
EC2L-34B
EC2L-42A
EC2L-42B
Strain