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(54) **POLYPEPTIDE DU SAF, GENE, ACTIVATEUR ET EXPRESSION DANS STREPTOMYCES**

(54) **SAF POLYPEPTIDE, GENE, PROMOTER AND USE FOR EXPRESSION IN STREPTOMYCES**

(57) Le clonage et la caractérisation d'un gène récemment isolé et désigné sous le nom de saf (facteur d'activation de métabolisme secondaire) sont divulgués. Ledit gène code un nouveau polypeptide d'amino-acide, désigné sous le nom de polypeptide SAF et modulant, directement ou indirectement, l'expression d'enzymes extracellulaires dans le Streptomyces. Des unités ou des fragments d'ADN codant ledit polypeptide SAF sont également divulgués, ainsi que des vecteurs contenant ledit ADN, des organismes hôtes transformés par l'introduction desdits vecteurs, et les procédés pour la préparation d'enzymes extracellulaires ou de polypeptides par la culture de tels organismes hôtes.

(57) The cloning and characterization of a newly isolated gene, referred to as saf (secondary metabolism activation factor) is disclosed. This gene encodes a new amino acid polypeptide, referred to as the SAF polypeptide, which directly or indirectly modulates the expression of extracellular enzymes in Streptomyces. DNA units or fragments which encode the SAF polypeptide are also disclosed as are vectors containing said DNA, host organisms transformed with such vectors, and processes for preparing extracellular enzymes or heterologous polypeptides by culturing such host organisms.

ABSTRACT OF THE DISCLOSURE

The cloning and characterization of a newly isolated gene, referred to as saf (secondary metabolism activation factor) is disclosed. This gene encodes a new amino acid polypeptide, referred to as the SAF polypeptide, which directly or indirectly modulates the expression of extracellular enzymes in Streptomyces. DNA units or fragments which encode the SAF polypeptide are also disclosed as are vectors containing said DNA, host organisms transformed with such vectors, and processes for preparing extracellular enzymes or heterologous polypeptides by culturing such host organisms.

SAF POLYPEPTIDE, GENE, PROMOTER AND USE FOR EXPRESSION IN STREPTOMYCES

Field of the Invention

The invention is in the field of biotechnology. More particularly, it relates to a new polypeptide increasing production of extracellular enzymes, DNA that codes for the polypeptide, a recombinant vector that includes the DNA, and a host organism transformed with the recombinant vector that includes the DNA encoding the polypeptide. Furthermore, the invention is directed to the promoter sequence of the gene encoding the new polypeptide. The present invention further relates to Streptomyces expression systems and methods for expressing foreign DNA sequences in Streptomyces and for secreting to the surrounding medium polypeptides and proteins coded for by those foreign DNA sequences.

Background of the Invention

The Streptomyces are well known producers of a variety of extracellular enzymes including proteases, phosphatases, xylanases, cellulases, amylases, lipases and nucleases.

In addition, members of the genus Streptomyces produce a large number of antibiotics, pigments and other secondary metabolites and have a complex pattern of differentiation resulting in the formation of spores. In batch cultures of Streptomyces there is usually a coincidence in the production of extracellular enzymes and the onset of antibiotic production and pigment biosynthesis and sporulation. All of these

processes are repressed by nutritional conditions favouring high growth rates and are derepressed by starvation of P, C or N sources. It is likely that enzyme secretion, formation of secondary metabolites and differentiation are completely independent but respond to similar triggering mechanisms.

Several genes of Streptomyces encoding extracellular enzymes have been cloned. These include agarase from Streptomyces coelicolor, endoglycosidase H from Streptomyces plicatus, xylanase from Streptomyces lividans, alpha-amylase from Streptomyces hygroscopicus, cellulase from Strep. spA2, beta-galactosidase from Strep. lividans and beta-lactamases from Strep. cacaoi, badius and fradiae.

However, the regulatory mechanisms which control expression of these genes are virtually unknown. In addition to specific regulatory mechanisms, such as induction of amylase by dextrins or maltotriose and carbon metabolite regulation of amylase or agarase, general mechanisms of derepression of several extracellular enzymes are likely to occur since simultaneous production of several polymeric-substrate degrading enzymes has been observed in Streptomyces following a nutritional down-shift. Such transacting regulatory genes have been found in Bacillus subtilis (J. BACTERIOL. 169:324-333,1987), Bacillus natto (J. BACTERIOL. 166:20-28,1986), and Bacillus licheniformis.

Positive regulatory genes affecting enzyme synthesis and/or secretion might be cloned by searching for increased secretion of extracellular enzymes in a poor secretory strain such as S.lividans.

Systems for expressing foreign DNA sequences in Streptomyces have previously been described in, for example, EP 148,552 and WO 88/07079. These systems use the endogenous promoters of

extracellular enzymes produced by Streptomyces.

Objects of the Invention

A principal object of this invention is to provide the cloning and characterization of a newly isolated gene, hereinafter referred to as saf (secondary metabolism activation factor) gene, encoding a new amino acid polypeptide (hereinafter referred to as SAF polypeptide). The SAF polypeptide modulates directly or indirectly expression of the genes for extracellular enzymes in Streptomyces by interacting with the control region of the structural genes for the extracellular enzymes.

A second aspect of the invention is DNA units or fragments comprising nucleotide sequences that upon expression encode the above described SAF polypeptide.

A third aspect of the invention is cloning vehicles (vectors) that include the above described DNA.

A fourth aspect of the invention is host organisms or cells transformed with the above described cloning vehicles, thereby resulting in increased production of extracellular enzymes or of a desired heterologous polypeptide.

A fifth aspect of the invention is a process for the preparation of extracellular enzymes or of a desired polypeptide by culturing a transformed host organism according to the present invention and recovering the product from the culture broth.

Still another aspect of the invention is the promoter of the saf gene.

It is yet another aspect of the present invention to produce

cloning vehicles (vectors) which include the promoter of the saf gene operatively linked to the secretion signal region of an endogenous extracellular enzyme, both of which are operatively linked to a foreign DNA sequence, as well as host organisms or cells transformed with such cloning vehicles, thereby resulting in expression and secretion of the foreign polypeptide encoded by the foreign DNA sequence.

Another aspect of the present invention is the integration of such a cloning vehicle carrying a foreign DNA sequence into the chromosomal DNA of Streptomyces. In a preferred embodiment, an expression vector containing the saf gene will also be incorporated into such transformed organisms.

These and other objects of the invention will be described in more detail in the further specification.

Summary of the Invention

A DNA fragment from Streptomyces griseus ATCC 10137 has been isolated that encodes a gene saf (secondary metabolism activation factor) which is involved in a common control mechanism for at least five extracellular enzymes in S.lividans and S. coelicolor. The saf gene is present in all Streptomyces tested, which suggests that this gene must have an important role in metabolism. Indeed, a DNA fragment containing a similar gene to saf has been cloned by us from Streptomyces griseus IMRU 3570, the candicidin producer strain. In some Streptomyces e.g., S.lividans and S.lactamdurans, the hybridization pattern suggests that the saf gene is present in several copies in the chromosomal DNA.

This is the first example of a gene involved in production of extracellular enzymes and sporulation in Streptomyces.

The saf gene encodes a polypeptide of 113 amino acids (the SAF

polypeptide); in vitro transcription-translation studies in E.coli showed that a protein of the correct size, about 15000 dalton, is formed. The SAF polypeptide has a strong positive charge (18 positively charged amino acids) and does not show a composition typical of a peptide leader or a transmembrane protein and, therefore, a direct effect on protein excretion appears to be unlikely. The positively charged protein may easily interact with DNA. In fact, a DNA-binding domain typical of several regulatory proteins (ANN. REV. BIOCHEM. 53:293-321, 1984) is observed in the SAF polypeptide. The saf promoter has unexpectedly been found to be more potent than natural promoter of extracellular enzymes. For example, the amylase gene expresses much greater quantities of amylase when the saf promoter is substituted for the natural amylase promoter. Thus, the saf promoter can be used to enhance the expression of any endogenous polypeptide or protein in place of that protein's natural promoter.

The SAF polypeptide has a pleiotropic effect, i.e., an effect affecting more than one pathway, on extracellular enzymes, on pigment production and on differentiation. In accordance with the invention, an increased production of endogenous (extracellular) proteins is achieved.

Broadly, the saf gene and corresponding polypeptide can be used to increase expression in Streptomyces of selected heterologous proteins by inserting the gene coding for the desired protein into a suitable cleavage site of the gene coding for an extracellular enzyme, the expression of which is enhanced by SAF, or a part thereof, transforming a Streptomyces host containing the saf gene with the recombined gene, and culturing the transformed bacteria to excrete the selected protein or portion thereof. Preferably, the heterologous DNA, under control of the saf promoter, is inserted (integrated) into the chromosomal DNA and an

expression vector containing DNA encoding the SAF polypeptide is concurrently inserted. The expression vector need not be integrated into the chromosomal DNA.

Pleiotropic genes affecting biosynthesis of antibiotics and differentiation in Streptomyces have been reported. A gene afsB which positively controls the production of A-factor, actinorhodin and prodigiosin in Streptomyces coelicolor and Streptomyces lividans was cloned from S.coelicolor A3(2) (Horinouchi et al., J. BACTERIOL. 155:1238-1248, 1983).

The saf gene is different from the afsB and in fact they have opposite effects on production of actinorhodin. They have in common that they have an amino acid sequence typical of DNA-binding proteins. Both afsB and saf genes have promoters without the -10 and -35 consensus sequences and are bounded by potential translational regulatory signals and by strong stem and loop structures that may act as transcriptional terminators (Horinouchi et al., J. BACTERIOL. 168:257-269, 1986). If the transcription forms a very stable loop, binding of the ribosomes will be precluded. Such attenuation-like mechanisms of control of gene expression have been found to modulate control of antibiotic resistance genes in streptomyces (Horinouchi et al., PROC. NATL. ACAD. SCI. USA 77:7079-7083, 1980). Since the saf gene might be considered as a "master" gene which switches on a variety of genes for enzyme excretion, its expression is probably tightly controlled in the cell. The enzymes stimulated by the saf gene are all involved in the degradation of complex polymers and therefor we speculate that this gene is part of a global regulatory system involved in the search for alternative nutrient sources, as suggested also in Bacillus (Henner at al. J. BACTERIOL. 170:296-300, 1988).

Brief Description of the Drawings

Fig. 1 Restriction maps of chromosomal DNA fragments from S.griseus ATCC 10137 clones in the BglII site of pIJ702 that carry the genetic determinant for alkaline phosphatase hyperproduction.

Fig. 2 Southern hybridization analysis of homology between the 1 kb BglII fragment of pULAD3 and genomic DNA of other Streptomyces species. (A) DNA fragments generated by BamHI digestion of genomic DNAs (Lanes 2 to 7). (B) Hybridization of DNAs shown in panel A, with the 1 kb fragment as the probe. Lanes: 1, HindIII digest of DNA giving fragments of 23, 9.59, 6.68, 4.29, 2.28, 1.94 and 0.58 kb; 2, S.lactamdurans; 3, S.acrimycini; 4, S.coelicolor 1157; 5, S.griseus 2212; 6, S.griseus 3570; 7, S.lividans 1326.

Fig. 3 Assays on Petri dishes of protease (A), amylase (B) and lipase (C) activities of S.lividans transformed with pIJ702 (left) and S.lividans transformed with pULAD3 (right)..

Fig. 4 Gene dosage effect on the expression of the saf gene. Production of protease (A) and amylase (B) by S.lividans transformed with pIJ702 (left), S.lividans transformed with pULAD3 (middle) and S.lividans transformed with pULAD30 (right).

Fig. 5 Trimming down of the saf gene. The 1 kb BglII fragment of pULAD3 was used as starting material. Extracellular enzyme production for all the plasmid constructions was measured on solid media plates as indicated in the description: -, wild type producer; ++, +++ and ++++ indicate different rates of hyperproduction. Short open arrows indicate the localization and direction of the transcription of the tyrosinase gene (mel) in plasmid pIJ702. Closed circles (•→) indicate the mel gene promoter; open

circles (·→) represent a putative clockwise promoter activity in pIJ702 and open squares (□→) indicate the saf promoter. The direction of transcription is shown by arrows. The ORF1 corresponds to the saf reading frame deduced from nucleotide sequence data (see Fig.6).

Fig. 6 Nucleotide sequence of the 1 kb BglI1 fragment of pULAD3 and deduced amino acid sequence of the saf gene product. The second putative ATG initiation codon is underlined. Inverted complementary repeat sequences are shown by convergent arrows. The wavy line indicates the amino acid sequence similar to DNA-binding domains of known DNA-binding proteins. The relevant restriction sites are shown. The nucleotides are numbered starting with the BglII site (numbers on the right).

Fig. 6A Restriction enzyme map of plasma constructs, pULTV100, pULTV110, pULTV90, pULTV95, pULA03, pULIVI

Fig. 7 Comparison of a region from the deduced amino acid sequence of the saf gene product with DNA-binding domains in known DNA-binding proteins. Data were taken from Pabo and Sauer (Pabo et al. ANN. REV. BIOCHEM. 53:293-321, 1984) and Horinouchi and Beppu (Horinouchi et al., GENETICS OF INDUSTRIAL MICROORGANISMS, p.41 Pliva, Zagreb, Yugoslavia, 1986).

Fig. 8 Repeated nucleotide sequences "upstream and "down-stream" of the saf gene.

Fig. 9 Repeated groups of nucleotides present in the saf gene.

Fig. 10 Plasmid pIJ2921.

Fig. 11 The complete sequence of nucleotides and inferred sequence of amino acids of the amylase gene of S.griseus. It is thought that cut BstEII is the optimum place for fusion of a foreign gene.

Fig. 12 The strategy used for cloning of the α-amylase gene

of S.griseus IMRU 3570 using the plasmid pIJ699.

Fig. 13 A series of graphs show α-amylase production in dishes by different plasmids in the course of time and in different media:
(△) MM + starch (1%) + thio
(□) MM 1 mMP + starch (1%) + thio
(■) MM without phosphate + starch (1%) + thio
(·) MM + starch (1%) + thio + IPTG
The bottom right-hand panel shows the production values of plasmid pULVD10 as compared with pULTV1, S.lividans and pULTV100 (see Fig. 6a) in the best production media. (x)
S.lividans (pIJ699) in MM without phosphate + 1% starch + thio: (•) pULTV1 in the previous mediums; (-) pULTV100 in the previous mediums; and (△) pULVD10 in MM 1 mMP + 1% starch + thio.

Fig. 14 A schematic representation of the construction of a recombinant Streptomyces cell containing a chromosomal gene producing a fusion protein including the CRF molecule and plasmids containing DNA coding for the SAF polypeptide.

Description of Preferred Embodiments

The work described herein was performed employing the following materials and methods.

Bacterial strains and plasmids. The Streptomyces and E.coli strains used in this study are listed in Table 1. Plasmids and phages are in Table 2.

Media and culture conditions. Streptomyces strains were grown in R2YE, minimal medium (MM), TSB (Difco) or YEME supplemented with 34% sucrose and 5 mM $MgCl_2$ (Hopwood et al., Genetic manipulation of Streptomyces. A LABORATORY MANUAL. The John

Innes Foundation, Norwich, U.K., 1985). Streptomyces strains were grown in triple baffled flasks at 28°C in a rotary shaker with an agitation of 220 rpm.

E.coli strains were grown in Luria Broth (LB) (Miller et al., EXPERIMENTS IN MOLECULAR GENETICS, p.433. Cold Spring Harbor Laboratories, New York, 1972) or Luria Agar (LA) at 37°C.

Enzyme assays in plates. Alkaline phosphatase (AP) assays for Streptomyces were carried out in PRMM medium (MM) without glucose and containing a reduced level [1mM] of phosphate supplemented with 30 mcg/ml of 5-bromo-4-chloro-3--indolylphosphate-p-toluidine (XP). Colonies producing AP were blue and those unable to produce it were white. For E.coli, B-XP medium was used (containing per litre: 10 g bactopectone, 12 g Trisma-base, 10 g NaCl, 20 g Difco agar, pH 7.5 and 40 mcg/ml of XP).

Amylase activity of Streptomyces colonies was assayed on MM (without glucose) supplemented with 1% starch. After 3 days of growth, the plates were exposed to iodine vapour. Zones of clearing around the colonies are due to starch degradation. Lipase activity was measured by growing the Streptomyces in MM supplemented with 2% (w/v) olive oil, 0.5% (w/v) Tween 80 and 0.5% (w/v)

Tween 20. After 3 days of growth the plates were flooded with 1 ml of 1M $CaCl_2$. The lipase production was observed as a precipitate of Ca^{2+}-fatty acid complex (Odera et al., FERMENT. TECHNOL. 64:363-371, 1986). Protease activity was assayed in MM (without glucose) supplemented with 0.5% casein and 10 mM $CaCl_2$. The enzyme activity was detected as zones of clearing around the colonies.

Agarase activity of S.coelicolor was assayed in MM without

glucose by flooding the plates with Gram's iodine solution. Beta-galactosidase activity was observed as a blue colour of the colonies growing on MM without glucose, and supplemented with X-gal (36 mcg/ml) and IPTG (10 mcg/ml).

DNA isolation. Total DNA from Streptomyces was prepared as described by Hopwood et al. (Hopwood et al., A LABORATORY MANUAL, The John Innes Foundation, Norwich, U.K., 1985). Plasmid DNA from Streptomyces or E.coli was isolated following the method of Kieser (Kieser et al., PLASMID 12:19-36, 1984).

Cloning procedures. Ten mcg of S.griseus ATCC 10137 chromosomal DNA and 0.5 mcg of pIJ702 were totally digested with BglII, and ligated for 12 h at 14°C using T4 DNA ligase. The ligation mixture was used directly for transformation. Subcloning of DNA fragments was carried out by digesting 1-2 mcg of plasmid DNA with adequate restriction enzyme(s) and the reaction products were separated by gel electrophoresis in low melting point agarose (LMPA). The required DNA bands were extracted using the CTAB-assisted method (Langridge et al., ANAL. BIOCHEM. 103:264271, 1980).

Transformation methods. Streptomyces strains were transformed as described by Hopwood et al. (Hopwood et al., A Laboratory Manual, The John Innes Foundation, Norwich, U.K., 1985). After transformation, protoplasts were plated on R2YE medium, and allowed to regenerate for 15-20 h at 30°C. Then, 1 ml of an aqueous solution of thiostrepton (300 mcg/ml) was poured over the plates, dried for 1 or 2 h and incubated for 2 or 3 days more. Transformants were replicated to PRMM medium containing thiostrepton (50 mcg/ml) and XP (30 mcg/ml).

Transformation of E.coli was done according to Cohen et al. (PROC. NATL. ACAD. SCI. USA 69:2110-2114, 1972). Transformants were selected on plates containing ampicillin (200 mcg/ml).

When required, X-gal (36 mcg/ml) and IPTG (10 mcg/ml) were added to LA plates.

Hybridization studies. Transfer of DNA from agarose gels to nitrocellulose filters and hybridizations were carried out as described by Hopwood et al. (A Laboratory Manual, The John Innes Foundation, Norwich, U.K., 1985). The 7.2 Kb BglII fragment from plasmid pULAD1 and the 1 Kb BglII fragment from plasmid pULAD3 were used as probes. Hybridizations were performed at 70-C for 24 h. The filters were washed twice for 30 min in 2 g SSC, 0.1% SDS and then twice again for 30 min in 0.2 g SSC, 0.1% at 70°C.

Nucleotide sequence analysis. The nucleotide sequence was determined by the chain termination method of Sanger et al. (PROC. NATL. ACAD. SCI. USA 74:5463:5467, 1977). The DNA fragments were subcloned into M13mp10 and M13mp11 to obtain the insert in either orientation. Ligation mixtures were transfected into competent E.coli JM103 cells and white plaques were screened for selection of inserts. The sequencing was performed in both strands using Amersham International plc. (U.K.) and Sequenase (United States Biochemical Corporation, USA) kits. All the fragments were sequenced using dGTP, but dITP was used instead of dGTP when needed. Reaction mixtures were separated on 6% or 8% polyacrylamide sequencing gels which were then exposed to X-ray film for autoradiography.

Promoter cloning. Fragments with transcription initiation activity were selected by using the multicopy promoter-probe plasmid pIJ486 (Ward et al., MOL. GEN. GENET. 203:468-478, 1986). Kanamycin (Km) resistant transformants were isolated by replication of colonies to MM containing 15 mcg/ml of Km.

In vitro transcription-translation. Plasmids pULAD300 and

pUC19 were transcribed and translated using the prokaryotic DNA directed translation kit from Amersham International plc. L(35S) methionine was used as radioactive label. 12.5% polyacrylamide gels containing sodium dodecyl sulphate were used to analyze the labelled proteins.

Modes of Carrying Out the Invention

The following detailed description will illustrate the invention:

Alkaline phosphatase production by different Streptomyces.

The alkaline phosphatase production in several solid media of ten different Streptomyces strains (listed in Table 1) was assayed. S.griseus IMRU 3570 and S.griseus ATCC 10137 were the best producers in all the media assayed. The best solid medium for alkaline phosphatase production was PRMM (containing 30 mcg/ml XP). In this medium, S.griseus IMRU 3570 and S.griseus ATCC 10137 showed a deep blue colour after 48 h of growth. S.lividans 1326 and S.coelicolor JI 2280 were poor alkaline phosphatase producers: after 90 h of growth on PRMM containing XP only a weak blue colour can be observed.

Cloning of a gene involved in alkaline phosphatase production.

Total DNA from S.griseus ATCC 10137 was digested with BglII, ligated to BglII-digested pIJ702 and the ligation mixture introduced by transformation into S.lividans 1326 protoplasts. The transformants were replicated to PRMM containing thiostrepton (50 mcg/ml) and XP (30 mcg/ml). One deep blue colony was found among 2,800 melanine negative transformants of S.lividans. The deep blue colony contained a pIJ702 derivative carrying a 7.2 kb BglII insert which was named pULAD1 (Fig. 1).

Plasmid pULAD1 was unstable in S.lividans and upon retransformation gave riss to white and blue colonies. All the

blue colonies contained the original pULAD1 and the white ones contained deleted forms of pULAD1 that were no longer studied. Since the instability might be caused by the large size of the insert in a plasmid such as pIJ702, which is known to have a certain instability, the 7.2 kb insert was subcloned into the 5 kb BglII fragment of plasmid pIJ699 (Kieser et al. GENE 65:83-91, 1988). Two plasmids, pULAD100 and pULAD101 were obtained with the insert in opposite orientation. Both plasmids were stable and the gene was expressed in both orientations in S.lividans.

Localization of the Saf gene.

The 7.2 Kb BglII fragment was partially digested with Sau3AI, and ligated to BglII-digested pIJ702. The ligation mixture was transformed into S.lividans protoplasts and transformants were replicated to PRMM containing XP. Plasmid DNA was isolated from several blue colonies. Four small plasmids, pULAD2, pULAD3, pULAD16 and pULAD18, carrying the determinant for alkaline phosphatase production, were studied in detail; they were stable, retransformed S.lividans protoplasts, and carried inserts of 2.4, 1, 2.1 and 1.9 Kb (Fig.1), respectively. The plasmid pULAD3 has the smallest insert (1 Kb) which can be rescued with BglII. When introduced into S.lividans, pULAD3 (deposition number I-859 on 19.05.89 with CNCM (Collection Nationale de Cultures de Microorganismes, 28 rue du Docteur Roux, F75724 Paris Cedex 15, France) under the Budapest Treaty and Rule 28 EPC) increased alkaline phosphatase production like pULAD1.

Hybridization with chromosomal DNA of several Streptomyces.

Total DNA from S.griseus ATCC 10137 digested with BamHI or BglII were hybridized to the 7.2 Kb BglII fragment from pULAD1 labelled by nick translation with (32p) dCTP. A 7.2 Kb BglII fragment homologous to the probe was present in S.griseus ATCC 10137 DNA digested with BglII, as expected. There was an

internal BamEHI site in the fragment that resulted in two clear hybridization bands (of 7.9 Kb and 9.4 Kb) when total DNA was digested with BamHI.

To study if the same gene was present in other Streptomyces, total DNA from S.acrimvcini, S.coelicolor 1157, S.griseus 2212, S.griseus 3570, S.lividans 1326 and S.lactamdurans NRRL 3802 were digested with BamHI and hybridized with the 1 Kb BalII fragment of pULAD3 as a probe. Hybridization with a 9.5 Kb common band was observed in the first four strains of Streptomyces cited above, whereas 4 and 5 weak hybridization bands were observed in the DNA of S.lividans and S.lactamdurans, respectively (Fig.2). In addition, hybridization was also observed with the DNA of S.albus G, S.coelicolor JI 2280, S.clavuligerus NRRL 3585 and S.fradiae ATCC 10475. No further attempts were made to characterize the hybridizing bands.

Lack of complementation of E.coli pho-mutants

Complementation of the E.coli phoA mutants E15 and AW1046 was tried to check if we had cloned the alkaline phosphatase structural gene from S.griseus ATCC 10137. The 7.2 Kb BglII fragment from pULAD1 and the 1 Kb BglII fragment from pULAD3 were subcloned separately in both orientations in BamHI-digested pUC19. All these plasmid constructions were verified in E.coli JM103 cells and then used to transform E.coli E15 (Sarthy et al., J. BACTERIOL 145:288-292, 1981) and E.coli AW1046. No blue colonies in B-XP were found suggesting that the complete structural gene for alkaline phosphatase is not present in the 7.2 kb fragment of s.griseus or that it is not expressed in E.coli. The structural gene for alkaline phosphatase (phoA) or Bacillus 11cheniformis (Hullett, F.M., J. BACTERIOL. 158:978-982, 1984) did not hybridize with the cloned 1 Kb fragment (data not shown).

In addition, the phoB (regulatory) E.coli mutant H2 (Kreuzer et al., GENETICS 81:459-468, 1975) was not complemented by either the 1 kb BglII fragment of pULAD3 or the 7.2 BglII fragment of pULAD1.

Overproduction of other extracellular enzymes.

When pULAD3 was used to retransform S.lividans protoplasts, a clear delayed pigmentation and sporulation was observed. These pleiotropic effects prompted us to study the role of this gene in protein secretion or in control of gene expression. As shown in Fig.3, several extracellular enzymes, including amylase, protease and lipase, were overproduced by S.lividans transformed with pULAD3. The ß-galactosidase production was also increased and its production started about 24-30 h earlier than in untransformed S.coelicolor. Due to the pleiotropic effects of the cloned gene on extracellular enzymes, on pigment production and on differentiation, this gene was named saf (secondary metabolism activation factor).

Gene dosage effect.

The copy number of pIJ702 is estimated to be 100 to 200 per chromosome. Although the exact copy number of pULAD3 was not determined, the intensity of both pIJ702 and pULAD3 bands suggested a similar copy number.

To study the gene dosage effect we subcloned the 1 kb BglII fragment of pULAD3 into the BamHI site of the low copy number plasmid pIJ61 (3 to 4 copies per cell) (Hopwood et al. A Laboratory Manual. The John Innes Foundation, Norwich, U.K.). This new plasmid was named pULAD30. Fig.4 shows that the production of extracellular enzymes by S.lividans transformed with pULAD30 clearly decreased with respect to S.lividans carrying pULAD3. In addition, S.lividans carrying pULAD30 showed a similar pigment production and sporulation pattern as untransformed S.lividans.

Expression in E.coli and trimming down of the gene.
Although Pho mutants of E.coli were not complemented by the saf gene, we observed that the 1 kb BalII insert of pULAD3, when subcloned in one direction in pUC19 (plasmid named pULAD300) was expressed in E.coli causing abnormal morphology when grown on solid media, but it was not expressed when inserted in opposite orientation (plasmid pULAD301). Plasmid pULAD300 contains the ORF (see below) downstream from the lacZ promoter. These results suggest that the saf gene is expressed in E.coli from the lacZ promoter present in pUC19.

In-vitro transcription-translation in E.coli assays (with pULAD300) were carried out and the products of the reaction were loaded in a 12.5% polyacrylamide gel. A band of MW 15000 was present in the lane corresponding to pULAD300 which was absent in the control pUC19 lane.

To locate precisely the saf gene it was decided to continue the splitting of the 1 Kb fragment of plasmid pULAD300. In these trials there was exploited the existence of unique cut sites for restriction enzymes SstI (nt 216), KpnI (nt 648) and Sa1GI (nt 936) within the 1 kb fragment. The different sub-fragments from the 1 kb insert of pULAD300 were cloned in a first step into pIJ2921, a derivative of pUC18 containing a modified polylinker flanked with Bg1II sites (see Fig. 10) and then rescued with Bg1II cohesive ends and cloned into Bg1II-digested pIJ702. Alkaline phosphatase, amylase and protease production was studied in Streptomyces lividans with all plasmid constructions. The results of these studies appear in outline form in Fig. 5 and the following conclusions have been extracted from their interpretation:

1. The 1 kb fragment contains the complete saf gene, including its own promoter, since it was expressed at a similar level in both orientations of plasmid pIJ702

(plasmid pULAD3 and pULAD4).

2. The saf gene seems to be located in the 648 nucleotides BglII-KpnI fragment (plasmids pULAD5 and pULAD6).
3. Fragment SstI-KpnI (432 nucleotide) seems to contain the genetic determinant for extracellular enzyme hyperproduction, but appears to have lost its promoting region, since it was expressed only in one orientation (plasmid pULAD10 and pULAD14), but not on the opposing one (plasmid pULAD9 and pULAD13).
4. The 215 nucleotides fragment BglII-SstI contains the saf gene promoter region.
5. All the effects on extracellular enzymes and on differentiation were kept or lost together, indicating that a single genic product was responsible for the action on all the enzymes.
6. A surprising aspect was the lack of expression of fragment SstI-KpnI (saf gene without promoter), starting from the tyrosinase promoter of the mel- gene present in pIJ702, which nevertheless was expressed in the opposite direction (clockwise direction). This finding implied that there was a fragment with promoter activity located before gene mel, and at the BglII cloning site (Bernan et al., GENE 37:101-110, 1985). Although such finding has not been published, it has occasionally been mentioned by some authors (Gil and Hopwood, GENE 25:119132, 1983). The possibility of a functional ORF in reverse direction (from Kpn1 -- SstI) was discarded, for several reasons:

A. Such ORF was not detected when the nucleotide sequence was analyzed.
B. If such ORF were to exist, it would be clear that it would have to carry its own promoter, since fragment BgnII-KpnI (nt 1 to nt 648) was expressed in both directions (plasmids pULAD5 and pULAD6). It did not make sense then, that the KpnI -- SstI fragment which would

carry the promoter in the KpnI zone could only express itself in one direction.

C. Expression in E.coli always took place when the promoter lacZ was in favor of (before) the proposed ORF, and never in the opposite direction.

Promoter activity of ORF1 upstream DNA fragment

In order to confirm the existence of a promoter in the BglII - -SstI fragment, as could be inferred from previous experi- carries the gene that codifies for aminoglycoside phosphotransferase (neo), without its promoter. The expression of this gene made S.lividans resistant to kanamycin and neomycin. When the fragment was subcloned in pIJ486 (with the SstI end next to neo gene), plasmid pIJ484::216 was created. It was observed that S.lividans transformed with pIJ486::216 grows in MM with over 100 mcg/ml Km while S.lividans transformed with pIJ486 did not grow in MM supplemented with 5 mg/ml Km. These results indicated that such fragment had promoter activity and supported the proposed ORF.

Other considerations that were deduced from the nucleotide sequence and probable ORF, included:

1. Gene saf was surrounded by two regions with inverted and complementary repeated sequences, which could form in mRNA very stable bonds G = -38.4 kcal, being gene saf, (nt 637 - 697) (Fig.8).
2. The sequenced fragment contains a high G+C percentage, as is usual in Streptomyces genes.
3. Before gene saf, between ATG (nt 219) and the repeated upstream structures, there is a very interesting nucleotide region: there are three pairs of repeated nucleotides (each one with 7 nt) (Fig.9). It is very likely that this region, especially the promoter zone, plays a very important part in the regulation of gene saf expression.

4. It is worth mentioning that the SAF protein contains 18 net positive charges. From a hypothetical perspective, this could imply the existence of a much greater affinity for DNA. Even in the deduced amino acid sequence, a very similar region to the domains of DNA-linking proteins can be observed (Pabo and Sauer, 1984) (Fig.7).

Nucleotide sequence of the saf gene.

The nucleotide sequence of the entire 1 kb BglII insert of pULAD3 was determined using plasmid pULAD300 as the starting material. Since M13mp10 and M13mp11 have no KpnI site, the fragments with KpnI ends were first subcloned into pUC19 and then rescued as EcoRI-HindIII fragments and introduced into mp10 and mp11 digested with EcoRI and HindIII. The complete nucleotide sequence of the active region shows an ORF1 of 339 nucleotides which encodes the putative SAF polypeptide (Fig. 6). There is only one possible open reading frame contained in the BglII-KpnI fragment starting with an ATG initiation codon at nt 183 and terminating with a TGA stop codon at nt 524 (ORF1 in Figs. 5 and 6). Since the SstI-KpnI insert, contained in plasmid pULAD14, showed a lower degree of activity than plasmids containing also the upstream region of the SstI site (pULAD3, pULAD4, pULAD4 and pULAD6), it is very likely that the ATG (nt 219) also in frame with the presumptive saf reading frame may act as the initiation codon in pULAD10 and pULAD14. No other alternative initiation triplets are possible since a TGA termination codon exists upstream of the first ATG. A long inverted repeat region, which may form a very stable stem and loop structure (G=-53.6 kcal) is present upstream of the saf gene, from nt 90 to nt 135. Another hyphenated inverted repeat sequence was observed downstream from the terminator triplet of ORF1, extending from nucleotides 637 to 697, (G = -38.4 kcal).

The saf gene has a high G+C content (76.3%) and a marked genes

(Hopwood et al., Regulation of Gene Expression 25 Years On, Cambridge University Press, p.251-276, 1986).

Promoter activity of the DNA fragment upstream of the ORF1.

Since plasmids lacking the BglII-SstI fragment (from nt 1 to nt 216) were only expressed in one orientation (plasmids pULAD10 and pULAD14), but not in the opposite (plasmids pULAD9 and pULAD13), it seemed likely that the ORF1 promoter was located in that fragment. The presence of a transcription initiating sequence in this region was confirmed by subcloning this fragment into the promoter-probe plasmid pIJ486 (Ward et al., MOL. GEN. GENET. 203:468:478, 1986) which carried a promoterless aminoglycoside phosphotransferase gene (neo). Expression of this gene confers kanamycin and neomycin resistance to S.lividans. The BglII-SstI fragment was subcloned into pIJ486 (the SstI end proximal to the neo gene) (plasmid named pIJ486::216). S.lividans transformed with pIJ486::216 was able to grow on MM containing more than 100 mcg/ml of km, whereas S.lividans carrying pIJ486 does not grow on MM with 5 mcg/ml of km. This result indicates that the BglII-SstI fragment has promoter activity. However, no typical "consensus" -10 or -35 regions were identified by nucleotide homology with other promoters of Streptomyces (Hopwood et al., Regulation of Gene Expression 25 Years On, Cambridge University Press, p.251-276. 1986).

Cloning of amylase gene.

The definitive sequence of the amylase gene (amy) of S.griseus IMRU 3570 has now been elucidated. The complete sequence of nucleotides and inferred sequence of amino acids of the amylase gene of S.griseus appears in Fig. 11. The protein with the leader peptide has 566 amino acids (from nucleotide 318 to 2155, both inclusive), which correspondg to a PM of 59,713 D. Plasmid pULTV1 containing the entire α-amylase gene has been constructed by digesting the DNA of S.ariseus IMRU 3570 with

Sau3A, isolating the fractions of 3 to 9 Kb and ligating to Bgl II digested pIJ699. After screening for α-amylase activity the pULTV1 plasmid was selected which contained the entire α-amylase gene. The construction of pULTV1 is set forth in Fig. 12. Another way of obtaining a plasmid with the entire α-amylase gene is to construct it from two smaller fragments. Among the plasmids obtained by cloning the entire DNA library of S.griseus are those with a partial gene starting at the 5' end and those with partial genes starting at the 3' end. From the former a 1.3 Kb fragment BamHI-SacI, which includes the 5' end of the gene can be obtained and from the latter a 1.2 Kb SacI-SalI fragment including the 3' end of the gene can be extracted. Both can then be linked to pIJ2921 treated with SalI-BamHI, to obtain a plasmid with the intact native α-amylase gene (pULTV200).

Superior potency of saf promoter.

Yo test the expression of the amy gene under the control of different promoters, the production of amylase by S.lividans JI66 was studied. The amy gene was placed under the control of different promoters and amylase production was compared to that produced using the native promoter (PamY). The promoters tested were that of the saf gene (Psaf), that of the Km^r gene (Pneo), that of the synthetase PABA gens (Ppab) and that of the E.coli ß-galactosidase gene (Plac). Plasmid pULTV220 containing the αamylase gene without promoter was obtalned by ligation of the EcoRI-Bg1II fragment, extracted from pULTv200, to the pUC18 digested with EcoRI-BamiI. The EcoRI-HindIII fragment from pULTV220 was ligated directly to the promoters of the saf, pab or Km^r genes to obtain the pULVD10, pULVA1 and pULTV80 plasmids, respectively. pULTV150, containing the promoter of lac was obtained directly by HindIII digestion of pULTV220 and ligation to the 5 Kb HindIII fragment of the

pIJ699.

The production studies of the resultant plasmids were carried out in dishes of MM + starch (1%) + thiostreptone, MM (1 mM P) + starch (1%) + thiostreptone, MM without phosphate + starch + thiostreptone and MM + starch (1%) + thlostreptone + IPTG. pIJ699, pULTV100 (See Fig. 6a) and pULTV1 were used as controls. The relative measurement of amylase production was considered to be the area of circular corona obtained around each colony when staining the growth medium with I_2.

The production graphs of the different constructions are set out in Fig. 13. Except in the controls, the highest production was achieved in MM (1 mM P) + starch + thiostreptone. It is clearly noticeable how the production peak was obtained with pULVD10 (which has the saf gene promoter) (1482 mm^2 as compared with the 1335 mm^2 of the second highest producer pULTV150, which bears the Plac promoter at 114 h of incubation).

In Fig. 13 (lower right-hand panel) the production values of pULVD10 are represented as compared with the controls in the best conditions so far known for each type of construction. The increase in pULVD10 production as compared with pULTV1 (native) is quite considerable: 1482 mm^2 as opposed to 1105 mm^2.

Thus, the saf gene promoter can be used as a substitute for other native promoters to improve expression of various endogenous Streptomyces polypeptides and proteins. Similarly, if a foreign DNA is inserted, the saf gene promoter will be expected to provide similar superior results as were demonstrated with amylase.

Expression of foreign polypeptide or protein.

To express a foreign polypeptide or protein the foreign DNA is preferably inserted at a suitable recognition site of an endogenous gene, preferably a gene encoding an extracellular enzyme so as to ensure secretion of the foreign polypeptide or protein. When the gene is the amylase gene, a suitable recognition site is the BstEII site (see Fig. 11). The foreign DNA is preferably inserted in such a way as to retain the secretion signals as much as possible from the extracellular enzyme gene. While these signals are predominantly on the leader sequence, there is evidence with respect to the amylase gene that the terminal carboxyl end is also important for secretion. Thus, it may be best to create a fusion protein by inserting the foreign DNA into the endogenous DNA, rather than removing the transcriptional part of the endogenous DNA and substituting the foreign DNA.

It is known that the saf gene controls the production of extracellular enzymes in the chromosomal DNA. Presumably, there are recognition sequences in the chromosomal DNA which recognize the SAF polypeptide and cause enhancement of extracellular enzyme production. The combination of the saf gene with the amylase gene on a plasmid does not cause increased production of amylase. Thus, it is preferred to place the foreign protein operatively linked to the secretion signal sequence of an endogenous extracellular enzyme which is controllable by SAF and, preferably, further operatively linked to the saf promoter (in the absence of the native promoter), into the chromosomal DNA as opposed to a plasmid. In this way, production of the foreign polypeptide or protein will be further enhanced by the native SAF. In a further preferred embodiment, the saf gene will be inserted by means of a plasmid into the same organisms in which the foreign DNA has been inserted into the chromosomal DNA. This will ensure enhanced secretion of the foreign polypeptide or protein.

DNA fragments may be cloned into the chromosomal DNA of Streptomyces by means of any of the known bacteriophage vectors for Streptomyces, such as ϕC31 or R4 (Chater et al, "Gene Cloning in Streptomyces" in Gene Cloning in Organisms Other Than E.coli, edited by P.H. Hofschneider et al, Springer-Verlag, Berlin, 1982, pages 87-95).

The diagram of Fig. 14 shows the preferred technique for obtaining secretion of a foreign protein, in this case CRF, from Streptomyces. KC 400 is a ϕ31 derivative which lacks the attachment site (att$^-$) and has the C$^+$ gene. See Chater, K.F. et al "Mutational cloning in Streptomyces and the isolation of antibiotic production genes," Gene, 26:67-78 (1983). The C$^+$ gene encodes the repressor necessary for maintaining the lysogenic state. The att site in the wild type phage directs the attachment of the phage to the host cell DNA and directs the liberation therefrom. Without the att site, the phage cannot be integrated into the host chromosome unless a homologous DNA fragment is cloned into it. Any endogenous gene in the host strain may be used for this purpose and, thus, it is merely designated as "gene X" in Fig. 14. The phage ϕ31 KC 400 is engineered by known techniques (see Hopwood, D.A. et al "Genetic manipulation of Streptomyces. A Laboratory Manual", The John Innes Foundation, Norwich, U.K. (1985)) to insert the homologous gene fragment (gene X) as well as a gene including the saf promoter and the endogenous α-amylase gene with the gene for CRF operatively linked to and in reading frame with the α-amylase gene. After infection of a host Streptomyces strain, preferably S.coelicolor A3(2) (available from the collection at the John Innes Institute, Norwich, U.K.), by this phage, the entire phage DNA will be inserted into the chromosomal DNA of the host cell, beginning at the homologous gene X, preferably using Campbell-type recombination. As indicated, all of the specific techniques for accomplishing this are within the skill of those of

ordinary skill in this art so that such insertion can take place without undue experimentation.

The cells with the inserted chromosomal gene are then treated to take up the pULAD3 plasmid. Such cells will express the SAF polypeptide which in turn will enhance the secretion of "α-amylase" by the chromosomal gene. The presence of the saf promoter on this gene will further enhance secretion of the "αamylase." The "α-amylase" secreted will include the CRF molecule fused thereto which can be readily separated by appropriate enzymatic digestion.

While α-amylase gene of Streptomyces has been specifically exemplified, it should be understood that for the purpose of enhancing expression through use of the saf promoter, the gene for any polypeptide or protein produced by the Streptomyces species being used can be modified by removing the native promoter and substituting the saf promoter. Similarly, the foreign polypeptide or protein can be inserted into any such endogenous gene. Preferably, however, the endogenous gene selected will be one which expresses the protein through the cell wall and into the culture medium, in which case it is important to retain the secretion signal sequence. The best results will be obtained when the gene being selected for insertion of the foreign gene is one which is controlled by SAF and the insertion is in the chromosomal DNA, preferably with the concurrent insertion of a plasmid containing the saf gene.

While CRF has been specifically mentioned, it should be understood that the foreign DNA sequence may be any non-Streptomyces-derived DNA sequence encoding a protein or polypeptide, particularly one of eukaryotic or viral origin. Examples of such eukaryotic and viral DNA sequences are sequences encoding human and animal leukocyte interferons

(IFN-α), fibroblast interferons (IFN-β), and immune interferons (IFN-γ), human insulin, human and animal growth and other hormones, such as corticotropin releasing factor (CRF), human serum albumin and various human blood factors and plasminogen activators, both tissue and urokinase, hepatitis B viral core and surface antigens, FMD viral antigens and other human, animal and viral polypeptides and proteins.

While the saf promoter is preferred, production of the foreign polypeptide or protein will further be enhanced by transforming the host cell with an expression vector containing the saf gene. This expression vector may be in the plasmid DNA of the cell. Thus, the endogenous promoter or any other promoter may be used and still obtain secretion of the foreign polypeptide or protein.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without departing from the generic concept, and therefore such adaptations and modifications are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation.

Table I. Bacterial Strains

Designation of	Relevant characteristics	Source reference
S.griseus ATCC 10137	wild type	ATCC
S.griseus JI 2212	wild type	JI
S.griseus IMRU 3570	wild type	IMRU
S.lividans JI 1326	wild type	JI
S.acrimycini JI 2236	wild type	JI
S.coelicolor JI 2280	CysE24, PabA1, str1	JI
S.coelicolor JI 1157	wild type	JI
S.clavuligerus NRRL 3585	wild type	NRRL
S.lactamdurans NRRL 3802	wild type	NRRL
S.albus G	wild type	JI
E.coli E15	phoA8	CGSC
E.coli AW1046	PhoA, phoC, tsZ::tn5, leu, Kan^R	1
E.coli H2 (E.coli CGSC 5259)	phoB52, pho-35	CGSC
E.coli JM103	proAB, lacZ M15	2

JI : Collection of microorganisms of the John Innes Institute. Coleny Lane, Norwich NR4 UH, United Kingdom.

ATCC : American Type Culture Collection.

IMRU : Waksmans Institute of Microbiology, Rutgers University, New Brunswick, N.J., USA.

NRRL : Northern Regional Research Laboratories, Peoria, ILL. USA.

CGSC : E.coli Genetic Stock Center

Table 2. Plasmids and Phages

Designation	Relevant characteristics	Source of reference
pIJ702	Thiostrepton resistance and melanine$^+$	3
pIJ699	Thistrepton, viomycin and Kanamycin resistances	4
pIJ61	Thiostrepton and neomycin resistances	5
pIJ486	Thiostrepton resistance and saf gene without promoter	6
pULAD series	Streptomyces and E.coli vectors carrying saf gene and/or adjacent sequences	This work
pIJ486::216	pIJ486 carrying the saf promoter region	This work
pUC19	bla$^+$, lacZpZoZ'	7
pIJ2921	bla$^+$, lacZpZoZ'	See Fig. 10
M13 mp10 and mp11	Phages for generation of single-stranded DNA	8
pULTV1 and 200	Streptomyces vectors carrying amylase gene	This work
pULTV220	Streptomyces vector carrying promoterless amylase gene	This work
pULVD10	Streptomyces vector carrying amylase gene with saf promoter	This work
pULVA1	Streptomyces vector carrying amylase gene with pab promoter	This work
pULTV80	Streptomyces vector carrying amylase gene with Km^r promoter	This work
pULTV150	Streptomyces vector carrying amylase gene with lac promoter	This work

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QUANTIFICATION OF THE CARRIER PROTEIN (AMYLASE) IN THE CULTURE BROTHS (SUPERNATANTS) OF CULTURES OF Streptomyces lividans TRANSFORMED WITH DIFFERENT PLASMIDS

We have used three different methods to determine the amount of protein secreted.

1. Spectrodensitometry of the protein bands in SDS-PAGE gels stained with silver or of the photographic negatives (films) of the same gels, using a Shimadzu spectrodensitometer.
2. Analysis of the amounts of different proteins using an HPLC equipped with a column C8 Aquapore RP-300 (200x 4.6 nm: Brownlee Labs, USA).
3. Analysis by HPLC equipped with a molecular Sieving (gel filtration) column Protein Pak 300 SW.

RESULTS

Spores of S. lividans [pULTV100], S. lividans [pULVD10] and S. lividans [pULTV80] were inoculated in medium Lechevalier + 1% starch. Maximal amylase activities were obtained respectively at 36, 60 and 60 h of fermentation. Total protein in the extracellular fluid (supernatant) was quantified by the Bradford method. 5 μg of protein of each broth were applied to SDS-PAGE and stained with silver.

1. Spectrodensitometry - Spectrodensitometry was carried out of the bands in SDS-PAGE (10% acrylamide) of supernatants of S. lividans [pULTV100] (5 μg of protein), S. lividans (pULVD10] (5 μg of protein, and S. lividans (pULTV80] (5 μg of protein).
2. HPLC in column Aquapore RP-300 - The proteins were eluted with 50 mM acetic acid-ammonium acetate buffer and a

gradient of methanol; 0-20 min 0% methanol; 30-35 min 100% methanol: 45 min 0% methanol. The amylase elutes with 100% methanol at min 33-34. Results are shown in Table 3.

3. HPLC in protein-Pak 300 SW - Supernatants of culture broths of the same construction as above were previously dialyzed and concentrated by liophylization. The proteins were eluted with acetic acid-ammonium acetate buffer with a flow of 0.5 ml/min. The amylase elutes as a peak centered at min 21-22.

Table 3

			α-amylase (%)			α-amylase protein (mg/l)		
			HPLC			HPLC		
Maximal Specific activity (nkatal/mg dr weight)		Protein concentration in the supernatant (mg/l)	Protein Pak 300 SW	Aquapore RP-300	Shimadzu (450 nm)	Protein Pak 300 SW	Aquapore RP-300	Shimadzu (450 nm)
pULTV100	25 (36 h)	143	58%	40%	49.6%	82.9	57.2	71
pULV010	450 (60 h)	965	86.03%	70.5%	84.2%	849.5	680	812
pULTV80	430 (60 h)	662	--	--	88.6%	--	--	586

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The embodiments of the invention in which exclusive property or privilege is claimed are defined as follows:

1. A substantially pure SAF (secondary metabolism activation factor) polypeptide obtainable from Streptomyces which polypeptide modulates directly or indirectly expression of genes for extracellular enzymes in Streptomyces by interacting with a control region of structural genes for the extra cellular enzymes having the following amino acid sequence:

Met Cys Ser Ala Val Val Ala Ala Ala Val Ser Ser Met Ser Arg
Arg Arg Arg Arg Ala Ser Ala Thr Arg Arg Ser Ala Ala Val Ser
Pro Pro His Thr Pro Tyr Gly Ser Gly Cys Ile Ser Ala Cys Ser
Trp His Ser Thr Ile Thr Gly His Arg Ala Gln Thr Arg Leu Ala
Ala Ser Ser Arg Ala Ser Arg Ala Ala Val Gly Ser Phe Asp Gly
Ala Lys Asn Arg Pro Ala Ser Ser Arg Arg Gln Ala Ala Ser Glu
Cys Gln Gly Pro Ala Ser Ser Ser Arg Ala Gly Val Arg Trp Ala
Gly Thr Ala Ala Asn Gly Arg Gly ter

and/or fragments thereof having the same activity.

2. A DNA sequence coding for a polypeptide according to claim 1.

3. A DNA sequence according to claim 2 and comprising the following nucleotide sequence:

ATG TGC TCG GCC GTC GTC GCG GCG GCC GTG AGC TCG ATG TCC CGC
CGT CGC CGC CGG GCC AGC GCC ACC CGG CGC TCC GCT GCG GTG AGC
CCG CCC CAC ACT CCG TAC CGC TCG GGC TGT ACT AGC GCG TGC TCC
TGG CAC TCC ACC ATC ACC GGA CAC CGG GCA CAG ACC CGC TTG GCC
GCC TCC TCG CGG GCC AGC CGG GCG GCG GTC GGC TCC TTC GAC GGG
GCG AAG AAC AGG CCG GCC TCG TCG CGG CGG CAG GCC GCA TCG GAA
TGC CAG GGG CCC GCG TCG TCC TCC CGT GCG GGG GTC CGC TGG GCC
GGA ACG GCG GCG AAC GGC AGA GGC TGA.

4. A DNA sequence which hybridizes with a nucleotide sequence according to claim 3 and which codes for a polypeptide having the activity of the SAF polypeptide according to claim 1.

5. A vector containing a DNA sequence according to claim 2.

6. A vector according to claim 5, wherein the DNA sequence is flanked by regulatory units.

7. A vector according to claim 6, wherein the regulatory unit upstream to the ATG has the following nucleotide sequence:

AGATCTCCTCGTCCCACCGGCTGTCGAAGCTCCGCGCCTACAGCGCCATCGACTT
CGACCGGGCGAAATAGGAAGCGGCCGGCGACAAAACGGGGAGGGGCGGGAAACGG
TGGTCCGTTTCCCGCCCCTGCCCGTAGGCCGTGCGCGTCCCGCCGGTGCGCGGCG
TTCAGCCCGCCGCCGCT.

8. A Streptomyces host cell transformed with a vector according to claim 5.

9. A process for preparing a polypeptide by culturing a host cell according to claim 8.

10. A vector containing a DNA sequence according to claim 3.

11. A vector according to clalm 10, wherein the DNA sequence is flanked by regulatory units.

12. A vector according to claim 11, wherein the regulatory unit upstream to the ATG has the following nucleotide sequence:

AGATCTCCTCGTCCCACCGGCTGTCGAAGCTCCGCGCCTACAGCGCCATCGACTT
CGACCGGGCGAAATAGGAAGCGGCCGGCGACAAAACGGGGAGGGGCGGGAAACGG
TGGTCCGTTTCCCGCCCCTGCCCGTAGGCCGTGCGCGTCCCGCCGGTGCGCGGCG
TTCAGCCCGCCGCCGCT.

13. A Streptomyces host cell transformed with a vector according to claim 10.

14. A process for preparing a polypeptide by culturing a host cell according to claim 13.

15. A vector containing a DNA sequence according to claim 4.

16. A vector according to claim 15, wherein the DNA sequence is flanked by regulatory units.

17. A vector according to claim 16, wherein the regulatory unit upstream to the ATG has the following nucleotide sequence:

AGATCTCCTCGTCCCACCGGCTGTCGAAGCTCCGCGCCTACAGCGCCATCGACTT
CGACCGGGCGAAATAGGAAGCGGCCGGCGACAAAACGGGGAGGGGCGGGAAACGG
TGGTCCGTTTCCCGCCCCTGCCCGTAGGCCGTGCGCGTCCCGCCGGTGCGCGGCG
TTCAGCCCGCCGCCGCT.

18. A Streptomyces host cell transformed with a vector according to claim 15.

19. A process for preparing a polypeptide by culturing a host cell according to claim 18.

20. Plasmid pULAD3 (CNCM 1-859).

21. A SAF promoter of the SAF gene encoding a polypeptide according to claim 1 having the following DNA sequence:
AGATCTCCTCGTCCCACCGGCTGTCGAAGCTCCGCGCCTACAGCGCCATCGACTT
CGACCGGGCGAAGTAGGAAGCGGCCGGCGACAAAACGGGGAGGGGCGGGAAACGG
TGGTCCGTTTCCCGCCCCTGCCCGTAGGCCGTGCGCGTCCCGCCGGTGCGCGGCG
TTCAGCCCGCCGCCGCT.

22. A cloning vehicle comprising a DNA sequence encoding a polypeptide or protein operatively linked to a promoter in accordance with claim 21.

23. A cloning vehicle in accordance with claim 22 wherein said DNA sequence is one which is endogenous to Streptomyces .

24. A cloning vehicle in accordance with claim 22, wherein said DNA sequence comprises all or part of a sequence encoding a polypeptide or protein endogenous to Streptomyces to which has been fused, in the same reading frame, a DNA sequence encoding a non-Streptomyces polypeptide or protein.

25. A Streptomyces host cell transformed with a cloning vehlcle in accordance with claim 22.

26. A Streptomyces host cell transformed with a cloning vehicle in accordance with claim 23.

27. A Streptomyces host cell transformed with a cloning vehlcle in accordance with claim 24.

28. A method of expressing a foreign DNA sequence in Streptomyces comprising operatively linking said foreign DNA sequence to a Streptomyces expression control sequence including the promoter in accordance with claim 21.

29. A method in accordance with claim 28, wherein the foreign DNA sequence and Streptomyces expression control sequence are inserted in the chromosomal DNA of the Streptomyces.

30. A method in accordance with claim 29, wherein the host Streptomyces further includes a plasmid capable of expressing a polypeptide having the activity of the SAF polypeptide according to claim 1.

31. A method in accordance with claim 28, wherein said foreign DNA sequence is operatively linked to said Streptomyces expression control sequence through a DNA

sequence encoding at least a part of the signal sequence of a protein or polypeptide secreted from Streptomyces, said DNA sequence encoding the signal sequence being expressed together with said foreign DNA sequence under the control of said Streptomyces expression control sequence to afford secretion of the protein or polypeptide coded for by the foreign DNA sequence.

32. The process of claims 9, 14 and 19 wherein the polypeptide is an extracellular polypeptide and the process further comprises the step of recovering the extracellular polypeptide after culturing the host cell.

FIG.1

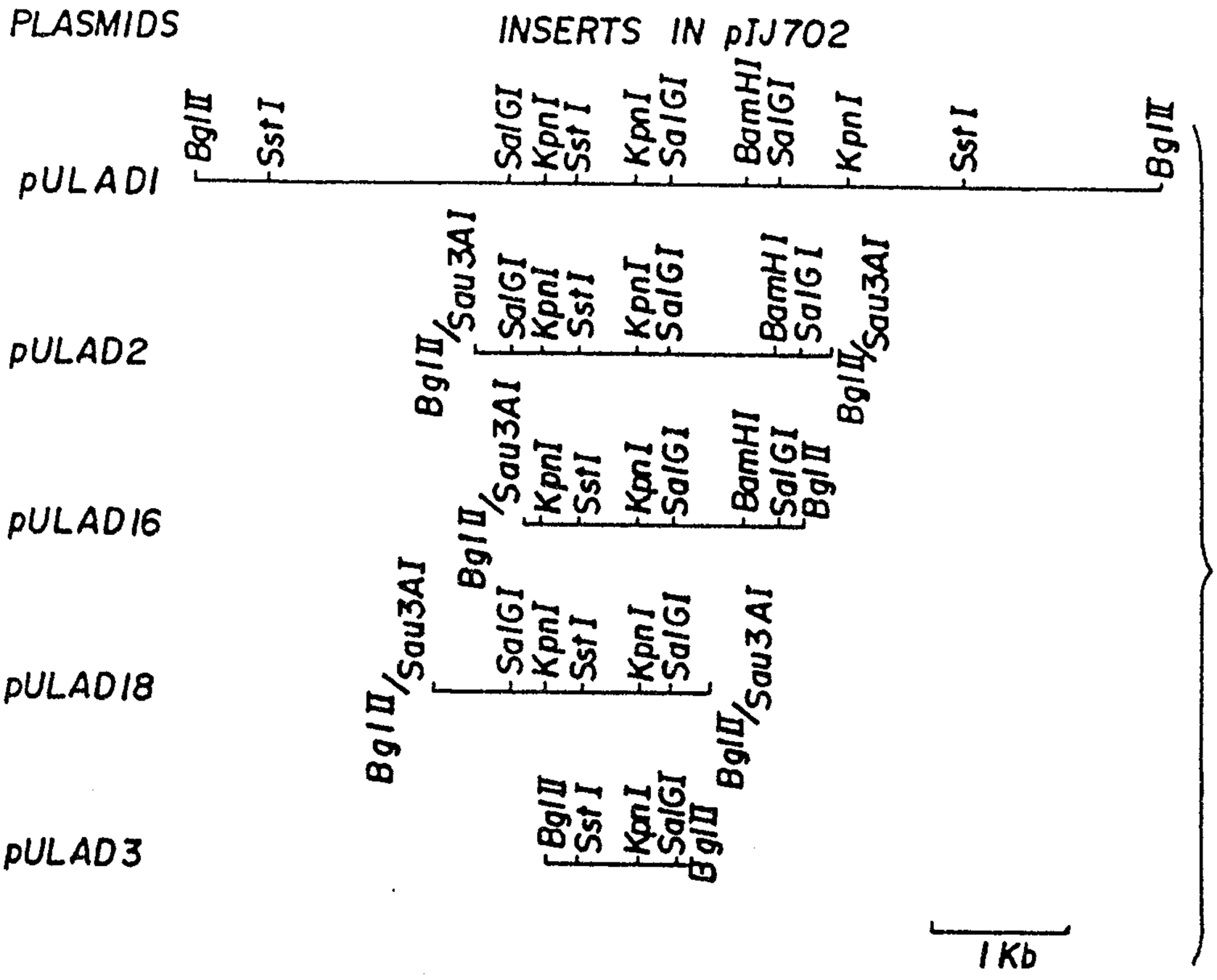
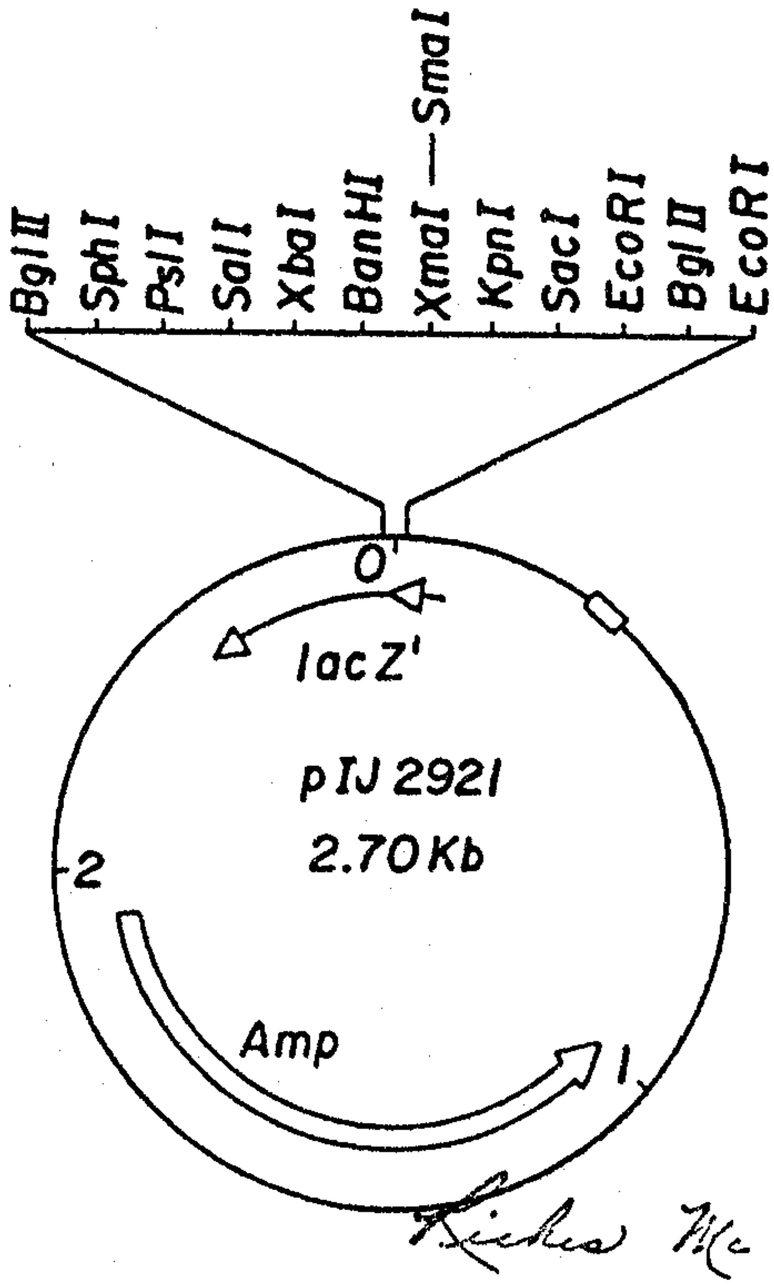
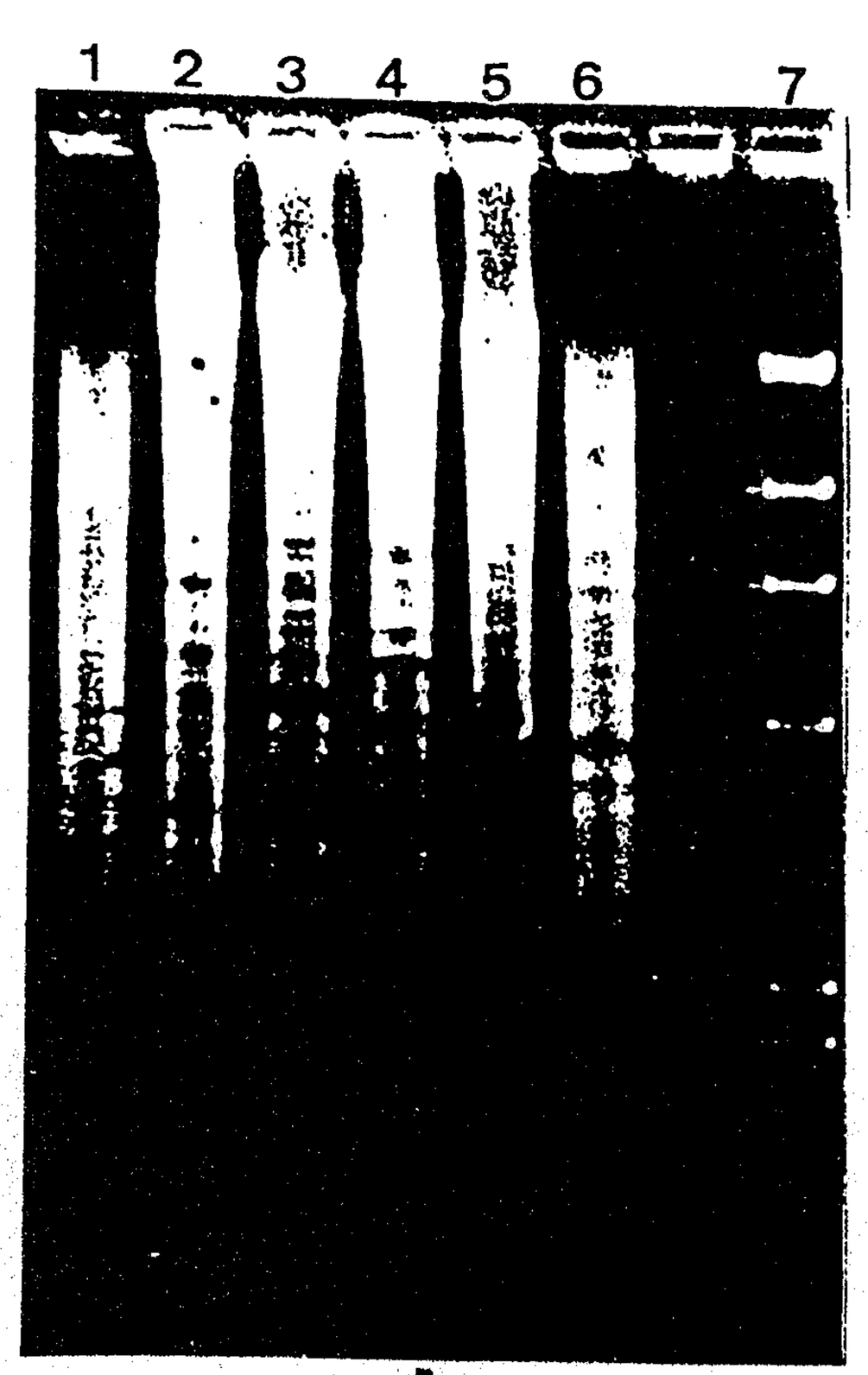
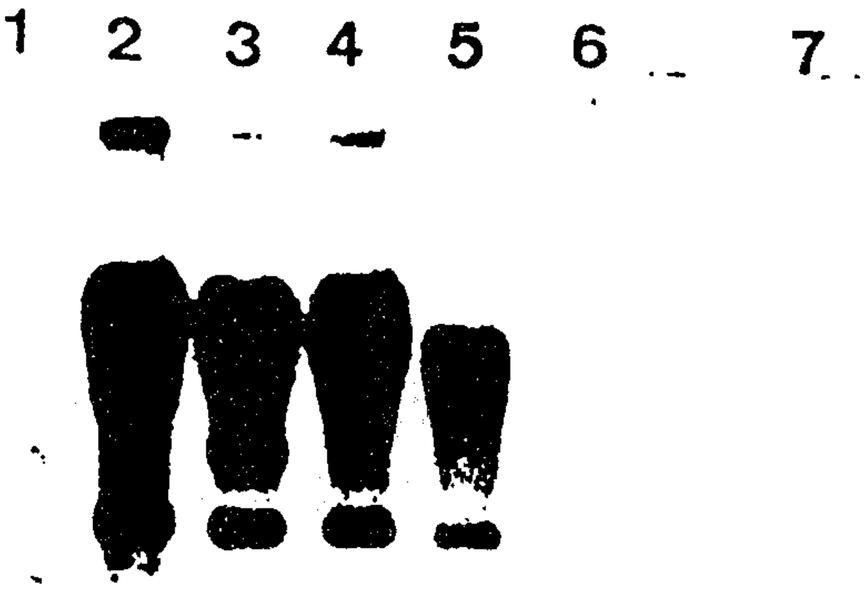

PLASMIDS
INSERTS IN pIJ702
pULAD1
pULAD2
pULAD16
pULAD18
pULAD3
BglII
SstI
SalGI
KpnI
SstI
KpnI
SalGI
BamHI
SalGI
KpnI
SstI
BglII
Sau3AI
1Kb

FIG.10


BglII
SphI
PstI
SalI
XbaI
BamHI
XmaI
SmaI
KpnI
SacI
EcoRI
BglII
EcoRI
O
lacZ'
pIJ2921
2.70Kb
2
1
Amp



A



B

FIG.2

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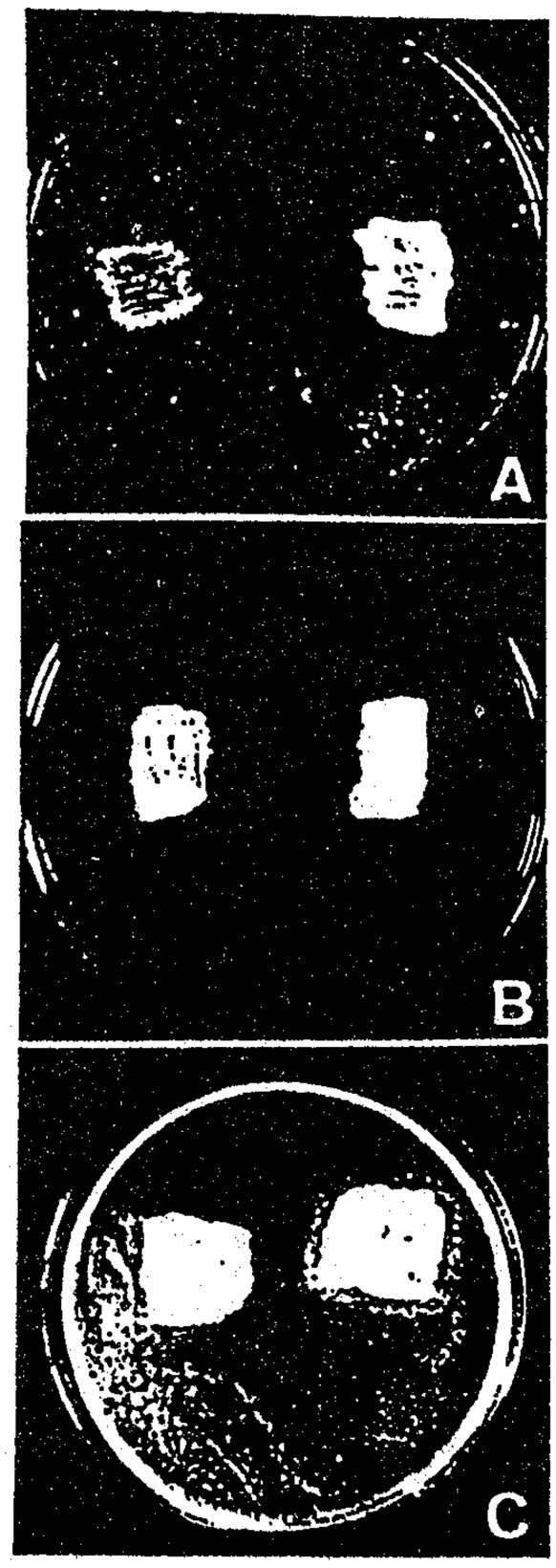

FIG.3

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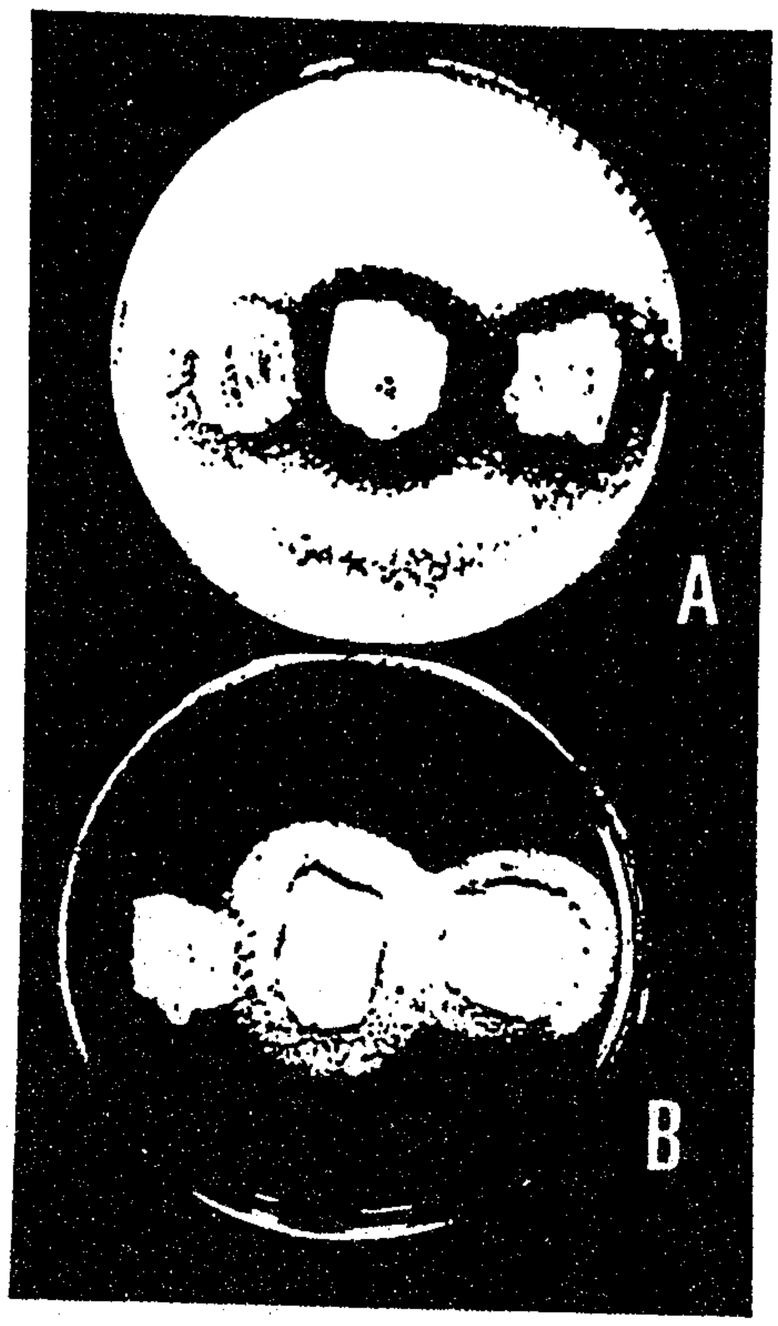

FIG.4

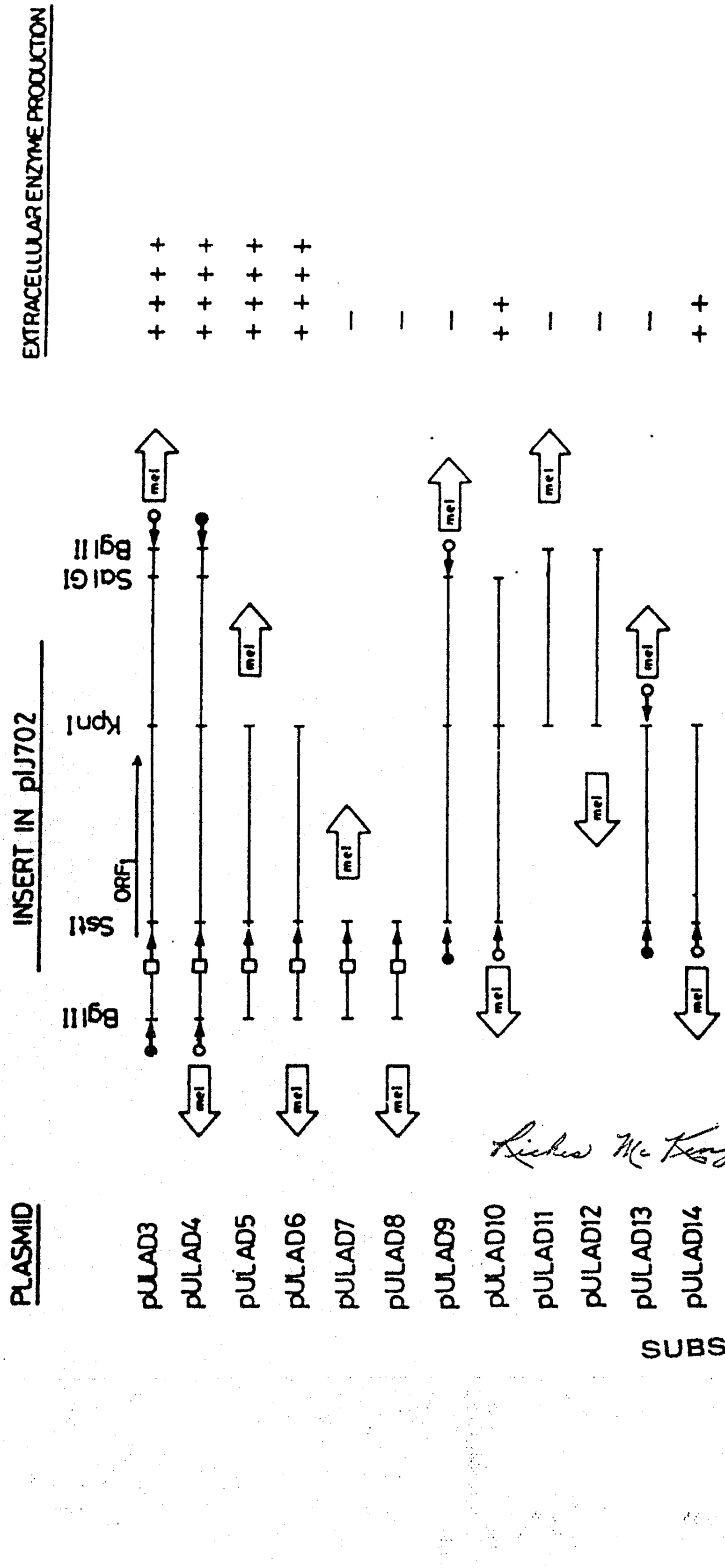

FIG 5

PLASMID
pULAD3
pULAD4
pULAD5
pULAD6
pULAD7
pULAD8
pULAD9
pULAD10
pULAD11
pULAD12
pULAD13
pULAD14

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FIG.6

```
 BglII
AGATCTCCTCGTCCCACCGGCTGTCGAAGCTCCGCGCCTACAGCGCCATCGACTTCGAC   59

CGGGCGAAGTAGGAAGCGGCCGGCGACAAAACGGGGAGGGGCGGGAAACGGTGGTCCGT  118

TTCCCGCCCCTGCCCGTAGGCCGTGCGCGTCCCGCCGGTGCGCGGCGTTCAGCCCGCCG  177

                                                        Sst
CCGCT            ATG TGC TCG GCC GTC GTC GCG GCG GCC GTG AGC  215
                 Met Cys Ser Ala Val Val Ala Ala Ala Val Ser

I
TCG ATG TCC CGC CGT CGC CGC CGG GCC AGC GCC ACC CGG CGC TCC  260
Ser Met Ser Arg Arg Arg Arg Arg Ala Ser Ala Thr Arg Arg Ser

GCT GCG GTG AGC CCG CCC CAC ACT CCG TAC GGC TCG GGC TGC ATC  305
Ala Ala Val Ser Pro Pro His Thr Pro Tyr Gly Ser Gly Cys Ile
```

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FIG.6 cont.

```
AGC GCG TGC TCC TGG CAC TCC ACC ATC ACC GGA CAC CGG GCA CAG   350
Ser Ala Cys Ser Trp His Ser Thr Ile Thr Gly His Arg Ala Gln

ACC CGC TTG GCC GCC TCC TCG CGG GCC AGC CGG GCG GCG GTC GGC   395
Thr Arg Leu Ala Ala Ser Ser Arg Ala Ser Arg Ala Ala Val Gly

TCC TTC GAC GGG GCG AAG AAC AGG CCG GCC TCG TCG CGG CGG CAG   440
Ser Phe Asp Gly Ala Lys Asn Arg Pro Ala Ser Ser Arg Arg Gln

GCC GCA TCG GAA TGC CAG GGG CCC GCG TCG TCC TCC CGT GCG GGG   485
Ala Ala Ser Glu Cys Gln Gly Pro Ala Ser Ser Ser Arg Ala Gly

GTC CGC TGG GCC GGA ACG GCG GCG AAC GGC AGA GGC TGA           524
Val Arg Trp Ala Gly Thr Ala Ala Asn Gly Arg Gly ter

TGCGGCGGTTGCAGCACGGTCTACTCCTGACGACGGCTACGGCTTCGCGAGGATGAGTC   583
```

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FIG.6 cont.

```
GATGCAGCTCTCCCTACCCGCTGTGCGTACGCCTAAGCACGGAGCGCCACCAGCGCGCG   642

  KpnI
GGGTACCGGAATGCGCCCCCGCGGCCTTTTGGCCGATTACCGGAGGTCGCGCGCCCCGC   701

CGTATACCGTGCGCACCACCGTATTCAGTGGCCGAGGTGTTTGCGCAGCCGCTGCTGGA   760

GGTCCGCCACGAACTTGCCCCGCTTCGGCCTGGCTTCCACGCTGCCGAACACGGAATAG   819

CCGTTGACCACGACGACCGGTGCTTCCGGGTCCGCCGATTCGAGCGTCCGCACGTCGAA   878

                                                        S
GGTGCCGAAGACGCCCGTGCCGCTGCCGCGCAGGGAAATGTTCTCCGGCACCCGGACGT   937

stI
CGACGCTGCCGAAGATGGACGTCGCATTGATAACGGTGAGGCGTTGCCCGAAAAGCGCT   996

       BglII
TCGGTGAGATCT                                                 1008
```

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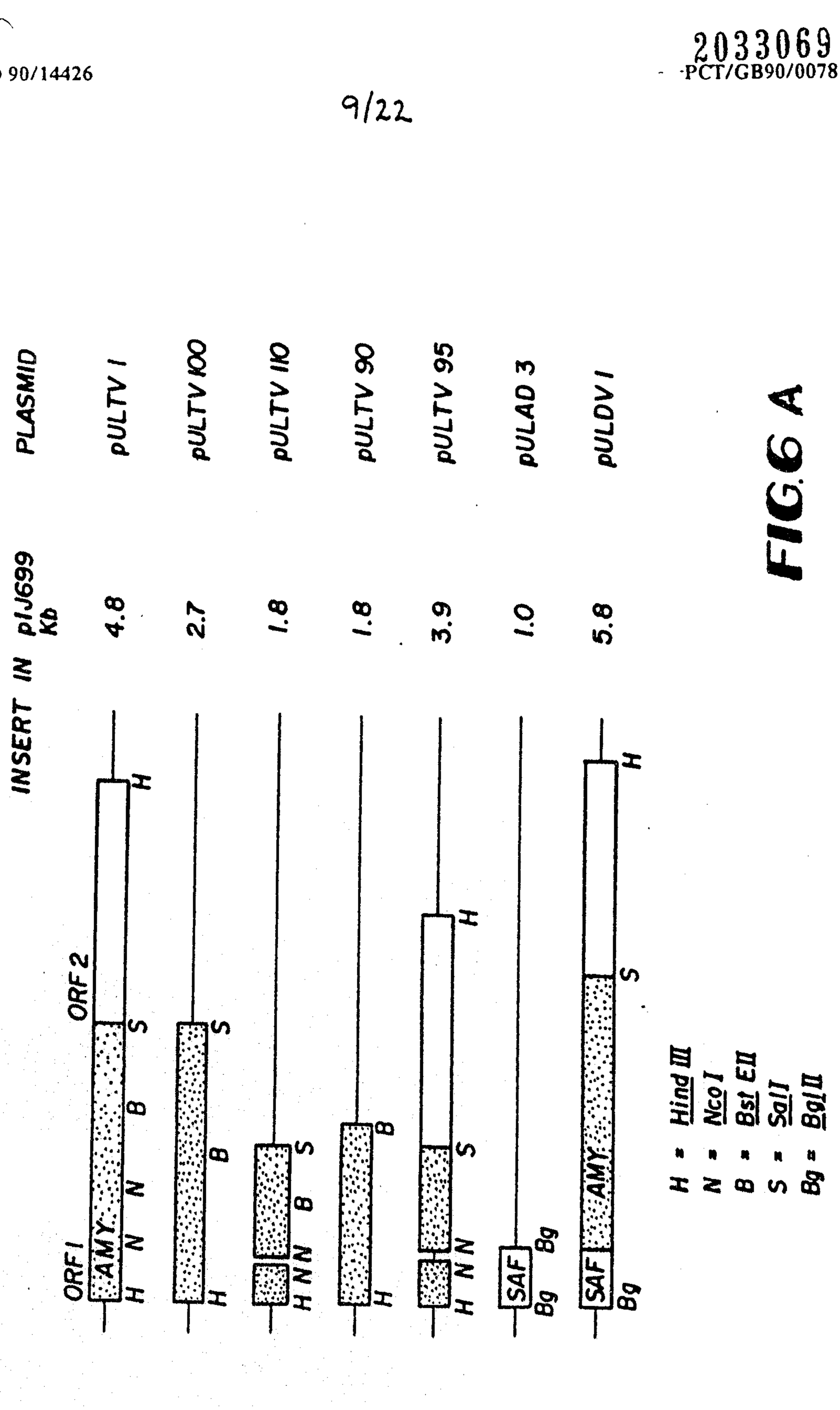

FIG. 6A

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λCI	Gln	Glu	Ser	Val	Ala	Asp	Lys	Met	Gly	Met	Gly	Gln	Ser	Gly	Val	Gly	Ala	Leu
λCro	Gln	Thr	Lys	Thr	Ala	Lys	Asp	Leu	Gly	Val	Tyr	Gln	Ser	Ala	Ile	Asn	Lys	Ala
LacI	Leu	Tyr	Asp	Val	Ala	Glu	Tyr	Ala	Gly	Val	Ser	Tyr	Gln	Thr	Val	Ser	Arg	Val
Lex	Ala	Ala	Glu	Ile	Ala	Gln	Arg	Leu	Gly	Phe	Arg	Ser	Pro	Asn	Ala	Ala	Glu	Glu
Lex R	Arg	Ala	Glu	Ile	Ala	Gln	Arg	Leu	Gly	Phe	Arg	Ser	Pro	Asn	Ala	Ala	Glu	Glu
P22Rep	Gln	Ala	Ala	Leu	Gly	Lys	Met	Val	Gly	Val	Ser	Asn	Val	Ala	Ile	Ser	Gln	Trp
CAP	Arg	Gln	Glu	Ile	Gly	Asn	Ile	Val	Gly	Cys	Ser	Arg	Glu	Thr	Val	Gly	Arg	Ile
Fnr	Arg	Gly	Asp	Ile	Gly	Asn	Tyr	Leu	Gly	Leu	Thr	Val	Glu	Thr	Ile	Ser	Arg	Leu
Saf	Arg	Ala	Ala	Val	Gly	Ser	Phe	Asp	Gly	Ala	Lys	Asn	Arg	Pro	Ala	Ser	Ser	Arg
$AfsB_a$	Gln	Arg	Arg	Tyr	Ala	Glu	Gly	Lys	Gly	Leu	Phe	His	Gln	Ala	Ile	Ala	Gly	Phe
$AfsB_b$	Glu	Gln	His	Asp	Ala	Gln	Glu	Val	Gly	Glu	Val	Arg	Ala	Leu	Leu	Ala	Arg	Ser

FIG.7

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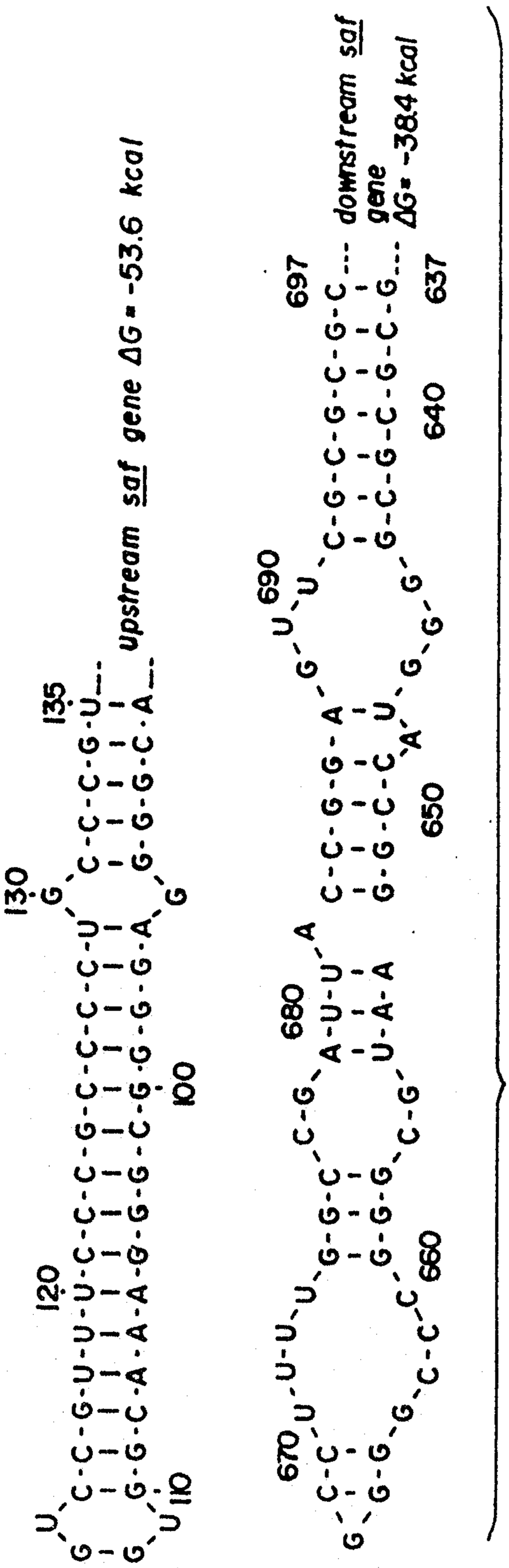
upstream saf gene ΔG = -53.6 kcal
downstream saf gene ΔG = -384 kcal

FIG8

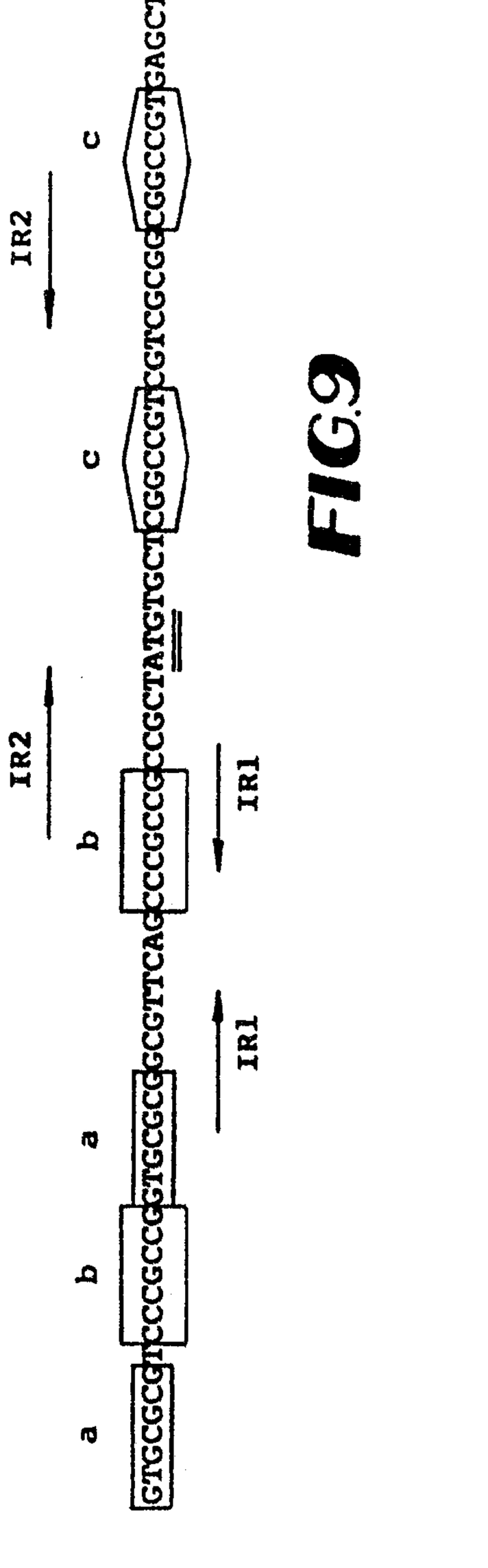
IR2
IR1
IR2
IR1
a
b
a
b
c
c

FIG9

FIG.11

```
MboI
GATCGCCCACCAGACCGTGCAGCGCACCGAGTTCGTCTTCCAGCCGGAGCTGGTGGTGCG   60
----.----+----.----+----.----+----.----+----.----+----.----+
 i  a  h  q  t  v  q  r  t  e  f  v  f  q  p  e  l  v  v  r

CGGCTCCACCGCCCAGTGGGTGCCCGGCGGCTGAGGGGACGTACGGGGCGCTTCGTCCGG  120
----.----+----.----+----.----+----.----+----.----+----.----+
 g  s  t  a  q  w  v  p  g  g
                                 ApaI
AGCCGCCCGCAATCTCTTGCAGAGGCTTGCGGGCCCTGCTGCCCGGCGCCCAACCCCCTT  180
----.----+----.----+----.----+----.----+----.----+----.----+
                                            SacII-KpnI
GATGTCCGTGGTGTTTCGGGTTCGTGTCCAAAGGGTTGACCGCGGGTACCGCTCGCTCTA  240
----.----+----.----+----.----+----.----+----.----+----.----+
                                             EcoRI
CGGTCTGCTTCGCGACGCTCCTTGCAGTTTTGCTGCAAGAGAATTCAGCCCTCCGCCCCC  300
----.----+----.----+----.----+----.----+----.----+----.----+
                                                   BglI
CGATCAGGAGGCACCACATGGCCCGCAGACTCGCCACCGCGTCCCTAGCCGTGCTGGCGG  360
----.----+----.----+----.----+----.----+----.----+----.----+
                 m  a  r  r  l  a  t  a  s  l  a  v  l  a  a
```

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FIG.11 cont.

```
                                                                  SmaI
CGGCCGCCACCGCCCTCACCGCGCCCACACCCGCCGCTGCCGCCCCGCCCGGGGCGAAGG   420
----.----+----.----+----.----+----.----+----.----+----.----+
 a  a  t  a  l  t  a  p  t  p  a  a  a  a  p  p  g  a  k  d

ACGTCACCGCCGTCCTCTTCGAGTGGAAGTTCGCCTCCGTAGCCCGCGCCTGCACCGACA   480
----.----+----.----+----.----+----.----+----.----+----.----+
 v  t  a  v  l  f  e  w  k  f  a  s  v  a  r  a  c  t  d  s
       ___BglI____
GCCTCGGCCCGGCCGGCTACGGATACGTCCAGGTCTCGCCGCCCCAGGAGCACATCCAGG   540
----.----+----.----+----.----+----.----+----.----+----.----+
 l  g  p  a  g  y  g  y  v  q  v  s  p  p  q  e  h  i  q  g

GCAGCCAGTGGTGGACCTCCTACCAGCCCGTCAGCTACAAGATCGCCGGACGGCTCGGCG   600
----.----+----.----+----.----+----.----+----.----+----.----+
 s  q  w  w  t  s  y  q  p  v  s  y  k  i  a  g  r  l  g  d
                    NcoI-SalI
ACCGCGCCGCCTTCAAGTCCATGGTCGACACCTGCCACGCGGCCGGCGTCAAGGTCGTCG   660
----.----+----.----+----.----+----.----+----.----+----.----+
 r  a  a  f  k  s  m  v  d  t  c  h  a  a  g  v  k  v  v  a
```

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FIG.11 cont.

SacII

```
CCGACTCGGTCATCAACCACATGGCCGCGGGTTCCGGCACCGGCACCGGCGGCAGCGCGT   720
----.----+----.----+----.----+----.----+----.----+----.----+----.----+
  d  s  v  i  n  h  m  a  a  g  s  g  t  g  t  g  g  s  a  y
```

SmaI

```
ACCAGAAGTACGACTACCCGGGCATCTGGTCCGGCGCCGACATGGACGACTGCCGCAGCG   780
----.----+----.----+----.----+----.----+----.----+----.----+----.----+
  q  k  y  d  y  p  g  i  w  s  g  a  d  m  d  d  c  r  s  e

AGATCAACGACTACGGCAACCGCGCCAACGTCCAGAACTGCGAACTGGTCGGCCTCGCCG   840
----.----+----.----+----.----+----.----+----.----+----.----+----.----+
  i  n  d  y  g  n  r  a  n  v  q  n  c  e  l  v  g  l  a  d

ACCTCGACACCGGTGAGCCGTACGTCCGCGACCGCATCGCCGCCTACCTCAACGACCTGC   900
----.----+----.----+----.----+----.----+----.----+----.----+----.----+
  l  d  t  g  e  p  y  v  r  d  r  i  a  a  y  l  n  d  l  l

TCTTGCTCGGCGTGGACGGCTTCCGCATCGACGCCGCCAAGCACATGCCCGCCGCCGACC   960
----.----+----.----+----.----+----.----+----.----+----.----+----.----+
  l  l  g  v  d  g  f  r  i  d  a  a  k  h  m  p  a  a  d  l
```

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FIG. 11 cont.

```
                 ___BglI____
TCACCGCCATCAAGGCCAAGGTCGGCAACGGGAGCACGTACTGGAAGCAGGAGGCCATCC 1020
----.----+----.----+----.----+----.----+----.----+----.----+
 t  a  i  k  a  k  v  g  n  g  s  t  y  w  k  q  e  a  i  h

ACGGCGCGGGCGAGGCCGTCCAGCCCAGCGAGTACCTCGGCACCGGCGACGTCCAGGAGT 1080
----.----+----.----+----.----+----.----+----.----+----.----+
 g  a  g  e  a  v  q  p  s  e  y  l  g  t  g  d  v  q  e  f

TCCGCTACGCCCGCGACCTCAAGCGGGTCTTCCAGAACGAGAACCTGGCCCACCTGAAGA 1140
----.----+----.----+----.----+----.----+----.----+----.----+
 r  y  a  r  d  l  k  r  v  f  q  n  e  n  l  a  h  l  k  n
                                                  SalI
ACTTCGGCGAGGACTGGGGCTACATGGCGAGCGGCAAGTCCGCCGTCTTCGTCGACAACC 1200
----.----+----.----+----.----+----.----+----.----+----.----+
 f  g  e  d  w  g  y  m  a  s  g  k  s  a  v  f  v  d  n  h

ACGACACCGAGCGGGGCGGCGACACCCTCAACTACAAGAACGGCTCCGCCTACACCCTCG 1260
----.----+----.----+----.----+----.----+----.----+----.----+
 d  t  e  r  g  g  d  t  l  n  y  k  n  g  s  a  y  t  l  a
```

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FIG.11 cont.

```
CCGGCGTCTTCATGCTGGCCTGGCCCTACGGCTCCCCGGACGTCCACTCCGGCTACGAGT 1320
----.----+----.----+----.----+----.----+----.----+----.----+
 g  v  f  m  l  a  w  p  y  g  s  p  d  v  h  s  g  y  e  f

TCACCGACCACGACGCCGGCCCGCCCAACGGCGGAACCGTCAACGCCTGCTACAGCGACG 1380
----.----+----.----+----.----+----.----+----.----+----.----+
 t  d  h  d  a  g  p  p  n  g  g  t  v  n  a  c  y  s  d  g
                              SacI      NcoI
GCTGGAAGTGCCAGCACGCCTGGCCCGAGCTCTCCTCCATGGTCGGCCTGCGCAACACCG 1440
----.----+----.----+----.----+----.----+----.----+----.----+
 w  k  c  q  h  a  w  p  e  l  s  s  m  v  g  l  r  n  t  a

CCTCCGGGCAGCCCGTCACCAACTGGTGGGACAACGGCGGCGACCAGATCGCCTTCGGCC 1500
----.----+----.----+----.----+----.----+----.----+----.----+
 s  g  q  p  v  t  n  w  w  d  n  g  g  d  q  i  a  f  g  r
SacII
GCGGCGACAAGGCGTACGTCGCCATCAACCACGAGGGCTCCGCGCTGAACCGCACCTTCC 1560
----.----+----.----+----.----+----.----+----.----+----.----+
 g  d  k  a  y  v  a  i  n  h  e  g  s  a  l  n  r  t  f  q
```

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FIG.11 cont.

```
        __BglI _
AGAGCGGCCTGCCCGGCGGCGCCTACTGCGACGTCCAGAGCGGCAGGTCCGTCACGGTCG 1620
----.----+----.----+----.----+----.----+----.----+----.----+
  s  g  l  p  g  g  a  y  c  d  v  q  s  g  r  s  v  t  v  g

GCTCCGACGGCACCTTCACCGCCACCGTCGCCGCCGGCACCGCCCTGGCCCTGCACACCG 1680
----.----+----.----+----.----+----.----+----.----+----.----+
  s  d  g  t  f  t  a  t  v  a  a  g  t  a  l  a  l  h  t  g
 ApaI
GGGCCCGTACCTGCTCCGGCGGCGGAACCGGCCCCGGCACCGGGCAGACCTCCGCCTCCT 1740
----.----+----.----+----.----+----.----+----.----+----.----+
  a  r  t  c  s  g  g  g  t  g  p  g  t  g  q  t  s  a  s  f
                                                BstEII
TCCACGTCAACGCCACCACCGCCTGGGGCGAGAACATCTACGTCACCGGTGACCAGGCCG 1800
----.----+----.----+----.----+----.----+----.----+----.----+
  h  v  n  a  t  t  a  w  g  e  n  i  y  v  t  g  d  q  a  a

CCCTCGGCAACTGGGACCCGGCCCGCGCCCTCAAGCTCGACCCGGCCGCCTACCCGGTGT 1860
----.----+----.----+----.----+----.----+----.----+----.----+
  l  g  n  w  d  p  a  r  a  l  k  l  d  p  a  a  y  p  v  w
```

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FIG.11 cont.

```
GGAAGCTCGACGTGCCGCTGGCCGCCGGAACCCCCTTCCAGTACAAGTACCTGCGCAAGG 1920
----.----+----.----+----.----+----.----+----.----+----.----+----.----+
  k   l   d   v   p   l   a   a   g   t   p   f   q   y   k   y   l   r   k   d

      SacII
ACGCCGCGGGGAAGGCCGTCTGGGAGTCCGGCGCCAACCGCACGGCGACCGTCGGCACCA 1980
----.----+----.----+----.----+----.----+----.----+----.----+----.----+
  a   a   g   k   a   v   w   e   s   g   a   n   r   t   a   t   v   g   t   t

CCGGCGCCCTCACCCTCAACGACACCTGGCGCGGCTGACCCCGCCCCCATCGGCCCGGCG 2040
----.----+----.----+----.----+----.----+----.----+----.----+----.----+
  g   a   l   t   i   n   d   t   w   r   g

          SacII
CCCGCCTCCCCGCGGACGCCGGGCCGCTGCCGCACCCGCCCCGCGAACCCCGCCCGGCGG 2100
----.----+----.----+----.----+----.----+----.----+----.----+----.----+

CCCCCGCCCCCGAAGGAGACCCGTCCCCGTGCGACGGACCACCATCCTCGCGGTGAGTCT 2160
----.----+----.----+----.----+----.----+----.----+----.----+----.----+
                                  m   r   r   t   t   i   l   a   v   s   l
```

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FIG.11 cont.

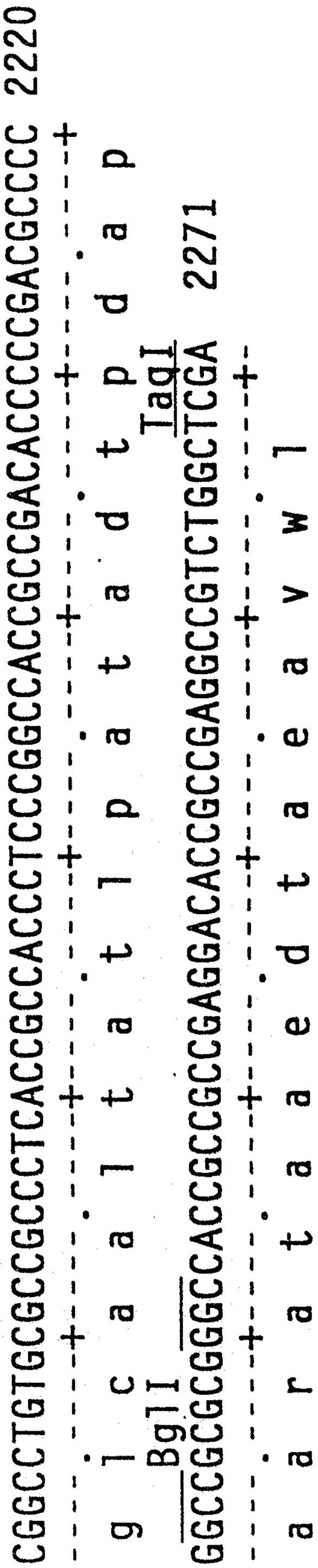

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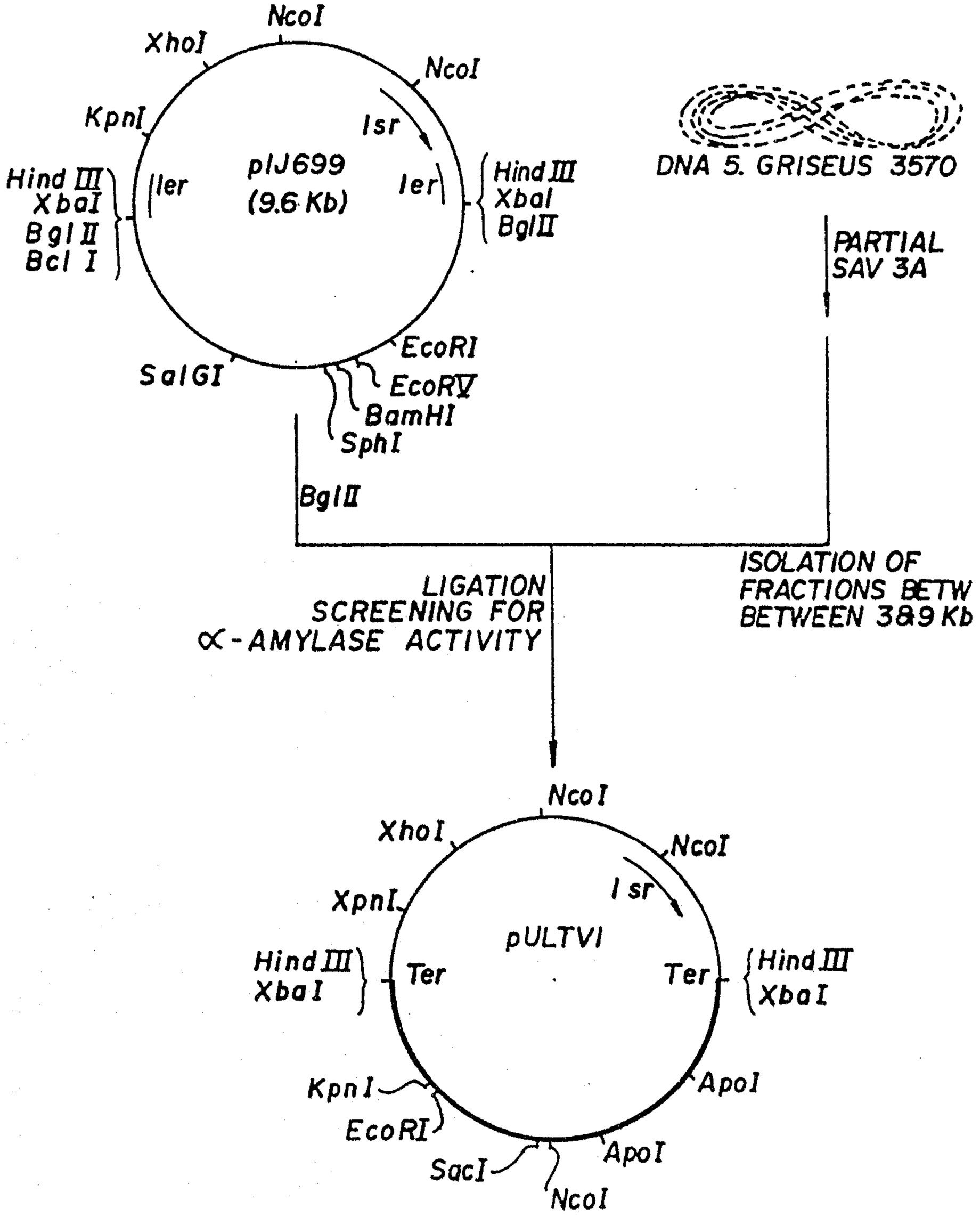
FIG.12



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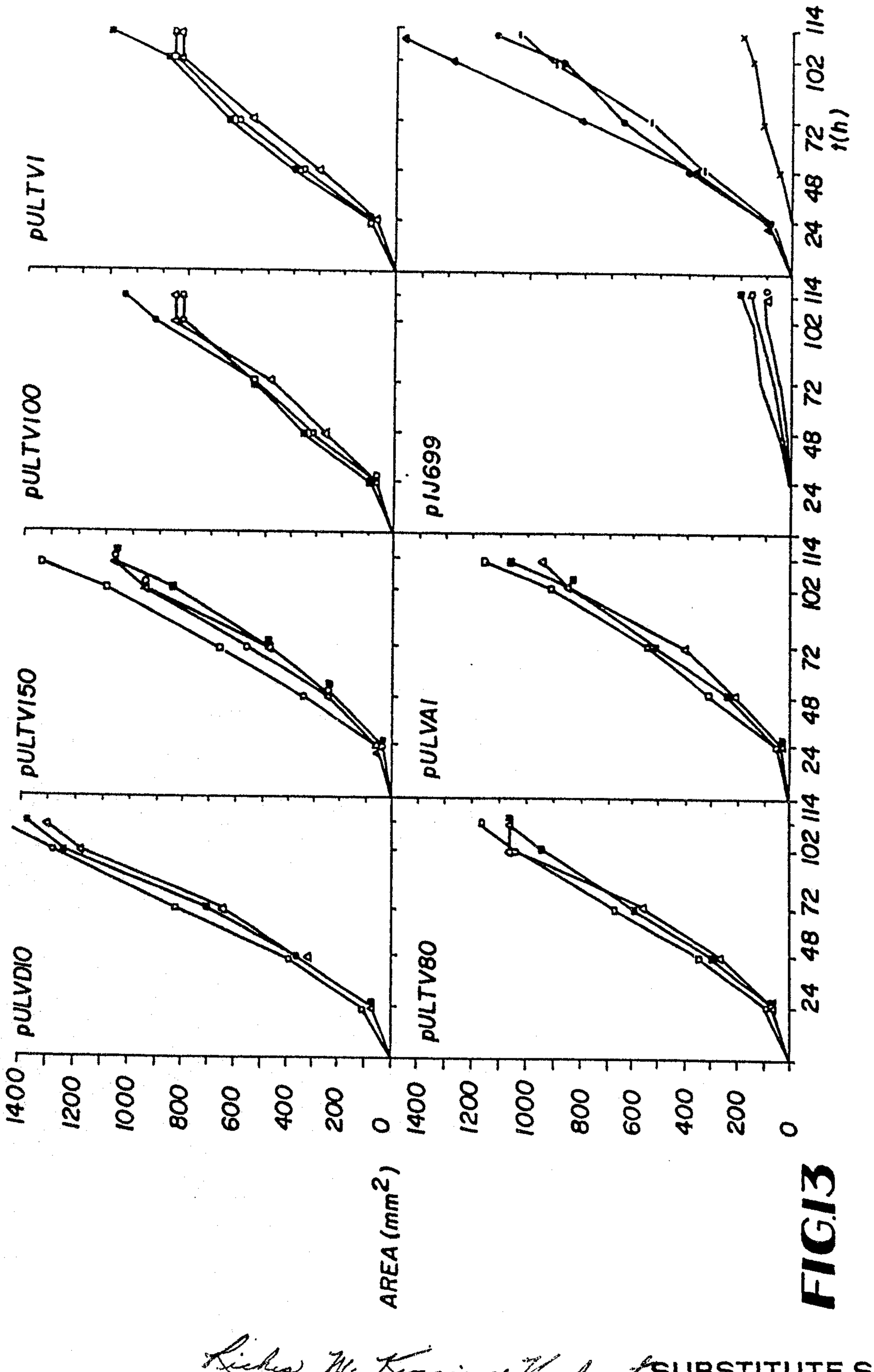
pULTV1
pULTV100
pIJ699
pULTV150
pULVAI
pULVD10
pULTV80
1400
1200
1000
800
600
400
200
0
24
48
72
102
114
t(h)
AREA (mm²)

FIG13

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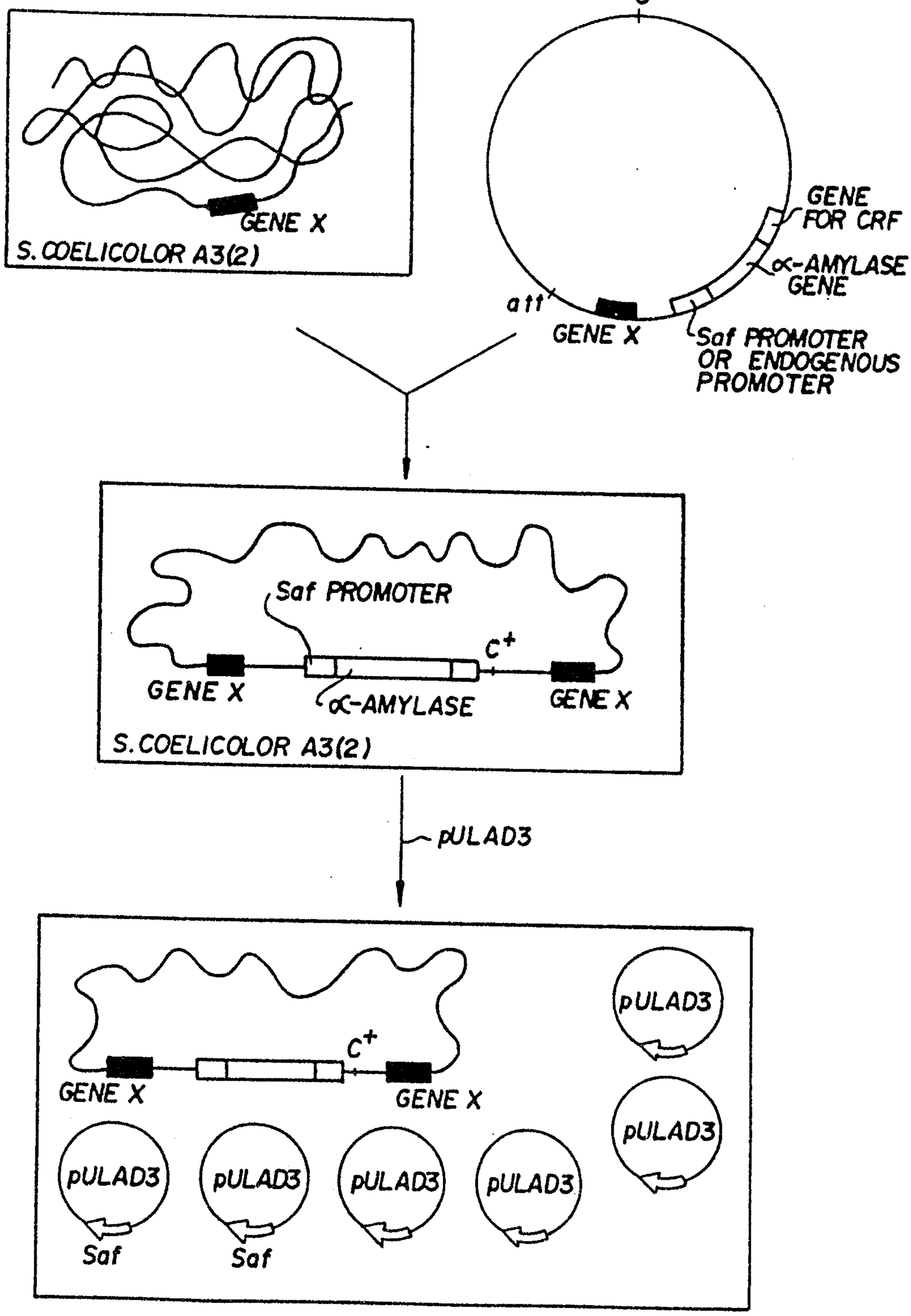
FIG.14



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