CONVENTION

AUSTRALIA

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APPLICATION FOR A STANDARD PATENT

628018

I/We Gist-Brocades NV

of Wateringseweg 1,
2611 XT,
Delft,
THE NETHERLANDS.

hereby apply for the grant of a standard patent for an invention entitled:

KLUYVEROMYCES AS A HOST STRAIN

which is described in the accompanying complete specification.

Details of basic application

Number of basic application: 078,539

Convention country in which basic application was filed: UNITED STATES OF AMERICA

Date of basic application : 28 July 1987

Address for Service:

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Dated: 26 July 1988

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By: David B Fitzpatrick

Our Ref : 101773
POF Code: 1219/1219

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DECLARATION FOR A PATENT APPLICATION

INSTRUCTIONS

(a) Insert "Convention" if applicable

(b) Insert FULL name(s) of applicant(s)

In support of the [a] Convention application made by [b]

GIST - BROCADES N.V., residing at 1, Wateringseweg, Delft, the Netherlands

(c) Insert "of addition" if applicable

(d) Insert TITLE of invention

(hereinafter called "applicant(~~s~~) for a patent [c] for an invention entitled [d]

Kluyveromyces as a host strain

(e) Insert FULL name(s) AND address(es) of declarant(s) (See headnote*)

I/~~We~~ [e] Hans Walter RAVEN of Everocken 21, Mijnsheerenland, the Netherlands

do solemnly and sincerely declare as follows:

~~I am/We are the applicant(s).~~

*(**or**, in the case of an application by a body corporate)*

1. I am/~~We are~~ authorized to make this declaration on behalf of the applicant(~~s~~).

~~2. I am/We are the actual inventor(s) of the invention.~~

~~*(or, where the applicant(s) is/are not the actual inventor(s))*~~

(f) Insert FULL name(s) AND address(es) of actual inventor(s)

2. [f] Johannes A. VAN DEN BERG, Hanegevecht 8, Reeuwijk, the Netherlands,
Albert J.J. VAN OOYEN, Laan van Heldenburg 23, Voorburg, the Netherlands
Krijn RIETVELD, Van der Waalsstraat 129, Vlaardingen, the Netherlands

(g) Recite how applicant(s) derive(s) title from actual inventor(s) (See headnote**)

~~is~~/are the actual inventor(s) of the invention and the facts upon which the applicant(~~s~~) ~~is~~/are entitled to make the application are as follows: [g]

The applicant is the assignee of the said invention from the actual inventors

(Note: *Paragraphs 3 and 4 apply **only** to Convention applications)*

(h) Insert country, filing date, and basic applicant(s) for the/or EACH basic application

3. The basic application(~~s~~) for patent or similar protection on which the application is based is/~~are~~ identified by **country, filing date, and basic applicant(s)** as follows: [h]

United States serial no. 078,539, filed on July 28, 1987 , by the said JOHANNES A. VAN DEN BERG, ALBERT J.J. VAN OOYEN and KRIJN RIETVELD.

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

(k) Insert PLACE of signing

(l) Insert DATE of signing

(m) Signature(s) of declarant(s)

Note: No legalization or other witness required

Declared at [k] Delft, the Netherlands

Dated [l] July 8 , 1988

[m] GIST - BROCADES N.V.

Hans Walter RAVEN

To: The Commissioner of Patents

P18/7/78.

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KLUYVEROMYCES AS A HOST STRAIN

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(56) Prior Art Documents
EP 96910
EP 96430
AU 15539/83

(57) Claim

1. A method for producing a polypeptide of interest in a Kluyveromyces host cell, said method comprising:

introducing into said host cell a DNA sequence encoding said polypeptide of interest and

growing said host cell comprising said DNA sequence in a culture medium whereby said polypeptide of interest, or part thereof, is secreted into the culture medium.

15. A transformed Kluyveromyces host cell comprising an expression cassette which comprises, in the direction of transcription, a transcriptional initiation regulatory region functional in said host cell, a signal sequence for secretion functional in said host cell joined in reading frame with a DNA sequence encoding a polypeptide of interest, and a transcriptional termination regulatory region functional in said host cell.

29. Plasmids pKS105, pGB901, pGBTPA1 and pGBHSA3 as hereinbefore described.

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COMPLETE SPECIFICATION
(ORIGINAL)

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APPLICANT'S REFERENCE: AUS-2390 Hu/fvdb

Name(s) of Applicant(s):

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Complete Specification for the invention entitled:

KLUYVEROMYCES AS A HOST STRAIN

Our Ref : 101773
POF Code: 1219/1219

The following statement is a full description of this invention, including the best method of performing it known to applicant(s):

KLUYVEROMYCES AS A HOST STRAIN

INTRODUCTION

Technical Field

This invention relates to methods for preparing and using Kluyveromyces for the production of polypeptides of interest which preferentially are secreted into the growth medium. The invention is exemplified by sequences useful in the production of chymosin and precursors thereof, tissue plasminogen activator (t-PA), and human serum albumin (HSA), in Kluyveromyces.

Background of the Invention

The bright promise of production of peptides in microorganisms has been tarnished by a number of factors. In many instances, where the peptide has been produced and retained in the cytoplasm, inclusion bodies have resulted requiring denaturation and renaturation of the protein, frequently with only partial or little success. In other instances, the peptide has been subjected to substantial degradation, so that not only are yields low, but also complicated mixtures are obtained which are difficult to separate. As a potential solution to these difficulties, the possibility of secretion of the desired peptide into the nutrient medium has been investigated. Secretion has met with limited success, since not all proteins have been found to be capable of secretion in the host which have been employed. Even when secreted, the processing of the peptide may result in a product which differs from the composition and/or conformation of the desired peptide. There is, therefore, substantial interest in being able to develop systems for the efficient and economic production of active peptides under conditions which allow for the use of the peptides in a wide variety of environments, both in vitro and in vivo.

RELEVANT LITERATURE

European Patent Application (EPA) 0096430 discloses Kluyveromyces as a host for cloning and expression of foreign genes. However, no mention was made of the capability of secreting proteins into the growth medium.

The leader sequence of amyloglucosidase for Aspergillus is described by Boyle et al., EMBO J. (1984) 3:1581-1585 and Innis et al., Science (1985) 228:21-26. Lactase promoters are described by Bruenig et al., Nucleic Acids Res. (1984) 12:2327-2341. The use of signal peptides associated with mating-type α-factor and of the enzymes invertase and acid phosphatase to direct the secretion of heterologous proteins in Saccharomyces has been described in EP-A-0123544 and by Smith et al., Science (1985) 229:1219.

Production of preprochymosin, prochymosin and chymosin in Saccharomyces has been studied by Mellor et al., Gene (1983) 24:1-14. When prochymosin is made intracellularly in Saccharomyces, only a low percentage of the prochymosin obtained is activatable. See Moir et al. in Developments in Industrial Biology (1985) 26:75-85; Mellor et al., Gene (1983) 24:1-14; Kingsman et al. in Biotechnology and Genetic Engineering Reviews Vol. 3 (1985) 376-418. The aggregated prochymosin produced by Saccharomyces required complicated methods of denaturation and renaturation to solubilize the prochymosin. See WO 83/04418 and EP-A-0114506.

~~SUMMARY OF THE INVENTION~~

~~Peptide production systems are provided comprising Kluyveromyces host strains, expression cassettes which include efficient transcriptional initiation and termination regions for use in Kluyveromyces and a gene, optionally containing a signal sequence for secretion, under the transcriptional and translational regulation of the regulatory regions. The cassettes are introduced into the Kluyveromyces host strain under conditions whereby the resulting transformants stably maintain the expression cassettes. Naturally~~

SUMMARY OF THE INVENTION

It is an object of the present invention to overcome, or at least alleviate, one or more of the difficulties related to the prior art namely to provide a method for preparing and using Kluyveromyces for the production of polypeptides of interest which are secreted into the growth medium.

Peptide production systems are provided comprising Kluyveromyces host strains, expression cassettes which include efficient transcriptional initiation and termination regions for use in Kluyveromyces and a gene, optionally containing a signal sequence for secretion, under the transcriptional and translational regulation of the regulatory regions. The cassettes are introduced into the Kluyveromyces host strain under conditions whereby the resulting transformants stably maintain the expression cassettes. Naturally occurring DNA and synthetic genes may be employed for the production of peptides of interest.

Accordingly the present invention provides a method for producing a polypeptide of interest in a Kluyveromyces host cell, said method comprising:

introducing into said host cell a DNA sequence encoding said polypeptide of interest and

growing said host cell comprising said DNA sequence in a culture medium whereby said polypeptide of interest, or part thereof, is secreted into the culture medium.

~~occurring DNA and synthetic genes may be employed for the production of peptides of interest.~~

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a diagram of the plasmid pGBTe418;

Figure 2 is a diagram of the plasmid pGB901;

Figure 3 is a description of the synthesized oligonucleotides for the signal sequence adapted from the amyloglucosidase signal sequence;

Figure 4 is a description of a synthetic signal sequence;

Figure 5 is an immunoblot showing the secretion of prochymosin by *K. lactis*;

Figure 6 is the sequence of the entire *BamHI* insert from pDM100PC comprising the fusion peptide of the α-factor of *S. cerevisiae* and prochymosin and transcriptional regulatory regions;

Figure 7 is a restriction map of plasmid pKS105;

Figure 8 shows the strategy used to design oligonucleotide probes used to identify *K. lactis* α-factor DNA;

Figure 9 is the complete sequence of a DNA fragment encoding the *K. lactis* α-factor;

Figure 10 is a description of plasmids employed for expression of the fusion of the α-factor signal sequence and the prochymosin structural gene;

Figure 11 shows the sequences around the junctions in α-factor/prochymosin fusions;

Figure 12 is the sequence of the *BamHI*/*SalI* insert of pAB309;

Figure 13 represents the sequences of the primers for mutagenesis of *K. lactis* α-factor leader DNA;

Figure 14 is a diagram of the plasmid pUCG418;

Figure 15 is a diagram of the plasmid pGBtPA1;

Figure 16 shows the secretion of human t-PA by *K. lactis* as analysed on a SDS-polyacrylamide gel overlayered with a plasminogen/fibrin-agarose gel;

Figure 17 is a diagram of the plasmid pGBtPA2;

Figure 18 is a diagram of the plasmid pGBHSA3;

Figure 19 shows the secretion of HSA by K. lactis as analysed on a 10% polyacrylamide gel.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the subject invention, expression cassettes are provided which allow for the efficient and economic production of polypeptides by Kluyveromyces yeast cells. The expression cassettes have transcriptional and translational regulatory sequences functional in a Kluyveromyces host cell and an open reading frame coding for a peptide of interest under the transcriptional and translational control of the regulatory regions. The open reading frame also may include a ~~leader~~ signal sequence recognized by the Kluyveromyces host which provides for secretion of the polypeptide into the growth medium. The Kluyveromyces cells used may be either laboratory or industrial strains.

The expression cassette will include in the 5'-3' direction of transcription, a transcriptional and translational initiation regulatory region, an open reading frame encoding a peptide of interest, desirably having a signal sequence for secretion recognized by Kluyveromyces, and a translational termination region. The expression cassette will further comprise a transcriptional termination regulatory region. The initiation and termination regulatory regions are functional in Kluyveromyces and provide for efficient expression of the peptide of interest without undesirable effects on the viability and proliferation of the Kluyveromyces host.

The transcriptional and translational initiation regulatory region may be homologous or heterologous to Kluyveromyces. Of particular interest are transcriptional initiation regions from genes which are present in Kluyveromyces or other yeast species, such as Saccharomyces, for example, cerevisiae, Schizosaccharomyces, Candida, etc., or other fungi, for example, filamentous fungi such as Aspergillus, Neurospora, Penicillium, etc. The transcriptional initiation regulatory regions may be obtained for example from genes in the glycolytic pathway, such as alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglucoisomerase, phosphoglycerate kinase, etc., or regulatable genes such as acid phosphatase, lactase, glucoamylase, etc.

Any one of a number of regulatory sequences may be preferred in a particular situation, depending upon whether constitutive or induced transcription is desired, the particular efficiency of the promoter in conjunction with the open reading frame of interest, the ability to join a strong promoter with a control region from a different promoter which allows for inducible transcription, ease of construction, and the like. These regulatory regions find ample precedent in the literature. See, for example, EP-A-0164566, incorporated herein by reference.

Secretion of heterologous proteins in genetically modified microorganisms is generally accomplished by one of two methods. In the first, the ~~leader~~ signal sequence is homologous to the protein; in the second, the ~~leader~~ signal sequence is homologous to the host organism. Other alternatives of particular interest in the present invention for secretion of heterologous proteins in Kluyveromyces include the use of a leader sequence heterologous to both Kluyveromyces and the peptide of interest, or a synthetic ~~leader~~ signal sequence specifically designed. The DNA encoding the former ~~leader~~ signal sequence can be obtained by isolation or made synthetically. Thus, the open reading frame usually will include a wild-type or mutated gene, where the signal sequence is normally associated with the remainder of the coding sequence, a hybrid or chimeric open reading frame, where the signal sequence is normally not associated with the remaining portion of the open reading frame, or a synthetic sequence, where the signal sequence and the remainder of the open reading frame are synthesized to provide for preferred codons, convenient restriction sites, novel amino acid sequences, and the like, or combinations thereof. Signal sequences which may be employed may be obtained from genes, such as α-factor, invertase, amyloglucosidase, native or wild type signal sequences present in structural genes and recognized by _Kluyveromyces_, _Saccharomyces_, other fungi, e.g. _Neurospora_, _Aspergillus_, and other eukaryotes.

Of particular interest is the use of a signal sequence which provides for secretion of the peptide of interest into the nutrient medium, rather than into the periplasmic space. For the most part, the signal sequence will be the 5'-terminus of the open reading frame. However, in some situations, it may be desirable to have the signal sequence internal to the open reading frame. For use of internal signal sequences for secretion, see U.S. Patent No. 4,338,397 and Perara and Lingappa, J. Cell Biology (1985) _101_:2292-2301. Genes into which the open reading frame of interest may be inserted include highly expressed constitutive genes, for example, genes encoding enzymes of the glycolytic pathway, or highly expressed regulatable genes such as lactase, amyloglucosidase, or the like.

For optimal gene expression, the nucleotide sequences surrounding the translational initiation codon ATG have been found to be important in yeast cells and in animal cells. For example, M. Kozak, Microbiol. Revs. (1983) _47_:1-45 has studied extensively the effect of these regions on the expression of insulin in COS cells. Similarly, specific nucleotides are found more frequently in highly expressed yeast proteins than others indicating the important effect of these nucleotides on the level of expression of these genes.

For optimal gene expression of exogenous genes it will be important to modify the nucleotide sequences surrounding the initiation codon ATG. This can be done by site-directed mutagenesis or by fusing the exogenous gene in frame to an endogenous _Kluyveromyces_ gene, preferably a highly expressed gene, such as the lactase gene.

Normally, it will be desirable to provide that the signal leader is cleaved from the peptide of interest during the secretory process, rather than subsequent to the secretory process, although either procedure may find use. Usually, the processing signal employed will be the processing signal naturally occurring with the signal sequence or a processing signal which has been modified from the naturally occurring one, which is still effective for providing for a peptide signal resulting in hydrolytic cleavage of the signal peptide and processing signal peptide from the peptide of interest. Various processing signals have been sequenced and defined, such as α-factor (see for example U.S. Patent No. 4,546,082, which is incorporated herein by reference), amyloglucosidase, α-amylase, etc. In some instances, other peptidase-recognized sequences may be employed which may required subsequent cleavage for isolation of the desired peptide. These sequences include dibasic peptides, e.g. KR, $(D)_4K$, and EA, which are cleaved by KEX2, bovine enterokinase, and a yeast membrane peptidase, respectively.

The peptide of interest may be native to the host or heterologous, being derived from prokaryotic or eukaryotic sources, which eukaryotic sources may involve fungi, protists, vertebrates, non-vertebrates, and the like. The peptide products may include enzymes, such as lactase, α-amylase, β-amylase, amyloglucosidase, chymosin, etc., mammalian peptides, such as hormones, interleukins, cytokines, cachexin, growth factors, e.g. platelet derived, epidermal, skeletal, etc., growth hormone, follicle stimulating hormone, interferons (α-, β-, and γ-), blood factors such as factor V, VI, VII, VIII (vW or c), IX, X, XI or XII, plasminogen activator (tissue or urinary), serum albumin, e.g. human serum albumin, colony growth factor (e.g. GM), erythropoietin, thaumatin, insulin, etc.

These structural genes may be obtained in a variety of ways. Where the amino acid sequence is known, the structural gene may be synthesized in whole or in part, particularly where it is desirable to provide yeast-preferred codons. Thus, all or a portion of the open reading frame may be synthesized using codons preferred by <u>Kluyveromyces</u>. Preferred codons may be determined by those codons which are found in the proteins produced in greatest amount by the

Kluyveromyces host e.g. glycolytic enzymes. Methods for synthesizing sequences and bringing the sequences together are well established in the literature. Where a portion of the open reading frame is synthesized, and a portion is derived from natural sources, the synthesized portion may serve as a bridge between two naturally occurring portions, or may provide a 3'-terminus or a 5'-terminus. Particularly where the signal sequence and the open reading frame encoding the peptide are derived from different genes, synthetic adaptors commonly will be employed. In other instances, linkers may be employed, where the various fragments may be inserted at different restriction sites or substituted for a sequence in the linker.

For the most part, some or all of the open reading frame will be from a natural source. Methods for identifying sequences of interest have found extensive exemplification in the literature, although in individual situations, different degrees of difficulty may be encountered. Various techniques involve the use of probes, where at least a portion of the naturally occurring amino acid sequence is known, where genomic or cDNA libraries may be searched for complementary sequences. Alternatively, differential transcription can be detected when the gene of interest can be induced or when cells are from the same host but of different differentiation, by comparing the messenger RNA's produced. Other techniques have also been exemplified.

The termination region may be derived from the 3'-region of the gene from which the initiation region was obtained or from a different gene. A large number of termination regions are known and have been found to be satisfactory in a variety of hosts from the same and different genera and species. The termination region is usually selected more as a matter of convenience rather than because of any particular property. Preferably, the termination region will be derived from a yeast gene, particularly Saccharomyces or Kluyveromyces.

In developing the expression cassette, the various fragments comprising the regulatory regions and open reading frame may be subjected to different processing conditions, such as ligation, restriction, resection, in vitro mutagenesis, primer repair, use of linkers and adaptors, and the like. Thus, nucleotide transitions, transversions, insertions, deletions, or the like, may be performed on the DNA which is employed in the regulatory regions and/or open reading frame.

During the construction of the expression cassette, the various fragments of the DNA will usually be cloned in an appropriate cloning vector, which allows for expansion of the DNA, modification of the DNA or manipulation by joining or removing of the sequences, linkers, or the like. Normally, the vectors will be capable of replication in at least a relatively high copy number in E. coli. A number of vectors are readily available for cloning, including such vectors as pBR322, pACYC184, pUC7-19, M13, Charon 4A, and the like.

The cloning vectors are characterized by having an efficient replication system functional in E. coli. Also, the cloning vector will have at least one unique restriction site, usually a plurality of unique restriction sites and may also include multiple restriction sites, particularly two of the same restriction sites for substitution. In addition, the cloning vector will have one or more markers which provide for selection for transformants. The markers will normally provide for resistance to cytotoxic agents such as antibiotics, heavy metals, toxins or the like, complementation of an auxotrophic host, or immunity to a phage. By appropriate restriction of the vector and cassette, and, as appropriate, modification of the ends, by chewing back or filling in overhangs, to provide for blunt ends, by addition of linkers, by tailing, complementary ends can be provided for ligation and joining of the vector to the expression cassette or component thereof.

After each manipulation of the DNA in the development of the cassette, the plasmid will be cloned and isolated and, as required, the particular cassette component analyzed as to its sequence to ensure that the proper sequence has been obtained. Depending upon the nature of the manipulation, the desired sequence may be excised from the plasmid and introduced into a different vector or the plasmid may be restricted and the expression cassette component manipulated, as appropriate.

In some instances a shuttle vector will be employed where the vector is capable of replication in different hosts requiring different replication systems. This may or may not require additional markers which are functional in the two hosts. Where such markers are required, these can be included in the vector, where the plasmid containing the cassette, the two replication systems, and the marker(s) may be transferred from one host to another, as required. In the present situation, the second replication system would be a replication system functional in <u>Kluyveromyces</u>. The replication systems which may be used may be derived from plasmids, e.g. pKD1 from <u>Kluyveromyces</u> <u>drosophilarum</u>, viruses, or the chromosome of <u>Kluyveromyces</u> or other species, particularly one associated with <u>Kluyveromyces</u>, such as <u>Saccharomyces</u>. Thus, replication systems include the replication system of the 2 micron plasmid found in <u>Saccharomyces</u> and an autonomously replicating sequence (ARS) gene, for example when used in conjunction with a centromere sequence, or the like. If desired, regions of homology may be provided to encourage integration of the expression cassette into the genome of the <u>Kluyveromyces</u> host.

Of particular interest in the constructs of the subject invention is a sequence derived from <u>Kluyveromyces</u> DNA chromosomes referred to as KARS, which provide for high transformation frequency. The KARS gene may be obtained by screening a library of <u>Kluyveromyces</u> DNA fragments for enhanced transformation efficiency. In this manner, fragments can be obtained which contain KARS sequences, which fragments can be further modified by restriction, resection, or primer repair, to provide a fragment of approximately 200 bp and not more than about 5000 bp, more usually from about 200 bp to 2000 bp which provides for enhanced transformation efficiency. The presence of the KARS gene can provide transformation of <u>K</u>. <u>lactis</u> auxotrophic species to prototophy at a frequency of at least about 10^3 per microgram of DNA, usually at a frequency of 10^4 per microgram of DNA or higher.

The manner of transformation of E. coli with the various DNA constructs (plasmids and viruses) for cloning is not critical to this invention. Conjugation, transduction, transfection or transformation, e.g. calcium chloride or phosphate mediated transformation, may be employed. By contrast, for yeast, for the most part the prior art has relied on transformation of protoplasts employing combinations of calcium ions and polyethylene glycol of from about 2000 to 8000, usually 4000 to 7000 daltons.

An alternative method of transformation involves growing Kluyveromyces in a standard yeast nutrient medium to a density of 1 to 25, desirably 4 to 10 OD_{610}. The Kluyveromyces cells are then harvested, washed and pretreated with chaotropic ions, particularly the alkali metal ions lithium, cesium or rubidium, particularly as the chloride or sulfate, more particularly the lithium salts, at concentrations of about 2 mM to 1M, preferably about 0.1 M. After incubating the cells with the chaotropic ion(s), the DNA is added and the incubation is prolonged for a short period of time at a moderate temperature, generally from about 20°C to 35°C, for about 5 to 60 min. Then polyethylene glycol is added, desirably at a concentration to about 25 to 50%, where the entire medium may be diluted by adding an equal volume of a polyethylene glycol concentrate to result in the desired final concentration. The polyethylene glycol will be of from about 2000 to 8000 daltons, preferably about 4000 to 7000 daltons. Incubation will generally be for a relatively short time, generally from about 5 to 60 min. Desirably, the incubation medium is subjected to a heat treatment of from about 1 to 10 min. at about 35°C to 45°C, preferably about 42°C.

For selection, any useful marker may be used, although the number of markers useful with Kluyveromyces is narrower than the markers used for Saccharomyces. Desirably, resistance to kanamycin and the aminoglycoside G418 are of interest, as well as complementation of a gene in the tryptophan metabolic pathway, particularly TRP1 or in the lactose metabolic pathway, particularly LAC4.

Although a marker for selection is highly desirable for convenience, other procedures for screening transformed cells have been described. See for example G. Reipen _et_ _al_., Current Genetics (1982) _5_:189-193. Besides the use of an indicator enzyme such as β-lactamase, transformed cells may be screened by the specific products they make. In the case of chymosin, for example, synthesis of the product may be determined by an immunological or an enzymatic method.

The vector used may be capable of extrachromosomal maintenance in _Kluyveromyces_ or result in integration into the _Kluyveromyces_ gene. It has been found that the 2 micron plasmid replication system from _Saccharomyces_ provides for extrachromosomal maintenance in _Kluyveromyces_. In addition, one may use a combination of a centromere, such as the _Saccharomyces_ _CEN_3 and a high transformation frequency sequence, such as _ARS_ or _KARS_. If selective maintenance is provided, such as complementation or resistance to an antibiotic to which _Kluyveromyces_ is susceptible, the ARS-like sequences will usually suffice for extrachromosomal maintenance.

For large scale fermentation even a small loss of plasmid stability will greatly affect the final yield of the desired protein. To increase the stability of recombinant molecules in host cells, for example _Kluyveromyces_, integration of the recombinant molecules into the host chromosome may be used.

Where integration is desired, it will usually be desirable to have a sequence homologous to a sequence of the chromosome of the host, so that homologous recombination may occur. It is understood, that random integration also occurs, so that the homologous sequence is optional. Where an homologous sequence is employed, the homologous sequence will usually be at least about 200 bp and may be 1000 bp or more. In addition, where integration is involved, one may wish to have amplification of the structural gene. Amplification has been achieved by providing for a gene in tandem with the desired structural gene, which provides for selective advantage for the host in a selective medium. Thus, the genes expressing dihydrofolate reductase, metallothioneins, thymidine kinase, etc., have proven useful in a variety of hosts to provide for amplification, where the gene provides protection from a toxin, such as methotrexate, heavy metals, such as copper and mercury, and the like.

Vectors of interest providing for stable replication include KARS vectors originating from K. lactis, e.g. pKARS12 and pKARS2, which plasmids comprise a K. lactis DNA fragment containing the KARS12 or KARS2 sequence in the S. cerevisiae plasmid YRp7. A vector employed for integration is, for example, pL4, a hybrid plasmid of the ARS1 carrying plasmid YRp7 and K. lactis XhoI DNA fragment carrying the LAC4 gene. See EP-A 0096430.

Plasmids of particular interest include plasmids having the 2 micron plasmid replication system, the LAC4 gene, the Tn601 and Tn5 kanamycin resistance gene, which also provides resistance to the antibiotic G418 in Kluyveromyces (Jimenez and Davis, Nature (1980) 287:869-871). This plasmid provides for autonomous replication in Kluyveromyces and can be selected for by resistance to G418 on regeneration plates containing glucose, sorbitol, and 0.2 µg/ml G418, while avoiding elevated concentrations of KCl, which interferes with the sensitivity of Kluyveromyces to G418. Preferred plasmids include the TRP1 gene, particularly from S. cerevisiae, the LAC4 gene, particularly from K. lactis, the Kan^R gene providing for resistance against antibiotic G418 from Tn5, or the like.

The subject vectors and constructs are introduced into an appropriate host for cloning and expression of the desired structural genes. After transformation, colonies will normally appear on regeneration medium within about 5 to 6 days. Where an antibiotic is employed for selection, the colonies should be screened to ensure the absence of spontaneous mutation to a resistant strain. Employing the plasmids and the methods of the subject invention, about 5%

of resistant colonies were found to contain the plasmid construct providing for at least about 4 transformants per µg of plasmid DNA. Where selection was based on the presence of the LAC4 gene, using plates containing lactose as the sole carbon source and 0.6M KCl as an osmotic stabilizer, all of the surviving colonies were found to be transformants and not spontaneous revertants. About 20 transformants were obtained after about 4 to 5 days of incubation at moderate temperature, e.g. 30°C.

As a host organism, <u>Kluyveromyces</u> is especially suitable for the production of heterologous proteins, for example for the production and extraction of the enzyme chymosin and its precursors preprochymosin, pseudochymosin and prochymosin, for human serum albumin (HSA), tissue plasminogen activator (t-PA) or mutant forms of t-PA, and thaumatin and its precursor forms. Although other organisms such as <u>Saccharomyces</u> produce prochymosin in reasonable amounts, the produced prochymosin cannot be extracted in an active or activatable form. We have surprisingly found that more than 90% of the total amount of the prochymosin produced by <u>Kluyveromyces</u> can be extracted in an active form with very simple standard techniques.

Any of the many <u>Kluyveromyces</u> species may be employed. Either laboratory or industrial, preferably industrial, strains may be used. By industrial species is intended, <u>Kluyveromyces</u> strains from organisms which may be isolated from natural sources or may be available from depositories or other sources or obtained by modification, e.g. mutation, of such strains. The industrial strains are characterized by being resistant to genetic exchange, being prototrophic or made prototrophic by a single gene being introduced into the host strain, and are usually selected for improved production of peptides. Among the <u>Kluyveromyces</u> species which may find use are <u>K. lactis</u>, <u>K. fragilis</u>, <u>K. bulgaricus</u>, <u>K. thermotolerans</u>, <u>K. marxianus</u>, etc. It should be further noted that the <u>Kluyveromyces</u> organisms are on the GRAS (Generally Recognized As Safe) list. Their use for production of products to be used <u>in vivo</u> or to be ingested normally will not require special governmental review and approval.

Both wild type and mutant Kluyveromyces, particularly Kluyveromyces lactis or Kluyveromyces fragilis may be employed as hosts. Hosts of particular interest include K. lactis SD11 lac4 trp1 and K. lactis SD69 lac4, and the wild-type strain CBS 2360 (see EP-A-0096430).

For maintaining selective pressure on the transformants for maintenance of the plasmids, selective media may be used, such as a yeast nitrogen-based medium, 2% lactose instead of glucose for K. lactis SD69 lac4 (PTY75-LAC4) and for K. lactis SD69 lac4 (pL4) and a yeast nitrogen-based medium (Difco) plus 2% glucose for K. lactis SD11 lac4 trp1 (pKARS12). See for the transformants mentioned, EP-A-0096430. Similarly, strains containing plasmids conferring antibiotic resistance, for example against G418, may be cultivated in a medium containing said antibiotic.

Where the hybrid plasmids are employed for large scale production of the desired protein, it would generally be useful to remove at least substantially all of the bacterial DNA sequences from the hybrid plasmids.

Depending upon the nature of the structural gene of interest, the expression product may remain in the cytoplasm of the host cell or be secreted. It has been found that not only the proteins that remain in the cell but also those that are secreted are soluble. Where the expression product is to remain in the host cell, it may generally be desirable to have an inducible transcription initiation region, so that until the transformant has reached a high density, there is little or no expression of the desired product. After sufficient time for the expression product to form, the cells may be isolated by conventional means, e.g. centrifugation, lysed and the product of interest isolated. Depending upon the nature and use of the product, the lysate may be subjected to various purification methods, such as chromatography, electrophoresis, solvent extraction, crystallization, or the like. The degree of purity may vary from about 50%, to 90% or higher, up to essential purity.

When the product is to be secreted, both constitutive and non-constitutive transcriptional initiation regions may be employed, depending on the fermentation process used for the production of the protein or polypeptide of interest. The expression product may be secreted into the culture medium, and produced on a continuous basis, where the medium is partially withdrawn, the desired product extracted, e.g., by affinity chromatography, or the like, and the spent medium discarded or recirculated by restoring essential components.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The following examples are offered by way of illustration and not by limitation.

EXPERIMENTAL

Example 1

Construction of chymosin expression plasmids containing a long lactase promoter sequence

A. Construction of pUCla56

Chromosomal DNA was isolated from Kluyveromyces lactis strain CBS 2360 (Das and Hollenberg, Current Genetics (1982) 5:123-128), cleaved with XhoI, and separated according to size on a sucrose gradient. Fractions containing the lactase gene were detected with a LAC4 probe from plasmid pK16 after spotting the DNA on a nitrocellulose filter (see EP-A-0096430, Example 16.C2). DNA containing the LAC4 gene was cloned into the SalI site of plasmid pPA153-215 (Andreoli, Mol. Gen. Genet. (1985) 199:372-380) giving rise to plasmid pPA31. An XbaI fragment of pPA31 containing the lactase gene was subcloned in the XbaI site of pUC19 (Yanisch-Perron et al., Gene (1985) 33:103-119) which yields plasmid pUCla56.

B. Introduction of the G418 resistance gene in the terminator of the lactase gene

The terminator fragment containing the G418 resistance marker was obtained from plasmid pGBTeG418. E. coli containing pGBTeG418 was deposited with Centraal Bureau voor Schimmelcultures under number CBS 184.87 on February 26, 1987. Plasmid pGBTeG418 (see Figure 1) consists of the plasmid pPA153-215, as described above, and a 5.6 kb fragment consisting of the 3.7 kb BamHI K. lactis lactase terminator fragment (Breuning et al., Nucl. Acid Res. (1984) 12:2327-2341) and the Tn5 gene (Reiss et al., EMBO J. (1984) 3:3317) conferring resistance to G418 under the direction of the promoter alcohol dehydrogenase I (ADH) from yeast, similar to that described by Bennetzen and Hall, J. Biol. Chem. (1982) 257:3018-3025.

C. Construction of plasmid pGB900 containing the G418 resistance gene and prochymosin encoding DNA

The 3.6 kb HindIII-XbaI fragment from plasmid pGBTeG418 containing the G418 resistance gene (see Example 1B) and the SalI-HindIII fragment containing the prochymosin gene from pGB123 (see EP-A-0096430) were ligated in pUC19 cleaved with SalI and XbaI. This yielded plasmid pGB900.

D. Construction of plasmid pGB901 (see Figure 2)

Plasmid pGB901 was constructed by ligating the following four fragments:

(1) a 3.6 kb XbaI-HaeII fragment containing the lactase promoter to about position -90 from the lactase ATG start codon isolated from pUCla56,

(2) a HaeII-SalI fragment extending from the above HaeII site to a SalI site, which was ligated to position -26 in a similar Bal31 experiment as described in Example 16.C2 of EP-A-0096430. However, in this experiment only a SalI

linker was used. This fragment has the following sequence:

```
   5'  TTAAC ACTTGAAATT TAGGAAAGAG CAGAATTTGG CAAAAAAAAT AAAAAAAAAA TAAACACG  3'
3'  CGCGAATTG TGAACTTTAA ATCCTTTCTC GTCTTAAACC GTTTTTTTTA TTTTTTTTTT ATTTGTGCAG CT  5'
```

(3) the 5.1 kb SalI-XbaI fragment containing prochymosin and G418 from pGB900 (see Example 1C),

(4) pUC19 cleaved with XbaI.

During the construction of the plasmid the CG sequence from the HaeII site was inadvertently removed, thereby creating a HindIII site at this position.

Prochymosin-encoding DNA is present in plasmid pGB901. This may readily be converted to plasmids with preprochymosin, pseudochymosin or chymosin DNA by using the SalI-BglII fragments from pGB 131, 122 or 124, respectively (see EP-A-0096430).

Example 2

Secretion of prochymosin from Kluyveromyces lactis transformants

To direct the synthesis of prochymosin in Kluyveromyces, plasmid pGB901 was used to transform K. lactis strains SD11 and CBS 2360 with similar results. The transformation was carried out essentially as described in Examples 4 and 14 of EP-A-0096430, by using intact plasmid DNA or plasmid DNA cut with restriction endonucleases. In the latter case restriction endonucleases were used which cut in the promoter region, e.g., SacII, NdeI, SnaBI or SpeI, or in the terminator region, e.g., EcoRV, or both the promoter and terminator regions.

K. lactis strain CBS 2360 was grown in 100 ml of YEPD-medium (1% yeast extract, 2% peptone, 2% glucose) containing 2.5 ml of a 6.7% yeast nitrogen base (Difco) solution to an OD_{610} of about 7. The cells were collected by centrifugation from 10 ml of the culture, washed with TE-buffer (10 mM Tris-HCl pH 7.5, 0.1 mM EDTA) and resuspended in 1 ml TE-buffer. An equal volume of 0.2 M lithium acetate was added and the mixture was incubated for 1 hr at 30°C in a shaking waterbath. Plasmid pGB901 (15 μg) was cut at the unique _Sac_II site in the lactase promoter, ethanol precipitated and resuspended in 15 μl TE-buffer. This DNA preparation was added to 100 μl of the pre-incubated cells and the incubation was prolonged for 30 minutes. Then an equal volume of 70% PEG 4000 was added and the mixture was incubated for 1 hr at the same temperature, followed by a heatshock of 5 minutes at 42°C. Then 1 ml of YEPD-medium was added and the cells were incubated for 1.5 hrs in a shaking waterbath of 30°C. Finally the cells were collected by centrifugation, resuspended in 300 μl YEPD and spread on agar plates containing 15 ml of YEPD agar with 300 μg/ml of G418, overlayered (1 hr before use) with 15 ml YEPD-agar without G418. Colonies were grown for 3 days at 30°C. _K_. _lactis_ strain SD11 was transformed in a similar way, only the initial G418 concentration in the selection plates was lowered to 150 μg/ml. In one of the experiments transformants of CBS 2360 were grown at 30°C in YEP-medium containing 2% galactose. After 60 hours, cells and medium were separated by centrifugation. Cells were disrupted by treatment with glass beads. Culture medium and cell extract were treated at pH 2 before assaying for chymosin activity (see Foltman, Methods in Enzymology (1970) _19_:421-426).

Cells were removed from cultures by centrifugation and the resulting supernatants were acidified to pH 2 by the addition of 1 M H_2SO_4 and incubated for 2 hours at room temperature. The solutions were then neutralized to pH 6 by the addition of 2 M Tris base. A 50 μl volume of an appropriate dilution was added to a suspension of 12% non-fat dry milk in 10 mM $CaCl_2$ and incubated at 37°C until a clot formed. A unit of chymosin activity is defined as the amount of active chymosin required to produce a clot in 10 min. under these conditions. The supernatant contained milk-clotting activity due to the production and secretion of prochymosin by _K_. _lactis_ transformants although no signal sequence for protein secretion was added to prochymosin. About 30-60% of the total prochymosin produced was found in the medium as determined by the above-described milk-clotting assay. Similar results were obtained when K. lactis strain SD11 was used.

Example 3

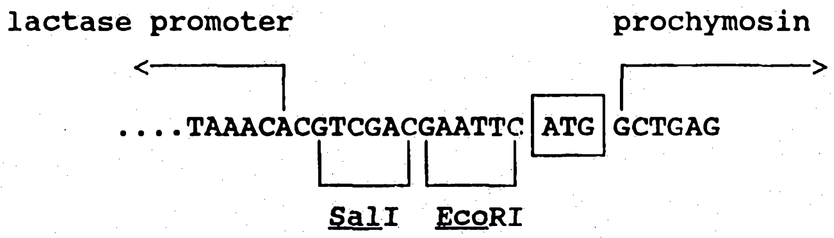
Lactase-chymosin fusion proteins giving enhanced chymosin production

By taking various SnaBI-SalI fragments (from a Bal31 experiment similar to the one described in Example 16.C2 of EP-A-0096430 but using a single SalI linker only) variants of pGB901 containing a fusion between the lactase and chymosin proteins were obtained (Table 1). The extra amino acids provided by lactase DNA and linker sequences can be removed, along with the pro sequence of prochymosin, by treatment with acid. It was observed that a fusion containing 4 amino acids from the lactase coding sequence (pGB902) resulted in enhanced chymosin production.

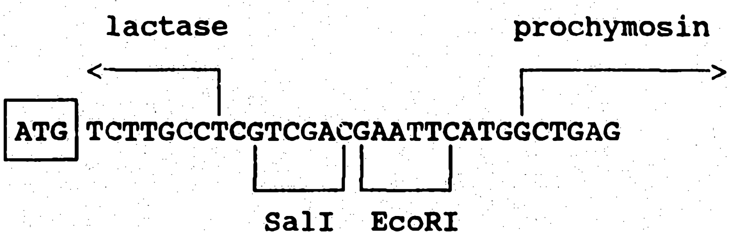
Table 1

Nucleotide sequence at the junction between the lactase promoter and prochymosin in pGB901 and pGB902

pGB901



pGB902



Protein synthesis starts at the boxed ATG codon.

Example 4

Expression of preprochymosin by Kluyveromyces transformants

The SalI site from the polylinker of pGB902 (see Example 3) was removed for convenience. pGB902 was partially digested with SalI, followed by a short incubation with Bal31 (Boehringer). Linear fragments were isolated from an agarose gel, ligated and transformed into *E. coli*. A correct plasmid, pGB903, was obtained. Restriction analysis showed that this plasmid also has the XbaI and HindIII sites removed from the polylinker.

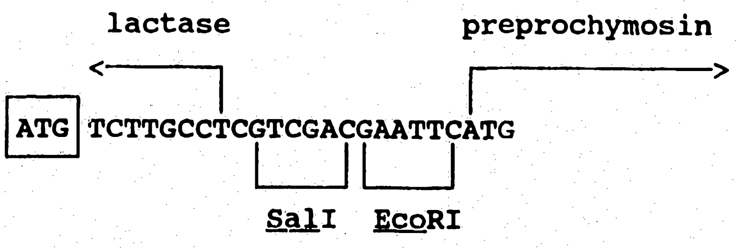
To construct a plasmid containing and expressing preprochymosin, plasmid pGB903 was digested with the restriction endonucleases SalI and BglII. The 11 kb DNA fragment was isolated from an agarose gel by electroelution. Similarly, plasmid pGB124 containing the preprochymosin gene (see EP-A-0096430, Example 16) was digested and the 0.3 kb SalI-BglII fragment containing the N-terminal part of the preprochymosin gene was isolated.

The 11 kb and the 0.3 kb DNA fragments were mixed, ligated with DNA ligase and transformed into *E. coli*. Plasmid **pGB904** was isolated which contained the preprochymosin gene fused to a small part of the lactase gene (Table 2).

Table 2

Nucleotide sequence at the junction between the lactase promoter and preprochymosin in pGB904

pGB904



Protein synthesis starts at the boxed ATG codon.

K. lactis CBS 2360 cells were transformed with pGB904, which had been linearized with SacII. Transformants were selected, grown and assayed for chymosin activity as described in Example 2. In the following Table 3 a comparison is made between the secretion of prochymosin from K. lactis CBS 2360 cells transformed with pGB902 (see Example 3) and with pGB904. (Pro)chymosin production is expressed in arbitrary units per ml of cells at OD_{610} of 200.

Table 3

Secretion of prochymosin by K. lactis cells transformed with pGB902 and pGB904

	pGB902		pGB904	
Transformant	Supernatant	Pellet	Supernatant	Pellet
1	3.2	<0.4	22.4	1.7
2	1.3	<0.4	33.3	3.0
3	7.1	1.4	28.0	2.3
4	4.4	0.66	53.8	5.8

Example 5

Secretion of prochymosin by Kluyveromyces using heterologous leader sequences

A. Chemical synthesis of an amyloglucosidase leader sequence and construction of a plasmid containing said leader sequence

The leader sequence of amyloglucosidase (AG) from Aspergillus awamori was published by Innis et al., Science (1985) 228:21-26. Based on the protein sequence, oligonucleotides were derived to permit insertion of the leader sequence in front of the prochymosin gene (see Figure 3).

The oligonucleotides were synthesized with an Applied Biosystems DNA synthesizer. The oligonucleotides were purified by electrophoresis on a denaturing polyacrylamide gel, then electroeluted from the gel.

Plasmid pGB903 (see Example 4) was cut at the unique SalI site. The oligonucleotides were hybridized at 65°C, 50°C and 37°C for one hour each in 2xSSC. The oligonucleotides had no phosphate at the 5' end to prevent formation of multimers. The DNA was ligated into the SalI site using T4 polynucleotide ligase. The ligation mixture was transformed into E. coli HB101. Twenty-four of the colonies were cultured and plasmid DNA isolated. One of the plasmids, pGB905, was shown to have the correct orientation of the oligonucleotides by restriction enzyme analysis. Plasmid pGB905 was transformed to K. lactis CBS 2360. (Pro)chymosin production was analyzed according to the procedure described in Example 2. The results are of the (pro)chymosin production, in arbitrary units/ml of cells at OD_{610} of 200, is shown in the following Table 4.

Table 4

Secretion of prochymosin by K. lactis cells transformed with pGB902 and pGB905

	pGB902		pGB905	
Transformant	Supernatant	Pellet	Supernatant	Pellet
1.	3.2	<0.4	60.6	<0.4
2.	1.3	<0.4	56.4	<0.4
3.	7.1	1.4	56.7	<0.4
4.	4.4	0.66	57.6	<0.4

B. Chemical synthesis of a novel synthetic leader sequence into construction of a plasmid containing the novel synthetic leader sequence.

A synthetic leader sequence was prepared which has a sequence different from any known leader sequence. Using this leader sequence, all prochymosin synthesized was secreted by Kluyveromyces as shown below.

This synthetic leader sequence was devised using frequently occurring amino acids from position -6 to +2 of the signal sequence cleavage site (Von Heyne, Eur. J. Biochem. (1983) 133:17-21). Frequently occurring yeast codons were also employed and extra nucleotides were incorporated in front of the ATG sequence to make up for the deletion of 26 nucleotides in pGB902. The oligonucleotides used and the resulting leader sequence are shown in Figure 5.

The synthetic leader sequence DNA was synthesized using an Applied Biosystems DNA synthesizer. The resulting oligonucleotides were run on a 40 cm long, 1 mm thick polyacrylamid gel, containing TBE buffer (50 mM Tris, 50 mM borate, 1mM EDTA, pH 8.3) and 7 M urea until the Bromophenol Blue marker had travelled 2/3 of the gel length. The DNA was visualized, eluted from the gel and precipitated with ethanol.

Also from pGB901 a derivative was made with a deletion around the SalI site resulting from the polylinker of pUC19. This was done by replacing the 0.5 kb SnaBI-BglII fragment from pGB903 by the corresponding fragment from pGB901. The resulting plasmid was cut at the unique SalI site. The oligonucleotides were hybridized at 65°C, 50°C and 37°C for one hour each in 2xSSC. The DNA was ligated into the SalI site using T4 polynucleotide ligase. The plasmid was then transformed into *E. coli* HB101. Of the colonies obtained, 24 were cultured and plasmid DNA isolated. One of the plasmids, pGB906, was shown to have the oligonucleotides in the correct orientation by restriction enzyme digestion. It was found that *K. lactis* CBS 2360 transformed with pGB906 secreted more than 95% of the prochymosin produced.

C. Analysis of chymosin protein produced by K. lactis transformed with pGB905

K. lactis CBS 2360 (pGB905) transformants were grown for 3 days at 30°C and samples were collected from the supernatant of the cultures. Protein samples were electrophoresed on a polyacrylamide gel according to Laemmli (Nature (1970) 227:680-685). Proteins were blotted onto a nitrocellulose filter according to the method of Towbin et al. (Proc. Natl. Acad. Sci. USA (1979) 76:4350-4354). Chymosin protein was detected by incubating the filter with a polyclonal antiserum against chymosin (Chr. Hansen), followed by donkey anti-rabbit antibodies coupled to a peroxidase (Amersham) and finally with 0.6 mg/ml 4-chloronaphthol and 0.015% hydrogen peroxide in a buffer solution (50 mM Tris-HCl pH 7.5, 0.9% NaCl) containing 40% methanol. Prochymosin excreted by the AG signal sequence is correctly cleaved after pH 2 treatment as demonstrated by this assay (Figure 5). Similar results were obtained with K. lactis CBS 2360 (pGB906) transformants.

Example 6

Construction of plasmids containing the Saccharomyces cerevisiae α-factor sequence for efficient secretion

A. Saccharomyces α-factor expression plasmids

1. Construction of Plasmids

pDM100-PC: The starting material was plasmid pGB163 (see EP-A-0096430, Example 16.C1). Plasmid pGB163 was digested with BamHI and ligated to an XbaI-BamHI, α-factor leader-prochymosin adaptor. The resulting mixture was then treated with PstI and a 96 bp fragment encoding the pro-α-factor processing site and the N-terminal region of prochymosin was isolated. A 1900 bp fragment encoding bovine prochymosin was isolated from plasmid pJS111 following digestion with PstI and SalI. Plasmid pJS111 is a pBR322 derivative containing the prochymosin gene from pGB163 under the regulatory control of the ADH-2 promoter and the glyceraldehyde 3-phosphate (GAPDH) terminator. The 1900 bp PstI to SalI fragment that was removed contains the prochymosin gene and the GAPDH terminator. The yeast GAPDH 49 gene promoter and transcription terminator are essentially as described by Travis, J. Biol. Chem. (1985) 260:4384-4389.

Plasmid pDM100, containing a fusion of the GAPDH promoter, the S. cerevisiae α-factor leader, and a synthetic gene for human -interferon flanked by XbaI and SalI sites and the α-factor terminator, was digested with XbaI and SalI, treated with alkaline phosphatase, then ligated to the 96 bp and 1900 bp fragments described above. The α-factor leader and terminator are essentially as described by Brake, Proc. Natl. Acad. Sci. USA (1984) 81:4642-4646. The resulting plasmid pDM100-PC was isolated and contained a fusion of the GAPDH promoter, the α-factor leader and prochymosin gene. The complete sequence of the BamHI insert is shown in Figure 6.

To allow selection of yeast transformants, two plasmids, pKS100 and pAB300, were constructed.

pKS100: Plasmid pKS100 was constructed by insertion into pDM100-PC of an 1170 bp HindIII fragment from YEp24 containing the S. cerevisiae URA3 gene.

pAB300: Plasmid pAB300 was constructed by insertion into pDM100-PC of a 3500 bp HindIII-SalI fragment from pGB901 containing the 3' region of the K. lactis LAC4 gene and the G418 resistance marker. The GAPDH/α-factor/prochymosin BamI insert in pDM100-PC is illustrated in Figure 6.

2. Transformation of K. lactis and S. cerevisiae

Plasmid pKS100 was digested at the BglII site in the prochymosin coding region and used to transform K. lactis strain KRN201-6. This strain is a derivative of strain 2UV21 (a lac4 trp1 ura3 [kil^o]) in which the lac4 gene has been replaced by the LAC4 promoter-prochymosin gene fusion from pGB901. Integration of pKS100 is thus targeted to the integrated prochymosin coding region. Plasmid pKS100 was also used to transform S. cerevisiae strain AB110 (α ura3 leu2 his4 pep4-3 [cir^o]), in this case targeting to the SacII site in the 3' region of the GAPDH gene.

The resulting transformants were grown to saturation in liquid YEPD medium, and the culture supernatants and cell lysates assayed for chymosin activity after activation at pH 2. As shown by the results summarized in Table 5 below, the K. lactis transformants efficiently secreted prochymosin into the medium, whereas the *S. cerevisiae* transformants secreted only a small fraction of the prochymosin produced.

Table 5

Prochymosin production in K. lactis and S. cerevisiae transformants

Strain	Chymosin Activity (relative units/ml culture) Cell Extract	Chymosin Activity (relative units/ml culture) Culture Supernatant
AB110	<0.25	<1.0
AB110::pKS100	15.5	2.3
KRN201-6	<0.25	<1.0
KRN201-6::pKS100	12.0	333.0

Plasmid pAB300 was used to transform *K. lactis* strain 2UV21 to G418 resistance, targeting integration to the *Eco*RV site in the 3' region of the LAC4 gene. These transformants were also found to efficiently secrete prochymosin into the culture medium as shown in Table 6 below.

Table 6

Prochymosin secretion from α-factor/prochymosin fusions

Host Strain	Transforming Plasmid	Secreted Chymosin Activity (relative units/ml culture)
2UV21	-	<2
KRN201-6	-	<2
KRN201-6	pKS100	385
2UV21	pAB300	294

B. Construction of LAC4 promoter/α-factor leader/ prochymosin fusions

In order to produce this fusion, two intermediate plasmids were constructed. Plasmid pDM100-PC was partially digested with PstI, ligated to a SalI-PstI adaptor encoding a portion of the α-factor leader and 26 bp of the region 5' to the LAC4 gene, and then digested with HindIII. A 1500 bp fragment was isolated from this mixture and then cloned into pUC18 digested with HindIII and SalI to produce **pKS102**.

A synthetic E. coli lac operator was ligated into the SalI site just 5' to the α-factor leader coding sequence in pKS102 to produce the plasmid **pKS103**. This was done because the LAC4 promoter/α-factor leader/prochymosin fusion may be toxic to E. coli.

A 490 bp SalI-BglII fragment from pKS103 was isolated and ligated to SalI-BglII-digested pJD15R. Plasmid pJD15R is derived from pGB901 by deletion of the SalI site in the pUC19 polylinker by filling-in to produce pJD15, and then recloning the 8800 bp XbaI fragment in the opposite orientation. From this reaction the plasmid **pKS105** was isolated. These plasmids are illustrated in Figure 7.

Plasmid pKS105 was then used to transform K. lactis strain CBS 2360 to G418 resistance, using the SacII site in the LAC4 5' region as a targeting site for the integrative transformation. Chymosin production is expressed in units per ml of cells at OD_{610} of 200, see Table 7 below.

Table 7

Secretion of Prochymosin by K. Lactis Cells Transformed with pKS105*

Transformant	Supernatant	Pellet
1	111	3.3
2	147	4.5
3	124	3.7
4	125	3.0

* Chymosin activity in relative units/ml culture.

Example 7

Construction of plasmids containing the Kluyveromyces lactis α-factor sequence for efficient secretion

A. Isolation and use of K. lactis α-factor signal sequence

Biological assays of culture supernatants were carried out as described (Julius et al., Cell (1983) 32:839) using as a tester strain the S. cerevisiae Mat a sst2-3 strain RC687. K. lactis strain CBS 141(α) was grown in a medium consisting of 0.5% glucose, 0.17% yeast nitrogen base without ammonium sulfate (Difco), and 0.002% ammonium sulfate. After removal of cells by centrifugation, acetic acid was added to the culture supernatant to a concentration of 0.1 M, and the supernatant was passed over a column of Bio-Rex 70 (Biorad). The column was washed with 0.1 M acetic acid and then the α-factor was eluted with 80% ethanol/10 mM HCl. The eluate was evaporated to dryness and then dissolved in 0.1% trifluoroacetic acid (TFA)/20% acetonitrile and applied to a reverse-phase HPLC guard column. The column was washed stepwise with solutions containing 0.1% TFA and 20%, 40%, 60% and 80% acetonitrile. The 60% fraction, containing the α-factor activity, was then applied to an analytical C-18 HPLC column and eluted with a gradient of 20% to 80% acetonitrile in 0.1% TFA. Fractions were collected and assayed for α-factor activity. The fractions containing α-factor activity were dried and subjected to amino acid sequence analysis.

B. Hybridization screening of plasmid libraries

Pools of oligonucleotides were labeled using γ-[^{32}P]-ATP and T4 polynucleotide kinase. These oligonucleotide probes were used to probe Southern blots or bacterial colonies at 42°C in the following hybridization solution: 4xSSC, 50 mM KH_2PO_4 pH 7, 1% sarkosyl, 10% dextran sulfate, 200 µg/ml sonicated, denatured salmon sperm DNA. Filters were washed in 2xSSC, 0.1% SDS at 42°C.

A plasmid library in the vector pJS109, containing inserts resulting from a limited Sau3AI digest of genomic DNA from K. lactis strain SD11 (a trp1 lac4), size-fractionated to purify fragments >5000 bp was screened with these probes by plating transformants of E. coli strain HB101 at a density of 500-2000 colonies per 80 mm plate of L-agar containing 100 μg/ml ampicillin. DNA was transferred from the colonies to nitrocellulose filters and these filters hybridized as described above. Areas on the original plates corresponding to regions of hybridization signals on the filters were picked, then replated and retested by hybridization to isolate single colonies with plasmids containing hybridizing sequences. Positive colonies were further tested by Southern blot analysis of DNA purified from small cultures.

Plasmids purified from hybridization-positive colonies were digested with a variety of restriction enzymes and the resulting fragments analyzed by Southern blot analysis using the same hybridization probes in order to identify restriction fragments of size suitable for DNA sequence analysis. Fragments thus identified were purified by agarose gel electrophoresis and cloned into appropriate MP18 and MP19 vectors. DNA sequence analysis was then performed.

C. Isolation of Kluyveromyces α-factor

The first 10 amino acids of the K. lactis α-factor showed a definite homology to that from S. cerevisiae, with 6 identical residues. This sequence is shown below:

Trp-Ser-Trp-Ile-Thr-Leu-Arg-Pro-Gly-Gln

This protein sequence was used to design a set of oligonucleotides deduced to be complementary to the structural gene for the corresponding structural gene as shown in Figure 8. Oligonucleotides including all of the possible codons for a segment of the α-factor peptide were synthesized as two pools of 96 and 48 different molecules.

These two pools were radioactively labeled using γ-[^{32}P]-ATP and T_4 polynucleotide kinase, and were each used to probe a Southern blot of restriction digests of K. lactis

DNA. Pool #2 gave strong hybridization to a single fragment and much weaker hybridization to a second fragment in several different digests. Thus, pool 2 was chosen to screen plasmid libraries of K. lactis genomic DNA.

Use of these probes to screen plasmid libraries resulted in the isolation of a number of hybridizing clones. DNA sequence analysis of one of these clones, αfk18b, showed it encodes an α-factor related peptide which bears a strong similarity to the precursor of the S. cerevisiae α-factor peptide. The hybridizing segment was located on a PstI-EcoRI fragment of about 1000 bp. The sequence of this fragment is shown in Figure 9. The K. lactis precursor contains only 2 sites for the addition of N-linked carbohydrate chains. In addition, the spacers of the K. lactis repeats are longer than those of the S. cerevisiae repeats and show a more diverse sequence with the pattern X-Ala/Pro rather than the Glu/Ala-Pro sequences found in S. cerevisiae. A comparison of the DNA sequences showed a strong degree of homology throughout the coding region.

D. Construction of Plasmids

A series of plasmids (shown in Figure 10) were constructed in order to provide a fusion of the K. lactis α-factor leader to prochymosin expressed under the transcriptional control of a strong promoter.

pAB307: A 673 bp SspI-EcoRI fragment from αfk18b (Figure 9) was modified by filling the EcoRI overhang by Klenow enzyme and addition of BglII linkers to the blunt ends. This fragment was then inserted into a BglII site joining the promoter and terminator regions of the S. cerevisiae glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH). This cassette was cloned as a BamHI fragment in pUC18, resulting in pAB307.

pAB309: Fusion of sequences encoding the α-leader and bovine prochymosin was then performed. First pAB307 was digested with NcoI and the cohesive ends made blunt by treatment with mung bean nuclease. The resulting product was then digested with SalI. To this was ligated a 2000 bp EcoRV-SalI fragment containing sequences encoding prochymosin and the *S. cerevisiae* transcriptional termination region. This fragment was derived from plasmid pJS111 in which a *Xba*I-*Bam*HI adaptor had been added to the 5' end of a fragment containing prochymosin cDNA fused to the *S. cerevisiae* GAPDH transcriptional termination region. This ligation mixture was used to transform *E. coli* strain HB101 and a transformant carrying the plasmid pAB309 was isolated. The sequences around the junction of this fusion are shown in Figure 11 and the sequence of the entire *Bam*HI-*Sal*I insert of pAB309 is shown in Figure 12.

pAB312: In order to obtain transformation of *K. lactis* strains, a 3560 bp *Hin*dIII fragment derived from pGB901 was inserted into pAB309 producing plasmid pAB312. The *Hin*dIII fragment contains the 3' region of the *K. lactis* *LAC4* gene and a fusion of the *S. cerevisiae* ADH1 promoter to the bacterial G418-resistance structural gene.

pAB313 and pAB314: A 1900 bp *Sac*I-*Hin*dIII was isolated from pAB309 and cloned into MP19 (Yanisch-Perron *et at.*, Gene (1985) *33*:103). Single-stranded phage DNA was prepared and used as a template for *in vitro* mutagenesis with one of the two oligonucleotide primers shown in Figure 13. The M13 phage MP19/αk11.5 and MP19/αk12.2 were prepared using Primer #1 and Primer #2, respectively.

Double-stranded RF DNA was prepared from these phages, and 1100 bp *Sac*I-*Stu*I fragments isolated from each. These fragments were ligated to a 7100 bp *Sac*I-*Stu*I fragment from pAB312. The resulting plasmids pAB313 and pAB314 were isolated with the sequence alterations illustrated in Figure 13.

E. Transformation of Kluyveromyces

Plasmid pAB312 was digested with *Eco*RV (to target integration to the *LAC4* region of the *K. lactis* genome) and was then used to transform *K. lactis* strain 2UV21 (*a ura3 trp*1 *lac*4 [kil^{o}]) to G418 resistance. The plasmids pAB313 and pAB314 were used to transform strain 2UV21 to G418 resistance. Cultures of transformants 2UV21::pAB312, 2UV21::pAB313 and 2UV21::pAB314 were grown and culture supernatants assayed for chymosin activity as above.

A number of these transformants, as well as an untransformed control strain, were grown for 36 hours in 1 ml of medium composed of 1% yeast extract, 2% peptone, 2% glucose, 0.17% yeast nitrogen base, 50 μg/ml tryptophan and 50 μg/ml uracil. Culture supernatants were then assayed for chymosin activity after acid activation. All of the transformants were found to secrete activatable chymosin. The results are shown in the following Table 8.

Table 8

Secretion of prochymosin in Kluyveromyces

Strain	Host	Plasmid	Chymosin Activity (relative units/ml culture)
2UV21	2UV21	-	<2
KRN303-1	2UV21	pAB312	256
KRN304-4	2UV21	pAB313	175
KRN305-2	2UV21	pAB314	206

Each of the transformants was found to secrete a single prochymosin-related species as judged by SDS polyacrylamide gel electrophoresis of trichloroacetic acid-precipitated culture supernatants. The prochymosin-related protein secreted by pAB312 transformants appeared to be of slightly higher molecular weight than those secreted by pAB313 and pAB314 transformants as determined by electrophoretic mobility.

The major species secreted by KRN303-1 and KRN304-4 were purified by preparative SDS polyacrylamide gel electrophoresis and subjected to gas phase amino acid sequence analysis. The N-terminal sequences of these species are given below.

KRN-303-1

1 5 10 15

Glu-Ala-Asp-Ala-Ser-His-His-Met-Ala-Glu-Ile-Thr-Arg-Ile-Pro

KRN304-4

```
1           5
Ala-Glu-Ile-Thr-Arg-Ile
```

These results indicate that the prochymosin-related species secreted by KRN303-1 has undergone no processing of the amino-terminal spacer sequence, while the species secreted from KRN304-4 has the authentic mature prochymosin amino terminus.

Example 8

Secretion of t-PA by Kluyveromyces lactis using an amyloglucosidase signal sequence

A. Cloning of tissue-type plasminogen activator cDNA

A cDNA coding for tissue-type plasminogen activator (t-PA) was obtained in a way similar to that described by Pennica *et al*. (Nature (1983) 301:214). DNA sequence analysis and restriction mapping confirmed the authenicity of the t-PA cDNA. For expression studies the 2.0 kb *Bgl*II fragment (see Pennica *et al*.), comprising almost the complete coding region for the mature protein and the 3' noncoding region, was used.

B. Introduction of the G418 resistance marker in pUC19

A DNA fragment comprising the Tn5 gene (Reiss *et al*., EMBO J. (1984) 3:3317), conferring resistance to G418 under the direction of the alcohol dehydrogenase I (ADHI) promoter from *S. cerevisiae*, similar to that described by Bennetzen and Hall, J. Biol. Chem. (1982) 257:3018, was inserted into the *Sma*I site of pUC19 (Yanisch-Perron *et al*., Gene (1985) 33:103). The obtained plasmid, **pUCG418**, is shown in Figure 14. *E. coli* containing pUCG418 was deposited at Centraal Bureau voor Schimmelcultures on December 4, 1987 under CBS 872.87.

C. Construction of pGBtPA1

In a few cloning steps **pGBtPA1** was constructed (see also Figure 15 and Table 9) containing the following elements:

(1) pUCG418 (see above) cut with *XbaI* and *HindIII*;

(2) the *XbaI*-*SalI* fragment from pGB901, containing the lactase promoter;

(3) synthetic DNA coding for the signal sequence of amyloglucosidase from *Aspergillus awamori* (Innis *et al*., Science (1985) 228:21). The sequence in front of the startcodon was chosen to remove the *SalI* site at the end of the lactase promoter fragment and further comprises part of the 5' noncoding region of the lactase gene;

(4) the 2.0 kb *BglII* fragment from the t-PA cDNA (see above);

(5) synthetic DNA, comprising part of the 3' noncoding region of the lactase gene.

Table 9

Schematic representation of plasmid pGBtPA1

```
                          XbaI
                          v
..........pUCG418.........TCTAGA.....lactase promoter.....


...........GTCGATCATCGAGAACTGAAAGATATGTCTTGCCTTATGTCTTTCAGA


TCCCTACTAGCTCTATCCGGTCTAGTTTGTACTGGTCTAGCTAACGTTATCTCCAAGAG

 SalI  BglII                        BglII
 v     v                            v
AGTCGACAGATCT.........t-PA cDNA......AGATCTGATATGAATTTATACT


TAGATAAGTATGTACTTAGATAAGTATGTACTTACAGGTATATTTCTATGAGATACTGA

                        HindIII
                        v
TGTATACATGCATGATAATATTTAAAGCTT
```

Protein synthesis starts at the underlined ATG codon.

D. Transformation of Kluyveromyces lactis and analysis of the transformants.

Transformation of K. lactis strain CBS 2360 with pGBtPA1 was performed as described in Example 2. The transformants and the control strain CBS 2360 were grown in YEPD-medium for about 64 hrs. at 30°C. Cells were separated from the culture medium by centrifugation. The cells were resuspended in a physiological salt solution at an OD_{610} of 300 and disrupted by shaking with glass beads for 3 minutes on a Vortex shaker at maximal speed. Cell debris was removed by centrifugation.

A clotlysis assay according to Wallen et al. (Biochim. Biophys. Acta (1982) 719:318) was performed in microtiter plates. A solution was made, containing 15 mM phosphate buffer (pH 7.3), 0.2 CU/ml plasminogen, 1.5 mg/ml fibrinogen and 0.04% gelatin. Into each well of a microtiter plate 10 μl thrombine (13.9 NIH units/ml), 25 μl sample and 65 μl of the plasminogen/fibrinogen solution were mixed. The reaction was followed by measuring the OD_{450} every 30 min. t-PA from melanoma cells (Kabi Vitrum) was used to provide a calibration curve within every microliter plate.

Table 10 shows the result of a typical analysis of 10 transformants. It is demonstrated that t-PA was found in the culture medium of K. lactis transformed with pGBtPA1.

Table 10

Clotlysis assay of the cultures from CBS 2360 and from CBS 2360 transformed with pGBtPA1

transformant	t-PA activity in supernatant
1	40 μg/l
2	6 μg/l
3	<3 μg/l
4	<3 μg/l
5	25 μg/l
6	3 μg/l
7	3 μg/l
8	<3 μg/l
9	3 μg/l
10	<3 μg/l
CBS 2360 1°)	<3 μg/l
CBS 2360 2°)	<3 μg/l
CBS 2360 3°)	<3 μg/l
CBS 2360 4°)	<3 μg/l
CBS 2360 5°)	<3 μg/l

In some of the cell extracts a slight t-PA activity ($\leq$ 3 μg/l) was found.

Analysis was also performed on SDS-polyacrylamide gels overlayered with a plasminogen/fibrin-agarose gel according to Granelli-Piperno and Reich (J. Exp. Med. (1978) <u>148</u>:223). 200 μl of the supernatant of a culture of CBS 2360 or CBS 2360 transformed with pGBtPA1 was precipitated with ethanol and resuspended in 20 μl sample buffer (62.5 mM Tris-HCl pH 6.8, 2% sodium dodecylsulphate, 10% glycerol, Bromophenol Blue). The samples were layered on the gels without prior boiling. The results (shown in Figure 16) demonstrate the secretion of human t-PA by <u>K</u>. <u>lactis</u>. Furthermore, it is clear that most of the secreted material is glycosylated.

The secretion of t-PA was also confirmed by using an ELISA with a monoclonal antibody against human t-PA (ESP5 purchased from Biopool) and by a chromogenic activity assay (a commercial test from Kabi Vitrum).

Example 9

Secretion of t-PA by Kluyveromyces lactis using the signal sequence from human serum albumin

A. Construction of pGBtPA2

In a few cloning steps **pGBtPA2** was constructed (see Figure 17 and Table 11), containing the following elements :

(1) pUCG418, cut with XbaI and HindIII;

(2) the XbaI-SalI fragment from pGB901, containing the lactase promoter;

(3) synthetic DNA coding for the prepro-region of human serum albumin;

(4) the 2.0 kb BglII fragment from the t-PA cDNA (see Example 8);

(5) synthetic DNA, comprising part of the 3' non-coding region of the lactase gene.

Table 11

Schematic representation of plasmid pGBtPA2

```
                                      XbaI
                                      v
..........pUCG418.........TCTAGA........lactase promoter..

             SalI
             v
.............GTCGACAAAAAATGAAGTGGGTTACCTTCATCTCCTTGTTGTTCTT

                                           BglII
                                           v
GTTCTCCTCCGCTTACTCCAGAGGTGTTTTCAGAAGAGGTGCTAGATCT..........

                                   EcoRV
                             BglII     XhoI
                             v     v    v
t-PA cDNA....................AGATCTGATATCTCGAGAATTTATACTTA


GATAAGTATGTACTTACAGGTATATTTCTATGAGATACTGATGTATACATGCATGATAA

      HindIII
      v
TATTTAAAGCTT.............
```

Protein synthesis starts at the underlined codon.

B. Transformation of K. lactis and analysis of the transformants

Transformation of CBS 2360 with pGBtPA2 was performed as described in Example 8. The transformants and the control strain were grown and treated as described in the previous Example. The results of the clotlysis assay are summarized in Table 12.

Table 12

Clotlysis assay of the cultures of CBS 2360 and CBS 2360 transformed with pGBtPA2

transformant	t-PA activity in the supernatant
1	25 μg/l
2	25 μg/l
3	<3 μg/l
4	<3 μg/l
5	6 μg/l
6	12 μg/l
7	<3 μg/l
8	<3 μg/l
9	<3 μg/l
10	<3 μg/l
CBS 2360 1°)	<3 μg/l
CBS 2360 2°)	<3 μg/l
CBS 2360 3°)	<3 μg/l
CBS 2360 4°)	<3 μg/l
CBS 2360 5°)	<3 μg/l

In some of the cell extracts a slight t-PA activity (< 3 μg/l) was found.

Example 10

Secretion of human serum albumin by Kluyveromyces lactis

A. Synthesis and cloning of the HSA cDNA

cDNA encoding human serum albumin was prepared according to the method of Okayama and Berg (Mol. Cell. Biol. (1982) 2:161) using mRNA prepared from human liver. The cDNA was cloned in a vector derived from pBR322 and transformed to E. coli. Screening of the transformants was performed using a oligodesoxyribonucleotide based on the sequence of the HSA cDNA clone of Lawn _et_ _al_., Nucleic Acids Res. (1981) _9_:6103). The selected cDNA clone was partially sequenced and compared to the sequence of Lawn _et_ _al_. This revealed that the first 5 nucleotides of the preproHSA coding region were absent, but the _Bst_EII site at nucleotides 9-15 of the coding region was still found. This _Bst_EII site and the _Hin_dIII site in the 3' noncoding region (see Lawn _et_ _al_., cited above) were used for subcloning in expression vectors.

B. Construction of pGBHSA1

In a few cloning-steps pGBHSA1 was constructed, containing the following elements:

(1) pUCG418 (see Example 8) cut with _Xba_I and _Hind_III;

(2) the _Xba_I-_Sal_I fragment from pGB901 (see Example 1);

(3) synthetic DNA (_Sal_I-_Hind_III fragment), comprising part of the 3' noncoding region of the lactase gene. The sequence of this fragment is given in Table 13.

Table 13

Sequence of the SalI - HindIII fragment of pGBHSA1

```
                    EcoRV
SalI  NotI    BglII      XhoI
v     v       v     v    v
 TCGACGCGGCCGCAGATCTGATATCTCGAGAATTTATACTTAGATAAGTATGTACTTACA
     GCGCCGGCGTCTAGACTATAGAGCTCTTAAATATGAATCTATTCATACATGAATGT

                                               HindIII
                                               v
GGTATATTTCTATGAGATACTGATGTATACATGCATGATAATATTTAA
CCATATAAAGATACTCTATGACTACATATGTACGTACTATTATAAATTTCGA
```

C. Construction of pGBHSA2

pGBHSA1 was cut with Sal I and EcoR V and synthetic DNA was inserted:

```
SalI              BstEII       StuI
v                 v            v
 TCGACAAAAATGAAGTGGGTAACCATCGATAGGCCTACTGGGCTCGAGATC
     GTTTTTACTTCACCCATTGGTAGCTATCCGGATGACCCGAGCTCTAG
```

The underlined ATG-codon indicate the initiation codon in the ultimate expression construct (pGBHSA3, see below).

The resulting plasmid was named **pGBHSA2**.

D. Construction of pGBHSA3

The HSA cDNA-clone was cut with HindIII and the sticky end was filled in, using Klenow DNA polymerase I. Subsequently the DNA was cut with BstEII and the BstEII-HindIII (filled in) fragment, containing almost the complete HSA coding region was purified. pGBHSA2 was digested with XhoI, the sticky ends were filled in using Klenow DNA polymerase I and digestion with BstEII was performed. In the resulting vector the HSA encoding fragment (BstEII-HindIII (filled in)) was inserted. The obtained plasmid, pGBHSA3, is shown in Figure 18.

E. Transformation of *Kluyveromyces lactis* and analysis of the transformants

Transformation of *K. lactis* strain 2360 with pGBHSA3 was performed as described in Example 2. Transformants and the control strain CBS 2360 were grown in YEPD medium for about 64 hrs at 30°C. The cells were separated from the culture medium by centrifugation. Samples of 30 μl were taken from the supernatants and analysed by electrophoresis on a 10% polyacrylamide gel according to Laemmli (Nature 227, 680 (1970)). The results shown in Figure 19 demonstrate that HSA is secreted into the culture medium by *K. lactis* cells transformed with pGBHSA3. There is also an indication that the secretion of other proteins is reduced in the HSA producing cells.

The above results demonstrate that one can obtain efficient, convenient expression of exogenous genes in Kluyveromyces strains. Furthermore, the Kluyveromyces strains appear to be particularly useful for providing highly efficient secretion and processing of a wide variety of proteins, as illustrated by the results with prochymosin. Constructs and vectors are provided which allow for the introduction of an exogenous gene under the regulatory control of efficient promoters in Kluyveromyces and, as desired, joining to signal sequences which provide for translocation of the exogenous gene, particularly secretion. Thus, a fermentation system is provided for commercial production of a wide variety of exogenous proteins in an active or activatable form.

The following organisms have been deposited with the American Type Culture Collection on June 30, 1987: 2UV21, ATCC Accession No. 20855; KRN201-6, ATCC Accession No. 20854; HB101 pAB307, ATCC Accession No. ~~67484~~ 67454; HB101 pAB312, ATCC Accession No. 67455.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

Although the foregoing invention has been described in some detail by way of illustration and example for purpose of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

The claims defining the invention are as follows:

1. A method for producing a polypeptide of interest in a <u>Kluyveromyces</u> host cell, said method comprising:

introducing into said host cell a DNA sequence encoding said polypeptide of interest and

growing said host cell comprising said DNA sequence in a culture medium whereby said polypeptide of interest, or part thereof, is secreted into the culture medium.

2. A method according to Claim 1, whereby said DNA sequence forms part of ~~a DNA construct~~ an expression cassette which is introduced into said host cell and which comprises, in the direction of transcription, a transcriptional initiation regulatory region functional in said host cell; said DNA sequence encoding said polypeptide of interest; and a transcriptional termination regulatory region functional in said host cell.

3. A method according to Claim 1 or 2, wherein a signal sequence for secretion heterologous to said host cell or to said polypeptide of interest, or to said host and to said polypeptide of interest is joined in reading frame to the 5' terminus of said DNA sequence, whereby said polypeptide of interest is secreted by said host cell.

4. A method according to any one of Claims 1 to 3, wherein said polypeptide of interest is an enzyme.

5. A method according to Claim 4, wherein said enzyme is chymosin, or a precursor thereof.

6. A method according to Claim 4, wherein said enzyme is tissue plasminogen activator (t-PA), or mutant forms thereof.

7. A method according to any one of the Claims 1 to 3, wherein said polypeptide of interest is human serum albumin (HSA).

8. A method according to any one of Claims 1 to 7, wherein said host cell is an industrial strain of *Kluyveromyces*.

9. A method according to any one of Claims 1 to 8, wherein said host cell is *K. lactis* or *K. fragilis*.

10. A method according to Claim 2, wherein said ~~DNA construct~~ expression cassette further comprises at least one of a selection marker, a replication system for autonomous replication of said DNA sequence, or a transformation efficiency enhancing sequence.

11. A method according to Claim 10, wherein said replication system is an autonomously replicating sequence (ARS).

12. A method according to Claim 11, wherein said autonomously replicating sequence is a *Kluyveromyces* autonomously replicating sequence (KARS).

13. A method according to Claim 10, wherein said selection marker is resistance to G418.

~~14. Use of *Kluyveromyces* as a host for the transformation~~ and expression of foreign genes and the secretion of the polypeptide encoded by said gene, or secretion of part of said polypeptide.

15. A transformed *Kluyveromyces* host cell comprising an expression cassette which comprises, in the direction of transcription, a transcriptional initiation regulatory region functional in said host cell, a signal sequence functional in said host cell joined in reading frame with a DNA sequence encoding a polypeptide of interest, and a transcriptional termination regulatory region functional in said host cell.

16. A cell according to Claim 15, wherein said signal ~~sequence is heterologous to said host cell or to said poly-~~

14. A method for the transformation and expression of a foreign gene and the secretion of the expressed polypeptide by said gene or part thereof comprising transferring and expressing said foreign gene in Kluyveromyces as a host.

15. A transformed Kluyveromyces host cell comprising an expression cassette which comprises, in the direction of transcription, a transcriptional initiation regulatory region functional in said host cell, a signal sequence for secretion functional in said host cell joined in reading frame with a DNA sequence encoding a polypeptide of interest, and a transcriptional termination regulatory region functional in said host cell.

16. A cell according to Claim 15, wherein said signal sequence for secretion is heterologous to said host cell or to said poly-

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peptide of interest or to said host cell and to said polypeptide of interest.

17. A cell according to Claim 16, wherein said signal sequence for secretion is the α-factor signal sequence form *Saccharomyces cerevisiae*.

18. A cell according to Claim 16, wherein said signal sequence for secretion is the amyloglucosidase signal sequence from *Aspergillus awamori*.

19. A cell according to any one of Claims 15 to 18, wherein said polypeptide of interest is chymosin, or a precursor thereof, t-PA or HSA.

20. A cell according to any one of Claims 15 to 19, wherein joined to said expression cassette is at least one of a selection marker, a replication system for autonomous replication of said DNA sequence, or a transformation efficiency enhancing sequence.

21. A cell according to Claim 20, wherein said replication system is an autonomously replicating sequence (ARS).

22. A cell according to Claim 21, wherein said autonomously replicating sequence is a *Kluyveromyces* autonomously replicating sequence (KARS).

23. A cell according to any one of Claims 20 to 22, wherein said selection marker is resistance to G418.

~~24. A DNA construct for use in a *Kluyveromyces* host cell comprising:~~

~~in the direction of transcription, a transcriptional initiation regulatory region functional in said host cell; a signal sequence functional in said host joined in reading frame with a DNA sequence encoding a polypeptide of interest; and a transcriptional termination regulatory region~~

24. An expression cassette when used in a Kluyveromyces host cell comprising:

in the direction of transcription, a transcriptional initiation regulatory region functional in said host cell; a signal sequence for secretion functional in said host joined in reading frame with a DNA sequence encoding a polypeptide of interest; and a transcriptional termination regulatory region functional in said host cell.

25. An expression cassette according to Claim 24, wherein said signal sequence is heterologous to said host cell or to said polypeptide of interest, or to said host cell and said polypeptide of interest.

26. An expression cassette according to Claim 24 or 25 further comprising:

at least one of a selection marker, a replication system for autonomous replication of said DNA construct, or a transformation efficiency enhancing sequence.

27. An expression cassette according to any one of Claims 24 to 26, wherein a DNA fragment encoding the signal sequence for secretion and the DNA sequence encoding the polypeptide of interest is fused in reading frame to a gene expressed in Kluyveromyces.

28. An expression cassette according to Claim 27, wherein said DNA sequence is fused to a second DNA sequence encoding at least four amino acids of the N-terminus of the lactase gene.

29. Plasmids pKS105, pGB901, pGBTPA1 and pGBHSA3 as hereinbefore described.

30. A method according to any one of Claims 1 to 13, wherein said polypeptide of interest or part thereof is isolated from the culture medium.

31. A method according to Claim 1 substantially as hereinbefore described with reference to any one of the examples.

32. A cell according to Claim 15 substantially as hereinbefore described with reference to any one of the examples.

33. An expression cassette according to Claim 24 substantially as hereinbefore described with reference to any one of the examples and accompanying figures 1 to 19.

DATED: 12 May 1992

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GIST-BROCADES N.V.

David B Fitzpatrick

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FIGURE 1

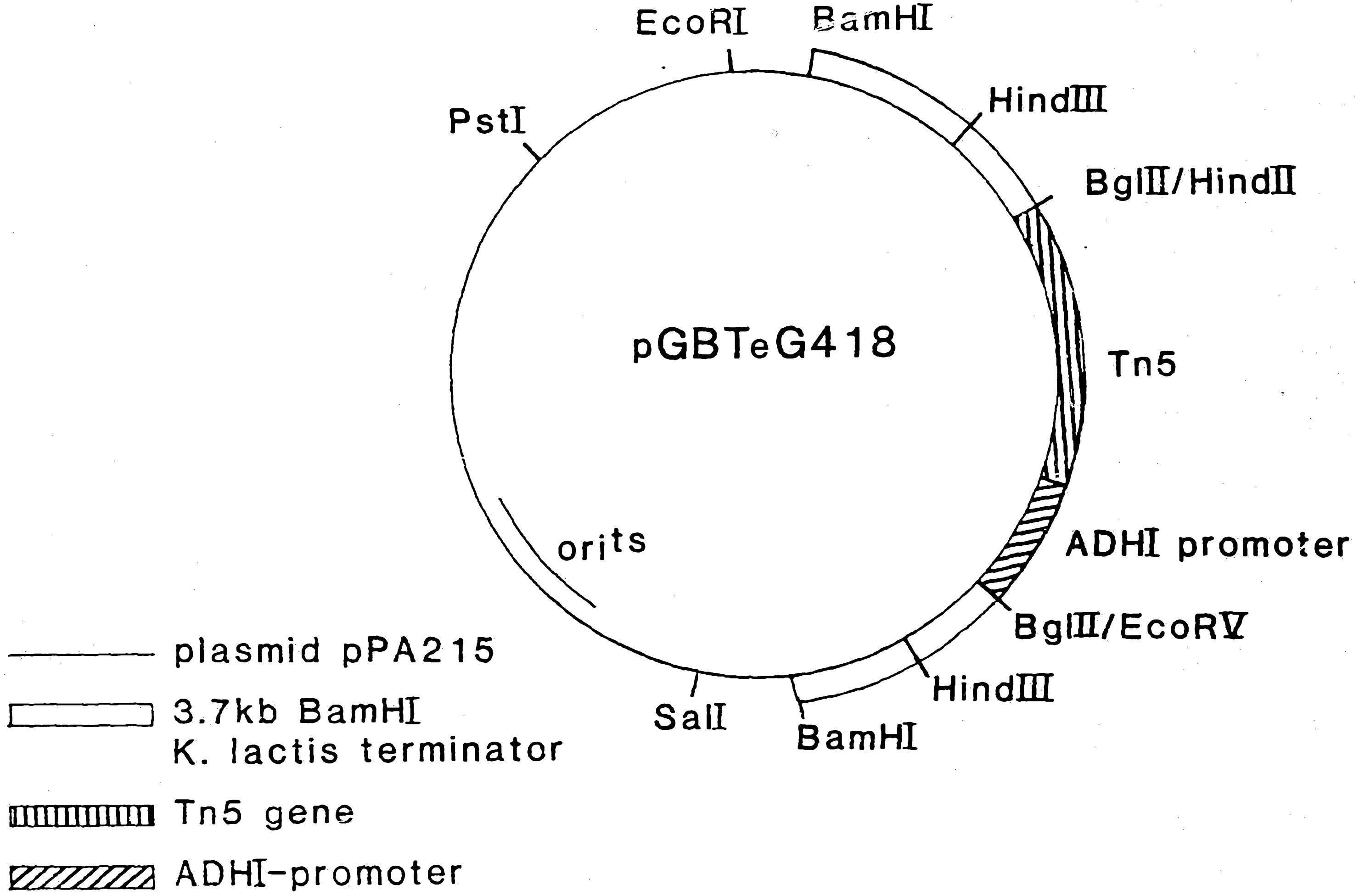

pGBTeG418
EcoRI
BamHI
HindIII
BglII/HindII
Tn5
ADHI promoter
BglII/EcoRV
HindIII
BamHI
SalI
orits
PstI
plasmid pPA215
3.7kb BamHI K. lactis terminator
Tn5 gene
ADHI-promoter

FIGURE 2

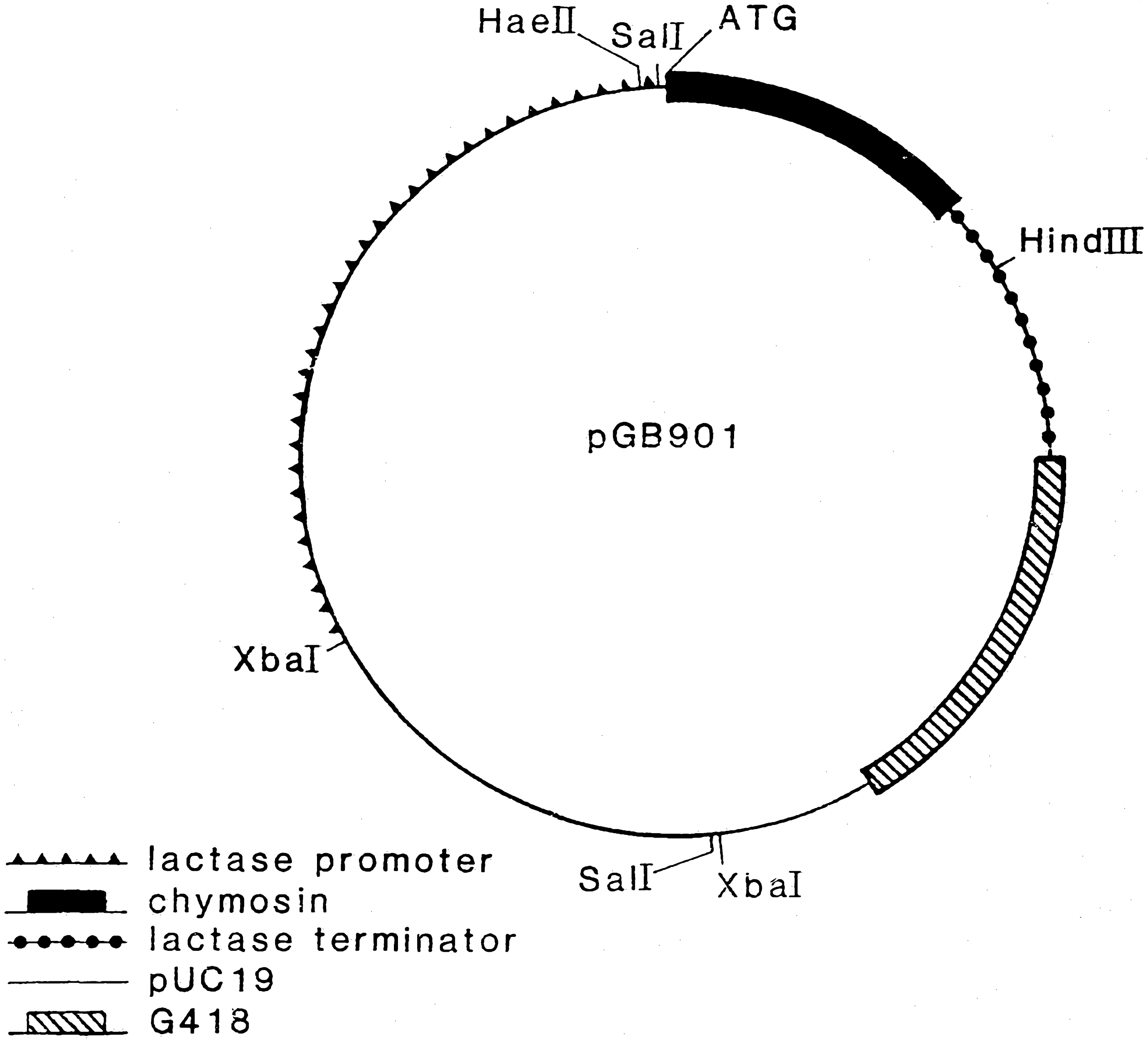

HaeII
SalI
ATG
HindIII
pGB901
XbaI
SalI
XbaI
lactase promoter
chymosin
lactase terminator
pUC19
G418

FIGURE 3

Oligonucleotides used for the synthesis of the amyloglucosidase leader and protein sequence of the putative leader.

A

(1) 5' TCGATATGTCTTTCAGATCCCTACTAGCTCTATCCG 3'

(2) 5' GTCTAGTTTGTACTGGTCTAGCTAACGTTATCTCCAAGAGAG 3'

(3) 5' TCGACTCTCTTGGAGATAACGTTAGCTAGACCAGTACAAACTAGACCGGATAG
AGCTAGTAGGGATCTGAAAGACATA 3'

B

Met Ser Phe Arg Ser Leu Leu Ala Leu Ser Gly Leu Val Cys Thr
Gly Leu Ala Asn Val Ile Ser Lys Arg

FIGURE 4

Oligonucleotides used for the synthesis of the synthetic leader and protein sequence of the putative leader.

A

(1) 5' TCGAATCTAATCTAAGTTTTAATTACAAAATGGCT 3'

(2) 5' TTCAGATCCTTGTTGGCTTTGTCCGGTTTGTCCTGTGGTGCTTTGGCTGCTCAAG 3'

(3) 5' TCGACTTGAGCAGCCAAAGCACCACAGGACAAACC 3'

(4) 5' GGACAAAGCCAACAAGGATCTGAAAGCCATTTTGTAATTAAAACTTAGATTAGAT 3'

B

Met Ala Phe Arg Ser Leu Leu Ala Leu Ser Gly Leu
Ser Cys Gly Ala Leu Ala Ala Glu

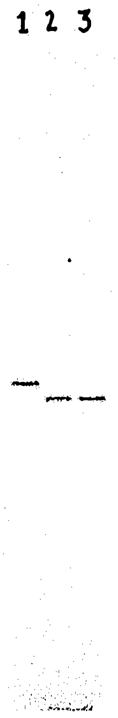


FIGURE 5

Immunoblot showing prochymosin secreted by the AG leader sequence. The experiment was performed as described in Example 5B. The supernatant from a culture of _K_. _lactis_ transformed with pGB905 was than treated with acid (pH 2) for 2 hours or 6 hours.

Lane 1: no treatment;
Lane 2: 2 hour treatment;
Lane 3: 6 hour treatment.

```
  1 GGATCCCCAGCTTAGTTCATAGGTCCATTCTCTTAGCGCAACTACAGAGAACAGGGGCACAA
    CCTAGGGGTCGAATCAAGTATCCAGGTAAGAGAATCGCGTTGATGTCTCTTGTCCCCGTGTT
    ^
    1 BAMHI,

 63   ACAGGCAAAAAACGGGCACAACCTCAATGGAGTGATGCAACCTGCCTGGAGTAAATGATG
      TGTCCGTTTTTTGCCCGTGTTGGAGTTACCTCACTACGTTGGACGGACCTCATTTACTAC

123   ACACAAGGCAATTGACCCACGCATGTATCTATCTCATTTTCTTACACCTTCTATTACCTT
      TGTGTTCCGTTAACTGGGTGCGTACATAGATAGAGTAAAAGAATGTGGAAGATAATGGAA

183   CTGCTCTCTCTGATTTGGAAAAAGCTGAAAAAAAAGGTTGAAACCAGTTCCCTGAAATTA
      GACGAGAGAGACTAAACCTTTTTCGACTTTTTTTTCCAACTTTGGTCAAGGGACTTTAAT
                                                             ^
      236 XMNI,

243   TTCCCCTACTTGACTAATAAGTATATAAAGACGGTAGGTATTGATTGTAATTCTGTAAAT
      AAGGGGATGAACTGATTATTCATATATTTCTGCCATCCATAACTAACATTAAGACATTTA

303   CTATTTCTTAAACTTCTTAAATTCTACTTTTATAGTTAGTCTTTTTTTTAGTTTTAAAAC
      GATAAAGAATTTGAAGAATTTAAGATGAAAATATCAATCAGAAAAAAAATCAAAATTTTG
                                                            ^
      355 AHA3,

                                                MetArgPheProSerIle
363   ACCAAGAACTTAGTTTCGAATAAACACACATAAACAAACACCATGAGATTTCCTTCAATT
      TGGTTCTTGAATCAAAGCTTATTTGTGTGTATTTGTTTGTGGTACTCTAAAGGAAGTTAA
                     ^
      377 ASU2,

      PheThrAlaValLeuPheAlaAlaSerSerAlaLeuAlaAlaProValAsnThrThrThr
423   TTTACTGCAGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACA
      AAATGACGTCAAAATAAGCGTCGTAGGAGGCGTAATCGACGAGGTCAGTTGTGATGTTGT
          ^
      427 PSTI,

      GluAspGluThrAlaGlnIleProAlaGluAlaValIleGlyTyrLeuAspLeuGluGly
483   GAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTTAGATTTAGAAGGG
      CTTCTACTTTGCCGTGTTTAAGGCCGACTTCGACAGTAGCCAATGAATCTAAATCTTCCC

      AspPheAspValAlaValLeuProPheSerAsnSerThrAsnAsnGlyLeuLeuPheIle
543   GATTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATA
      CTAAAGCTACAACGACAAAACGGTAAAAGGTTGTCGTGTTTATTGCCCAATAACAAATAT

      AsnThrThrIleAlaSerIleAlaAlaLysGluGluGlyValSerLeuAspLysArgAla
603   AATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTCTAGATAAAAGAGCT
      TTATGATGATAACGGTCGTAACGACGATTTCTTCTTCCCCATAGAGATCTATTTTCTCGA
                                                    ^
      647 XBAI,
```

GAP/Alpha-Factor/Prochymosin Insert in pDM100PC

FIGURE 6 (Sheet 1 of 5)

```
       GluIleThrArgIleProLeuTyrLysGlyLysSerLeuArgLysAlaLeuLysGluHis
 663   GAGATCACCAGGATCCCTCTGTACAAAGGCAAGTCTCTGAGGAAGGCGCTGAAGGAGCAT
       CTCTAGTGGTCCTAGGGAGACATGTTTCCGTTCAGAGACTCCTTCCGCGACTTCCTCGTA
                  ^

       673 BAMHI,

       GlyLeuLeuGluAspPheLeuGlnLysGlnGlnTyrGlyIleSerSerLysTyrSerGly
 723   GGGCTTCTGGAGGACTTCCTGCAGAAACAGCAGTATGGCATCAGCAGCAAGTACTCCGGC
       CCCGAAGACCTCCTGAAGGACGTCTTTGTCGTCATACCGTAGTCGTCGTTCATGAGGCCG
                          ^                               ^

       741 PSTI, 772 SCAI,

       PheGlyGluValAlaSerValProLeuThrAsnTyrProAspSerGlnTyrPheGlyLys
 783   TTCGGGGAGGTGGCCAGCGTCCCCCTGACCAACTACCCGGACAGTCAGTACTTTGGGAAG
       AAGCCCCTCCACCGGTCGCAGGGGGACTGGTTGATGGGCCTGTCAGTCATGAAACCCTTC
                  ^                                    ^          ^

       793 BALI, 829 SCAI, 841 BGL2,

       IleTyrLeuGlyThrProProGlnGluPheThrValLeuPheAspThrGlySerSerAsp
 843   ATCTACCTCGGGACCCCGCCCCAGGAGTTCACCGTGCTGTTTGACACTGGCTCCTCTGAC
       TAGATGGAGCCCTGGGGCGGGGTCCTCAAGTGGCACGACAAACTGTGACCGAGGAGACTG

       PheTrpValProSerMetTyrCysLysSerAsnAlaCysLysAsnHisGlnArgPheAsp
 903   TTCTGGGTACCCTCTATGTACTGCAAGAGCAATGCCTGCAAAAACCACCAGCGCTTCGAC
       AAGACCCATGGGAGATACATGACGTTCTCGTTACGGACGTTTTTGGTGGTCGCGAAGCTG
            ^

       908 KPNI,

       ProArgLysSerSerThrPheGlnAsnLeuGlyLysProLeuSerIleHisTyrGlyThr
 963   CCGAGAAAGTCGTCCACCTTCCAGAACCTGGGCAAGCCCCTGTCTATCCACTACGGGACA
       GGCTCTTTCAGCAGGTGGAAGGTCTTGGACCCGTTCGGGGACAGATAGGTGATGCCCTGT
                            ^^

       983 PFLM1, 984 ALWN1,

       GlySerMetGlnGlyIleLeuGlyTyrAspThrValThrValSerAsnIleValAspIle
1023   GGCAGCATGCAGGGCATCCTGGGCTATGACACCGTCACTGTCTCCAACATTGTGGACATC
       CCGTCGTACGTCCCGTAGGACCCGATACTGTGGCAGTGACAGAGGTTGTAACACCTGTAG
           ^                       ^               ^

       1027 SPHI, 1050 TTH3I, 1066 BSTXI,

       GlnGlnThrValGlyLeuSerThrAspGluProGlyAspValPheThrTyrAlaGluPhe
1083   CAGCAGACAGTAGGCCTGAGCACCGACGAGCCCGGGGACGTCTTCACCTATGCCGAATTC
       GTCGTCTGTCATCCGGACTCGTGGCTGCTCGGGCCCCTGCAGAAGTGGATACGGCTTAAG
                  ^                   ^     ^                  ^

       1094 STUI, 1113 SMAI XMAI, 1119 AAT2, 1137 ECORI,

       AspGlyIleLeuGlyMetAlaTyrProSerLeuAlaSerGluTyrSerThrSerValPhe
1143   GACGGGATCCTGGGGATGGCCTACCCCTCGCTCGCCTCAGAGTACTCGACATCCGTGTTT
       CTGCCCTAGGACCCCTACCGGATGGGGAGCGAGCGGAGTCTCATGAGCTGTAGGCACAAA
           ^                                    ^

       1147 BAMHI, 1183 SCAI,
```

FIGURE 6 (Sheet 2 of 5)

```
       AspAsnMetMetAsnArgHisLeuValAlaGlnAspLeuPheSerValTyrMetAspArg
1203   GACAACATGATGAACAGGCACCTGGTGGCCCAAGACCTGTTCTCGGTTTACATGGACAGG
       CTGTTGTACTACTTGTCCGTGGACCACCGGGTTCTGGACAAGAGCCAAATGTACCTGTCC
                          ^

       1221 DRA3,

       AsnGlyGlnGluSerMetLeuThrLeuGlyProIleAspProSerTyrTyrThrGlySer
1263   AATGGCCAGGAGAGCATGCTCACGCTGGGGCCCATCGACCCGTCCTACTACACAGGGTCC
       TTACCGGTCCTCTCGTACGAGTGCGACCCCGGGTAGCTGGGCAGGATGATGTGTCCCAGG
         ^           ^             ^

       1265 BALI, 1276 SPHI, 1290 APAI,

       LeuHisTrpValProValThrValGlnGlnTyrTrpGlnPheThrValAspSerValThr
1323   CTGCATTGGGTGCCCGTGACAGTGCAGCAGTACTGGCAGTTCACTGTGGACAGTGTCACC
       GACGTAACCCACGGGCACTGTCACGTCGTCATGACCGTCAAGTGACACCTGTCACAGTGG
                                    ^                    ^        ^

       1351 SCAI, 1371 TTH3I, 1380 HGIE2,

       IleSerGlyValValValProCysGluGlyGlyCysGlnAlaIleLeuAspThrGlyThr
1383   ATCAGCGGTGTGGTTGTGCCCTGTGAGGGTGGCTGTCAGGCCATCCTGGACACGGGCACC
       TAGTCGCCACACCAACACGGGACACTCCCACCGACAGTCCGGTAGGACCTGTGCCCGTGG

       SerLysLeuValGlyProSerSerAspIleLeuAsnIleGlnGlnProIleGlyAlaThr
1443   TCCAAGCTGGTCGGGCCCAGCAGCGACATCCTCAACATCCAGCAGCCCATTGGAGCCACA
       AGGTTCGACCAGCCCGGGTCGTCGCTGTAGGAGTTGTAGGTCGTCGGGTAACCTCGGTGT
                   ^

       1455 APAI,

       GlnAsnGlnTyrGlyAspPheAspIleAspCysAspAsnLeuSerTyrMetProThrVal
1503   CAGAACCAGTACGGTGATTTTGACATCGACTGCGACAACCTGAGCTACATGCCCACTGTG
       GTCTTGGTCATGCCACTAAAACTGTAGCTGACGCTGTTGGACTCGATGTACGGGTGACAC

       ValPheGluIleAsnGlyLysIleTyrProLeuThrProSerAlaTyrThrSerGlnAsp
1563   GTCTTTGAGATCAATGGCAAAATCTACCCACTGACCCCCTCCGCCTATACCAGCCAGGAC
       CAGAAACTCTAGTTACCGTTTTAGATGGGTGACTGGGGGAGGCGGATATGGTCGGTCCTG

       GlnGlyPheCysThrSerGlyPheGlnSerGluAsnHisSerGlnLysTrpIleLeuGly
1623   CAGGGCTTCTGTACCAGTGGCTTCCAGAGTGAAAATCATTCCCAGAAATGGATCCTGGGG
       GTCCCGAAGACATGGTCACCGAAGGTCTCACTTTTAGTAAGGGTCTTTACCTAGGACCCC
                                                          ^

       1672 BAMHI,

       AspValPheIleArgGluTyrTyrSerValPheAspArgProAsnAsnLeuValGlyLeu
1683   GATGTTTTCATCCGAGAGTATTACAGCGTCTTTGACAGGCCCAACAACCTCGTGGGGCTG
       CTACAAAAGTAGGCTCTCATAATGTCGCAGAAACTGTCCGGGTTGTTGGAGCACCCCGAC
                                                                  ^

       1741 BALI,

       AlaLysAlaIleOP
1743   GCCAAAGCCATCTGATCTCGACTTGGTTGAACACGTTGCCAAGGCTTAAGTGAATTTACT
       CGGTTTCGGTAGACTAGAGCTGAACCAACTTGTGCAACGGTTCCGAATTCACTTAAATGA
                                                     ^            ^

       1787 AFL2, 1802 AHA3,
```

FIGURE 6 (Sheet 3 of 5)

```
1803   TTAAAGTCTTGCATTTAAATAAATTTTCTTTTTATAGCTTTATGACTTAGTTTCAATTTA
       AATTTCAGAACGTAAATTTATTTAAAAGAAAAATATCGAAATACTGAATCAAAGTTAAAT
                    ^
       1816 AHA3,

1863   TATACTATTTTAATGACATTTTCGATTCATTGATTGAAAGCTTTGTGTTTTTTCTTGATG
       ATATGATAAAATTACTGTAAAAGCTAAGTAACTAACTTTCGAAACACAAAAAAGAACTAC
                                            ^
       1900 HIND3,

1923   CGCTATTGCATTGTTCTTGTCTTTTTCGCCACATGTAATATCTGTAGTAGATACCTGATA
       GCGATAACGTAACAAGAACAGAAAAAGCGGTGTACATTATAGACATCATCTATGGACTAT

1983   CATTGTGGATGCTGAGTGAAATTTTAGTTAATAATGGAGGCGCTCTTAATAATTTTGGGG
       GTAACACCTACGACTCACTTTAAAATCAATTATTACCTCCGCGAGAATTATTAAAACCCC

2043   ATATTGGCTTTTTTTTTTAAAGTTTACAAATGAATTTTTTCCGCCAGGATAACGATTCTG
       TATAACCGAAAAAAAAAATTTCAAATGTTTACTTAAAAAAGGCGGTCCTATTGCTAAGAC
                      ^               ^
       2058 AHA3, 2074 XMNI,

2103   AAGTTACTCTTAGCGTTCCTATCGGTACAGCCATCAAATCATGCCTATAAATCATGCCTA
       TTCAATGAGAATCGCAAGGATAGCCATGTCGGTAGTTTAGTACGGATATTTAGTACGGAT

2163   TATTTGCGTGCAGTCAGTATCATCTACATGAAAAAAACTCCCGCAATTTCTTATAGAATA
       ATAAACGCACGTCAGTCATAGTAGATGTACTTTTTTTGAGGGCGTTAAAGAATATCTTAT

2223   CGTTGAAAATTAAATGTACGCGCCAAGATAAGATAACATATATCTAGCTAGATGCAGTAA
       GCAACTTTTAATTTACATGCGCGGTTCTATTCTATTGTATATAGATCGATCTACGTCATT

2283   TATACACAGATTCCCGCGGACGTGGGAAGGAAAAAATTAGATAACAAAATCTGAGTGATA
       ATATGTGTCTAAGGGCGCCTGCACCCTTCCTTTTTTAATCTATTGTTTTAGACTCACTAT
                    ^
       2296 SAC2,

2343   TGGAAATTCCGCTGTATAGCTCATATCTTTCCCTTCAACACCAGAAATGTAAAAATCTTG
       ACCTTTAAGGCGACATATCGAGTATAGAAAGGGAAGTTGTGGTCTTTACATTTTTAGAAC

2403   TTACGAAGGATCTTTTTGCTAATGTTTCTCGCTCAATCCTCATTTCTTCCCTACGAAGAG
       AATGCTTCCTAGAAAAACGATTACAAAGAGCGAGTTAGGAGTAAAGAAGGGATGCTTCTC

2463   TCAAATCTACTTGTTTTCTGCCGGTATCAAGATCCATATCTTCTAGTTTCACCATCAAAG
       AGTTTAGATGAACAAAAGACGGCCATAGTTCTAGGTATAGAAGATCAAAGTGGTAGTTTC

2523   TCCAATTTCTAGTATACAGTTTATGTCCCAACGTAACAGACAATCAAAATTGGAAAGGAT
       AGGTTAAAGATCATATGTCAAATACAGGGTTGCATTGTCTGTTAGTTTTAACCTTTCCTA
                  ^
       2534 SNAI XCA1,

2583   AAGTATCCTTCAAAGAATGATTCTGCGCTGGCTCCTGAACCGCCTAATGGAACAGAGAA
       TTCATAGGAAGTTTCTTACTAAGACGCGACCGAGGACTTGGCGGATTACCCTTGTCTCTT
```

FIGURE 6 (Sheet 4 of 5)

```
1803    TTAAAGTCTTGCATTTAAATAAATTTTCTTTTTATAGCTTTATGACTTAGTTTCAATTTA
        AATTTCAGAACGTAAATTTATTTAAAAGAAAAATATCGAAATACTGAATCAAAGTTAAAT
                     ^

        1816 AHA3,

1863    TATACTATTTTAATGACATTTTCGATTCATTGATTGAAAGCTTTGTGTTTTTTCTTGATG
        ATATGATAAAATTACTGTAAAAGCTAAGTAACTAACTTTCGAAACACAAAAAAGAACTAC
                                              ^

        1900 HIND3,

1923    CGCTATTGCATTGTTCTTGTCTTTTTCGCCACATGTAATATCTGTAGTAGATACCTGATA
        GCGATAACGTAACAAGAACAGAAAAAGCGGTGTACATTATAGACATCATCTATGGACTAT

1983    CATTGTGGATGCTGAGTGAAATTTTAGTTAATAATGGAGGCGCTCTTAATAATTTTGGGG
        GTAACACCTACGACTCACTTTAAAATCAATTATTACCTCCGCGAGAATTATTAAAACCCC

2043    ATATTGGCTTTTTTTTTTAAAGTTTACAAATGAATTTTTTCCGCCAGGATAACGATTCTG
        TATAACCGAAAAAAAAAATTTCAAATGTTTACTTAAAAAAGGCGGTCCTATTGCTAAGAC
                        ^               ^

        2058 AHA3, 2074 XMNI,

2103    AAGTTACTCTTAGCGTTCCTATCGGTACAGCCATCAAATCATGCCTATAAATCATGCCTA
        TTCAATGAGAATCGCAAGGATAGCCATGTCGGTAGTTTAGTACGGATATTTAGTACGGAT

2163    TATTTGCGTGCAGTCAGTATCATCTACATGAAAAAAACTCCCGCAATTTCTTATAGAATA
        ATAAACGCACGTCAGTCATAGTAGATGTACTTTTTTTGAGGGCGTTAAAGAATATCTTAT

2223    CGTTGAAAATTAAATGTACGCGCCAAGATAAGATAACATATATCTAGCTAGATGCAGTAA
        GCAACTTTTAATTTACATGCGCGGTTCTATTCTATTGTATATAGATCGATCTACGTCATT

2283    TATACACAGATTCCCGCGGACGTGGGAAGGAAAAAATTAGATAACAAAATCTGAGTGATA
        ATATGTGTCTAAGGGCGCCTGCACCCTTCCTTTTTTAATCTATTGTTTTAGACTCACTAT
                      ^

        2296 SAC2,

2343    TGGAAATTCCGCTGTATAGCTCATATCTTTCCCTTCAACACCAGAAATGTAAAAATCTTG
        ACCTTTAAGGCGACATATCGAGTATAGAAAGGGAAGTTGTGGTCTTTACATTTTTAGAAC

2403    TTACGAAGGATCTTTTTGCTAATGTTTCTCGCTCAATCCTCATTTCTTCCCTACGAAGAG
        AATGCTTCCTAGAAAAACGATTACAAAGAGCGAGTTAGGAGTAAAGAAGGGATGCTTCTC

2463    TCAAATCTACTTGTTTTCTGCCGGTATCAAGATCCATATCTTCTAGTTTCACCATCAAAG
        AGTTTAGATGAACAAAAGACGGCCATAGTTCTAGGTATAGAAGATCAAAGTGGTAGTTTC

2523    TCCAATTTCTAGTATACAGTTTATGTCCCAACGTAACAGACAATCAAAATTGGAAAGGAT
        AGGTTAAAGATCATATGTCAAATACAGGGTTGCATTGTCTGTTAGTTTTAACCTTTCCTA
                    ^

        2534 SNAI XCA1,

2583    AAGTATCCTTCAAAGAATGATTCTGCGCTGGCTCCTGAACCGCCTAATGGGAACAGAGAA
        TTCATAGGAAGTTTCTTACTAAGACGCGACCGAGGACTTGGCGGATTACCCTTGTCTCTT
```

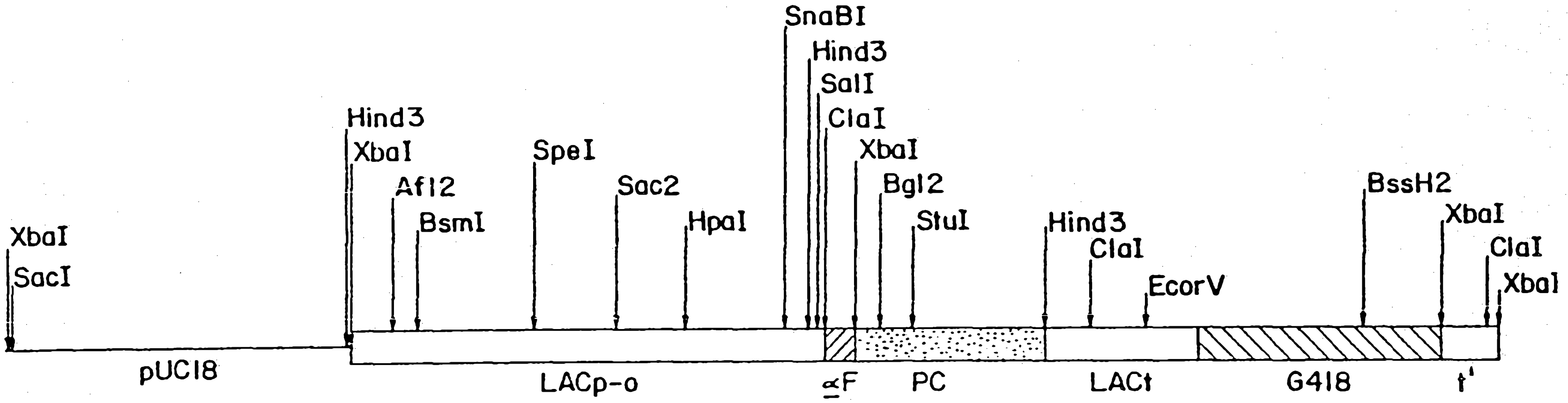
FIGURE 6 (Sheet 4 of 5)

```
2643      GTCCAAAACGATGCTATAAGAACCAGAAATAAAACGATAAAACCATACCAGGATCGGTCG
          CAGGTTTTGCTACGATATTCTTGGTCTTTATTTTGCTATTTTGGTATGGTCCTAGCCAGC
                                                          ^         ^
          2689 HGIE2, 2699 SALI,

2703      ACTTTGTTCCCACTGTACTTTTAGCTCGTACAAAATACAATATACTTTTCATTTCTCCGT
          TGAAACAAGGGTGACATGAAAATCGAGCATGTTTTATGTTATATGAAAAGTAAAGAGGCA

2763      AAACAACATGTTTTCCCATGTAATATCCTTTTCTATTTTTCGTTCCGTTACCAACTTTAC
          TTTGTTGTACAAAAGGGTACATTATAGGAAAAGATAAAAAGCAAGGCAATGGTTGAAATG

2823      ACATACTTTATATAGCTATTCACTTCTATACACTAAAAAACTAAGACAATTTTAATTTTG
          TGTATGAAATATATCGATAAGTGAAGATATGTGATTTTTTGATTCTGTTAAAATTAAAAC

2883      CTGCCTGCCATATTTCAATTTGTTATAAATTCCTATAATTTATCCTATTAGTAGCTAAAA
          GACGGACGGTATAAAGTTAAACAATATTTAAGGATATTAAATAGGATAATCATCGATTTT

2943      AAAGATGAATGTGAATCGAATCCTAAGAGAATTCGGATCC
          TTTCTACTTACACTTAGCTTAGGATTCTCTTAAGCCTAGG
                                        ^     ^
          2971 ECORI, 2977 BAMHI,
```

FIGURE 6 (Sheet 5 of 5)

8 7 92 20148

FIGURE 7



pKS105
11775 bp

```
Trp Ser Trp Ile Thr Leu
TGG QZV TGG ATL ACN YTI
```

Code for ambiguous bases:

Q: A or T
R: A or G
S: A or C
Y: T or C
Z: C or G

L: A,T, or C
N: any base
V: T or C if preceded by AG; else any base
I: A or G if preceded by TT; else any base
P: A or G if preceded by AG; else any base

```
            3'                     5'
POOL #1      ACC AGG ACC TAG TGG GA
                   A       A   A  A
                   T       T   T
                   C           C

            3'                     5'
POOL #2      ACC TCG ACC TAG TGG GA
                   A       A   A  A
                           T   T
                               C
```

FIGURE 8

```
  1 CTGCAGTTTGTGAATCGTAAGACAGTGACATTTTTAGAGGTTGTTATCTGTTTAAGACGAAA
    GACGTCAAACACTTAGCATTCTGTCACTGTAAAAATCTCCAACAATAGACAAATTCTGCTTT
    ^
    1 PSTI

 63   TGGTTTGCTGTTCAAGCTCACTGGGTGATCGGATTTCGGGAAAATTCATATATAAAGGAC
      ACCAAACGACAAGTTCGAGTGACCCACTAGCCTAAAGCCCTTTTAAGTATATATTTCCTG
                        ^
      81 DRA3

123   CCTTGATTGATAGGATGTTATGGTATTGTTCTAAGTTTGTTTCAATAGTAATTTCAATAT
      GGAACTAACTATCCTACAATACCATAACAAGATTCAAACAAAGTTATCATTAAAGTTATA

183   AGTATATTAGAACAAGCAAACCAGAGCATCTAAAGCCCAACTCGTCTGATCTTTTTCTGT
      TCATATAATCTTGTTCGTTTGGTCTCGTAGATTTCGGGTTGAGCAGACTAGAAAAAGACA

243   CTTTATTATCCTGAACTTCACCTTAATCTAAATTATACAAACCCAACTATCCAATTTGAA
      GAAATAATAGGACTTGAAGTGGAATTAGATTTAATATGTTTGGGTTGATAGGTTAAACTT

                  MetLysPheSerThrIleLeuAlaAlaSerThrAlaLeuIleSerVal
303   CTATCCAATATTATGAAATTCTCTACTATATTAGCCGCATCTACTGCTTTAATTTCCGTT
      GATAGGTTATAATACTTTAAGAGATGATATAATCGGCGTAGATGACGAAATTAAAGGCAA
            ^
      309 SSPI

      ValMetAlaAlaProValSerThrGluThrAspIleAspAspLeuProIleSerValPro
363   GTTATGGCTGCTCCAGTTTCTACCGAAACTGACATCGACGATCTTCCAATTTCGGTTCCA
      CAATACCGACGAGGTCAAAGATGGCTTTGACTGTAGCTGCTAGAAGGTTAAAGCCAAGGT

      GluGluAlaLeuIleGlyPheIleAspLeuThrGlyAspGluValSerLeuLeuProVal
423   GAAGAAGCCTTGATTGGATTCATTGACTTAACCGGGGATGAAGTTTCCTTGTTGCCTGTT
      CTTCTTCGGAACTAACCTAAGTAACTGAATTGGCCCCTACTTCAAAGGAACAACGGACAA

      AsnAsnGlyThrHisThrGlyIleLeuPheLeuAsnThrThrIleAlaGluAlaAlaPhe
483   AATAACGGAACCCACACTGGTATTCTATTCTTAAACACCACCATCGCTGAAGCTGCTTTC
      TTATTGCCTTGGGTGTGACCATAAGATAAGAATTTGTGGTGGTAGCGACTTCGACGAAAG
               ^
      492 HGIE2

      AlaAspLysAspAspLeuLysLysArgGluAlaAspAlaSerProTrpSerTrpIleThr
543   GCTGACAAGGATGATTTGAAGAAAAGAGAAGCCGATGCTTCCCCATGGAGTTGGATTACT
      CGACTGTTCCTACTAAACTTCTTTTCTCTTCGGCTACGAAGGGGTACCTCAACCTAATGA
                                                ^
      585 BSTXI, NCOI

      LeuArgProGlyGlnProIlePheLysArgGluAlaAsnAlaAspAlaAsnAlaGluAla
603   CTAAGACCTGGTCAACCAATCTTTAAAAGAGAAGCCAACGCTGACGCTAATGCTGAAGCA
      GATTCTGGACCAGTTGGTTAGAAATTTTCTCTTCGGTTGCGACTGCGATTACGACTTCGT
          ^                ^
      607 TTH3I, 624 AHA3
```

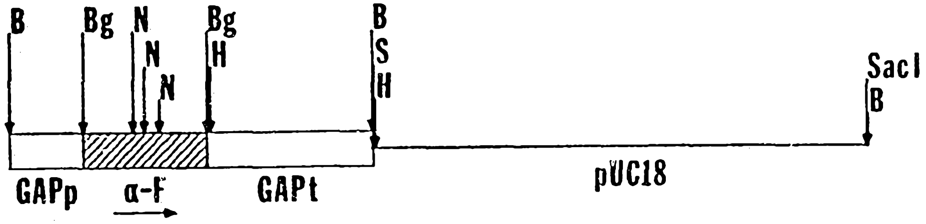
FIGURE 9 (Sheet 1 of 2)

```
     SerProTrpSerTrpIleThrLeuArgProGlyGlnProIlePheLysArgGluAlaAsn
663  TCCCCATGGAGCTGGATTACTCTAAGACCTGGTCAACCGATCTTTAAGAGAGAGGCTAAT
     AGGGGTACCTCGACCTAATGAGATTCTGGACCAGTTGGCTAGAAATTCTCTCTCCGATTA
        ^                     ^

     666 BSTXI, NCOI, 688 TTH3I

     AlaAspAlaAsnAlaAspAlaSerProTrpSerTrpIleThrLeuArgProGlyGlnPro
723  GCTGATGCCAATGCAGATGCCTCCCCATGGAGCTGGATCACTCTAAGACCTGGTCAACCA
     CGACTACGGTTACGTCTACGGAGGGGTACCTCGACCTAGTGAGATTCTGGACCAGTTGGT
                             ^                     ^

     747 BSTXI, NCOI, 769 TTH3I

     IlePheLysArgGluAlaAsnProGluAlaGluAlaAspAlaLysProSerAlaTrpSer
783  ATCTTTAAAAGAGAAGCCAACCCTGAGGCCGAGGCTGATGCCAAACCTAGTGCTTGGAGT
     TAGAAATTTTCTCTTCGGTTGGGACTCCGGCTCCGACTACGGTTTGGATCACGAACCTCA
        ^                 ^

     786 AHA3, 804 MST2

     TrpIleThrLeuArgProGlyGlnProIlePheOP
843  TGGATTACATTAAGACCTGGCCAACCAATTTTCTGAATTAGAAGGAAATTGACTTTTTGA
     ACCTAATGTAATTCTGGACCGGTTGGTTAAAAGACTTAATCTTCCTTTAACTGAAAAACT
                      ^

     860 BALI

903  CTCGTTTTCCAATGCGTCTATCTAATTTCTTCCAAAAGACAATACCCATCTTCCTTATAC
     GAGCAAAAGGTTACGCAGATAGATTAAAGAAGGTTTTCTGTTATGGGTAGAAGGAATATG

963  TTTTTTTATTTATCCAAACGAATTC
     AAAAAAATAAATAGGTTTGCTTAAG
                          ^

     982 ECORI
```

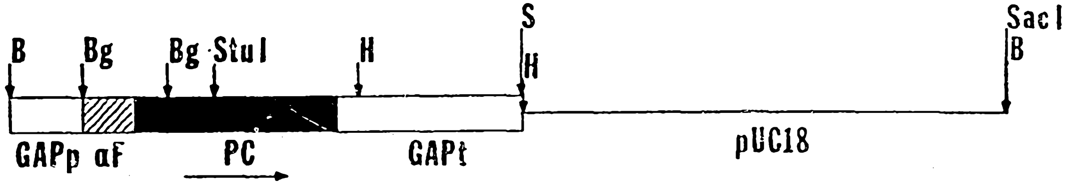
FIGURE 9 (Sheet 2 of 2)

28 7 88 20148

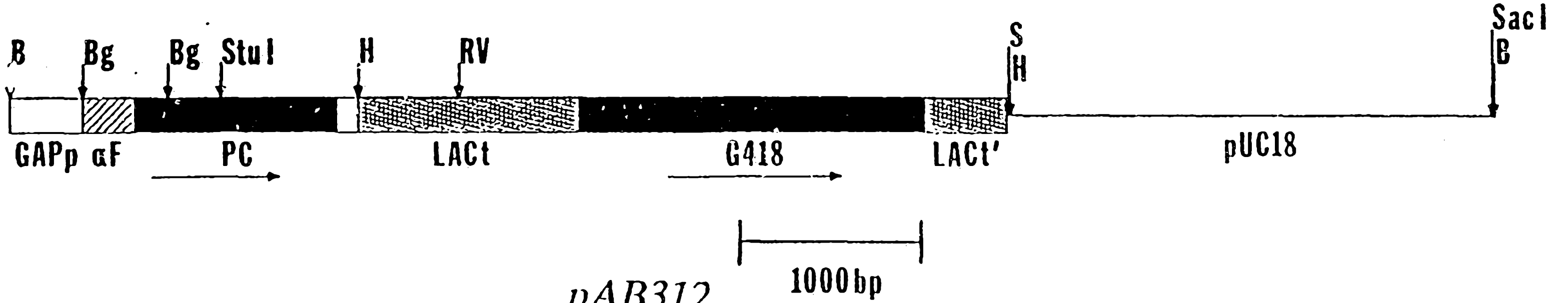
FIGURE 10



pAB307



pAB309



pAB312

```
        <---α-Factor Leader--><----(Spacer)-Prochymosin------------>

pAB309, AspAspLeuLysLysArgGluAlaAspAlaSerHisHisMetAlaGluIleThrArgIle
pAB312  GATGATTTGAAGAAAAGAGAAGCCGATGCTTCCCATCATATGGCTGAGATCACCAGGATC
        CTACTAAACTTCTTTTCTCTTCGGCTACGAAGGGTAGTATACCGACTCTAGTGGTCCTAG


        AspAspLeuLysLysArgAlaGluIleThrArgIleProLeuTyrLysGly
pAB313  GATGATTTGAAGAAAAGAGCTGAGATCACCAGGATCCCTCTGTACAAAGGC
        CTACTAAACTTCTTTTCTCGACTCTAGTGGTCCTAGGGAGACATGTTTCCG


        AspAspLeuLysLysArgAlaGluIleThrArgIleProLeuTyrLysGly
pAB314  GATGATTTGAAGAAGCGCGCTGAGATCACCAGGATCCCTCTGTACAAAGGC
        CTACTAAACTTCTTCGCGCGACTCTAGTGGTCCTAGGGAGACATGTTTCCG
                      ------
                      BssHII
```

FIGURE 11 Sequences Around the Junctions in Alpha-Factor Leader/Prochymosin Fusions

pAB309 BamHI/SalI Insert in pUC18

```
  1  GGATCCCCAGCTTAGTTCATAGGTCCATTCTCTTAGCGCAACTACAGAGAACAGGGGCAC
     CCTAGGGGTCGAATCAAGTATCCAGGTAAGAGAATCGCGTTGATGTCTCTTGTCCCCGTG
     ^

     1 BAMHI,

 61  AAACAGGCAAAAAACGGGCACAACCTCAATGGAGTGATGCAACCTGCCTGGAGTAAATGA
     TTTGTCCGTTTTTTGCCCGTGTTGGAGTTACCTCACTACGTTGGACGGACCTCATTTACT

121  TGACACAAGGCAATTGACCCACGCATGTATCTATCTCATTTTCTTACACCTTCTATTACC
     ACTGTGTTCCGTTAACTGGGTGCGTACATAGATAGAGTAAAAGAATGTGGAAGATAATGG

181  TTCTGCTCTCTCTGATTTGGAAAAAGCTGAAAAAAAAGGTTGAAACCAGTTCCCTGAAAT
     AAGACGAGAGAGACTAAACCTTTTTCGACTTTTTTTTCCAACTTTGGTCAAGGGACTTTA
                                                            ^

     236 XMNI,

241  TATTCCCCTACTTGACTAATAAGTATATAAAGACGGTAGGTATTGATTGTAATTCTGTAA
     ATAAGGGGATGAACTGATTATTCATATATTTCTGCCATCCATAACTAACATTAAGACATT

301  ATCTATTTCTTAAACTTCTTAAATTCTACTTTTATAGTTAGTCTTTTTTTTAGTTTTAAA
     TAGATAAAGAATTTGAAGAATTTAAGATGAAAATATCAATCAGAAAAAAAATCAAAATTT
                                                           ^

     355 AHA3,

                                                     MetLysPheSer
361  ACACCAAGAACTTAGTTTCGAATAAACACACATAAACAGATCTTCATTATGAAATTCTCT
     TGTGGTTCTTGAATCAAAGCTTATTTGTGTGTATTTGTCTAGAAGTAATACTTTAAGAGA
                     ^                    ^

     377 ASU2, 398 BGL2,

     ThrIleLeuAlaAlaSerThrAlaLeuIleSerValValMetAlaAlaProValSerThr
421  ACTATATTAGCCGCATCTACTGCTTTAATTTCCGTTGTTATGGCTGCTCCAGTTTCTACC
     TGATATAATCGGCGTAGATGACGAAATTAAAGGCAACAATACCGACGAGGTCAAAGATGG

     GluThrAspIleAspAspLeuProIleSerValProGluGluAlaLeuIleGlyPheIle
481  GAAACTGACATCGACGATCTTCCAATTTCGGTTCCAGAAGAAGCCTTGATTGGATTCATT
     CTTTGACTGTAGCTGCTAGAAGGTTAAAGCCAAGGTCTTCTTCGGAACTAACCTAAGTAA

     AspLeuThrGlyAspGluValSerLeuLeuProValAsnAsnGlyThrHisThrGlyIle
541  GACTTAACCGGGGATGAAGTTTCCTTGTTGCCTGTTAATAACGGAACCCACACTGGTATT
     CTGAATTGGCCCCTACTTCAAAGGAACAACGGACAATTATTGCCTTGGGTGTGACCATAA
                                                  ^

     586 HGIE2,
```

FIGURE 12 (Sheet 1 of 5)

```
      LeuPheLeuAsnThrThrIleAlaGluAlaAlaPheAlaAspLysAspAspLeuLysLys
601   CTATTCTTAAACACCACCATCGCTGAAGCTGCTTTCGCTGACAAGGATGATTTGAAGAAA
      GATAAGAATTTGTGGTGGTAGCGACTTCGACGAAAGCGACTGTTCCTACTAAACTTCTTT

      ArgGluAlaAspAlaSerHisHisMetAlaGluIleThrArgIleProLeuTyrLysGly
661   AGAGAAGCCGATGCTTCCCATCATATGGCTGAGATCACCAGGATCCCTCTGTACAAAGGC
      TCTCTTCGGCTACGAAGGGTAGTATACCGACTCTAGTGGTCCTAGGGAGACATGTTTCCG
                           ^                  ^

      682 NDEI, 701 BAMHI,

      LysSerLeuArgLysAlaLeuLysGluHisGlyLeuLeuGluAspPheLeuGlnLysGln
721   AAGTCTCTGAGGAAGGCGCTGAAGGAGCATGGGCTTCTGGAGGACTTCCTGCAGAAACAG
      TTCAGAGACTCCTTCCGCGACTTCCTCGTACCCGAAGACCTCCTGAAGGACGTCTTTGTC
                                                      ^

      769 PSTI,

      GlnTyrGlyIleSerSerLysTyrSerGlyPheGlyGluValAlaSerValProLeuThr
781   CAGTATGGCATCAGCAGCAAGTACTCCGGCTTCGGGGAGGTGGCCAGCGTCCCCCTGACC
      GTCATACCGTAGTCGTCGTTCATGAGGCCGAAGCCCCTCCACCGGTCGCAGGGGGACTGG
                         ^                    ^                 ^

      800 SCAI, 821 BALI, 839 BSTXI,

      AsnTyrLeuAspSerGlnTyrPheGlyLysIleTyrLeuGlyThrProProGlnGluPhe
841   AACTACCTGGACAGTCAGTACTTTGGGAAGATCTACCTCGGGACCCCGCCCCAGGAGTTC
      TTGATGGACCTGTCAGTCATGAAACCCTTCTAGATGGAGCCCTGGGGCGGGGTCCTCAAG
                      ^           ^

      857 SCAI, 869 BGL2,

      ThrValLeuPheAspThrGlySerSerAspPheTrpValProSerIleTyrCysLysSer
901   ACCGTGCTGTTTGACACTGGCTCCTCTGACTTCTGGGTACCCTCTATCTACTGCAAGAGC
      TGGCACGACAAACTGTGACCGAGGAGACTGAAGACCCATGGGAGATAGATGACGTTCTCG
                                         ^

      936 KPNI,

      AsnAlaCysLysAsnHisGlnArgPheAspProArgLysSerSerThrPheGlnAsnLeu
961   AATGCCTGCAAAAACCACCAGCGCTTCGACCCGAGAAAGTCGTCCACCTTCCAGAACCTG
      TTACGGACGTTTTTGGTGGTCGCGAAGCTGGGCTCTTTCAGCAGGTGGAAGGTCTTGGAC

      GlyLysProLeuSerIleHisTyrGlyThrGlySerMetGlnGlyIleLeuGlyTyrAsp
1021  GGCAAGCCCCTGTCTATCCACTACGGGACAGGCAGCATGCAGGGCATCCTGGGCTATGAC
      CCGTTCGGGGACAGATAGGTGATGCCCTGTCCGTCGTACGTCCCGTAGGACCCGATACTG
                                        ^                      ^

      1055 SPHI, 1078 TTH3I,

      ThrValThrValSerAsnIleValAspIleGlnGlnThrValGlyLeuSerThrGlnGlu
1081  ACCGTCACTGTCTCCAACATTGTGGACATCCAGCAGACAGTAGGCCTGAGCACCCAGGAG
      TGGCAGTGACAGAGGTTGTAACACCTGTAGGTCGTCTGTCATCCGGACTCGTGGGTCCTC
                   ^                           ^

      1094 BSTXI, 1122 STUI,
```

FIGURE 12 (Sheet 2 of 5)

```
      ProGlyAspValPheThrTyrAlaGluPheAspGlyIleLeuGlyMetAlaTyrProSer
1141  CCCGGGGACGTCTTCACCTATGCCGAATTCGACGGGATCCTGGGGATGGCCTACCCCTCG
      GGGCCCCTGCAGAAGTGGATACGGCTTAAGCTGCCCTAGGACCCCTACCGGATGGGGAGC
      ^     ^                 ^         ^

      1141 SMAI, 1147 AAT2, 1165 ECORI, 1175 BAMHI,

      LeuAlaSerGluTyrSerIleProValPheAspAsnMetMetAsnArgHisLeuValAla
1201  CTCGCCTCAGAGTACTCGATACCCGTGTTTGACAACATGATGAACAGGCACCTGGTGGCC
      GAGCGGAGTCTCATGAGCTATGGGCACAAACTGTTGTACTACTTGTCCGTGGACCACCGG
                ^

      1211 SCAI,

      GlnAspLeuPheSerValTyrMetAspArgAsnGlyGlnGluSerMetLeuThrLeuGly
1261  CAAGACCTGTTCTCGGTTTACATGGACAGGAATGGCCAGGAGAGCATGCTCACGCTGGGG
      GTTCTGGACAAGAGCCAAATGTACCTGTCCTTACCGGTCCTCTCGTACGAGTGCGACCCC
                                      ^          ^

      1293 BALI, 1304 SPHI,

      AlaIleAspProSerTyrTyrThrGlySerLeuHisTrpValProValThrValGlnGln
1321  GCCATCGACCCGTCCTACTACACAGGGTCCCTGCATTGGGTGCCCGTGACAGTGCAGCAG
      CGGTAGCTGGGCAGGATGATGTGTCCCAGGGACGTAACCCACGGGCACTGTCACGTCGTC
                                                                ^

      1379 SCAI,

      TyrTrpGlnPheThrValAspSerValThrIleSerGlyValValValAlaCysGluGly
1381  TACTGGCAGTTCACTGTGGACAGTGTCACCATCAGCGGTGTGGTTGTGGCCTGTGAGGGT
      ATGACCGTCAAGTGACACCTGTCACAGTGGTAGTCGCCACACCAACACCGGACACTCCCA
                        ^        ^

      1399 TTH3I, 1408 HGIE2,

      GlyCysGlnAlaIleLeuAspThrGlyThrSerLysLeuValGlyProSerSerAspIle
1441  GGCTGTCAGGCCATCCTGGACACGGGCACCTCCAAGCTGGTCGGGCCCAGCAGCGACATC
      CCGACAGTCCGGTAGGACCTGTGCCCGTGGAGGTTCGACCAGCCCGGGTCGTCGCTGTAG
                                                ^

      1483 APAI,

      LeuAsnIleGlnGlnAlaIleGlyAlaThrGlnAsnGlnTyrGlyGluPheAspIleAsp
1501  CTCAACATCCAGCAGGCCATTGGAGCCACACAGAACCAGTACGGTGAGTTTGACATCGAC
      GAGTTGTAGGTCGTCCGGTAACCTCGGTGTGTCTTGGTCATGCCACTCAAACTGTAGCTG

      CysAspAsnLeuSerTyrMetProThrValValPheGluIleAsnGlyLysMetTyrPro
1561  TGCGACAACCTGAGCTACATGCCCACTGTGGTCTTTGAGATCAATGGCAAAATGTACCCA
      ACGCTGTTGGACTCGATGTACGGGTGACACCAGAAACTCTAGTTACCGTTTTACATGGGT

      LeuThrProSerAlaTyrThrSerGlnAspGlnGlyPheCysThrSerGlyPheGlnSer
1621  CTGACCCCCTCCGCCTATACCAGCCAGGACCAGGGCTTCTGTACCAGTGGCTTCCAGAGT
      GACTGGGGGAGGCGGATATGGTCGGTCCTGGTCCCGAAGACATGGTCACCGAAGGTCTCA
```

FIGURE 12 (Sheet 3 of 5)

```
       GluAsnHisSerGlnLysTrpIleLeuGlyAspValPheIleArgGluTyrTyrSerVal
1681   GAAAATCATTCCCAGAAATGGATCCTGGGGATGTTTTCATCCGAGAGTATTACAGCGTC
       CTTTTAGTAAGGGTCTTTACCTAGGACCCCCTACAAAAGTAGGCTCTCATAATGTCGCAG
                          ^

       1700 BAMHI,

       PheAspArgAlaAsnAsnLeuValGlyLeuAlaLysAlaIleOP
1741   TTTGACAGGGCCAACAACCTCGTGGGGCTGGCCAAAGCCATCTGATCTCGACTTGGTTGA
       AAACTGTCCCGGTTGTTGGAGCACCCCGACCGGTTTCGGTAGACTAGAGCTGAACCAACT
                                   ^

       1769 BALI,

1801   ACACGTTGCCAAGGCTTAAGTGAATTTACTTTAAAGTCTTGCATTTAAATAAATTTTCTT
       TGTGCAACGGTTCCGAATTCACTTAAATGAAATTTCAGAACGTAAATTTATTTAAAAGAA
                     ^              ^             ^

       1815 AFL2, 1830 AHA3, 1844 AHA3,

1861   TTTATAGCTTTATGACTTAGTTTCAATTTATATACTATTTTAATGACATTTTCGATTCAT
       AAATATCGAAATACTGAATCAAAGTTAAATATATGATAAAATTACTGTAAAAGCTAAGTA

1921   TGATTGAAAGCTTTGTGTTTTTTCTTGATGCGCTATTGCATTGTTCTTGTCTTTTTCGCC
       ACTAACTTTCGAAACACAAAAAAGAACTACGCGATAACGTAACAAGAACAGAAAAAGCGG
              ^

       1928 HIND3,

1981   ACATGTAATATCTGTAGTAGATACCTGATACATTGTGGATGCTGAGTGAAATTTTAGTTA
       TGTACATTATAGACATCATCTATGGACTATGTAACACCTACGACTCACTTTAAAATCAAT

2041   ATAATGGAGGCGCTCTTAATAATTTTGGGGATATTGGCTTTTTTTTTTAAAGTTTACAAA
       TATTACCTCCGCGAGAATTATTAAAACCCCTATAACCGAAAAAAAAAATTTCAAATGTTT
                                                    ^

       2086 AHA3,

2101   TGAATTTTTTCCGCCAGGATAACGATTCTGAAGTTACTCTTAGCGTTCCTATCGGTACAG
       ACTTAAAAAAGGCGGTCCTATTGCTAAGACTTCAATGAGAATCGCAAGGATAGCCATGTC
        ^

       2102 XMNI,

2161   CCATCAAATCATGCCTATAAATCATGCCTATATTTGCGTGCAGTCAGTATCATCTACATG
       GGTAGTTTAGTACGGATATTTAGTACGGATATAAACGCACGTCAGTCATAGTAGATGTAC

2221   AAAAAAACTCCCGCAATTTCTTATAGAATACGTTGAAAATTAAATGTACGCGCCAAGATA
       TTTTTTTGAGGGCGTTAAAGAATATCTTATGCAACTTTTAATTTACATGCGCGGTTCTAT

2281   AGATAACATATATCTAGCTAGATGCAGTAATATACACAGATTCCCGCGGACGTGGGAAGG
       TCTATTGTATATAGATCGATCTACGTCATTATATGTGTCTAAGGGCGCCTGCACCCTTCC
                                                  ^

       2324 SAC2,
```

FIGURE 12 (Sheet 4 of 5)

```
2341   AAAAAATTAGATAACAAAATCTGAGTGATATGGAAATTCCGCTGTATAGCTCATATCTTT
       TTTTTTAATCTATTGTTTTAGACTCACTATACCTTTAAGGCGACATATCGAGTATAGAAA

2401   CCCTTCAACACCAGAAATGTAAAAATCTTGTTACGAAGGATCTTTTTGCTAATGTTTCTC
       GGGAAGTTGTGGTCTTTACATTTTTAGAACAATGCTTCCTAGAAAAACGATTACAAAGAG

2461   GCTCAATCCTCATTTCTTCCCTACGAAGAGTCAAATCTACTTGTTTTCTGCCGGTATCAA
       CGAGTTAGGAGTAAAGAAGGGATGCTTCTCAGTTTAGATGAACAAAAGACGGCCATAGTT

2521   GATCCATATCTTCTAGTTTCACCATCAAAGTCCAATTTCTAGTATACAGTTTATGTCCCA
       CTAGGTATAGAAGATCAAAGTGGTAGTTTCAGGTTAAAGATCATATGTCAAATACAGGGT
                                                ^
       2562 SNAI,

2581   ACGTAACAGACAATCAAAATTGGAAAGGATAAGTATCCTTCAAAGAATGATTCTGCGCTG
       TGCATTGTCTGTTAGTTTTAACCTTTCCTATTCATAGGAAGTTTCTTACTAAGACGCGAC

2641   GCTCCTGAACCGCCTAATGGGAACAGAGAAGTCCAAAACGATGCTATAAGAACCAGAAAT
       CGAGGACTTGGCGGATTACCCTTGTCTCTTCAGGTTTTGCTACGATATTCTTGGTCTTTA

2701   AAAACGATAAAACCATACCAGGATCC
       TTTTGCTATTTTGGTATGGTCCTAGG
                           ^
       2721 BAMHI,
```

FIGURE 12 (Sheet 5 of 5)

Primer #1: Spacer Deletion

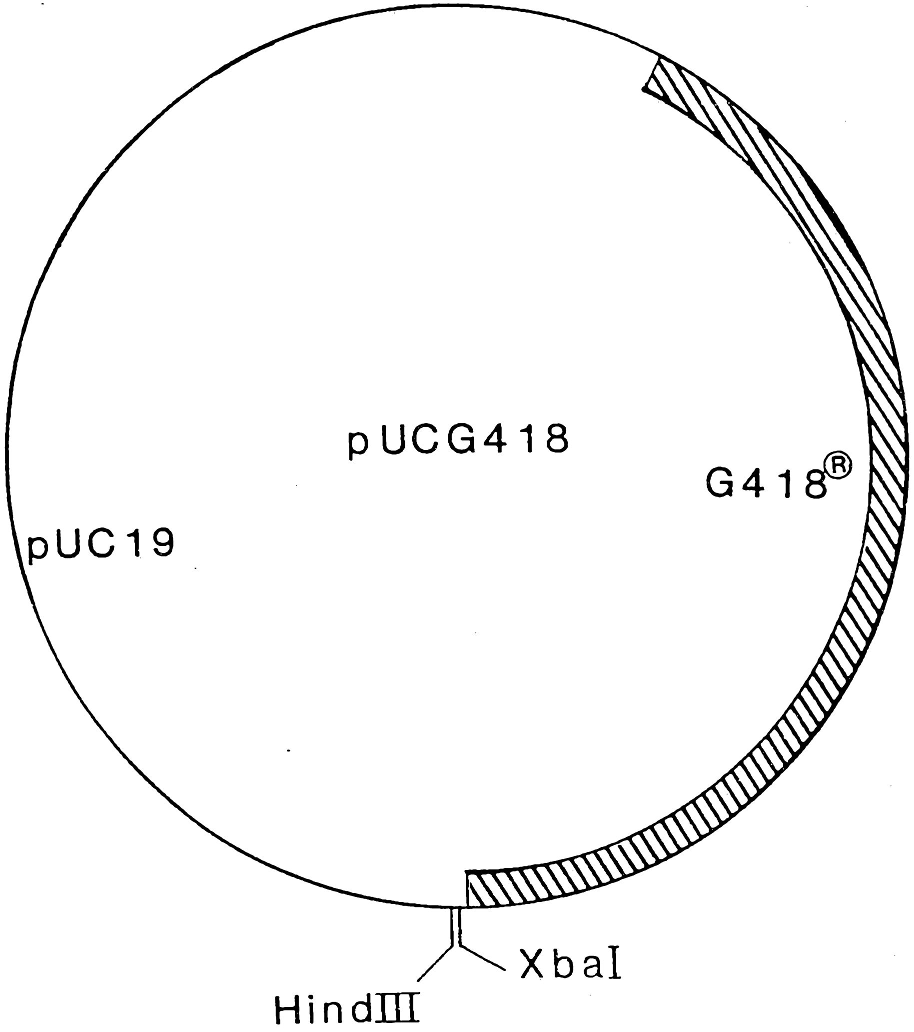
5' 3'
GATTTGAAGAAAAGAGCTGAGATCACCAGG

Primer #2: Spacer Deletion + BssHII Site

5' 3'
GATTTGAAGAAGCGCGCTGAGATCACCAGG
BssHII

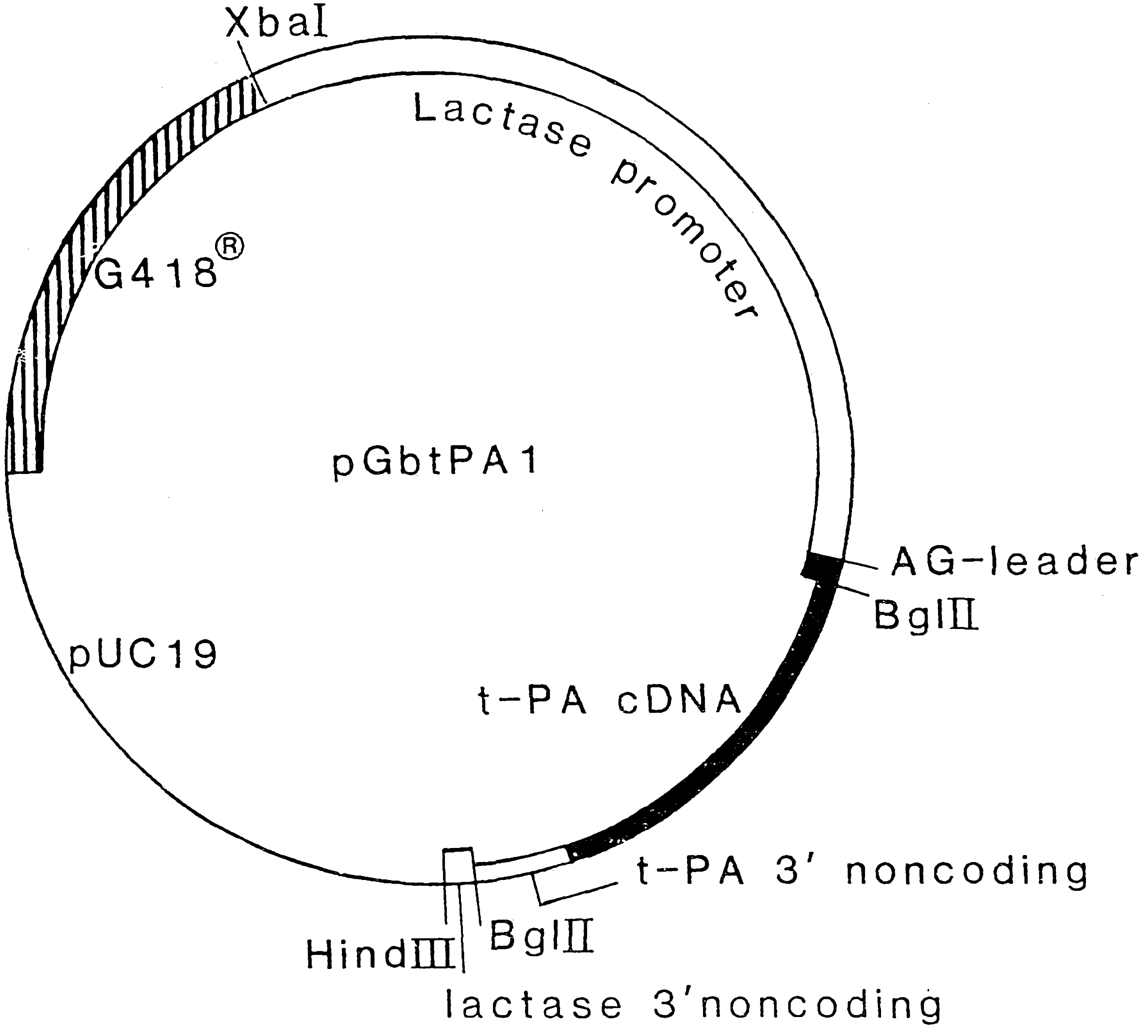
FIGURE 13 **Primers for Mutagenesis of the *K. lactis* α-Factor Leader**

FIGURE 14



28 7 88 20148

FIGURE 15



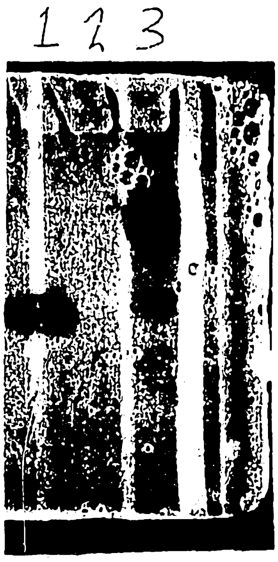
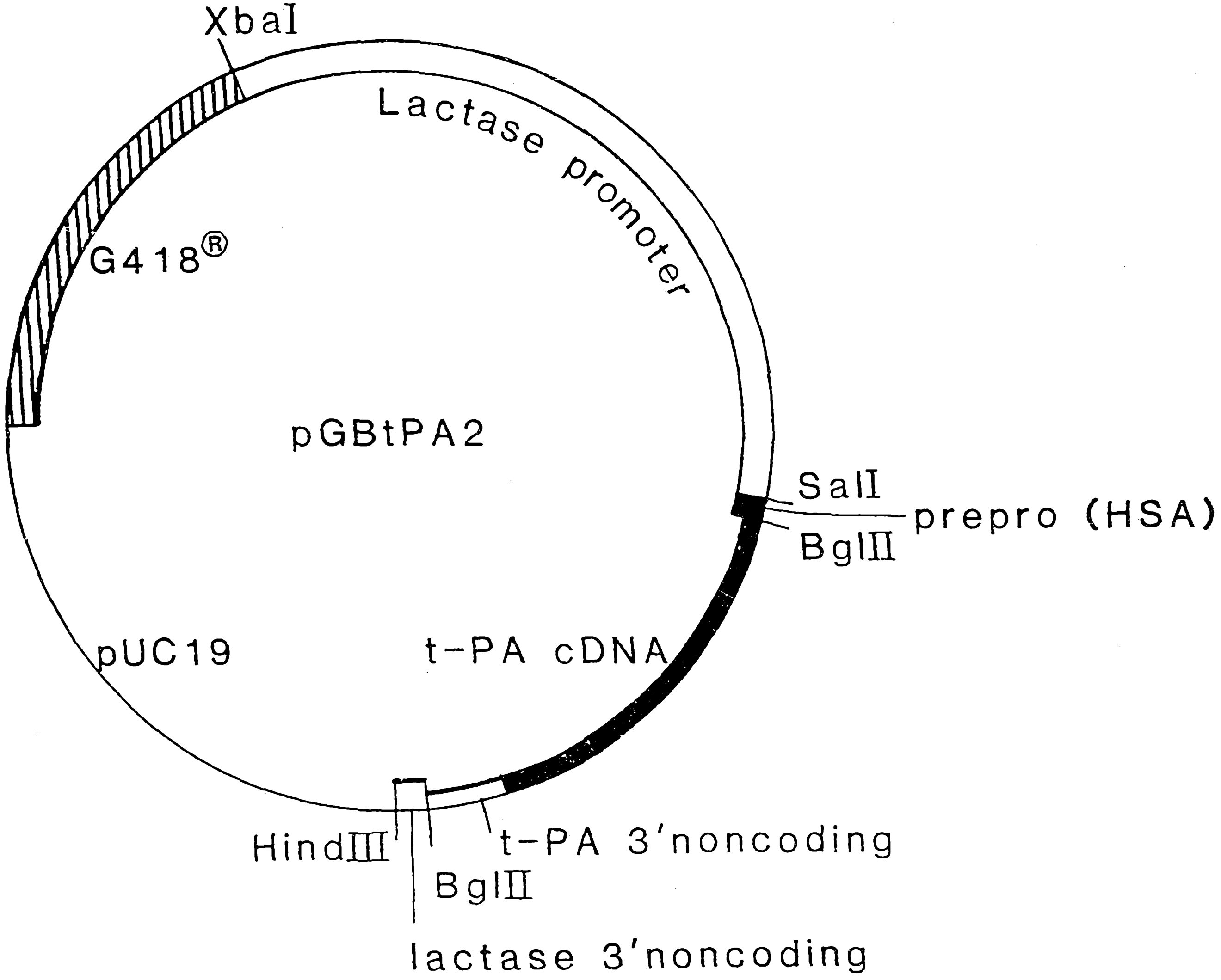


FIGURE 16

Polyacrylamide gel overlayered with a plasminogen/fibrin-agarose gel. Lane 1 contains 20 ng t-PA produced by melanoma cells (Kabi-Vitrum). In lane 2 a sample prepared from the supernatant of a CBS 2360 culture was applied and in lane 3 a sample prepared from the supernatant of a culture from CBS 2360 transformed with pGBtPA1.

FIGURE 17


XbaI
Lactase promoter
G418®
pGBtPA2
SalI
prepro (HSA)
BglII
pUC19
t-PA cDNA
HindIII
t-PA 3'noncoding
BglII
lactase 3'noncoding

28 7 88 20148

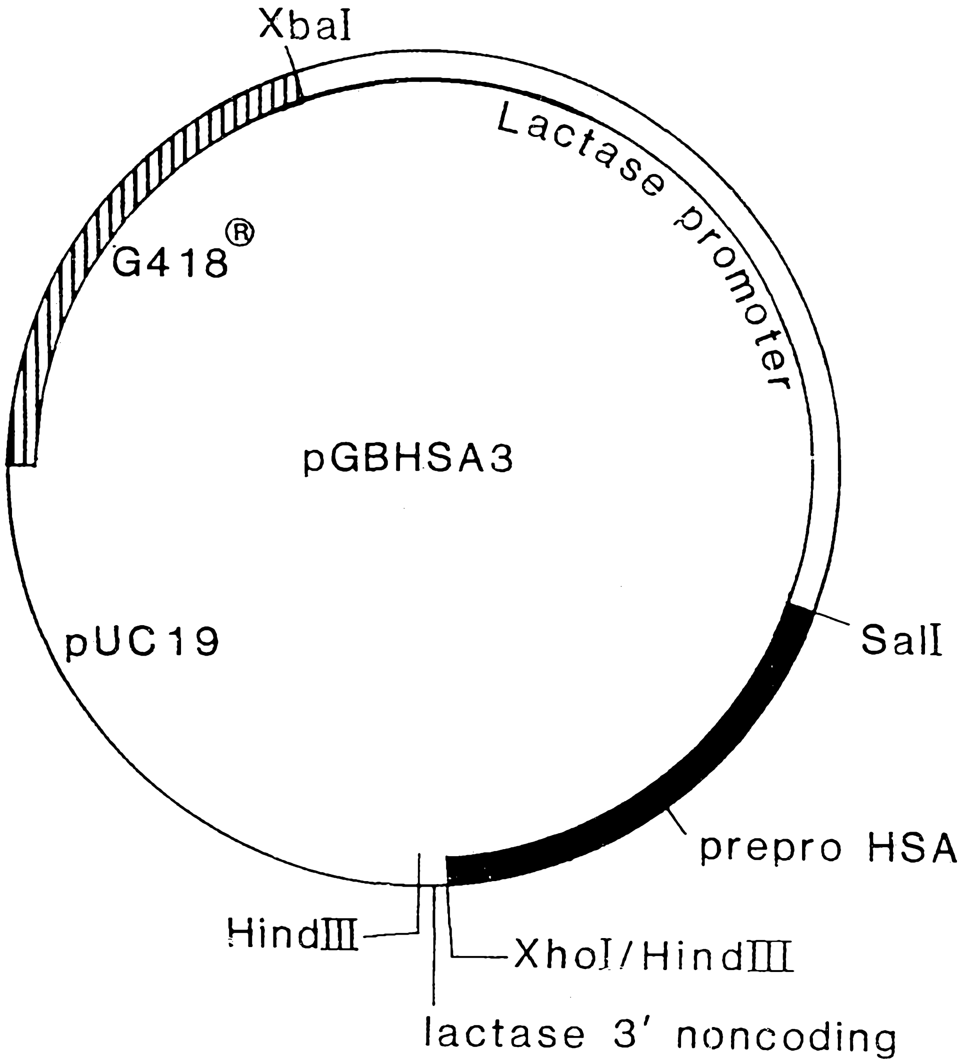


FIGURE 18

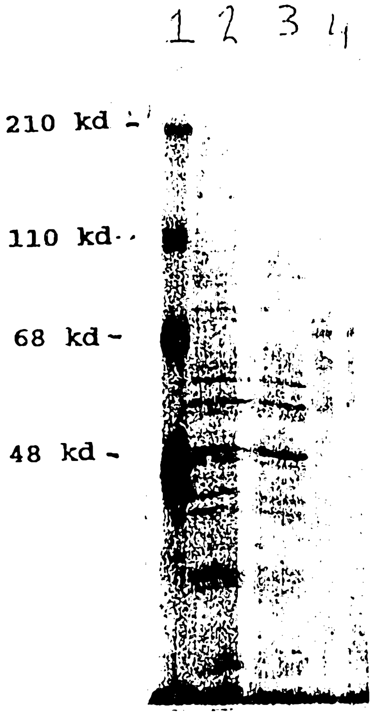


FIGURE 19

Analysis of the supernatant of cultures of CBS 2360 and CBS 2360 transformed with pGBHSA3 on a 10% polyacrylamide gel according to Leammli. Lane 1 contains marker proteins (the molecular weights are indicated), lane 2 and 3 samples from the supernatants of the control strain CBS 2360 and lane 4 a sample from the supernatant of one of the transformants.