

COMMONWEALTH OF AUSTRALIA

617057

PATENTS ACT 1952

APPLICATION FOR A STANDARD PATENT

Eli Lilly and Company, a corporation organised and existing under the laws of the State of Indiana, of Lilly Corporate Center, Indianapolis, Indiana, 46285, UNITED STATES OF AMERICA, hereby apply for the grant of a standard patent for an invention entitled:

Control of Aberrant Expression Vector Accumulation During Fermentation Procedures

which is described in the accompanying complete specification.

Details of basic application(s):-

<u>Basic Applic. No:</u>	<u>Country:</u>	<u>Application Date:</u>
371,579	US	26 June 1989

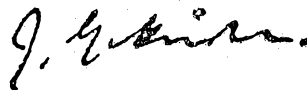
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DATED this TWENTY SECOND day of JUNE 1990

Eli Lilly and Company

By:



Registered Patent Attorney

TO: THE COMMISSIONER OF PATENTS
OUR REF: 131993
S&F CODE: 53710

SO15634 22/06/90

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COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

DECLARATION IN SUPPORT OF A
CONVENTION APPLICATION FOR A PATENT

In support of the Convention Application made for a patent for an invention entitled: CONTROL OF ABERRANT EXPRESSION VECTOR ACCUMULATION DURING FERMENTATION PROCEDURES I, Leroy Whitaker, Lilly Corporate Center, City of Indianapolis, State of Indiana 46285, United States of America do solemnly and sincerely declare as follows:

1. I am authorized by ELI LILLY AND COMPANY the applicant for the patent to make this declaration on its behalf.

2. The basic application as defined by Section 141 of the Act was/~~were~~ made in United States of America

on 26 June 1989

by William Lawrence Muth

3. William Lawrence Muth of
450 Leaning Tree Road, Greenwood, Indiana 46142
United States of America

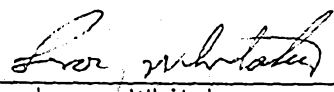
is ~~are~~ the actual inventor~~s~~ of the invention and the facts upon which the applicant is entitled to make the application are as follows:

The said applicant is the assignee of the actual inventor~~s~~.

4. The basic application(~~s~~) referred to in paragraph 2 of this Declaration was/~~were~~ the first application(~~s~~) made in a Convention country in respect of the invention(~~s~~) the subject of the said application.

DECLARED at Indianapolis, Indiana this 23rd day of May, 1990

ELI LILLY AND COMPANY

By 
Leroy Whitaker
Assistant Secretary and
General Patent Counsel

TO: THE COMMISSIONER OF PATENTS
AUSTRALIA

FORM 10

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

COMPLETE SPECIFICATION

(ORIGINAL) **617057**

FOR OFFICE USE:

Class Int Class

Complete Specification Lodged:
Accepted:
Published:

Priority:

Related Art:

Name and Address
of Applicant: Eli Lilly and Company
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Complete Specification for the invention entitled:

Control of Aberrant Expression Vector Accumulation During
Fermentation Procedures

The following statement is a full description of this invention, including the
best method of performing it known to me/us

CONTROL OF ABERRANT EXPRESSION VECTOR
ACCUMULATION DURING FERMENTATION PROCEDURES

5

Fermentation processes have been used for decades in the production of antibiotics and much of this science has proven useful for genetically engineered protein production. Unlike traditional fermentation procedures which involve the culturing of pristine microbes which produce and often secrete the molecule of interest, the genetically altered cells are often unreliable in the maintenance of the expression vector and subsequently in the production of the desired product.

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Extrachromosomal elements such as plasmids and other expression vectors are frequently lost when they offer no growth or survival benefit to the host cell. During fermentation processes, host cells which have deleted the extrachromosomal elements no longer suffer the metabolic drain of maintaining these expression vectors and are thus unimpeded in their rate of growth. In a short period of time, host cells which do not contain these extrachromosomal elements will overgrow those host cells whose growth is retarded by expression and accumulation of the product of interest. This process of negative selection toward unadvantageous genetic material has been documented for years in studies of drug-resistant bacteria. It is well established that removal of the antibiotic, towards which

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resistance is conferred by a plasmid encoded gene, gives rise to a population of bacteria with the drug-resistant plasmid maintained in only a small percentage of the bacteria.

5 Antibiotic resistance genes are often engineered into expression vectors to allow selection of transformants and transfectants and to provide selective pressure toward retention of the expression system during prolonged culturing. The selective pressure
10 exerted by the antibiotic ensures maintenance of the drug resistant phenotype, but does not preclude the appearance of plasmids which confer antibiotic resistance but lack the capacity to form the product of interest. Production of an extraneous protein is a
15 metabolic drain on the cells expressing and accumulating the polypeptide product of interest and results in a selection pressure towards cells which no longer form the product.

 The present invention comprises the addition
20 of an antibiotic or combination of antibiotics at bacteriostatic or bactericidal concentrations to the fermentation mixture at approximately the time product expression is induced. The addition of bacteriostatic or bactericidal concentrations of an antibiotic at
25 approximately the time of product induction will effect all cells; however, cells producing product are already impeded in cell division capacity and therefore the addition of antibiotic functions to prevent outgrowth of aberrant expression systems.

30 The use of antibiotics for purposes of this invention has not been previously disclosed. The prior

art has revealed that antibiotics are frequently used to eliminate plasmids and other extrachromosomal elements from host cells. Michel-Brian^c et.al., 1986, J. Antimicrob. Chemother. 18: 667-674 reported the use of
5 several 4-quinolone derivatives including cinoxacin and novobiocin to eliminate various drug resistant plasmids from members of Enterobacteriaceae. Numerous other references including: Trevors, 1986, FEMS Microbiol. Rev. 32: 149-157 ; Wolfson, et.al., 1982, J. Bacteriol.,
10 152: 338-344 ; and Weissner and Wiedemann, 1987, J. Antimicrob. Chemother., 18: 575-584 similarly teach the use of antibiotics for plasmid curing. The present invention differs significantly from the prior art use of antibiotics for expression vector elimination;
15 indeed, this would be contrary to the purpose of the present invention.

Until the present invention, no method was known for ensuring that the cells in a fermentation process were not overgrown by cells, which having lost the capacity for product expression, were able to grow
20 at accelerated rates.

For purposes of the present invention as disclosed and claimed herein, the following terms are as described below.

25 Host Cell - A cell suitable for transformation or transfection with an expression vector.

30 Expression System - A combination of host cell and expression vector capable of expressing a polypeptide product of interest.

Induction - Fermentation conditions appropriate to cause increased expression of the polypeptide product of interest.

5 Expression - Transcription and translation of the product of interest.

Fermentation - Aerobic or anaerobic conditions appropriate for the growth of microorganisms.

10

Bacteristatic - A concentration of an antibiotic which prevents microbial cell division.

15 Bactericidal - A concentration of an antibiotic which kills actively growing microbial cells.

Aberrant Expression Vector - An expression vector which due to deletion of its regulatory or coding regions no longer expresses the product of interest.

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Aberrant Expression System - A host cell containing an aberrant expression vector.

25 Fidelity - Ability of an expression vector to maintain product expression.

Bovine Growth Hormone Derivative - A polypeptide product having bioactivity similar to bovine growth hormone.

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Expression Vector - Any agent capable of autonomous replication or genomic integration, including but not limited to plasmids, comprising a DNA molecule into which one or more transcriptional and translational activating sequence(s), and a gene encoding a polypeptide of interest have been incorporated in such a manner as to allow expression of the gene encoding a polypeptide of interest.

The following figures are provided to promote an understanding of the invention.

Figure 1 - is a flowchart detailing the construction of plasmid pL32.

Figure 2 - is a restriction site and function map of plasmid pNM789.

Figure 3 details the construction of plasmid 120.

Figure 4 is a flowchart detailing the construction of plasmid pL110.

Figure 5 is a restriction site and function map of plasmid pCZR125.

Figure 6 illustrates the relationship between the induction of product expression and production of EK-bGH. Cinoxacin was added at approximately the six hour time point.

The present invention relates to a method for ensuring expression vector fidelity in fermentation processes utilizing recombinant expression vectors. When expression systems are induced for product expression, a negative selective pressure is exerted upon all cells which produce an extraneous protein. The selective pressure toward a lack of product expression

often results in an accumulation of deletant expression vectors. The prior art usage of antibiotics in fermentations does not function to control deletant expression vectors except as related to maintenance of drug-resistant phenotypes.

5 The present invention comprises the addition of an antibiotic or combination of antibiotics at bacteristatic or bactericidal concentrations to the fermentation mixture at approximately the time product expression is induced. The addition of bacteristatic or bactericidal concentrations of an antibiotic at approximately the time of product induction will effect all cells; however, cells producing product are already impeded in cell division capacity and therefore the addition of an antibiotic functions to prevent outgrowth of aberrant expression systems.

10 The use of an antibiotic resistance gene on an expression system is known in the art and is desirable, in that the appropriate resistance gene provides a selective pressure towards retention of the expression vector. Examples 1, 2, 3, and 4, while illustrating the present invention also embody the use of an antibiotic resistance selection system which utilizes tetracycline in the fermentation medium and the presence of a tetracycline resistance gene on the expression vector. It is critical to note that the prior art use of antibiotics ensures merely the retention of an antibiotic resistant phenotype.

15 The expression system used to illustrate the method of the present invention is a thermoinducible expression system providing high level expression of a

bovine growth hormone (bGH) derivative. Construction of the expression vector pCZR125 encoding EK-bGH, the bGH derivative used to illustrate the method of the present invention, was constructed from a wide variety of publicly available starting materials.

Plasmid pCZR125 was constructed by first isolating plasmid pKC283 from E. coli K12 BE1201/pKC283. This culture may be obtained from the NRRL under accession number NRRL B-15830. Plasmid pKC283 comprises a hybrid lpp-pL promoter of bacteriophage λ . This plasmid is obtained from E. coli K12 BE1201 cells because these cells comprise a temperature sensitive ci repressor integrated into the cellular DNA. The unneeded lacZ portion of plasmid pKC283 was excised by first digesting the plasmid with restriction enzyme PvuII. Specific DNA linkers were then added to the digested DNA to convert the PvuII sites into a single XhoI site, which created plasmid pKC283PX. Detailed descriptions of the isolation of plasmids pKC283 and pKC283PX are presented respectively in Examples 6 and 7. Restriction site and function maps of plasmids pKC283 and pKC283PX are presented in Figure 1 of the accompanying drawings. As explained in Example 8, plasmid pKC283PX is transformed into E. coli K12 MO(λ^+). E. coli K12 MO(λ^+) is available from the NRRL under the accession number NRRL B-15993.

Plasmid pKC283PX was next digested with restriction enzymes BglII and XhoI. After the vector was purified, DNA linkers with BglII and XhoI ends were ligated into the vector to form plasmid pKC283-L. The BglII-XhoI linker also contained an XbaI site. The

XhoI site of plasmid pKC283-L was next converted into a BamHI site. This was accomplished by a total digestion of plasmid pKC283-L with restriction enzyme XhoI, followed by treatment with Klenow, then addition of BamHI linkers, to form plasmid pKC283-LB. Detailed descriptions of the construction of plasmids pKC283-L and pKC283-LB are presented respectively in Examples 9 and 10. Restriction site and function maps of plasmids pKC283-L and pKC283-LB are presented in Figure 1 of the accompanying drawings.

The extraneous E. coli DNA was next excised from plasmid pKC283PX by total digestion with restriction enzyme SalI, followed by treatment of the ~4.0 kb vector with Klenow, then addition of EcoRI linkers. Upon recircularization via ligation, this formed plasmid pKC283PRS. Plasmid pKC283PRS was then digested with restriction enzymes PstI and SphI and the ~0.85 kb PstI-SphI restriction fragment was isolated. In an analogous manner, plasmid pKC283-LB was digested with restriction enzymes PstI and SphI and the ~3.0 kb fragment was isolated. The ~0.85 kb PstI-SphI fragment of pKC283PRS was then ligated into the ~3.0 kb PstI-SphI vector fragment of pKC283-LB to form plasmid pL32. Detailed descriptions of the construction of plasmids pKC283PRS and pL32 are presented in Example 11. Restriction site and function maps of plasmids pKC283PRS and pL32 are presented in Figure 1 of the accompanying drawings.

Next, plasmid pNM789 is obtained from the NRRL in E. coli K12 RV308/pNM789 under the accession number B-18216. A restriction site and function map of plasmid

pNM789 is presented in Figure 2 of the accompanying drawings. Plasmid pNM789 was partially digested with restriction enzyme PvuII, fully digested with restriction enzyme BamHI, then treated with alkaline phosphatase. Next, a new PvuII-BamHI linker was ligated into the digested, phosphatased vector pNM789 to form plasmid 120. Plasmid 120 was then totally digested with restriction enzymes XbaI and BamHI and the ~0.6 kb XbaI-BamHI EK-bGH-encoding restriction fragment was isolated. Plasmid pL32 was also digested with restriction enzymes XbaI and BamHI and the ~3.9 kb vector fragment was isolated. The ~0.6 kb XbaI-BamHI fragment of plasmid 120 was then ligated into the ~3.9 kb vector fragment of plasmid pL32 to form plasmid pL47. Detailed descriptions of the construction of plasmids 120 and pL47 are presented in Examples 11 and 12. Restriction site and function maps of plasmids 120 and pL47 are presented respectively in Figures 3 and 4 of the accompanying drawings.

Plasmid pPR12 comprises the temperature-sensitive pL repressor gene cI857 and the plasmid pBR322 tetracycline resistance-conferring gene. Plasmid pPR12 is disclosed and claimed in U.S. Patent No. 4,436,815, issued 13 March, 1984. A restriction site and function map of plasmid pPR12 is presented in Figure 4 of the accompanying drawings. The EcoRI site was removed from plasmid pPR12 by first totally digesting the plasmid with restriction enzyme EcoRI, followed by treatment with Klenow. The vector was then recircularized by ligation to form plasmid pBR12ΔR1. Plasmid pPR12ΔR1 was then digested with restriction enzyme AvaI and

treated with Klenow. The AvaI-digested, Klenow treated pPR12AR1 was next ligated to EcoRI linkers, cut with restriction enzyme EcoRI, then recircularized to form plasmid pPR12AR1. A detailed description of the construction of plasmid pPR12AR1 is presented in Example 13. A restriction site and function map of plasmid pPR12AR1 is presented in Figure 4 of the accompanying drawings.

The ~2.9 kb PstI-EcoRI restriction fragment of plasmid pPR12AR1 was isolated after the plasmid was first digested with restriction enzymes PstI and EcoRI. Plasmid pL47 was digested with restriction enzymes PstI and BamHI and the ~2.7 kb PstI-BamHI restriction fragment was isolated. In a separate reaction, plasmid pL47 was digested with restriction enzymes EcoRI and BamHI and the ~1.03 kb EcoRI-BamHI fragment was isolated. The ~2.7 kb PstI-BamHI and ~1.03 kb EcoRI-BamHI restriction fragments of plasmid pL47 were ligated to the ~2.9 kb PstI-EcoRI restriction fragment of plasmid pPR12AR1 to form plasmid pL110. A detailed description of the construction of plasmid pL110 is presented in Example 14. A restriction site and function map of plasmid pL110 is presented in Figure 4 of the accompanying drawings.

Plasmid pL110 was digested with the restriction enzymes XbaI and BamHI and the ~5.8 kb fragment was isolated. The 5.8 kb fragment of plasmid pL110 was then ligated to a synthetic DNA sequence, as described in Example 15. The presence of the first cistron promotes higher level expression of met-EK-bGH product.

A restriction site and function map of plasmid pCZR125 is presented in Figure 5 of the accompanying drawings.

E. coli RV308 can be obtained from the Northern Regional Research Laboratories in lyophilized form under the accession number NRRL B-15624. E. coli RV308 host cells were transformed with plasmid pCZR125 to generate the expression system used throughout this disclosure to exemplify the various embodiments of the present invention.

10 The E. coli K12 RV308/pCZR125 expression systems are preferably maintained at temperatures of about 33°C during the early stages of the fermentation process wherein the expression systems are merely replicating in order to achieve sufficient cell numbers for optimal product expression. When sufficient cell numbers are present, the temperature is raised to induce expression of the EK-bGH. Figure 6 illustrates the induction of EK-bGH production. The time period of 0 to about six hours represents the time allowed for the expression systems to replicate to sufficient numbers for optimal fermentative production. At about the six hour time point, the temperature is elevated thereby inactivating the temperature sensitive repressor. Upon

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inactivation of the repressor high level product expression begins.

When thermoinducible expression systems are used, antibiotics added for purposes of the present invention are added at about the time the temperature of the fermentation mixture is increased. Skilled artisans, while realizing the broad applicability of the present invention, will realize that a kinetic analysis of expression vector replication and product expression would be required when expression systems other than those used illustratively herein are used for purposes of the present invention. The present invention teaches that when antibiotics are added at bacteristatic or bactericidal concentrations, at approximately the time at which product expression is induced, the accumulation of aberrant expression systems is prevented.

A thermoinducible expression system was used merely to illustrate the present invention. The present invention is in no way restricted to the use of this type of expression system the details of which were provided merely to promote an understanding of the invention. Any expression system which expresses and subsequently accumulates a recombinant product is at a selective disadvantage in a fermentation process and therefore would benefit from the teachings of the present invention.

Thus, it should be noted that the present invention is not limited to thermoinducible systems. Expression systems in which product expression is driven by inducible promoters such as, for example, the trp, lac, and the tac promoters, are examples of other promoters providing further embodiments of the invention.

Induction of product expression using expression vectors utilizing the aforementioned promoters follows the addition of derepressors, substances which negate the effect of repressor proteins. For example, addition of 3-indolylacetic acid to expression systems using the trp promoter results in the derepression of the promoter and subsequent expression of the gene encoding the product of interest. Expression systems using the lac or tac promoters are likewise derepressed upon addition of isopropyl- β -D-thiogalactoside. The use of the present invention in these expression systems would comprise the addition of bacteriostatic or bactericidal concentrations of antibiotics at about the time product expression is induced through addition of the appropriate derepressor.

Skilled artisans are aware that temperature sensitive mutants of many repressors are known in the art. Induction of product expression in systems using these temperature sensitive regulatory systems occurs when culture conditions are elevated. Maniatis *et al.*, "Molecular Cloning-A Laboratory Manual", 1982, Cold Spring Harbor, NY, reviews various expression systems and discusses mechanisms of their induction. Application of the present invention to any expression system utilizing a prokaryotic host cell transformed or transfected with an expression vector requires only the addition of bacteriostatic or bactericidal concentration of an antibiotic at approximately the time when product expression is induced.

The term "approximately the time" includes brief time periods preceding and subsequent to the induction of product expression. Prior to induction of

product expression, there exists a time period wherein the expression systems are merely allowed to replicate. The "seeding" of a fermentation process with less than optimal numbers of expression systems is common in the art of fermentation. Thus, for purposes of the present invention, the addition of bacteristatic or bactericidal concentrations of at least one antibiotic occurs when sufficient expression vectors are present and may briefly precede the induction of product expression.

5 Likewise, the addition of bacteristatic or bactericidal concentrations of at least one antibiotic can occur subsequent to product induction. Addition of bacteristatic or bactericidal concentrations of at least one antibiotic is also included in the "approximately the time" term, if the antibiotic is added within a brief period following product induction. The brief period following induction of product expression, during which addition of bacteristatic or bactericidal concentrations of at least one antibiotic can be performed to practice the present invention, precedes the time point in the fermentation process wherein the accumulation of the product of interest within cells expressing the product places them at a disadvantage in terms of cell division and thus encourages the outgrowth of cells which are not expressing the product. Generally, the addition of antibiotic to practice the present invention will be made within about two hours of induction of product expression, and preferably within about one hour of product induction.

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30 DNA synthesis inhibitors such as, for example: nitrofurans, acrosaxacin, novobiocin oxolinic acid,

pipemedic acid, piromidic acid, norfloxacin, anthra-
mycin, bleomycin, nitroimidazoles, and especially
cinoxacin are most preferred for purposes of the present
invention. Other antibiotics which are of use in the
5 present invention include cell wall inhibitors, such as,
for example: penicillins, cephalosporins, vancomycin,
cycloserine, alafosfolin, clavulanic acid, bacitracin,
moenomycin, and ristocetin, and especially ampicillin.
Vitamin analogs, such as, for example: sulfonamides and
10 trimethoprim; and enzyme inhibitors such as, for example,
clavulanic acid are also suitable for purposes of the
invention and therefore are included in the scope
thereof. Those skilled in the art will recognize that
the aforementioned antibiotics are merely illustrative
15 of antibiotics suitable for purposes of the invention
and that substitutions with various other antibiotics is
possible without exceeding the scope of the present
invention.

The present invention has been practiced in
20 both defined and complex media formulations. Complex
media are preferred for use in the present invention.
Examples 1 and 3 illustrate the method of the invention
in defined and complex media respectively, while
Example 2 serves as a control group for the first
25 Example and Example 4 serves as the control for Example
3. Those skilled in the art realize that the medium used
in a fermentation process is critical to maximizing
product expression. Nutritional requirements vary from
expression system to expression system and a variety of
30 media including, for example, those discussed in
Maniatis, et al., Molecular Cloning - A Laboratory

Manual, 1982, Cold Spring Harbor, NY, should be evaluated to determine the best medium for the particular expression system used in practicing the present invention.

5 Results of the fermentations detailed in
Examples 1, 2, 3, and 4 are summarized in Table 1. The
data on deletant expression vectors were obtained from
restriction endonuclease analysis of expression vectors
isolated from the fermentative mixture at the end of
10 fermentation. The use of restriction analysis to
characterize expression vectors is well known in the
art. The methodology for the generation and the inter-
pretation of restriction endonuclease mapping of expres-
sion vectors is well known in the art. Maniatis, Fritsch,
15 and Sambrook in "Molecular Cloning - A Laboratory Manual",
1982, Cold Spring Harbour Laboratory, Cold Spring Harbor,
NY, provides an excellent review of this subject.

TABLE I

Example	Medium	Cinoxacin (20 mcg/ml)	Dry Cell Wt. (g/L)	Relative Potency (non- dimensional)	Deletants
1.	Defined	Yes	9.50	0.649	No
2.	Defined	No	8.38	1.019	Yes
3.	Complex	Yes	13.52	0.904	No
4.	Complex	No	9.62	1.103	Yes

Table I clearly demonstrates that an antibiotic, when used in accordance with the teachings of the present invention, prevents the accumulation of deletant expression vectors during fermentation processes, utilizing either defined or preferably complex medium formulations.

The relative potency values are an index of bovine growth factor activity. Monitoring of bovine growth hormone derivative production was accomplished by a combination of chromatographic and bio-assay procedures.

The concentration of the bGH derivative in the fermentation samples was determined by comparison to reference EK-bGH using standard HPLC techniques. The activity of EK-bGH in the samples was determined using the bio-assay as taught in Example 5. Skilled artisans

will appreciate that a bio-assay is essential for initial determinations of any biomolecule. Biochemical and immunochemical assays are useful in determining total amounts of bGH; however bio-assays provide information on the amount of functional material. Bioassays are a preferred method of determining bGH activity in samples. When bioassay results are consistently found to be in agreement with HPLC analysis, or for example, radio-immunoassay or enzyme-linked immunosorbent assays it is preferable to rely on these inexpensive and less time consuming analyses. The relative potency estimates reported in Table I and Figure 6 derive from an HPLC analysis which has been found to correlate well with bio-assay data.

Complex media are preferred for use in the present invention as reference to Table I will illustrate. A modest depression in bovine growth factor production was observed when complex medium was supplemented with cinoxacin at approximately the start of product expression. A substantial depression in EK-bGH production was observed when a defined medium was used. Growth of expression systems in defined medium and supplementing the defined medium to make it a complex medium at approximately the time when antibiotics are added and product expression is induced is also contemplated in the present invention and included in the scope thereof.

The data obtained with cinoxacin merely illustrates the invention and those skilled in the art will realize that any antibiotic which does not disrupt expression of the recombinant polypeptide product of

interest would be appropriate for purposes of the invention and therefore is included within the scope thereof.

5 Cinoxacin is a preferred antibiotic for practicing the present invention. Those skilled in the art will realize that the effective concentration range of antibiotics comprises the bacteristatic or bactericidal range of the particular antibiotic or antibiotics being used. The effective antibiotic concentration
10 range is readily determined by the skilled artisan and will depend on the particular expression systems, the cell density of the expression systems, the particular antibiotic or antibiotics used, as well as the fermentation conditions.

15 When cinoxacin is used to practice the present invention in fermentations utilizing the E. coli RV308/pCZR125 expression system for EK-bGH production, the preferred cinoxacin concentration is in the range of from about 5 ug/ml to about 50 ug/ml with about 10 ug/ml
20 to about 20 ug/ml being especially preferred. The preferred concentrations stated above relate to the fermentative production of EK-bGH in the RV308/pCZR125 expression system and are merely illustrative of the present invention and are not limiting upon the scope
25 thereof.

To further illustrate the method of the invention, fermentations were conducted using ampicillin and novobiocin (both obtained from Sigma Chemical Co., St. Louis, MO. 63178) in the E. coli RV308/pCZR125
30 EK-bGH fermentation process. Ampicillin was used at a concentration of about 25 mcg/ml, while novobiocin was used at about 50 mcg/ml. Both of the aforementioned

antibiotics, when added at approximately the time the product expression was induced, were effective in preventing the accumulation of deletant expression vectors. The concentrations of the antibiotics used are merely illustrative and skilled artisans realize that concentrations of an antibiotic used for purposes of the invention is limited only to those concentrations which comprise the bacteristatic or bactericidal range of said antibiotic.

10 The numerous antibiotics which are suitable for purposes of the invention, the compatibility of the present invention with a variety of media, and the applicability of the method of the invention to numerous host cell/expression vector systems indicate that the present invention is a significant advance in the technical art of fermentation.

15 The host cell used for illustrating the present invention was E. coli K12 RV308 and this strain is preferred. Skilled artisans realize that other host cells, such as, for example: Other Enterobacteria, Bacillus species, and Streptomyces species would also be amenable to the method of the present invention.

20 The Examples disclosed to illustrate the present invention are merely representative of polypeptide products which can be produced using the present invention. A bovine growth hormone derivative is the polypeptide product used in the examples to illustrate the invention, however skilled artisans will recognize that other polypeptide products of interest encoded on expression vectors would be suitable for use in the present invention, such as, for example: β -galactosidase,

human pre-proinsulin, human proinsulin, human insulin A-chain, human insulin B-chain, non-human insulin, human growth hormone, insulin like growth factors, bovine growth hormone, human interferon, non-human interferon, 5 viral antigens, urokinase, tissue-type plasminogen activator, interleukins 1-6, colony stimulating factors, erythropoetin, and the like.

The Examples set forth below are intended to assist in a further understanding of the invention. 10 Particular materials employed, species, and conditions, are intended to be further illustrative of the invention and not limiting of the reasonable scope thereof.

EXAMPLE 1

15 The present invention has been illustrated using cinoxacin, (available from Eli Lilly and Co., Indianapolis, IN 46285) to prevent the appearance of detectable levels of deletant plasmids during a fermentative process for production of a bovine growth 20 hormone derivative.

A medium formulation described by Domach, et. al., 1984, Biotechnology and Bioengineering, vol. xxvi :203-206 was used for purposes of teaching the invention, said medium consisting of: Ammonium Sulfate Technical 25 Grade, 0.125% (VanWaters & Rogers, 7425 E. 30th St. Indianapolis, IN 46219;) Potassium Phosphate Monobasic, 0.150% (Ulrich Chemical Inc., 3111 North Post Rd., Indianapolis, IN 46226) Sodium Chloride, 0.001% (Central Indiana Supply Co., P.O. Box 762, Indianapolis, IN 30 46206); Potassium Phosphate, Dibasic, 0.3% (Ulrich Chemical Inc., Terre Haute, IN 47808) Ferrous Sulfate,

0.1% (Mays Chemical Co., Inc. Indianapolis, IN 46226); Sodium EDTA-7H₂O, 0.00372% (Sigma Chemical Co., St. Louis, MO 63178); Magnesium Sulfate for Fermentation, 0.01% (VanWaters & Rogers, Indianapolis, IN 46219);
5 Glucose (80%), 0.85%. Thiamine, 0.0005% (SST Corporation, Clifton, NJ 07012); and Tetracycline 0.0015% (Sigma Chemical Co., St. Louis, MO 63178) were added as supplements to the basic medium of Donach et al. for nutritional and selective purposes respectively.

10 The transformed microbes were cultivated under conditions suitable for growth until numbers sufficient for optimal product expression were obtained. Expression of the recombinant polypeptide product was induced and at approximately the same time sterile filtered
15 cinoxacin was added to achieve a final concentration of 20 mcg/ml. Fermentation continued in the presence of the cinoxacin and expression of the recombinant polypeptide product was monitored by sampling the fermentation mixture and assaying for bovine growth hormone
20 activity. A representative fermentation is illustrated in Figure 6.

EXAMPLE 2

25 Another fermentation was performed in substantial accord with the procedure of Example 1. In this procedure, cinoxacin was not added, thereby serving as a control group and allowing the determination as to the extent to which cinoxacin can prevent deletant expression vector accumulation.

30

EXAMPLE 3

The method of the present invention was further illustrated using a complex medium prepared by addition of 0.5% w/v autolyzed yeast (Difco Laboratories, Inc., Detroit, MI. 48232) to the medium of Example 1. Cells were cultured under conditions appropriate for growth until sufficient cell numbers were attained. Product expression was induced and at approximately the same time, cinoxacin was added to achieve a final concentration of 20 mcg/ml. Fermentation was allowed to continue in the presence of cinoxacin.

EXAMPLE 4

Another fermentation was performed in substantial accord with the procedure of Example 3. In this procedure, cinoxacin was not added, thereby serving as a control group and allowing the determination as to the extent to which cinoxacin can prevent deletant expression vector accumulation.

EXAMPLE 5Bioassay of Bovine Growth Hormone Activity

Bovine growth hormone activity was analyzed for biological activity using the following "Tibia Assay".

Female rats, hypophysectomized at 25 days of age, are given, in addition to rat chow, 5% glucose water ad libitum for the first 48 hours following hypophysectomy after which normal tap water is used. On the 7th day (32 days of age) after hypophysectomy animals that are obviously sick or weak are eliminated. All remaining rats are earmarked and weighed. At day

14 (39 days of age) after hypophysectomy the rats are weighed again. Rats that have gained more than 10 grams during the seven-day period and those weighing in excess of 120 grams are eliminated.

5 The test compound is dissolved in a minimum of 0.01 M NaHCO_3 , pH 8.0 and then is brought to the desired final volume by addition of physiological saline (0.1 N NaCl) or distilled water.

10 On the 14th day after hypophysectomy (39 days of age) the animals are randomized into control and treatment groups. One group serves as control and receives vehicle only. All treatments are administered at a volume of 0.1 ml by subcutaneous injection once daily for 4 days. Individual body weights are recorded at the beginning of the assay. Approximately 18-24 hours following the fourth injection, body weights are again recorded, and the rats are sacrificed by carbon dioxide asphyxiation. The sella turcica of each rat is visually checked for completeness of hypophysectomy.

15 The right tibia of each rat is removed, dissected free from soft tissue, and split in a midsagittal plane with a scalpel to expose a cross-section of the proximal epiphyseal cartilage plate.

20 The tibias so removed and split are subjected to the following histological preparation. Bone halves are

- 25
- (1) Fixed in 10% neutral formalin for at least 72 hours.
 - (2) Washed thoroughly in deionized water for one hour.
 - (3) Immersed in acetone for one hour.
- 30

- 5
- (4) Washed again in deionized water for one hour.
 - (5) Immersed in 2% silver nitrate for exactly 2 minutes and then immediately placed in deionized water while being exposed to strong light for 6 minutes (calcified parts appear dark brown).
 - (6) Rapidly removed from water and submerged immediately and fixed in 10% sodium thiosulfate for about 30 seconds.
 - (7) Washed thoroughly in deionized water for 30 minutes and maintained in deionized water until reading.

10

Following reading, the bones are stored in 15 80% ethanol in the dark.

The width of the uncalcified epiphyseal cartilage is determined. A binocular dissecting scope with a 10X ocular lens (reticle micrometer eyepiece with 0.1 mm divisions) and a 4X objective lens is used for determining the cartilage plate width. The separate readings are made across the epiphyseal plate and recorded on the data sheet. Using a scope having the above lens configuration, the width of the epiphyseal plate in micra is calculated by multiplying each value by 25 (with this lens configuration, 1 mm on a ruler is covered by 40 small divisions on the reticle; therefore, one division = 25 microns). The arithmetic means of all ten readings for each bone are calculated. The mean of all bones within a treatment group are also calculated.

20

25

30

The bovine growth hormone is compared against the vehicle as Control and a sample of pituitary source standard bovine growth hormone obtained from the National Pituitary Agency (Compound II).

5

EXAMPLE 6

Isolation of Plasmid pKC283

10 Lyophilis of E. coli K12 BE1201/pKC283 are obtained from the Northern Regional Research Laboratory, Peoria, Illinois 61604, under the accession number NRRL B-15830. The lyophilis are decanted into tubes containing 10 ml LB medium (10 g Bacto-tryptone, 5 g
15 Bacto-yeast extract, and 10 g NaCl per liter; pH is adjusted to 7.5) and incubated two hours at 32°C, at which time the cultures are made 50 µg/ml in ampicillin and then incubated at 32°C overnight. The E. coli K12 BE1201/pKC283 cells were cultured at 32°C, because
20 the cells comprise a temperature-sensitive cI repressor gene integrated into the cellular DNA. When cells that comprise a wild-type lambda pL repressor gene or do not comprise a lambda pL promoter are utilized in this plasmid isolation procedure, as described in subsequent
25 Examples herein, the temperature of incubation is 37°C.

A small portion of the overnight culture is placed on LB-agar (LB medium with 15 g/l Bacto-agar) plates containing 50 µg/ml ampicillin in a manner so as to obtain a single colony isolate of E. coli K12
30 BE1201/pKC283. The single colony obtained was inoculated into 10 ml of LB medium containing 50 µg/ml

ampicillin and incubated overnight at 32°C with vigorous shaking. The 10 ml overnight culture was inoculated into 500 ml LB medium containing 50 µg/ml ampicillin and incubated at 32°C with vigorous shaking until the culture reached stationary phase.

The following procedure is adapted from Maniatis *et al.*, 1982, Molecular Cloning (Cold Spring Harbor Laboratory).

The cells were harvested by centrifugation at 4000 g for 10 minutes at 4°C, and the supernatant was discarded. The cell pellet was washed in 100 ml of ice-cold STE buffer (0.1 M NaCl; 10 mM Tris-HCl, pH 7.8; and 1 mM EDTA). After washing, the cell pellet was resuspended in 10 ml of Solution 1 (50 mM glucose; 25 mM Tris-HCl, pH 8.0; and 10 mM EDTA) containing 5 mg/ml lysozyme and left at room temperature for 10 minutes. Twenty ml of Solution 2 (0.2 N NaOH and 1% SDS) were then added to the lysozyme-treated cells, and the solution was gently mixed by inversion. The mixture was incubated on ice for 10 minutes.

Fifteen ml of ice-cold 5 M potassium acetate, pH 4.8, were added to the lysed-cell mixture and the solution mixed by inversion. The solution was incubated on ice for 10 minutes. The 5 M potassium acetate solution was prepared by adding 11.5 ml of glacial acetic acid to 28.5 ml of water and 60 ml of 5 M potassium acetate; the resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

The lysed cell mixture was centrifuged in a Beckman SW27 (or its equivalent) at 20,000 rpm for 20 minutes at 4°C. The cell DNA and debris formed a pellet

on the bottom of the tube. About 36 ml of supernatant were recovered, and 0.6 volumes of isopropanol were added, mixed, and the resulting solution left at room temperature for 15 minutes. The plasmid DNA was collected by centrifugation at 12,000 g for 30 minutes at room temperature. The supernatant was discarded, and the DNA pellet was washed with 70% ethanol at room temperature. The ethanol wash was decanted, and the pellet was dried in a vacuum desiccator. The pellet was then resuspended in 8 ml of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA).

Eight grams of CsCl were added to the DNA solution. About 0.8 ml of a 10 mg/ml solution of ethidium bromide in water were added for each 10 ml of CsCl-DNA solution. The final density of the solution was about 1.55 g/ml, and the ethidium bromide concentration was about 600 µg/ml. The solution was transferred to a Beckman Type 50 centrifuge tube, filled to the top with paraffin oil, sealed, and centrifuged at 45,000 rpm for 24 hours at 20°C. After centrifugation, two bands of DNA were visible in ordinary light. After removing the cap from the tube, the lower DNA band was removed by using a syringe with a #21 hypodermic needle inserted through the side of the centrifuge tube.

The ethidium bromide was removed by several extractions with water-saturated 1-butanol. The CsCl was removed by dialysis against TE buffer. After extractions with buffered phenol and then chloroform, the DNA was precipitated, washed with 70% ethanol, and dried. About 1 mg of plasmid pKC283 was obtained and stored at 4°C in TE buffer at a concentration of about

1 µg/µl. A restriction site and function map of plasmid pKC283 is presented in Figure 1 of the accompanying drawings.

5

EXAMPLE 7Construction of Plasmid pKC283PX

About 10 µl of the plasmid pKC283 DNA prepared in Example 1 were mixed with 20 µl 10 X medium-salt restriction buffer (500 mM NaCl; 100 mM Tris-HCl, pH 7.5; 100 mM MgCl₂; and 10 mM DTT), 20 µl 1 mg/ml BSA, 5 µl restriction enzyme PvuII (~50 Units, as defined by Bethesda Research Laboratories (BRL), from which all restriction enzymes used herein were obtained), and 145 µl of water, and the resulting reaction was incubated at 37°C for 2 hours. Restriction enzyme reactions described herein were routinely terminated by phenol and then chloroform extractions, which were followed by precipitation of the DNA, an ethanol wash, and resuspension of the DNA in TE buffer. After terminating the PvuII digestion as described above, the PvuII-digested plasmid pKC283 DNA was precipitated and then resuspended in 5 µl of TE buffer.

About 600 picomoles (pM) of XhoI linkers (5'-CCTCGAGG-3') were kinased in a mixture containing 10 µl 5 X Kinase Buffer (300 mM Tris-HCl, pH 7.8; 50 mM MgCl₂; and 25 mM DTT), 5 µl 5 mM ATP, 24 µl H₂O, 0.5 µl of T4 polynucleotide kinase (about 2.5 units as defined by P-L Biochemicals), 5 µl 1 mg/ml BSA, and 5 µl of 10 mM spermidine by incubating the mixture at 37°C for 30 minutes.

About 12.5 μ l of the kinased XhoI linkers were added to the 5 μ l of PvuII-digested plasmid pKC283 DNA, and then 2.5 μ l of 10 X ligase buffer (300 mM Tris-HCl, pH 7.6; 100 mM MgCl₂; and 50 mM DTT), 2.5 μ l of 1 mg/ml BSA, 7 μ l of 5 mM ATP, 2.5 μ l (about 2.5 units as defined by P-L Biochemicals) of T4 DNA ligase, 2.5 μ l of 10 mM spermidine, and 3 μ l of water were added to the DNA. The resulting ligation reaction was incubated at 4°C overnight. After the ligation reaction, the reaction mixture was adjusted to have the composition of high-salt buffer (0.1 M NaCl; 0.05 M Tris-HCl, pH 7.5; 10.0 mM MgCl₂; and 1 mM DTT). About 10 μ l (100 units) of restriction enzyme XhoI were added to the mixture, and the resulting reaction was incubated at 37°C for 2 hours.

The reaction was terminated, and the XhoI-digested DNA was precipitated, resuspended, and ligated as described above, except that no XhoI linkers were added to the ligation mixture. The ligated DNA constituted the desired plasmid pKC283PX. A restriction site and function map of plasmid pKC283PX is presented in Figure 1 of the accompanying drawings.

EXAMPLE 8

Construction of E. coli K12 MO(λ^+)/pKC283PX

E. coli K12 MO(λ^+) can be obtained from the Northern Regional Research Laboratories in lyophilized form under the accession number NRRL B-15993. E. coli K12 MO(λ^+) comprises the wild-type lambda pL cI repressor gene, so that transcription from the hybrid pL-lpp

promoter of the present invention does not occur in E. coli K12 MO(λ^+) cells. The lyophils are reconstituted, single colonies of MO(λ^+) are isolated, and a 10 ml overnight culture of the MO(λ^+) cells is prepared in
5 substantial accordance with the procedure of Example 6, except that the temperature of incubation is 37°C and no ampicillin is used in the growth media.

Fifty μ l of the overnight culture were used to inoculate 5 ml of LB media which also contained 10 mM
10 MgSO₄ and 10 mM MgCl₂. The culture was incubated at 37°C overnight with vigorous shaking. The following morning, the culture was diluted to 200 ml with LB media containing 10 mM MgSO₄ and 10 mM MgCl₂. The diluted culture was incubated at 37°C with vigorous
15 shaking until the absorbance at 550 nm (A_{550}) was about 0.5, which indicated a cell density of about 1×10^8 cells/ml. The culture was cooled for ten minutes in an ice-water bath, and the cells were then collected by centrifugation at 4000 g for 10 minutes at 4°C. The
20 cell pellet was resuspended in 100 ml of cold 10 mM MgSO₄ and then immediately re-pelleted by centrifugation. The cell pellet was resuspended in 100 ml of 30 mM CaCl₂ and incubated on ice for 20 minutes.

The cells were again collected by centri-
25 fugation and resuspended in 10 ml of 30 mM CaCl₂. A one-half ml aliquot of the cells was added to the ligated DNA prepared in Example 7; the DNA had been made 30 mM in CaCl₂. The cell-DNA mixture was incubated on ice for one hour, heat-shocked at 42°C for 90 seconds,
30 and then chilled on ice for about two minutes. The cell-DNA mixture was diluted into 10 ml of LB media in

5'-GATCTATTA ACTCAATCTAGAC-3'
 |||||
 3'-ATAATTGAGTTAGATCTGAGCT-5'

5

The linker depicted above was synthesized from single-stranded deoxyoligonucleotides by procedures well known in the art. The single-stranded deoxyoligonucleotides can be synthesized with commercially available instruments, such as the 380A DNA Synthesizer marketed by Applied Biosystems (850 Lincoln Centre Drive, Foster City, CA 94404), which utilizes phosphoramidite chemistry. Other procedures for synthesizing DNA are also known in the art. The conventional modified phosphotriester method of synthesizing single stranded DNA is described in Itakura et al., 1977, Science 198:1056 and in Crea et al., 1978, Proc. Nat. Acad. Sci. USA 75: 5765. In addition, an especially preferred method of synthesizing DNA is disclosed in Hsiung et al., 1983, Nucleic Acid Research 11:3227 and Narang et al., 1980, Methods in Enzymology 68:90.

20

The linker and BglII-XhoI-digested plasmid pKC283PX were ligated in substantial accordance with the procedure of Example 7. The ligated DNA constituted the desired plasmid pKC283-L. A restriction site and function map of plasmid pKC283-L is presented in Figure 1 of the accompanying drawings. The plasmid pKC283-L DNA was used to transform E. coli K12 MO(λ^+) and the resulting E. coli K12 MO(λ^+)/pKC283-L transformants were identified in substantial accordance with the procedure of Example 8.

25

30

EXAMPLE 10Construction of *E. coli* K12 MO(λ^+)/pKC283-LB

5 About 10 μ g of plasmid pKC283-L DNA, prepared
in substantial accordance with the procedures of Example
1, were dissolved in 20 μ l 10X high-salt buffer, 20 μ l 1
mg/ml BSA, 5 μ l (\sim 50 units) restriction enzyme XhoI,
and 155 μ l of H₂O, and the resulting reaction was
10 incubated at 37°C for two hours. The XhoI-digested
plasmid pKC283-L DNA was then precipitated from the
reaction mixture by the addition of three volumes of
95% ethanol and one-tenth volume of 3 M sodium acetate,
incubation in a dry ice-ethanol bath for five minutes,
and centrifugation. The resulting DNA pellet was washed
15 with 70% ethanol, dried, and resuspended in 2 μ l 10X
nick-translation buffer (0.5 M Tris-HCl, pH 7.2; 0.1
M MgSO₄; and 1 mM DTT), 1 μ l of a solution 2 mM in each
of the deoxynucleotide triphosphates, 15 μ l of H₂O, 1 μ l
(\sim 6 units as defined by P-L Biochemicals) of Klenow,
20 which is the large fragment of *E. coli* DNA polymerase I,
and 1 μ l of 1 mg/ml BSA. The resulting reaction was
incubated at 25°C for 30 minutes; the reaction was
stopped by incubating the solution at 70°C for five
minutes.

25 BamHI linkers (5'-CGGGATCCCG-3') were kinased
and ligated to the XhoI-digested, Klenow-treated plasmid
pKC283-L DNA in substantial accordance with the pro-
cedure of Example 7. After the ligation reaction,
the DNA was digested with about 100 units of BamHI for
30 about 2 hours at 37°C in high-salt buffer. After the
BamHI digestion, the DNA was prepared for ligation in
substantial accordance with the procedure of Example 2.

The ~5.9 kb BamHI restriction fragment was circularized by ligation and transformed into E. coli K12 MO(λ^+) in substantial accordance with the procedures of Examples 7 and 8. The E. coli K12 MO(λ^+)/pKC283-LB transformants were identified, and then plasmid pKC283-LB DNA was prepared in substantial accordance with the procedure of Example 6. A restriction site and function map of plasmid pKC283-LB is presented in Figure 1 of the accompanying drawings.

EXAMPLE 11

Construction of E. coli K12 MO(λ^+)/pL32

About 10 μ g of plasmid pKC283PX were digested with restriction enzyme SalI in high-salt buffer, treated with Klenow, and ligated to EcoRI linkers (5'-GAGGAATTCCTC-3') in substantial accordance with the procedure of Example 10, with the exception of the starting plasmid, restriction enzymes, and linkers used. After digestion with restriction enzyme EcoRI, which results in the excision of ~2.1 kb of DNA, the ~4.0 kb EcoRI restriction fragment was circularized by ligation to yield plasmid pKC283PRS. The ligated DNA was used to transform E. coli K12 MO(λ^+) in substantial accordance with the procedure of Example 8. After the E. coli K12 MO(λ^+)/pKC283PRS transformants were identified, plasmid pKC283PRS DNA was prepared in substantial accordance with the procedure of Example 6. A restriction site and function map of plasmid pKC283PRS is presented in Figure 2 of the accompanying drawings.

About 10 μ g of plasmid pKC283PRS were digested in 200 μ l of high-salt buffer with about 50 units each

of restriction enzymes PstI and SphI. After incubating the reaction at 37°C for about 2 hours, the reaction mixture was electrophoresed on a 0.6% low-gelling-temperature agarose (FMC Corporation, Marine Colloids Division, Rockland, Maine 04841) gel for 2-3 hours at 5 ~130 V and ~75 mA in Tris-Acetate buffer.

The gel was stained in a dilute solution of ethidium bromide, and the band of DNA constituting the ~0.85 kb PstI-SphI restriction fragment, which was 10 visualized with long-wave UV light, was cut from the gel in a small segment. The volume of the segment was determined by weight and density of the segment, and an equal volume of 10 mM Tris-HCl, pH 7.6, was added to the tube containing the segment. The segment was then 15 melted by incubation at 72°C. About 1 ug of the ~0.85 kb PstI-SphI restriction fragment of plasmid pKC283PRS was obtained in a volume of about 100 µl. In an analogous manner, plasmid pKC283-LB was digested with restriction enzymes PstI and SphI, and the resulting 20 ~3.0 kb restriction fragment was isolated by agarose gel electrophoresis and prepared for ligation.

The ~0.85 kb PstI-SphI restriction fragment of plasmid pKC283PRS was ligated to the ~3.0 kb PstI-SphI restriction fragment of plasmid pKC283-LB in substantial accordance with the procedure of Example 7. The ligated 25 DNA constituted the desired plasmid pL32. A restriction site and function map of plasmid pL32 is presented in Figure 1 of the accompanying drawings. Plasmid pL32 was transformed into E. coli K12 MO(λ^+) cells in substantial accordance with the procedure of Example 8. Plasmid 30 pL32 DNA was prepared from the E. coli K12 MO(λ^+)/pL32

transformants in substantial accordance with the procedure of Example 6. Analysis of the plasmid pL32 DNA demonstrated that more than one EcoRI linker attached to the Klenow-treated, SalI ends of plasmid pKC283PX. The presence of more than one EcoRI linker does not affect the utility of plasmid pL32 or derivatives of plasmid pL32 and can be detected by the presence of an XhoI restriction site, which is generated whenever two of the EcoRI linkers are ligated together. Alternatively, plasmid pL32 may be constructed by carrying out the SalI-EcoRI excision and ligation of the first paragraph of this example upon plasmid pKC283-LB.

EXAMPLE 12

Construction of *E. coli* K12 MO(λ^+)/pL47

E. coli K12 RV308/pNM789 can be obtained from the Northern Regional Research Laboratories in lyophilized form under the accession number NRRL B-18216. A restriction site and function map of pNM789 is presented in Figure 3 of the accompanying drawings. Plasmid DNA is extracted from the culture in substantial accordance with the teaching of Example 6, except that the temperature of incubation is 37°C. Ten micrograms of pNM789 are suspended in 200 μ l PvuII buffer (50 mM Tris-HCl (pH 7.5), 60 mM NaCl and 6mM MgCl₂). One unit of PvuII is added and the reaction mix is incubated for 5 minutes at 37°C. The enzyme is inactivated by heating 10 minutes at 65°C. 30 μ l of 10X BamHI buffer (200 mM Tris-HCl (pH 8.0), 1M NaCl and 70 mM MgCl₂), 70 μ l H₂O and 10 units of BamHI are next added and the reaction is

incubated for 1 hour at 37°C. This is followed by the addition of 5 units of alkaline phosphatase and incubation for 1 hour at 65°C. The DNA fragments are separated on a 1 percent agarose gel, and a DNA fragment (Figure 4) the size of a single cut fragment is purified.

A DNA linker with a blunt end and a BamHI end is synthesized in substantial accordance with the teaching of Example 9. This linker (shown in Figure 4) has the following structure:

10
 5'-CTGTGCCTTCTAG-3'
 |||||
 3'-GACACGGAAGATCCTAG-5'

15 The linker is kinased and ligated into the BamHI-PvuII digested plasmid pNM789 in substantial accordance with the teaching of Example 9. This ligation mixture is used to transform E. coli K12 RV308 cells and plasmid isolation is performed upon these transformants in substantial accordance with the teaching of Example 8. Several plasmids are selected which contain the appropriate size PvuII fragment (494bp) and XbaI-BamHI fragment (628bp). The sequence of at least two of these is determined by sequencing from the BamHI site toward the unique SmaI site and one clone is selected with the desired sequence. This intermediate plasmid is designated plasmid 120. A schematic outline of this procedure and a restriction site and function map of plasmid 120 is presented in Figure 4 of the accompanying drawings.

30 To isolate the EK-bGH-encoding DNA, about 10 µg of plasmid 120 were digested in 200 µl of

high-salt buffer containing about 50 units each of restriction enzymes XbaI and BamHI. The digestion products were separated by agarose gel electrophoresis, and the ~0.6 kb XbaI-BamHI restriction fragment which
5 encodes EK-bGH was isolated and prepared for ligation in substantial accordance with the procedure of Example 11.

Plasmid pL32 was also digested with restriction enzymes XbaI and BamHI, and the ~3.9 kb restriction fragment was isolated and prepared for ligation. The
10 ~3.9 kb XbaI-BamHI restriction fragment of plasmid pL32 was ligated to the ~0.6 kb XbaI-BamHI restriction fragment of plasmid 120 in substantial accordance with the procedure of Example 7 to yield plasmid pL47.

A restriction site and function map of plasmid pL47 is presented in Figure 5 of the accompanying drawings. Plasmid pL47 was transformed into E. coli K12 MO(λ^+) in
15 substantial accordance with the procedure of Example 8, and the E. coli K12 MO(λ^+)/pL47 transformants were identified. Plasmid pL47 DNA was prepared from the transformants in substantial accordance with the pro-
20 cedures of Example 6.

EXAMPLE 13

Construction of E. coli K12 RV308/pPR12AR1

25 Plasmid pPR12 comprises the temperature-sensitive pL repressor gene cI857 and the plasmid pBR322 tetracycline resistance-conferring gene. Plasmid pPR12 is disclosed and claimed in U.S. Patent #4,436,815,
30 issued 13 March 1984. A restriction site and function map of plasmid pPR12 is presented in Figure 5 of the accompanying drawings.

About 10 μ g of plasmid pPR12 were digested with about 50 units of restriction enzyme EcoRI in 200 μ l of high-salt buffer at 37°C for two hours. The EcoRI-digested plasmid pPR12 DNA was precipitated and
5 treated with Klenow in substantial accordance with the procedure of Example 10. After the Klenow reaction, the EcoRI-digested, Klenow-treated plasmid pPR12 DNA was recircularized by ligation in substantial accordance with the procedure of Example 7. The ligated DNA, which
10 constituted the desired plasmid pPR12AR1, was used to transform E. coli K12 RV308 in substantial accordance with the procedure of Example 8, except that selection was based on tetracycline (5 μ g/ml) resistance, not ampicillin resistance. E. coli K12 RV308 is available
15 from the NRRL under the accession number NRRL B-15624. After the E. coli K12 RV308/pPR12AR1 transformants were identified, plasmid pPR12AR1 DNA was prepared from the transformants in substantial accordance with the procedure of Example 6.

20 About 10 μ g of plasmid pPR12AR1 were digested with about 50 units of restriction enzyme AvaI in 200 μ l of medium-salt buffer at 37°C for 2 hours. The AvaI-digested plasmid pPR12AR1 DNA was precipitated and treated with Klenow in substantial accordance with the
25 procedure of Example 10. After the Klenow reaction, the AvaI-digested, Klenow-treated plasmid pPR12AR1 DNA was ligated to EcoRI linkers (5'-GAGGAATTCCTC-3') in substantial accordance with the procedure of Example 7. After the linker ligation, the DNA was precipitated
30 and then resuspended in about 200 μ l of high-salt buffer containing about 50 units of restriction enzyme EcoRI.

The resulting reaction was incubated at 37°C for about 2 hours. After the EcoRI digestion, the reaction mixture was loaded onto an agarose gel, and the ~5.1 kb EcoRI restriction fragment was purified in substantial accordance with the procedure of Example 11. The ~5.1 kb EcoRI restriction fragment was recircularized by ligation in substantial accordance with the procedure of Example 2. The ligated DNA constituted the desired plasmid pPR12AR1. The plasmid pPR12AR1 DNA was transformed into E. coli K12 RV308 in substantial accordance with the procedure of Example 8, except that selection was based on tetracycline resistance, not ampicillin resistance. After identifying the E. coli K12 RV308/pPR12AR1 transformants, plasmid pPR12AR1 DNA was prepared in substantial accordance with the procedure of Example 6. A restriction site and function map of plasmid pPR12AR1 is presented in Figure 5 of the accompanying drawings.

EXAMPLE 14

Construction of E. coli K12 RV308/pL110

About 10 µg of plasmid pPR12AR1 DNA were suspended in about 200 ml of high-salt buffer containing about 50 units each of restriction enzymes PstI and EcoRI, and the digestion reaction was incubated at 37°C for about 2 hours. The reaction mixture was then loaded onto an agarose gel, and the ~2.9 kb PstI-EcoRI restriction fragment of plasmid pPR12AR1 was isolated and prepared for ligation in substantial accordance with the procedure of Example 11.

About 10 µg of plasmid pL47 were digested with

restriction enzymes PstI and BamHI in 200 ul of high-salt buffer at 37°C for two hours. The PstI-BamHI-digested DNA was loaded onto an agarose gel, and the ~2.7 kb PstI-BamHI restriction fragment that comprised the
5 origin of replication and a portion of the ampicillin resistance-conferring gene was isolated and prepared for ligation in substantial accordance with the procedure of Example 11. In a separate reaction, about 10 ug of plasmid pL47 DNA were digested with restriction enzymes
10 EcoRI and BamHI in 200 ul of high-salt buffer at 37°C for two hours, and the ~1.03 kb EcoRI-BamHI restriction fragment that comprised the novel transcriptional and translational activating sequence and the EK-bGH-encoding DNA was isolated and prepared for ligation in
15 substantial accordance with the procedure of Example 11. The ~2 ug of the ~1.03 kb EcoRI-BamHI restriction fragment obtained were used in the construction of plasmid pL110.

The ~2.7 kb PstI-BamHI and ~1.03 kb EcoRI-BamHI restriction fragments of plasmid pL47 were ligated
20 to the ~2.9 kb PstI-EcoRI restriction fragment of plasmid pPR12AR1 to construct plasmid pL110, and the ligated DNA was used to transform E. coli K12 RV308 in substantial accordance with the procedure of Examples 7
25 and 3, except that tetracycline resistance, not ampicillin resistance, was used as the basis for selecting transformants.

Two PstI restriction enzyme recognition sites are present in the EK-bGH coding region that are not
30 depicted in the restriction site and function maps presented in the accompanying drawings. A restriction

site and function map of plasmid pL110 is presented in Figure 5 of the accompanying drawings.

EXAMPLE 15

5

Construction of Plasmid pCZR125

10 About 26 ug of plasmid pL110 was digested with XbaI as follows. 10X XbaI buffer consists of 600mM Tris-HCl, 100mM MgCl₂, 1 M NaCl, and 10mM 2-mercaptoethanol, pH 7.5 (at 37°C). 50 ul of 10X XbaI buffer, 15 ul of Xba I (10U/ul), and 185 ul of H₂O were added to the 250 ul of water containing about 25 ug of plasmid pL110. The digestion proceeded at 37°C for 1 hour. XbaI digested pL110 was then extracted in phenol, a 1/10 volume 3M CH₃COO-Na was added, 3 volumes of ethanol were added; the mixture was incubated in a dry ice-ethanol bath for 5 minutes, and then centrifuged. 15 The precipitated DNA was resuspended in 50 ul H₂O.

The XbaI digested plasmid pL110 was digested with BamHI as follows. 0.2 ul of BamHI (10 U/ul), 20 10 ul of BamHI buffer (100mM Tris-HCl, 50mM MgCl₂, 1 M NaCl, and 10mM 2-Mercaptoethanol, pH 8.0 [at 37°C], and 90 ul of H₂O was added to the 50 ul of XbaI digested pL110 obtained hereinabove. The digest proceeded for 5 minutes at 37°C. The digested pL110 was extracted in phenol, a 1/10 volumes of CH₃COONa+ was added, followed 25 by addition of 3 volumes of ethanol. Precipitated DNA was resuspended in 50 ul of 10mM Tris, 1mM EDTA, pH 8.0 buffer.

30 The XbaI and BamHI digested pL110 was then loaded onto an agarose gel and the DNA band at about 5.8 kb was isolated. Plasmid pCZR125 was produced by

ligating the ~5.8 kb fragment of pL110 to an XbaI to
NdeI linker and a synthetic gene encoding EK-bovine
 growth hormone, which contains an NdeI site on its 5'
 end and a BamHI site on its 3' end. The XbaI to NdeI
 5 sequence was produced using standard oligonucleotide
 sequence methodology and consists of the following
 sequence:

10 5' CTAGAGGGTATTAATAATGTATATTGATTTAATAAGGAGGAAT^TCA 3'
 ||||||||||||||||||||||||||||||||||||||||||||
 TCCATAATTATTACATATAACTAAAATTATTCCTCCTTATTAGTAT 5'

The above sequence was constructed by
 chemical synthesis of both strand, followed by mixing
 to allow hybridization. The gene encoding EK bGH was
 15 constructed from 16 chemically synthesized pieces of
 single-stranded DNA, ranging from 71 to 83 nucleotides
 long, which together comprise both complementary
 strands of the entire gene. The synthesis was done by
 Battelle using an Applied Biosystems (ABS) machine and
 20 consists of the following sequence:

5' TATGTTCCCATTTGGATGATGATGATAAGTTCCCAGCCATGTCCTT
 ||||||||||||||||||||||||||||||||||||||||||||
 25 ACAAGGGTAACCTACTACTACTATTCAAGGGTCGGTACAGGAA

 GTCCGGCCTGTTTGCCAACGCTGTGCTCCGGGCTCAGCACCTGCATCAGCTGGCTGCTGA
 ||||||||||||||||||||||||||||||||||||||||||||
 30 CAGCCCGACAAACGGTTGCGACACGAGGCCGAGTCGTGGACGTAGTCGACCGACACT

 CACCTTCAAAGAGTTTGAGCGCACCTACATCCCGGAGGGACAGAGATACTCCATCCAGAA
 ||||||||||||||||||||||||||||||||||||||||||||
 GTGGAAGTTTCTCAAACCTCGCGTGGATGTAGGGCCTCCCTGTCTCTATGAGGTAGGTCTT

 35 CACCCAGGTTGCCTTCTGCTTCTCTGAAACCATCCCGGCCCCACGGGCAAGAATGAGGC
 ||||||||||||||||||||||||||||||||||||||||||||
 GTGGTCCAACGGAAGACGAAGAGACTTTGGTAGGGCCGGGGGTGCCCGTTCTTACTCCG

5 CCAGCAGAAATCAGACTTGGAGCTGCTTCGCATCTCACTGCTCCTCATCCAGTCGTGGCT
 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 GGTCTCTTTAGTCTGAACCTCGACGAAGCGTAGAGTGACGAGGAGTAGGTGACACCGA

 TGGGCCCTGCAGTTCCTCAGCAGAGTCTTCACCAACAGCTTGGTGTGGCACCTCGGA
 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 ACCCGGGGACGTCAAGGAGTCGTCTCAGAAGTGGTTGTCGAACCACAAACCGTGGAGCCT

 10 CCGTGTCTATGAGAAGCTGAAGGACCTGGAGGAAGGCATCCTGGCCCTGATGCGGGAGCT
 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 GGCACAGATACTCTTCGACTTCTGGACCTCCTCCGTAGGACCGGGACTACGCCCTCGA

 GGAAGATGGCACCCCGGGCTGGGCAGATCCTCAAGCAGACCTATGACAAATTTGACAC
 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 CCTTCTACCGTGGGGGGCCGACCCGTCTAGGAGTTCGTCTGGATACTGTTAAACTGTG

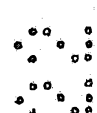
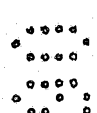
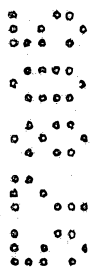
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 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 20 TTTGTACGCGTCACTGCTGCGCGACGAGTTCCTGATGCCAGACGAGAGGACGAAGCCCT

 GGACCTGCATAAGACGGAGACGTACCTGAGGGTCATGAAGTGCCGCCGCTTCGGGGAGGC
 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 CCTGGACGTATTCTGCCTCTGCATGGACTCCAGTACTTCACGGCGGCGAAGCCCTCCG

 25 CAGCTGTGCCTTCTAG 3'
 ||||||||||||||
 GTCGACACGGAAGATCCTAG 5'

30 Construction of plasmid pCZR125 was accom-
 plished by ligation of the following site components
 ~0.28 ug of the 5.8 kb fragment obtained from plasmid
 pL110 after complete digestion with XbaI and partial
 digestion with BamHI in a total volume of 2 ul, ~0.18 ug
 35 of the synthetic gene encoding a bovine growth factor
 derivative which has a 5' termini corresponding to a
XbaI site and a 3' termini corresponding to a BamHI site
 in a total volume of 2.5 ul, 8.75 picomoles of the
 chemically synthesized XbaI to NdeI linker in 1 ul. The
 40 plasmid components were added to 6 ul of 5x ligation
 buffer: 250 mM Tris-HCl, 50 mM MgCl₂, 5 mM ATP, 5 mM

DTT, 25% v/v polyethylene glycol 8,000, pH 7.6, 2 ul of ligase, and 16.5 ul of H₂O. The ligation mixture was incubated overnight at 16°C. The circularized plasmid pCZR125 was then used to transform E. coli RV308 cells
5 in substantial accord with the method of Example 8.



~~WE CLAIM:~~

The claims defining the invention are as follows:

1. A method of controlling aberrant expression vector replication comprising adding at least a bacteriostatic concentration of antibiotic to a fermentation mixture at approximately the time at which product expression is induced.
5
2. The method of Claim 1, wherein said antibiotic is selected from the group consisting of DNA synthesis inhibitors, cell wall synthesis inhibitors, enzyme inhibitors, and vitamin analogs.
10
3. The method of Claim 2 in which said antibiotic is a DNA synthesis inhibitor.
15
4. The method of Claim 3 wherein said DNA synthesis inhibitor is selected from the group consisting of a nitrofurantoin, cinoxacin, acrosaxacin, novobiocin, oxolinic acid, pipemidic acid, piromidic acid, norfloxacin, anthramycin, and bleomycin.
20
5. The method of Claim 4 wherein said DNA synthesis inhibitor is cinoxacin or novobiocin.
25
6. The method of Claim 2 wherein said antibiotic is a cell wall synthesis inhibitor.

7. The method of Claim 6 wherein said cell-wall synthesis inhibitor is selected from the group consisting of penicillins, cephalosporins, vancomycin, cycloserine, alafosfolin, fosfomicin, clavulanic acid,
5 bacitracin, moenomycin, and ristocetin.

8. The method of Claim 7 in which said cell wall synthesis inhibitor is ampicillin.

10 9. The method of Claim 2 in which said anti-biotic is an enzyme inhibitor.

10. The method of Claim 9 wherein said enzyme inhibitor is clavulanic acid.

11. A method of controlling aberrant expression vector replication substantially as hereinbefore described with reference to any one of the Examples.

DATED this EIGHTH day of JUNE 1990
Eli Lilly and Company

Patent Attorneys for the Applicant
SPRUSON & FERGUSON

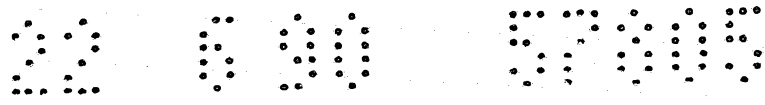


FIG. 1
Construction of Plasmid pL32

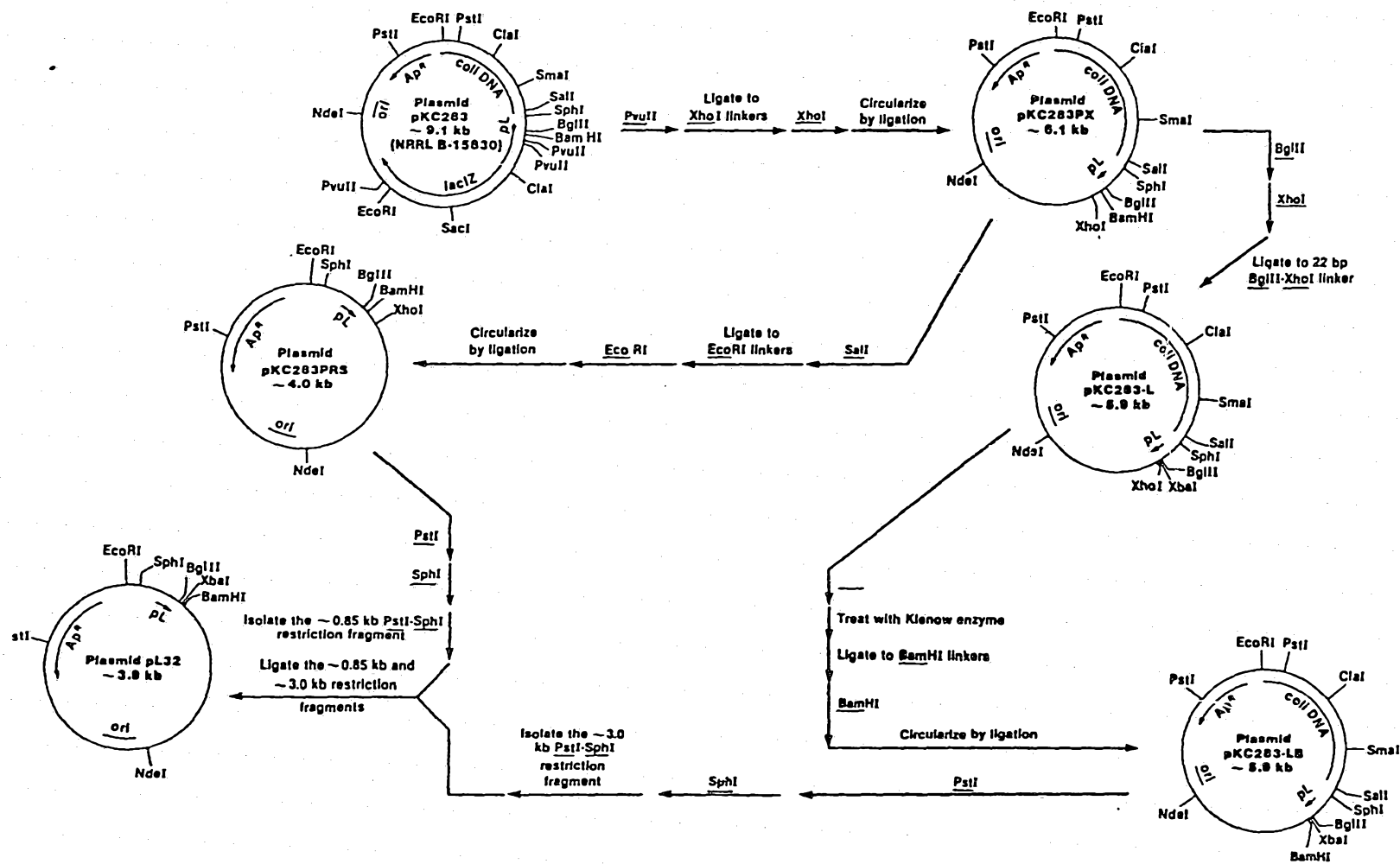


FIG.2
Restriction Site and Function Map of
Plasmid pNM789

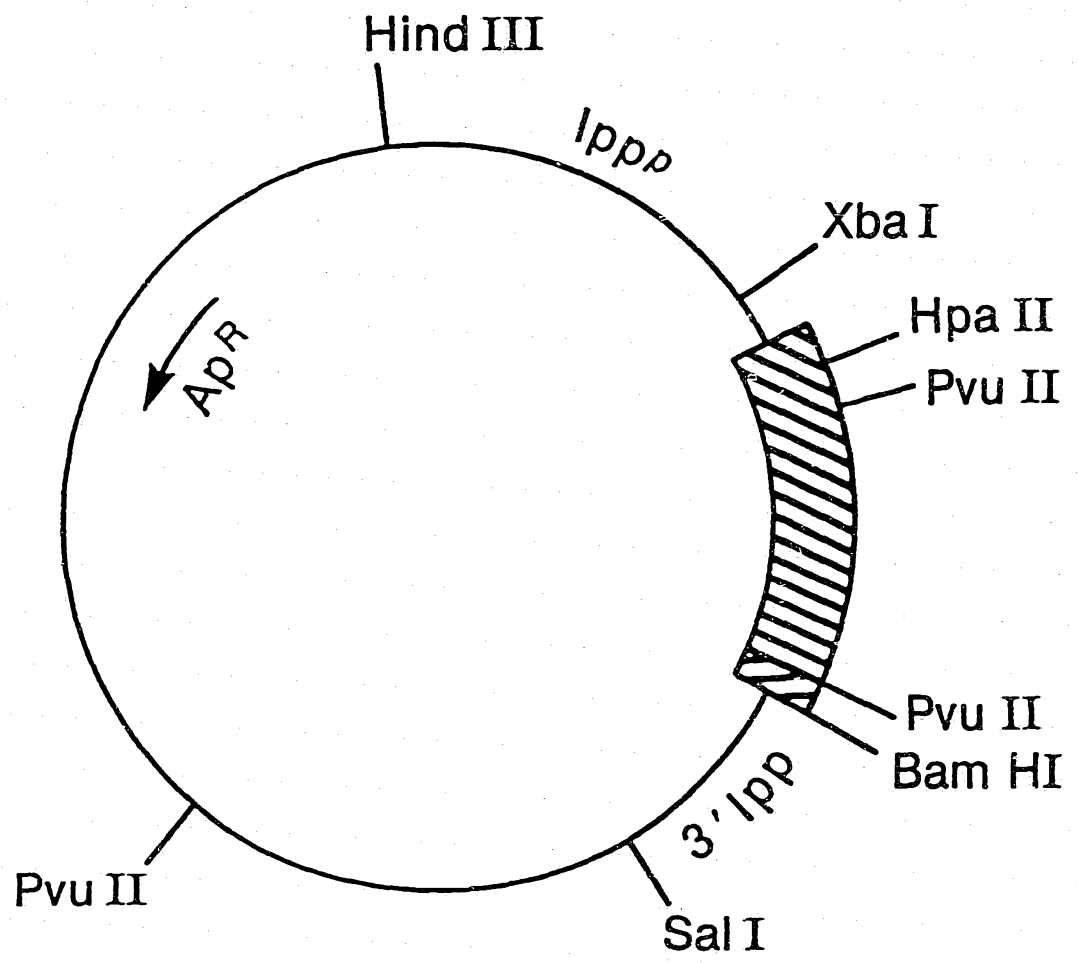
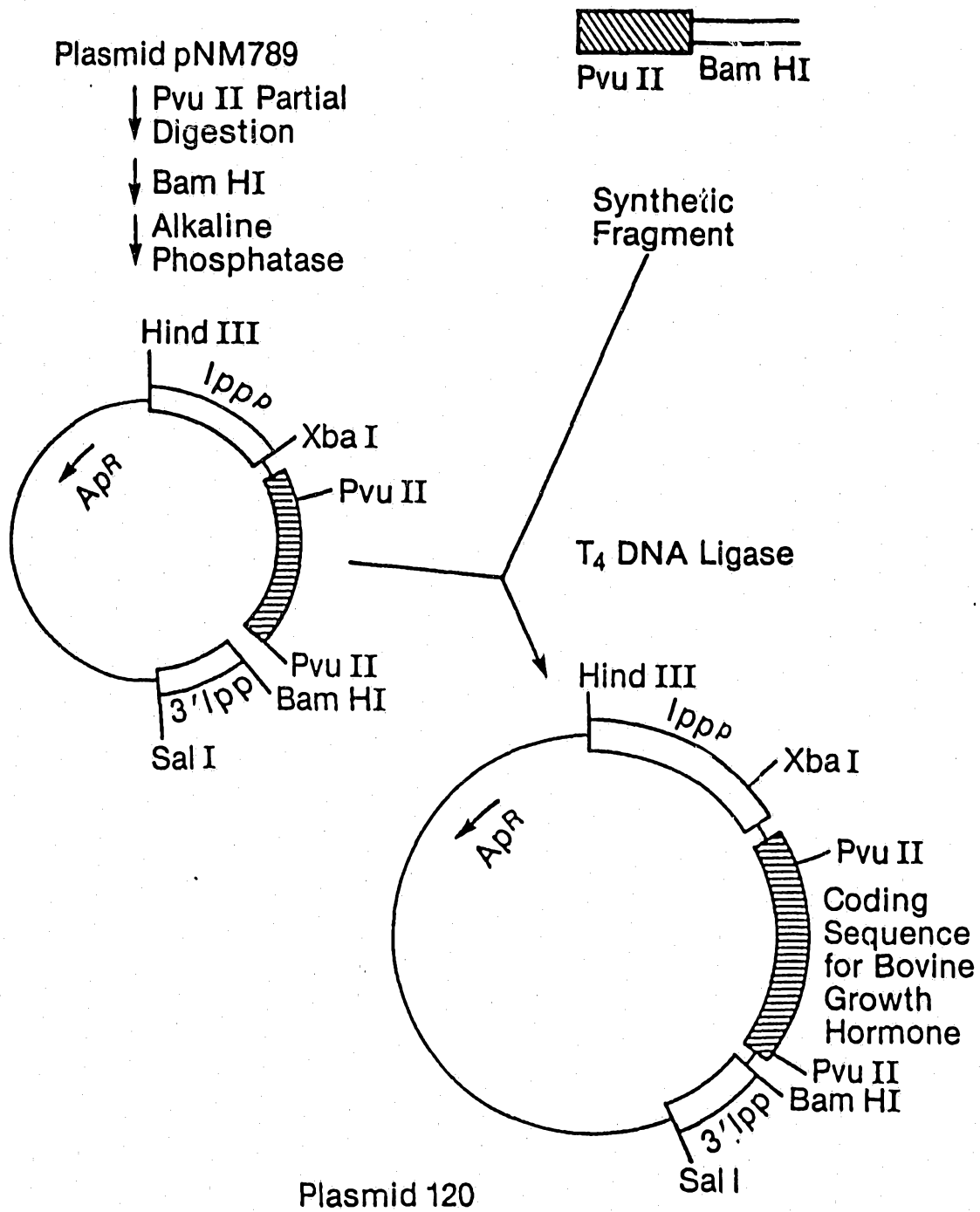


FIG.3



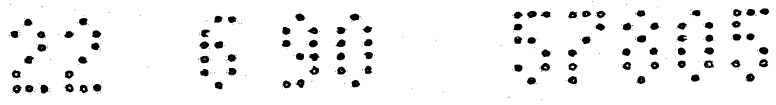


FIG. 4
Construction of Plasmid pL110

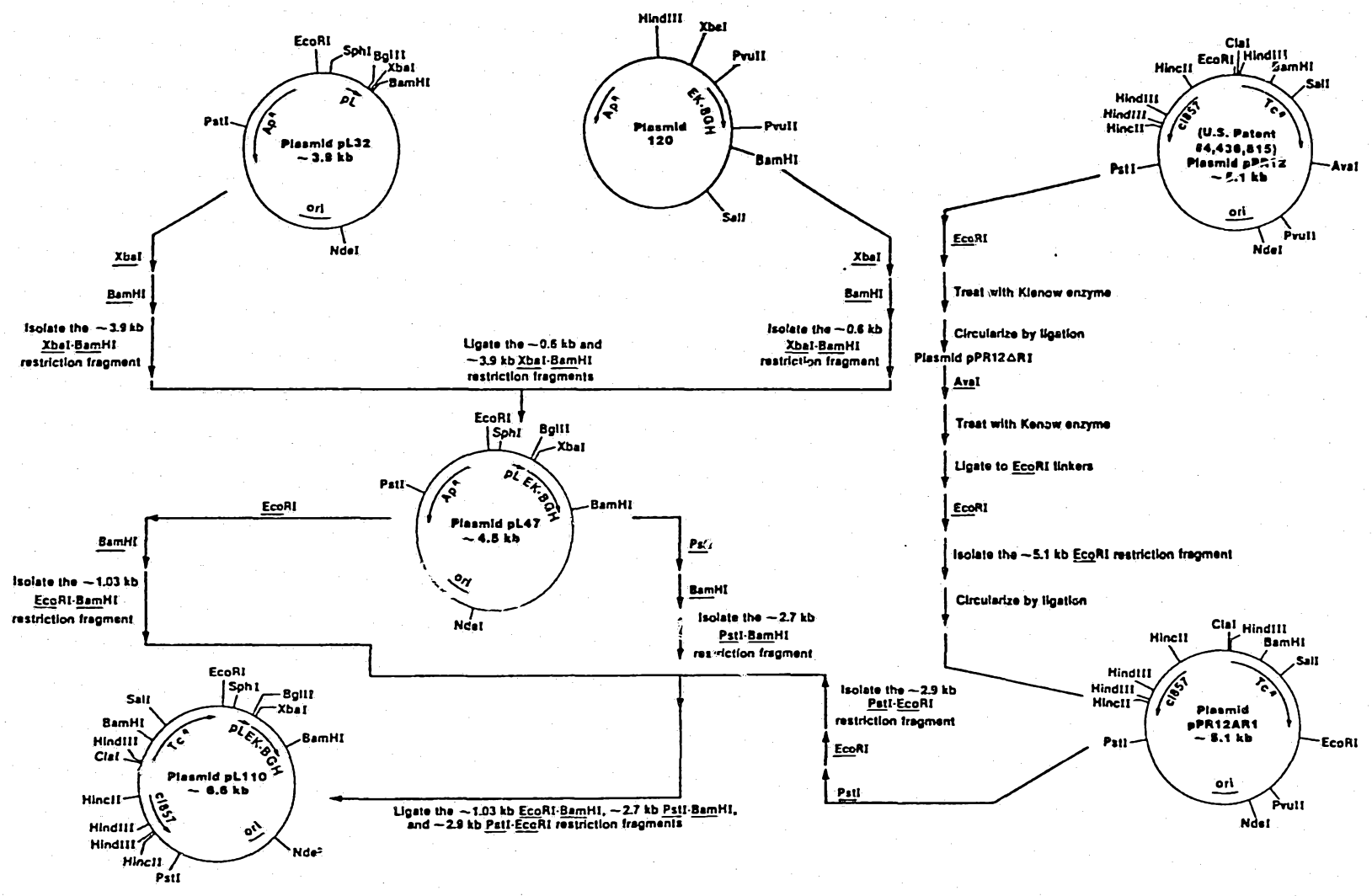


FIG.5
Restriction Site and Function Map
pCZR125

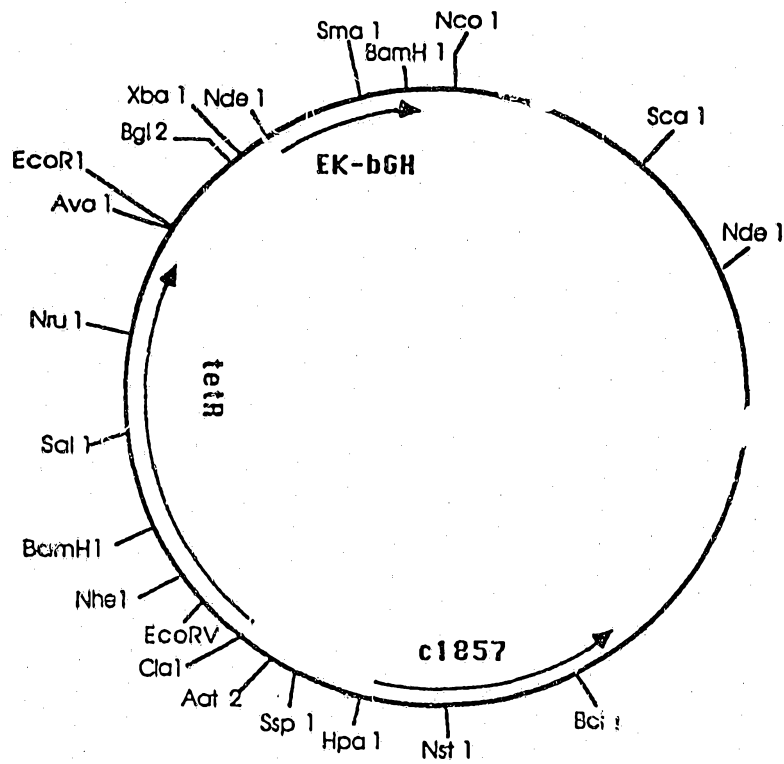


FIG.6
A Fermentation Process Using Cinoxacin to Control Aberrant Expression Vector Replication During Production of EK-bGH

