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TRANSFORMED THEREWITH

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![Diagram of plasmid structure]

(57) Abstract

Chimeric plasmids capable of transforming bacteria and yeast. The plasmids carry the Cm (chloramphenicol resistance) gene and the Tc (tetracycline resistance) gene as selectable markers. The Cm gene allows the plasmids to be selected for in wild-type strains of yeast, including Saccharomyces cerevisiae. The Tc gene allows heterologous genes cloned into the plasmids to be selected for in bacteria, including Escherichia coli.
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CHIMERIC PLASMIDS THAT REPLICATE IN BACTERIA AND YEAST AND MICROORGANISMS TRANSFORMED THEREWITH

This invention relates to molecular biology and, more particularly, to the so-called art of recombinant DNA. Specifically, the invention relates to the construction of improved chimeric plasmids that replicate in bacteria and yeast. These chimeric plasmids carry genetic markers which make them especially suitable for industrial yeast production of cloned gene products.

The invention discloses two unique genetically engineered plasmids: plasmid pV85 and plasmid pVB15. E. coli strains containing these two plasmids have been deposited with the American Type Culture Collection (ATCC), Rockville, Maryland, USA. The E. coli strain containing plasmid pV85 has been assigned ATCC number 31804; the E. coli strain containing plasmid pVB15 has been assigned ATCC number 31803. The strains with the plasmids have been deposited at the ATCC under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of the Patent Procedure. The strains will be maintained, and are available to persons entitled to receive them, in accordance with said Treaty and the rules promulgated thereunder.

As is well known, recent advances in the rapidly developing field of recombinant DNA technology make it possible to construct chimeric plasmids which allow a variety of cloned genes to be expressed in microorganisms. Some of these plasmids replicate in more than one type of microorganisms, e.g., in both bacteria and yeast. K. Struhl et al, Proceedings of the National Academy of Science (U.S.) 76:1035-1039 (1979).

However, the plasmids disclosed thus far are not suitable for a large scale industrial yeast production of cloned gene products because of the types of genetic markers used to select for the presence of the plasmids in bacteria and yeast. For example, some of the plasmids (vectors) disclosed by Struhl et al, supra, use the his3, trpl and ura3 genes as selectable markers. Use of these genes as markers means that the plasmids are only detectable in auxotrophic
strains of yeast. As a result, commercially available wild-type strains of yeast will have to be mutated to his3-, trp1- and ura3- strains before the transformed microorganisms are detectable. Such a mutational requirement severely limits the industrial usefulness of these plasmids. Therefore, it is an object of the present invention to create chimeric plasmids carrying selectable gene markers detectable in commercially available wild-type strains of yeast.

Another plasmid disclosed by Struhl et al, supra, uses resistance to the antibiotic tetracycline as a selectable marker. The gene coding for the protein that confers tetracycline resistance is only expressed in bacteria; thus use of this marker does not allow the plasmid to be selected for in yeast. As a result, the industrial usefulness of this plasmid is also severely limited. Therefore, it is an object of the present invention to create an industrially useful chimeric plasmid carrying a genetic marker which allows the plasmid to be selected for in yeast.

J. Cohen et al, Proceedings of the National Academy of Science (U.S.) 77:1078-1082 (1980), disclosed the construction of a chimeric plasmid carrying the E. coli R factor-derived chloramphenicol resistance gene. Although these investigators are able to demonstrate expression of this gene in the yeast S. cerevisiae, their vector does not carry a second marker gene which easily allows genes cloned into the plasmid to be selected for in bacteria. Lack of such a second marker limits the industrial usefulness of such a plasmid. Therefore, it is an object of the present invention to create a chimeric plasmid carrying a second marker gene which easily allows genes cloned into the plasmid to be selected for in bacteria.

A. Jimenez and J. Davies, Nature 287:869-871 (1980), disclose use of antibiotic G418 as a selective agent. Although these authors allude to the advantages of such a system, Jimenez and Davies do not use a plasmid containing a second gene marker, coding for a protein conferring resistance to a second antibiotic, to allow genes cloned into the plasmid to be selected for in bacteria. As with the plasmid used by Cohen et al, supra, lack of such a second marker also
limits the industrial usefulness of the Jimenez and Davies plasmid as a cloning vector. Also, at the present time antibiotic G418 is very much more expensive than other antibiotics that might be used for selection. Having the selection of cells transformed by the plasmid depend upon a product that is as expensive as G418 severely limits the industrial usefulness of the plasmid. Therefore, it is an object of the present invention to create chimeric plasmids carrying marker genes conferring resistance to antibiotics that are commercially available at prices which do not significantly limit use of the plasmid in large scale industrial fermentations. Other objects of the invention will become apparent to those skilled in the art from the following description, taken in connection with the accompanying drawings wherein:

FIGURE 1 is a diagram of **E. coli** plasmid pACYC184 which indicates the approximate locations of the Cm and Tc genes;

FIGURE 2 is a diagram of chimeric plasmid pBG3 which indicates some of the restriction endonucleases that can be used to remove the yeast *arsl* DNA fragment;

FIGURE 3 is a diagram of **E. coli** plasmid pACYC184 which indicates the nucleotide kilobase pairs that result when the plasmid is digested with various restriction endonucleases;

FIGURE 4 is a diagram of chimeric plasmid pVB5 which indicates the nucleotide kilobase pairs that result when the plasmid is digested with various restriction endonucleases;

FIGURE 5 is a diagram of chimeric plasmid pVB15 which indicates the nucleotide kilobase pairs that result when the plasmid is digested with various restriction endonucleases;

FIGURE 6 is a diagram of the yeast *arsl* DNA fragment which indicates the nucleotide kilobase pairs that result when the fragment is digested with various restriction endonucleases; and

FIGURE 7 is a diagram of an agarose gel of restriction enzyme digests of plasmids pACYC184, pVB5, pVB15 and pLC544.
Very generally the invention involves construction of chimeric plasmids which can replicate in yeast and bacteria. Such plasmids carry one genetic marker that allows genes cloned in the plasmids to be selected for in bacteria, and another that allows the plasmids themselves to be selected for yeast. The genetic markers are genes coding for proteins that confer resistance to common, low cost, commercially available antibiotics. Thus the plasmids are especially suitable for industrial production of cloned gene products in yeast because they do not require the use of auxotrophic strains.

The plasmids are constructed by combining an *E. coli* plasmid and a fragment of *S. cerevisiae* yeast DNA. The *E. coli* plasmid carries all of the genes necessary for replication of the plasmid in *E. coli*. It also carries the Tc gene (coding for a protein that confers resistance to tetracycline) and the Om gene (coding for a protein that confers resistance to chloramphenicol). The Tc gene is used as a marker to allow genes cloned into the plasmids to be selected for in bacteria. The Om gene is used as a marker to allow the plasmids to be selected for in wild-type strains of *S. cerevisiae*. The fragment of *S. cerevisiae* yeast DNA contains all of the genes necessary for replication of the plasmid in this species of yeast. The plasmids thus formed, and disclosed herein, have been designated plasmid pVB5 (ATCC No. 31804) and plasmid pVB15 (ATCC No. 31803). The two plasmids differ from one another only in the orientation of the yeast DNA fragment in the *E. coli* plasmid. Both plasmid pVB5 and pVB15 transform *E. coli* bacteria and *S. cerevisiae* yeast.

In the most preferred form of the present invention both plasmids are constructed by combining a segment of yeast DNA, arsL, characterized by K. Stinchcomb et al, *Nature* 282:39-43 (1979), with *E. coli* plasmid pACYC184, characterized by A. Chang and S. Cohen, *J. Bacteriol.* 134:1141-1156 (1978). In the disclosed embodiment, plasmids pVB5 and pVB15 are constructed by ligating the arsL yeast DNA fragment with *E. coli* plasmid pACYC184. The bacterial DNA and the yeast DNA could be combined by other means known to the art, such as insertion of the fragments into a third vector.
E. coli plasmid pACYC184 contains all of the genes necessary for replication in E. coli. It also contains the gene Cm, which codes for the protein that confers resistance to the common antibiotic chloramphenicol, and the gene Tc, which codes for the protein that confers resistance to the common antibiotic tetracycline. The gene Cm can be functionally expressed in both E. coli and yeast; thus it provides an excellent genetic marker for selecting for the presence of the plasmids in both organisms. The gene Tc is important because it provides an excellent means of screening for the insertion of additional DNA sequences (cloned genes) into the plasmids. The gene Tc is functionally expressed in E. coli. Both chloramphenicol and tetracycline are available commercially at sufficiently low cost for large scale industrial utilization of plasmids carrying these resistance markers.

PLASMID CONSTRUCTION

E. coli plasmid pACYC184 DNA was prepared for use by digesting 2.6 μg of pACYC184 DNA with four units of AvaI endonuclease in AL buffer to a final volume of 339 μl, for 1 hour at 37°C. The components of AL buffer are listed infra. The digestion mixture was heated at 65°C for 5 minutes to inactivate the endonuclease. The DNA was precipitated from the solution with cold 100% ethanol and then dried by lyophilization.

The action of endonuclease AvaI results in single stranded, so-called "sticky" ends being left on the cut ends of the digested pACYC184 plasmid. These "sticky" ends were converted to so-called "blunt" ends by complementary base addition catalyzed by the large fragment of E. coli DNA polymerase I. This conversion was accomplished by resuspending the lyophilized DNA in 10 μl of modified ligation buffer (described infra) and then heating the DNA to 70°C for 2 minutes in order to open the "sticky" ends. The mixture was cooled to 0°C on ice and 1 μl of DNA polymerase I - large fragment (0.7 units per μl) was added. After 5 minutes, the mixture was heated at 70°C for an additional 5 minutes to inactivate the polymerase. The mixture
was then frozen at -20°C for later use. *E. coli* plasmid pACYC184 is diagramed in FIGURE 1.

In order to eliminate the single stranded ends of the yeast ars1 DNA fragment, it was incorporated into a plasmid known as pBG3. This plasmid is diagramed in FIGURE 2. The ars1 fragment was then removed from plasmid pBG3 by digesting 14 µg of plasmid pBG3 with 20 units of SmaI endonuclease. Digestion was carried out in R buffer plus 6 mM KCl, to a final volume of 800 µl, for 1 hour at 37°C. The DNA fragments were then separated by electrophoresis on a 0.8% agarose gel (4 tracks at 200 µl per track). The bands of DNA corresponding to the ars1 fragment were eluted from the gel and precipitated with ethanol using standard procedures. Since the pBG3 plasmid was digested with SmaI, an endonuclease which produces "blunt" ends rather than "sticky" ends, no further modification of the ars1 fragment was necessary.

Plasmid pACYC184 was then ligated with the ars1 DNA fragment by mixing 2.5 µg of pACYC184 DNA with 4.8 µg of the ars1 fragment, in the presence of 1 µl of 10 mM ATP and T4 ligase in ligation buffer to a final volume of 11 µl. The DNA ligation reaction mixture was incubated at 16°C for 21 hours and then the 11 µl was diluted with 14 µl of TE buffer for use in transformation of *E. coli* strain JA300. Ligation buffer and TE buffer are described infra.

TRANSFORMATION OF JA300

Transformation of *E. coli* strain JA300 was carried out according to standard procedures except that, prior to plating, the cells were centrifuged and resuspended in 0.4 µl of VB medium. Cells from the transformation mix were plated on VB medium, VB medium plus chloramphenicol (20 µg per µl) and on No. 454 medium, a complex complete growth medium. Non-transformed JA300 cells were also plated on VB medium. VB medium and No. 454 medium are described infra. It should be noted the VB medium contains casamino acids which lack the amino acid tryptophan.
JA300 is a tryptophan-requiring, chloramphenicol-sensitive strain of *E. coli*. The *arsl* DNA fragment contains the yeast *trpl* gene; this gene can be expressed in *E. coli* and can make the JA300 strain tryptophan non-requiring (*trp*⁺). Therefore, a simple determination of the frequency of desired transformants can be made by comparing the difference in the number of tryptophan non-requiring colonies between untreated and DNA-treated JA300 cells. In one experiment, DNA treatment resulted in an 8-fold increase in *trp*⁺ cells: 1.6 trp⁺/10⁸ cells in the untreated cell sample versus 13.4 trp⁺/10⁸ cells in the DNA-treated sample.

CHARACTERIZATION OF PLASMID DNA IN THE *E. COLI* TRANSFORMANTS

Approximately 90% of the *trp*⁺ *E. coli* cells were probably transformants containing the desired plasmid. 82 colonies from the transformation plates (VB medium and VB medium plus chloramphenicol) were picked and retested for the tryptophan requirement and the chloramphenicol resistance. Of the 84 colonies picked, 72 were *trp*⁺ and chloramphenicol-resistant. Twenty-five of these picked strains were checked for plasmids using a quick plasmid screen procedure and then compared to two purified plasmid DNAs, i.e., plasmid pACYC184 (4.0 kilobase pairs) and plasmid pHOS101 (5.1 kilobase pairs). One Kbp is 1,000 nucleotide base pairs. *E. coli* plasmid pACYC184 plus the yeast *arsl* fragment would result in a plasmid containing about 5.45 Kbp. Twenty-one of the 25 strains examined contain plasmids equal or greater in size than the 5.45 Kbp expected; the larger plasmids are most likely multimers. Six of these plasmids were examined in more detail by restriction endonuclease digestion followed by agarose gel electrophoresis. Two plasmids, designated as plasmid pVB5 and plasmid pVB15, appear to result from ligation of the *arsl* yeast fragment with *E. coli* plasmid pACYC184. The plasmids are identical except for the orientation of the yeast *arsl* fragment in the *E. coli* DNA.

FIGURE 3 is a diagram of *E. coli* plasmid pACYC184 showing the position of some of the restriction endonuclease digestion sites, and the DNA segments generated by digestion with these restriction endonucleases when used either alone or in combination. Chimeric
plasmids pVB5 and pVB15 are diagramed in FIGURES 4 and 5 respectively. The map for E. coli plasmid pACYC184 is the same as that published previously by Chang and Cohen, supra, except for the addition of the AvaI restriction site. A map for the arsI segment of the yeast DNA has been published by G. Tschumper and J. Carbon, Gene 10:157-166 (1980) and is diagramed with a few of the restriction endonuclease sites in FIGURE 6. The maps of plasmid pVB5 and pVB15 differ in the orientation of the arsI fragment in the AvaI site of plasmid pACYC184. The assignment of plasmid pVB5 and plasmid pVB15 to their appropriate maps was determined by agarose gel analysis of the restriction endonuclease digests.

A set of restriction endonuclease digests of plasmids pACYC184, pVB5, pVB15, and pLCS44 separated on agarose gel is diagramed in FIGURE 7. Tracks 10, 11 and 12 are digests of three of the plasmids with the endonuclease BamHI. Since BamHI cuts each plasmid only once, it was determined that the size of pVB5 and pVB15 are between 4.5 and 6.5 Kbp, based on the molecular weight standards in track number 1.

Digestion of E. coli plasmid pACYC184 with the endonuclease EcoRI (track number 9) again produces a single full length fragment; however digestion with both EcoRI and AvaI (track number 8) produces two fragments of approximately 2.77 and 1.20 Kbp. Digestion of pVB5 (track number 7) and pVB15 (track number 6) with EcoRI produces three bands; two of the bands correspond to the bands in the EcoRI-AvaI digest of pACYC184 (track number 8) and the third corresponds to the 1.45 Kbp band in track 5 which is known to be the arsI fragment. Thus the data is compatible with the genetic maps shown in FIGURES 4 and 5. The insertion site of the arsI fragment into pACYC184 was confirmed by digestion with the restriction endonuclease HincII. HincII cut pACYC184 in two places, resulting in fragments of 2.87 and 1.10 Kbp. Digestion of pVB5 and pVB15 with HincII produced three fragments, one of 2.87 Kbp and two smaller pieces. Since the third HincII site is known to be in the arsI DNA segment, the size of the two small fragments allowed the orientation of the arsI fragment in the plasmid to be determined.
TRANSFORMATION OF YEAST WITH PLASMIDS pVB5 and pVB15

After the pVB series of plasmids had been constructed and characterized with the quick plasmid screen and restriction enzyme digests, plasmids pVB5 and pVB15 were chosen for use in the transformation experiments because they contained the ars1-trp1 DNA inserted in opposite directions in plasmid pACYC184.

Yeast strain C483 was chosen for transformation. Strain C483 is a haploid S. cerevisiae of mating type a that is defective in a gene for tryptophan biosynthesis (trp1) and cannot ferment galactose (gal2) or maltose (mal). Since yeast strain C483 cannot grow without tryptophan being added to the medium, transformation frequencies with plasmids pVB5 and pVB15 were measured by monitoring growth of DNA-treated cells on media without tryptophan. For purposes of this disclosure, minimal media without tryptophan is called selective media and minimal media with added tryptophan is called complete media.

The transformation experiments involved three steps: protoplasting the yeast, i.e., removing the yeast cell walls; exposing them to DNA; and treating them with an agent that causes the uptake of the plasmid DNA. The source of the DNA was either plasmid pVB5 or pVB15. Approximately 2.3 x 10^8 protoplasts from strain C483 were incubated with 10 μg of plasmid DNA from pVB5 or pVB15 for 15 minutes at room temperature. In order to encourage the uptake of the plasmid DNA, polyethylene glycol (PEG) was added and the mixture incubated for an additional 40 minutes. The polyethylene glycol is a 44% solution in 10 mM tris-HCl plus 10 mM CaCl₂ at pH 7.5. Dilution and plating on either selective or complete media followed directly after the 40 minute incubation in PEG.

Protoplasts that were transformed with plasmid pVB5 or pVB15 were plated on selective medium, as were protoplasts that had been through the same series of treatments but had never been exposed to plasmid DNA. These untransformed protoplasts were controls that showed the frequency with which strain C483 reverted from requiring
tryptophan to being able to make its own. For purposes of this disclosure the C483 cells now able to synthesize tryptophan are referred to as back-mutants.

Transformation with plasmids pVB5 and pVB15 led to titers of 6.3 x 10^4 and 5.48 x 10^4 transformants per 2.3 x 10^8 protoplasts respectively; the back-mutant titer was 1.1 x 10^3 reverting per 2.3 x 10^8 protoplasts. In other words, transformation with plasmids pVB5 or pVB15 led to a 50-60 fold increase in the occurrence of colonies on plates without tryptophan. Expressed in a slightly different way, plasmid pVB5 yielded 6.19 x 10^3 colonies per µg of DNA added and plasmid pVB15 yielded 5.37 x 10^3 colonies/µg DNA added.

Both plasmids pVB5 and pVB15 contain the yeast *ars1* DNA fragment. Plasmids containing this fragment are expected to transform yeast at a frequency of 5-50 x 10^3 colonies per µg of DNA (D. Stinchcomb et al, Proceedings of the National Academy of Science (U.S.) 77:4559-4563 (1980)). The results of this disclosure concur with the Stinchcomb et al expectation. The frequencies of transformation for plasmid pVB5 and pVB15 are not substantially different; thus it appears that the orientation of the *ars1-trp1* DNA fragment in the pVB plasmids does not significantly influence transformation.

Complete medium was used for plating further controls for this experiment. The complete medium demonstrated the protoplasts' ability to regenerate cell walls and form colonies. These complete medium controls indicated that the protoplasts are healthy and that approximately 5.8% of those present in the original mix were capable of forming colonies on minimal media. This percentage is called the regeneration efficiency; the normal regeneration efficiency range for yeast strain C483 is between 5 and 20%. Thus this experiment demonstrates that plasmid pVB5 and pVB15 transformed yeast strain C483 at frequencies consistent with those expected for plasmids carrying an *ars1* fragment, and that orientation of insertion of the *ars1-trp1* DNA does not appear to significantly influence transformation frequencies.
To show that the chloramphenicol resistance gene could be used as a selectable marker in yeast, we plated cells of strain C483 and cells of strain C483 transformed with pVB5 on chloramphenicol gradient plates. The gradient plates were prepared in 90 mm square petri dishes (nominally 100 mm). A 25 ml portion of minimal media containing tryptophan was mixed with 0.5 ml of DMSO containing 0.7 mg of chloramphenicol and was allowed to solidify in the petri dishes on a slanted table. An additional 25 ml of minimal media containing tryptophan was added while the plates were horizontal. The gradient plates were allowed to equilibrate overnight. After spotting both strains on the gradient plates, we incubated the plates at 30°C. The plates were checked after 2 and 5 days of incubation, and at both times, the strain transformed with pVB5 grew at significantly higher concentrations of chloramphenicol than did the untransformed strain. This difference is satisfactory for selection of transformants on the basis of resistance to chloramphenicol.

It may be seen, therefore, that the invention discloses chimeric plasmids capable of transforming E. coli bacteria and S. cerevisiae yeast. The plasmids are especially suitable for industrial production of cloned gene products in yeast because they can be used with wild-type strains of S. cerevisiae. The plasmids carry the Om and Tc genes as selectable markers. The Om gene allows the plasmids to be selected for in S. cerevisiae; the Tc gene allows heterologous genes cloned into the plasmids to be selected for in E. coli.

Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.
MEDIA AND BUFFERS

AL Buffer (10x concentration)

100 mM Tris-HCl, pH 7.9
79 mM MgCl₂
5 600 mM NaCl
60 mM β-mercaptoethanol

Prepare as follows:
17 ml DDI water
10 ml 1M Tris-HCl pH 7.9
10 ml 1M MgCl₂
60 ml 1M NaCl

Autoclave for 20 minutes.
After autoclaving add 6.0 ml of 1M β-mercaptoethanol

Ligation Buffer (10x concentration)

15 500 mM tris-HCl, pH 7.4
10 mM EDTA, pH 8.1
100 mM MgCl₂
100 mM dithiothreitol

Modified Ligation Buffer

20 10 μl 10x ligation buffer
10 μl 100 μM dATP
10 μl 100 μM dGTP
10 μl 100 μM dCTP
10 μl 100 μM dTTP
25 50 μl glass distilled H₂O
1 μl 1% bovine serum albumin
R Buffer (10x concentration)

100 mM Tris-HCl, pH 7.8
70 mM MgCl₂
60 mM β-mercaptoethanol

1 mg/ml gelatin

Prepare as follows:
20 ml DDI water
10 ml 1M Tris-HCl pH 7.8
7 ml 1M MgCl₂

0.1g gelatin (heat to dissolve gelatin)

Add DDI water to 94 ml.
Autoclave 20 minutes.
After autoclaving add 6.0 ml of 1M β-mercaptoethanol

TE Buffer (10x concentration)

100 mM Tris-HCl, pH 8.0
1 mM EDTA

VB Medium

0.2 g MgSO₄·7H₂O
2.0 g Citric Acid · H₂O
10.0 g K₂HPO₄
3.5 g Na(NH₄)HPO₄ · 4H₂O
844 ml distilled water

After autoclaving add:
25 ml sterile 40% glucose

25 100 ml sterile 2% casamino acids
1 ml sterile 1% vitamin B1
10 ml sterile 0.1% thymidine
20 ml sterile 0.1% chloramphenicol

#454 Medium
Difco Antibiotic Medium #2

5 gms
Composition:
Bacto Beef Extract 1.5gm
Bacto Yeast Extract 3gm
Bacto Peptone 6gm

10 gms
Bacto Agar 15gm

Water 1000 ml

Autoclave.
CLAIMS:

1. Plasmid pVB5.


3. A gram-negative bacterium transformed with a plasmid selected from the group consisting of pVB5 and pVB15.

4. A bacterium according to Claim 3 which is of species Eschericia coli.

5. A bacterium according to Claim 4 which is transformed with plasmid pVB5 and a culture of which is deposited in the American Type Culture Collection under deposit no. 31804.

6. A bacterium according to Claim 4 which is transformed with plasmid pVB15 and a culture of which is deposited in the American Type Culture Collection under deposit no. 31803.

7. A yeast cell transformed with a plasmid selected from the group consisting of pVB5 and pVB15.

8. A yeast cell according to Claim 7 which is of species Saccharomyces cerevisiae.
FIG. 7
DIAGRAM OF AGAROSE GEL OF RESTRICTION ENZYME DIGESTS OF pACYC184, pVBI5, pVB5 AND pLC544

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<tr>
<th>TRACKS</th>
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TRACK
1. DNA MOLECULAR WEIGHT STANDARDS
2. pVBI5 DIGESTED WITH HincII
3. pVB5 DIGESTED WITH HincII
4. pACYC184 DIGESTED WITH HincII
5. pLC544 DIGESTED WITH EcoRI
6. pVBI5 DIGESTED WITH EcoRI
7. pVB5 DIGESTED WITH EcoRI
8. pACYC184 DIGESTED WITH EcoRI AND AvaI
9. pACYC184 DIGESTED WITH EcoRI
10. pVBI5 DIGESTED WITH BamHI
11. pVB5 DIGESTED WITH BamHI
12. pACYC184 DIGESTED WITH BamHI
#### INTERNATIONAL SEARCH REPORT

**International Application No:** PCT/US 84/00867

**I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)**

According to International Patent Classification (IPC) or to both National Classification and IPC:

- IPC: C 12 N 15/00; C 12 N 1/20; C 12 N 1/18 // (C 12 N 1/18; C 12 R 1/865) (C 12 N 1/20; C 12 R 1/19)

**II. FIELDS SEARCHED**

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<th>Classification System</th>
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Documentation Searched other than Minimum Documentation to the extent that such Documents are Included in the Fields Searched

**III. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
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<tr>
<th>Category</th>
<th>Citation of Document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to Claim No.</th>
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<tr>
<td>E</td>
<td>US, A, 4477571 (S. CHANG et al., CETUS CORP.) 16 October 1984 see the entire document</td>
<td>1-8</td>
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<td>A</td>
<td>Current Topics in Microbiology and Immunology, vol. 96, 1982 C.P. Hollenberg: &quot;Cloning with 2.4M DNA vectors and the expression of foreign genes in Saccharomyces cerevisiae&quot;, pages 119-144</td>
<td></td>
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**IV. CERTIFICATION**

Date of the Actual Completion of the International Search: 30th January 1985

International Searching Authority: EUROPEAN PATENT OFFICE

Date of Mailing of this International Search Report: 2 FEB. 1985

Signature of Authorized Officer: G.L.M. Kromberg

Form PCT/ISA/210 (second sheet) (January 1985)
### III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

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