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Bradley J. Kleinsasser

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CONSTRUCTION AND CHARACTERIZATION OF LAC CONTAINING PLASMIDS AND THE TRANSFORMATION OF ESCHERICHIA COLI AND SACCHAROMYCES CEREVISIAE WITH THESE PLASMIDS

BY

BRADLEY J. KLEINSASSER

A thesis submitted in partial fulfillment of the requirements for the degree Master of Science Major in Microbiology

South Dakota State University 1984
CONSTRUCTION AND CHARACTERIZATION OF
LAC CONTAINING PLASMIDS AND THE
TRANSFORMATION OF ESCHERICHIA COLI
AND SACCHAROMYCES CEREVISIAE WITH
THESE PLASMIDS

This thesis is approved as a creditable and
independent investigation by a candidate for the degree,
Master of Science, and is acceptable for meeting the thesis
requirements for this degree. Acceptance of this thesis
does not imply that the conclusions reached by the candidate
are necessarily the conclusions of the major department.

Carl A. Westby
Thesis Advisor

Date

Head, Microbiology

Date
ACKNOWLEDGMENTS

I wish to thank Dr. Carl Westby for "the chance" and for help in preparing this manuscript.

I wish to acknowledge the late John McAdaragh, he cared.

Many thanks to Anand Rao for his patience and help with the word processor.

Special appreciation is extended to Cindy Peterson for her assistance and encouragement.

Sincere gratitude is felt for my parents, Lewis and Florene Kleinsasser, for their endless support.
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INTRODUCTION AND LITERATURE REVIEW

OVERVIEW OF GENETIC ENGINEERING

The science of genetic engineering or direct genetic manipulation required the availability of four basic procedures. These procedures are: i) a way to cut and join DNA molecules, ii) a carrier or vector for genes that can replicate both itself and the foreign DNA it harbors, iii) a way of gaining entrance for this carrier and its foreign DNA into a host cell, and iv) a way of selecting the cells which had received the chimera DNA from among a large cell population which had not.

A major breakthrough in the first procedure was made in 1970 with the discovery of a gene cutting enzyme (HindII) from Haemophilus influenza serotype d. This was the first of many class II restriction endonucleases that were subsequently discovered (50). The Haemophilus enzyme was first found and described by Smith et al (78,46). Class II enzymes differ from the previously reported Class I enzymes described by Meselson and Yuan (52) and Linn and Arber (47). Both class I and the class II enzymes recognize a specific nucleotide sequence on DNA but Class I enzymes cut at sites different from the site used for recognition. Class II enzymes, on the other hand, including Bam HI, KPNI, and HindIII cut only at the nucleotide sequence originally recognized. Fragments resulting from Class II restriction
enzymes when separated by agarose gel electrophoresis (72) can be visualized and therefore recovered, by staining the gel with ethidium bromide and viewing the stained DNA bands under ultra-violet irradiation.

Previously (1967) DNA ligases had been discovered simultaneously by several independent groups (32,84,57,31,23). This made it possible to covalently join together DNA strands having either complementary ends (68) or with T-4 ligase, blunt ends (68).

The role of R factor plasmid DNA in the antibiotic resistance of *Escherichia coli* prompted an intensive study of this and other plasmids (53,55,22,74,73) during the 1960s and early 1970s. The discovery by Cohen (20), that foreign DNA could be inserted into a single restriction site (EcoRI) located on a plasmid (pSC101) and the recombinant DNA propagated successfully in *E. coli*, lead quickly to the development of specialized plasmid vectors for the cloning of DNA. Many of these developed vectors such as pMB9 and pBR322 are derivatives of the plasmid ColE1 (40,63,11), are present at 30-50 copies per cell under normal growth conditions, and can be amplified to greater copy numbers with chloramphenicol.

Transformation was the process exploited for host uptake of recombinant DNA. For an overview of microbial transformation the reader is referred to other reviews (49,24,39). One of the first developments in transformation
relating directly to genetic engineering was the discovery of Morton, Mandel and Higa (49). They found that treatment of E. coli with calcium salts enabled the bacterium to take up viral DNA. Cohen and coworkers subsequently found that E. coli treated with calcium chloride would take up purified R factor DNA and transformants carrying this plasmid could be selected by growth on suitable antibiotic containing media (21).

Since the beginning, gene engineering has relied mainly on the bacterium E. coli as the host for receiving and maintaining chimeric DNA molecules. The plasmid pBR322 has been the most versatile and widely used vector for cloning double stranded DNA in E. coli (11). The size of pBR322 DNA is relatively small, 4,362 base pairs (54). Smaller plasmids like pBR322 are less susceptible to physical damage, have fewer multiple restriction sites, and generally produce higher copy numbers in the host (50). The plasmid pBR322 was derived from the R factor ColE1 and replicates under relaxed control, that is it replicates independently of host chromosome replication (56) and can therefore be amplified. Chloramphenicol, an agent which binds to the 50S subunit of ribosomes and so prevents protein synthesis (50), was found by Clewell (19) to amplify (increase) ColE1 derived plasmid copy numbers from an average of 24 copies per cell to 3,000. Plasmid pBR322 DNA carries genes for ampicillin and tetracycline resistance and
these serve as convenient selective markers.

**TRANSFORMATION OF _SACCHAROMYCES CEREVISIAE_**

Although most gene engineering has been carried out on _E. coli_, interest was generated by Sinclair et al. (76) and Guerineau et al. (36) for cloning in _Saccharomyces cerevisiae_ from reports by these investigators of yeast circular plasmid DNA molecules with a 2um contour length (76,36). These 2um circles have the same density as yeast nuclear DNA (76,36), and are usually present in copy numbers of from 50 to 100 molecules per cell. They comprise approximately 4% of the total yeast DNA (15) and contain an estimated 6,000 to 7,000 nucleotide base pairs (3). The 2um DNA also referred to as SCpI is not nuclear (18) but cytoplasmic (48) and is self replicating with one origin of replication (60). The SCpI plasmid structure has been studied extensively and described in detail (48,45,37).

Hinnen and coworkers in 1978 demonstrated transformation of _S. cerevisiae_ with (42) a ColE1 derived plasmid carrying a segment of DNA from the yeast chromosome with the LEU2 gene. Since then considerable effort has been put forth in developing _S. cerevisiae_ into a more suitable host for DNA cloning. As a result of this work it was found that many yeast genes including LEU2, HIS3, and URA3 transform yeast in an integrative manner and at a low frequency (42,43) while other genes, for example the trpl
gen, transform yeast at higher frequencies but in a non-integrative manner (81). The term "integrative" and "non-integrative transformation" means the plasmid associated gene during transformation either becomes integrated into the yeast chromosome, or does not integrate but instead remains as an autonomously replicating molecule (42). Any gene, it was later found, when associated with 2um circle DNA gave a much higher transformation efficiency than was obtained without the association. Plasmids carrying 2um circle DNA, in addition to giving higher transformation efficiencies, exhibit both forms of transformation and in the non integrative form they have an average copy number of 5 to 10 plasmids per yeast cell. These plasmids also replicate as a stable chromosomally integrated structure (81). The plasmid molecules YEp-13 and pZ-1000 both carry 2um DNA. The presence of 2um DNA in the plasmid has improved the efficiency of what previously had been poor transformation efficiency genes. For example, the LEU2 gene from yeast chromosome 3 (41) which encodes for the biosynthetic enzyme, B-isopropylmalate dehydrogenase (62,41), and was first cloned and selected in E. coli (62), transforms the leu AH22 yeast strain at a frequency of 1.0 $\times 10^{-7}$ transformants per viable cell (42). The LEU2 gene in association with yeast 2um circle DNA was later shown to be transformed with the much higher frequency of 5 $\times 10^{-4}$ to 3 $\times 10^{-3}$ transformants per viable cell (7).
USE OF LAC OPERON GENES

The lac operon of *E. coli* is a segment of DNA on the chromosome containing three genes designated Z, Y, and A. These are adjacent to a promoter and operator region. The Z gene specifies the amino acid sequence of beta-galactosidase. The Y gene specifies the sequence of beta-galactosidase permease, and the A gene specifies the amino acid sequence of B-thiogalactosidase acetyltransferase. An I gene, although not part of the lac operon controls it by functioning as a repressor gene. The I gene is transcribed and translated into a repressor protein. This repressor binds to the operator region just downstream from the promoter preventing transcription of the Z, Y, and A genes. The inducer lactose (actually allolactose) if present combines with the repressor at a separate site, changing the configuration of the repressor so that it can no longer bind to the operator. This permits transcription (initiated at the promoter site) and subsequent translation of the Z, Y and A genes.

Interest in the *E. coli* lac operon for cloning in yeast was quite natural owing to the previously widespread use of lac5 (*E. coli* promoter, operator, and Z gene) as a general indicator of transformation success in *E. coli* (9). The lac5 segment produces beta-galactosidase constitutively in *E. coli* which in the presence of the chromogenic substrate, 5-chloro-4-bromo-3-indolyl-B-D-
galactoside, (XG), forms vivid blue colonies on agar. Insertions of foreign DNA into lac5 stop production of beta-galactosidase and therefore indicate that insertion of foreign DNA has taken place.

Lac operons are currently being used to further modify ColEl derived plasmids carrying the 2um circle DNA (34). Recently the insertion of an E. coli lac Z gene and Kluyveromyces lactis LAC4 gene into plasmids intended for S. cerevisiae transformation have been described (59,29).

Purposes of this study were to obtain sufficient lambda lac5 DNA for plasmid insertion. This recombinant plasmid was then to be used for transformation of E. Coli and S. cerevisiae. The ultimate goal was to obtain lactose fermenting strains of S. cerevisiae.

MATERIALS AND METHODS

Microbial strains and plasmids used in this study are described in Tables 1 and 2 respectively.

**Escherichia coli** GROWTH MEDIA

The *Escherichia coli* bacteriophage host Y-mel (see Table 1) was grown in NZYDT medium containing per liter: 5g NaCl, 2g MgCl. 7H O, 10 g "NZ amine A" (type A hydrolysate of casein from Hum-Ko-Sheffield division of Kraftco Corp., West Lynnhurst, N.J. 07071), 5g yeast extract (Difco), 0.04g thymidine (Sigma), 0.01g diamino pimelic acid (Sigma), and when the medium was used to make plates or slants 20 g of
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> K802</td>
<td>galK2, galT22, metB1, lacY1, supE44, hsdR2</td>
<td>ATCC</td>
</tr>
<tr>
<td>ATCC no. 33526</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> Ymel</td>
<td>pro, sul11, F</td>
<td>F. Blattner</td>
</tr>
<tr>
<td><em>E. coli</em> CSH18</td>
<td>*(lac pro), supE, thi, <em>(F' lacZ pro)</em> A+B+</td>
<td>F. Blattner</td>
</tr>
<tr>
<td><em>E. coli</em> LG90</td>
<td>F, <em>(lac pro XIII)</em></td>
<td>ATCC</td>
</tr>
<tr>
<td>ATCC no. 33858</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> MC1066</td>
<td>leuB6 *(lacI POZY)*X74 trpC9830 pyrF::Tn5 *(Kmr) strA</td>
<td>T. Zamb</td>
</tr>
<tr>
<td><em>E. coli</em> HB101</td>
<td>F, leuB6, proA2, recA13, thi-1, ara-14, lacY1, galK2, xyl-5, mtl-1, rpsL20, *-, supE44 [hsdS20]r-, m-</td>
<td>N. Alexander</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>a, leu2-3, leu2-112, his4-519, kanl</td>
<td>N. Alexander</td>
</tr>
<tr>
<td>AH22</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>leu2, ura3, his, kan</td>
<td>T. Zamb</td>
</tr>
<tr>
<td>SSU10</td>
<td></td>
<td>NRRL</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td></td>
<td>NRRL</td>
</tr>
<tr>
<td>Y-2034</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y-1572</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lambda bacteriophage</td>
<td>lac5 KH100 BW1 nin5</td>
<td>F. Blattner</td>
</tr>
<tr>
<td>Cl857S7 lac5</td>
<td></td>
<td>QSR80</td>
</tr>
</tbody>
</table>
agar was added. All other E. coli strains were grown in either Luria Bertani medium (LB) (27) or in basic growth medium which contains per liter minus the carbohydrate, 5g Casamino acids with vitamins (Difco), 7g K HPO$_4$, 1g $(NH_4)SO_4$, 0.5g Na citrate, 0.15g thiamine hydrochloride, and 0.1g MgSO$_4$·7H$_2$O, pH 7.0. The carbohydrate source used in all of the above media was either 2% D-glucose or 1 to 2% lactose. Concentrated solutions of these were autoclaved separately and then after cooling were aseptically added to solutions of the above (autoclaved) to the desired concentration. Antibiotic supplements of ampicillin and tetracycline were 50 and 25 μg/ml, respectively for all growth media. These antibiotics were filter sterilized separately before being added to the media.
SACCHAROMYCES CEREVISIAE GROWTH MEDIA

Yeast strains (see Table 1) were grown either in YPD medium containing per liter 10g Bacto yeast extract (Difco), 20g Bacto peptone (Difco) and 20g D-glucose or they were grown in SD Minimal medium (70). Media used for growing E. coli and yeast auxotrophs (see Table 1) were supplemented with 30 ug/ml L-leucine and 20 ug/ml L-histidine

TABLE 2. Plasmids used for DNA Transformation

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Size (daltons)</th>
<th>Markers</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBR322</td>
<td>2.8 x 10^6 d</td>
<td>amp, tet</td>
<td>B. Pilacinski</td>
</tr>
<tr>
<td>pBR325</td>
<td>3.6 x 10^6 d</td>
<td>amp, tet</td>
<td>B. Pilacinski</td>
</tr>
<tr>
<td>YEp13</td>
<td>6.96 x 10^6 d</td>
<td>amp, tet</td>
<td>N. Alexander</td>
</tr>
<tr>
<td>pZ1000</td>
<td>15.0 x 10^6 d*</td>
<td>amp</td>
<td>T. Zamb</td>
</tr>
</tbody>
</table>

1. tet represents tetracycline resistance gene
2. amp represents ampicillin resistance gene
3. d represents daltons
4. * represents an approximate value
hydrochloride. Both were sterilized by autoclaving for 15 min at 121°C and 15 psi.

**Y-MEL AND K-802 GENOTYPE VERIFICATION**

Lambda bacteriophage C1857S7 lac5 was propagated using Y-mel (see Table 1) as the phage host. Isolated 24 h Y-mel colonies grown on NZYDT agar at 37°C were suspended in 1 ml of Q80 buffer (0.01M tris hydrochloride pH 7.4, 0.01M NaCl) and one loop of the suspension was used to streak duplicate NZYDT slants and to inoculate each of 5 different plates containing AB agar with various supplements (F. Blattner, Laboratory of Genetics, University of Wisconsin, Madison Wisconsin 53706). AB agar was supplemented here with various mixtures of biotin (B), diaminopimelic acid (D), thymidine (T), and nalidixic acid (N). All AB agar supplements were obtained through Sigma. Y-mel, if it has retained the correct genotype (see Table 1), forms red-purple colonies turning metallic green at 37°C on AB/BDT, AB/BD, AB/BT, and AB/DT but does not grow on AB/BDTN (F. Blattner). Hence wherever this pattern was observed, the duplicate NZYDT slant cultures were saved. The verification procedure was then repeated with growth from these slants to ensure the correct genotype.

For further genotype assurance, NZYDT cultures of Y-mel were tested for their ability to grow in the absence of L-proline. Cells from a single overnight colony were
suspended in 1 ml of Q80 buffer and a single loopful was used to inoculate each of two plates of the following basal salts medium containing per liter: 1.0 g KCl, 0.2 g Na₂SO₄, 7.0 g KH₂PO₄, 0.01 g CaCl₂, 0.6 g NH₄Cl, and when the medium was used to make plates, 20 g/liter agar was added. The basal salts medium in one set of plates contained L-proline (Sigma) at a concentration of 15 mg/liter while the basal salts medium of the other plates lacked L-proline. Proline was added prior to autoclaving the medium. The carbohydrate source for both media was 5 g/liter D-glucose autoclaved separately in a concentrated solution and added aseptically. The plates were scored for growth after incubating for 7 d at 37 C in a wet box and only those NZYDT slant cultures were saved that would not grow in the absence of L-proline. Following completion of the proline growth test, Y-mel stock cultures on NZYDT agar were made from verified slant cultures and these were stored at 4 C. Every four weeks a new slant was prepared from a verified Y-mel slant.

Lambda bacteriophage C1857S7 was propagated using K-802 (see Table 1) as the phage host. An isolated 24 h K-802 colony grown on NZYDT agar at 37 C was suspended in 1 ml of Q80 buffer. A single loopful of this suspension was then used to inoculate duplicate NZYDT slants and 6 plates containing basal salts medium plus, respectively; 5 g/liter lactose (L), 5 g/liter D-galactose (G), 15 mg/liter L-methionine (M),(Sigma), 5 g/liter lactose with 15 mg/liter L-
methionine (LM), 5g/liter lactose with 5g/liter D-galactose (LG), and 5g/liter D-galactose with 15mg/liter L-methionine (GM).

The six plates were incubated for 7 days in a 37°C wet box and at the end of that time the plates were scored for growth. Slants were saved when the pattern of growth was (L), minus; (G), minus; (M), minus; (LM), slow growth; (LG), minus; and (GM), slow growth; respectively.

BACTERIOPHAGE PLAQUE ISOLATION, PRIMARY STOCK PREPARATION AND TITRATION

Upon receipt of a phage stock suspension containing bacteriophage Cl857S7 lac5 (F. Blattner) (see Table 1), plaque isolation was carried out to ensure phage stock purity. For this procedure 0.1 ml of an overnight stationary culture of Y-mel grown on NZYDT was mixed with 0.2 ml of a mixture containing 125 ul of the phage stock suspension and 1.0 ml of plaque storage buffer (PSB). PSB contains 0.01M tris hydrochloride pH 7.4, 0.10 M NaCl, 0.01 M MgCl₂, and 0.05% gelatin. To this was added 0.1 ml MgCa₂ solution (0.01 M MgCl₂, 0.01 M CaCl₂). The combined solution was thoroughly mixed (vortex mixer) and incubated for 10 min at 37°C. Finally, the components were mixed (vortex mixer) into 3 ml of NZYDT molten agar and the total was overlaid onto a NZYDT plate. The plate, after solidification, was inverted and incubated for 18 h in a 37°C wet box. Following incubation a core of agar

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containing a single plaque was removed with a pasteur pipette. The phage agar plug was broken apart and suspended in 1.0 ml of PSB and allowed to soak for 15 min after which 0.1 ml of a stationary Y-mel culture grown in NYZDT and 0.1 ml of MgCa solution were added. The mixture was diluted with 50 ml of NZYDT broth in a 250 ml Erlenmeyer flask and the flask was incubated at 37 C and 140 rev/min on a New Brunswick (Model G-25) gyrotory shaker for 22 h. After incubation the lysate was treated with 0.5 ml of chloroform and the mixture was clarified by centrifugation at 3,015 x g for 10 min at 4 C. The supernatant was decanted and enough gelatin was added to bring the solution (primary phage stock A) to 0.01% concentration.

Titering of phage stocks was accomplished by mixing duplicate 0.1 ml aliquots of ten fold serial dilutions of primary phage stock A with 0.1 ml samples of both MgCa and an overnight stationary culture of Y-mel grown on NZYDT. The serial dilution for each duplicate was carried out to 10^-15 and the diluent was PSB. Following the addition of Y-mel, the dilutions were lightly mixed (vortex mixer), incubated for 10 min at 37 C, and the contents were added to molten NZ top agar which contains per liter 5 g NaCl, 2 g MgCl_2 . 7H O, 10 g "NZ amine A", and 7.5 g agar. The inoculated top agar was poured over NZYDT plates and after solidification the plates were incubated right side up in a 37 C wet box for 18 h and the resultant plaques were
counted.

Plaque purification, primary phage stock production, and titering procedures were also carried out under similar conditions for Cl857S7 lambda bacteriophage phage stock A using the host cell K-802.

To ensure pure phage stocks of both phage types Cl85757 lac5 and Cl85757 a second plaque (derived from Phage Stock A) of both types was harvested and used by the above procedure for a second phage stock production (Primary Phage Stock B). All phage stock solutions were titered, made to contain 0.01% gelatin and stored at 4 C.

Cl857S7 LAC5 LARGE SCALE INFECTION, GROWTH AND RECOVERY

A scaled up version of the method used for primary phage stock production was employed for large scale production. Titers obtained in large scale production varied depending upon the initial phage and host cell concentration. The components for large scale phage growth consisted of 0.3 ml of a stationary Y-mel culture grown in NZYDT, 0.3 ml of MgCa solution and 5.0 x 10^7 phage in 0.39 ml of NZYDT (see Table 3). The suspension was lightly mixed (vortex mixer) and incubated at 37 C for 10 min. Each of four 2-liter flasks containing one liter of NZYDT broth were inoculated with replicates of this mixture and the flasks were incubated at 37 C on a gyrotory shaker at 160 rpm. The phage were harvested at maximum titer as determined by the
development of a "fine splintery" appearance in the flasks (lysed cells) and a subsequent increase in absorbance at 520 nm due to the proliferation of phage resistant cells (D. Daniels, personal communication, University of Wisconsin, Madison, Wisconsin). This usually occurred 18 h after the start of incubation.

Before harvesting the phage, 2 ml of chloroform was placed into each flask and the incubation was continued as before for an additional 30 min. Following the incubation, the contents of each flask were decanted from the chloroform and placed into a single 4-liter flask. Crystalline DNAse and RNAse were added to the phage lysate to a final concentration each of 1.0 ug/ml. This was followed by incubation for one h at room temperature with slow stirring on a magnetic stir plate. Enough solid NaCl crystals were then added to the lysate to make it 1.0 M in concentration after which the lysate was cooled to 4 °C by stationary incubation for 3 h at 4 °C. Debris was removed from the lysate by centrifugation at 11,000 x g for 30 min at 4 °C. Following centrifugation the supernatant was decanted and filtered through several layers of cheesecloth to remove any larger particles or remaining debris. Solid polyethyleneglycol (Fisher p-156 Carbowax 8000) was added to make a final concentration of 10% w/v. The mixture was stirred at room temperature until it had reached the ambient temperature and then was cooled to 4 °C overnight while
mixing slowly on a magnetic stir plate.

PURIFICATION AND CONCENTRATION OF BACTERIOPHAGE

Phage particles were precipitated by centrifugation at 11,000 x g at 4 C for 30 min. The phage precipitates from each of four 280 ml centrifuge bottles that were used were combined and resuspended in 20 ml of (SM) buffer. SM buffer contains per liter: 5.8 g NaCl, 2.0 g MgSO$_4$·7H$_2$O, 2.7 g tris hydrochloride, pH 7.5; and 0.1 g gelatin. An equal volume of chloroform was next added and the resulting suspension was mixed thoroughly (vortex mixer for 30 s). Centrifugation followed at 1,600 x g for 15 min at 4 C. Following centrifugation, the supernatant containing the phage particles was collected with a 5 ml pipette down to but not including the white interface. The volume of the recovered supernatant was measured and 0.5 g solid cesium chloride (high purity from KBI Inc.) was added for every ml of solution. The supernatant was placed on top of a step gradient consisting of 2 ml steps of 1.45, 1.5 and 1.7 g/ml (top to bottom, respectively) cesium chloride dissolved in SM buffer. Centrifugation followed at 100,000 x g for 2 h at 4 C (International Preparative Ultracentrifuge Model B-60 using an SB283 rotor). Following centrifugation approximately 3 ml of fluid was collected (by side puncture) from each of the six tubes beginning at the middle of the 1.5 g/ml step. The 3 ml samples from each tube were
combined and a refractive index reading of the mixture was taken using a Bausch & Lomb ABBE-3L refractometer. The refractive index reading was converted into a density value (g/ml) and this was compared to known phage density values showing that the collected fluid contained the phage. Enough solid cesium chloride was then added to adjust the mixture to a density of 1.50 g/ml and the contents was divided in half and put in two centrifuge tubes. Centrifugation followed using the SB283 rotor at 200,000 x g for 24 h at 4°C. A bluish band was visible in each of the two tubes after centrifugation and upon side puncture collection, the material showed a refractive index reading of 1.380. The collected fluid (2 ml) containing the phage particles from this band was placed in a pretreated dialysis membrane sack (50) and the contents dialysed against two 1 liter changes of buffer at 4°C for an initial 2 h and a later 12 h period. The dialysis buffer consisted of 10 mM NaCl, 50 mM tris hydrochloride, pH 8.0, and 10 mM MgCl₂.₆H₂O.

Extraction of the bacteriophage DNA followed dialysis and was accomplished following a procedure in "Molecular Cloning" (50). Proteinase K (50 ug/ml) was used here in place of pronase and all centrifugation steps were carried out using an HS4 Sorvall rotor at 6,927 x g for 10 min at room temperature. The resulting DNA containing solution was stored at -20°C.
C1857S7 LAC5 HindIII RESTRICTION AND RECOVERY OF THE 25,737 BASE PAIR FRAGMENT

The Lambda phage DNA was restricted with HindIII endonuclease (lot #860-20R New England Biolabs) with the following volumes and concentrations per restriction digest, 16 ul H2O, 4.7 ug Lambda DNA (as determined by absorbance at 260 nm) in 20 ul of TE buffer, 2 ul HindIII enzyme (24 units), and 4 ul medium salt restriction buffer (27). Each tube was capped then lightly mixed (vortex mixer) and incubated at 37 C for 1.5 h. Following restriction, the digest solution was adjusted to 5% glycerol by the addition of a 1 to 1 mixture (v/v) of concentrated glycerol and water. Next the tube contents were mixed lightly and heated to 70 C in a water bath for 10 min. The restriction digest was then placed into sample wells (2.33 ug restricted DNA per well) of a 0.6% agarose gel. The gel was formed by adding 1.2 g of Sea Plaque agarose (FMC Corp) to 200 ml of TB buffer pH 8.3 (27), and heating the mixture to 80 C for 15 to 20 min, cooling it to 37 C and pouring it into a horizontal electrophoresis bed (Model 850 electrophoresis apparatus, Aquebogue Machine Shop, Aquebogue, NY) which was precooled to 8 C with tap water. The samples were electrophoresed on the agarose gel (22 cm in length) while it was submerged in TB buffer at 3.2 volts per cm for 11 h. Unless stated otherwise, all gels were run under these conditions. Following electrophoresis the gel (as were all
other gels) was stained for DNA visualization in TB buffer containing 1.0 ug/ml ethidium bromide (Sigma) for one h at room temperature. The gel was then destained by two consecutive 500 ml changes of deionized water allowing 20 min to elapse between each change. The gel, after destaining, was placed over a 302 nm UV source (TM-40 Transilluminator, Ultra Violet Products, Inc. San Gabriel California) for DNA visualization. Photographs of stained DNA bands in the gel were taken using a Crown Graphic camera with Polaroid Type 55 film through a Series VI Wratten filter K-2 with a two min exposure.

The band containing the largest fragment of the HindIII digested Lambda DNA (the 25,737 base pair fragment) (28) was excised from the gel and trimmed of any excess agarose. The trimmed HindIII Fragment gel strip was melted in a 65 C water bath for 10 min after which the volume of the molten agar was determined and 5 volumes of a solution consisting of 20 mM tris hydrochloride, pH 8.0; and 1 mM Na EDTA was added. This solution was heated for an additional 20 min then extracted twice with equal volumes of equilibrated phenol (50) followed by extraction with an equal volume of a 24 to 1 mixture (v/v) of chloroform and iso-amyl alcohol (50). A final extraction was carried out with an equal volume of water saturated ethyl ether. In the extractions, phases were separated by centrifugation at 10,444 x g for 30 min at room temperature. The volume of
the HindIII Fragment solution (110 ml) was reduced by flash evaporation (Precision Scientific) (approximately 30 ml at a time) in a 125 ml siliconized flask at a partial vacuum of 24 to 26 psi at 50 C. Evaporation was continued until a total fluid volume of 7.0 ml was obtained at which time enough 2.5 M sodium acetate, pH 5.2, was added to give the sample a final concentration of 0.25M. Ethanol was added next (2 volumes of 95%) and the resulting solution was chilled to -70 C for one h. The ethanol solution was centrifuged at 100,000 x g for 24 min at 0 C (SB283 rotor) to precipitate the DNA. The pellet, following centrifugation, was rinsed with 5 ml of 0 C 70% ethanol, allowed to air dry at room temperature, and finally the DNA was dissolved overnight in 200 ul of low salt buffer. Low salt buffer contains 0.2 M NaCl, 20 mM tris hydrochloride, pH 7.4; and 1 mM Na EDTA. The DNA was next purified and concentrated using Elutip-d units (Schleicher and Schuell Inc., Keene, N.H.) following the manufacturer's directions. To the elutip eluate was added 2.5 volumes of 95% ethanol. The solution was then chilled to -70 C for 60 min and the DNA precipitated by centrifugation at 75,000 x g (SB405 rotor) at 0 C for 24 min. The resulting pellet was allowed to air dry and was then resuspended in 100 ul of TE buffer.

**RESTRICTION OF HindIII (25,737 BASE PAIR) FRAGMENT WITH KpnI AND RECOVERY OF THE 6,925 BASE PAIR FRAGMENT**

The recovered HindIII fragment (see above) was
restricted (5 identical digests in total) with KPNI using
the following solutions and volumes per restriction digest,
16 ul H2O, 4 ul of 10X low salt restriction buffer (27), 6 ul KPNI (54 units) (New England Biolabs), 12 ug DNA
suspended in 20 ul of TE buffer, and 2 ul of a 5.0 mg/ml
bovine serum albumin fraction (pentax 5, P.L. Biochemicals).
The 5 tubes containing the restriction digest were vortexed
lightly and incubated at 37 C for 3 h. Following incubation
the digest was adjusted to 5% glycerol (v/v) and heated to
70 °C for 8 min. The restricted DNA was electrophoresed
under the same conditions as previously described on page
11. Three bands were visible after staining the gel with
ethidium bromide and illuminating it under 302 nm light.
The second of these bands (the lac operon containing
fragment, 6,925 base pairs) (28) was excised from the gel
and trimmed of excess agarose. The agarose band was stored
at 4 °C until it could be combined with another preparation
of the fragment, as described below.

A second method for the recovery of the 25,737 base
pair HindIII Fragment was used. The DNA after restriction
with HindIII enzyme was electrophoresed using the same
procedure as described previously. Here, however a 0.6%
agarose gel (Sea Chem agarose LE) was prepared by boiling
the agarose in TB buffer for several minutes and cooling the
solution to 50 °C before pouring. The 27,737 base pair
fragment was recovered by electroelution into pretreated
dialysis membrane bags and was purified by direct extraction (50). The DNA recovered by this method was restricted with KPNI with the same procedure used for the KPNI restriction digests above. Electrophoresis of the restriction digest followed using a 0.6% Sea Plaque agarose gel. The second band containing the 6,925 base pair fragment was excised from the gel, trimmed of extraneous gel and combined with the 6,925 base pair restriction fragment prepared by the first method.

The combined agarose gel material with the KPNI fragments from both procedures was melted at 65°C and the volume determined. A solution containing 20 mM tris hydrochloride pH 8.0, and 1 mM Na EDTA was added (5 volumes) and the resulting mixture was heated for 10 min at 65°C. The fluid was then extracted (direct extraction) (50) to remove most of the agarose. The DNA was then ethanol precipitated as above (page 21), resuspended in 200 ul of low salt buffer and purified and concentrated by elutip-d elution (following the manufacturers directions). After elution, the DNA was precipitated with ethanol by the same procedure used for the 25,737 base pair fragment following elutip-d purification. The DNA containing pellet was dried for several min under a vacuum of approximately 29 psi and then resuspended in 10 ul of TE buffer.
REMOVAL OF THE 3' OVERLAPPING END OF THE 6,925 BASE PAIR FRAGMENT

The protruding 3' end of the 6,925 base pair fragment, resulting from KPNI restriction, was removed (blunt end made). This was done with the exonuclease activity of bacteriophage T-4 DNA polymerase (lot 3, New England Biolabs) using a procedure described in "Molecular Cloning" (50). The DNA was then separated from the exonuclease reaction mixture after being extracted with an equal volume of 1 part equilibrated phenol to 1 part chloroform. This was done by adding enough 2.5 M sodium acetate (pH 5.2) to make the reaction mixture 0.25 M, combining this with 2.5 volumes of 95% ethanol and chilling the mixture to -70°C for 60 min to make the DNA insoluble. Centrifugation followed for 15 min at 4°C in a Beckman model 152 microfuge to sediment the DNA. After centrifugation, the DNA pellet was rinsed with 100 ul of 0°C 70% ethanol, dried under vacuum and dissolved in 10 ul of TE buffer.

FILLING IN THE 5' END OF THE 6,925 BASE PAIR FRAGMENT

The protruding 5’ end of the 6,925 base pair fragment removed of its 3’ overlapping end (see above on page 24) was made blunt ended by the polymerizing activity of DNA polymerase I (large or Klenow fragment, New England Biolabs) using a procedure in "Molecular Cloning" (50). All 2’-deoxy nucleotide 5’-triphosphates, as sodium salts used in this procedure were obtained from PL Biochemicals, Inc.
Upon polymerization of the DNA and inactivation of the enzyme, the mixture was extracted, ethanol precipitated, rinsed, dried under vacuum, and finally resuspended in 10 ul of TE buffer as above.

PHOSPHORYLATION AND ADDITION OF LINKERS TO BLUNT ENDED 6,925 BASE PAIR FRAGMENT

Phosphorylation of the 5' linker ends was carried out with 1.5 ug of linker DNA and 2 units of T-4 polynucleotide kinase (New England Biolabs). This was in an 8 ul final volume reaction mixture containing 10 mM ATP. A procedure in "Molecular Cloning" was used (50) to accomplish the phosphorylation. Each linker or decaoligonucleotide was composed of 10 bases, d(C-G-G-G-A-T-C-C-C-G), (New England Biolabs lot 7-64). Adenosine 5'-triphosphate (disodium salt) used for this procedure was from PL Biochemicals Inc and the 10X linker kinase buffer contained 2 mg/ml bovine serum albumin instead of gelatin. The mixture was incubated for 60 min at 37 C to bring about the phosphorylation.

Following incubation, the 6,925 base pair blunt ended fragment suspended in 10 ul of TE buffer (see above), was added to the linker kinase mixture. T-4 DNA ligase (New England Biolabs) was also added at this time (1 ul containing 400 units) to covalently connect the phosphorylated linkers to the fragment. After incubation at room temperature for 6 h, 1 ul 0.5 M Na EDTA was added and this was followed by extraction, and ethanol
precipitation as discussed above ("Removal of 3' overlapping end of 6,925 base pair fragment", on page 24).

**BamHI RESTRICTION OF LINKERED 6,925 BASE PAIR FRAGMENT**

The DNA pellet from above was dissolved in 40 ul of TE buffer, and to this was added 5 ul of medium salt buffer and 8 to 9 units (1 ul) of BamHI restriction enzyme (PL Biochemicals). Incubation was carried out at 37 C for 3 h after which 1 ul of 0.5 M Na EDTA was added and the mixture extracted as above (page 24) with a phenol chloroform mixture. The 47 ul of solution following the extraction was first adjusted to 5% glycerol, then was heated at 60 C for 10 min and finally was placed on a 0.6% low melt agarose gel (Sea Plaque). Electrophoresis was carried out under the same conditions as described previously.

**PLASMID DNA SEPARATION AND PURIFICATION**

Plasmid separations were carried out by the following procedure. Cells from an isolated single colony of strain HB101, MC1066, or CSH18 grown overnight on LB agar with 50 ug/ml ampicillin were inoculated into 100 ml of LB broth containing 50 ug/ml ampicillin in a 500 ml Ehrlemeyer o flask. Flasks were incubated at 37 C in a New Brunswick Model 25 gyrotory shaker at 160 rev/min until the cultures reached the stationary phase. A 10 ml aliquot of the stationary culture was used to inoculate each of 5 identical two liter flasks containing 1000 ml of LB broth with 20
ug/ml ampicillin and these flasks were incubated as before. The contents of one of the 5 flasks was used to follow the parallel increase in the cell density of all the flasks (Bausch & Lomb spectronic-20). When the proper optical density of 0.3 at 550 nm was reached, 100 ug/ml powdered chloramphenicol (Sigma) was added to each flask and the flasks were incubated as before for an additional 30 h. The flask contents used for obtaining optical density samples was discarded at this point.

The cells from each liter of culture were pelleted (8,000 x g, 5 min, 4 C) in separate 280 ml centrifuge bottles. The procedures described from hereon are for the amount of cells harvested from each liter of culture although 4 liters of culture were actually processed. All steps were carried out unless specified using solutions at 4 C. Cells after centrifugation were resuspended in 100 ml of wash buffer (10 mM tris hydrochloride, pH 8.0, 1 mM Na EDTA) and were pelleted as before. The cells were resuspended in 25 ml of protoplasting buffer (50 mM tris hydrochloride, pH 8.0, 25% sucrose) and then were incubated on ice for 10 min after which 5 ml lysozyme (12 mg/ml, Sigma grade 1) dissolved in protoplasting buffer was added. The contents were mixed by inversion several times and then incubated on ice for 30 min. After incubation, 6.3 ml of 0.5 M Na EDTA, pH 8.0, was added and the suspension was inverted several times and incubated on ice for 5 min. This
was followed by the addition of 36.5 ml of lysis buffer (0.2% v/v triton X 100 from Sigma, 50 mM Na$_2$EDTA, pH 8.0) and incubation on ice for 10 min. The mixture was next centrifuged at 43,542 \times g for 60 min at 4°C to remove debris and nonlysed cells. The clear non-viscous supernatant present after centrifugation was decanted down to but not including the gelatinous pellet. The volume of this supernatant fluid was determined and 5 \mu g/ml RNase added.

RNase was dissolved in a minimum of water, heated to 100°C for 10 min and cooled slowly to room temperature prior to use. The addition of RNase was followed by static incubation at 37°C for 60 min after which 25 \mu g/ml solid proteinase K (type XI from Sigma) was added and incubation was continued for an additional 60 min.

The RNase and proteinase treated supernatant was extracted following incubation with equal volumes of chloroform : phenol : isoamyl alcohol in a 24 : 24 : 1 ratio, respectively. The aqueous layer was recovered and extracted with an equal volume of chloroform : isoamyl alcohol in a 24 : 1 ratio, respectively. One volume of isopropanol was added to the recovered aqueous phase and the solution was chilled to -70°C for 20 min. All phase separations for the extraction procedures were accomplished by centrifugation at 4,080 \times g at room temperature for 5 min. The solution was next subjected to centrifugation at 13,218 \times g for 30 min at 4°C. The supernatant was decanted.
and the pellet, after air drying, was slowly dissolved in 16 ml of TE buffer by shaking gently overnight in a New Brunswick (model G-76) gyrotory shaker 100 rev/min at room temperature.

The following day, solid cesium chloride (high purity from Kawecki-Berylco Industries Inc.) was added and the density adjusted to 1.59 g/ml. The resulting fluid was placed into centrifuge tubes (one 38.5 ml tube when using the Ti-60 rotor and two 12 ml tubes when using the SB283 rotor). Concentrated ethidium bromide (10 mg/ml) was next added by layering 20 ul on top of the 38.5 ml centrifuge tube or 10 ul on the 12 ml gradients. The tubes were then centrifuged at 200,000 x g for 40 h at 16 C (SB283 rotor on an IEC centrifuge) or they were centrifuged at 161,100 x g for 40 h at 16 C (Ti-60 rotor on a Beckman Model L centrifuge).

Bands that developed upon centrifugation were visualized using 302 nm light. They were collected by either bottom or side puncture using an 18 guage needle. The refractive index was determined for each band. Next ethidium bromide was removed by extraction with isopropanol saturated with aqueous 5 M NaCl, 10 mM tris hydrochloride, pH 8.5; and 1 mM Na EDTA (27). Dialysis to remove the cesium chloride was carried out sequentially against three, 1000 ml changes of TE buffer over a period of two days at 4 C. Purity of DNA preparations and concentration of the DNA
present was determined using a Beckman Model DU spectrophotometer. It was assumed that an absorption of 1.0 at 260 nm corresponds to 50 ug/ml DNA. This is true if the 280/260 nm absorbance ratio is less than 0.55, indicating minimal protein contamination (45). The 280/260 ratio was checked to verify minimal protein contamination. Some DNA preparations required an additional phenol extraction followed by ethanol precipitation to arrive at an acceptably low protein concentration.

Typical DNA preparations (including plasmid preparations for pBR325, YEp2, pBR322, YEpl3, and pZ1000) ranged from 200 to 350 ug of DNA/ml.

YEAST TRANSFORMATION PROCEDURES

YEpl3 and pZ1000 plasmid DNA was introduced into various yeast strains (SSU10, AH22, Y-2034 and Y-1572) by the following procedures. Growth from an isolated colony of the yeast strain to be transformed was taken from a YPD plate incubated overnight and inoculated into 5 ml of YPD broth. Incubation was carried out statically overnight at \( 30 \) C, resulting in a stationary phase culture. Duplicate 100 ml quantities of YPD in 250 ml flasks were each inoculated with 2 ml from this stationary culture. The flasks were incubated at \( 30 \) C on a gyrotory shaker at 160 rev/min.

While the flasks were incubating, regeneration agars
(see below) were prepared and minimal medium plates made previously were warmed to 47 C. Minimal medium contains 1 M D-sorbitol, 2% D-glucose, 6.7 g/liter Difco yeast nitrogen base without amino acids but with sulfate and 2% agar. Ingredients were autoclaved separately and then combined.

Two types of regeneration agar were prepared and used, regeneration agar containing the amino acids L-leucine and L-isoleucine and regeneration agar without any L-leucine or L-isoleucine, hereafter referred to as regen agar, and regen agar, respectively. Regen agar contains per liter 158.6 g D-sorbitol; 8.7 ml YPD broth; 17.4 g D-glucose, 18 mg each of adenine sulfate, uracil, L-tryptophane, L-histidine hydrochloride, L-arginine, and L-methionine; 27 mg each of L-tryosine and L-lysine hydrochloride; 45 mg L-phenylalanine; 90 mg each of L-glutamic acid, L-aspartic acid, L-valine, L-threonine, and L-serine; 6.82 g Bacto yeast nitrogen base without amino acids and with ammonium sulfate and 3% agar. Regen agar contains the same ingredients plus, per liter, 27 mg L-leucine and 27 mg L-isoleucine. All individual media constituents were autoclaved separately, and then were combined aseptically. Regen agars were stored at 47 C until required but after 24 h all unused regen agars were discarded.

Yeast cells were collected from one of the flasks for transformation when the absorbance in a duplicate flask at 520 nm was approximately 3.0. This corresponds to 1.0 to
2.0 x 10^7 cells/ml (as determined from a previous growth curve). The cells were harvested by centrifugation at 9,179 x g for 7 min at room temperature. The pelleted cells were washed by resuspending them in 10 ml of 1 M D-sorbitol and then they were sedimented at 3,015 x g for 5 min at room temperature. The wash step was repeated and the pelleted cells were resuspended in 2 ml of 1 M D-sorbitol to which was added an additional 2 ml of 1 M D-sorbitol containing 1.0 mg of Zymolase 5000 (Kirin Brewery Co.). Incubation followed at 37°C with intermittent shaking for one hour. Following incubation the degree of spheroplasting was determined by mixing approximately 10-20 ul of cells with a drop of 10% w/v sodium dodecyl sulfate (SDS) and examining the preparation under a phase contrast microscope. Incubation was extended if less than 90% of the cells lysed to form ghosts.

Successful spheroplasting was followed by centrifugation of the cells at 1,930 x g for 5 min at room temperature. Cells were next gently resuspended in 2 ml of 1 M D-sorbitol after which was added an additional 8 ml. Centrifugation followed under the same conditions and then the pellet was resuspended in 9 ml of a mixture containing 9 parts 1 M D-sorbitol to 1 part 100 mM tris hydrochloride (pH 7.5) with 100 mM CaCl_2. Next centrifugation at 1,930 x g for 5 min at room temperature was carried out followed by resuspension of the cells in 9 ml of the same mixture. At
this point two 10 ul samples (controls) of cells were removed, one sample was added to 10 ml of molten regen agar and the other was added to 10 ml of regen agar. The cells were mixed into the agars by inversion and plated as overlays (approximately 5 ml per plate) on minimal medium plates. The overlays were allowed to solidify and the plates were afterwards inverted and incubated at 30°C.

Plasmid DNA, 4 ug in 11.5 to 20 ul TE buffer (preparation described in "Plasmid DNA Preparation and Purification"), was added to the remaining cells, and the mixture was stirred gently and incubated statically for 15 min at room temperature. After incubation, 5 ml of a mixture containing 9 parts of 44% polyethylene glycol (Sigma PEG 4000) and 1 part tris: CaCl (100 mM tris, pH 7.5; 100 mM CaCl ) was added and the suspension was mixed gently and incubated statically at room temperature for 10 minutes. The cells were then sedimented by centrifugation at 1,930 x g for 3 min at room temperature. The pellet was resuspended in 5 ml of a mixture containing 9 parts of 1 M D-sorbitol and 1 part tris: CaCl . A 20 ul sample of the plasmid treated cells was removed for a positive control at this point. It was mixed with 10 ml of molten regen agar and the mixture was overlaid on several minimal medium plates. The treated cells remaining were added to 90 ml of molten regen agar which was then inverted several times and overlaid on approximately 15 plates as above. All plates
from the above procedure were inverted and incubated at 0
30°C.

A number of yeast colonies visible after several
days of incubation on the regen agar plates were checked
for beta-galactosidase production by plating growth samples
on regen indicator plates containing 5-bromo-4-chloro-3
indolyl, B-D galactoside "XG" (from Bachem Inc.). Indication
plates contained regen agar with 80 ug/ml "XG" (dissolved
in 2 ml of dimethyl formamide per liter of regen medium).
The carbohydrate source was generally 2% D-glucose, however,
4% lactose and combinations of the two were also used.
Sensitive blue plates were also used for screening
transformed yeast colonies for the production of beta-
galactosidase. Sensitive blue medium contains per liter
13.6g KH\(PO_4\), 2.0 g (NH\(_4\))\(SO_4\), 4.2 g KOH; 0.2 g MgSO\(\cdot\)\(_4\)
\(2\cdot\)\(_4\)\(_2\cdot\)\(_4\)
\(2\cdot\)\(_2\)
H\(_2\)O, 0.53 mg FeCl\(_3\)\(\cdot\)6 H\(_2\)O, 0.4 mg thiamine hydrochloride,
0.02 mg D-biotin, 0.48 mg pantothentic acid, 8.0 mg
inositol, 0.4 mg pyridoxine, 30 mg histidine hydrochloride,
40 mg "XG" dissolved in 2 ml of dimethyl formamide, 20 g D-
glucose, and 20g agar. The vitamins were autoclaved
separately from the glucose and agar which were autoclaved
together. All of the other medium components were mixed
and autoclaved separately (except "XG" which was added last
and was not autoclaved). After cooling to 60°C all
components were combined and mixed. Both the indicator
and the sensitive blue plates once inoculated were incubated
(after being wrapped in cellophane) at 30°C.

**E. coli Transformation Procedures**

Transformation was accomplished for *E. coli* strains CSH18 and LG90 using a calcium chloride procedure (50) with the following changes and modifications. The amount of plasmid DNA was 119 instead of 40 ng of DNA in 0.5 μl of TE buffer. Basic growth medium was added (1 ml) to each tube after the 2 min 42°C heating step instead of LB. Basic growth medium contains per liter 5 g Difco casamino acids with vitamins, 7 g K HPO₄, 3 g KH₂PO₄, 1.0 g (NH₄)₂SO₄, 0.5 g Na citrate·2H₂O, 0.15 g thiamine hydrochloride, 0.1 g MgSO₄·7H₂O, and 10 g lactose. The Basic Growth Medium (added at step 8 in 1 ml quantities) was separated into two parts, one part was adjusted to 50 μg/ml ampicillin while the other (to be used for transformation controls) received no ampicillin. Basic Growth agar plates without ampicillin and containing 40 μg/ml "XG" (added by dissolving 40 mg XG in 2 ml of Dimethyl formamide per 3 liters of basic growth agar) were used with the spreading (in place of the top agar) procedure. Plates were left at room temperature until most of the fluid was absorbed by the agar and were then inverted and incubated at 37°C for several days. Basic growth agar plates without ampicillin and containing 40 μg/ml XG and a 1% glucose concentration in place of lactose were also used as control plates.
RESULTS

TITERS OF LAMBDA C1857S7 LAC5 FROM ESCHERICHIA COLI Y-MEL

The initial objective of my research was to obtain a sufficient quantity of lambda C1857S7 lac5 so as to have adequate lac5 DNA for yeast and E. coli transformation. Tests were therefore made to determine the level of phage inoculum required for maximal post infection titers. Table 3 shows the results obtained following the procedure described on pages 15-16 for large scale phage production. All lysate titers were determined (in duplicate) on 1 liter Y-mel cultures grown in NZYDT broth at 18 h post infection. Table 3 shows that $5.0 \times 10^7$ infecting phage particles on $3.5 \times 10^7$ Y-mel cells gave the greatest post infection titer, namely $3.8 \times 10^10$ particles/ml.

LARGE SCALE LAMBDA C1857S7 LAC5 GROWTH AND PARTICLE RECOVERY

The procedure used for titering (Table 3) was employed to obtain a large enough population of C1857S7 lac5 for adequate lac5 transforming DNA. The phage inoculum (from phage stock B) for each liter of broth culture was $5.0 \times 10^7$ particles and 4 liters of E. coli Y-mel (containing $3.5 \times 10^7$ cells each) were infected.

Large scale inoculation, growth and recovery of Lambda C1857S7 lac5 progressed well as monitored by titers collected at various steps during the procedure. The final
TABLE 3. Titers obtained from various sized lambda C1857S7 lac5 infections of Escherichia coli Y-mel

<table>
<thead>
<tr>
<th>Infecting phage particles</th>
<th>Post infection titer particles/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>5.0 x 10</td>
<td>7.0 x 10</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
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<tr>
<td>5.0 x 10</td>
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<td>3</td>
<td>8</td>
</tr>
<tr>
<td>5.0 x 10</td>
<td>4.5 x 10</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>5.0 x 10</td>
<td>4.8 x 10</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>5.0 x 10</td>
<td>1.5 x 10</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>5.0 x 10</td>
<td>2.6 x 10</td>
</tr>
<tr>
<td>7b</td>
<td>10</td>
</tr>
<tr>
<td>5.0 x 10</td>
<td>3.8 x 10</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>5.0 x 10</td>
<td>3.1 x 10</td>
</tr>
</tbody>
</table>

Lambda C1857S7 lac5 particles were from phage stock B, a concentrate which had $1.3 \times 10^{10}$ particles/ml. All infected E. coli Y-mel cultures had a starting population of approximately $3.5 \times 10^7$ (in 0.3 ml).

This was the concentration used for all further procedures.
titer was $1.41 \times 10^{10}$ phage/ml. Some problems were, however, encountered at the 3 step block gradient procedure (pages 17-18 "Materials and Methods"). Phage particles typically sediment upon centrifugation of this sort of gradient as a hazy bluish band between the 1.45 and 1.50 g/ml cesium chloride interface (D. Daniels). Repeated centrifugation runs, however, did not yield bands at this location. The gradients instead had a slightly hazy area between densities of 1.50 and 1.15 g/ml. This fluid when collected (3ml per gradient) and combined proved to contain the phage. It had a refractive index of 1.379 and a density of 1.479 g/ml.

Several other bands were present in the step gradients besides the hazy band. One of these, a hazy band located at the 1.7 and 1.5 g/ml interface did not contain phage and was probably bacterial proteins. Another band of cellular debris (probably lipid containing) was found between the mineral oil region and the 1.15 g/ml interface.

The phage containing fluid from 6 replicate step gradients was adjusted with solid cesium chloride to 1.5 g/ml and the mixture was centrifuged for 24 h to form a new gradient. This gradient had a single hazy blue band with a refractive index of 1.380 and a density of 1.490 g/ml. The blue band contained the phage which were further purified and concentrated according to the procedure described in "Materials and Methods" (page 18). The final product (3.5
12 ml) was a dense suspension of phage (6.5 x 10\(^7\) phage/ml). The percentage of phage particles recovered, (with post 10 infection titer of 1.41 x 10\(^7\)) was 40.34%. It was calculated as follows:

\[
\text{percent recovery} = \frac{12 \times (6.5 \times 10^7 \text{ phage/ml}) \times (3.5 \text{ ml})}{10 \times (1.41 \times 10^7 \text{ phage/ml}) \times (4000 \text{ ml})} \times 100 = 40.34\%
\]

PURIFICATION OF LAMBDA C1857S7 LAC5 DNA

Lambda C1857S7 lac5 genome DNA was recovered from the dense phage suspension by a method referenced on page 18 of "Materials and Methods". Following recovery, the phage DNA concentration and its degree of protein contamination were determined from absorbance readings at 280 and 260 nm. The absorbance readings at 280 and 260 nm were 0.235 and 0.466, respectively, and the 280/260 ratio was therefore 0.504. This ratio indicated the C1857S7 lac5 DNA preparation contained minimal protein contamination. By multiplying the value of the 260 nm reading (corrected for dilution) times the 50 ug/ml value expected for each increase of 1.0 in absorbance (45), a final DNA concentration of 233 ug/ml was calculated. The total DNA recovery was 815.5 ug.

MAKING FRAGMENTS FROM LAMBDA C1857S7 LAC5 GENOME WITH HINDIII

A restriction endonuclease digest was made of
Cl857S7 lac5 DNA. The intention was to separate lac5 DNA from other non lac DNA. To achieve this, the DNA was restricted with HindIII. The resulting 25,737 base pair lac5 fragment was recovered by agarose gel electrophoresis for further manipulation.

MOLECULAR WEIGHTS OF HINDIII LAMBDA Cl857S7 LAC5 DNA FRAGMENTS

Molecular weight estimates of Lambda Cl857S7 lac5 DNA fragments obtained by HindIII digestion were obtained. This was accomplished by coelectrophoresing the digest (0.8ug) in parallel with known fragments of Lambda Cl857S7 and following electrophoresis, comparing the distances moved by the known fragments with the digest fragments. A commercially obtained HindIII digest of Cl857S7 DNA was used as the source of the known fragments (0.9 ug/geltrack) when such comparisons were made.

Figure 1 shows a power curve fit (P. Evenson, personal communication, Dept. of Plant Science, South Dakota State University, Brookings, South Dakota). Most of the necessary information was obtained by relating fragment molecular weights to distance moved in the agarose gel. (F. Sanger, personal communication, Post Graduate School of Medicine, University of Cambridge, Cambridge, England.) In this curve, logarithmic values for both fragment molecular weight and the distance traveled were used and it was assumed that the distance traveled by each
fragment was proportional to its molecular weight. The molecular weight values for the stars in Figure 1 were independently determined (F. Sanger). The solid curve shows the (see Figure 1) projected molecular weights of Cl857S7 HindIII generated DNA fragments. These projected values have a calculated $r$ of 0.98. The curve shown in Figure 1 was used to determine molecular weights of unknown Lambda DNA and plasmid fragments.

The number of base pairs and the molecular weights of HindIII lambda DNA fragments are given in Table 4. Values here are for strains Cl857S7 and Cl857S7 lac5. Figure 2 shows an agarose gel electrophoresis separation of the fragments listed in Table 4. Origins A and C of the gel before electrophoresis contained HindIII restricted Cl857S7 lac5 and Cl857S7 DNA respectively. Origin B contained unrestricted Cl857S7 lac5 DNA.

**OBTAINING THE 25,737 BASE PAIR LAC5 SEGMENT**

The 25,737 base pair (16.73 x 10$^6$ daltons) fragment (see Figure 2 and Table 4) resulting from HindIII restriction of Lambda Cl857S7 lac5 DNA was assumed to contain the lac5 substitution region. The basis for this assumption can be found in the "Discussion" section of this paper under "comparison of Cl857S7 and Cl857S7 lac5 genomes". The next task following identification of the 25,737 base pair fragment was to scale up the procedure to
Fig. 1. Power Curve Fit relating molecular weight in daltons to distance migrated through agarose gel of Lambda C1857S7 Hind III restriction fragments. The solid line connects values representing projected molecular weights determined from the equation

\[ y = ax^n \]

where:

- \( y \) is equal to fragment molecular weight in daltons
- \( x \) is equal to distance fragment migrated through agarose gel in millimeters
- \( a \) is a constant equal to 7,826,358
- \( b \) is a power function equal to (-)1.92

Stars show values representing the actual molecular weights (F. Sanger)
TABLE 4. Sizes of HindIII fragments of Cl857S7 and Cl857S7 lac5

<table>
<thead>
<tr>
<th>Fragment #</th>
<th>C1857S7</th>
<th></th>
<th>C1857S7 lac5</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Base Pairs</td>
<td>Mol a Wt.</td>
<td>Base Pairs</td>
<td>Mol b Wt.</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>125</td>
<td>0.08 x 10</td>
<td>125</td>
<td>0.08 x 10</td>
</tr>
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<td>564</td>
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<td>0.37 x 10</td>
</tr>
<tr>
<td>6</td>
<td>2,028</td>
<td>1.32 x 10</td>
<td>0</td>
<td>0</td>
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<td>4,731</td>
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</tr>
<tr>
<td></td>
<td>48,516</td>
<td>31.5 x 10</td>
<td>49,095</td>
<td>31.91 x 10</td>
</tr>
</tbody>
</table>

a The values given are independently determined molecular weights (F. Sanger).

b The molecular weight values for fragments 2–5 and #8 are from references 9, 25 and 28. The method for determining the molecular weight for fragment #1 is described in the text, here a molecular weight of 650 daltons per base pair was assumed. The size of fragment #7 is an approximate value based on various restriction maps (28).
Fig. 2. Agarose gel electrophoresis of DNA from Lambda Cl857S7 and Cl857S7 lac5. Prior to electrophoresis the origins at A and C received HindIII restricted Cl857S7 lac5 (0.5 ug) and Cl857S7 (0.9 ug) DNA, respectively, while the origin at track B received 0.5 ug unrestricted Cl857S7 lac5 DNA. Numbers give 6 molecular weights in daltons x 10⁶ of the DNA 6 fragment of each band. The 16.73 x 10⁶ dalton DNA (track A) contained lac5. The DNA of band #8 (see Table 4) migrated off the end of the gel and so was not observed. Gels were stained with 1 ug/ml ethidium bromide.
obtain sufficient lac5 DNA for plasmid insertion and transformation. Figure 3 shows the lower one half of a 0.6% agarose gel slab which was electrophoresed to separate the lac5 containing fragment of HindIII restricted Cl857S7 lac5 DNA from the other 6 fragments.

A total of 47 ug of HindIII Cl857S7 lac5 DNA was electrophoresed along 10 replicate tracks in two gels. A Lambda Cl857S7 HindIII digest was included in one sector of this gel to provide known molecular weight markers. It is not shown in Figure 3. Replicate gel bands containing the lac5 DNA (25,737 base pair fragment) were cut out of the agarose gel for DNA recovery.

The initial method used to recover the DNA from the gel was electroelution into dialysis bags (50). This method was, however, unsatisfactory and several other methods were tested before a satisfactory one was found. The unsatisfactory methods included electroelution of DNA from the gel while inside dialysis membrane bags, direct extraction from low melting temperature agarose (50), and pushing molten low melting agarose containing the DNA through an Elutip-d unit. The method finally found through trial and error for successful DNA recovery is described on pages 20-21 of "Materials and Methods". The key to the success of this procedure referred to as "combination purification" was the use of low temperature melting agarose for electrophoresis. Contaminating protein was acceptably
Fig. 3. Preparative agarose gel electrophoresis used to separate lac5 DNA (25,737 base pairs) from the other HindIII generated fragments of Cl857S7 lac5 Lambda genome. The gel contained 0.6% agarose (Sea Plaque) Prior to electrophoresis the origins at A-J each received 2.35 ug of Cl857S7 lac5 DNA. Numbers give 6 molecular weights in daltons x 10 for DNA in each 6 band. The 16.73 x 10 dalton DNA in tracks A-J contained lac5. All electrophoretic and staining conditions were identical to those in Figure 2.
low with this method and the DNA was pure enough for restriction.

RECOVERY OF 6,925 BASE PAIR LAC DNA FROM 25,737 BASE PAIR FRAGMENT

The 25,737 base pair lac DNA containing fragment recovered by "combination purification" was restricted with KpnI endonuclease (page 21-23 of "Materials and Methods") to remove the lac5 DNA segment (6,925 base pairs) from non-lac DNA. KpnI restriction of lac DNA was successful but the number of enzyme units required for good yields was at least double the recommended number of units (New England Biolabs technical data, number 142) for the level of DNA used. In addition the required time of incubation was longer than that recommended (see "Materials and Methods" pages 21-22).

Fragments resulting from KpnI digestion of the 25,737 base pair piece of DNA were separated by electrophoresis on a 0.6% agarose gel. Figure 4 shows the results. The 6,925 base pair lac DNA segment migrated about the same distance as the third fragment of HindIII digested C1857S7 DNA. The 6,925 base pair lac5 DNA segment was recovered from the gel (five replicates) as described on pages 21-23 of "Materials and Methods".

ADDING LINKERS TO LAC DNA

The 6,925 base pair lac segment had its 3'
Fig. 4. Preparative agarose gel electrophoresis used to separate 6,925 base pair lac DNA from other KpnI generated segments of the 25,737 base pair fragment. Prior to electrophoresis the origin at A received 0.9 µg of HindIII restricted C1857S7 DNA. The origins at B-F received at that time 2 µg each of the KpnI restricted 25,737 base pair fragment. The agarose gel concentration was 0.6% and after electrophoresis it was stained with 1 µg/ml ethidium bromide. The 25,737 base pair fragment was heated to 60°C for 10 min following its preparation with HindIII to denature the 12 base-pair cos site. Bp represents base pairs. The 6,925 bp DNA contained lac5.
overlapping end removed with exonuclease activity and its 5' overlapping end filled in with nucleotides by polymerase activity. The procedures are described on pages 24-25 in "Materials and Methods" and were necessary before blunt ended linker DNA could be added on to each end.

In order to be covalently joined to each end of the lac5 segment, the commercially used linker (decaoligonucleotide) had to be first 5' phosphorylated. This phosphorylation was accomplished with T-4 DNA kinase as described on page 25 of "Materials and Methods". A trial of the phosphorylation step and the subsequent ligation were performed beforehand using just linker DNA. This was to ensure that all procedures worked properly before lac DNA was used. Figure 5 shows the results of this trial. Two samples (1.5 ug) of linker DNA were electrophoresed on a 0.8% agarose gel. Both samples had previously been phosphorylated but the sample shown from origin A had, as opposed to that from origin B, also been treated with T4 DNA ligase. Origin C contained 0.9 ug of HindIII restricted Cl857S7 DNA to provide known molecular weight markers. As can be seen from Figure 5 the phosphorylated but unligated linkers from origin B migrated at a faster rate than any of the Cl857S7 HindIII standard fragments. This indicated that the linkers were of small molecular weight. The phosphorylated and ligase treated linkers from origin A, on the other hand, migrated as higher molecular weight bands.
This indicated the linkers were ligated to each other to form oligomers and polymers of varying length.

The linking of each end of the 6,925 base pair lac segment, which followed the successful trial, was accomplished in the same way that self-linkering of the decaoligonucleotides had occurred. It was necessary then to get rid of any extra and possibly interfering (50) linkers (joined end to end) and provide BamHI ends. Therefore, the linker ed, lac DNA was treated with BamHI which staggeredly cuts the linker at mid-section. It was the intent to separate the released linkers from the larger 6,925 base pair segment by electrophoresis. Unfortunately before, during, or after this electrophoresis, the 6,925 base pair segment was lost. The exact step at which the lac DNA was lost is not known with certainty but it probably occurred following ethanol precipitation of the DNA. A problem was encountered with decanting away the ethanol from precipitated lac DNA. The ethanol would not pour out of the small microfuge tube that was used, making it necessary to tease the ethanol with a small pipete to the rim of the tube where it was removed by a gentle flicking of the tube. It was most likely here where the loss of the lac DNA occurred because after the ethanol was removed, no pelleted material was observed at the bottom of the tube. Electrophoresis (after BAMHI restriction) of the remaining material in the tube verified the loss.
All further work on inserting the lac operon into a plasmid had to be discontinued at this point since there was insufficient time to start over again and complete the procedures necessary to insert lac5 into the intended plasmid, YEpl3. It was therefore decided to use a lac plasmid that fortunately became available at that time, (pZ1000) from T. Zamb (Molecular Genetics Inc., Minnetonka Minnesota), and pursue yeast transformation with this lac plasmid.

PRIMARY PLASMID PURIFICATION

PZ1000 (source of leu gene and lac operon) and YEpl3 (source of leu gene) plasmids were obtained from their respective \( E. coli \) hosts MC1066 and HB101 in amounts sufficient for transformation. The procedures for this referred to hereafter as "primary purification" are described in "Materials and Methods" (pages 26-30) and yielded approximately 350 ug/ml of plasmid DNAs.

A BamHI restriction digest of YEpl3 DNA (prior to secondary purification) was made to determine the amount of covalently closed circular DNA present in the preparation. The digest (1.8 ug) was electrophoresed on a 0.6% agarose slab gel and the results are shown in Figure 6. Also shown in Figure 6 are the electrophoretic patterns of unrestricted YEpl3 and a HindIII digest of C1857S7 DNA. YEpl3 monomer and multimers contain a single BamHI restriction site which
when cleaved with BamHI endonuclease decircularizes to form a linear molecule. This linear form (Figure 6) exhibits a different electrophoretic pattern than its circular precursor, monomer or multimer forms. (N. Alexander, personal communication, Northern Regional Research Service, Peoria, Illinois).

ADDITIONAL PURIFICATION OF YEpl3

Although transformation of S. cerevisiae was accomplished successfully with the primary purified YEpl3 plasmid preparation (see Table 5), further purification of the plasmid was carried out (before the loss of the lac5 fragment). The purpose was to obtain a preparation with mostly the closed circular form so as to enhance the number of correct lac segment insertions into the BamHI restriction site of the plasmid. Plasmid purification was accomplished by preparative electrophoresis on 0.6% agarose gels. The sample DNA which migrated farthest through the gel contained the plasmid in a covalently closed circular form. This DNA was recovered by electroleution (50) and was then extracted, ethanol precipitated and dissolved in TE buffer (200 ul) as described on pages 21 of "Materials and Methods". This solution was found to contain 125 ug of DNA/ml.

TRANSFORMATION OF YEAST FOR LEU GENE WITH pZ1000 and YEpl3

The initial aim of yeast transformation was to
determine whether the pZ1000 plasmid was taken up and any of the genes expressed. The easiest of these genes to check for since it was a yeast gene and would most likely be expressed in yeast transformants was the leu gene. It was deemed less likely that the bacterial lac operon of pZ1000 would be expressed in yeast without special manipulations. Therefore 2 leu strains of S. cerevisiae, SSU10 and AH22, were exposed to pZ1000 ("primary purified, page 26-30 in "Materials and Methods") and leu transformants were selected for on regen minimal medium. YEp13, another plasmid that carries the leu gene, was tested in a similar way with the 2 yeast leucine auxotrophs. Both plasmids are capable of transforming either E. coli or S. cerevisiae (14), (T. Zamb personal communication). It can be seen from Table 5, where the results are shown that the leu yeast strains after transformation with either plasmid showed leu transformants as evidenced by colonies growing on regen agar where no leucine was present. Figure 7 shows 3 day old colonies of S. cerevisiae SSU10 growing on regen agar after the inoculum was first transformed with pZ1000.

Following transformation and incubation, cells from randomly selected colonies of the two strains were placed on XG "indicator plates" with lactose, glucose or no carbohydrate ("Materials and Methods", page 34). The purpose was to screen for the production of beta-galactosidase on the
remote chance that the lac operon would be expressed under one of the conditions. The plates were incubated at 30°C for 3-7 d and then were checked for blue colonies, indicative of beta-galactosidase. However, no colonies or growth were observed on any of the plates except those with glucose, and here the colonies (of SSU10 and AH22) were not blue colored.

TRANSFORMATION OF LAC _ESCHERICHIA COLI_ CSH18 AND LG-90 WITH pZ1000 AND YEpl3 PLASMID DNA

At this point it seemed possible that although the pZ1000 plasmid was incorporated by SSU10 and AH22 and the leu gene was recognized, the lac operon was not functional. It was therefore necessary to test the plasmid for a functional lac operon. This was accomplished by exposing the lac _E. coli_ strains CSH18 and LG90 to pZ1000 and checking for blue colored pZ1000 transformants on lactose XG agar. Controls included CSH18 and LG90 transformed with a non-lac plasmid (YEpl3) and growth checks of non-transformed + CSH18, LG90 and wild type _E. coli_ (lac) (ATCC No. 25922). Transformation was carried out as described on page 35 of "Materials and Methods". The results of the transformation tests, shown in Table 6, indicate that the pZ1000 plasmid have functional lac Z and Y genes. This is shown by the ability of pZ1000 transformed CSH18 and LG90 to grow on lactose forming blue colored colonies on XG agar. This occurred even when ampicillin was also present in the medium
indicating that the lac operon was not by itself but was on the pZ1000 plasmid which carries an amp gene. The results in Table 6 also indicate that the pZ1000 plasmid transformed CSH18 and LG90 E. coli to ampicillin resistance as well as for the lac operon. CSH18 and LG90 transformed with YEp13 as expected did not grow on lactose XG agar and wild type E. coli which is known to carry a lac operon on the bacterial chromosome and be ampicillin sensitive formed blue colonies when ampicillin was absent.

The CSH18 transformants carrying the pZ1000 plasmid were streaked for isolation on basic growth agar. Figure 8 shows what happened in one case. Figure 8A illustrates the results for non transformed CSH18 cells and Figure 8B illustrates the results for pZ1000 transformed CSH18 cells. The basic growth agar contained glucose as the sole carbohydrate source and XG as a beta-galactosidase indicator.

SACCHAROMYCES CEREVISIAE TRANSFORMATION WITH PZ1000 PLASMID FROM CSH18

PZ1000 DNA was amplified in and purified from a pZ1000 transformed strain of E. coli CSH18 which had tested positive for beta-galactosidase. The plasmid amplification and separation procedures used are outlined in "Materials and Methods" on pages 26-30. The plasmid preparation resulting from this procedure (referred to hereafter as checked pZ1000) contained 300 ug of DNA/ml. It was used to
transform SSU10 and AH22 strains of *S. cerevisiae* using the procedure detailed in "Materials and Methods" on pages 30-34. It was anticipated that transformation using plasmid DNA from an *E. coli* strain known to carry a functioning lac positive plasmid (pZ1000) would increase the chance of obtaining beta-galactosidase positive yeast transformants.

Transformed cells and non transformed controls of SSU10 and AH22 were plated on regen agar and after 3-7 days of incubation at 30°C, the plates were screened for growth. Both strains showed colonies on regen agar indicating that + leu transformants had resulted. This presumably occurred by plasmid uptake and recognition by the yeast strains. Cells + from a number of these leu transformant colonies were subcultured on squared sectors of several sensitive blue plates ("Materials and Methods" page 34) and the plates were incubated at 30°C. Blue colonies from transformed SSU10 and AH22 subcultures were observed after 3-7 days of incubation thus indicating that beta-galactosidase was indeed produced. Figure 9A and 9B show two plates of transformed SSU10 and AH22 colonies on sensitive blue agar. Beta-galactosidase production is indicated here by the presence of a blue coloration surrounding several of the colonies. Two other strains of *S. cerevisiae* Y-1572 and Y-2034, were exposed to pZ1000 and tested in the same way as SSU10 and AH22. Both strains tested negative for beta-galactosidase production. Y-1572 and Y-2034 are, unlike AH22 and SSU10, prototrophic for leucine.
Fig. 5. Agarose gel electrophoresis used to determine the effectiveness of phosphorylation and ligation of trial DNA. Prior to electrophoresis the origin at C received 0.9 ug of a HindIII digest of Lambda C1857S7 DNA to provide known molecular weight markers. Origins at A and B received 1.5 ug each of phosphorylated linker trial DNA. The trial DNA at origin A had in addition been treated with T-4 DNA ligase.
Fig. 6. Agarose gel electrophoresis of BamHI digest of YEpl3 plasmid. Prior to electrophoresis the origin A received 1.8 ug of YEpl3 DNA (10,700 bp) restricted with BamHI. Origin B received 1.35 ug of C1857S7 DNA restricted with HindIII, and origin C received 1.8 ug of unrestricted YEpl3 DNA. The gel contained 0.6% agarose. The gel was stained in 1 ug/ml ethidium bromide. All other electrophoretic conditions were identical to those in Figure 2. Lines point to and identify the various plasmid forms present.
TABLE 5. Transformation of leu* *Saccharomyces cerevisiae* strains to leu+ with YEpl3 and pZ1000 plasmids

<table>
<thead>
<tr>
<th>PLASMID TP</th>
<th>GROWTH OF YEAST strain:</th>
<th>SSU10</th>
<th>AH22</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Regen agar</td>
<td>Regen agar</td>
</tr>
<tr>
<td>pZ1000</td>
<td>(+) (+) (+) (+) (+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YEpl3</td>
<td>(+) (+) ND ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>(+) (-) (+) (-)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a A (+) symbol indicates presence of colonies on agar after 3 to 7 days at 30 C. A (-) symbol indicates the absence of growth. ND means "not done". Regen is regeneration agar ++ without L-leucine or L-isoleucine and Regen is regeneration agar with these amino acids. The composition of regeneration agar is described in "Materials and Methods".

b TP is "transforming principle".
Fig. 7. Leu+ transformed colonies of *Saccharomyces cerevisiae* SSU10. Colonies are shown 3 days after an SSU10 inoculum of approximately $1.5 \times 10^9$ cells was exposed to 4 ug of pZ1000 DNA and the exposed cells plated on regen agar. The plate was incubated at 30°C. The composition of regen agar is described in "Materials and Methods".
TABLE 6. The ability of pZ1000 and YEpl3 transformed 
*Escherichia coli* to grow on lactose as the sole 
carbon source and produce beta-galactosidase

<table>
<thead>
<tr>
<th>GROWTH OF ESCHERICHIA COLI</th>
</tr>
</thead>
<tbody>
<tr>
<td>strain: CSH18</td>
</tr>
<tr>
<td>plasmid</td>
</tr>
<tr>
<td>Ampicillin</td>
</tr>
<tr>
<td>No ampicillin</td>
</tr>
</tbody>
</table>

* A (+) symbol indicates the presence of colonies on agar 
after several days of incubation at 37°C. A (-) symbol 
indicates the absence of growth. An asterisk indicates blue 
colored colonies were formed. Basic growth medium described 
in "Materials and Methods" on page 35 was used throughout. 
It contained 40 ug/ml XG. The basic growth medium also 
contained 1% lactose as the sole carbohydrate source. 
Ampicillin when present was at a concentration of 50 ug/ml.
Fig. 8. Growth of untransformed and pZ1000 transformed E. coli CSH18 on basic growth agar. The basic growth agar contained 1% glucose and 40 ug/ml XG. The other ingredients of basic growth agar are described on page 35 of "Materials and Methods". Incubation was for several days at 37 C. Figures 8A and 8B are non-transformed and pZ1000 transformed CSH18, respectively.
Fig. 9. Growth of pZ1000 transformed *Saccharomyces cerevisiae* SSU10 and AH22 on sensitive blue plates. Sensitive blue agar used here contained 40 ug/ml of XG. Other sensitive blue ingredients are described on page 34 of "Materials and Methods". Each sector on each plate was inoculated with cells from randomly selected SSU10 or AH22 colonies from regen agar plates. Incubation was at 30 C for 3 to 7 days. Figures 9A and 9B show pZ1000 transformed AH22 and SSU10, respectively.
9A. transformed AH22

9B. transformed SSU10
DISCUSSION

OVERALL OBJECTIVES

The first objective of this study was to recover the lac5 portion of the lambda Cl857S7 lac5 genome. This was accomplished A.) by growing and harvesting a large quantity of the phage B.) recovering the genome from the phage particles in sufficient quantity C.) restricting the genome and the lac containing fragment, and D.) purifying the lac operon segment by agarose gel electrophoresis. The next objective was to insert the lac operon, after it had been linkered to short molecular weight DNA and BamHI restricted, into BamHI restricted YEp13 plasmid. Unfortunately the preparation was lost at this stage and the objective of transforming \textit{S. cerevisiae} with this preparation was prevented. The final intent of these procedures was to bring about beta-galactosidase enzyme production within the yeast.

Demonstration of \textit{S. cerevisiae} transformation by \textit{E. coli} yeast hybrid plasmids was another objective of the study. The plasmids tested were YEp13 and pZ1000 and the plasmid marker gene used was the leu gene. Transformation of lac \textit{E. coli} and \textit{S. cerevisiae} with the lac Z and Y genes via the plasmid pZ1000 was the final objective of this research.
CHOICE OF LAMBDA AS THE LAC OPERON SOURCE

Bacteriophage have become an indispensable tool for molecular geneticists. When using phage to amplify foreign DNA sequences the compatibility of the sequence with E. coli metabolism is not as critical as it would be with plasmid amplification since the lytic cycle occurs rapidly (9). Recently many changes have been made in various Lambda strains which distinguish them from Lambda papa the generally accepted wild type strain (25,30). These changes have in general been directed at making Lambda a more suitable vector for gene cloning by the addition and deletion of various restriction sites. Restriction sites were changed by adding point mutations, substitutions, and deletions into the dispensable one third of the phage genome and even into certain of the essential regions of the genome. Only about 66% of the phage genome is essential for the lytic propagation of the phage (85). Using these lytic phage it has become possible to obtain many individual, highly accurate copies of genes or foreign DNA sequences.

For my research the lytic phage Lambda C1857S7 lac5 was selected as the best source for the lac operon. Phage amplification of the lac5 segment was chosen over bacterial amplification because greater copy numbers could be obtained using the lytic phage system with lesser amounts of interfering non-lac DNA. The lac5 segment (plac5) originated in E. coli but was isolated and purified by
Shapiro et al (69) and was placed into the non essential genome region of various Lambda phage including Cl857S7 (9). The lac5 region in Lambda Cl857S7 lac5 has a functional promotor, operator, and lac Z gene but lacks a functional I (repressor) Y and A genes (69). Consequently in an I, Z or lac deletion host, lac5 imparts constituitivity for beta-galactosidase. This was another reason for choosing lac5 over the operon from E. coli. Cl857S7 lac5 beside having the lac5 region also carries a ts mutation that leaves the phage CI gene product thermolabile, and a deletion that removes the transcription termination site t (referred to as nin 5). This deletion allows delayed early transcription to be independent of the N-gene product. The N-protein is usually required for lytic growth, however, with the t site deleted, lytic growth can occur without it. Cl857S7 lac5 also has a mutation in the S protein (the R and S proteins are required for lysis of the host cell (38) which allows more phage particles to assemble prior to lysis and thus increases yield (33).

COMPARISON OF LAMBDA Cl857S7 AND Cl857S7 LAC5 GENOMES

The Cl857S7 and Cl857S7 lac5 lambda strains have very similar genomes but with several significant differences. The similarities and differences are shown in Figure 10. When constructing the Cl857S7 lac5 strain a fragment of approximately 4,168 base pairs (26) was removed
from the extreme left end of the replaceable region of the Cl857S7 genome. Into this region was substituted a fragment with an approximately equivalent number of base pairs and containing the lac region of *E. coli* (9). This substitution eliminated one HindIII site (the one closest to the cos L site) of the Cl857S7 genome and in so doing changed the pattern and size of fragments generated with HindIII restriction. Cl857S7 DNA upon restriction with HindIII endonuclease yields 8 fragments (see Table 4). The fragment sizes are well documented and the fragments themselves have been marketed (New England Biolabs) as molecular weight standards. The Cl857S7 lac5 genome when restricted under the same conditions yields only 7 fragments (see Figure 10) and one of these, fragment 1, has a larger molecular weight. The number of base pairs for fragment 1 was increased from 23,130 in Cl857S7 to 25,737 in Cl857S7 lac5 and fragment number 6 in Cl857S7 is no longer present in Cl857S7 lac5 at the same site. A base pair estimate of Cl857S7 lac5 fragment 1 was made by using a restriction map of charon 1 phage (28). Fragment 6 was not removed in its entirety when the lac5 substitution was made in Cl857S7, however, as previously mentioned, the HindIII recognition sequence was lost. Any fragment 6 base pairs not removed were assumed to end up as part of fragment 1 of Cl857S7 lac5.
Fig. 10. Comparative restriction maps of Lambda

C1857S7 and C1857S7 lac5 genomes

--- represents double stranded DNA of Lambda C1857 and C1857S7 lac5

--.-- represents KpnI restriction site

--::-- represents HindIII restriction site

cos(L) and cos(R) represent the 12 nucleotide (single stranded DNA) cohesive ends formed during replication.

Numbers 1 through 8 and 1 through 7 represent HindIII generated fragments from the largest to smallest for C1857S7 and C1857S7 lac5, respectively. ( ) represents the lac5 substitution region.
\begin{align*}
\cos(L) & \quad 1. & 6. & 5. & 7. & 8. \\
& \quad 2. & \ \backslash / & 3. & 4. \\
\end{align*}

\text{C1857S7}

\begin{align*}
\cos(L) & \quad 1. & 5. & 6. & 7. \\
& \quad 2. & \ \backslash / & 3. & 4. \\
\end{align*}

\text{C1857S7 lac5}
Fig. 11. Fragment 1 (25,737 base pairs) from HindIII restriction of C1857S7 lac5 DNA showing the 2 KpnI sites. Numbers 1 through 3 represent fragments with the greatest to the smallest number of base pairs formed upon KpnI restriction of fragment 1. The (TCGAA) sequence is the overlapping 5' end of fragment 1 resulting from HindIII restriction.

- - - - - - - - - represents double stranded DNA
- - - - - - - - - represents each KpnI restriction site
REMOVAL OF THE 25,737 BASE PAIR LAC5 FRAGMENT FROM LAMBDA C1857S7 LAC5 GENOME

Fragment 1 containing lac5 (Figure 10) was separated from the rest of the C1857S7 lac5 genome by cutting the lambda genome with HindIII and purifying the 25,737 base pair lac5 fragment. The fragment is shown in Figure 11. Other workers had previously separated the lac5 segment for transformation of yeast (59). These workers restricted the Lambda genome with BamHI endonuclease, recovered the lac5 segment (23,097 base pairs) and inserted it into the BamHI site of the G10-53 plasmid (59).

RECOVERY OF THE 6,925 BASE PAIR SEGMENT

The 25,737 base pair lac5 segment upon KpnI restriction yielded 3 fragments. One of these, the lac5 6,925 base pair segment, was recovered using the "combination purification", and direct extraction following electroelution.

The first successful KpnI restriction of the 25,737 base pair segment produced results which at first were quite puzzling. Agarose gel electrophoresis of the digest yielded a band containing a 4,731 base pair piece of DNA. This band initially could not be explained since KpnI restriction of the 25,737 base pair fragment was expected to generate only 3 fragment sizes (28) instead of 4 none of which should have had that base pair count. Figure 4 of "Results" shows a restriction digest electrophoresed later
Fig. 12. Fragment #2 (6,925 base pairs) from KpnI restriction of the 25,737 base pair HindIII fragment. Overlapping ends on the left and right termini are from KpnI and HindIII restriction, respectively.
5'  C  -----------------------------A
3'  CATGG  -----------------------------TTCGAA  5'

2
Fig. 13. KpnI fragment #2 (6,925 base pairs) after 3’ end removal by T-4 DNA polymerase in the presence of all four deoxyribonucleoside triphosphates (dNTPs).
5' C --------------- A 3'
3' G --------------- TTCGAA 5'
Fig. 14. KpnI fragment #2 (6,925 base pairs) after fill in (blunt ends made) of the 5' end using the activity of the Klenow fragment of DNA polymerase I in the presence of all four deoxyribonucleoside triphosphates.
5' C -------------------------------------AAAGCTT 3'
3' G -------------------------------------TTTCGAA 5'
which does not contain the 4,731 base pair band. The difference between the way the DNAs were handled was a single heat treatment step normally included at the end of all restriction digestions to inactivate the restriction enzyme. This heat treatment step omitted in the first case brings about denaturation of a 12 base pair complementary region called the cos site (cohesive ends) (50). The cos sites tend to pair up spontaneously in purified, concentrated DNA preparations of the lambda genome resulting in circularized DNA. HindIII restricted lambda DNA if not heat treated maintains the cohesive ends (cos L and cos R) in the double stranded form. This form causes the two restriction fragments that are connected by the cos site (fragments 1 and 4, see Figure 10) to migrate together during electrophoresis to form one band. The bands shown in Figure 4 of "Results" are the expected ones when the Lambda DNA has been properly heat treated.

PUTTING BLUNT ENDS ON THE LAC OPERON

The 6,925 base pair lac5 fragment since it was the product of both HindIII and KpnI restrictions carried dissimilar sticky ends. In addition, one of the ends was 5' overlapping and the other was 3' overlapping. These end differences plus the fact that blunt ends can be ligated (68) led to the decision to blunt both ends of the lac5 fragment. Figures 13-14 show the steps used for blunt ending
the 6,925 base pair lac5 segment. Figure 13 indicates how
the 3' overlap of the lac5 segment is blunt ended by the
exonuclease activity of T-4 DNA polymerase. Figure 14 shows
how the HindIII generated 5' overlap is filled in by the DNA
polymerizing activity of the Klenow fragment of _E. coli_ DNA
polymerase I.

**ADDING LINKERS TO THE BLUNT ENDED LAC OPERON**

The blunt ended lac segment (6,925 base pairs)
could have been placed in the YEpl3 plasmid directly by
forming blunt ends at the BamHI site of the YEpl3 plasmid.
This would have resulted, however, in an insert which could
not have been recovered. Using instead lac5 connected at
both ends to linkers with internal BamHI recognition
sequences made it possible to recover the insert at a later
time by BamHI treatment. Linker DNA (decaoligonucleotide
see Figure 15A) must have 5' phosphate ends (see Figure 15B)
if T-4 ligase is to covalently attach it to an insert such
as lac5. Linker phosphorylation was accomplished with T-4
kinase. Upon 5' phosphorylation, linker DNA (see Figure 16)
was covalently attached to the lac segment with T-4 ligase.
The "linkered" lac5 segment was then digested with BamHI to
create sticky ends to match the ends at the YEpl3 BamHI
site. The BamHI restriction of the "linkered" lac5 segment
in addition to guaranteeing a linear form of lac5 (instead
of a circular form, see Figure 17) served to remove excess
linkers in the reaction mixture and at both ends of the lac segment.

PROPOSED JOINING OF THE LINKERED LAC5 SEGMENT TO YEP13 PLASMID

As mentioned in "Results", the linker ed lac5 segment was lost. It was the intention, until that happened to get expression of lac5 in \textit{S. cerevisiae}. The linker ed lac5 segment for this purpose would have to be put into a suitable vector such as YEp13. YEp13 is a 10,700 base pair plasmid (14) consisting of pBR322, the LEU2 gene of yeast, and a DNA fragment containing a yeast origin of replication from \textit{2um} circle DNA (14). This plasmid because it contains the pBR322 sequence can replicate in \textit{E. coli} and because it contains the \textit{2um} circle DNA is also capable of replicating in \textit{S. cerevisiae}. YEp13 plasmid retains the ampicillin and tetracycline resistance genes of pBR322 and has a single \textit{BamHI} restriction site in the \textit{tet} gene. The presence of the yeast LEU2 gene in YEp13 would have facilitated the screening of yeast transformants. Leu prototrophs could have been selected from among leu auxotrophs.

The linear linker ed lac5 (6,925 base pair) fragment was to be inserted in \textit{BamHI} restricted YEp13 and covalently joined to the plasmid with T-4 ligase (see Figure 18). Thereafter it was the intention to amplify the lac5 segment in a \textit{lac E. coli} strain. The amplified recombinant DNA could then have been used to transform \textit{leu} yeast and
Fig. 15. The synthetic, oligonucleotide linker added on to each end of lac5. The diagram in "A" illustrates the non-phosphorylated form of the linker. The BamHI recognition sequence for restriction cleavage is indicated by an (\ or /) symbol. The diagram in "B" illustrates the 5′phosphorylated form of the linker.
| 5' | CGGGATCCCG | 3' |
|----|-------------------|
| 3' | GCCCTAGGGC       | 5' |

A.

\[
\begin{array}{c}
\text{5' CGGGATCCCG 3'} \\
\text{3' GCCCTAGGGC 5'} \\
\end{array}
\]

B.

\[
\begin{array}{c}
\text{5' pCGGGATCCCG 3'} \\
\text{3' GCCCTAGGGGp 5'} \\
\end{array}
\]
Fig. 16. The 6,925 base pair lac5 segment (indicated inside brackets and shown by itself in Fig. 14) with linkers covalently attached. The ... symbolizes additional linkers which may or may not be present. The (\ or /) symbols have the same meaning as in Fig. 15.
\[ 5'... \text{pCGGGATCCCG}[C \:\cdots \:\cdots \:\cdots AAGCTT]}CGGGATCCCG ... 3'
\]
\[ 3'... \text{GCCCTAGGGC}[G \:\cdots \:\cdots \:\cdots TTGCAA]}GCCCTAGGGGCp... 5' \]
Fig. 17. Linkered lac5 after BamHI restriction. The DNA molecule shown in Figure 16 was converted to the single, linkered BamHI form shown here by restriction with BamHI endonuclease. The base pairs within brackets represent the original blunt ended 6,925 base pair lac5 segment and base pairs outside of the brackets represent BamHI restricted linkers.
(6,925 bp lac5 operon)

5' GATCCCG[------AAGCTT]CGG
3' GGC[---------TTGCAA]GCCCTAG

5' GATCCCG[------AAGCTT]CGG
3' GGC[---------TTGCAA]GCCCTAG
the leu transformants checked for beta-galactosidase.

**TRANSFORMATION OF YEAST FOR LEU2 GENE**

**PZ1000** is a derivative of pMC1403 which was itself derived from pBR322. An insertion of lac Z, Y and part of the A gene derived from *E. coli* into pMC1403 allows for lac gene expression in *E. coli*. The first 8 codons of the lac Z gene have been omitted. Other modifications of pBR322 made in constructing pMC1403 include deletions to remove particular restriction sites and additions or substitutions to add various restriction sites to certain regions of the plasmid. PZ1000 consists of the pMC1403 plasmid but has in addition an approximate 800 base pair random yeast fragment downstream from lacZ and the entire LEU2 gene upstream of the lac complex. (T. Zamb). An *E. coli* ampicillin resistance gene is also present. The plasmid is depicted in Figure 19. In my research, the initial source of pZ1000 DNA for *S. cerevisiae* transformation was from *E. coli* strain MC1066 grown on ampicillin containing LB medium. The antibiotic was included in the growth medium to select for cells carrying the plasmid but no attempt was made to directly select for the lac operon or for LEU2. Plasmid DNA so obtained was then used to transform two leu yeast strains, AH22 and SSU10. Transformants were selected by growth on regen- agar which contains no leucine. Prototrophs were
obtained but none tested lac by the beta-galactosidase assay procedure using indicator agar.

The LEU2 gene was, as indicated above, selected as the yeast transformation marker in this phase of the study. This gene when associated with 2um circular DNA as in YEpl3 and pZ1000 has been found by others to transform leu2 yeast strains to leu with an efficiency of $10^3$ to $10^4$ transformants per ug of DNA (14,7). This is similar to the $1.0 \times 10^3$ transformants observed in the present research.

In addition to being chosen for its high transformation efficiency, the 2um associated LEU2 gene was also chosen because of its ability to transform yeast in both the stable integrative, and the less stable but plasmid retrievable non-integrative forms (42). The final rationale for the selection of this marker was the availability of several stable leu yeast recipient strains, namely AH22 and SSU10. The AH22 strain contains two different mutations in its LEU2 gene and has a reversion rate of less than $10^{-10}$ (42). The SSU10 strain also carries a stable leu marker (Zamb, T.).

TRANSFORMATION OF ESCHERICHIA COLI FOR LAC OPERON WITH PZ1000

It was assumed that leu2 yeast transformants carried the pZ1000 plasmid and therefore possessed the lac operon. Since they surprisingly tested out negative for beta-galactosidase, it was necessary to go back and check the plasmid transforming principle for the lac operon. This was
Fig. 18. Plasmid YEpl3 with the 6,925 base pair lac5 segment-linker complex inserted into the BamHI restriction site. Bases indicated with red represent linker DNA, bases in green indicate lac5 DNA segment, and bases in black represent YEpl3 DNA. Amp r and tet r represent ampicillin and tetracycline resistance genes respectively. The yeast leucine gene is located within the YEpl3 sequence.
Fig. 19. Diagram of pZ1000 plasmid. This plasmid was derived from a pMC1403 plasmid. Amp represents an ampicillin resistance gene. lac Z, Y and A are explained in text. Leu2 represents the yeast leucine gene.
done by transforming lac $\mathbf{E}$. coli strains with the plasmid and plating out the transformants on XG agar. Lac transformant colonies did appear here indicating lac was indeed present in the plasmid.

My study is not the only one where the lac operon has been utilized, refer to (6) for a partial review. Some investigators (5, 13) have used the Z gene of the lac operon as an indicator of other genes. In these studies the Z gene produced or did not produce betagalactosidase when fusions, deletions, or DNA rearrangements had taken place.

**TRANSFORMATION OF YEAST FOR LAC OPERON WITH PZ1000**

Plasmid DNA from a pZ1000 transformed $\mathbf{E}$. coli strain positive for beta-galactosidase was used to transform AH22 and SSU10 yeast strains. Selection for transformants was on medium lacking leucine. Some of the transformants obtained were found to possess beta-galactosidase indicating they carried the lac operon, supposedly in the pZ1000 plasmid.

The rate of plasmid transformation in yeast is somewhat dependent on the form of the plasmid DNA molecule, with increasing transformation efficiency linked to the linear form (43). This might explain the present findings where some leu $^+$ pZ1000 transformants were lac $^-$. Broken linearized DNA molecules may have been taken up by the yeast cells here and although able to code for $B$-isopropylmalate dehydrogenase (LEU2 gene product) they might not have been
able to successfully code for betagalactosidase. The ratio of beta-galactosidase positive yeast transformants to leu transformants was not determined, however, all yeast and bacterial transformation control plates were negative.

A 31,400 base pair LAC5 containing plasmid designated G10.53.lac was made by J. Panthier et al (59). This plasmid also had a URA3 sequence (originally a low transformation frequency gene) associated with a 2um circle DNA fragment inserted into an EcoRI site. Panthier found colonies of yeast producing beta-galactosidase among ura3 transformants. The betagalactosidase producing yeast, however, had a low level of plasmid expression and produced only about 30 active betagalactosidase molecules per yeast cell. In comparing the beta-galactosidases produced by G10.53 lac transformed yeast and E. coli, Panthier showed that the two enzymes were identical electrophoretically, immunologically and in their sedimentation behavior in the ultracentrifuge.

One aim in my attempt to construct a lac plasmid complex was to keep it as small as possible. The reason for this was to increase the plasmids average copy number per cell and thereby hopefully increase levels of beta-galactosidase. A relationship between small plasmid size and higher copy numbers has generally been found (50). The plasmid lac complex envisioned in my research would have contained 17,625 base pairs. Besides having a beneficial effect
upon copy numbers it also seemed possible that a smaller plasmid would have a greater chance of becoming chromosomally incorporated into the yeast cell genome. This may have further increased expression of lac.

**SUMMARY AND RECOMMENDATIONS**

Lambda strain Cl857S7 lac5 was grown on *E. coli* Y-mel for large scale production of the virus genome. Comparisons made between restriction fragments of Cl857S7 lac5 and Cl857S7 DNA separated by agarose gel electrophoresis demonstrated that lac5 was indeed present in the Cl857S7 lac5 genome. Lac5 separation from the Cl857S7 lac5 genome was accomplished with HindIII. HindIII cut the genome into 7 fragments and one of these, a 25,737 base pair piece, contained lac5. This fragment was purified and concentrated and served as a substrate for size reduction by KPNI. The purpose was to obtain a lac5 fragment with less extraneous DNA. The result was a 6,925 base pair piece of DNA containing lac5. This was separated from other fragments by agarose gel electrophoresis. The 3' overlapping end of the 6,925 base pair fragment was removed by T-4 DNA polymerase exonuclease activity. The 5' overlapping end was filled in with nucleotides using the polymerizing activity of DNA polymerase I (Klenow fragment). The purpose of treating the overlapping ends was to obtain blunt ended lac5 for linking. Linker DNA fragments
(decaoligonucleotides) were 5' end phosphorylated using T-4 polynucleotide kinase. Phosphorylated linker DNA was treated with T-4 DNA ligase to ascertain the amount of blunt end ligation that had occurred with the viral enzyme. Once ligation was confirmed through self ligation of the linkers, the blunt ended 6,925 base pair fragment was linker to the decaoligonucleotide in a similar manner. The resulting linker lac5 fragment was restricted with BamHI to produce overlapping ends suitable for insertion into the plasmid vector YEpl3. Unfortunately the preparation was lost at this stage.

The plasmids YEpl3 and pZ1000 amplified and not amplified (using E. coli strains HB101 MC1066 and CSH18) by chloramphenicol were isolated in sufficient quantity for bacterial transformation. A separate plasmid purification to enhance the ratio of covalently closed circular DNA was also performed on YEpl3. The plasmid preparations were restricted and the fragments separated by agarose gel electrophoresis for identification and quantification purposes.

YEpl3 and pZ1000 were used to transform various E. coli strains so as to study changes in phenotypic response brought about by plasmid DNA presence and thereby verify plasmid gene function. No differences were found between chloramphenicol amplified and non amplified plasmid preparations from E. coli and S. cerevisiae. Phenotypic
analysis of various E. coli strains transformed with YEpl3 and pZ1000 demonstrated that these plasmids have functional promoters and marker genes and in the case of pZ1000, a functional lac operon. YEpl3 and pZ1000 were also used to transform two S. cerevisiae leucine auxotrophs, AH22 and SSU10. Analysis of the two transformed yeast strains indicated that they carried the plasmids and the plasmids had functional yeast promoters, leu markers and for pZ1000, an active bacterial lac operon. Prototrophic industrial yeast strains were also exposed to YEpl3 and pZ1000 but no lac transformants were recovered.

Experience gained from the yeast studies indicated that indicator agar (page 34) should not be used in the future for detecting yeast lac transformants but that instead it should be replaced by sensitive blue agar.

For the transformation of industrial yeast strains which as a rule are not auxotrophic, a method of selection different from that used in my research is necessary. Selection of transformants from among a population of plasmid treated cells based on beta-galactosidase production is unlikely because most of the cells are not transformed. These nontransformed cells are not selected against and tend to overgrow and thereby mask any transformants that may be present. Furthermore, typical bacterial markers such as the Amp and Tet resistance genes on pBR322 and YEpl3 plasmids are of no value for selection
of transformants among eukaryotic cells because they are not expressed. There has been preliminary research at the University of Wisconsin which addresses this transformant selection problem and offers promise (26). This research focuses on an antibiotic (G413) which when in proper concentration inhibits many prokaryotic and eukaryotic cells but is inactivated by a plasmid coded enzyme which phosphorylates the antibiotic.

Another approach which would allow for the screening of transformants from non transformants would be to use auxotrophs for transformation. These strains are not now available, however, and would first have to be obtained by mutagenesis. Such mutants would have to possess a chromosomal mutation in the same gene carried by the plasmid (eg leu2 gene). There are several drawbacks to this approach (A) most industrial yeast strains are polyploid making it unlikely that mutations could be created simultaneously at the various loci of the same gene (B) each yeast strain so obtained should have 2 stable point mutations (a double mutation) in each gene used for selection to ensure a low reversion frequency (43), and (C) auxotrophic industrial strains may lose some of their growth potential, fecundity and/or alcohol producing efficiency after auxotrophic producing mutagenesis.
LITERATURE CITED


