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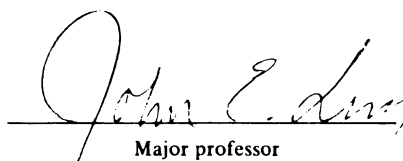
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**TRANSFORMATION OF MUCOR BY A PLASMID SHUTTLE VECTOR WITH A
DOMINANT SELECTABLE MARKER : THE BENOMYL RESISTANCE GENE**

by

Juili L. Lin

A THESIS

submitted to
Michigan State University
in partial fulfillment of the requirements
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ABSTRACT

TRANSFORMATION OF MUCOR BY A PLASMID SHUTTLE VECTOR WITH A
DOMINANT SELECTABLE MARKER : THE BENOMYL RESISTANCE GENE

By

Juili Lillian Lin

A transformation system was developed in the filamentous fungous Mucor racemosus by complementation of the leucine auxotrophic mutant strain R7B with the Leu1 gene from wild type cells. To apply this transformation system to industrially important species of Mucor, a dominant selectable marker, the Ben^R gene, which encodes a benomyl resistant β -tubulin protein from N. crassa was inserted into the plasmid shuttle vector pLeu4. The correct construction of pMBen plasmids was confirmed with restriction endonuclease analysis. These constructs were transformed into protoplasts of M. racemosus R7B. The presence of unaltered pMBen plasmids in Mucor transformant cells was confirmed with Southern analysis. No significant increase in the resistance level to benomyl was observed in cells transformed with pMBen1 and pMBen2 when compared with resistance levels of the non-transformed host strain. Further studies will focus on the regulation of Ben^R gene expression in Mucor cells.

DEDICATION

To Lin, T. C. and Lin, J. - my dear parents

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INTRODUCTION

Mucor, filamentous fungi of the class of Zygomycetes, have received the attention of academic researchers because of their ability to undergo cellular morphogenesis (Cihlar, 1985). Various species of Mucor are important to the food industry because of the production of extracellular enzymes which are used world wide in food processing (Crueger & Crueger, 1982). Hydrolytic enzyme production also results in this group of organism being leading causative agents of food spoilage.

Although a large quantity of biochemical research data has accumulated on Mucor, the absence of an efficient sexual recombination system for genetic analysis and manipulation (Gauger, 1965; Schipper, 1978) made it desirable to develop a transformation system and introduce molecular genetics to enhance the study of these organisms. One species, Mucor racemosus, has a haploid vegetative phase and a small genome (10^7 bp) which made it particularly convenient to target for molecular genetic study. Recently a transformation system was developed in this organism, based on the complementation of a leucine auxotrophic mutant strain, M. racemosus R7B, by

a homologous gene (Leu1) from wild type cells. This transformation system has limitations in practical application, because it can only be used for transformation of this particular mutant strain. A transformation system useful for other Mucor species is necessary at this stage. A dominant selectable marker for transformation is highly desirable because it can be selected in a wide variety of genetic backgrounds. For research on morphogenesis, this approach can save the time of generating complex mutant strains ordinarily needed for studies involving gene complementation, gene disruption and gene replacement. To the food industry, this approach will provide a simpler method to utilize the tools of molecular genetics to study the control of spoilage organisms (M. circinelloides, M. piriformis, M. racemosus) and an opportunity to do genetic engineering on enzyme producing strains or protein engineering to alter the functionality of enzymes used in food processing.

Benomyl is a fungicide which has been used for a long time (Davidse, 1988). Recently, a benomyl resistance gene, Ben^R, which is a mutated β - tubulin gene from a Neurospora crassa benomyl resistant strain, was cloned and its nucleotide sequence determined. This benomyl resistance gene has been used as a dominant selectable marker for the transformation of fungi including N. crassa and Gaeumannomyces graminis (Henson, 1988). In preliminary experiments conducted in our

laboratory, two strains of M. racemosus 1216B - wild type and R7B, were found to be sensitive to benomyl at a minimum inhibitory concentration (M.I.C.) of 50 μ g benomyl/ml for cells grown in the dark and 100 μ g benomyl/ml for cells grown in the light.

Based on these preliminary data, our goal for this project was to utilize the benomyl resistance gene (Ben^R) from Neurospora crassa as a dominant selectable marker to transform Mucor racemosus and to measure the functional expression of this heterologous gene in Mucor transformants. We hypothesize that the expression of the N. crassa Ben^R gene in M. racemosus will increase their resistance to benomyl.

HISTORICAL REVIEW

1. BIOTECHNOLOGY AND THE FOOD INDUSTRY

Food Processing Enzymes and Food Additives

The food processing industry is the oldest and the largest industry using biotechnological processes. Biotechnology can be described as the controlled and deliberate application of simple biological agents, living or dead cells, or cell components, in technically useful operations, either of productive manufacture or as service operations (Bu'lock, 1987). The use of biotechnology started more than 8000 years ago with alcoholic beverage, vinegar, sourdough and cheese production by "natural" microbial and enzyme processes (Knorr, 1985). Modern biotechnology began with Weizmann's development of a practical acetone-butanol fermentation process in 1915 (Bu'lock, 1987) aided by the development of microbiology in the late 19th century. Biotechnological processes are used to mass-produce other fermented products like ethanol, food and bakery products, animal feed, and food additives like antibiotics, organic acids, nucleic acids, vitamins, single-cell protein, and processing aids including enzymes like hydrolyases, proteolyases, carbohydrases, and lipases

(Godfrey, 1983). From an economic point of view, in 1985, United Kingdom (UK) consumers spent \$ 36 billion on food, which is 20% of their total consumption. The output of the food industry in that year was about 17.5% of total gross manufacturing output (King and Cheetham, 1987). The food processing industry, having annual sales of \$ 30 billion in the UK, and \$ 300 billion in the United States, is the largest user of biotechnological processes (Table 1, pg.6) (Knorr, et al, 1985). The application of biotechnology can reduce the manufacturing cost and improve the quality of products in the food industry (Newell, 1986).

In general, an industrial process can be implemented either by chemical synthesis or bio-conversion which involves the use of living cells or enzyme systems in chemical modifications. Biocoverion is often preferable because of high substrate specificity, regiospecificity (site specificity), stereospecificity, and mild reaction conditions (Crueger and Crueger, 1984). Today, with the aid of biotechnology, the production of many food additives and food processing enzymes is more economical and efficient. For example, in 1954, world consumption of methionine was a few million pounds at a cost of almost \$ 3.00 per pound, while in current years, methionine production has soared to over 200 million pounds per year, with a price of \$ 1.70 per pound (Paul, 1981).

Table 1. World Market Value of Selected Biotechnology-based Products

Products	Market size (millions U.S. \$)		Primary end use
	1981	1990 (estimated)	
Amino acids	1.9×10^3 1.8×10^3	2.2×10^3	Feed additive, food enrichment and flavoring agent, feed preservative
Citric acid			Food additive, processing aid
Enzymes	310 to 400	1.5×10^3	Processing aid
Vitamins	668		Feed and food additive, food enrichment agent
Baker's yeast	1.1×10^3		Food additive, enrichment agent
Beer	27×10^3	44×10^3	Beverage
Cheese			Food
Fermented foods	3.5×10^3	6×10^3	Food
Miso			Food
Soy sauce			Food

(Knorr and Sinskey, 1985)

Genetic Engineering

Modern biotechnology has entered a new phase with the discovery of tools for genetic engineering beginning in 1960. The ability to manipulate genetic material by molecular biologists has made it possible theoretically to construct DNA molecules containing genes and regulatory elements from any cell type and to clone and propagate these new molecules in suitable host cells (Bu'lock, 1987). Genetic engineering combines recombinant DNA technology, which allows improved protein production, and protein engineering, which allows the improvement of protein properties and the creation of new products (Lin, 1986).

Recombinant DNA technology, which breaks and rejoins DNA molecules from different species, has widened the range of end-products that can be considered for commercialization to include even those from unusual organisms, plants and animals via heterologous gene cloning and expression (Bu'lock, 1987; Pitcher, 1986). High yields can be achieved in desired host organisms by increasing the copy number of the desired gene in the host organism or by regulating the level of gene expression (Pitcher, 1986). The cost of end-product recovery can be reduced by the utilization of a secretion system in the host to generate a high production level of active extracellular end-product. The production of unwanted proteins, nucleic acids or other by-products can be eliminated by specific deletion or by changing to a host organism without

these disadvantages. For example, bacterial secretion of calf rennin was difficult to achieve, but scientists successfully achieved the secretion of properly processed prochymosin, a precursor of calf rennin, from filamentous fungi (Pitcher, 1986).

Protein engineering manipulates the primary structure of a protein by synthesis of a novel DNA sequence or by site-directed oligonucleotide mutagenesis (Craik, 1985; Dalbadie-McFarland, et al 1983; Zolkr and Smith, 1983). Modification of the structure of a protein can improve the activities and functionalities of a protein including substrate specificity, chemical stability, thermal stability, pH optimum, catalytic activity or to create entirely new protein products (Pitcher, 1986; Kang, 1985; Lin, 1986). Advances in protein engineering require the continual convergence of several technologies, such as protein crystallography, X-ray diffraction, sophisticated computer graphic systems and systematic analysis of protein folding and subunit association (Estell, 1985). A seemingly minor alteration at the nucleic acid level can have profound effects on the behavior characteristics of a protein used in processing and can improve the processing procedure or the texture, flavor, and color of the processed food.

Several enzymes, some showing modified catalytic

properties, have been investigated using recombinant DNA and site-directed mutagenesis techniques. A good example was reported by Wells, et al. (1985) and concerned the improvement in chemical oxidation resistance of subtilisin by site-directed mutagenesis. Research on engineering of caseins, which are functional milk proteins and serve as the basis for a major segment of dairy industry, are progressing (King, 1987; Kang, 1985).

Industrial Applications The development of biotechnology using the technology of genetic engineering may lead this world to better health, more food, more economical energy sources, cheaper and more efficient industrial processes and reduced pollution (Prentis, 1984). The application of genetic engineering can reduce the cost of manufacturing by cloning an engineered gene into host cells which can utilize cheap substrates, grow faster, and achieve highly regulated production of a secreted end-product. Table 2. (pg.11) shows the development of genetic engineering to solve some problems in food production. For example, CPC-International (1984) has submitted to the Food and Drug Administration (FDA) the first petition for generally recognized as safe (GRAS) status for production of a food grade enzyme, α -amylase, in an engineered strain of Bacillus subtilis (Pitcher, 1986). Genecor is working on commercial production of rennin by secreting filamentous fungi such as

Aspergillus and Trichoderma. This enzyme has received much attention as a candidate for the commercialization of recombinant DNA technology. Significant progress in constructing amylolytic strains of yeast has demonstrated their potential for ethanol or alcoholic beverage production utilizing starch as a substrate (Tubb,1986). As shown in Table 3. (pg.12), almost all food processing enzymes and additives have been targeted by genetic engineering techniques (Lin, 1986). The pharmaceutical industry has also utilized genetic engineering successfully for years. Numerous mammalian gene products including antiviral, antitumor and antidiabetic agents and growth promoting factors have been developed, expanded and are being commercialized (Lin, 1986).

2. HETEROLOGOUS GENE EXPRESSION / SECRETION SYSTEMS

Genetic engineering technology made possible the use of microorganisms for large-scale synthesis of industrial proteins from a variety of sources including mammalian, animal, and plant cells or other microorganisms. Strain selection and mutagenesis can be used to improve the production of an endogenous (homologous) protein (Momos and Furaya, 1980 ; Miyagawa, et al,1986). However, introduction of a functional gene for a heterologous protein into a suitable host microorganism, has the potential to achieve more efficient production (Lin, 1986). While, every protein has

Table 2. Some Proposed Genetic Engineering Solutions to Problems in Food Production

Problem	Solution
Availability of calf rennin for cheesemaking is limited	Induce microorganisms to produce calf rennin by interspecies gene transfer of the calf gene to yeasts or fungi
Environmental problems are caused by disposal of large volumes of lactose-containing whey Many enzymes lack stability for use in food processing	Transfer the lactase gene from <i>Escherichia coli</i> into yeast, which is able to use sugars for fermentation Engineer minor structural changes in enzyme molecule that lead to new and stronger internal bonding patterns by pinpoint alteration of the structural gene Amplify the number of genes coding for those proteins to increase protein or enzyme output
Microorganisms and plant cells produce desirable proteins and enzymes, but at levels too low for commercial utility Frost damage limits the growth season of many fruits and vegetables	Genetically modify <i>Pseudomonas syringae</i> to delete one of its proteins that promotes ice nucleation; spray organism on plant to compete with natural flora Transfer gene for an enzyme that catabolizes herbicide into plant or genetically modify herbicide target site Identify genes for these enzymes to understand how their appearance is regulated. Use this knowledge to design improved cultivars and storage and handling techniques
Herbicides are toxic to desirable crops as well as to weeds Appearance of softening enzymes in fruits and vegetables such as pectinases and cellulases leads to senescence and short shelf life	Pinpoint the lipoxxygenase gene to understand its regulation, and develop methods to limit its expression Amplify genes coding for proteins containing low levels of these amino acids for increased protein production; evaluate nutritional value and functionality of expressed proteins
Frozen vegetables sometimes become rancid due to the action of the enzyme lipoxxygenase Important grains and legumes are deficient in essential amino acids; e.g., soybean is low in sulfur-containing amino acids, and corn is low in lysine	

(Wasserman, 1988)

Table 3. Food Processing Enzymes and Food Additives used in the U.S. Food Industry that Benefit from Genetic Engineering Technology

Category	Example
Food Processing Enzymes	
Starch processing	α -Amylase β -Amylase Glucoamylase Glucose isomerase Pullulanase
Dairy products	Rennin Lipase Lactase
Brewing	Amylases Proteases
Wine/fruit/vegetable processing Fuel alcohol	Pectinases Amylases Glucoamylase
Food Additives	
Low-calorie products	Aspartame Thaumatococcus
Flavor enhancers	Glutamic acid 5'-Ribonucleotides
Human and animal diet supplements	Amino acids Vitamins
Stabilizing agents	Xanthan gum
Preservatives	Cecropin

(Lin, 1986)

its own unique properties, so does every host / vector secretion system have unique properties (Van Brunt, 1987). The systems used for heterologous gene expression include E. coli, Bacillus (prokaryotic cells), yeast, filamentous fungi, plant tissue and mammalian cells (eukaryotic cells).

Prokaryotes E. coli has been used as a host to synthesize large quantities of recombinant proteins for a long time. E. coli cultures grow relatively rapidly by utilizing cheap nutrient sources and can express complementary-DNA (c-DNA) of foreign genes with the aid of constitutive or regulated promoters (like the promoters from lac, trp genes, Edens, et al, 1982; Emtage, et al, 1985) for over production (Lin, 1986). But E. coli does not have the ability to properly perform the posttranslational modifications that many eukaryotic proteins require to be fully functional. Furthermore, E. coli, is a Gram-negative bacterium and secretes end-products into the periplasmic space of the cell (Hsiung, et al, 1986) if the proper E. coli secretion signal peptides are fused to the amino terminus of the protein. The expression of rat proinsulin, human proinsulin, human immunoglobulin light chain, and human growth hormone (hGH) (Chang, 1987; Hsing, 1986) have been achieved, but the end-products need to be recovered by osmotic shock and other procedures. This increases the cost of product recovery.

The other commonly used prokaryotic host is Bacillus, a Gram-positive bacterium. Bacillus, which has been used in fermentation industry for a long time (Ehrlich, 1978), is a GRAS strain, and is able to secrete the end-product into the growth medium. This may reduce the end-product purification cost. The expression of the α -amylase gene from B. amyloliquefaciens in B. subtilis has improved production 2,500 fold over that level obtained previously (Wasserman, 1984). But Bacillus also is not able to properly posttranslationally modify preproteins translated from eukaryotic genes (Van Brunt, 1986).

Eukaryotes The yeast, Saccharomyces cerevisiae, a lower eukaryote, is another microorganism which is widely used in the food industry (Tubb, 1986). Many studies have been done on heterologous protein production in yeast (Kingsman, 1985). Eukaryotic proteins including calf prochymosin (Mellor et al, 1983), and interferon gamma (IFN- γ) (Derynck, Singh and Goeddel, 1983) which are produced by E. coli in an inactive state, are produced as soluble, biologically active proteins in S. cerevisiae. Yeast cells appear to be very stringent about the sequence-specific recognition of splice junctions of Hn-RNA (Dobson, et al, 1982). Yeast cells are not able to correctly process the Hn-RNA of the Aspergillus glucoamylase gene (Innis, et al, 1985), the tRNA^{Tyr} gene of *Xenopus* oocytes (Nishikura, 1982) or the Schizosaccharomyces tRNA^{Ser} gene

(Greer, et al, 1987). So, a c-DNA copy of the foreign gene must be cloned into yeast to make expression possible. S. cerevisiae requires yeast derived expression signals; a promoter region, which initiates and regulates gene transcription; and a termination region, which stabilizes the message, to generate efficient transcription. The copy number of plasmid vectors in yeast was relatively low until the discovery of the 2 μ m plasmid, which leads to high copy number. The secretion system of yeast cells is very similar to that in higher eukaryotic cells. A functional secretion signal sequence can be fused to the heterologous gene to ensure the correct posttranscriptional modification and cotranslational transport through the endoplasmic reticulum resulting in secretion of a biologically functional end-product (Kingsman, et al, 1985). Most of the heterologous proteins produced in yeast are rich in mannose (Penttila, et al, 1987) and seem to be hyper-glycosylated, whereas homologously produced mammalian glycoproteins contain a variety of glycosyl residues with complex branching (Staneloni and Leloir, 1982). It is unlikely that mammalian proteins, which require complex and specific carbohydrate modifications, will be biologically functional when synthesized in yeast. Intensive research to solve the inherent obstacles of these microbial hosts is continuing.

One group of organisms with great potential for synthesis

/ secretion of heterologous proteins is the filamentous fungi. Filamentous fungi are eukaryotic cells which naturally secrete vast quantities of extracellular products including amylase, cellulase, protease, rennin, pectinase, lipase, antibiotics and citric acid (Montenecourt, 1985; Cullen and Leong, 1986; Crueger and Crueger, 1984). These products are widely used in food processing and as food additives. For instance, glucoamylase, one of the largest bulk enzymes in the world, is produced by industrial strains of Aspergillus niger. These strains can naturally produce up to 20 grams of enzyme per liter of growth medium from a single copy of the glucoamylase gene (Van Brunt, 1986). Another group of industrially useful filamentous fungi are Mucor, which produce a variety of extracellular enzymes (Van Heeswirck, 1986). Strains of M. pusillus Var. Lindt and M. miehei are used world wide for production of rennin, which is second on the list of industrial enzymes in terms of volume of sales (Crueger and Crueger, 1982).

Filamentous fungi are also good at the expression and secretion of biologically active mammalian proteins like bovine chymosin (Cullen, et al, 1987), human interferon (Gwynne, et al, 1987) and human tissue plasminogen activator (Upshall, 1987). The filamentous fungi can correctly perform posttranscriptional processing of m-RNA, and often posttranslationally modify heterologous polypeptides to

functional proteins. The development of a heterologous protein secretion system in GRAS strains of filamentous fungi is one significant approach to advance modern biotechnology.

3. FUNGAL GENETIC SYSTEM

Sexual and Parasexual Recombination In microbes, recombination of DNA molecules can take place by a wide variety of mechanisms, resulting in a complete chromosome at one extreme to substitution of a single gene, or part of a gene, at the other. The various recombination processes, with examples of microbes to which they apply, (with an emphasis upon industrial organisms), are listed in Table 4 (pg.18). Sexual reproduction in eukaryotic microbes is akin to classic breeding techniques in plants and animals which have resulted in widespread successes in agriculture and horticulture. The most notable commercial successes have been in genetic improvement of yeast involved in baking, brewing, wine making and single cell protein (Miwa, et al., 1978; Johnston & Oberman, 1979; Spencer & Spencer, 1983; Snow, 1983). The majority of eukaryotic micro-organisms of industrial importance, however, do not appear to possess sexual capabilities. Nevertheless, recombinants can be produced in many filamentous fungi by exploiting 'parasexual' mechanisms based upon mitotic rather than meiotic events. An industrial

Table 4. Various Processes of Genetic Recombination
Underlying Construction of New Recombination Strains
of Industrial Micro-organisms

<i>Type of microbe</i>	<i>Recombination process</i>	<i>Examples where applied</i>
Eukaryotic	Sexual hybridisation	<i>Saccharomyces</i> yeast
	Parasexual breeding	<i>Aspergillus</i> spp. <i>Acremonium chrysogenum</i>
Eukaryotic and prokaryotic	Protoplast fusion	<i>Candida tropicalis</i> <i>Bacillus</i> spp.
	Native DNA transformation	<i>Streptomyces</i> spp. <i>Bacillus subtilis</i> <i>Neurospora crassa</i>
	Gene cloning, vector transformation	<i>Bacillus</i> <i>Streptomyces</i> <i>Saccharomyces</i>
	Gene cloning, transfection Transduction Conjugation, plasmid transfer	} <i>Bacillus</i> , <i>Streptomyces</i>
Prokaryotic		

(Johnston, 1985)

strain of Aspergillus niger used for the production of the enzyme amyloglucosidase, is a parasexual recombinant of a high yielding strain and a lower yield strain with superior filtration characteristics (Ball, et al., 1978). A self-diploid strain obtained by inbreeding a production strain of A. niger produces improved yields of citric acid (Das & Roy, 1978).

Notwithstanding some impressive results of sexual and parasexual breeding, a major advance in the construction of recombinant strains has ensued from the process of protoplast fusion. The principal barrier to cellular or hyphal fusion in many cases is the cell (or hyphal) wall, and its removal, under appropriate conditions, frequently permits fusion between normally incompatible strains, species, genera and even phyla. Protoplast fusion has been used to produce new strains of industrial microbes, e.g. Acremonium chrysogenum (Hamlyn & Ball, 1979), Penicillium chrysogenum (Queener & Baltz, 1979), Streptomyces (Hopwood, 1981), brewing yeasts (Russell & Stewart, 1979) and distillery yeasts (Mowatt, et al., 1983). Interspecific hybrids have included P. chrysogenum / P. cyaneo-fulvum (Queener & Baltz, 1979) and intergeneric crosses including those of Candida tropicalis / Saccharomycopsis fibuligera (Provost, et al., 1978) and Candida utilis / Trichoderma reesei (Heslot, 1980).

Molecular Genetics

The major disadvantage of these various genetic techniques, including protoplast fusion, is the combining of large numbers of genes from both parental strains into one cell. Thus if a range of strain characteristics is of importance in the industrial process, it is very probable that the majority of recombinants obtained is inferior to the parental production strain or strains. Often, improvement of only a single characteristic is sought and, in the simplest case, this will be controlled by a single gene. It is therefore highly desirable to attempt addition of only this one gene to the parental strain. The mechanisms of molecular genetics including transduction, transformation and transfection afford such a possibility. The procedures of genetic engineering not only satisfy this objective but, in addition, potentially allow transfer of a gene or genes between any two organisms, whether related or unrelated.

Genes, normally located on chromosomes, are also found extrachromosomally as part of DNA molecules existing as plasmids or in organelles such as mitochondria. Genes may be transferred between some micro-organisms by transduction and transformation. In transduction, genes of a donor bacterium are carried into a recipient strain by incorporation into infective viruses (bacteriophages). Phage are of great importance as vector molecules in genetic engineering.

Transformation Systems-Overview Genetic transformation was used initially in bacterial cells and involves the uptake and expression of genes from a donor strain in a recipient organism. The preparation of protoplasts, however, improved uptake in eukaryotic cells as did the discovery that PEG promotes transformation in addition to protoplast fusion.

In transformation, vectors serve as a vehicle to carry the foreign DNA into the host cell and help to propagate that DNA fragment. The essential steps for in vitro transformation procedures are summarized in Figure 1 (pg.23, 24). Once inside the host cell, fragments of DNA may either be integrated into chromosomes or alternatively circularize to form extrachromosomal molecules which behave as plasmids.

Selectable Markers To facilitate selection of the foreign DNA in transformed cells, the vector, generally a plasmid, must carry a gene whose expression in the host offers a selective growth advantage. To date, two approaches used for selection have been the complementation of a recessive mutation in the auxotrophic host strain and, the use of markers whose presence can be selected in wild-type cells, so - called dominant transformation markers (Rine and Carlson, 1986).

Figure 1. Microbial Transformation Procedures
(Prentis, 1984)

- (1) fragmentation of DNA extracted from a particular organism, usually by digestion with 'restriction' endonuclease enzymes.
- (2) joining of individual fragments into suitable vector DNA molecules, such as plasmids or viruses, often by the action of ligase enzymes.
- (3) introduction of recombinant DNA molecules into appropriate cells by transformation, transfection or transduction.
- (4) selection for cells, containing recombinant DNA by means of selectable markers.

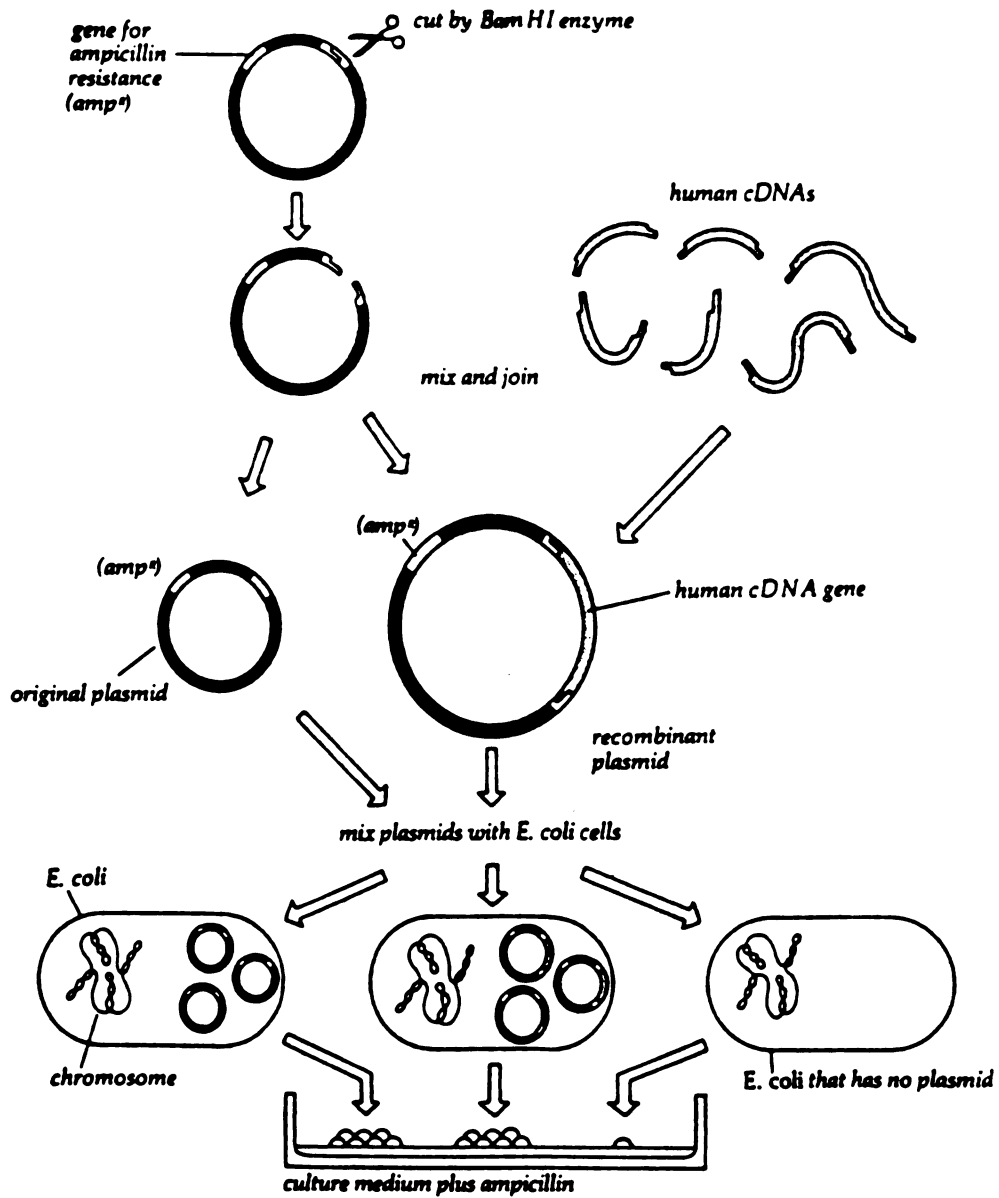


Figure 1. Microbial Transformation Procedures (Prentis, 1984)

Auxotrophic Markers Transformation of nutritional mutants with wild-type genes has been the main approach. For this procedure to be successful, it is necessary to have an appropriate, prototrophic gene and a stable, auxotrophic mutant (Turner and Ballance, 1986). Recessive auxotrophic mutants are usually obtained by mutagenesis of the wild type strain, and selection of mutants by Lederberg's replica plating technique (Crueger and Crueger, 1982). The wild type prototrophic gene corresponding to the auxotrophic mutation can be cloned by complementation of the mutant strain with a DNA library generated from wild type DNA.

Dominant Selectable Markers Dominant selectable markers are ideal in any transformation system, because their use avoids the need to construct complex mutant strains for use as recipients. Host strain construction is relatively tedious, and very time consuming, especially in organisms lacking a sexual recombination system. Antibiotic resistance genes are widely used as dominant selectable markers in prokaryotic and eukaryotic transformation systems. The antibiotic resistance gene may come from resistant mutants of the wild strain, or from other resistant microorganisms.

Fungal Transformation Systems

Yeast In yeast, host strains that are auxotrophic

for amino acids such as leucine, tryptophan and histidine or bases such as uracil, can be used with cloning vectors that contain the appropriate yeast gene for complementation. (Botstein, et al., 1979; Beach and Nurse, 1981; Beggs, 1978; Hinnen. et al., 1978; Hitzeman, et al., 1980). However, industrially-developed yeast strains are generally polyploid (triploid or higher) and vectors used to transform them should bear a positive selection characteristic (like, resistance to toxic substances) that does not require prior genetic modification of the host strain (Saunders, G., et al., 1986). The use of the hygromycin B resistance gene, the G418 resistance gene and the copper resistance gene solved this problem (Kaster, 1984; Butt, 1984). The CUP1 vectors, encoding metallothionein proteins which confer copper resistance, have been successfully introduced into brewing yeast strains (Henderson, 1985; Butt, 1987).

Filamentous Fungi The development of vectors for transformation of filamentous fungi have, to a large extent, lagged behind those developed in yeast, but in general have followed the same basic strategies. In Neurospora crassa, the first vectors described used the N. crassa qa-2⁺ gene. This gene encodes the catabolic enzyme dehydroquinase and can be used to functionally complement a strain of N. crassa with qa-2⁻ and arom-9⁻ mutations, (lacking both catabolic and biosynthetic dehydroquinase activity) (Case, 1979). Later in

1984, another auxotrophic selectable marker, the *am* gene of *N. Crassa*, which codes for NADP - specific glutamate dehydrogenase (GDH), was used to transform *am* mutant strains to prototrophy (Kinsey, 1984). Recently the development of benomyl resistance gene as a dominant selectable marker was found to be ideal for transformation of *N. crassa* (Orbach, 1986).

The genetics of *Aspergillus nidulans* have been studied in some detail since Pontecorvo, et al., (1953) first described the system. A number of selectable markers has been developed for transformation of the filamentous fungi *Aspergillus niger* and *Aspergillus nidulans*. In most cases selection of transformants is based on complementation of auxotrophic mutants. A plasmid carrying the *N. crassa* *pyr4* gene (encoding orotidine - 5' - phosphate decarboxylase), was used to functionally complement an *A. nidulans* *pyrG* mutant (Ballance, 1983). Other auxotrophic mutants (from the same organism) such as the *trpC* mutant, which lacks phosphoribosylanthranilate isomerase activity (by the trifunctional *trpC* gene) (Yelton, 1984); and the *ArgB* mutant, which lacks ornithine transcarbamylase (Buxton, 1985) have been successfully used as recipient hosts.

Some dominant selectable markers do not require special mutant strains. For example, the *amdS* gene allows growth on

acetamide or acrylamide as the sole nitrogen source (Tilburn, 1983; Kelly, 1985). But this marker can be used only in strains that have no requirement for nitrogen-containing compounds interfering with the amds selection (Punt, 1987). Another useful dominant selectable marker is the *oliC31* gene, which encodes an oligomycin resistant variant of the mitochondrial ATP synthase subunit 9 and confers oligomycin resistance (Ward and Turner, 1986; Ward, Wilkinson and Turner, 1986). This gene should prove useful as a marker for gene replacement or disruption experiments or transformation of Aspergillus strains lacking auxotrophic lesions (Ward, Wilkinson and Turner, 1986). However, this gene is probably species - specific since it requires the formation of a functional oligomycin - resistant ATP synthase complex (Punt, et al., 1987). In 1987, a new dominant selectable marker used for transformation of A. nidulans transformation was reported. This marker, the E. coli hmb (hygromycin B) gene, encodes phosphotransferase hph which phosphorylates and inactivates the antibiotic, hygromycin B (Malpartida, 1983; Gritz and Davies, 1983). Expression of the hph gene was controlled by A. nidulans glyceraldehyde -3- phosphate dehydrogenase gene (*gdh*) and *trpC* expression signals in A. nidulans transformation (Cullen, 1987; Punt, 1987). Fusions of fungal promoters with the hph gene have also been used to transform S. cerevisiae (Gritz and Daves, 1983), and most, recently, several filamentous fungi, e.g. Cephalosporium acremonium

(Queener, et al., 1985), Ustilago maydis (Cullen, 1987), and Cochliobolus hoterostrophus (Yoder, et al., 1986). Currently, another prokaryotic dominant selectable marker, the neomycin resistance gene, has been used successfully in transformation of lower eukaryotes, animal cells and plant cells (Reiss, 1984). This gene encodes a neomycin phosphotransferase II (NPT II) which confer the resistance to aminoglycoside antibiotics like Kanamycin (Km), gentamycin and neomycin. This gene has been successfully used in transformation of fungi including Absidia glauca (Wostemeyer, 1987) and Phycomyces blakesleeanus (zygomycetes) (Revuelta, 1986), Cephalosporium acremonium (Skatrud, 1987), and the slime mold Dictyostelium discoideum (Hirth, 1982).

4. MUCOR - FILAMENTOUS FUNGI OF THE CLASS ZYGOMYCETES

Food Industry Species of Mucor produce a variety of extracellular enzymes including amylase (Adams, 1976), rennin (Arima, 1968, Ottesen, 1970), cellulase (Somkuti, 1974) and lipase (Somkuti, 1968). Strains of M. pusillus var. Lindt and M. miehei are used in the production of rennin, which is the second most important enzyme used in the food industry (Crueger and Crueger, 1982). Species of Mucor are also important to the food production industry because of food spoilage (Pitt, 1985). Mucor is ubiquitous and grows rapidly

in nature. M. circinelloides has been reported to spoil cheese (Northolt, 1980) and yams (*Dioscoria* sp.; Ogundana, 1972). M. piriformis was reported to be a destructive pathogen of fresh straw berries (Lowings, 1956; Harris, 1980). M. racemosus is responsible for a spongy soft rot of cool stored sweet potatoes, potatoes and citrus (Chupp, 1960).

Morphogenesis The biochemistry and molecular biology of Mucor rouxii and Mucor racemosus have been studied because of their ability to undergo physiological alternations between yeast and hyphae. It is a particularly attractive system since the yeast - to - hyphae transition can be either induced (Bartnicki - Garcia, 1962; Mooney, 1976) or inhibited (Larsen, 1974; Ito, 1982) in a variety of ways. A large quantity of biochemical and molecular research data has accumulated about macromolecular biosynthesis, enzyme activity and expression, and posttranslational modification of proteins (Cihlar, 1985).

Transformation The absence of an efficient sexual recombination system for genetic analysis and manipulation of Mucor (Gauger, 1965; Schipper, 1978) made it desirable to develop a transformation system and introduce molecular genetics to the study of these organisms. One species, M. racemosus, has a haploid vegetative phase and has a small genome (10^7 bp) which made it particularly convenient to

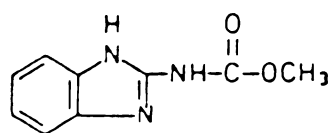
target for molecular genetic study. A high frequency transformation system was developed in this organism using protoplasts of a leucine auxotrophic mutant of M. racemosus strain R7B and a shuttle vector pLeu4, which is able to propagate in bacterial cells and in M. racemosus (Van Heeswijck, 1984). The plasmid shuttle vector pLeu4 contains a fragment of DNA from E. coli plasmid pUC 13 and a fragment of homologous DNA containing the functional Leu1 gene from a wild type strain of M. racemosus. The application of this transformation system is very limited because the construction of Leu⁻ mutant strains for use as recipients in transformation is tedious and very time consuming. This transformation system cannot be currently used to study most other important strains of Mucor including the food spoilage causing strains or enzyme producing strains which are less amenable to genetic manipulation. Owing to the drawbacks and limitations in the use of this transformation system, we sought to develop a dominant selectable marker for use in Mucor, which can be used in wild-type genetic background.

Resistances to antibiotics or fungicides are potentially very useful dominant selectable markers. The development of a dominant selectable marker for transformation of Mucor will provide a mechanism to utilize the tools of molecular genetics such as gene cloning by complementation, gene disruption and gene replacement in the study of either dimorphism or food

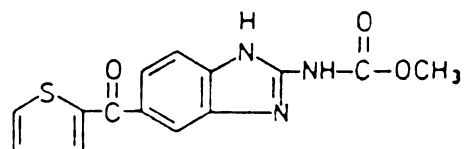
spoilage in Mucor species. This system also will enhance our ability to genetically engineer the GRAS industrial strains of Mucor. For example, microbial rennins produced by Mucor are temperature stable, remaining active in the curd after precipitation and subsequently causing harmful proteolysis (Cruger and Cruger, 1982). The advances of biotechnology in genetic engineering provides the opportunity to alter the heat stability of this protein.

5. BENOMYL RESISTANCE GENE FROM N. CRASSA AS A DOMINANT SELECTABLE MARKER IN TRANSFORMATION OF M. RACEMOSUS

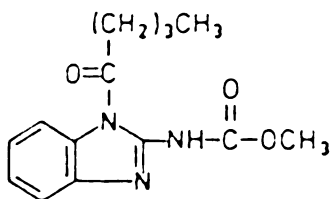
Benomyl (Figure 2, pg.32) is one of the benzimidazole fungicides which have been used in agriculture for a long time (Davidse, 1988). The benzimidazoles bind to tubulin, which is a heterodimer. Its subunits, usually designated α -tubulin and β -tubulin, each have molecular weights of ca 50,000 Daltons (Dustin, 1984). Benomyl binding to tubulin interferes with the normal assembly of microtubules, causing their gradual disappearance as the tubulins disassemble (Davidse, 1986). The inhibition of microtubule assembly disturbs a great number of cellular processes that involve microtubules, including mitosis, meiosis, DNA synthesis (by inactivating the spindle in animal and plant cells); intracellular transport of molecules, particles, and organelles; maintenance of cell



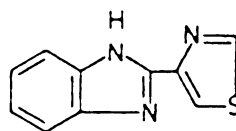
carbendazim



nocodazole



benomyl



thiabendazole

Figure 2. Chemical Structures of Antifungal Benzimidazoles

(Davidse, 1988)

shape, and cell mobility through ciliar and flagellar action. Benomyl induces nuclear instability in A. nidulans diploids (Hastie, 1970). In benzimidazole-treated germinating conidia of A. nidulans, spindle formation does not take place (Kunkel, 1980). Under light and electron microscopy, the disappearance of microtubules in carbendazim treated hyphal tip cells of Fusarium acuminatum causes the displacement of mitochondria from hyphal epics, reduces linear growth rate and arrests metaphase of mitosis (Howard, 1980; 1977). Benomyl also induces multipolar germination in N. crassa due to the inhibition of nuclear migration into multiple germ tubes. The germ tubes stop growing, swell and emit several branches (Can Caesar-Ton, 1988).

In 1978, Sheir-Neiss, et al. suggested that the structure of β -tubulin is the major determinant for binding of benzimidazoles. Recently, a benomyl resistance gene, Ben^R, was cloned, and its nucleotide sequence determined (Orbach, 1986). The Ben^R gene is a mutated β -tubulin gene from a benomyl resistant strain of N. crassa and encodes a protein of 447 - amino acid residues. The mutation responsible for benomyl resistance comes from a single base change in the DNA sequence (UUC to UAC), which results in a phenylalanine-to-tyrosine change at amino acid position 167. This benomyl resistance gene has been used as a dominant selectable marker for the transformation of N. crassa at a frequency of $>10^3$

transformants per μg of DNA. The amino acid sequence of N. crassa β -tubulin has been found to be highly homologous to the sequence of other tubulin proteins. Homology to S. cerevisiae, S. pombe, chicken, Trypanosoma brucei, and Chlamydomonas reinhardtii protein is 76, 77, 83, 78, and 79%, respectively (Orbach, 1986). This high level of sequence conservation of β -tubulins from different organisms suggested that the benomyl resistance gene might be used as a dominant selectable marker in transformation of other organisms including Mucor. In fact, the Ben^R gene has been successfully used in the transformation of Gaeumannomyces graminis (Henson, 1988).

In preliminary experiments conducted in our laboratory, two strains of M. racemosus 1216B - wild type and R7B, were found to be sensitive to benomyl at a minimum inhibitory concentration (M.I.C.) of 50, 25 $\mu\text{g}/\text{ml}$ for cells grown in the dark and 100 $\mu\text{g}/\text{ml}$ for cells grown in the light. Sensitivity of the organism to moderate levels of benomyl suggests that the benomyl resistance gene from N. crassa may be a useful dominant selectable marker to improve the transformation system of Mucor. Our goal for this research project was to utilize a vector containing the N. crassa benomyl resistance gene to transform Mucor cells, and to test the expression of this heterologous gene in Mucor transformants.

MATERIALS AND METHODS

1. MICROBIAL STRAINS

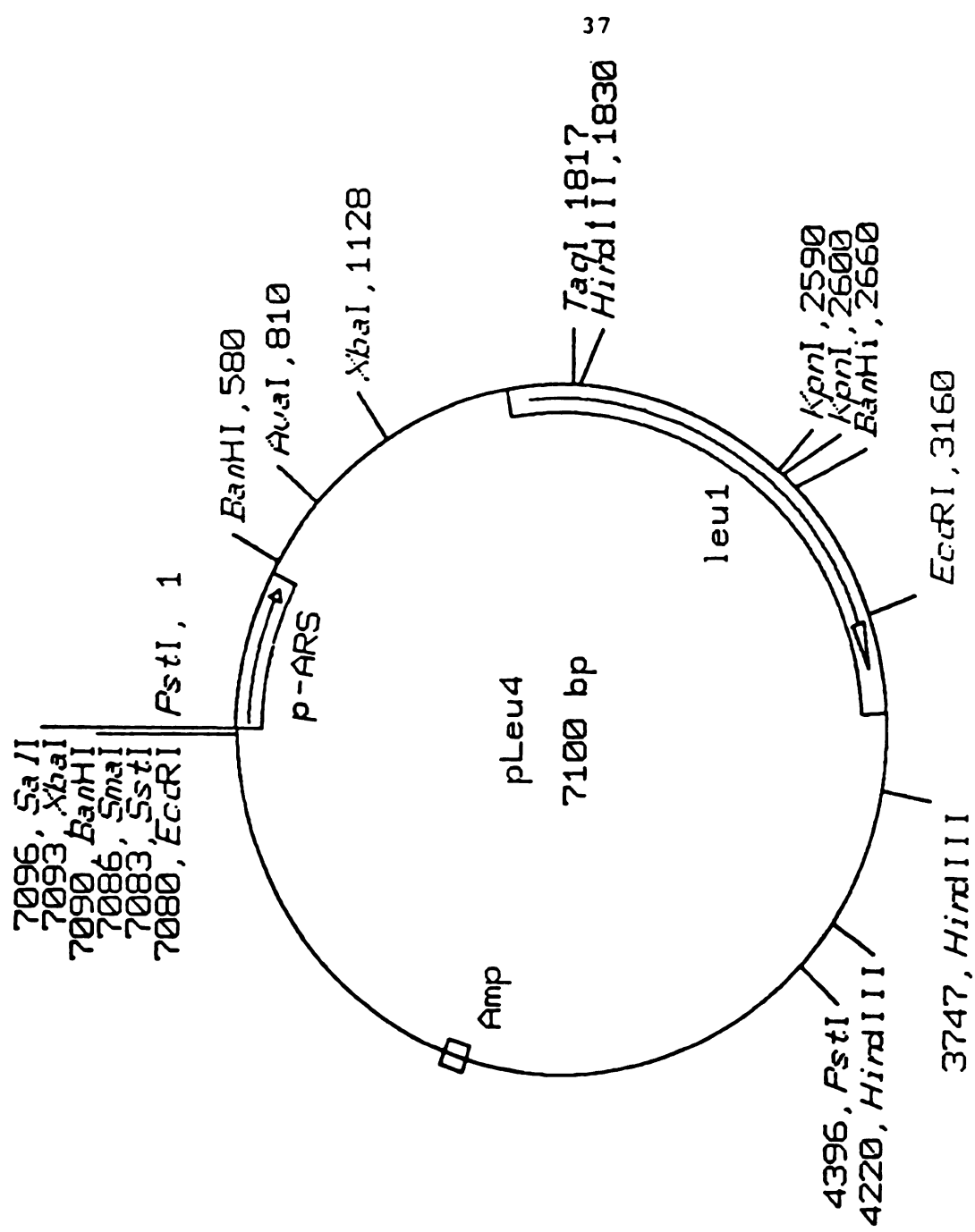
Escherichia coli strain JM83 (asa (pro-lac) rpsL, thi phi80 dlacZ M15) E. coli strain LE392 (F-, hsd R514 supE44 supF58 lacY1 gal K2 galT22 met B1 trpR55) and E. coli strain DH 5 α (endA1 hsdR17(r_K⁺m_K⁺) supE44 thi-1 recA1 gyrA relA1 ϕ 80lacZ M15 (lacZYA-argF)_{U169}) were used as recipient strains in bacterial transformation experiments and for propagation of vector molecules.

Mucor racemosus ATCC 1216B R7B (Leu-) (abbreviated as M. racemosus R7B), a leucine auxotrophic mutant strain derived from the wild type parent strain (M. racemosus [circinelloides] ATCC 1216B (abbreviated as M. racemosus 1216B), Van Heeswijck, A. ,1984), was used as recipient strain in the Mucor transformations.

2. PLASMIDS

Plasmid pLeu4 (Figure 3, pg.36, 37) is a Mucor / E. coli shuttle vector constructed from pUC13 containing the β -

Figure 3. Plasmid pLeu4



lactamase gene (Amp^R) for the selection of ampicillin resistance in E. coli, and a 4.4 Kb PstI restriction fragment of M. racemosus genomic DNA which carries a leucine biosynthetic gene (Leu1) as a selectable marker for transformation of M. racemosus 1216B R7B (leucine auxotroph) and a putative autonomous replication sequence (ARS) (Roncero, et al., 1988).

Plasmid pBT3 (Figure 4 pg.39, 40) is a pUC12 vector which contains a 3.1 Kb HindIII restriction fragment from the genomic DNA of a benomyl resistant Neurospora crassa strain Bml511 (r)a (Orbach, et al, 1986), encoding a benomyl resistant β -tubulin protein.

Plasmid pMCup1-A (Figure 5, pg.41, 42) is a pUC8 vector which contains a 3.3 Kb Eco RV - Nru II restriction fragment from the genomic DNA of M. racemosus strain 1216B (Luis Sosa, 1987, personal communication) thought to encode a metallothionein protein conferring copper resistance.

3. CHEMICALS

Chemicals used in these experiments were from Sigma Chemical company with the exception of those specifically described. Restriction endonucleases, T4 DNA ligase ,DNA polymeraseI (Klenow fragment), 2'-deoxyribonucleotides, and

Figure 4. Plasmid pBT3

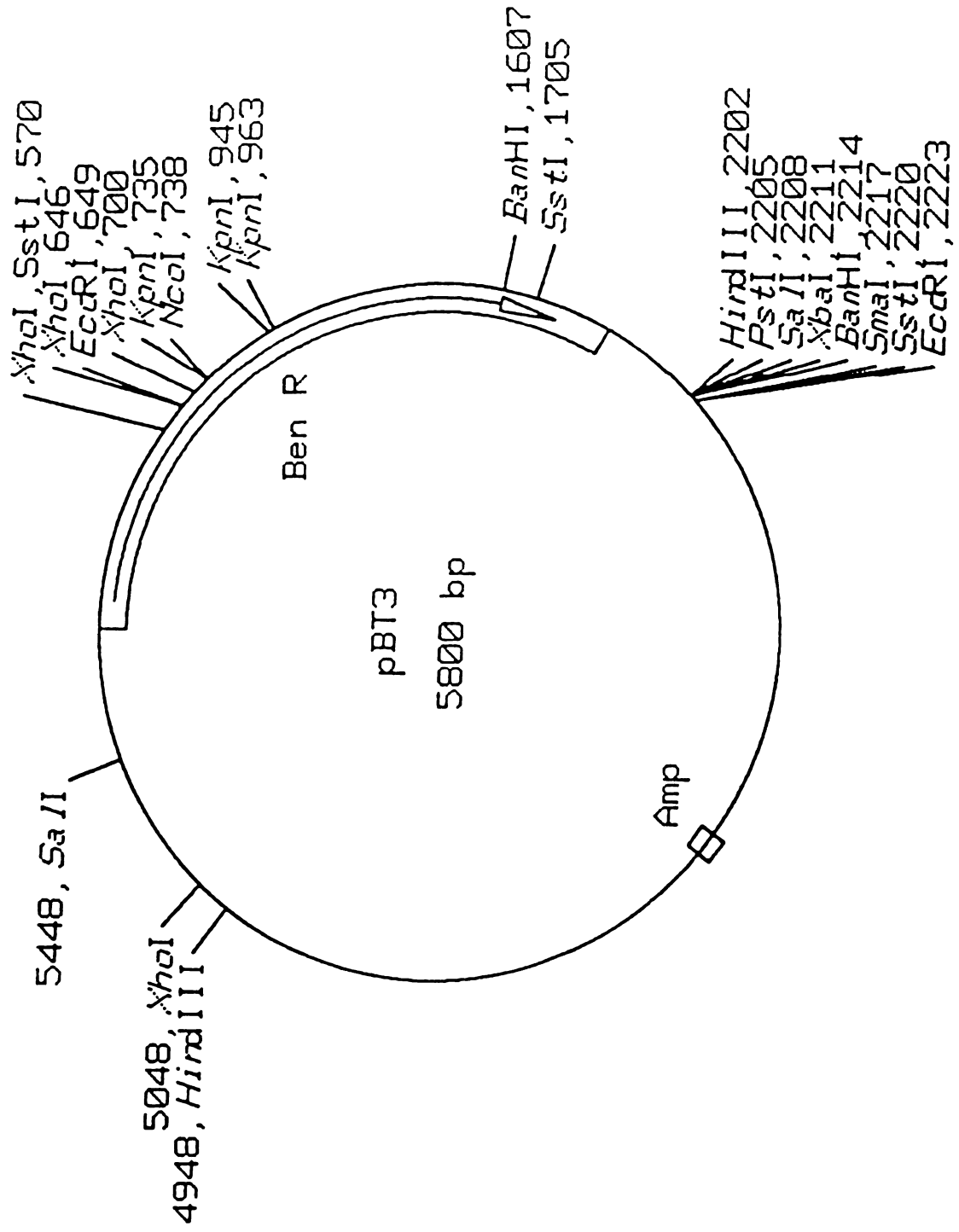
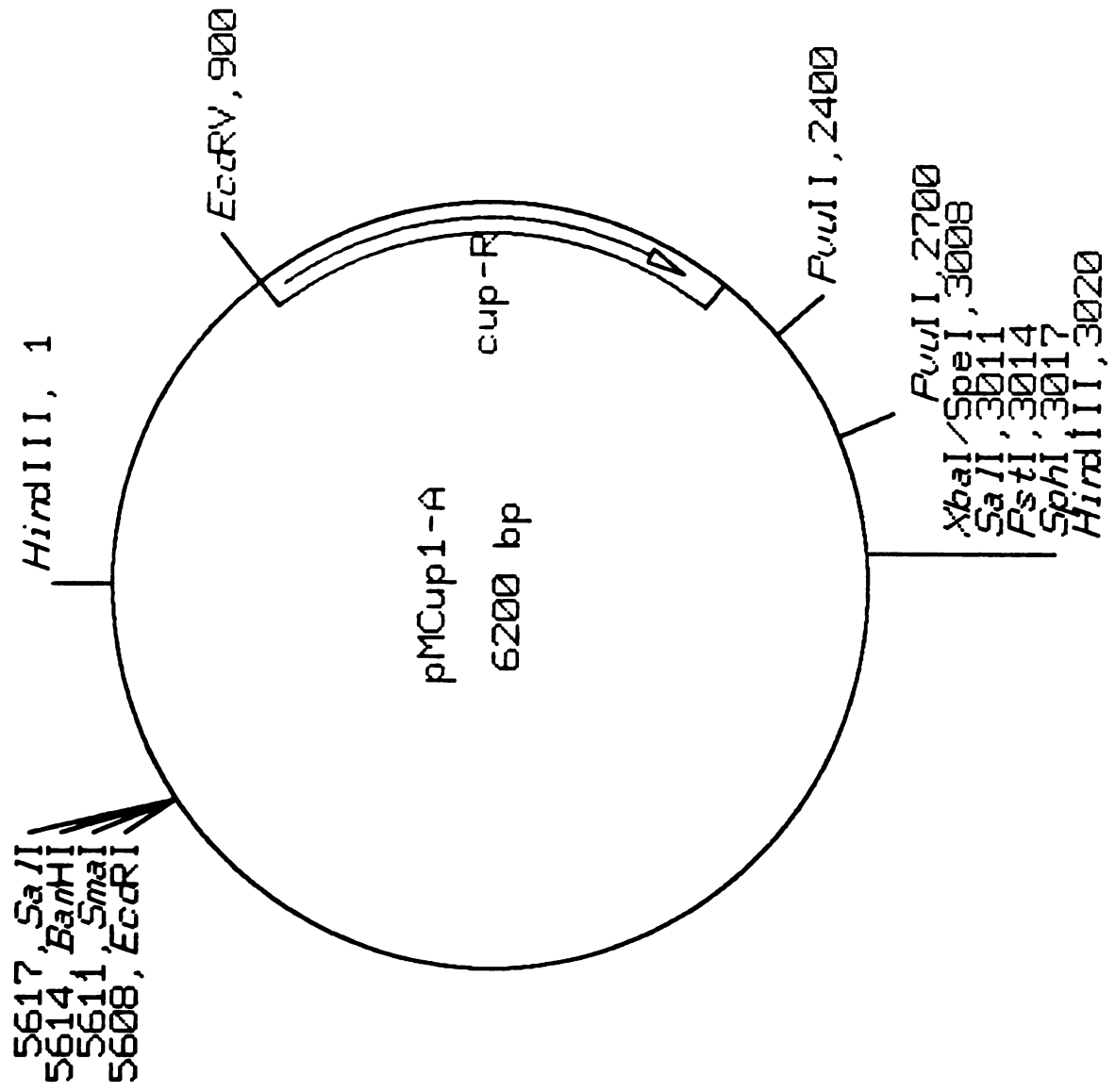


Figure 5. Plasmid pMCup1-A



buffers for these reactions were obtained from Boehringer Mannheim Biochemicals. [α -P³²] dGTP (>6000 Ci/mmol) was obtained from New England Nuclear (USA). Polyethylene glycol 4000 (PEG 4000) was obtained from Fluka AG (Switzerland). Novozyme 234 (prepared from Trichoderma harzianum, containing mainly α -1,3-glucanase activity) was from Novo Industries.

4. GROWTH MEDIA AND GROWTH CONDITIONS FOR E. COLI AND M. RACEMOSUS

E. coli cultures were grown in Luria-Bertani (LB; Maniatis, et al, 1982) medium at 37°C with vigorous shaking (rotary shaking platform, 250 rpm; rotating wheel, 100 rpm). E. coli strains which contained plasmids conferring ampicillin resistance were propagated on LB medium supplemented with 100 μ g/ml of ampicillin. E. coli strains which produced β -galactosidase from pUC plasmids were identified by supplementing LB media with the chromogenic substrate, 5-bromo-4-chloro-3-indolyl- β -D-galactoside (x-gal, Sigma) to a final concentration of 40 μ g/ml.

YPG (complete) medium (1 Liter) contained 10 g of bacto-peptone, 3 g of bacto yeast extract and 20 g of glucose. The pH was adjusted to 4.5 with sulfuric acid. YNB (minimal defined) medium (1 Liter) contained 0.5 g yeast nitrogen base,

1.5 g ammonium sulfate and 1.5 g glutamic acid. The pH was adjusted to 4.5 or 3.0 (depending on the different procedures) with sulfuric acid. After autoclave sterilization, sterile glucose (1%, final concentration), thiamine (1 µg/ml, final concentration) and niacin (1 µg/ml, final concentration) were added (Van Heeswijck, 1984).

Sporangiospore Preparations M. racemosus 1216B
and M. racemosus 1216B R7B were grown on YPG media or YNB media enriched with 1mM leucine. Cells transformed with pLeu4 derived plasmids were grown on YNB (minimal) media without leucine to select for the functional Leu1 gene on the plasmids. Asexual sporangiospores were inoculated onto the center of solid growth medium and incubated at room temperature, under room atmosphere, with normal room light for 7 days to generate large spore stocks. The sporangiospores were harvested with ice-cold sterile distilled water by scraping the surface of the mycelium with a sterile glass rod (Paznokas and Sypherd, 1975). The spores were diluted with YNB broth medium, quantitated with a hemacytometer, and used to inoculate fresh cultures or stored at -20°C after addition of glycerol (20%, V/V).

5. THE MINIMUM INHIBITORY CONCENTRATION OF BENOMYL OR COPPER FOR MUCOR

The minimum inhibitory concentration (MIC) of benomyl or copper for Mucor were determined with YNB media plus leucine supplemented with various concentrations of the inhibitors. Approximately 1×10^2 viable sporangiospores of M. racemosus 1216B and M. racemosus 1216B R7B were inoculated onto the YNB medium. Duplicate plates were incubated at room temperature under normal room light for 1 week, and individual colonies counted.

6. TRANSFORMATION AND SELECTION OF E. COLI TRANSFORMANTS

Transformation of E. coli strains JM83, JM101 and DH5 α were performed as described in Maniatis et al (1982). Log phase cells were resuspended in a solution containing calcium chloride (100 mM). Exposure to calcium ions renders the cells able to take up DNA (competent cells). Plasmid DNA (1-4 μ g) was mixed with 200 μ l of competent cells for 30 minutes on ice. The competent cells were then heat shocked at 42°C for 2 minutes to allow the DNA to efficiently enter the cells. The cells were grown 60 minutes at 37°C with shaking in 1 ml LB media to allow cell recovery and the synthesis of plasmid-encoded β -lactamase, which cleaves the β -lactam ring of ampicillin and confers resistance to ampicillin. Cells were then plated on LB media containing 100 μ g/ml of ampicillin to allow the selection of plasmid containing colonies.

7. THE IDENTIFICATION OF RECOMBINANT PLASMIDS IN E. COLI

E. coli transformants containing the desired recombinant plasmid were identified by colony hybridization (Maniatis, T., et al, 1982 , Grunstein and Hogness 1975) with ^{32}P -labeled DNA fragments as probes. This procedure was used to screen small numbers (100-200) of bacterial colonies. The colonies were simultaneously consolidated onto a master agar plate and onto a nitrocellulose filter laid on the surface of a second agar plate. After growth overnight, the cells on the nitrocellulose filter were lysed, denatured with Southern base (1.5 M NaCl and 0.5 M NaOH) and neutralized with Southern neutralizer (1 M Tris-Cl and 1.5 M NaCl, pH adjusted to 8.0 with HCl). The DNA liberated from these colonies was fixed to the filter by baking at 80°C for 2 hours under vacuum. Hybridizations were performed at 37°C in a hybridization solution which contained 5X SSC (20X SSC contains 3M sodium chloride and 0.3M sodium citrate, pH 7.0), 5X Denhardt's (1L of 50X Denhardt's solution contains 10 g ficoll, 10 g polyvinylpyrrolidone, 10 g bovine serum albumin [Pentax Fraction V] in 1 L of distilled water), 50% formamide, 0.1% SDS, 5mM EDTA AND 100 $\mu\text{g/ml}$ denatured salmon sperm DNA. After two non-specific washes (at 2X SSC, room temperature, for 15 minutes) and one high stringency final wash (0.5 X SSC, at 65°C for 1 hour), the filters were analyzed by autoradiography. Colonies, whose DNA gave positive

autoradiography results, were recovered from the master plates.

8. ISOLATION OF PLASMID DNA FROM E. COLI

Minipreps Plasmid DNA was isolated by the alkaline lysis method using a rapid, small-scale protocol described in Maniatis (et al, 1982). DNase-free RNase (20 $\mu\text{g/ml}$) was added to digest contaminating RNA. The plasmid DNA was stored in TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) buffer at -20°C to prevent DNase degradation.

Large Scale Plasmid DNA Isolation We used the large scale plasmid isolation method described by Maniatis et al (1982). A small volume of overnight culture (0.1 ml) was added to 25 ml of LB containing ampicillin (100 $\mu\text{g/ml}$) in a 100 ml pyrex erlenmeyer flask, and incubated at 37°C with vigorous shaking until the growth of cells reached midlog phase (A_{600} equals 0.4). This culture was added to 500 ml of LB (containing 100 $\mu\text{g/ml}$ ampicillin) in a pyrex erlenmeyer flask (2 liter) for overnight incubation. The chloramphenical amplification procedure was not used, because amplification does not significantly improve in the recovery of pMB-1 derived pUC vectors.

After alkaline lysis, the plasmid DNA was purified by

equilibrium centrifugation on cesium chloride - ethidium bromide density gradients. After isoamyl alcohol extraction and dialysis, the plasmid DNA was stored in TE buffer at 4°C.

9. RESTRICTION ANALYSIS OF PLASMID DNA

Approximately 0.6 to 0.8 µg of plasmid DNA was digested with restriction endonucleases according to directions provided by the manufacturer. The DNA fragments were separated according to size by electrophoresis through 0.8% agarose gels together with Lamda-DNA/HindIII size marker. Gels were stained with ethidium bromide (0.5 µg/ml) and photographed under UV light.

10. PROTOPLAST FORMATION AND TRANSFORMATION OF MUCOR

Protoplast formation and transformation of Mucor racemosus 1216B strain R7B were based on methods by Van Heeswijck (1984,1984) with the following modifications.

Viable sporangiospores (10^7) of M. racemosus 1216B strain R7B were suspended in 50 ml of YNB broth (enriched with 1mM of leucine) and germinated at 28°C under vigorous shaking for 5.5 hours until the germ tubes were 3 to 5 spore diameters in length. Germlings were harvested by filtration through nylon cloth (mesh size 35 µm), washed twice with 0.5 M

sorbitol, and resuspended in 0.5 M sorbitol, 0.01 M-sodium phosphate buffer pH 6.5. Chitosanase (200 units) and Novozyme (2 mg) were added to 10^7 germlings in 1 ml of buffer. After 40 minutes of incubation at 28°C with mild shaking, protoplasts were generated. Protoplasts were collected by centrifugation (400 X g for 8 minutes at room temperature), and washed twice with 0.5 M sorbitol and once with 0.5 M sorbitol in MOPS buffer, then resuspended with 0.5 M sorbitol in MOPS (3-N-morpholino propane sulphonic acid) buffer (10 mM MOPS pH 6.3, 50 mM CaCl_2) to a final volume of 1 ml. The presence of protoplasts was confirmed by adding distilled water to a small sample. Protoplasts were observed to burst on a hemacytometer slide under a light microscope.

Plasmid DNA was pretreated with 1 mg of heparin in 20 μl of 0.5 M-sorbitol in MOPS buffer for 20 minutes on ice. Plasmid DNA (1 to 4 μg) was added into 0.2 ml of protoplast suspension containing 20 μl of 40% (W/V) PEG 4000 in MOPS buffer, and incubated on ice for 30 minutes. Then 2.5 ml of 40% PEG 4000 in MOPS buffer was added, and the mixture was incubated at room temperature for 25 minutes to complete the transformation. Cell suspensions were washed twice with 20 ml of 0.5 M sorbitol in MOPS and centrifuged at 400 X g at room temperature for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 5 ml of YPG broth (pH 4.5) with 0.5 M sorbitol, and incubated at room

temperature for 30 minutes to allow cells to recover. The cells were washed once with 0.5 M sorbitol and resuspended in 5 ml of YNB broth pH 4.5 with 0.5 M sorbitol. Aliquots of these cell suspensions (0.1 ml and 1 ml) were added to a 9 ml YNB (pH 3.0, with 0.5 M sorbitol) agar overlay (1% w/v agar). Duplicate samples were plated on freshly made YNB medium (pH 3.0) with 0.5 M sorbitol. The pH value of the medium was reduced to limit the colony size of transformants.

11. SINGLE COLONY ISOLATION OF MUCOR TRANSFORMANTS

Sporangiospores of putative transformants were harvested with 200 μ l of ice cold sterile distilled water by carefully scraping the surface of mycelia of each isolated colony with a sterile platinum loop. Dilutions of these spore suspensions (1 and 10^{-1}) were plated on YNB plates, which were incubated at room temperature in an anaerobic jar (BBL GasPak-anaerobic system) for 5 days to isolate single yeast colonies. One well-grown single colony was selected from each plate and reinoculated onto YNB medium to generate a sporangiospore stock.

12. ISOLATION OF GENOMIC AND PLASMID DNA FROM MUCOR

DNA isolation from Mucor strains was based on methods by Van Heeswijck (1984) with the following modification. Viable

sporangiospores (5×10^7) of each clone were inoculated into 250 ml of YNB minimum broth, and incubated at 28°C with shaking for 10 hours under air (0.6 liters / minute). The mycelia were harvested with filtration (Whatman 1mm), and ground under liquid nitrogen in a mortar with a pestle. Then 8 mls of ice-cold TES (contains 100 mM Tris pH 8.0, 150 mM sodium chloride, 100 mM EDTA, 0.1% SDS) was added to the frozen powder and mixed for 1 minute. This suspension was extracted successively with equal volumes of phenol saturated with TES, phenol saturated with 100 mM Tris (pH 8.0), and phenol / chloroform : isoamylalcohol (25 / 24:1) solution for 1 hour, respectively, on a rocker mixing platform. The aqueous phase from each extraction was separated from the organic phase by centrifugation (6800 X g, 10 minutes at 4°C). DNA was precipitated by addition of NaOAC (final concentration is 0.25 M) and 2 volumes of 100% ethanol followed by storage overnight at -20°C. The DNA was pelleted by centrifugation (6800 X g, 10 minutes at 4°C), dried under vacuum, and resuspended in 1 ml of TE with 50 µg/ml of RNase and 100 µg/ml of proteinase K. The solution was incubated at 37°C for 2 hours. This DNA solution was then extracted with an equal volume of phenol / chloroform : isoamylalcohol (25 / 24:1) solution for 5 minutes. After centrifugation (14,000 X g, 4°C for 20 minutes), the aqueous phase was removed. The DNA was precipitated, pelleted by centrifugation and vacuum dried as described above. The pellet was resuspended in 800 µl of TE.

The DNA concentration was measured with a spectrophotometer (1 unit [A_{260}] equals 40 μ g DNA/ml). The concentration of a small quantities of DNA was estimated by comparison of the fluorescence of DNA bands with fluorescence of known DNA standards by agarose gel electrophoresis and staining with ethidium bromide. DNA samples were stored frozen at -20°C .

13. SOUTHERN ANALYSIS

Southern analysis of Mucor genomic DNA and plasmid DNA samples was based on the transfer technique described by Southern (1975). DNA was separated according to size by electrophoresis through a 0.8% agarose gel. The DNA in the gel was denatured, neutralized, transferred and immobilized on a nitrocellulose filter (BA85, from Schleicher & Schuell FRG) with the same solutions as described in colony hybridization. DNA fragments to be used as probes were labeled with [α - ^{32}P]-dGTP by the random primer method (Feinberg and Vogelstein, 1983). The DNA attached to the filter was then hybridized to the denatured ^{32}P -labeled DNA probe. Hybridization was carried out at 37°C - 40°C in hybridization solution which contained 5X SSC, 5X Denhardt's solution, 50% formamide, 0.1% SDS, 5 mM EDTA and 100 μ g/ml of denatured salmon sperm DNA for 18 hours. After two non-specific washes (2X SSC, room temperature for 15 minutes) and the one final wash (1 hour, at 65°C , 3X, 0.5x, or 0.1X SSC depending on

stringency needed), filters were autoradiographed to locate the position of target bands complementary to the radioactive probe.

RESULTS

1. SENSITIVITY OF Mucor racemosus TO BENOMYL OR COPPER

A prerequisite for the use of the benomyl resistance gene (Ben^R) as a selectable marker in transformation of Mucor racemosus is the sensitivity of the host strain to this drug. Approximately 1×10^2 viable sporangiospores of M. racemosus were inoculated on YNB medium (1216B) and YNB plus leucine medium (R7B) respectively, containing different amounts of benomyl (0, 10, 20, 50, or 100 μg benomyl/ml). Growth of M. racemosus 1216B and M. racemosus R7B was observed after 6 days on all plates except those containing 50 μg benomyl/ml for cells incubated in the dark, and on plates containing 100 μg benomyl/ml for cells incubated in the light. These data suggested the M.I.C. to benomyl of both the wild strain (1216B) and the leucine auxotrophic mutant strain (R7B) of Mucor was between 20 and 50 μg benomyl/ml for cells grown in the dark and was between 50 and 100 μg benomyl/ml for cells grown in the light. In order to be able to detect a small increase in resistance level of pMBen transformants to benomyl, it was desired to determine the M.I.C. more accurately. Approximately 1×10^2 viable sporangiospores of M.

racemosus were inoculated onto YNB medium (1216B) and YNB plus leucine medium (R7B), containing 0, 50, 60, 70, 80, 90, 100, 110, 120 or 130 μg benomyl/ml for cells grown in the light, and 0, 20, 23, 27, 30, 33, 37, or 40 μg benomyl/ml for cells grown in the dark. Growth was observed after 6 days (and maintained up to 15 days) on all plates except those containing 100 μg or more benomyl/ml for cells incubated in light, and on plates containing 30 μg and more benomyl/ml for cells incubated in the dark. These data indicated that the M.I.C. of benomyl to both the wild strain (1216B) and the leucine auxotrophic mutant strain (R7B) of Mucor was between 27 and 30 μg benomyl/ml in the dark and was between 90 and 100 μg benomyl/ml in the light.

The sensitivity of M. racemosus to copper was tested in similar fashion on YNB (1216B) and YNB plus leucine (R7B) media containing 0, 0.1, 0.5, 1, 2, 10, or 20 mM copper. The data suggested that the M.I.C. of copper to both M. racemosus 1216B and M. racemosus R7B was 2.0 mM after 6 days of growth.

The resistance of M. racemosus to hygromycin B was also tested in similar fashion on YNB (1216B) and YNB plus leucine (R7B) media containing 0, 100, 200, or 500 mg/ml hygromycin B. The data indicated that these two Mucor strains were resistant to hygromycin B at 500 mg/ml. Because these two Mucor strains were resistant to this high dose of hygromycin

B, it was not suitable to use hygromycin B as a selectable marker in transformation.

2. THE HOMOLOGY OF THE β - TUBULIN GENES OF M. RACEMOSUS TO THE N. CRASSA BENOMYL RESISTANCE GENE

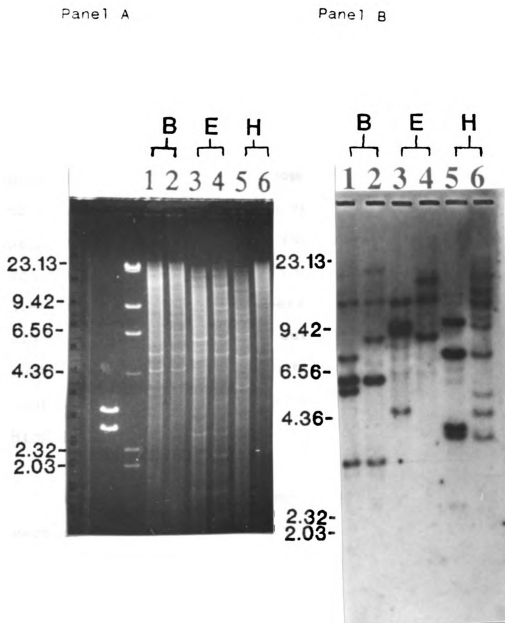
The amino acid sequence of the Ben^R gene from N. crassa is highly homologous to genes encoding β - tubulins in other organisms. To determine the degree of homology of this Ben^R gene of N. crassa to that of M. racemosus, a DNA fragment containing the Ben^R gene from N. crassa was purified, radiolabeled and hybridized to a nitrocellulose paper carrying genomic DNA of M. racemosus R7B and 1216B digested with BamHI, EcoRI, and HindIII. The nitrocellulose filter was washed at medium stringency (2X SSC, 0.1% SDS, at 65°C for 1 hour), and autoradiographed (Figure 6, pg.57). There were more than 3 DNA fragments in R7B and 1216B which hybridized to the benomyl resistance gene. The Southern analysis data suggested that the Ben^R gene from N. crassa has some (medium) homology with several DNA fragments in the genome of M. racemosus. Medina, et al., (1988) reported that there are 3 β -tubulin genes in the M. racemosus genome. Figure 6. (pg. 57) revealed several restriction site polymorphisms between the β -tubulins of M. racemosus 1216B and M. racemosus R7B, the mutant strain derived from M. racemosus 1216B. The differences between the β -tubulins in these strains indicated that they differ from

Figure 6. Southern analysis of genomic DNA of M. racemosus strains 1216B and R7B with ³²P-labelled 2.6 kb SalI fragment from plasmid pBT3, which contains the Ben^R gene.

Panel A Genomic DNA (20-40 ug) was digested with different endonucleases , then analyzed by electrophoresis on a 0.8 % agarose gel. Lane 1, 3, 5 : genomic DNA of M. racemosus 1216B digested with BamHI, EcoRI, and HindIII endonucleases. Lane 2, 4, 6 : genomic DNA of M. racemosus R7B digested with BamHI, EcoRI, and HindIII endonuclease.

Panel B The genomic DNA on agarose gel was transferred onto a nitrocellulose paper, hybridized with the Ben^R probe, washed at medium stringency, and exposed to X-ray film for 40 hours at -70°C.

Figure 6.



each other in more regions of the genome than the Leu1 gene.

3. RESTRICTION ANALYSIS AND SOUTHERN ANALYSIS OF PLASMID PMCUP1-A

The plasmid pMCup1-A is a pUC based vector, which contains a 3.3 kb HindIII fragment from the genome of M. racemosus thought to carry the gene encoding a metallothionein protein. This protein binds copper ions and enhance resistance to copper. Restriction analysis of pMCup1-A with HindIII, SalI, and PstI endonucleases revealed that the plasmid was not constructed as indicated by the supplier (L. Sosa, gift). A 3.8 kb fragment was generated with SalI endonuclease digestion, while PstI only cut this plasmid once. Further restriction analysis (data not shown) revealed that pMCup1-A contains an unknown 500 bp DNA fragment between SalI and HindIII site.

We then obtained a second plasmid, pMCup1-B, a pUC based vector, thought to contain a 1.3 kb EcoRV / NruII fragment subclone of the original 3.3 kb Hind III fragment of pMCup1-A. This 1.3 kb EcoRV / NruII fragment was isolated, radio-labeled and used as a probe in Southern hybridization analysis of pLeu4 and pMCup1-A (Figure 7, pg.60,61.) The 1.3 kb EcoRV / NruII probe hybridized to all bands containing pUC sequences including a 7.1 kb fragment derived from pLeu4 in lane 1, a

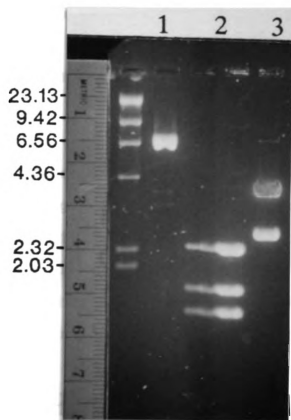
Figure 7. Southern analysis of plasmid DNA with ^{32}P -labelled 1.3 kb fragment from plasmid pMCup1-B

Panel A Plasmid DNA was digested with different endonucleases , then analyzed by electrophoresis on 1 % agarose gel. Lane 1 : plasmid DNA of pLeu4 digested with SalI endonuclease. Lane 2 : plasmid DNA of pMCup1-A digested with EcoRV and PvuII endonucleases. Lane 3 : plasmid DNA of pMCup1-A digested with SalI endonuclease.

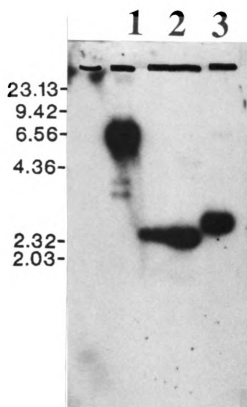
Panel B The plasmid DNA on the agarose gel was transferred to a nitrocellulose paper, hybridized with 1.3kb fragment from plasmid pMCup1-B, washed at high stringency, and exposed to X-ray film for 20 hours at -70°C .

Figure 7.

Panel A



Panel B



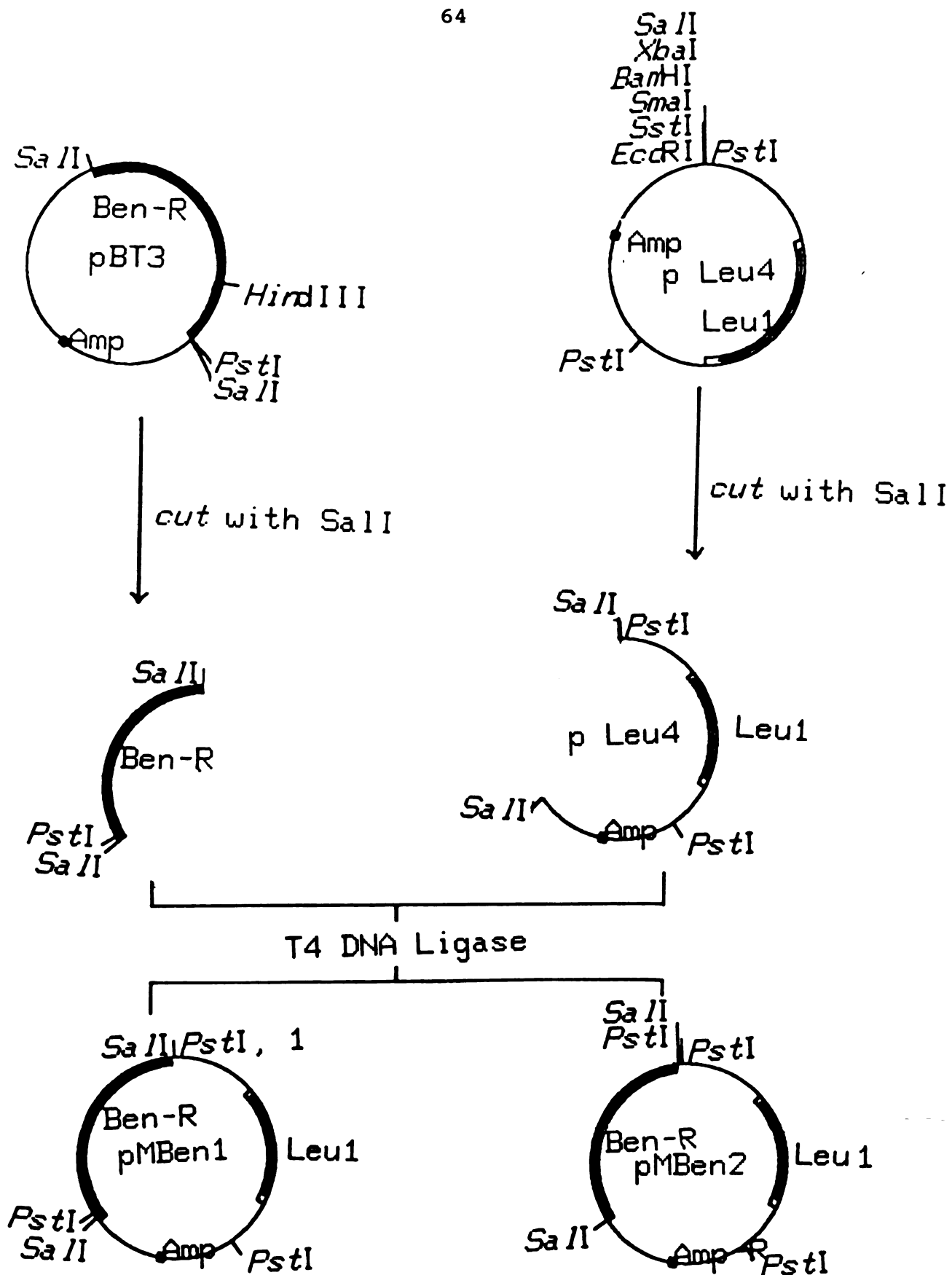
2.35 kb fragment derived from pUC 8 in lane 2, and a 2.7 kb pUC derived fragment in lane 3. We have not confirmed that pMCup1-A and pMCup1-B contained the M. racemosus Cup gene. The use of the copper resistance gene as a dominant selectable marker for Mucor transformation was abandoned awaiting verification of these two plasmids.

4. PMBEN PLASMID VECTORS; CONSTRUCTION AND ANALYSIS

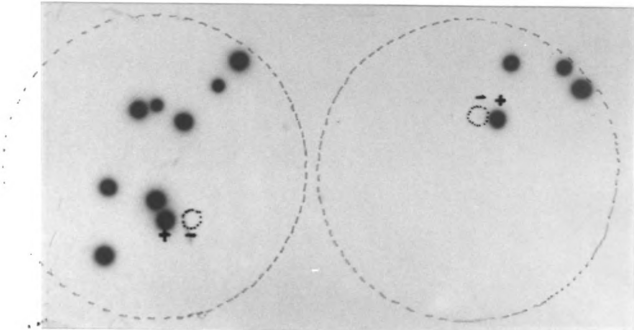
The pMBen plasmid vectors were constructed as described in Figure 8 (pg. 63, 64) A 2.6 kb SalI restriction fragment from pBT3 (Orbach, 1986) containing the benomyl resistance gene (Ben^R) from N. crassa was found to be sufficient to transform N. crassa to benomyl resistance at high frequency (Orbach, 1986). Translation of the Ben^R gene starts 352 base pairs downstream from the SalI site, and stops 300 base pairs upstream from the HindIII site. The 2.6 kb SalI insert and a 3.1 kb HindIII insert which also contains the benomyl resistance gene were generated from pBT3 by digestions with SalI and HindIII respectively. These DNA fragments were gel purified by electrophoresis on a 0.8 % agarose gel, and by electroelution (the electroelution apparatus is from International Biotechnologies, Inc.). The recessed 3'ends of the 3.1 kb HindIII fragment were filled in with dNTPs using the Klenow fragment of E. coli DNA polymerase I (Maniatis, 1982) to generate blunt ended DNA fragments which

Figure 8. Construction of Plasmids pMBen1 and pMBen2

The plasmids pBT3 and pLeu4 are described in MATERIALS AND METHODS. The region representing particular DNA fragments are labeled :  , Ben^R gene;  ,Leu1 gene;  ,Amp^R; ampicillin resistance gene.



were then combined with SmaI linearized pLeu4 DNA and T4 DNA ligase. The vector pLeu4 was also digested with SalI and combined with the 2.6 Kb SalI fragment. Ligation of vector and insert was carried out at a ratio between insert fragments and linearized vectors of 3:1 for the SalI sticky-end ligation and 5:1 for SmaI blunt-end ligation. The newly constructed plasmids were transformed into E. coli DH 5 α to propagate the vector molecules, and to screen for clones with the desired construction. Recombinants containing the N. crassa Ben^R gene were identified by colony hybridization with a radio-labeled 2.6 kb SalI fragment from pBT3 as probe. As shown in Figure 9 (pg.66), 8 of 121 sticky - end recombinants (obtained upon subcloning of the 2.6 kb SalI fragment) and 3 of 50 blunt - end recombinants (ontained upon subcloning of blunt ended 3.1 kb HindIII fragment) were detected to contain Ben^R inserts. After single colony isolation, the plasmid DNA of these positive recombinants was isolated with the "miniprep" method. Samples of the resulting plasmid were digested with XbaI and PstI restriction endonucleases and analyzed by electrophoresis on a 0.8 % agarose gel. XbaI cuts 3 times in the pLeu4 vector, while PstI cuts twice in pLeu4 and once in the Ben^R insert. The PstI digests were designed to reveal the orientation of insertion by the generation of 3 DNA fragment : 4.4 kb, 2.7 kb, 2.6 kb in one orientation and 3 DNA fragment : 5.3 kb, 4.4 kb and a non-detectable 10bp fragment in the other orientation. Restriction analysis suggested that of 8



STICKY-END LIGATION 8/121

BLUNT-END LIGATION 3/51

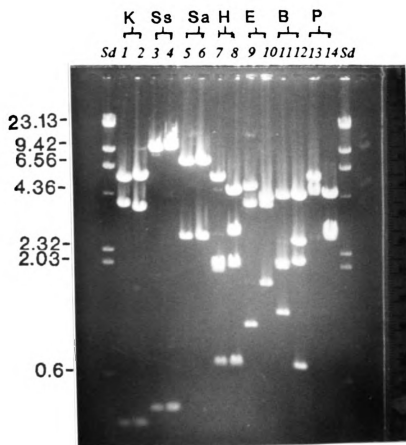
Figure 9. Colony hybridization with the Ben^R gene

The *E. coli* cells grown on nitrocellulose paper were lysed, denatured, neutralized and hybridized with the Ben^R probes to identify the cells which has correct recombinant plasmids. Filters were washed at high stringency and exposed to X-ray film for 6 hours at room temperature. "+" represents the colony containing pBT3 as a positive control. "-" represents the colony containing pLeu4 as a negative control.

Figure 10. Restriction analysis of pMBen1 and pMBen2

Plasmid DNA of pMBen1 and pMBen2 was digested with different endonucleases and analyzed by electrophoresis on 0.8% agarose gel. Lane 1, 3, 5, 7, 9, 11, 13 : plasmid DNA of pMBen2 digested with KpnI, SstI, SalI, HindIII, EcoRI, BamHI and PstI endonuclease . Lane 2, 4, 6, 8, 10, 12, 14 : plasmid DNA of pMBen1 digested with KpnI, SstI, SalI, HindIII, EcoRI, BamHI and PstI endonuclease .

Figure 10.



SalI recombinants, 2 clones contained the 2.6 kb SalI/ Ben^R gene in one orientation (named pMBen1) and 5 clones contained the 2.6 kb SalI/ Ben^R gene in the opposite orientation (named pMBen2). One clone did not show the predicted restriction map and was discarded.

One clone from each orientation was selected for detailed restriction analysis with 7 different endonucleases including PstI, BamHI, EcoRI, HindIII, SalI, SacI (SstI), and KpnI (Figure 10, pg.67, 68). Based on these data, the predicated restriction maps of pMBen1 and pMBen2 plasmids were confirmed (shown in Figure 11, pg.70 and Figure 12, pg.72).

Plasmid DNA of recombinant clones containing pMBen1 and pMBen2 was isolated from E. coli cells using large-scale protocol and cesium chloride density gradients. This pure DNA was then used to transform protoplasts of M. racemosus R7B.

5. TRANSFORMATION OF MUCOR RACEMOSUS

Initial experiments were designed to demonstrate our capability to transform the filamentous fungus M. racemosus. Approximately 1×10^5 viable sporangiospores of M. racemosus R7B were inoculated onto each of 20 YNB agar plates to test the spontaneous reversion frequency of this Leu⁻ strain to the

Figure 11. Plasmid pMBen1

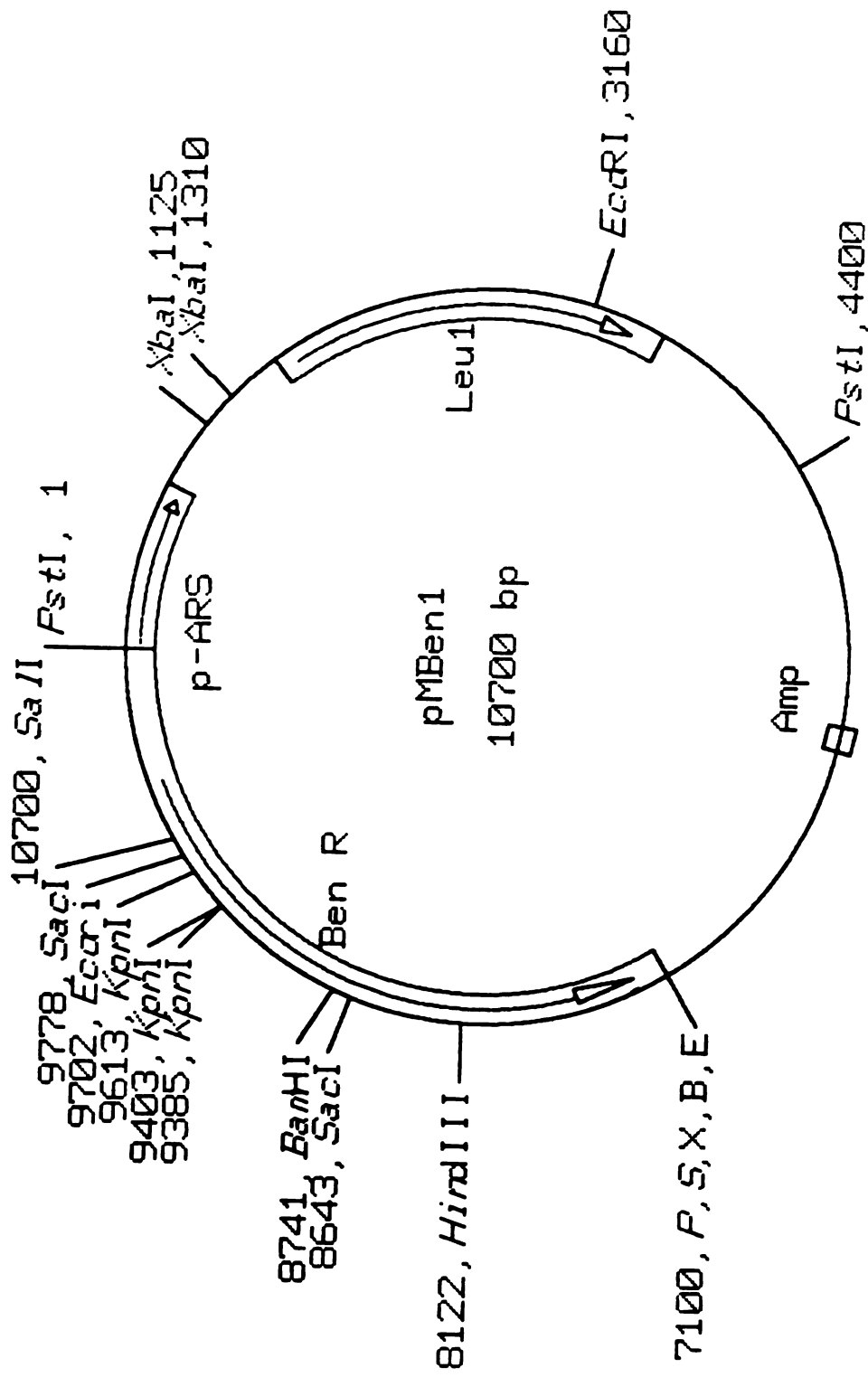
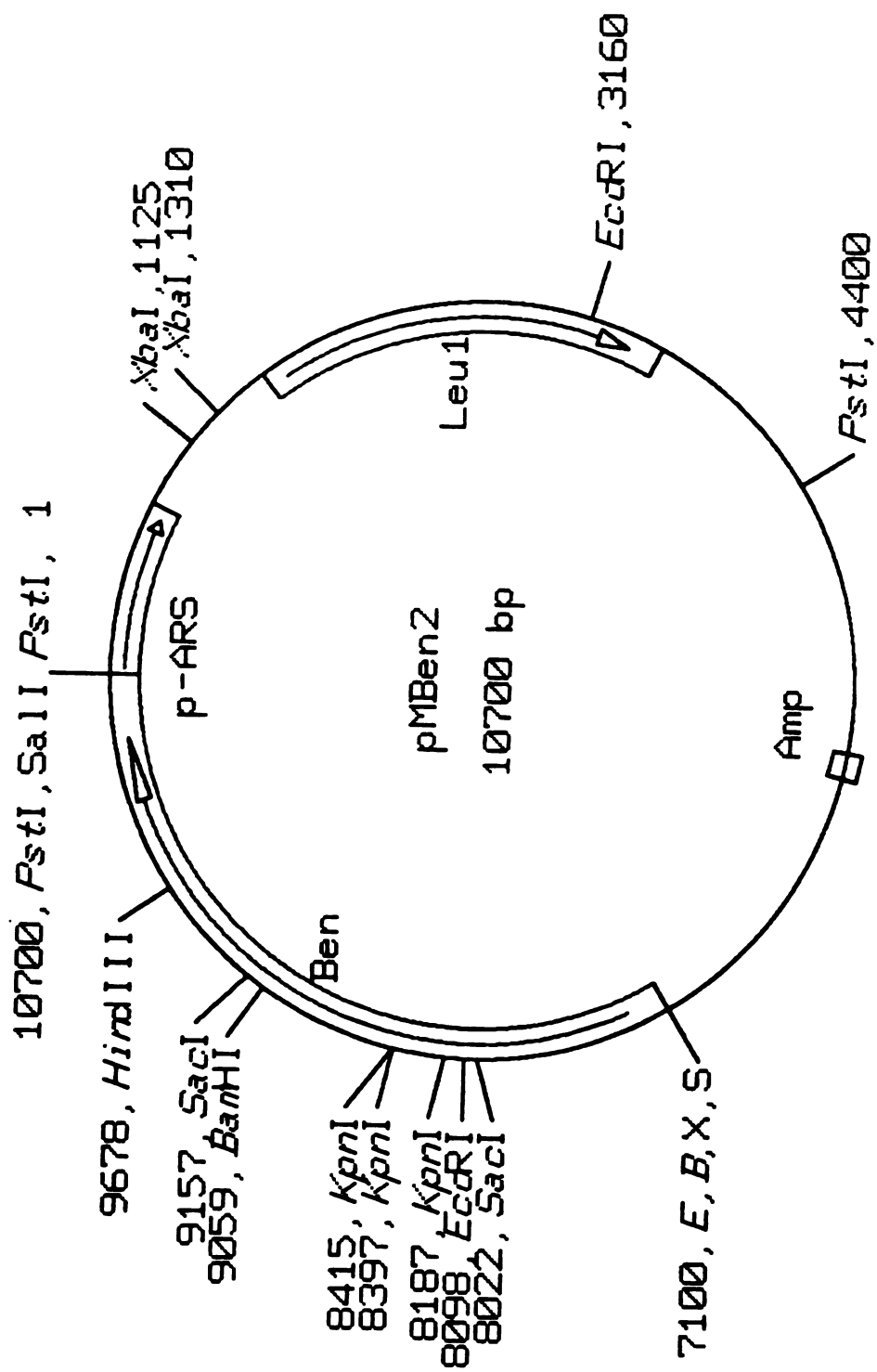


Figure 12. Plasmid pMBen2



Leu⁺ phenotype. No M. racemosus colonies appeared after 7 days. The spontaneous reversion frequency of this Leu⁻ strain is less than $1/2 \times 10^6$. This makes the detection of transformants possible because the transformation frequency of R7B with pLeu4 is 600 transformants/ 3.2×10^6 viable protoplasts (Van Heeswijck, 1984).

Protoplasts of M. racemosus R7B were generated as described in MATERIALS AND METHODS. Approximately 60 % of the sporangiospores of M. racemosus R7B inoculated in YPG broth germinated and produced germ tubes 3-5 spore diameters in length after five and a half hours of incubation. Approximately 50 % of the total protoplasts were generated from germlings after 40 minutes of cell wall digestion with chitosanase and Novozyme 234. Because the mycelia of Mucor are unicellular, not every protoplast generated has a nucleus. Only the protoplasts with a nucleus are able to regenerate cell wall and grow colonies on YNB plus leucine plates by the second day. The regeneration efficiency of M. racemosus R7B was 60 %, (ie., 60% of protoplasts grew under nonselective conditions).

pLeu4 Vector Protoplasts were transformed with the plasmid pLeu4. Prototrophic colonies appeared 2-3 days after transformation and were capable of sporulation. The transformation frequency was 50 colonies/ μg DNA/ 10^6

protoplasts. No prototrophic colonies grew when no pLeu4 DNA was added to protoplasts in the transformation experiment. Sporangiospores of putative transformants were harvested and reinoculated into YNB (minimal media). The ability of transformants to grow suggested that the acquired Leu⁺ phenotype was stable under selective pressure. Spore stocks were produced from 5 of the putative transformants. Three stable putative transformants were then further characterized by Southern analysis (see below).

pMBen Vectors In order to increase the number of transformants generated, more protoplasts were used in transformation experiments. To eliminate confusion due to isolation of revertants, the spontaneous reversion frequency of the R7B Leu⁻ strain back to Leu⁺ was tested again and was found to be less than $1/9 \times 10^7$. Approximately 2×10^6 protoplasts of R7B were mixed with 1-3 μg pMBen1 and pMBen2 DNA. Transformant colonies appeared 3-4 days after transformation. Since cells in these colonies must synthesize their own leucine, they grow more slowly than protoplasts regenerated on medium containing leucine. Twenty four prototrophic colonies were obtained with pMBen-1 and 12 prototrophic colonies were obtained with pMBen-2. The transformation frequency of pMBen1 was 30 colonies/ μg DNA/ 2×10^6 protoplasts and that of pMBen2 was 50 colonies/ μg DNA/ 2×10^6 protoplasts. Increasing the number of protoplasts used in an experiment

increased the total number of transformants obtained but did not increase the transformation efficiency.

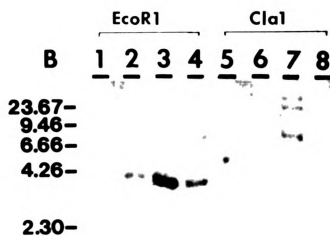
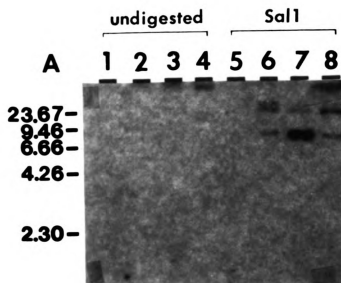
Putative transformants were single colony isolated by growing as yeasts under CO₂ (see Materials and Methods). Sporangiospores of 5 pMBen1 and 5 pMBen2 transformants were harvested and reinoculated onto YNB plates. All transformants were able to grow and sporulate. Two clones containing pMBen1 , B1-3 and B1-5, and two clones containing pMBen2 , B2-1 and B2-5, were chosen to conduct further analysis. Approximately 1×10^2 of viable sporangiospores of B2-1 were plated on YNB and YNB plus leucine duplicate plates. Only 5% of the spores were able to grow in YNB plates while 99% of spores were able to grow in YNB plus leucine plates. This result suggested that the transformants were mitotically or segregationally unstable. Only 5% of spores apparently received at least one copy of the plasmid and the Leu1 gene.

6. SOUTHERN ANALYSIS OF M. RACEMOSUS TRANSFORMED WITH PLASMIDS

pLeu4 Transformants Genomic DNA of 3 transformants (T1, T2, T3) and non-transformed cells (R7B) was isolated and digested with different restriction endonucleases. The DNA fragments were separated by electrophoresis on a 0.7% agarose gel and transferred onto nitrocellulose paper for Southern

Figure 13. Southern analysis of genomic DNA of M. racemosus R7B and 3 pLeu4 transformants with ³²P-labelled pUC9 plasmid probe.

Genomic DNA (20-40 ug) was digested with different endonucleases, analyzed on by electrophoresis on a 0.7 % agarose gel, transferred onto a nitrocellulose paper and hybridized with ³²P-labeled pUC9 probe. Filters were washed at high stringency and exposed to X-ray film for 42 hours (Panel A) and 22 hours (Panel B) at -80°C. Lane 1, 5 of Panel A and Panel B : genomic DNA of M. racemosus R7B undigested, digested with SalI, EcoRI, and ClaI endonucleases . Lane 2, 6 of Panel A and Panel B : genomic DNA of pLeu4 transformant T1 undigested, digested with SalI, EcoRI, and ClaI endonucleases . Lane 3, 7 of Panel A and Panel B : genomic DNA of pLeu4 transformant T2 undigested, digested with SalI, EcoRI, and ClaI endonuclease . Lane 4, 8 of Panel A and Panel B : genomic DNA of pLeu4 transformant T3 undigested, digested with SalI, EcoRI, and ClaI endonuclease .



hybridization with a radio-labeled pUC9 probe (Figure 13, pg.77, 78). The pUC9 DNA is nearly identical to the pUC13 backbone of pLeu4, and has no homologous sequences in the genome of the R7B host. The probe hybridized to the genomic DNA of the transformants (Lane 2, 3, 4, 6, 7, 8 on panel A and Lane 2, 3, 4, 6, 7, 8 on panel B), but not to DNA of non-transformed R7B cells. Therefore, only cells containing the pLeu4 plasmid hybridized to this probe as expected. These results confirmed the entry of pLeu4 plasmid into R7B host strain during transformation. The pUC9 probe hybridized to 3.8 Kb and 7.1 Kb EcoRI restriction fragments (Lane 2, 3, 4 on panel B) as predicted. This data suggested that there was no DNA rearrangement after the plasmids were transformed and propagated in the M. racemosus host.

pMBen Transformants Transformants B1-3, B1-5, thought to contain pMBen1, and B2-1, B2-5 thought to contain pMBen2, were chosen to do Southern Analysis. Genomic DNA of these 4 clones was isolated from cells and digested with ClaI, SalI, and PstI endonucleases, separated on a 0.8 % agarose gel and transferred to nitrocellulose paper. The nitrocellulose was probed with the 2.6 Kb SalI Ben^R insert and pUC 19, which is nearly identical to the pUC12 backbone of the pLeu4 vector. Filters were washed under high stringency conditions and autoradiographed (Figure 14, pg.80, 81 and Figure 15, pg.82, 83). ClaI endonuclease was shown previously to have no

Figure 14. Southern analysis of pMBen plasmids DNA and genomic DNA of M. racemosus R7B and 3 pMBen1 and pMBen2 transformants with ³²P-labeled 2.7 kb SalI fragment (Ben^R gene) from plasmid pBT3 .

Panel A-1 Plasmid DNA of pMBen1 and pMBen2 was digested with different endonucleases and analyzed by electrophoresis on 0.8 % agarose gel. Lane 1, 2, 3 : plasmid DNA of pMBen1 undigested, digested with SalI, and PstI endonucleases. Lane 4 : plasmid DNA of pMBen2 digested with PstI endonuclease.

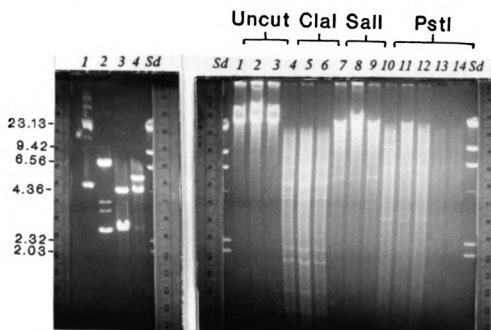
Panel A-2 Genomic DNA (20-40 ug) was digested with different endonucleases, then analyzed by electrophoresis on a 0.8% agarose gel. Lane 1, 4, 7, 10 : genomic DNA of pMBen1 transformant B1-5 undigested, digested with ClaI, SalI, and PstI endonucleases. Lane 2, 5, 8, 11 of : genomic DNA of pMBen2 transformant B2-1 undigested, digested with ClaI, SalI, and PstI endonucleases. Lane 3, 6, 9, 12 : genomic DNA of pMBen2 transformant B2-5 undigested, digested with ClaI, SalI, and PstI endonucleases. Lane 13 : genomic DNA of M. racemosus R7B digested with PstI endonuclease. Lane 14 : genomic DNA of M. racemosus 1216B digested with PstI endonuclease.

Panel B The plasmid and genomic DNA on agarose gel was transferred onto a nitrocellulose paper and hybridized with ³²P-labelled 2.7 kb SalI fragment (Ben^R gene) from plasmid pBT3. Filters were washed under high stringency and exposed to X-ray film for 30 minutes at -70°C.

Figure 14.

Panel A-1

Panel A-2



Panel B

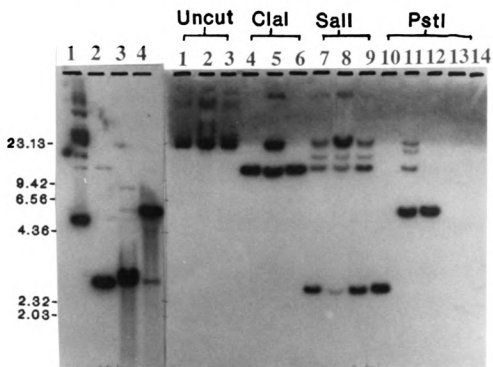


Figure 15. Southern analysis of pMBen plasmid DNA and genomic DNA of *M. racemosus* R7B and 3 pMBen1 and pMBen2 transformants with ³²P-labelled 2.6 kb pUC19 probe.

Panel A-1 Plasmid DNA of pMBen1 and pMBen2 was digested with different endonucleases and analyzed by electrophoresis on a 0.8 % agarose gel. Lane 1, 2, 3 : plasmid DNA of pMBen1 undigested, digested with SalI, and PstI endonucleases. Lane 4 : plasmid DNA of pMBen2 digested with PstI endonuclease.

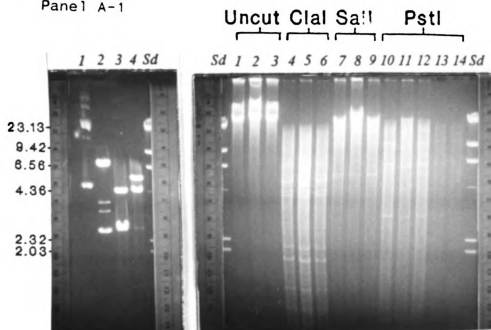
Panel A-2 Genomic DNA (20-40 ug) was digested with different endonucleases, then analyzed by electrophoresis on 0.8% agarose gel. Lane 1, 4, 7, 10 : genomic DNA of pMBen1 transformant B1-5 undigested, digested with ClaI, SalI, and PstI endonucleases. Lane 2, 5, 8, 11 : genomic DNA of pMBen2 transformant B2-1 undigested, digested with ClaI, SalI, and PstI endonucleases. Lane 3, 6, 9, 12 : genomic DNA of pMBen2 transformant B2-5 undigested, digested with ClaI, SalI, and PstI endonucleases. Lane 13 : genomic DNA of *M. racemosus* R7B digested with PstI endonuclease. Lane 14 : genomic DNA of *M. racemosus* 1216B digested with PstI endonuclease.

Panel B The plasmid and genomic DNA on agarose gel was transferred onto a nitrocellulose paper, hybridized with pUC19 probe, washed at high stringency, and exposed to X-ray film for 30 minutes at -70°C.

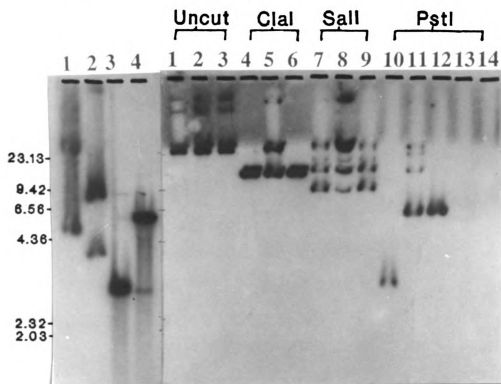
Figure 15.

Panel A-2

Panel A-1



Panel B



restriction sites in the pMBen plasmid (data not shown) but was assumed to cut in the genomic DNA of Mucor. The SalI endonuclease cuts the Ben^R insert out from pMBen plasmids. PstI was used to distinguish between pMBen1 and pMBen2 (see restriction maps in Figure 11, pg.70, 71, and Figure 12, pg.72, 73). The Ben^R probe hybridized to DNA of each of the Ben^R transformants under high stringency conditions, but not to that of the R7B host strain or to the M. racemosus 1216B (wild type). The pUC19 probe hybridized to DNA of each of the Ben^R transformants under high stringency condition, but not to that of the R7B host strain or to the M. racemosus 1216B (wild type). The ability of these putative transformants to grow on YNB plates combined with the results of Southern analysis confirmed the presence of pMBen plasmid in the host strain after transformation.

The hybridization patterns of pure pMBen plasmid DNA and pMBen plasmids in the transformants' DNA were the same. These data indicated no detectable gene rearrangement after the plasmids were transformed and propagated in Mucor host. These results were similar to those reported for other M. racemosus plasmids by Van Heeswijck (1986).

7. RESISTANCE OF PMBEN TRANSFORMANTS TO BENOMYL

To determine if the Ben^R gene from N. crassa was

functionally expressed in M. racemosus transformants, we measured the level of resistance to benomyl in 4 transformant clones. Approximately 200 and 2000 viable spores of M. racemosus R7B, M. racemosus 1216B, and 4 pMBen transformant clones were inoculated onto YNB plus leucine medium (for M. racemosus R7B only) and YNB medium, containing benomyl at final concentrations of 0, 80, 90, 100, 110, 120 or 130 μg benomyl/ml. Growth of the 4 pMBen transformants, M. racemosus 1216B, and M. racemosus R7B was observed after 6 days (and maintained to 15 days) on all plates except those containing 100 μg or more benomyl/ml (incubated in the light). These data suggested that the M.I.C. of benomyl to M. racemosus 1216B, R7B and 4 chosen transformants is between 90 and 100 μg benomyl/ml when grown in light. There was no significant increase in resistance to benomyl in pMBen transformants. The colonies of transformant B2-1 grown in YNB medium versus in YNB plus leucine were also compared. There were consistently fewer colonies of B2-1 grown in YNB medium than that in YNB plus leucine medium. This result suggested that the transformants were mitotically or segregationally unstable as mentioned before in Result 5 (pg.77).

DISCUSSION

In this work, transformation of protoplasts of M. racemosus R7B, a leucine auxotrophic mutant was accomplished using a shuttle vector, pLeu4, containing the Leu1 gene as a selectable marker. In order to improve this transformation system, our goal was to construct pMBen vectors containing the Ben^R gene from N. crassa as a dominant selectable marker and to test the functional expression of this heterologous gene in Mucor cells transformed with the construct. The plasmids pMBen1 and pMBen2 were constructed and used to transform M. racemosus R7B. Southern analysis of the genomic DNA of 4 transformants suggested that the Ben^R gene was present in the cells with no obvious DNA rearrangements. Although the pMBen plasmids were shown to be present in transformants cells, there was no significant increase in the resistance level of pMBen transformants detected when compared with the resistance level to benomyl of the host strain (without pMBen), indicating that the Ben^R gene was not functionally expressed in Mucor.

The M.I.C. to benomyl of both the wild type strain (1216B) and the leucine auxotrophic mutant strain (R7B) of

Mucor was between 27 and 30 μg benomyl/ml for cells grown in the dark and was between 90 and 100 μg benomyl/ml for cells grown in the light. To determine the reason for the observed differences in M.I.C., two sets YNB and YNB plus leucine media containing 50 μg /ml benomyl were prepared. One set was stored in the dark and the other in the light for 7 days. The media were inoculated with spores of M. racemosus 1216B and R7B, and incubated under the opposite conditions (ie. the media which were stored in dark were incubated in light, and vice versa). After 15 days of incubation, 32 colonies grew on the medium incubated in light while there was zero growth on the set incubated in the dark. These data suggested that the breakdown of benomyl by light was not the reason why these two Mucor strains grew better in light, but that light contributed as an important factor in the improved growth of M. racemosus. One possibility is that cells grow more slowly in the dark, and are consequently more sensitive to benomyl. Since Mucor has the ability to adapt to antibiotics such as cycloheximide, trichodermin, and amphotericin B (Linz and Sypherd, personal communication), it is also possible that the Mucor cells adapted to benomyl more rapidly in the light, so they appeared to be more resistant to benomyl.

The transformation efficiency of M. racemosus R7B with pLeu4 was 500 transformants/ μg DNA/ 10^7 protoplasts; with pMF25, 250 transformants/ μg DNA/ 10^7 protoplasts were obtained;

with pMF29, 64 transformants/ μg DNA/ 10^7 protoplasts were obtained (Chiu, M.S. Thesis, 1988) and with pMBen1 and pMBen2, 150 and 250 transformants/ μg DNA/ 10^7 protoplasts were obtained. In our laboratory, the more protoplasts that were added to the transformation reaction, the more transformants were obtained. However, there was no significant change in the efficiency of transformation.

The Leu⁺ phenotype of M. racemosus R7B transformed with pLeu4 or pMBen was stable after spores were reinoculated onto YNB plates (ie, selective pressure). However only 5% of the spores generated from cells growing on YNB were able to grow when plated under this selective pressure. Transformants tended to lose their plasmids at an even faster rate when they were grown on YPG (rich medium) containing leucine (without selective pressure). These results suggested that there is mitotic instability either during cell growth or during sporulation. One possible reason for this observation could be that the plasmids do not integrate into genome of the host cell, so they would not be segregated equally to daughter cells during mitosis. Generally, there are at least three possible fates of a plasmid which enters the host cell. A plasmid may replicate autonomously. It may integrate into the chromosomal DNA randomly by nonhomologous recombination or it may integrate into chromosomal DNA at specific sites via homologous recombination. The results of our Southern

analysis shown previously indicated there was no detectable DNA rearrangement after plasmids were transferred into host cells. This confirmed results reported by Van Heeswijck (1984), who also demonstrated that the pLeu4 plasmid can be rescued by transformation of E. coli with Mucor genomic DNA. The plasmid obtained from E. coli is unchanged from the original plasmid. These experimental data supported the notion that pLeu4 derived plasmids replicate autonomously. Another possible explanation of the results observed in the Southern analysis is that the transformed plasmid integrates into the genome at one location and duplicates tandemly. The results seen in Southern analysis could be the same in this situation. In support of this explanation, plasmid probes used in Southern analysis of transformant DNA digested with ClaI endonucleases (which does not cut in the plasmid) hybridized to smaller DNA fragments than in undigested DNA, suggesting integration of plasmid DNA.

It is possible that most of the pLeu4 derived plasmids replicate autonomously in Mucor while a few plasmid molecules integrate into the genome of host cells. Under these conditions, the low number of integrated plasmid may not be detected in our Southern analysis. Our laboratory is currently testing this hypothesis. Transformant cells were grown without selective pressure for several generations, and were transferred onto selective medium to generate isolated

colonies. This protocol was repeated several times. Since there is a high level of segregational instability in Mucor, cells grown in the absence of selective pressure will rapidly lose their plasmids. If any plasmid DNA integrated into genome of host, it should be detectable after inoculation of spores back onto YNB plates. Whether pLeu4 derived plasmids integrate into the Mucor genome or not is an interesting question. More future research is required to fully answer this question.

The presence of pMBen plasmid in transformants was confirmed by Southern analysis with the Ben^R gene and pUC19 probes. The Leu1 gene on pMBen vectors was expressed in R7B cells enabling them to grow on YNB media lacking leucine. However, the resistance level of the transformant strains of B1-3, B1-5, B2-1, and B2-5 to benomyl was not improved when directly compared with the sensitivity level of the R7B host. The Ben^R gene from N. crassa has been expressed functionally in fungi other than N. crassa. The functional expression of a heterologous gene in a eukaryotic host entails a complicated series of events. First, the gene must be transcribed to produce a stable premessage (Hn-RNA), which requires posttranscriptional modification to generate a stable m-RNA. Then the m-RNA must be translated into a protein. In many cases, further posttranslational processing is necessary to produce a functional end-product.

The Ben^R gene was confirmed to be present in Mucor cells in apparently unaltered form, but was not functioning to improve the benomyl resistance level. This could be due to one of many possible reasons such as : 1) no transcription of the Ben^R gene, 2) no stable message (m-RNA) produced, because the enzyme system in Mucor could not properly process the Hn-RNA, (there are 6 introns in the Ben^R gene), 3. the m-RNA was not translated, 4. lacking the translated protein, 5. the produced β -tubulin protein was not incorporated into microtubules in M. racemosus. The first question to ask is whether this heterologous Ben^R gene was expressed in M. racemosus transformant cells or not (eg., was there a stable message transcribed from this gene?). Northern analysis of RNA isolated from transformant cells using a Ben^R gene probe will answer this question. If no stable message is detected, then, presumably a homologous promoter, and a homologous transcription terminator will produce a functional mRNA in M. racemosus. Currently, the promoter from M. racemosus TEF-1, promoter from a gene which encodes elongation factor 1 α (Linz, et al., 1986), and the Leu1 promoter are being characterized for possible use with the Ben^R gene.

If stable message is detected in Northern analysis, then we must determine whether a functional β -tubulin protein is translated from this message. Specific antibody to the β -tubulin protein will be useful in this regard.

If the heterologous benomyl resistant protein can not incorporate in the microtubules in Mucor to improve benomyl resistance in transformed cells, then a homologous benomyl resistance gene needs to be cloned. This homologous resistance gene could come from a benomyl resistant Mucor strain (through spontaneous mutation or through mutagenesis), or by genetic engineering of a wild type β -tubulin gene from Mucor.

SUMMARY

Our goals for this research project were to insert the N. crassa Ben^R gene, a dominant selectable marker, into pLeu4, to transform Mucor cells with this construct, and to test the expression of this heterologous gene in Mucor transformants. The correct construction of pMBen1 and pMBen2 was confirmed with restriction endonuclease analysis. The plasmids pMBen1 and pMBen2 were used to transform M. racemosus R7B. Southern analysis of the genomic DNA of 4 transformants suggested that the Ben^R gene was present in transformants and that there were no obvious DNA rearrangements in the Ben^R gene. Although unaltered pMBen plasmids were present in the transformants, no significant increase in the resistance level to benomyl of pMBen transformants was detected when compared with the resistance level to benomyl of the host strain (without pMBen). Further studies will focus on the transcription of the ben^R gene in Mucor cells to determine if the heterologous N. crassa promoter drives transcription in M. racemosus.

REFERENCES

- Adams, P.R., 1976. Amylase production by Mucor miehei and Mucor pusillus. Mycologia. 68:934-938.
- Arima, K.S., et al., 1968. Milk-clotting enzyme from microorganisms. V. Purification and crystallization of Mucor rennin from Mucor pusillus. Var. Lindt. Appl. Microbiol. 16:1727-1733.
- Ball, C., et al., 1978. Improvement in amyloglucosidase production following genetic recombination of A. niger strains. Eur. J. appl. Microbiol. Technol. 5:95-102.
- Ballance, D.J., et al., 1983. Transformation of A. nidulans by the orotidine-5'-phosphate decarboxylase gene of N. crassa. Biochemical and Biophysical Research communications 112:284-289.
- Bartnicki-Garcia, S. and W. N., 1962. Nutrition, growth and morphogenesis of Mucor rouvii. J. Bacteriol. 84:841-858.
- Beach, D. and P. Nurse, 1981. High-frequency transformation of the fission yeast S. pombe. Natural, 290:140-142.
- Beggs, J. D., 1978. Transformation of yeast by a replicating hybrid plasmid. Nature, 275:104-108.
- Berse, B., et al., 1983. Cloning and characterization of the

- ornithine carbamoyltransferase gene from A. nidulans.
- Botstein, D., et al., 1979. Sterile host yeasts (SHY); A eukaryotic system of biological containment for recombinant DNA experiments. *Gene* 8:17-24.
- Bu'lock, J., 1987. Basic biotechnology. Academic press.
- Burchfield, H.P., 1977. Residue analysis in Anti fungal compounds Chap.14:463-501.
- Butt, T.R., et al, 1984. Cloning and expression of a yeast copper metallothionein gene. *Gene* 27:23-33.
- Butt, T.R., et al., 1987. Yeast metallothionein and applications in biotechnology. *Microbiological Reviews* 51-3:351.
- Buvton, F.P., et al., 1985. Transformation of A. niger using the arg B gene of Aspergillus nidulans. *Gene* 37:207-214.
- Case, M.E. et al., 1979. Efficient transformation of N. crassa by utilizing hybrid plasmid DNA. *Pro. Natl. Acad. Sci. U.S.A.* 76:5259-5263.
- Chang, C.N. et al., 1987. High-level secretion of human growth by E. coli. *Gene* 55:189-196.
- Chupp, C. and A. F. S. 1960. Vegetable diseases and their control. New York:Ronald Press Co. pg.639.
- Cihlar, R.L., 1985. Morphogenesis and dimorphism of Mucor Gene Manipulations in fungi Chap.18:449-464.
- Craik, C.S. 1985. Use of oligonucleotides for site specific mutagenesis. *Biotechniques* 3:12.

- Cullen, D. and S. L., 1986. Recent advances in the molecular genetics of industrial filamentous fungi.
- Cullen, D. et al., 1987. Controlled expression and secretion of Bovine chymosin in A. nidulans. Bio/Tech: 5: Apr: 369.
- Cullen, D. Leong, S. A., et al, 1987. Transformation of Aspergillus nidulans with the hygromycin-resistance gene, hph. Gene 57:21-26.
- Dalbadie-McFarland, G.D., et al., 1982. Oligonucleotide-directed mutagenesis as a general and powerful method for studies of protein function. Proc. Natl. Acad. Sci. USA 79:6409.
- Das, A. and P. R., 1978. Improved production of citric acid by a diploid strain of A. niger. Can. J. Microbiol. 24:622-625.
- Davidse, L.C., 1986. Benzimidazole fungicides: mechanism of action and biological impact. Ann. Rev. Phytopathol. 24:43-65.
- Davidse, L.C., 1988. Biochemical aspects of benzimidazole fungicides-action and resistance. Modern selective fungicides. Chap, 6:245-258.
- Derynck, R., et al., 1983. Expression of the human interferon α CDNA in yeast. Nucleic Acids Research 11: 1819-1837.
- Dobson, M.J. et al., 1982. Conservation of high efficiency promoter sequences in S. cerevisiae. Nucleic Acid

- Research 10:2625-2636.
- Dustin, P. ed. 1984. Microtubules. Berlin: Springer Verlag:482.
- Ehrlich, S.D. et al., 1978. B. subtilis as a host for DNA cloning. Genetic Engineering 2:25-32.
- Esmon, P.C. et al., 1987. Structure, assembly, and secretion of octameric invertase. J. Biol. Chem. 262:4387-4394.
- Estell, D.A., et al., 1985. Engineering an enzyme by site-directed mutagenesis to be resistant to chemical oxidation. J. Biol. Chem. 260 (11):6518.
- Feinberg, Andrew P. and Vogelstein Bert, 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Analytical Biochemistry 132, 6-13.
- Gauger. W., 1965. The germination of zygosporos of Mucor hiemalis Mycologia. 57:634-641.
- Godfrey, T, J.R., 1983. Industrial enzymology.
- Goeddel, D.H., et al., 1979. Direct expression in E. coli of a DNA sequence coding for human growth hormone. Nature 281:544-548.
- Greer, C.L., et al., 1987. Substrate recognition and identification of splice sites by the tRNA-splicing endonuclease and ligase from S. cerevisiae. Molecular and cellular Biol. 7:76-84.
- Gritz, L., et al., 1983. Plasmid encoded hygromycin B resistances the sequence of hygromycin B

- phosphotransferase gene and its expression in E. coli and S. cerevisiae. Gene 25:179-188.
- Gwynne, D.I. et al., 1987. Genetically engineered secretion of active human interferon and a bacterial endoglucanase from A. nidulans. Bio/Tech. 5:July:713.
- Harris, J.E. and C. D., 1980. Distribution of Mucor piriformis, Sexualis and R. stolonifer in relation to their spoilage of strawberries. Trans. Br. mycol. Soc. 75:445-450.
- Hastie, A.C., 1970. Benlate-induced instability of A. nidulans diploids. Nature 226:771.
- Henderson, R.C.A., et al., 1985. The transformation of brewing yeasts with a plasmid containing the gene for copper resistance. Current Genetic 9:133-138.
- Henson, J.M., et al., 1988. Transformation of Gaeumannomyces graminis to benomyl resistance. Curr. Genet. 14:113-117.
- Hinnen, et al., 1978, Transformation of yeast , Proc. Natl. Acad. Sci. U.S.A. 75:1929-1933.
- Hirth, K.P., et al., 1982. A DNA-mediated transformation system for Dictyostelium discoideum Proc. Natl. Acad. U.S.A. 79:7356-7360.
- Hitzeman, R.A, et al., 1980. Isolation and Characterization of the yeast 3-phosphoglycerokinase gene (PGK) by an immunological screening technique. J. Biol. Chem. 255:12073-12080.

- Howard, R.J., J. R. A., 1977. Effects of MBC on hyphal tip organization, growth and mitosis of F. acuminatum, and their antagonism by D₂O. *Protoplasma* 92:195-120.
- Howard, R.J., J. R. A., 1980. Cytoplasmic microtubules and fungal morphogenesis: Ultrastructural effects of methyl benzimidazol-2-yl carbamate determined by freeze-substitution of hyphal tip cells. *J. Cell Biol.* 87:55-64.
- Hsiung, H.M., et al., 1986. High-level expression, efficient secretion and folding of human growth hormone in E. coli. *Biotechnology* 4:991.
- Innis, M.A., et al., 1985. Expression, glycosylation and secretion of an *Aspergillus* glucoamylase by S. cerevisiae. *Science* 228:21-26.
- Ito, E., et al., 1982. Lipid Synthesis during morphogenesis in Mucor racemosus. *J. Bacteriol.* 152:880-887.
- Johnston, J.R., H. O., 1979. Yeast genetics in industry. *Progr. Ind. Microbiol.* 15:151-205.
- Johnston, J.R., 1985. Strain selection and improvement *Microbiology of Fermented Foods*: 271-288.
- Kang, Young and T.R., 1985. Genetic engineering of caseins. *Food Biotech-nology*: October:89-94.
- Kaster, K.R., et al., 1983. Analysis of a bacterial hygromycin B resistance gene by transcriptional and translational fusions and by DNA sequencing. *Nucl. Acids Res.* 11:6895-6911.

- Kaster, K.V., et al., 1984. Hygromycin B resistance as dominant selectable marker in yeast. *Current Genetic* 8:353-358.
- Kelly, J.M., et al., 1985. Transformation of A. niger by the amdS gene of A. nidulans. *The EMBO Journal* 4:475- 479.
- Kevei, 1977. Interspecific hybridization between A. nidulans and A. rigulosus by fusion of somatic protoplasts *J. Gen. Microbiol.* 102:255-262.
- King, J. 1986. Genetic analysis of protein folding pathways *Bio/technology* 4:297.
- King, R.D. and Cheetham P.S.J., 1987. *Food Biotechnology*, Elsevier applied science.
- Kingsman, S.M., 1985. Heterologous gene expression in S. cerevisiae *Biotechnology & genetic engineering reviews* 3:377-416.
- Kinnaird, J.H., et al., 1982. Cloning of the am gene of N. crassa through the use of a synthetic DNA probe. *Gene* 20:387-396.
- Kinsey, J.A., 1985. Neurospora plasmids. *Gene manipulations in fungi*. Chap. 9:245-256.
- Knorr, D. and Sinskey, A. J., 1985. *Biotechnology in Food Production and Processing*. Science. V.229. 1224-1229.
- Knorr, D., Ed. *Impact of biotechnology on production and processing*. (Dekker, New York, in press.)
- Kunkel, W., 1980. Antimitotische Aktivitat Von Methylbenzimidazol-2-yl carbamat (MBC). I. Licht-,

- elektronenmikroskopische und physiologische
Untersuchungen an Keimenden Konidien Von A. nidulans.
Z. Allg Mikrobiol 20:113-120.
- Larson, A.D. and P. S. S. 1974. Cyclic adenosine 3', 5'-
monophosphate and morphogenesis in Mucor racemosus J.
Bacteriol 117:432-438.
- Lin, Y.L., 1986. Genetic engineering and process development
for production of food processing enzymes and
additives. Food Tech. V.40. NO.10. 104-112.
- Linz, J.E. and M. Orlowski, 1982. Stored mRNA in
sporangiospores of the fungus M. racemosus. J.
Bacteriol. 150:1138-1144.
- Linz, J.E. and M. Orlowski, 1982. Stored sporangiospores of
the fungus M. racemosus. Abstracts of the Annual
meetings of the American Society for Microbiology.
- Linz, J.E. and M. Orlowski, 1983. Selective translation of
mRNA during sporangiospore morphogenesis in the fungus
Mucor. Abstracts of the Annual meetings of the American
Society for Microbiology.
- Linz, J.E., and M. Orlowski, 1984. Differential gene
expression during aerobic germination of M. racemosus
sporangiospores. J. Bacteriol. 159:965-972.
- Linz, J.E. et al., 1986. Three for the elongation factor EF-
1 α in M. racemosus. Molecular and Cellular Biology
6:593-600.
- Linz, J.E., 1986, The primary structure and functional

- domains of an elongation factor-1 α from M. racemosus.
J. Biological Chemistry 261:15022-15029.
- Lowings, P.H., 1956. The fungal contamination of Kentish
strawberry fruits in 1955. Appl. Microbiol. 4:84-88.
- Maniatis, T., Fritsch, E. F., Sambrook, J., 1982. Molecular
Cloning (a laboratory manual) CSH press.
- Mellor, J., et al., 1983. Efficient synthesis of
enzymatically active calf chymosin S. cerevisiae. Gene
24:1-14.
- Miwa, H., et al., 1978. Breeding of Saccharomyces yeast for
improved growth-rate on acetate medium. J. Ferment.
Technol. 56:539-545.
- Miyagawa, K. et al., 1986. Cloning of Bacillus subtilis IMP
dehydrogenase gene and its application to increased
production of guanosine. Biotechnology 4:225.
- Momose, H. and Furaya, A. 1980. New genetic approaches to
industrial microorganisms. Chapt. 9 in "Molecular
breeding and genetics of applied microorganisms" ed.
K. Sagaguchi and M. Okanishi, p.139. Kodansha Ltd.,
Tokyo, Japan.
- Mooney, D.T. and P. S. S., 1976. Volatile factor involved in
the dimorphism of Mucor racemosus. J. Bacteriol.
126:1266-1270.
- Newell, N. 1986. Large U.S. food firms will take a wait and
see approach to applications of biotechnology. Gen.
Eng. News 6:4.

- Nishikura, K., et al., 1982. Genetic analysis of the processing of a spliced tRNA. The EMBO J. 1:263-268.
- Northolt, M.D., et al., 1980. Fungal growth and the presence of sterigmatocystin in hard cheese. J. Ass. off. anal. Chem. 63:115-119.
- Ogundana, S.K., 1972. The post-harvest decay of yam tubers and its preliminary control in Nigeria. In Biodeterioration of Materials. 2:481-492.
- Orbach, M.J., E. B. Porro and C Yanofsky, 1986. Cloning and characterization of the gene for β -tubulin from a benomyl resistant mutant of Neurospora crassa and its use as a dominant selectable marker. Mol. Cell Bio. Vol.6 NO.7.
- Ottesen, M. and W. R., 1970. The isolation and partial characterization of an acid protease produced by Mucor miehei. compt. Rend. Trav. Lab. Carlsberg 37:301-325.
- Paul, J.K. 1989. Genetic Engineering applications for industry. nde. press.
- Paznokas, J.L., and P. S. Syphard, 1975. Respiratory capacity, cyclic adenosine 3',5'-monophosphate, and morphogenesis of Mucor racemosus. J. Bacterial. 124:134-139.
- Pennttila, M.E., et al., 1987. Construction of brewer's yeast secreting fungal endo-beta-glucanase. Curr. Genet. 12:413-420.
- Pitt, J.I. and Hocking, A. D., 1985. Fungi and Food

- Spoilage, Chap 6. Zygomycetes: 143-167.
- Pontecorvo, G., et al., 1953. The genetics of Aspergillus nidulans. Adv. Gent. 5:141-238.
- Prentis, S., 1984. Genetic engineering-reweaving the threads of life. Biotechnology:36-61.
- Prentis, S., 1984. Biotechnology - a new industrial revolution. New York press.
- Punt, P.J., et al., 1987. Transformation of Aspergillus based on the hygromycin B resistance marker from E. coli. Gene 56:117-124.
- Reiss, B., et al., 1984. Protein Fusions with the kanamycin resistance gene from transposon Tn5.
- Revuelta, J.L., et al., 1986. Transformation of Phycomyces blakesleeanus to G418 resistance by an autonomously replicating plasmid. Proc. Natl. Acad. Sci. U.S.A. 83:7344-7347.
- Rigby, P.W.J., 1982. Expression of cloned genes in eukaryotic cells using vector systems derived from viral replicons. Genetic engineering 3:83-134.
- Rine, J. and M. Carlson, 1986. S. cerevisiae as a paradigm for molecular genetics of fungi. Gene manipulations in fungi Chap. 5.
- Roncero, M.I.G., L.P.Jepsen, P.Stroman and R. Van Heeswijck.,1988. Characterization of a 4.4 Kb PstI DNA fragment from M. circinelloides which contains a leucine gene and a ARS element. (Personal

communication)

- Saunders, G., et al., 1986. Fungal cloning vectors. *Trends in Biotechnology* 4:93-98.
- Schipper, M.A.A., 1978. On certain species of Mucor with a key to all accepted species. *Studies in Mycology* 17:1-52.
- Sheir-Neiss, G., et al., 1978. Identification of a gene for β -tubulin in A. nidulans. *Cell* 15:639-649.
- Skatrud, P.L., et al., 1987. Efficient integrative transformation of Cephalosporium acremonium. *Curr. Genet.* 12:337-348.
- Snow, R., 1983. Genetic improvement of wine yeast. In *Yeast Genetics: Fundamental and Applied Aspects*, Eds. J.F.T. Spencer, D.M. Spencer and A. R. W. Smith, Eds. Springer-Verlag, New York, pg.439-459.
- Somkuti, G.A., et al., 1968, Lipase activity of Mucor pusillus. *Appl. Microbiol.* 16:617-619.
- Somkuti, G.A., 1974. Synthesis of cellulase by Mucor pusillus and Mucor miehei. *J. Gen. Microbiol.* 81:1-6.
- Spencer and Spencer. 1983. Genetic improvement of industrial yeasts. *Ann. Rev. Microbiol.* 37:121-142.
- Staneloni, R.J., et al., 1982. The biosynthetic pathway of the asparagine-linked oligosaccharides of glycoproteins. *critical Rev. in Bio. Chem.* 12:298-326.
- Strnadova, K. 1976. A method of preparation and application of nitrous acid as a mutagen in Claviceps purpurca.

- Folia Microbiologica (Prague) 21:455-458.
- The Can Caesar-Ton, 1988. Induction of multiple germ tubes in N. crassa by antitubulin agents. European J. of Cell Biology 46:68-79.
- Thompson, R., 1982. Plasmid and M13 vectors. Genetic Engineering 3:2-41.
- Tilburn, J. et al., 1983. Transformation by integration in A. nidulans Gene 26:205-221.
- Tubb, R.S., 1986. Genetics of ethanol-producing microorganisms CRC Critical Reviews in Biotechnology, Volumn 1, Issue 3:241-261.
- Turner, G. and D. J. Ballance, 1986. Cloning and Transformation in Aspergillus. Gene manipulations in fungi Chap. 10.
- Upshall, A., et al., 1987. Secretion of active human tissue plasminogen activator from the filamentous fungus A. nidulans. Bio/Tech. 5:Dec:1301-1305.
- Van Apeldoorn, J.H.F., 1981. Biotechnology- a Dutch perspective P.47-50. STT publication.
- Van Brunt, J. 1987. Host system pointers. Bio/technology 5:664.
- Van Brunt, J., 1989. New clues on folding. Bio/technology. 7:324.
- Van Heeswijck, Robyn, and M. I. G. R., 1984. High frequency transformation of mucor with recombinant plasmid DNA. Carlsberg Res. Commun. 49:691-702.

- Van Heeswijck, R., 1984. The formation of protoplast from Mucor species. Carlsberg Res. Commun. Vol.49. 597-609.
- Ward, M. and G. Turner. 1986. The ATP synthase subunit 9 gene of A. nidulans : sequence and transcription. Mol. Gen. Genet. 205:331-338.
- Ward, M., B. W. and G. T., 1986. Transformation of A. nidulans with a cloned, oligomycin-resistant ATP synthase subunit 9 gene. Mol. Gen. Genet. 202:265-270.
- Wasserman, B.P. 1984. Thermostable enzyme production. Food Tech. 38, Feb:78-88.
- Wasserman, B.P., 1988. Food biotechnology. Food Tech. V.42. No.1. 133-146.
- Watson, J.D., et al., 1983. Recombinant DNA; A short course pg.176 and 200. W.H. Freeman and Co., New York.
- Wayne H. Pitcher. 1986. Genetic modification of enzymes used in food processing. Food Tech. V.40. No.10. 62-69.
- Wostemeyer, J., et al., 1987. Neomycin resistance as a dominantly selectable marker for transformation of the Zygomycetes Absidia glauca. Curr. Genet. 12:626-627.
- Wulf Crueger and Anneliese Crueger. 1984. Biotechnology : a textbook of industrial microbiology. Science Tech, Inc.
- Yelton, M.M., et al., 1984. Transformation of A. nidulans by using a trpC plasmid. Proc. Natl. Acad. Sci. U.S.A. 81:1470-1474.
- Yoder, O.C., et al., 1986. Technology for molecular cloning of fungal virulence genes. Bailey, J. A. (Ed.), Biology

and molecular biology of plant-pathogen interactions.

NATO ASI Sing, H. Cell Biology 1:371-384.

Zoller and Smith, 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. Methods in Enzymol. 100:468.

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