COMPLEMENTATION OF A *REM* MUTANT IN *USTILAGO MAYDIS*

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We accept this thesis as conforming
to the required standard

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ABSTRACT

Ustilago maydis is a fungal pathogen of corn (*Zea mays*). Its complex life cycle consists of three cell types: (1) non-pathogenic, yeast-like haploids, (2) mycelial, pathogenic dikaryons, and (3) diploid cells produced upon karyogamy within the plant. Mating and pathogenicity in *U. maydis* are under the control of two loci, *a* and *b*. Heterozygousity at both loci is required for fusion of cells, pathogenesis, and subsequent development of the fungus within the plant.

The aim of this project was to identify genes, other than *a* and *b*, that are involved in the pathogenesis of *U. maydis*. To this end, mycelial haploid mutants were isolated by UV mutagenesis of a yeast-like strain. A single mutant (designated 87-18) was selected for further study due to the strength and stability of the mycelial phenotype. This mutation was named *rem* (repressor of mycelial phenotype). The phenotype of mutant 87-18 was remarkable because the strain grows with a filamentous morphology in both solid and liquid media. This phenotype is not demonstrated by any of the cell types of *U. maydis*, regardless of the condition of the *a* and *b* loci. Pathogenicity assays indicate that 87-18, which was derived from an auxotrophic strain, was not solopathogenic, but could cause disease when co-inoculated with a compatible wild-type strain. Prototrophic, mutant progeny of this cross were not solopathogenic.

To clone the gene(s) responsible for the *rem* phenotype, a wild-type genomic library was constructed in the cosmid vector, pJW42. Transformation of the mutant 87-18 with the cosmid library yielded six yeast-like colonies. Cosmid DNA was isolated from three of these transformants and restriction digest patterns indicated that the insert DNA in the three cosmids was highly similar. Cosmid DNA gave 100% yeast-like transformants when reintroduced into strain 87-18. Thus, these results indicate the isolation of a DNA sequence

that complements the mutation of strain 87-18. This complementing DNA provides the starting material for a molecular analysis of the determination of cell morphology in *U. maydis*.

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1. INTRODUCTION

1.1 Literature Review

1.1.1 *Pathology of Ustilago maydis.*

Ustilago maydis is a Basiodiomycete of the order Ustilagenales, the smut fungi. Infection of the host *Zea mays* (corn), results in a range of symptoms in the plant including stunted growth, chlorosis and anthocyanin production. At advanced stages of infection, plant cells are stimulated to divide and enlarge. This proliferation results in characteristic tumor-like galls on the ears, stems, and tassels. Fungal hyphae differentiate within these galls producing masses of black spores, from which the name of the disease, corn smut, is derived. Similar pathology is found among other members of Ustilaginales (Agrios, 1988): *Tilletia* causes bunt of wheat; *Sphacelotheca* causes smut of sorghum; members of the genus *Ustilago* are responsible of smuts of barley (*U. hordei*, *U. nuda*, *U. nigra*), oats (*U.avenae*), wheat (*U. tritici*) and sugarcane (*U. scitaminea*).

Distribution of *U. maydis* is world wide; however, it is particularly prevalent in warm, moderately dry areas where infection rates of 2-10% can result in reduced yields and significant economic loss (Agrios, 1988). Control of corn smut is exerted mainly through the use of resistant host varieties and through sanitation procedures, i.e. removal of smutted plants from the field.

1.1.2 *Ustilago maydis as a Model System.*

Ustilago maydis has been used as a model system to study many aspects of eukaryotic genetics including mechanisms of recombination (Holliday et.al., 1974), nitrogen metabolism (Lewis and Fincham, 1970), the genetics of interstrain inhibition (Ganesa et.al., 1991) and regulation of development (reviewed in Froeliger and Kronstad, 1990). Interest in the developmental genetics of *U. maydis* stems from the fact that self recognition, differentiation and pathogenicity are interrelated processes and reflect general pathways of development which are shared among other pathogenic and economically beneficial fungi.

There are several practical reasons for choosing *U. maydis* as a model system for studying the genetics of mating and pathogenicity. 1. Diploid teliospores produced in mature galls can be germinated *in vitro* allowing Mendelian analyses of meiotic products. 2. Mating reactions can be scored outside the plant using growth media supplemented with 1% activated charcoal (charcoal mating plates). 3. Haploid and diploid cells can be grown vegetatively and this facilitates the application of standard genetic and molecular techniques. Several molecular tools have been recently developed: efficient transformation (Wang et.al. 1988); autonomously replicating vectors (Tsukuda et.al. 1988); metabolic and antibiotic resistance genes for selection markers (Wang et.al., 1988; Banks and Taylor, 1988); and gene disruption (Kronstad et.al., 1989; Fotheringham and Holloman, 1989).

Furthermore, the mating type genes have recently been cloned and sequenced (Kronstad and Leong, 1989; Froeliger and Leong, 1991) facilitating characterization of pathogenicity, mating and dimorphism at the molecular level.

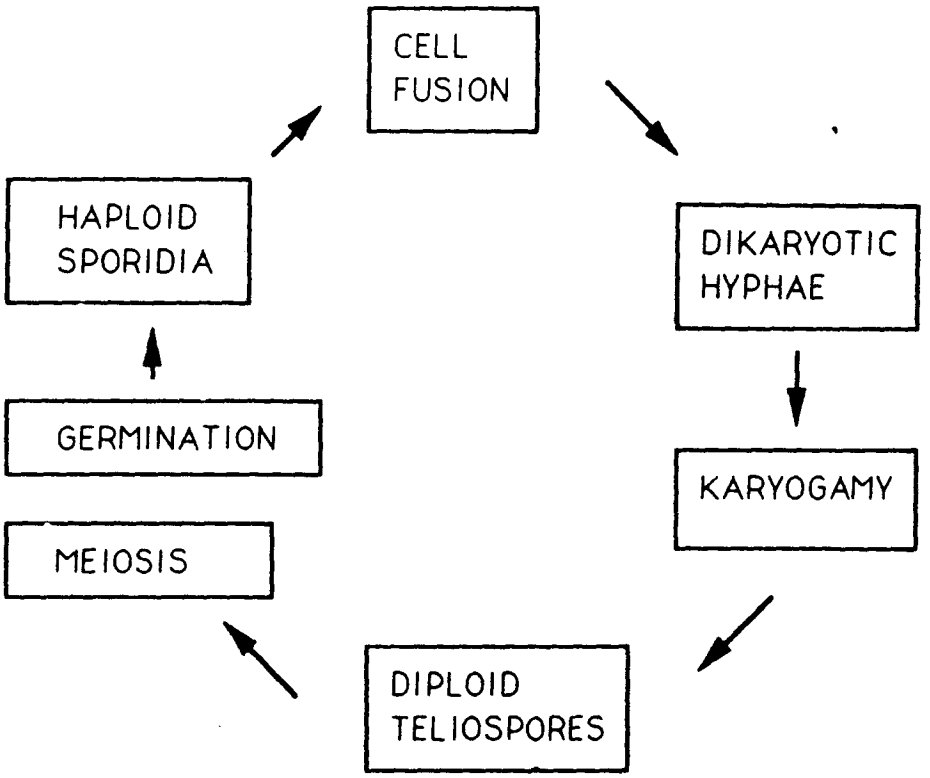
Two significant disadvantages to working with *U. maydis* cannot be overlooked. Firstly, it is not possible to culture all cell types on artificial media. The dikaryon is unstable and readily breaks down into haploid products. Furthermore, inoculation of the plant is prerequisite to completion of the life cycle because teliospore formation and meiosis cannot be induced *in vitro*. As a result, pathogenicity tests and classical genetic analyses require approximately one month to complete. A second disadvantage to working with *U. maydis* is that information concerning general genetics is limited. Molecular analysis of the *U. maydis* karyotype using orthogonal-field-alteration gel electrophoresis (OFAGE) has revealed at least 20 chromosome sized DNA segments (Kinscherf and Leong, 1988). However, Mendelian segregation analyses have defined only 4 linkage groups (Holliday, 1974). Although several genes have been mapped, comprehensive linkage data is unavailable (see Holliday, 1961a and 1974; Kinscherf and Leong, 1988).

1.1.3 The Life Cycle of Ustilago maydis.

Figure 1 outlines the main aspects of the *U. maydis* life cycle. There are three cell types. Haploids are

Figure 1.

Life Cycle of *Ustilago maydis*



Haploid sporidia with compatible mating-types fuse in the host plant to form an infectious dikaryon. The parasitic dikaryon grows as a white mycelium within the meristematic tissue of the plant. Karyogamy within the dikaryon results in diploid cells which subsequently develop into teliospores. Germination of the teliospores is characterized by the outgrowth of a basidium in which meiosis takes place. Haploid products of meiosis, called sporidia, are released and continue the cell cycle.

saprophytic, uninucleate cells that are non-pathogenic and form colonies with a smooth, yeast-like morphology. Haploid cells of compatible mating-type fuse in the host plant, producing an infectious dikaryon. The dikaryon is parasitic and grows as a white mycelium within the meristematic tissue of the plant. Karyogamy within the dikaryon results in diploid cells which subsequently develop into teliospores. The black spores are released from plant galls and dispersed by wind. Germination of teliospores is characterized by the outgrowth of a basidium, or promycelium, in which meiosis takes place. Haploid products of meiosis, called basidiospores or sporidia, are released from the basidium. Fusion of sporidia continues the cell cycle.

Although production of teliospores is dependent upon infection of the plant, portions of the *U. maydis* life cycle can be simulated *in vitro*. On charcoal mating plates, yeast-like haploid cells of compatible mating-type fuse to produce dikaryotic mycelium. This reaction is a positive mating test and is indicative of pathogenicity. Diploid strains can also be constructed *in vitro* by mating compatible haploid strains carrying complementing auxotrophic markers and selecting for prototrophic diploids on minimal media (Puhalla, 1969). Diploids formed in this manner are yeast-like and non-pathogenic, or mycelial and pathogenic, depending on the mating-type genes. Strains which cause disease in the plant, when inoculated as a pure culture, are termed "solopathogens".

Mating tests on charcoal plates and characterization of growth morphology (as yeast-like or mycelial) are standard *in vitro* assays used to determine mating-type and to predict the pathogenicity of a given cross or strain.

1.1.4 The Mating System of Ustilago maydis

Ustilago maydis is a heterothallic, or self-sterile organism; sexual reproduction requires the fusion of cells of compatible mating-type. Compatibility is defined by 2 mating-type genes designated *a* and *b*. There are two alleles of the *a* mating-type gene (*a*1 and *a*2) (Rowell and DeVay, 1954). Based on natural population surveys, the number of *b* alleles has been estimated at 25 (*b*1, *b*2, *b*3...*b*n) (Silva, 1972). Heterozygousity at *a* and *b* is required for fusion of haploid cells and subsequent development of pathogenesis. This type of system is termed "tetrapolar" because each mating event involves four loci. *Schizophyllum commune* and *Coprinus cinereus* are examples of other filamentous fungi which have tetrapolar mating systems.

Regulation of development in *U. maydis* presents an intriguing problem, both for the fungus and for the researcher. How do cells recognize compatible mating partners, i.e., distinguish among 25 different *b* alleles, and how does this recognition lead to the activation of genes required for mycelial growth and pathogenicity? Although the complete regulatory pathway has not been elucidated, genetic

and molecular experiments have begun to clarify the roles played by the *a* and *b* genes in *U. maydis* development and differentiation. The remainder of this section and the next section will describe several of these experiments.

Preliminary studies concerning the functional roles of the *a* and *b* compatibility factors involved *in vitro* crosses of haploid strains using coleoptile extracts from corn seedlings to induce fusion between mating partners (Rowell, 1955). In these experiments, haploid cells fused if they were heterozygous with respect to the *a* gene (a≠). Strains homozygous with respect to *a* (*a*=) did not fuse. Morphology and vigor of dikaryotic hyphae produced upon successful fusion was dependent upon the *b* alleles. Hyphae heterozygous for *b* (*b*≠) were straight and grew rapidly whereas hyphae homozygous for *b* (*b*=) were short, grew slowly and were particularly unstable. It was concluded that the *a* gene governs fusion of cells and the *b* gene regulates growth and stability of the resulting dikaryon. These results were further substantiated by analysis of diploid strains. Puhalla (1968) isolated *a*=*b*≠ and *a*≠*b*= diploids from immature gall tissue. Strains which were heterozygous at *b* were mycelial and pathogenic; strains homozygous at *b* were yeast-like and non-pathogenic. In a similar experiment, Puhalla (1969) constructed diploids *in vitro* by mating haploids carrying complementary auxotrophic markers and selecting for diploid prototrophs on minimal media. In these experiments, *a*≠*b*= diploids were yeast-like and non-pathogenic whereas a≠b≠

diploids were mycelial and pathogenic. The results demonstrate that, in diploids, heterozygousity at *b* is required for mycelial growth and pathogenicity and, subsequent to cell fusion, the *a* alleles do not play a vital role in differentiation.

Additional evidence for *a* and *b* gene function has been gained from the analysis of strains with altered complements of *b* genes. Strains heterozygous for *b* (*a*/*b*1*b*2) have been obtained by two methods, (1) isolation of haploid sporidia in which meiosis failed to separate *b* alleles (Holliday, 1961b) and (2) by transforming a second *b* allele into a haploid strain (Kronstad and Leong, 1989). In both cases, *a*/*b*1*b*2 strains were mycelial on charcoal plates and weakly pathogenic when inoculated into plants. These results indicate that heterozygousity at the *b* locus is sufficient to induce pathogenesis in a haploid strain and further substantiate the conclusion that the *a* gene governs cell recognition and the *b* gene regulates development of the dikaryon and pathogenesis.

Isolation and characterization of mutants is a standard method of determining gene function. There have been two reports of mutation at the *U. maydis b* mating type locus. In the first report, Day et.al. (1971) mutagenized a yeast-like diploid strain (genotype *a*≠*b*=) with UV light and isolated mycelial mutants. When inoculated into plants, 30% of the mycelial mutants were solopathogenic. Teliospores produced in galls upon infection with mutant strains, showed defects

in meiosis and basidiospore formation; haploid progeny were produced which carried either the wild type or mutant *(bmut)* *b* allele. Haploids carrying *bmut* were compatible with themselves, their progenitor strain and with other *bmut* strains, when associated with different *a* alleles. Pathogenicity tests with pure cultures of *bmut* haploids showed that all auxotrophic *bmut* strains were non-pathogenic whereas prototrophic *bmut* strains were solopathogenic. The results of this experiment are significant because they demonstrate the isolation of constitutive mutations at the *b* locus and show that such mutations can produce pathogenic strains which do not require heterozygousity at the *a* or *b* loci. Furthermore, these results corroborate earlier conclusions that the *b* gene regulates mycelial growth and pathogenicity and extend the range of *b* activity to the control of meiosis and basidiospore formation.

A second report of mutation at the *b* locus describes the inactivation of *b* gene activity by direct gene replacement (Kronstad and Leong, 1990). A null mutation of the *b* gene in haploid strains does not affect cell viability, demonstrating that the *b* product is non-essential. However, haploids lacking *b* gene activity are unable to participate in mating reactions and are non-pathogenic when crossed with compatible wild-type haploids. Diploid strains which are heterozygous at the *b* locus (*a1a2/b1b2*) are normally solopathogenic and are mycelial on charcoal media. A null mutation at one of the *b* alleles results in yeast-like growth and loss of

pathogenicity. The results suggest that the *b* gene product plays a positive regulatory role in the activation of genes required for mycelial growth and pathogenicity. The reasoning behind this conclusion is that lack of *b* function in haploids is not sufficicient to induce mycelial growth and pathogenicity (Kronstad and Leong, 1990).

Genetic analyses, such those described above, support the conclusions that the *U. maydis* mating-type factors, *a* and *b*, have the following functional roles: The *a* gene governs fusion of compatible cells, possibly by a pheromone/ receptor system similar to that found in *S. cerevisiae*. The *b* gene regulates pathogenicity, growth and development of the dikaryon, meiosis and basidiospore formation.

1.1.5 Molecular Analyses of the b gene.

Analyses of mating and pathogenicity in *U. maydis* have been greatly facilitated by the cloning and molecular characterization of the *b* mating type gene. A total of 7 *b* alleles have been isolated by complementation, hybridization and polymerase chain reaction (PCR) amplification (Kronstad and Leong, 1989, 1990; Schulz et.al. 1990).

To clone the *b*1 allele, a diploid strain homozygous at *b* (*a1a2/b2b2*) was transformed with a cosmid library constructed from a *a1b1* haploid strain. Transformants were screened for mycelial growth which is characteristic of strains heterozygous for *b*. To isolate *b2*, hybridization probes were prepared from the cloned *b*1 allele and used to screen a

second cosmid library constructed from an *a2b2* haploid strain (Kronstad and Leong, 1989). An additional four alleles (*b*H, *b*J, *b*K, *b*L) were obtained by PCR amplification using primers based on *b*1 and *b*2 alleles (Kronstad and Leong, 1990). A similar strategy was employed by Schulz et al. (1990) to isolate 4 *b* alleles: *b*1, *b*2, *b*3, *b*4. The *b*3 and *b*H clones appear to be isolates of the same allele.

All *b* alleles are present on 8.5 kb *BamHI* fragments (with the exception of one allele which is present on a 7.0 kb *BamHI* fragment). Restriction enzyme analysis of *b*1 and *b*2 reveal identical restriction sites in a 6.0 kb region of the cloned allele and polymorphisms in the remaining 2.5 kb region. The coding sequence has been localized to the 2.5 kb polymorphic region by subcloning and Tn5 transposon mutagenesis (Kronstad and Leong, 1990). Nucleotide sequence data of the 2.5 kb region has revealed approximately 90% identity among cloned *b* alleles and a 1230 bp open reading frame (ORF). Searches of data bases failed to detect significant homologies among the *b* allele sequences and documented gene sequences.

Analysis of the predicted amino acid sequence encoded by the *U. maydis b* gene ORF has revealed clues to the functional role of the *b* gene and has provided the basis for models proposed to explain how the *b* alleles interact to regulate genes required for pathogenicity and mycelial growth. The putative *b* protein is 410 amino acids long and has a variable amino terminus and a constant carboxyl terminus. A short

stretch of basic amino acids is similar in sequence to the nuclear localization region of the mouse glucocorticoid receptor and to histone genes (Kronstad and Leong, 1990; see Hunt, 1989 for references on nuclear localization sequences). This suggests that the active *b* product resides in the nucleus. Kronstad and Leong (1990) suggest a model in which *b* allele activity is regulated by nuclear localization of the *b* gene products. In such a system, conformational differences between *b* protein homodimers and heterodimers determine intracellular location of the active species and thereby ultimately regulate *b* activity.

The carboxyl terminus of the *b* protein contains four amino acids (W-F-N-R) which are invariant among eukaryotic homeodomain proteins as well as six out of eight amino acids which are highly conserved in homeodomain proteins (Schulz et.al., 1990; see Scott et.al., 1989 for review of homeodomain proteins). This suggests that the *b* gene products may function as a manner similar to that of other eukaryotic homeodomain proteins such as the MAT*a*1 and MATα2 products of *Saccharomyces cerevisiae* (Dranginis, 1990). Schulz et.al. (1990) have proposed two general models in which *b* protein dimers regulate transcription of dikaryon-specific genes by binding to specific sequences of DNA. In the first model, variable regions of the *b* proteins regulate specificity and activity of dimers. In the second model, constant regions govern formation of dimers, and variable regions determine activity.

The models discussed above are not mutually exclusive. In fact, together they suggest that the *U. maydis b* gene participates in a regulatory system which is similar to systems described for other eukaryotic organisms. The models also suggest a role for regulatory factors other than the *a* and *b* genes. For example, transcriptional activators or repressors, accessory proteins, and/or cytoplasmic anchoring proteins may interact with *b* proteins to regulate genes involved in pathogenicity and mating. Genes for such factors have not yet been identified.

1.2 Research Outline.

The aim of research described in this thesis is to identify genes, other than the *a* and *b* mating type genes, which regulate mycelial growth and pathogenicity in *Ustilago maydis*. The stategy used to achieve this aim is outlined in Figure 2. A haploid strain, which grows with a yeast-like morphology, was mutagenized with UV light and mycelial mutants were isolated. Mutants were characterized with respect to stability of mycelial morphology, dominance of the mutation and pathogenicity. A single recessive mutant was chosen for further study and the mutation was complemented with a cosmid library constructed from a wild-type haploid strain.

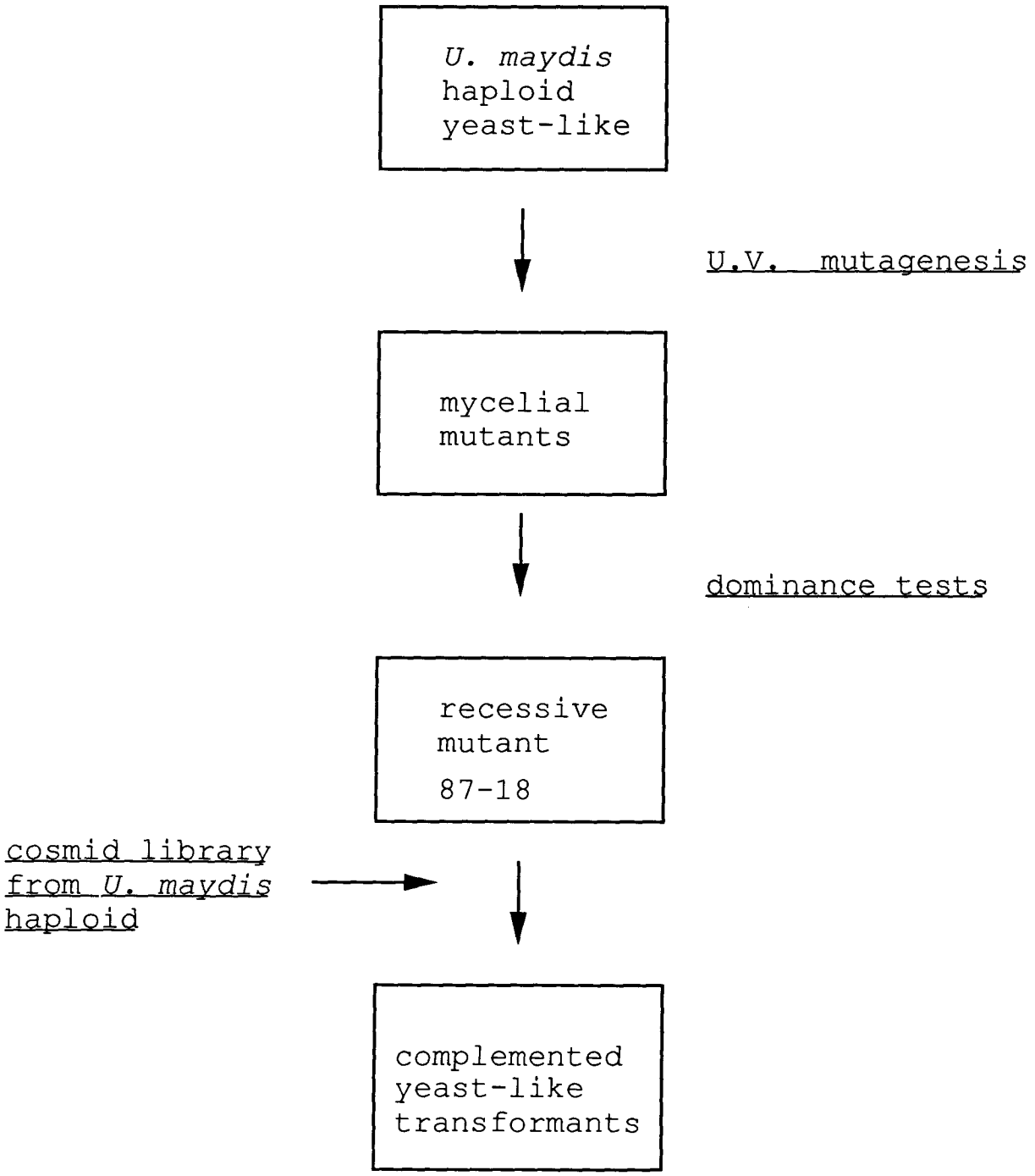
The rationale for this approach was as follows. It has been shown previously that spontaneous mycelial mutants can be isolated at a high frequency from *U. maydis* haploid strains (eg. Stakman et.al., 1929, cited in Rowell and Devay, 1954, and Stakman et.al., 1943b). Mycelial mutants have also been isolated from a yeast-like diploid strain by UV mutagenesis (Day et.al., 1971). Preliminary mutagenesis experiments in this laboratory have corroborated these results: UV mutagenesis of a yeast-like haploid strain yielded mycelial mutants at a high frequency. These experiments suggested that haploid strains are capable of expressing genes required for mycelial growth and pathogenicity. The fact that haploid strains, heterozygous at b, (a/b1b2) are weakly mycelial and pathogenic (Kronstad and Leong, 1989) further substantiates this conclusion.

Cloning by complementation has become a standard technique for isolating genes in fungi (see Fincham, 1989) and has been used to clone mating type genes such as RME1 (regulator of meiosis) of *S. cerevisiae* (Mitchell and Herskowitz, 1986).

These precedents support the experimental plan outlined in Figure 2 to identify genes involved in mycelial growth and pathogenicity by complementation of a mycelial haploid mutant.

Figure 2

Complementation of a *rem* mutant: Project Strategy



2. MATERIALS

2.1 Strains.

The strains of *Ustilago maydis* employed are listed in Table 1. All strains were obtained from R. Holliday except d410, 031 and 032 which have been previously described (Kronstad and Leong, 1989). *E. coli* strain DH5α (F$^-$, *end*A1, *hsd*R17(r_k^-, m_k^+), *sup*E44, *thi*$^-$, *rec*A1, *gyr*A96, *rel*A1, ϕ80d*lac*Z M15) was used for all DNA manipulations.

2.2 Media.

Media for *U. maydis* cultures have been previously described (Holliday, 1974). Media for *E. coli* and YEPS media are described in Sambrook et.al. (1989) and in information provided with Gigapack packaging extract (Stratagene). The following abbreviations have been adopted: complete medium, CM; double complete medium, DCM; double complete medium with 0.8M sorbitol, DCMS; double complete medium with activated charcoal, DCMC; hygromycin B, hyg; ampicillin, amp; potato dextrose agar, PDA.

2.3 DNA.

The cosmid vector pJW42 (Leong, et.al., 1991) was used for the preparation of the cosmid library (Figure 3).

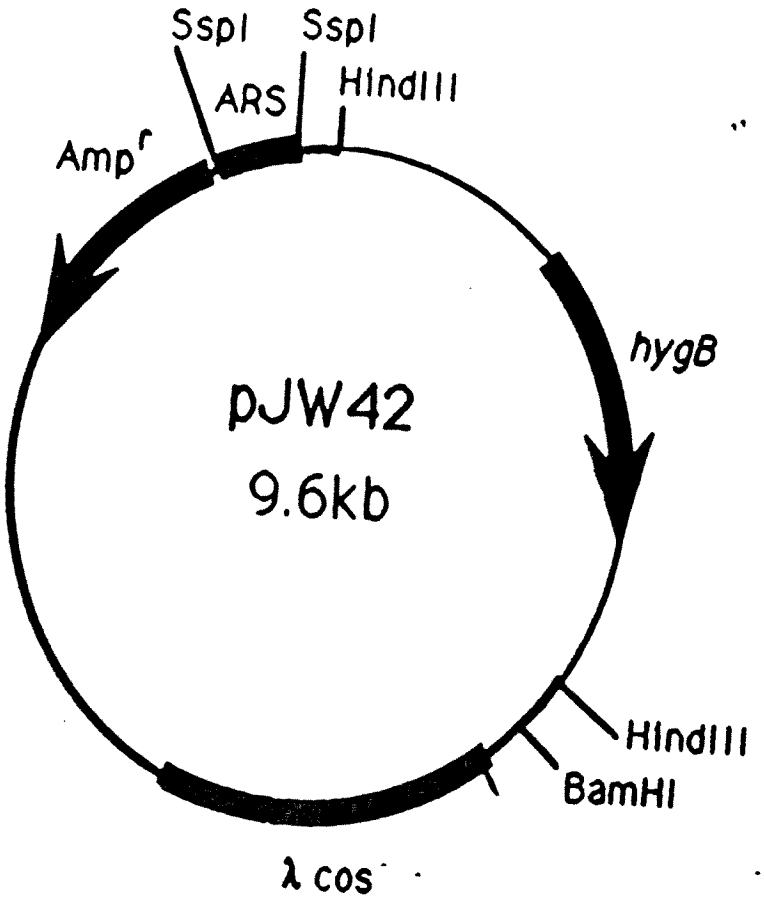
Table 1

List of *U. maydis* Strains

STRAIN	GENOTYPE
001	*a*2 *b*2
002	*a*1 *b*1
031	*a*1 *b*2
032	*a*2 *b*1
87	*a*2 *b*2 ad1-1 leu1-1
272	*a*1 *b*2 pan 1-1 inos 1-3 nar 1-1 pyr 1-1
d410	*a*2 *b*2 ad1-1 leu1-1 x *a*1 *b*2 pan1-1 inos1-3 nar1-1 pyr1-1

Figure 3.

Cosmid vector pJW42



3. METHODS

3.1 Mutagenesis.

Cultures of *U. maydis* strains 87 and 272 were grown to log phase in 10 mls of complete medium. Cells were harvested, washed twice with sterile water and resuspended to a final volume of 3 mls. Cells were counted using a hemacytometer. 1 ml aliquots of culture (cell density of approximately 10^7 cells/ml) were exposed to U.V. light for 3 minutes with constant stirring to achieve approximately 90% cell death. Mutagenized cultures were plated on DCMC at a density of 200-500 colonies per 100mm plate; the plates were wrapped in parafilm and incubated at 30°C for 3-4 days. Colonies were observed under 16x magnification and putative mycelial mutants were regrown on DCMC at 30°C. Stock cultures of isolated mutants were maintained at -70C° in 0.8 mls of complete medium with 0.07 mls DMSO.

3.2 Characterization of Mutants.

3.2.1 Morphology and Stability

Mycelial mutants isolated by UV mutagenesis were scored for strength and stability of mycelial phenotype after overnight growth at 30°C on charcoal plates. Temperature sensitivity was assessed by comparing colony morphology on DCMC at room temperature and at 30°C.

Microscopic examination of cell morphology was done using phase contrast microscopy and a BIORAD MRC 500 confocal laser scanning microscope. To produce a confocal image, an overnight culture of 87-18 cells was stained in a solution of 25% ethanol, 15 mM $MgCl_2$, and 100 mM mithramycin for 10 minutes. A fluorescent image was taken using a 488 nm filter block and was merged with a phase contrast image using BIORAD software.

3.2 2 Dominance and Complementation Tests.

Dominance and complementation tests were adapted from Puhalla (1969). Fresh cultures of haploids carrying complementary auxotrophic markers were streaked on top of one another on DCMC: parental strain x mutant for dominance tests; mutant x mutant for complementation tests. A sterile strip of Whatman No.1 filter paper was placed across the inoculated agar and the wrapped plates were incubated at 30°C for 3-4 days. The filter paper strips were then inverted onto minimal media plates and incubated at 30°C for 5-15 days. Diploids formed by mating between haploid strains grew outward from the periphery of the filter paper. This growing cell material was transferred onto minimal media plates and incubated 3-5 days at 30°C. Diploids isolated in this manner were then streaked onto DCMC plates to access the mycelial phenotype. Diploids produced with recessive mutants were crossed with tester *U. maydis* strains 032 and 002 on DCMC plates to confirm the presence of both *a* alleles.

3.2.3 Pathogenicity Tests.

The pathogenicity of strains was tested by inoculating one week old corn seedlings ("Golden Bantam" variety) with cell suspensions using a 1 ml syringe and a 26 gauge needle. Approximately 100 µl of suspensions containing 10^8 cells for single strain inoculations and 10^6 cells of each culture in crosses were injected per seedling. Plants were grown in a Conviron growth chamber with a 12 hour photo-period, an average temperature of 26°C during the day and 21°C at night, relative humidity of 70%, and an average light intensity of 350 µeinsteins/m^2/s.

Plants were harvested after approximately 3 weeks growth. To analyze meiotic products of teliospores, galls were ground in 1.5% copper sulphate, strained through cheese cloth and kept at room temperature for 18-24 hours (Puhalla, 1968). Spore suspensions were washed in water, counted, plated on CM or PDA germination plates and incubated at 30°C for 36-48 hours. Plates were flooded with sterile water and meiotic progeny were replated on CM or DCMC. Plates were incubated at 30°C overnight. Single colonies were transferred to DCMC plates, and colony morphology was assessed after overnight growth at 30°C.

3.3 Complementation of rem mutant 87-18.

3.3.1. Construction of Cosmid Library.

3.3.1a. Isolation of genomic DNA.

Protoplasts of *U. maydis* strain 001 were prepared by cell wall digestion with Novozyme 234 following the method of Wang et.al. (1988). Protoplasts were treated with 0.4 mg/ml proteinase K for 30 minutes, then lysed in 3% sodium N-lauroyl sarcosinate at 65°C for 3 hours. The lysate was layered onto a 15-50% sucrose step gradient and centrifuged in Beckman SW28 rotor at 24,000 RPM, 3.5 hours. 20°C. The bottom 8-10 mls of the gradient was removed and dialyzed against TE (10 mM Tris pH 8.0, 1 mM Na_2 EDTA) buffer at 4°C for 48 hours. The resulting solution was concentrated on solid sucrose then redialysed in TE for 24 hours. The genomic DNA preparation was treated the Boehringer RNase (DNase-free) at a concentration of 4 units/ml, incubated at 37°C for 2 hours, then dialyzed against TE at 4°C overnight. The concentration of the DNA was measured at OD_{260} and the molecular weight was estimated on a 0.3% agrarose gel.

The genomic DNA preparation was divided into 200 µl aliquots and digested for 20, 25, 30, and 35 minutes using 0.05 units of *Sau* 3A1. The reaction was stopped by the addition of EDTA to 10 mM and incubation for 20 minutes at 65°C. Fractions were pooled, extracted twice with phenol-chloroform, and dissolved in 200 µl of TE. The digestion mixture was layered on to a 5-20% NaCl linear gradient and centrifuged in a Beckman SW41 rotor at 37,000 RPM, for 4.5

hours at 25°C (in a Beckman model L8-80 centrifuge). Fractions of 0.25 mls were collected and aliquots were run on a 0.3% agarose gel to estimate molecular weight. Fragments in the size range of 30-40 kb were pooled, precipitated in 95% ethanol and resuspended in TE.

3.3.1b Ligation of Vector and Insert DNA

The cosmid pJW42 was digested to completion with *Bam* H1 and treated with 0.5 units of calf intestinal phosphatase for 1 hour at 37°C. The reaction was stopped by the addition of EDTA to 10 mM and incubation at 65°C for 15 minutes. The DNA was extracted twice with phenol-chloroform, precipitated with ethanol and dissolved in 5 µl of TE. A molar ratio of 2:1, vector to target DNA, was ligated in a reaction volume of 5.0 µl at 16°C for 16 hours. The total DNA concentration in the ligation reaction was 450 ng/µl.

3.3.1c. Packaging.

Gigapack packaging extracts were obtained from Stratagene. The packaging protocol was followed as described in the instruction manual supplied by the manufacturer. The supernatant was titred by adsorbing 25 µl of OD_{600}= 0.5 *E. coli* DH5α cells to 10^{o}, 10^{-2}, 10^{-4}, and 10^{-6} dilutions of packaging extract. Plates (NZY with 50 µg/ml amp) were incubated at 37°C overnight.

3.3.1d. Isolation of Cosmid DNA.

Minipreps. A total of 18 colonies were picked from NZY amp (50 μg/ml) transformation plates. Cosmid DNA was prepared as described by Sambrook et.al. (1989). Cosmid DNA was digested with *BamHI* and run on a 0.5% agarose gel to check the size and the diversity of the inserts.

Library Amplification. An aliquot of packaging extract sufficient to produce approximately 10^5 *E. coli* transformants was adsorbed to *E. coli* DH5α cells, plated on 150mm NZY amp (50 μg/ml) plates and incubated overnight at 37°C. Plates were flooded with 10 mls of NZY amp (50 μg/ml) broth and used to inoculate 250 mls of NZY amp (50 μg/ml) broth in Fernbach flasks. Cultures were grown for 5 hours at 37°C. Cosmid DNA was extracted as described by Ish-Horowicz and Burke (1981). Preparations were purified by CsCl centrifugation as described by Sambrook et al. (1989), and dissolved in TE to a concentration of 1 μg/ml as determined by OD_{260} measurements.

3.3.2 Transformation of the rem Mutant 87-18.

Mycelial mutant 87-18 was transformed to hygromycin resistance with the cosmid library prepared from *U. maydis* strain 001 and subsequently screened for yeast-like transformants. Control transformations using vector DNA without inserts or no DNA were also performed.

The transformation protocol was adapted from Tsukuda et al. (1988). Cultures were grown in YEPS media with 10%

sucrose to log phase (OD_{600} of approximately 1), harvested, washed in SCS buffer (20 mM Na citrate pH5.8, 1M sorbitol) and resuspended in 1ml SCS with 20-50 units/ml, filter sterilized, Novozyme 234. Protoplasting was monitored under 40x magnification and cultures were harvested when 50-90% of cells had protoplasted. Preparations were washed once in SCS and once in STC buffer (10 mM Tris HCl pH7.5, 0.1M $CaCl_2$, 1M sorbitol), then resuspended in 1 ml ice cold STC. Protoplasts were counted and frozen at -70°C in 50 µl aliquots with 5% DMSO.

DNA for transformation was incubated with 15 µg heparin (15 mg/ml solution in SCS) for 15 minutes on ice. Approximately 10^6 protoplasts (in 50 µl) were added to the DNA mixture and incubation continued for 10 minutes on ice. 0.5 mls of 40% PEG 4000 in STC was added to protoplasts and incubation continued for 15 minutes. To recover protoplasts, 0.5 mls STC was added and protoplasts were pelleted in microfuge at 5000 RPM for 5 minutes. Cells were resuspended in 200 µl of DCMS and plated immediately on transformation plates (12 mls DCMS, 750 µg/ml hyg, and 12 mls DCMS top agar). Plates were incubated at 30°C for 4-7 days and transformants were picked onto CM (350 µg/ml hyg) plates.

Colonies were screened for yeast-like phenotype after growth on DCMC (350 µg/ml hyg) at 30°C for 1-2 days. The plates were sealed with parafilm. Putative yeast-like transformants were tested for reversion by growing cultures to saturation in non-selective media (5 mls YEPS 10% sucrose)

and then plating on DCMC lacking hygromycin. The phenotype was noted and single colonies were tested for hygromycin resistance by transfer to DCMC (350 μg/ml hyg).

3.3.3 Cosmid Rescue and Re-Transformation.

Cosmids were rescued from putatively, complemented mutants by isolating cosmid DNA, re-packaging into lambda phage heads and transfecting *E.coli* DH5α.

Cosmid DNA was isolated from yeast-like transformants using a method adapted from Sambrook et al. (1989). Mutants were grown to log phase in 50 mls YEPS with 10% sucrose and 350 μg/ml hyg. Cells were converted into protoplasts as described for the transformation procedure, then lysed in 0.15M EDTA, 2% SDS and 1.25M NaCl. Chromosomal DNA and debris were removed by centrifugation at 30,000 RPM for 30 min. in a Beckman Vac 50 rotor. The DNA is the supernatant was extracted with phenol:chloroform:isoamyl alcohol, precipitated in 100% ethanol and dissolved in 30 μl TE.

Isolated cosmid DNA was packaged into phage heads, transfected into *E. coli* DH5α using Stratagene Gigapack packaging extracts, purified by CsCl extraction and re-transformed into 87-18 protoplasts as described previously in this section.

The morphology of transformants was tested after overnight growth at 30°C on DCMC (with 350 μg/ml hyg). Cosmids were cured from transformants as decribed above.

4. RESULTS

4.1 Mutagenesis.

The results of mutagenesis of *U. maydis* and screening for mutants with a mycelial phenotype are summarized in Table 2. Cultures of strains 87 and 272 were mutagenized to approximately 10% survival. The mutants were detected at a frequency of approximately 10^{-3} (1:1000 mutant:survivor) for both strains. A total of 34 mutants of strain 87 and 68 mutants of strain 272 were isolated. This type of mutant was named *rem* (*re*pressor of *m*ycelial phenotype).

4.2 Characterization of Mutants.

4.2.1 Morphology and Stability.

The morphology of all mycelial mutants was assessed after overnight growth on DCMC at 30°C. Among the mutants isolated, two morphological categories were apparent: weak mycelial phenotype and strong mycelial phenotype. Photographs of mutants representing these classes are shown in Figure 4.

Mutants with a strong mycelial phenotype were tested for stability and temperature sensitivity as described in Methods (section 3.2.1). Most mutants were unstable to some degree; non-mycelial revertants were observed at varying frequencies when single mycelial colonies were streaked on DCMC.

Table 2
Results of Mutagenesis and Screening

strain	total # mutants	phenotype		stability	
		weak	strong	#stable	# ts
87	34	9	25	2	6
272	68	37	31	12	7

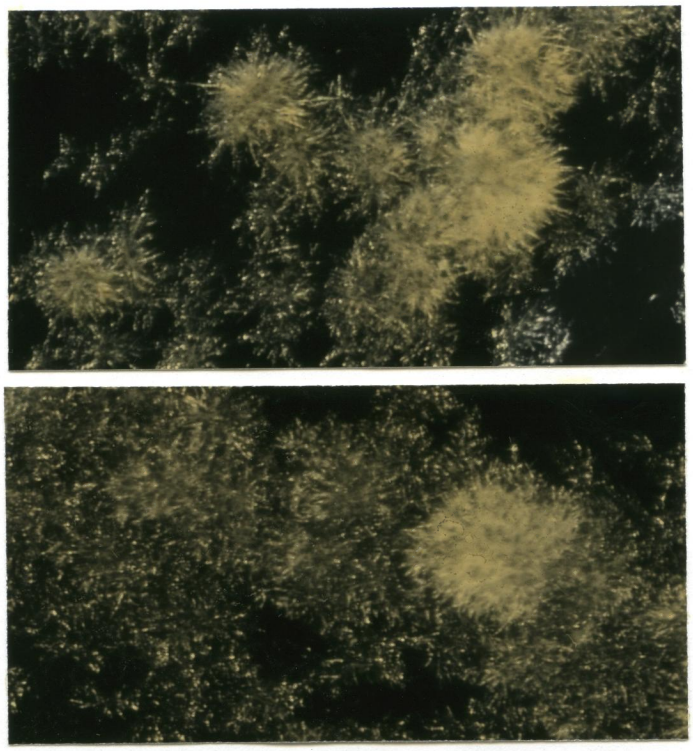
Table 3
Results of Dominance Tests

mutant strain	# mutants tested	# 2N formed	# recessive (yeast-like)
87	21	9	9
272	31	18	18

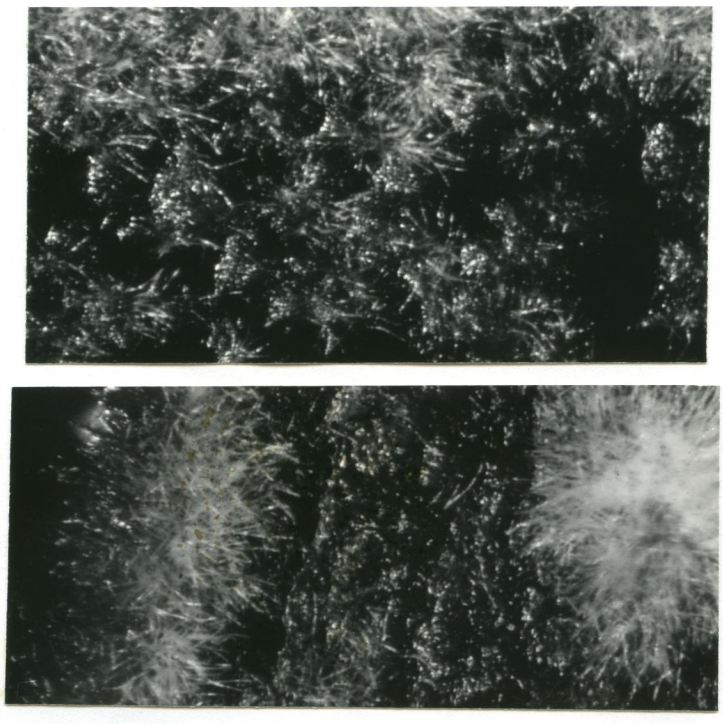
Figure 4.

Examples of weak and strong mycelial mutants. All mutants were grown overnight at 30°C on DCMC. 50x magnification.

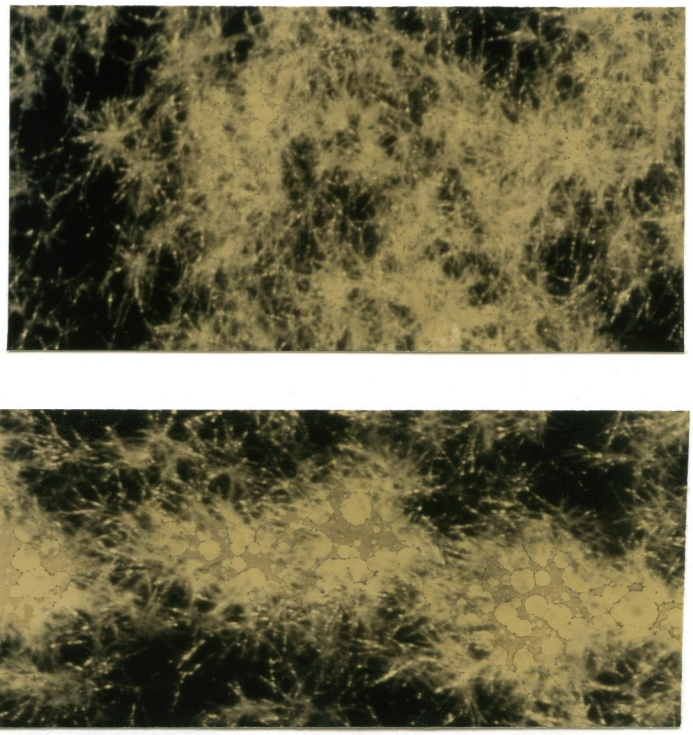
4A. Examples of weakly mycelial mutants of strain 87



4B. Examples of weakly mycelial mutants of strain 272.



4C. Examples of strongly mycelial mutants of strain 87.



4D. Examples of strongly mycelial mutants of strain 272.

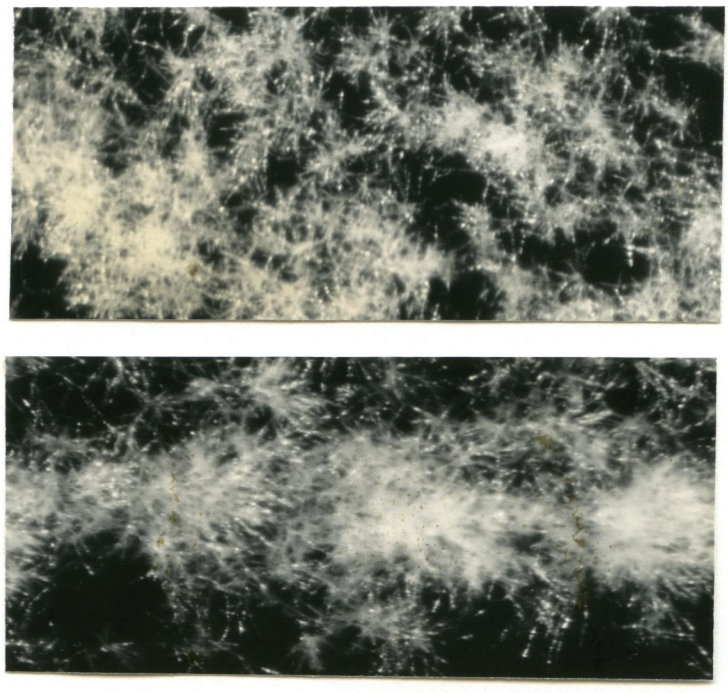
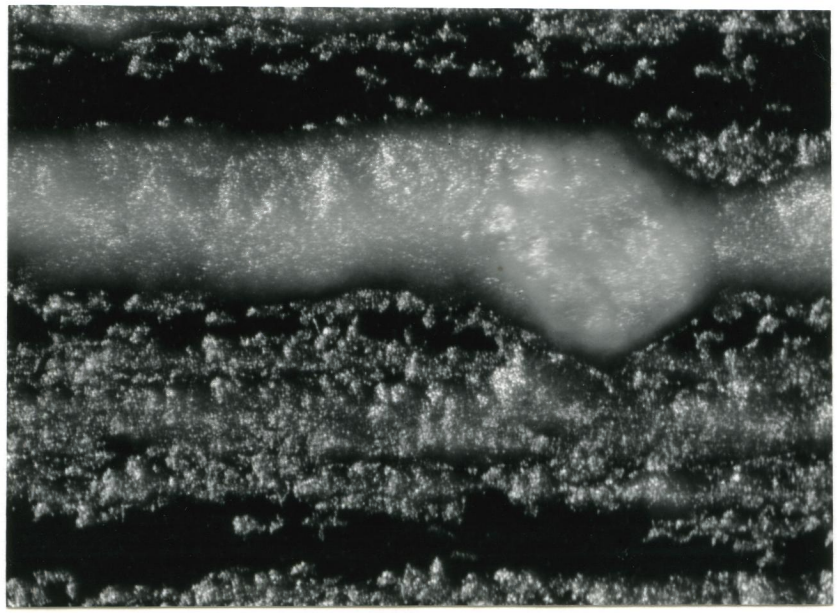


Figure 5.

Morphology of strain 87 (parental strain) and strain 87-18 (mutant) on charcoal mating plates.

5A. Yeast-like phenotype of strain 87 (parental strain) on charcoal media (overnight growth at 30°C).

25x magnification.



5B. Mycelial phenotype of strain 87-18 (mutant) on charcoal media (overnight growth at 30°C).

50x magnification.

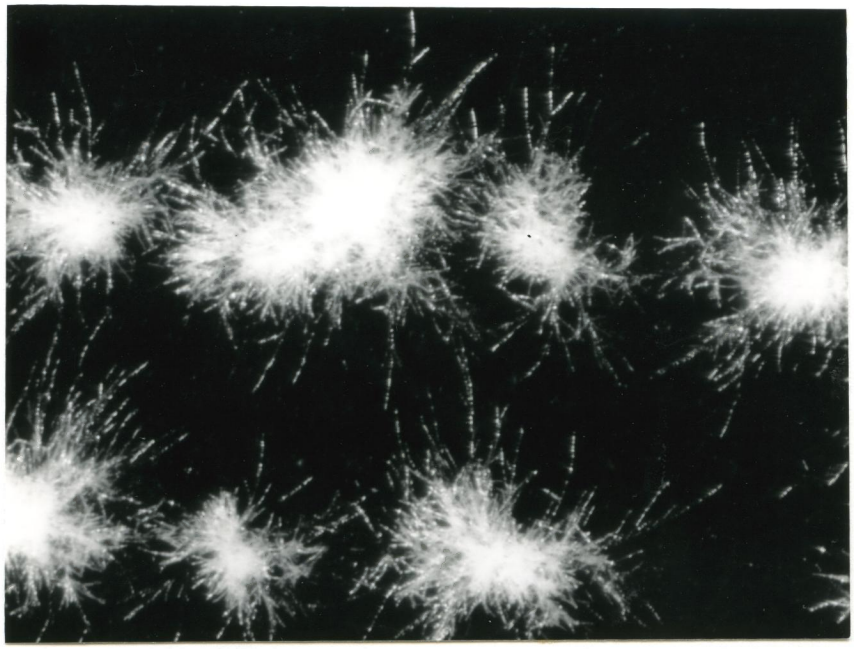
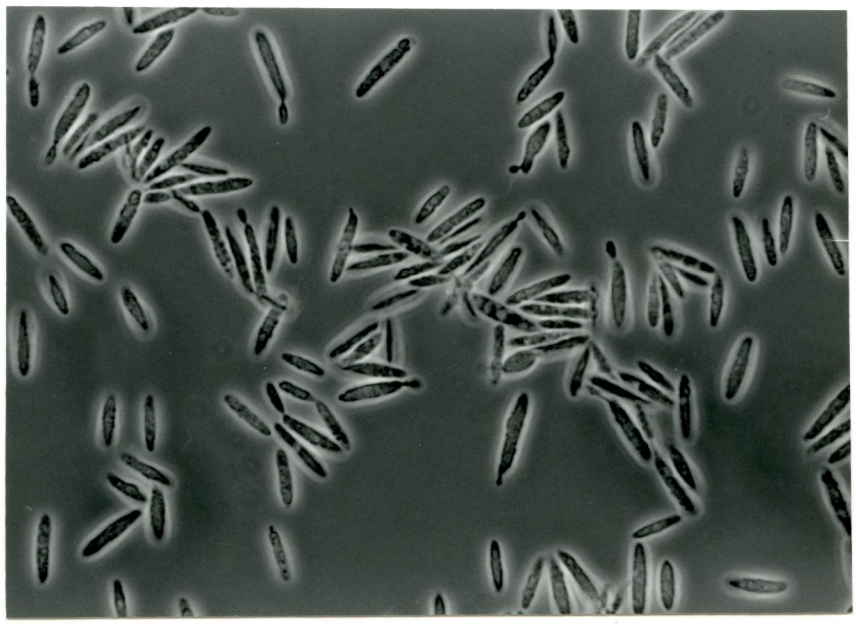


Figure 6.

Morphology of strain 87 (parental strain) and strain 87-18 (mutant) in liquid media.

6A. Yeast like phenotype of strain 87 (parental strain) in liquid DCM (overnight growth at 30°C). 1000x magnification, phase contrast microscopy.

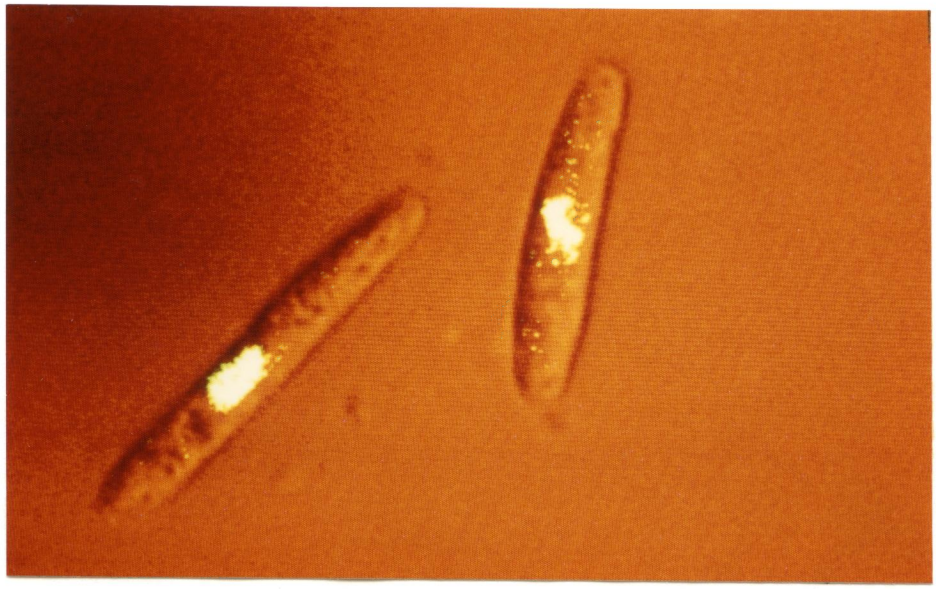


6B. Mycelial phenotype of strain 87-18 (mutant) in liquid DCM (overnight growth at 30°C). 1000x magnification, phase contrast microscopy

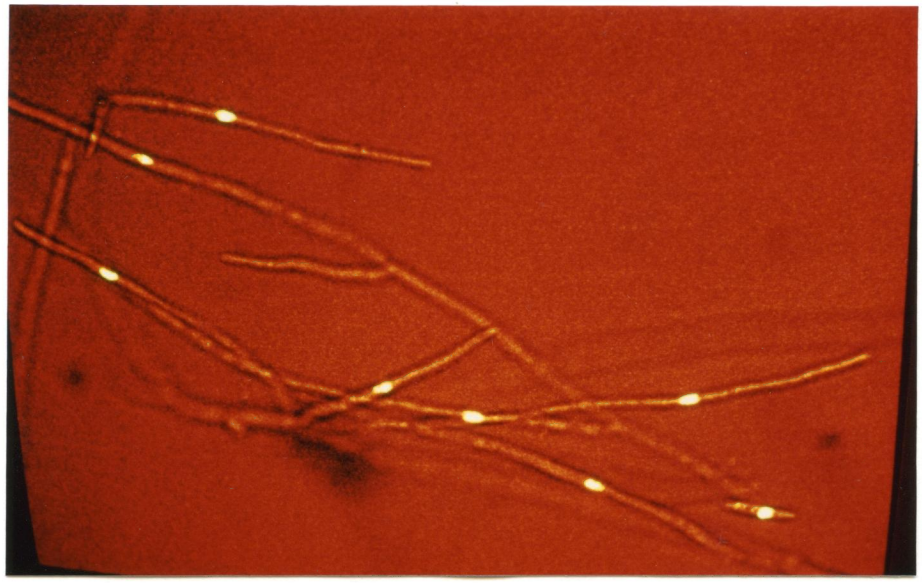
Figure 7.

Nuclear staining of strain 87 (parental strain) and strain 87-18 (mutant). Confocal image of 87-18 mycelium showing distribution of nuclei throughout mycelium. Nuclei are stained with mithramycin and fluoresce green under a 488 nm filter.

7A. Strain 87 (parental strain).



7B. Strain 87-18 (mutant).



However, 2 mutants of strain 87 and 12 mutants of strain 272 were stable throughout initial screening on solid charcoal medium.

Temperature sensitivity of the mycelial phenotype was assessed by comparing mutant phenotypes at 30°C and room temperature. A mutant was considered to be temperature sensitive if it was mycelial at 30°C and yeast-like at room temperature. Temperature sensitivity was difficult to determine due to instability and variability of the mycelial phenotype; i.e., all mutants showed some change in morphology when grown at room temperature. None of the stable mutants were temperature sensitive; however, 6 unstable mutants of strain 87 and 7 unstable mutants of strain 272 were temperature sensitive upon initial screening. These were not examined further.

A single mutant of strain 87 (designated 87-18) was chosen for further study due to the strength and relative stability of the mycelial phenotype. The filamentous morphology of 87-18 is compared with the yeast-like morphology of the parental strain 87 in Figures 5-7.

4.2.2 Dominance and Complementation Tests.

The results of dominance tests are summarized in Table 3. These tests proved to be difficult and time-consuming due to inefficient formation of diploids between compatible haploids. In contrast, appropriate combinations of wild-type haploid cells readily fuse to form diploids using the method

of Puhalla (1969). After approximately 300 attempts, a total of 27 dominance tests were completed (9% success rate). In all cases, the resulting diploid was non-mycelial. This result suggested that the *rem* mutation was recessive. Mutant 87-18 was among the 9 recessive mutants of strain 87 (see Table 3). Diploidy was confirmed by a positive mating reaction between the diploid (*a*1/*a*2 *b*2/*b*2) and tester strains 002 (*a*1 *b*1) and 032 (*a*2 *b*1), indicating the presence of both *a* alleles in the diploid. Negative mating reactions were obtained in crosses with the diploid and tester strains 001 (*a*2 *b*2) and 031 (*a*1 *b*2).

Complementation tests were performed with the aim of approximating the number of genes associated with the mutant, mycelial phenotype and possibly accounting for the apparent groups of morphological types. The tests involve crossing compatible recessive mutants to form diploids. Complementing mutations will result in a yeast-like diploid whereas non-complementing mutations will produce a mycelial diploid. The success of these tests is dependent upon the outcome of dominance tests (because recessive mutations are required) and efficient formation of diploids between two mutant strains. As discussed above, dominance tests involving diploid formation between wild-type and mutant strains were slow and inefficient. Furthermore, matings between two mutants proved to be more difficult than matings between mutants and parental strains. For these reasons, complementation tests were abandoned after repeated attempts

with marginal success. Approximately 500 complemetation tests were attempted. Of these tests, 9 were successful (2% success rate). The diploids formed in all cases were yeast-like. Mutant 87-18 was not successfully crossed with any of the mutants from strain 272.

4.2.3 Pathogenicity Tests

Pathogenicity tests were performed to address the following questions: 1. Is the *rem* mutant, 87-18, solopathogenic? 2. Will strain 87-18, when mixed with a compatible mating partner (strain 002) cause disease? 3. What frequency of mycelial progeny result from a cross between 87-18 and 002? 4. Are mycelial progeny that are prototrophic solopathogenic? 5. Is a diploid formed by crossing 87-18 with wild-type strain 272 pathogenic?

The results of pathogenicity tests are summarized in Table 4. No symptoms were observed upon inoculation of seedlings with monocultures of 87 (parental strain) or 87-18. Crosses of 87 (parental strain) x 002 resulted in large galls and teliospore production in 95% of the crosses. This result was expected of a cross between yeast-like strains of mating genotypes *a1b1* x *a2b2*. Crosses of 87-18 x 002 resulted in teliospore production in 40% of the plants, small galls in 10%, and no symptoms in 50% of inoculated plants. Reasons for this result are suggested in the Discussion (section 5.2.1)

Table 4. Results of Pathogenicity Tests.

CROSS	approx. # of plants inoculated	SYMPTOMS*
87	40	100% A
87-18	45	100% A
87 x 002	35	95% C 5% A
87-18 x 002	50	50% A 40% C 10% B
87-18 x 001	10	100% A
001 x 002	15	93% C 7% A
d410	10	100% A
87-18/272 diploid	20	100% A
prototrophic mycelial progeny of 87-18 x 002	40	100% A

*Expressed as approximate percentage of total plants inoculated according to the following scale:

A= no symptoms; healthy plant

B= small galls on leaves and stem

C= large galls and teliospore production

Pathogenic crosses of *U. maydis* strains will result in teliopsore production in the infected plants. Teliopsores produced upon inoculation of plants with 87 x 002 and 87-18 x 002 crosses were germinated to analyse meiotic progeny, as described in Methods (section 3.2.3). Two experiments were performed to examine the morphology of meiotic progeny. 1. In the first experiment, teliospores were germinated on CM. Upon germination, progeny cells were washed from plates, counted and regrown on CM plates. Single colonies were transferred from CM plates to DCMC plates to assess colony morphology. Using this procedure, all progeny resulting from 87 (parental strain) x 002 crosses displayed the yeast-like morphology expected of haploid strains. Progeny from 87-18 x 002 crosses were yeast-like at frequencies ranging from 53-99% (1-47% mycelial) in 4 separate trials. 2. In a second experiment, teliospores were germinated on PDA plates. Upon germination, meiotic progeny were washed from plates, counted and regrown directly on DCMC plates. Using this procedure, morphological differences among colonies could be detected on charcoal (DCMC) plates at early stages of colony development. DCMC plates were incubated at 30°C for approximately 18 hours. All progeny resulting from 87 (parental strain) x 002 crosses were yeast-like. Progeny resulting from 87-18 x 002 crosses were of two morphological types: yeast-like colonies and very small mycelial colonies. The approximate ratio of yeast-like:mycelial colonies was 1:1 where colony density on

DCMC plates was low. In areas of high colony density, yeast-like colonies predominated.

If a single locus is responsible for the *rem* phenotype, a 1:1 ratio of mycelial:yeast-like progeny is expected from a cross of 87-18 x 002. Explanations for the ratios reported above, are suggested in the Discussion (section 5.2.1).

Haploid, mycelial progeny were tested for prototrophy on minimal media. A total of 6 prototrophic mycelial colonies were isolated. Each prototrophic strain was inoculated as a monoculture into corn seedlings. Symptoms did not result from any of these inoculations.

Inoculation of plants with diploid strain d410 (272 x 87) and the diploid constructed with 87-18 and 272 did not result in symptoms.

4.3 Complementation of rem 87-18.

4.3.1 Construction of Cosmid Library.

A cosmid library was constructed using the autonomously replicating, cosmid vector pJW42 and *Sau* 3A1 partial digests of genomic DNA from *U. maydis* strain 001. Insert DNA fragments were ligated into the unique *BamHI* site of pJW42, packaged *in vitro* and transfected into *E. coli* DH5α. The size of the library was estimated at 10^6 clones, each containing an insert of 29-41kb. The size and diversity of the inserts were determined by *BamHI* digestion of cosmid miniprep DNA from 18 clones. The size of the *U. maydis*

haploid genome has been estimated to be 10^4 kb (Fotheringham and Holloman, 1989). Using the formula N=ln (1-p)/ln (1-x/y), where x=average size of insert DNA and y= size of genome, it was calculated that a library of approximately 1300 clones with average inserts of 35 kb would be adequate to represent the entire genome of strain 001 with a probability of .99. The library constructed in the experiments described was therefore considered to be of sufficient size for the purpose of complementing the mutation in strain 87-18.

4.3.2 Transformation of the rem Mutant 87-18.

The *rem* mutant, 87-18, was transformed with the complete cosmid library from *U. maydis* wild type strain 001. Transformants were selected for hygromycin resistance and screened for non-mycelial phenotype.

Several significant problems were encountered throughout the transformation and screening procedures. The compact colonies of hyphae produced by *rem* mutants in liquid culture proved to be more difficult to protoplast than the yeast-like cells of wild type haploid and diploid cells. This problem was partially overcome by increasing the sucrose concentration in liquid media to promote growth of yeast-like cells. The following combinations of sugars and media were assayed for effects on cell morphology: sucrose, glucose and sorbitol at 1%, 5%, 10% and 20% in YEPS and DCM. Growth in DCM consistently resulted in a strong mycelial phenotype

whereas growth in YEPS with glucose or sucrose favored yeast-like growth. Addition of 10% sucrose was most favorable to yeast-like growth. Addition of sorbitol did not have a significant effect on cell morphology. It has been previously reported that high sugar and salt concentrations promote yeast-like growth of *U. maydis* (Kernkamp, 1939). The morphology of 87-18 in YEPS with 10% sucrose and in DCM is shown in Figure 8.

A second factor influencing the rate of protoplasting was the concentration and age of the preparation of the cell wall degrading enzyme, Novozyme 234. Solutions freshly prepared at a concentration of 20 units/ml were found to be most efficient. Tranformation frequencies ranged from 50-150 transformants per μg of cosmid DNA.

A total of 1859 hygromycin resistant transformants were isolated. Of these transformants, 209 possessed a yeast-like morphology. It was suspected that the majority of the yeast-like transformants were revertants rather than complemented mutants and further transformation experiments of 87-18 were not performed. To distinguish between reversion and complementation, cosmids were cured from all 209 yeast-like transformants by growing cultures to saturation in non-selective media. Loss of hygromycin resistance corresponded with a recovery of the mutant, mycelial phenotype in 6 of the 209 transformants. This result indicated that the cosmid was responsible for complementation of the mycelial phenotype in these 6 transformants. The remaining 203 yeast-like

transformants remained yeast-like upon loss of the cosmid and were assumed to be revertants. As a control experiment, protoplasts of mutants 87-18 were transformed with the vector pJW42 containing no insert DNA. Of the 199 transformants obtained, 90% were mycelial.

To determine if the *rem* mutation resided at the *b* locus, 87-18 protoplasts were transformed with the wild type *b*1 allele contained in the integrative cosmid vector, pCU3 (Kronstad and Leong, 1989). A total of 8 transformants were isolated; all were mycelial. The lack of complementation suggests that the *rem* locus is different from the *b* locus (see Discussion section 5.2.2).

4.3.3 Cosmid Rescue and Re-Transformation.

Cosmid DNA was isolated from 3 of the 6 putatively complemented transformants using the protocol described in Methods (section 3.3.3). The cosmids were recovered by packaging total DNA from the transformants into λ phage particles and subsequent transfection of *E. coli*. Attempts to isolate cosmids using standard alkaline lysis procedures to prepare *U. maydis* DNA, and $CaCl_2$ transformation or electroporation of *E. coli* were unsuccessful, presumably due to the large size of the cosmid.

BamHI and *EcoRI* restriction digest patterns of the three cosmids (designated A, B, C) were indistinguishable from each other on a 0.5% agarose gel. This suggests that the cosmid inserts are identical or highly similar. The gel is shown in

Figure 9. *rem* cosmid digests are compared with *BamHI* and *Eco RI* digests of the cosmid pCU3 which has an insert fragment containing the *U. maydis b*1 allele (Kronstad and Leong, 1989). Lack of similarity in the restriction patterns provides further evidence that the *rem* cosmid does not carry the *b* gene.

Protoplasts of 87-18 were transformed with each of the isolated cosmids. In each case, all transformants were yeast-like (wild-type phenotype). Cosmids were cured from transformants as described above. Loss of hygromycin resistance correlated with a recovery of the mutant mycelial phenotype, indicating that complementation was imparted by cosmid DNA. Protoplasts of 87-18 transformed with pJW42 containing no insert DNA were mycelial (mutant phenotype). Figure 10 compares the morphology of transformants carrying a complementing cosmid and transformants carrying pJW42 with no insert DNA.

Figure 8.

Morphology of mutant 87-18 in liquid media. Cultures were grown in 5 mls of media at 30°C overnight. The tube on the left shows 87-18 grown in YEPS with 10% sucrose. Cells grow primarily in a yeast-like morphology, simliar to the parental strain 87. The tube on the right shows 87-18 grown in DCM. Cells grow primarily as aggregates of hyphae.

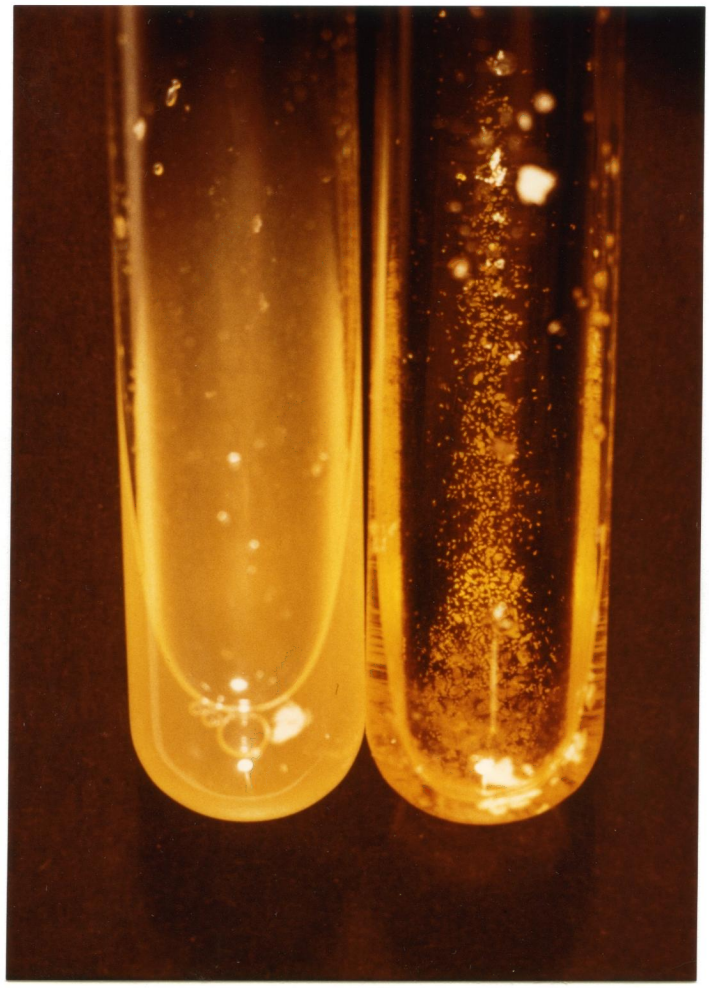
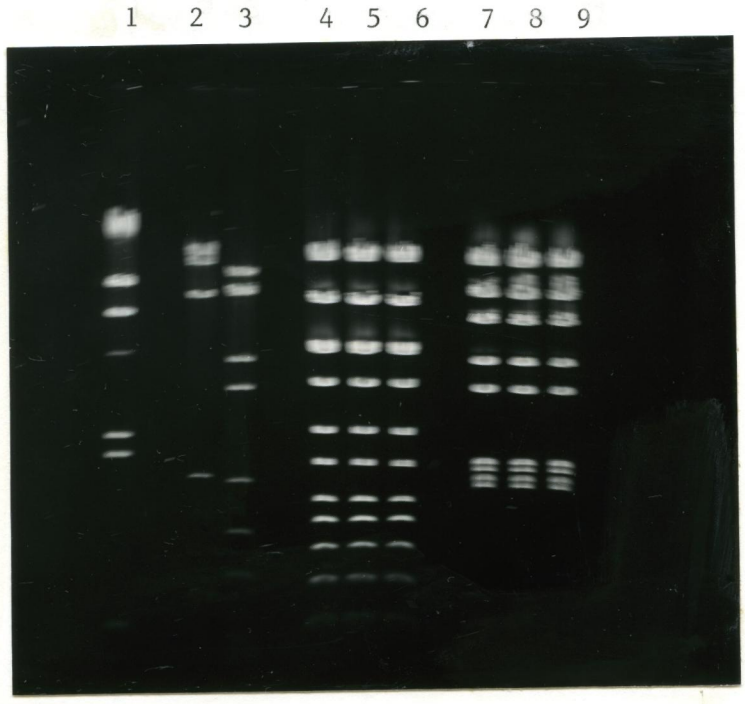


Figure 9.

Restriction digests of *rem* cosmids A, B., and C.

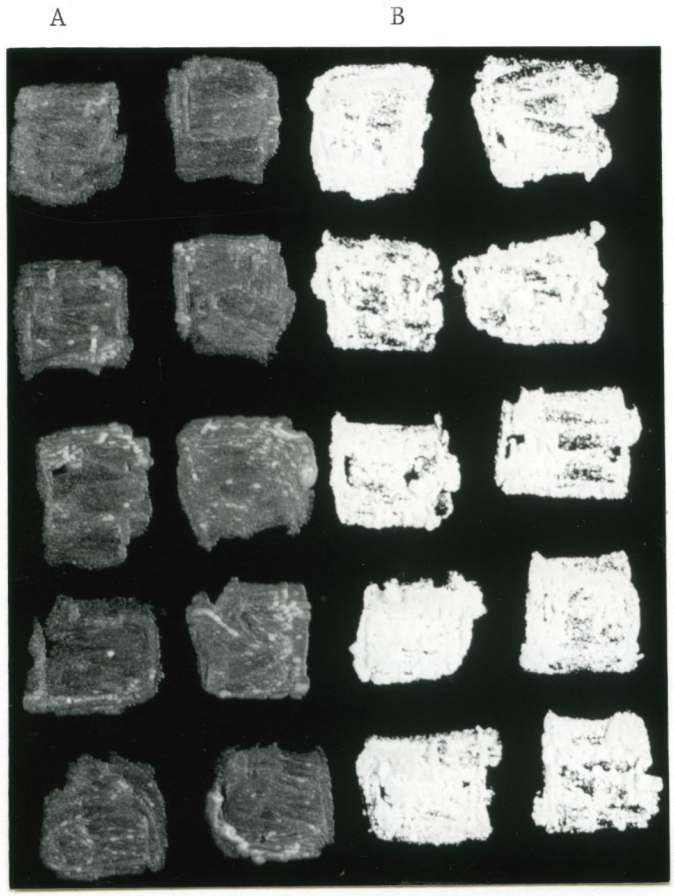


Lanes:

1. lambda DNA cut with *HindIII*.
2. Cosmid pUC3 with *b*1 insert, cut with *BamHI*
3. Cosmid pUC3 with *b*1 insert, cut with *EcoRI*
4-6. *rem* cosmids, A, B, C, cut with *BamHI*
7-9. *rem* cosmids, A, B, C, cut with *EcoRI*

Figure 10.

Morphology of Complemented vs. Non-Complemented *rem* Mutant.



A. Non-complemented *rem* mutant: 87-18 transformants carrying pJW42 with no insert DNA.

B. Complemented *rem* mutant: 87-18 transformants carrying pJW42 with complementing insert DNA.

5. Discussion

5.1 Summary of Results

The aim of the experiments described in this thesis was to identify genes which are involved in the switch from yeast-like to mycelial growth in *U. maydis*. To this end, mycelial mutants have been isolated by UV mutagenesis of haploid strains 87 and 272. The majority of these mutants were unstable and revert to yeast-like morphology. A single mutant of strain 87 (designated 87-18) was chosen for further characterization based on the strength and relative stability of the mycelial phenotype. The mutation was named *rem* (*r*epressor of *m*ycelial phenotype). Pathogenicity tests indicate that the *rem* mutant is not solopathogenic. However, when *rem* is co-inoculated with a compatible mating type strain, regular symptoms of pathogenicity are produced in the plant.

To complement the *rem* mutation, a cosmid library was constructed using the vector pJW42 and 30-40 kb inserts of DNA from a wild-type haploid strain. Transformation of the library into the *rem* mutant resulted in 6 complemented transformants. Cosmid DNA has been rescued from 3 of these transformants; restriction digests indicate that the insert DNA is highly similar. Re-transformation of cosmid DNA into 87-18 gave 100% yeast-like colonies.

5.2 Discussion of Results

5.2.1 Mutagenesis and Characterization of Mutants

A total of 102 mycelial mutants were isolated from haploid strains 87 and 272. These mutants fell into two morphological categories: weak mycelial phenotype and strong mycelial phenotype. There are several possible explanations for this result: 1. More than one gene may be involved in switch from yeast-like to mycelial growth. This conclusion is supported by experiments by Banuett (1991) in which several phenotypic classes of mycelial reactions were found upon mating a mutant *a1b1* strain with an *a2b2* strain. 2. The mutation may be leaky in some *rem* mutants; e.g., weak mycelial mutants may result from partial expression or activity of the *rem* gene. 3. The morphological classes of mycelial mutants and the observed temperature sensitivity of mycelial growth may be attributed, in part, to a range of environmental factors such as light intensity, carbon dioxide levels, and media composition, which frequently cause phenotypic variability in *U. maydis* (Christensen, 1963).

Most of the mutants isolated in these experiments were unstable and readily reverted to yeast-like growth on charcoal plates. Mutants which were relatively stable throughout the initial screening on solid media were unstable when grown in liquid culture. Mutant 87-18, which was used for complementation studies, was stable on charcoal plates but reverted to yeast-like growth in YEPS media. Instability of spontaneous mycelial haploid mutants has been previously

reported (Schmitt, 1940) and competition experiments by Jackson et.al. (1952) indicate that yeast-like growth is favored in liquid media. High reversion frequencies may therefore be explained in part by a selective pressure for cells to grow in a yeast-like morphology.

Dominance and complementation tests were performed according to the method of Puhalla (1969). The aim of these tests was to determine the dominance/recessiveness of the *rem* mutation and to establish complementation groups among the mutants. Conclusive results for these assays require the successful formation of prototrophic diploids between mutant and parental strains (for dominance tests) and between two mutant strains (for complementation tests). These tests proved to be inefficient and time consuming. A total of 27 dominance tests were completed. In all cases, the diploid formed was yeast-like, indicating that the *rem* mutation is recessive. Diploidy was confirmed in each test by (1) growth on minimal media and (2) the presence of both *a* alleles.

Dominance tests with the mutant 87-18 indicated a recessive mutation. Recessiveness was further confirmed in this case by successful complementation of the mutation with a cosmid library containing DNA from a wild-type strain.

A total of 9 complementation tests were completed. In all cases the resulting diploid was yeast-like. This result suggests that the strains tested in each of the 9 crosses carry different mutations. An alternative explanation is

that selective pressure favors the formation of diploids between yeast-like revertants rather than between mycelial mutants. This explanation is supported by the observation that diploids were more readily formed between two yeast-like parental strains (eg. wt 272 x wt 87) and that dominance tests were more successful than complementation tests (9% and 2% success rate, respectively). Furthermore, inefficient formation of diploids with strains carrying the *pan* 1-1 marker was reported by Puhalla (1969). The auxotrophic state of strain 272 (*pan* 1-1, *inos* 1-3, *nar* 1-1, *pyr* 1-1) may therefore effect diploid formation in the dominance and complementation tests reported here.

Pathogenicity tests were performed with the aim of answering the following questions: 1. Is the rem mutant, 87-18, solopathogenic? 2. Will strain 87-18, when mixed with a compatible mating partner (strain 002) cause disease? 3. What frequency of mycelial progeny result from a cross between strain 87-18 and strain 002? 4. Are mycelial progeny that are prototrophic solopathogenic? 5. Is a yeast-like diploid constructed between 87-18 and wild-type strain 272 pathogenic?

Inoculation of seedlings with monocultures of the mutant 87-18 did not result in pathogenicity; plants remained completely healthy. This result may be explained by the auxotrophic state of the parent strain 87 (*ad*1-1, *leu*1-1). Inhibition of pathogenicity by mutations causing auxotrophy,

particularly those causing an adenine requirement, is well documented (Holliday, 1961a,b). Alternatively, the *rem* mutation may cause a mycelial phenotype via a pathway that is not directly related to normal dikaryon formation or that is not sufficient to cause pathogenicity. For example, a mutation in a cell wall biosynthetic gene may cause a phenotypic change without altering pathogenicity.

Co-inoculation of mutant 87-18 (mating type *a2b2*) with compatible strain 002 (mating type *a1b1*) resulted in large galls and teliospore production in 40% of inoculated plants, small galls in 10% of plants and no symptoms in 50% of inoculated plants. In comparison, co-inoculation of parental strain 87 with strain 002 resulted in severe infection in 95% of inoculated plants. The reduced rate of infection which was observed with the mutant strain, may be attributed to the constitutively mycelial morphology of strain 87-18. The following points support this reasoning: 1. The majority of hyphal cells may be inviable (Day and Anagnostakis, 1971a) or may be unable to participate in mating. 2. Cell counts of hyphal cells are inaccurate and, therefore the appropriate ratio of strains in the cross is difficult to obtain.

The reduced infection rate of the 87-18 x 002 cross may also be attributed to the *rem* mutation itself. Previous experiments have described mutations in *U. maydis* which affect pathogenicity (Stakman et al., 1943a,b; Day et al., 1971; Banuett, 1991).

Progeny of successful crosses between 87-18 and 002 were isolated and analysed according to the method of Holliday (1961a). If the *rem* mutation resides in at a single locus, a 1:1 segregation of mutant:parental morphology is expected among the progeny. Mutant-like colonies were isolated at frequencies of 1%-47% in 4 separate trials, using Hollidays's method. Several factors may account for low recovery of mutant-like progeny. 1. Mycelial colonies grow at a much slower rate than yeast-like colonies. It is therefore possible that filamentous cells were overgrown by yeast-like cells and are under-represented on plates. This conclusion is supported by experiments in which progeny were transferred directly from germination plates to charcoal medium. Using this procedure, yeast-like and small mycelial colonies were observed at an approximate ratio of 1:1, at low colony density. At high colony density, yeast-like colonies predominated. 2. Auxotrophic markers such as *leu* 1-1 tend to disrupt meiosis and cause abberant segregation ratios for all markers in a cross (Holliday, 1974). 3. More than one gene may be involved in the *rem* mutation. However, the result of 47% mutant-like colonies in one trial suggests that a mutation in a single locus is responsible for the *rem* phenotype.

A total of 6 prototrophic mycelial progeny from a cross between 87-18 and 002 were inoculated into plants. Symptoms did not result from any of these infections. Possible reasons for this result have been suggested above, e.g., (1)

a physical barrier may exist between the hyphal cells of the mutant and the plant cells and (2) the *rem* mutation may not affect pathogenicity, but may cause mycelial growth via an alternative pathway.

Inoculation of seedlings with the diploid strain constructed by mating mutant 87-18 with strain 272 did not result in pathogenicity. This combination would not normally be pathogenic because the diploid strain is homozygous for *b;* the presence of the recessive mutation in one partner clearly did not alter pathogenicity.

5.2.2 Complementation of rem Mutation

To complement the mutation leading to the *rem* phenotype, a cosmid library was constructed using the cosmid vector pJW42 and *Sau*3A1 partial digests of genomic DNA from wild-type *U. maydis* strain 001. The library was transformed into mutant 87-18 and 1859 transformants were screened. Of these transformants, 203 were determined to be revertants. Reasons for high reversion frequencies are discussed above.

A total of 6 complemented transformants were isolated. Cosmid DNA has been rescued from 3/6 of these transformants and restriction digests indicate that insert DNA is highly similar. This high degree of similarity suggests that all or most of the insert sequence is required for complementation of the *rem* mutation. An alternative or additional explanation is that this particular cosmid was selectively amplified during the library amplification procedure.

To determine if the *rem* mutation resides at the *b* locus, mutant 87-18 was transformed with the wild-type *b*1 allele on the integrative cosmid vector, pCU3 (Kronstad and Leong, 1989). A total of 8 transformants were isolated; all were mycelial. Lack of complementation suggests that the *rem* locus is different from the *b* locus. This result has been further confirmed by Southern blot analysis: cosmid DNA containing the insert which complements the *rem* mutation was probed with DNA from the *U. maydis* *a*1,*a*2 *b*1 and *b*2 alleles. Lack of hybridization in all cases indicates that the *rem* locus in distinct from both the *a* and *b* loci (G. Bakkeren, unpublished results). Furthermore, the restriction digestion patterns of cosmid DNA carrying the *b* locus are very different from the patterns of the cosmids which complement the mutation (shown in Figure 9).

5.3 Models for *rem* Function

Based on our current knowledge of the *U. maydis* mating system and on our understanding of mating systems in other fungi, several roles for *rem* function may be hypothesized.

1. The *rem* gene may directly effect cell morphology by encoding structural proteins or enzymes involved in cell wall biosynthesis, e.g. chitin synthases, glucanases or chitinases. In *S. cerevisiae*, growth morphology is altered by mutations in a number of genes that control cell budding patterns (reviewed by Drubin, 1991). In *U. maydis*, the *rem* gene may determine cell morphology by a similar mechanism.

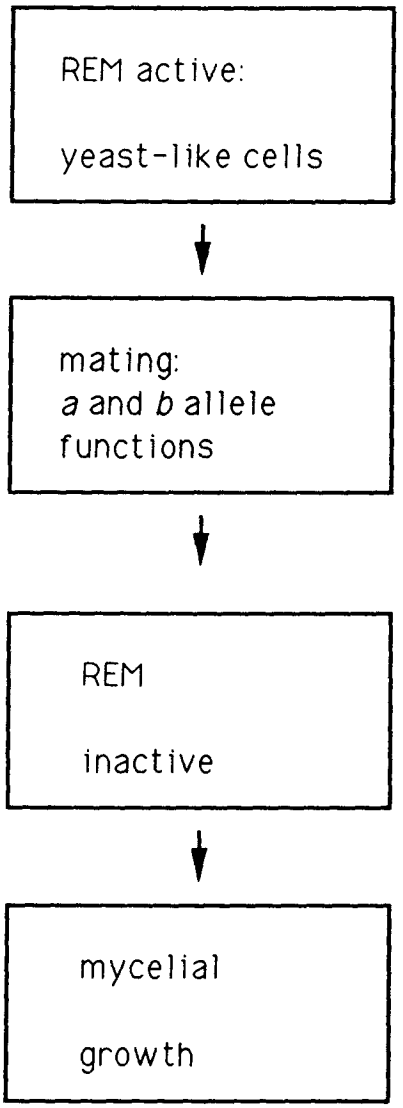
For example, the default growth type may be filamentous cells; the *rem* product may function to establish a specific bud site, thereby allowing yeast-like growth to occur.

2. Models for *b* allele regulation have been proposed in which *b* activity is a function of the intracellular location of the *b* gene products (Kronstad and Leong, 1990). This type of model suggests that the *rem* gene product may function as a cytoplasmic anchoring protein or as another accessory protein which regulates localization of a *b* protein dimer. A precedent for such a model is the nuclear localization of the glucocorticoid receptor; a candidate for a cytoplasmic anchoring protein, in this case, is the heat shock protein, HSP90 (Picard et al., 1988).

3. The *rem* gene product may function as a transcriptional regulator. This type of model is illustrated in Figure 11 and is predicated on the mating system of the budding yeast, *Saccharomyces cerevisiae* (reviewed by Herskowitz, 1989). In *S. cerevisiae*, meiosis and sporulation are inhibited by the *RME1* (repressor of meiosis) product. This repressor is expressed only in haploid cells. In diploids, *RME1* expression is inhibited by the products of the mating-type genes, the a1/α2 heterodimer. Inhibition of *RME1* leads to meiosis and sporulation (Mitchell and Herskowitz, 1986).

An analogous system can be envisioned in *U. maydis*. The *rem* gene product may be expressed only in haploid cells, where it functions to repress genes required for mycelial

Figure 11.
Model for *rem* Activity.



Model for *rem* gene activity: In yeast-like haploid cells, *rem* is active and genes for mycelial growth are repressed. Upon mating of compatible cells, products of *a* and *b* genes function to turn off *rem* activity and thereby allow expression of the mycelial phenotype.

growth and pathogenicity. In diploid cells, *rem* expression may be inhibited by a *b* protein heterodimer, thereby turning on the genes required for the switch from yeast-like to mycelial growth. The phenotype of the *rem* mutant, i.e. a recessive and gain-of-function phenotype, is consistent with the phenotype expected of a mutation in a repressor gene.

5.4 Conclusion and Further Research

Experiments in this thesis report the isolation, characterization and complementation of a *U. maydis* mutant with a novel phenotype. The *rem* mutation causes a dramatic change in the morphology of haploid cells: parental cells grow in a smooth yeast-like morphology; *rem* mutants are constitutively mycelial. The gene(s) responsible for the *rem* mutation are therefore key to understanding the regulation of dikaryon development in *U. maydis*.

Isolation of a cosmid which complements a *rem* mutant is an initial step in elucidating the role which *rem* plays in the formation of mycelium. Further characterization of the complementing DNA will require sub-cloning of the insert fragment and the sequencing of key regions. It is also important to confirm that the cloned gene is responsible for the *rem* mutation. This could be achieved by tagging the cloned *rem* gene with a selectable marker and integrating this sequence into the chromosome. If the integrant strain is

then taken through a cross, co-segregation should occur between the *rem* gene and the marker gene.

Classical genetic analyses (e.g. complementation tests) of the *rem* mutant are difficult due to its constitutive mycelial phenotype (mating tests on plates cannot be scored) and inefficient mating with compatible strains. However, the question of complementation groups may be addressed by transforming the cloned *rem* gene into a representative number of *rem* mutants to look for complementation. In this way, it may be possible to determine whether additional genes are involved in mycelial growth of *U. maydis*.

The aim of the work presented in this thesis was to identify genes required for mycelial growth of *U. maydis*. The isolation of constitutively mycelial *rem* mutants and the cloning of complementing DNA are initial steps towards this goal. Further investigation of the *rem* mutation will elucidate key steps in the regulation of mating, dimorphism and pathogenesis of *Ustilago maydis*.

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