Morphological, genetic and molecular analysis of the mating process in Ustilago hordei
by Alfredo Dick Martinez-Espinoza

A thesis submitted in partial fulfillment of the requirements for the degree Doctor of Philosophy in Plant Pathology
Montana State University

Abstract:
Ustilago hordei is a pathogenic basidiomycete that causes covered smut of barley. Mating in U. hordei, which is controlled by a single locus with two alleles, results in the conversion of haploid, non-pathogenic yeast-like sporidia to dikaryotic, pathogenic mycelium. Mating is one of the most important steps in the U. hordei life cycle, initiating the sexual phase of this fungus. An improved medium to test the mating reaction and genetic complementation was developed. The addition of activated charcoal to a variety of media enhanced the stability and intensity of the mating reaction. Complementation assays using minimal media containing activated charcoal demonstrated allelism of auxotrophic mutations. The ease and reliability of the mating test allowed for rapid identification of the mating type of unknown isolates and progeny of crosses, and also provided a dependable procedure for performing complementation assays. A comprehensive morphological analysis of the mating process was performed. Evidence of mating-type specific, diffusible, small molecular weight factors was found when sporidia separated by a dialysis membrane were capable of inducing conjugation tube formation by cells of the opposite mating-type. This indicates that these compounds were necessary for recognition of opposite mating-type cells. Genetic control of the mating process was analyzed by the use of mating defective mutants, which were isolated using charcoal-containing media. These mutants were phenotypically characterized and arranged into several classes. These classes indicate the complexity of the genetic control of mating in U. hordei. Molecular analysis of the mating process is also described. DNA fragments from U. maydis which encode pheromones (mfa1 and mfa2) and pheromone receptor (pra1 and pra2) hybridize with U. hordei DNA. Infa1, mfa2, and pra2-like fragments were cloned from a U. hordei A (MAT-1) mating-type strain. Mating-type of U. hordei is proposed to be determined by specific sequences that encode a pheromone and a pheromone receptor, which serve as mechanisms of cell-to-cell recognition in the initial stages of the mating process.

MORPHOLOGICAL, GENETIC AND MOLECULAR ANALYSIS OF THE MATING PROCESS IN *USTILAGO HORDEI*

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Alfredo Dick Martinez-Espinoza

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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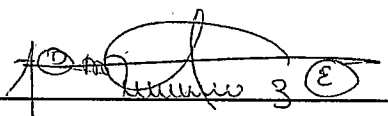
Signature 

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TO MY WIFE ELVIA
TO MY DAUGHTER ERIKA
TO MY PARENTS LUCITA AND NICANOR

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ABSTRACT

Ustilago hordei is a pathogenic basidiomycete that causes covered smut of barley. Mating in *U. hordei*, which is controlled by a single locus with two alleles, results in the conversion of haploid, non-pathogenic yeast-like sporidia to dikaryotic, pathogenic mycelium. Mating is one of the most important steps in the *U. hordei* life cycle, initiating the sexual phase of this fungus. An improved medium to test the mating reaction and genetic complementation was developed. The addition of activated charcoal to a variety of media enhanced the stability and intensity of the mating reaction. Complementation assays using minimal media containing activated charcoal demonstrated allelism of auxotrophic mutations. The ease and reliability of the mating test allowed for rapid identification of the mating type of unknown isolates and progeny of crosses, and also provided a dependable procedure for performing complementation assays. A comprehensive morphological analysis of the mating process was performed. Evidence of mating-type specific, diffusible, small molecular weight factors was found when sporidia separated by a dialysis membrane were capable of inducing conjugation tube formation by cells of the opposite mating-type. This indicates that these compounds were necessary for recognition of opposite mating-type cells. Genetic control of the mating process was analyzed by the use of mating defective mutants, which were isolated using charcoal-containing media. These mutants were phenotypically characterized and arranged into several classes. These classes indicate the complexity of the genetic control of mating in *U. hordei*. Molecular analysis of the mating process is also described. DNA fragments from *U. maydis* which encode pheromones (*mfa*$_1$ and *mfa*$_2$) and pheromone receptor (*pra*$_1$ and *pra*$_2$) hybridize with *U. hordei* DNA. *mfa*$_1$, *mfa*$_2$, and *pra*$_2$-like fragments were cloned from a *U. hordei A (MAT-1)* mating-type strain. Mating-type of *U. hordei* is proposed to be determined by specific sequences that encode a pheromone and a pheromone receptor, which serve as mechanisms of cell-to-cell recognition in the initial stages of the mating process.

CHAPTER 1

LITERATURE REVIEW

The Pathogen

Economic Importance of the Pathogen

Covered smut of barley, caused by *Ustilago hordei*, is distributed worldwide and seems to be more extensively dispersed than either true loose smut or semi-loose smut (Mathre, 1982). Losses from the disease in areas where seed treatment is not practiced are economically important (Mathre, 1982). Grain quality and yield reduction represent the main monetary losses. Barley with more than a small percentage of heads infected with covered smut is not acceptable according to federal grain standards (Mathre, 1982; Zillinsky, 1983).

In areas of the Middle East and Africa where growers sow untreated seed, covered smut continues to cause economic losses (Mathre, 1982). In the prairie provinces of Canada, including Manitoba, Saskatchewan and Alberta, overall yield losses from barley smuts were calculated to range from 0.7%-1.4% during the years 1978-1982, representing considerable monetary losses. The largest economical loss on the prairies of Canada was $17.6 million in 1981. Other major losses in those provinces include $1.1 million in 1980, $10.9 million in 1978 and $5.8 million in 1979. The maximum incidence of *U. hordei* was in 1980 at 0.9%. In addition, up to 25% of the plants were infected with

U. hordei in several fields in 1980 and 1982 (Thomas, 1984). In 1983-1988, again in the prairie provinces of Canada, annual losses were between 0.7% to 1.6% representing economic losses of $8.8 to $13.2 million. *U. hordei* was the most significant smut in Canada in 1987, having the highest level of infection in that year compared to other smuts. In addition, up to 40% covered smut infection was observed in a field in 1987 in Manitoba and 30% infection in a field in Alberta in 1988 (Thomas, 1989a) being the most important smut in that year in Alberta (Krehm and Beauchamp, 1989). *U. hordei* was present in the provinces of Saskatchewan and Manitoba in 1989 (Weller and Rossnell, 1990) with an average of 70% of the fields infected and an average incidence of infection of 0.8% (Thomas, 1990). It is thought that economic losses occur throughout the world due to smuts, and particularly to covered smut. However, strict surveys like those carried out in Canada have not been widely implemented.

Taxonomy and Description of the Pathogen

The causal organism of covered smut is *Ustilago hordei*, a fungus classified in the division *Eumycota*, and the subdivision *Basidiomycotina*. It belongs in the class *Hemibasidiomycetes* in which the promycelium usually has crosswalls, the order *Ustilaginales* where the promycelium has lateral sporidia at septa, and the genus *Ustilago* (Webster, 1989).

The teliospores or resting structures of the fungus are globose, subspherical to spherical, 5-8 μm in diameter, brown to dark brown, with a smooth surface and a lighter colored side which is presumably

where germination takes place (Tapke, 1937). The teliospores germinate forming a four-celled basidium called the promycelium from which four oblong to ovate hyaline basidiospores termed sporidia grow. Sporidia are 9-11 μm long and 4-6 μm wide, and represent the haploid phase of the disease (Dinoor and Person, 1969). They bud in a yeast-like fashion to produce numerous secondary sporidia (Fisher and Holton, 1957); fusion of sporidia of opposite mating-type results in the infectious dikaryotic mycelium (Dinoor and Person, 1969; Mathre, 1982).

Teliospores germinate on standard mycological media (Vogel, 1956; Lade, 1967; Holliday, 1974; Hellmann and Christ, 1991), including water agar (Henry et al., 1988, personal observation). Some reports state that peptone plays a role in growth of the fungus (Fisher and Holton, 1957). Maltose, sucrose and glucose seem to be the best carbon sources (Fisher and Holton, 1957), and nitrate is the best nitrogen source. The pH of the medium does not appear to have a strong influence on teliospore germination or growth of the fungus (Fisher and Holton, 1957). Teliospores germinate to produce the promycelium in which meiosis occurs and results in four haploid sporidia which continuously produce haploid, uninucleate sporidia. Although early studies reported teliospore formation on artificial media (Fisher and Holton, 1957), formation of diploid teliospores occurs exclusively in the plant (Thomas, 1988).

Host Range

U. hordei has been reported to occur naturally on rye in Siberia, and on several grasses including *Agropyron cristatum* and *Elymus glaucus*

in the USA (Aisworth and Sampson, 1950). Artificial inoculations using paired sporidial cultures of covered smut from these hosts produced infection on the following grasses: *Agropyron caninum*, *Elymus canadiensis*, *Elymus glaucus*, *Elymus sibirieus*, *Hordeum nodosum* and *Sitanion jabatum* (Fisher, 1939). Other hosts reported include *Agropyron semiconstatum*, *Avena byzantina*, *Avena fatua*, *Avena orientalis*, *Avena sativa*, *Elymus canadiensis*, *Elymus excelsus*, *Elymus glaucus*, *Hordeum bulbosum*, *Hordeum vulgare*, *Hordeum vulgare* var. trifurcatum, several *Hordeum* spp. and *Secale cereale* (Zundel, 1953).

Symptoms of Covered Smut

The characteristic symptom of the disease is a purple- black fungal mass of teliospores (sorus) of about 6-10 mm in length. The teliospores are contained within a rather persistent membrane that surrounds the seeds on the entire head of the host (Dickson, 1956). The sorus is enclosed in the floral bracts of the spikelet which frequently appears at maturation of the host (Fisher and Holton, 1957). Smutted heads tend to emerge later than healthy heads or in many cases are trapped in the sheath of the flag leaf. Occasionally, depending on environmental conditions and physiological races, smut teliospores also develop in leaf blades as long streaks or in nodal tissue (Mathre, 1982; Gaudet and Kiesling, 1991).

Disease Cycle

The smutted heads are broken and crushed during harvest, depositing inoculum in the soil and on the surface of healthy seeds. Teliospores are the resting stage and survive in this form between

growing seasons. Teliospores germinate at the same time that seeds germinate (Mathre, 1982). Germination of teliospores in the soil is enhanced by moderate moisture (50%) and cool soil temperatures (10-15°C) (Tapke, 1948). *U. hordei* sporidia were found on hulls of germinating seeds and the infective dikaryotic mycelium colonized the seeds and germinating tissue (Kozar, 1969b). Infection occurred through the coleoptile, and the mycelium advanced through the host tissue and became established behind the growing point where a profuse, thick, branched mycelium was observed. Plants developed more infected tillers when the pathogen penetrated the entire length of the coleoptile (Thomas, 1976; Kozar, 1979b). As the plant grows, the mycelium maintains its position within the growing point until flowering time. Then the fungus permeates the tissue of the ovary and teliospore formation begins by segmentation and rounding of intercellular mycelium that forms the mass of teliospores that replace the seed. The teliospores are contained within a persistent membrane that surrounds the entire head and are released at harvest (Mathre, 1982).

Physiological Specialization

Physiological specialization describes the traits of morphologically similar species that differ from each other in one or more physiologic characters. Specialized races of *U. hordei*, based on pathogenicity, were first indicated in 1924 when 5 different "biological" forms were identified on 4 barley cultivars, Nepal, Hannchen, Texas winter, and Summit (Faris, 1924). Two distinct physiological races were subsequently found in Canada which were

readily distinguished by their reaction on Eureka and Hannchen (Aamodt and Johnston, 1935). From 200 collections of *Ustilago hordei* from 26 states, 8 races were determined based on the differential reaction to 5 barley cultivars. Race 6 was found in 21 of 26 states and in 114 of the 200 collections. Later, in Canada, evidence of 4 new races was reported (Fisher and Holton, 1957). However, three of them proved to be the same when tested on more cultivars.

Thirteen physiological races were identified in 1945 (Tapke, 1945), 5 from a collection of 244 isolates and 8 from previous studies from a total of 444 collections from 33 states. Race 6 was widespread and comprised 61.3% of total collections. The most prevalent races were 1, 5 and 6. In California and Washington, race 6 was the most prevalent. Not infrequently, minor differences in size, teliospore color, spore wall smoothness, compactness of the smutted heads and spore mass, degree of awn destruction and degree of smutted head emergence from the boot have been associated with the different physiological races of *U. hordei*.

Fourteen races are now recognized for *U. hordei* after a new race with a unique pathogenicity pattern was obtained in an inbreeding study of race 8 (Pedersen and Kiesling, 1979).

Control

U. hordei has been adequately controlled by chemical seed treatments. In the early 1900's, formaldehyde was used as a seed disinfectant which gave complete control. However, formaldehyde, besides being a health hazard, causes injury to the seed if not

properly applied (Fisher and Holton, 1957). Later, organic mercuric compounds such as Ceresan (ethyl mercury chloride) were used effectively to control covered smut. However, these compounds were not widely accepted due to health hazards and high cost (Fisher and Holton, 1957). To date, effective control of covered smut can be achieved with several fungicides, including benomyl (Alcock, 1980) and carboxin (Ballinger, 1980). Formulations containing Carboxin and triadimenol also gave complete control (Johnston and Mathre, 1980; Sheridan, 1980).

Because of the success of chemical seed treatment, little effort has been placed on the development of resistant cultivars. However, there are many cultivars that have resistance to some of the 14 physiological races including Excelsior, Hannchen, Himalaya, Lion, Nepal, Pannier, and Trebi (Tapke, 1945). Other resistant cultivars include the six-rowed hulled types Atlas, Sacramento, Velvet, Shaw, Sol and Success, the two-rowed hulled types Spartan, Golden, Pheasant and Horn and the hulless types Himalaya, New Era, Russian and Burbank (Aamodt and Johnston, 1935). Other cultivars resistant to several races of the fungus include BJ28, and C163 (Bedi and Singh, 1972), BHD22, EB582, EB570, EB905, EB 2100, EB 2505, EB 2507, EC24882 and K572/11 all of which seem to be immune to 5 races of *U. hordei* (Shrivastava and Shrivastava, 1976).

Major gene resistance is conditioned by a single dominant gene in some lines, while in others two or three independent factors are known (Fullerton and Nielsen, 1974; Thomas, 1988). Minor genes influencing partial resistance are also known (Emara and Sidhu, 1974). Partial resistance is expressed as partial infection in which not all heads of

the plants become infected (Groth et al., 1976; Mathre, 1982). However, depth of sowing, soil type and temperature at which inoculated plants are grown can also influence the occurrence of partial smutting (Groth et al., 1976; Mathre, 1982).

Genetic mechanisms of host resistance will be described in detail in the following sections.

Genetics of *Ustilago hordei*

Mutation and Genetic Complementation

The *Ustilago hordei* life cycle is particularly well suited for genetic studies. In the asexual cycle of *U. hordei*, haploid, yeast-like, non-pathogenic cells, which are the result of meiosis from the resting sexual spores (teliospores), are produced. Daughter sporidia are produced by budding. Mutation of *U. hordei* has proven to be performed with relative ease. Mutation rate, lethality and interactive influences affecting mutation were determined by Hood (1968), including the effect of the stage of the cell cycle and repair processes.

Hood (1968) isolated three hundred and twenty three auxotrophic mutants using UV irradiation and selection on minimal media. Three hundred were identified with respect to their special nutritional requirements. Genetic analyses of ninety of these mutants were made using crosses between mutants and wild-type strains. This analysis showed that haploid segregants had the same nutritional requirement as the parents, indicating stability of the mutation (Hood, 1968).

Mutation using UV irradiation combined with inositol starvation seemed to increase the mutation frequency, compared with irradiation

alone (Thomas, 1972). 1.9% of the survivors using UV irradiation followed by inositol starvation were found to carry a nutritional requirement in addition to inositol, whereas 0.11% and 0.17% were recovered using UV irradiation by itself and 0.27% by inositol starvation alone. From these mutants, 118 required an exogenous source of amino acid including serine, leucine, isoleucine and valine, methionine, arginine, lysine, phenylalanine, histidine, and tryptophan. Thirty-six mutants required adenine and 58 required vitamins including niacin, pyridoxin, pantothenic acid and biotin. Interestingly, 1140 mutants required proline in addition to inositol (Thomas, 1972).

Fifty-two auxotrophic, morphological and temperature sensitive-mutants were isolated following UV irradiation, N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and ethyl methane sulfonate (EMS) (Henry et al., 1988). Auxotrophic strains included 3 adenine (*ade*) mutants and 9 vitamin-requiring strains including nicotinic acid, pantothenic acid, inositol and pyridoxine. Twenty-three amino acid-requiring strains were auxotrophic for arginine, phenylalanine, proline, tryptophane, lysine, histidine, valine, arginine/valine, methionine/lysine, lysine/nicotinic acid, and valine/inositol. In this study 10 morphological (*morph*) mutants were identified by their cauliflower-like appearance rather than the wet yeast-like colony typical of the wild type. These mutants had greatly reduced numbers of sporidia, which were distributed in chains rather than solitary sporidia. Four temperature-sensitive mutants were also obtained. Two mutants were shown to be heat sensitive and two were cold sensitive (Henry et al., 1988).

Fungicide resistant strains have also been obtained. Benomyl, chloroneb, thiabendazole and triadimenol resistant strains were isolated using UV irradiation and MNNG treatments. The inheritance of fungicide resistance was determined (Henry et al., 1985a; Henry et al., 1985b; Henry et al., 1987).

Complementation studies in *U. hordei* have been used to determine allelism of auxotrophic mutations. Genetic complementation tests with 58 arginine requiring mutants were performed (Dinoor and Person, 1969). Dikaryotic hyphae formed when haploid lines of the opposite mating-type containing complementary mutations were mixed on minimal media. Ten different complementation groups were identified, indicating that mutations in 10 different loci which control arginine biosynthesis were obtained (Dinoor and Person, 1969). Henry et al. (1988) performed genetic complementation tests on *U. hordei* auxotrophic mutants, and of twenty one compatible pairs with different biochemical requirements, seven did not complement (i.e., form dikaryotic mycelium). However, some did form mycelium when tested with the standard mating types, indicating some interference with heterokaryon formation between those mutants. However, of the 13 compatible pairs with similar biochemical requirements, four did not complement, indicating allelism (Henry et al, 1988).

Interspecific Hybridization and Echinulation

The fact that several smuts parasitize the same host, e.g. *U. nigra*, *U. hordei* and *U. nuda* on barley and *U. kolleri* and *U. avenea* on oats, and the probability that these fungal species coexist in

nature leads to ample opportunity for cross-hybridization in nature. Interspecific hybridization can result in the development of new physiological (pathogenic) races or species. Intra- and inter-specific hybridization have been repeatedly demonstrated (Holton, 1931; Fisher, 1951).

U. hordei seems to hybridize readily with other species including *U. nigra* (Fisher, 1951). Spore cell wall morphology or ornamentation, i.e., smooth, echinulated or wart-like characteristics, has been used as a genetic marker to measure interspecific hybridization. The first hybrids reported were between *U. hordei* and *U. medians* (*U. nigra*) using monosporidial lines of each fungus (Allison, 1935). Head type was intermediate with sori enclosed in a durable but rather loose membrane, and all teliospores were echinulated. Factors for mating-type, pathogenicity, head type and spore cell morphology segregated independently (Allison, 1935).

U. hordei and *U. bullata* which infect both *Agropyron* and *Elymus spp.* also hybridized under experimental conditions (Fisher, 1951). Nine hybrids were recovered; the F_1 spores were echinulated and lighter colored on two sides (*U. hordei* has small, 5-8 μm long smooth teliospores which are lighter colored on one side while *U. bullata* has longer, 8-12 μm, echinulated, dark spores). Teliospore progeny were characterized, and one F_2 hybrid yielded the same spore wall morphology as the F_1, but several F_2 hybrids yielded diverse spore wall morphology including (1) small echinulated spores which were lightly colored on one side, (2) small smooth spores which were lighter on two sides, (3) small smooth spores which were lightly colored on one side,

(4) large echinulated spores with one side lightly colored, (5) large spores with uniformly colored walls, and (6) large smooth spores with uniformly colored walls. Pathogenicity was the same as in the parents (Fisher, 1951).

Hybrids between *U. hordei*, *U. nigra*, *U. avenea* and *U. kolleri* have been obtained using a common host *Agropyron tsukushiense* var. *transciens* (Huang and Nielsen, 1984). Genetic relationships of these species were based on their ability to cross and the distribution of genes for spore morphology. *U. hordei* strains from 10 collections from 10 countries on 5 continents had an echinulation genotype E_1e_2 resulting in smooth spores. All hybrids between *U. hordei* (smooth spores, E_1e_2) and *U. kolleri* genotype e_1E_2 (smooth spores) had echinulated teliospores. E_1 and E_2 acted as dominant and complementary genes. Teliospore hybrids between *U. nigra* (in which echinulation is conditioned by two dominant, complementary genes E_1 and E_2) and *U. hordei* (E_1e_2), segregated as if echinulation was controlled by a single dominant gene, where E_2 from *U. nigra* was complementing e_2. Huang and Nielsen, (1984) concluded that a diploid teliospore is echinulated only if it carries both dominant E_1 and E_2 genes. Size of echinulations among hybrids between *U. hordei* and *U. nigra* seems to be affected by a recessive gene(s) found in *U. hordei* that modifies the expression of the major genes (E_1E_2) required to produce echinulation when backcrossed to the smooth-spored parent (*U. hordei*). Echinulation is not affected by the microenvironment. Sorus type seems to be associated with teliospore ornamentation. The least prominent echinulation had a sorus type like that of *U. hordei* while more

prominent echinulation had a sorus type like *U. nigra* (Thomas, 1989b). Recently, hybrids between *U. kolleri* (echinulated spores) and *U. hordei* (smooth spores) were found to possess surface ornamentation which was only 30% of the diameter of normal echinulations and only visible using scanning electron microscopy (Thomas, 1991).

Somatic Recombination

Evidence for parasexual recombination in *U. hordei* has been described (Kozar, 1969a; Megginson and Person, 1974). Compatible combinations of mutant sporidia requiring isoleucine and valine were used in one experiment. Isolation of mononucleated sporidia from excised infected plant tissue grew in minimal media, suggesting that the novel genotypes recovered arose via somatic recombination. Evidence of diploidization was found as tested by DNA content, ability to grow in minimal media and results from the Bauch test (Kozar, 1969a).

Somatic recombination was probably responsible for the genotypes of teliospores after inoculation with a mixture of three different haploid sporidia (Megginson and Person, 1974). The teliospores contained the genetic markers (arg-, leu- and mating type) from all three parents. Somatic recombination was assumed, since inoculations were arranged in a way that the parasitic phase would be completed only if an unusual event had taken place. In addition, parasexual recombination was assumed responsible for the spontaneous appearance of niacin deficiency in sporidial progeny (Megginson and Person, 1974).

Virulence

Genetic analyses of virulence of *U. hordei* on barley supported a gene-for-gene relationship in the *Ustilago-Hordeum* system (Person et al., 1986). Early experiments using tetrad analysis to follow segregation of virulence in *U. hordei* suggested that a single recessive allele for virulence was responsible for a level of infection of 5% in two cultivars of barley. Later it was demonstrated that virulence was due to recessive alleles at different loci for each of the cultivars Excelsior, Lion, Pannier, Trebi, while the gene for virulence on Hannchen was dominant (Thomas, 1988).

Specific genes for virulence of *U. hordei*, *Uhv1* through *Uhv6*, have been identified. *Uhv1* and *Uhv2* are effective against resistance genes in barley on a gene-for-gene basis, *Uhv1* against *UhR1* in Hannchen and Vantage and *Uhv2* against *UhR2* in Excelsior. *Uhv3* is a recessive gene, apparently linked to *Uhv2* for virulence on Nepal and Pannier. *Uhv4* and *Uhv5* are duplicate recessive genes at two different loci that control virulence on Lion and Plush (Thomas, 1976).

Despite the polygenic effects that modify avirulence genes (Emara and Sidhu, 1976), environmental influences (Ebba and Person, 1969; Thomas, 1988), and presence of genes influencing virulence (Christ and Person, 1986), the gene-for-gene hypothesis is still the most viable explanation for the basic compatibility or incompatibility of the barley-*U. hordei* interaction (Christ and Person, 1986).

Aggressiveness

The term aggressiveness has been used to describe the variation in degree of infection of susceptible cultivars by the pathogen. The genetic determinants of aggressiveness in *U. hordei* have been and still are the subject of intense study (Thomas, 1988).

Several compatible sporidial lines of *U. hordei* have been found to infect barley cultivars at rates ranging from 1.7% to 41.9% infection (Christ and Person, 1986). This variability was due primarily to additive genetic effects and, to a lesser degree, to genetic interactions such as dominance and epistasis (Christ and Person, 1986). Genetic modification of the expression of *Uhv1* on the cultivar Vantage was studied using two teliospore types. Teliospores homozygous for *Uhv1* were selfed in all possible combinations. Forty-three percent of the infection variability was caused by additive genes and 22% by non-additive genes. Alleles for greater aggressiveness were dominant and heterosis was evident (Emara and Sidhu, 1974). Infection ranging from 15% to 90% on the variety Hannchen due to the effect of *Uhv1* was reported (Thomas, 1988). Twenty-eight percent of this variation was found to be due to *Uhv1*. The rest of the variation was due to the environment (Thomas, 1988). Aggressiveness was also examined on the cultivar Odessa (Thomas, 1988). Race 10 appeared to be homozygous for genes affecting aggressiveness, another dikaryon from unrelated sporidia was heterozygous for polygenic factors affecting aggressiveness. Sixty percent of the genetic effect was additive and 40% was non-additive. One factor affecting aggressiveness appeared to be linked to the mating type locus (Thomas, 1988).

All the studies mentioned above used percentage of infection as the only criterion for aggressiveness. However, variation in aggressiveness among and within races of *U. hordei* was recently reported (Gaudet and Kiesling, 1991) using other components than percent of infection. Aggressiveness was measured according to several components including peduncle compactation, extent of sorus formation in heads, leaves and nodes and plant dwarfing. Aggressiveness should be determined using several criteria besides percentage of infection (Gaudet and Kiesling, 1991).

Molecular Genetics

Two dimensional, isoelectric-focusing/SDS polyacrylamide gel electrophoresis was used to compare polypeptide extracts from teliospores of several *Ustilago spp*, including *U. hordei* (Kim et al., 1984). The same technique was applied to *U. hordei* isolates with known genes for virulence. An initial correlation of a dominant allele for avirulence with a polypeptide was later modified when tetrads from a heterozygote were backcrossed to the virulent parent. The polypeptide segregated independently from avirulence (Thomas, 1991).

Isozyme variation has also been used to try to define races using starch gel electrophoresis (Hellman and Christ, 1991). Isozymes from different races were obtained and single bands (alleles) were detected for aconitase, adenylate kinase, glucose-6-phosphate dehydrogenase, phosphoglucose isomerase, 6-phosphogluconate dehydrogenase and peptidase. Two alleles were found for isocitrate dehydrogenase and malate dehydrogenase and 3 alleles for phosphoglucomutase. However,

races could not be separated solely by means of this isozyme analysis (Hellmann and Christ, 1991).

One approach toward molecular isolation of fungal genes is to develop a transformation system that allows cloned DNA sequences to be introduced and expressed in fungal cells. A genetic transformation system for *Ustilago hordei* has been reported (Holden et al., 1988). This method consists of the preparation of spheroplasts of *U. hordei* by the use of Novozyme 234 utilizing early log phase cultures, transforming with the plasmid pHL1 linearized at the *Xho*I site. The transformation was confirmed by Southern blots of DNA extracted from the transformants. Transformation frequencies of 10 to 50 resistant colonies per microgram of DNA per 2 x 10^7 spheroplasts were obtained. The majority of the transformed cells retained resistance to hygromycin B through many mitotic divisions (Holden et al., 1988). An improved transformation procedure has also been described (Duncan and Pope, 1990). Protoplasts were recovered after treatment with KCl buffer solution and Novozyme 234. Stabilized protoplasts were treated with the plasmid pCM54 which contains the hygromycyn resistant gene and an autonomously replicating sequence (ARS) from *U. maydis*. The reported transformation rate was 3 to 14 times higher than that previously reported. However, no Southern blot hybridization was done to conclusively demonstrate transformation.

Electrophoretic karyotypes have been determined for monosporidial strains of the 14 races of *Ustilago hordei*. Chromosomal DNA of sporidia was separated by contour-clamped homogeneous electric-field gel electrophoresis (CHEF) (McCluskey and Mills, 1990). A unique

karyotype was observed for each strain. The number of bands for each race varied from a minimum of 15 to a maximum of 19 with approximate length distributions from 170 to 3150 kb. Karyotypes were conserved among members of individual tetrads and between tetrads representing the same race. Southern hybridization was used to map genomic fragments and heterologous conserved genes to chromosomes (McCluskey and Mills, 1990). Electrophoretic karyotyping has also been reported without the need of making protoplasts (McCluskey et al., 1990). Genome plasticity using probes from chromosome-specific DNA libraries was also determined. The demonstration that most single copy probes hybridized with chromosomes of similar size suggests that the variability in karyotype is not generated by translocation (McCluskey and Mills, 1991). Karyotype analysis has led to new approaches to study fungi whose cytogenetics can not be studied by conventional means. In *U. hordei* karyotyping has helped to improve the knowledge of the cytology of this fungus, i.e., number and sizes of chromosomes (Thomas, 1991).

Mating-Type Genetics

Sexuality or mating in fungi has been recognized as one of the most interesting aspects of the biology of this group of organisms. Mating has been found to be very diverse in a wide variety of fungi (Raper, 1966). Different mechanisms have evolved in various groups of fungi to favor cross-mating which enhances outbreeding or promotes inbreeding to preserve the most fit genotypes (Fincham et al., 1979).

Sexual dimorphism is one of these mechanisms of sexuality and it occurs in a number of forms. Most of these cases occur in the *Phycomycetes*. However, this feature is relatively uncommon in fungi as a group (Raper, 1966).

Incompatibility systems are far more prevalent as a means of determining mycelial interactions. There are two basic types of incompatibility among fungi, *heterothallism* (*homogenic incompatibility*) and *homothallism* (*heterogenic incompatibility*). *Heterothallism* prevents self-fertilization, thus restricting inbreeding and promoting outbreeding, while *homothallism* favors inbreeding and self-fertility (Raper, 1966; Fincham et al., 1979).

There are a number of important differences between incompatibility systems. These systems can be distinguished by two major criteria; number of loci and number of alleles at each locus. *Bipolar* and *tetrapolar* are terms that refer to systems with either one or two loci and describe the number of mating types possible among the progeny of a single cross. A bipolar cross yields 2 mating types, while a tetrapolar cross yields 4 mating types. *Biallelic* and *multiallelic* are terms used to distinguish systems with two alleles per locus or those with an large number of alleles.

The types of incompatibility systems that occur in various groups of fungi may be generalized. *Phycomycetes*, especially those belonging to the order *Mucorales*, have a bipolar, biallelic mating system. The *Ascomycete* yeasts have a bipolar biallelic system while the *Euascomycetes* have a similar bipolar, biallelic system but each cell has the capability of acting as either male or female. *Basidiomycetes*

in the *Uredinales* also have a bipolar biallelic system with each cell having the capability of acting as either male or female. In the *Ustilaginales*, some species (i.e., *U. hordei*) have a bipolar, biallelic system while other species (i.e., *U. maydis*) have a tetrapolar system which is biallelic for one locus and multiallelic for the other locus. In either case, mating types are morphologically identical. *Basidiomycetes* in the *Hymenomycetes* and *Gasteromycetes* have either a bipolar or tetrapolar multiallelic mating-type system (Raper, 1966).

There are extensive literature reviews on the control of heterothallism or sexuality in fungi (Whitehouse, 1949; Burnett, 1956; Olive, 1958; Raper, 1959; Raper, 1960; Raper, 1966; Nasmyth, 1982) which cover mechanisms of regulation, characteristics of the systems, hypotheses, terminology and biological significance of the mating process. In addition, there are several mating systems that have been well characterized and the molecular mechanisms have been described. A brief review of these systems will be discussed here to give a better understanding of mating. The mating process for several smuts will be also described.

Saccharomyces cerevisiae, an ascogenous yeast, has a bipolar mating system. Mating is determined by the *MAT* locus. Cells with *MATa* are referred to as *a* cells while α cells carry the *MAT*α locus. Cells of the opposite mating type can mate while cells of the same mating type cannot. Recognition of cells of the opposite mating type is accomplished by secretion of *a* and α pheromones which bind to receptors on the surface of opposite mating-type cells. Cells fuse to produce an a/α diploid which carries the *MATa* and the *MAT*α alleles (Herskowitz,

1988). Switching from one allele to other at the *MAT* locus can occur in haploid cells; thus a colony which begins from a single cell of one mating type can quickly become a mixture of both mating types. This is due to two silent loci designated *HMLα* and *HMRa* in which copies of α and *a* reside regardless of the information contained at the *MAT* locus (Hicks et al., 1979; Herskowitz, 1988). Switching is induced by a dominant allele *HO* which encodes an endonuclease that acts on these loci. In the switching process, a copy of *HML* or *HMR* replaces the *MAT* allele which changes the mating-type. The "cassette" model has been suggested for this process in which *MAT* is the active "cassette" or locus carrying the information which identifies the mating-type of the cell. *HML* and *HMR* are silent "cassettes". Switching occurs when the active "cassette" is replaced by information from the opposite mating-type silent "cassette" (Nasmyth, 1982; Herskowitz, 1988).

The structure of the mating-type locus has been elucidated for *S. cerevisiae* (Nasmyth et al., 1981; Draginis, 1990). *MATa* and *MATα* contain unique DNA sequences *Ya* and *Yα*, but also contain similar regions common to both mating-types called *W* and *X*. The *Y* region is flanked by a *Z1* and a *Z2* region which are common to both mating types. Unique transcripts from *Ya* and *Yα*, called *a1* and *a2* and *α1* and *α2*; code for DNA binding proteins that control several target genes (Nasmyth et al, 1981). *a*-specific genes are expressed in *a* cells but are repressed in α cells. *a*-specific genes also include structural genes which are induced by exposure to α factor. α-specific genes, which include the *a*-factor receptor gene, are induced by *α1* and expressed on α cells (Draginis, 1990). Other molecular mechanisms of regulation of the

"active" and "silent" cassettes have been described, including the *SIR* (Silent Information Regulator) locus, which represses the silent cassette (Draginis, 1990), and regulation of the *HO* gene which initiates the switching. Similarities to the "cassette" model of *S. cereviceae* have been found in the fission yeast *Schizosaccharomyces pombe* (Kelly et al., 1988). Molecular techniques of restriction analysis, Southern blot hybridization and heteroduplex analysis revealed that mating switching occurs by transposition (Beach, 1983). Nucleotide sequences and the mechanism of regulation have been described (Kelly et al., 1988).

The Ascomycete heterothallic fungus, *Neurospora crassa*, has two mating types designated *A* and *a*. The mating type alleles *A* and *a* control mating in the sexual cycle and also function in heterokaryon incompatibility in the vegetative cycle. The molecular mechanisms of mating type control have been elucidated and the mating-type locus has been cloned. The mating-type clones contain unique DNA segments that are flanked by common DNA sequences (Glass et al., 1988).

In the *Hymenomycetes* and *Gasteromycetes*, which belong in the *Basidiomycetes*, two loci systems are prevalent. Compatibility is controlled by the action of two independent loci *A* and *B* (Raper et al., 1958; Day, 1960). In the *Hymenomycetes* each mating-type locus has a large number of alleles. In *Schizophyllum commune* 96 *A* alleles and 56 *B* alleles were found in a worldwide survey (Raper et al., 1958). These loci actually consist of four multiallelic genes, $A\alpha$, $A\beta$, $B\alpha$, and $B\beta$ with 9, 32, 9 and 9 alleles respectively estimated to exist worldwide (Raper, 1959; Raper, 1966, Ullrich, 1978). The four mating type genes

are linked in two pairs, *AαBβ* and *AβBα*, on two separate chromosomes. Complete sexual development occurs only when two strains with allelic differences at *Aα* and *Aß* and *Bα* and *Bß* mate. A series of distinct developmental events is initiated to convert the two haploid strains into a fertile heterokaryon, with *A* and *B* alleles controlling part of the development (Giasson et al, 1989). *A* alleles regulate nuclear pairing, nuclear conjugation, cellular division, and hook cell formation. *B* alleles regulate septal dissolution and nuclear migration (Raper, 1966). Two *Aα* mating-type alleles have been cloned (*Aα1* and *Aα4*) from *Schizophyllum* using chromosome walking with a cosmid library. Transforming DNA is active in the trans configuration, suggesting that *Aα* encodes a diffusible product. Restriction mapping analysis has shown that *Aα1* and *Aα4* are located in the same physical region of this chromosome but within a subregion that contains extensive sequence divergence (Giasson et al., 1989).

In the *Ustilaginales* incompatibility systems are defined as being either bipolar, one locus with two alleles, or tetrapolar, biallelic for one locus and multiallelic for the other locus.

Ustilago violacea, the anther smut fungus which systemically infects many species of the *Caryophyllaceae* family, has a bipolar mating-type locus termed a_1 and a_2 , which act as a "developmental master switch" controlling the entry into several pathways including vegetative budding, conjugation, dikaryotic hyphae and sporulation (Day and Garber, 1988). Mating-type alleles a_1 and a_2 remain inactive at high temperatures (20-30°C) or in the presence of cations, such that cells continue to bud vegetatively under these conditions (Day, 1979).

When cells containing the two mating-type alleles are brought together, developmental changes occur, going from a saprophytic, yeast-like form to a mycelial form which is characteristic of the parasitic phase (Castle and Day, 1989). Host products can activate or induce mating and mycelium formation (Castle and Day, 1984). One of these products has been isolated and characterized using HLPC, UV absorption spectroscopy and mass spectrometry. The compound has been identified as α-tocopherol (vitamin E). Natural α-tocopherol, synthetic α-tocopherol and other tocopheroles (β, gamma, σ) are very active at low concentrations. It appears that α-tocopherol acts on the mating-type locus affecting gene regulation (Castle and Day, 1984), inducing mycelial formation only in compatible mixtures of haploid sporidia. During conjugation of *U. violacea,* cells of the opposite mating type first pair tightly and then develop a conjugation tube or bridge between them. Recognition of opposite mating-type cells seems to be established by long fine hairs or fimbriae. Fimbriae are not essential for cell pairing but may be involved in later stages of conjugation including development and direction of conjugation tube growth toward opposite mating-type cells. This suggests that fimbriae serve as communication structures between cells or in transporting inducer molecules for the initiation of conjugation tube development (Day and Poon, 1975; Day, 1976; Day and Garber, 1988).

Ustilago maydis or corn smut is a heterothallic fungus with tetrapolar mating-type loci and is another example where the mating-type genetics have been well characterized. Two distinct forms characterize the *U. maydis* life cycle, a unicellular haploid form which

is non-pathogenic and divides as yeast-like cells (sporidia) and the dikaryotic filamentous form which is pathogenic and whose growth is dependent on the living corn plant where it causes tumors on the leaves, tassels and ears (Christensen, 1963). The transitions between forms are governed by two separated loci, the *a* locus and the *b* locus. Formation of the filamentous dikaryon is only possible when haploid cells heterozygous for both the *a* and *b* loci are present. If either identical *a* or identical *b* alleles are present, the filamentous dikaryon form is not developed. The *a* locus has two alleles, a_1 and a_2, and has been proposed to control the fusion of haploid cells (Rowell and DeVay, 1954; Rowell, 1955; Holliday, 1961). Recently, the two alleles of the *a* locus have been cloned (Froelinger and Leong, 1991; Bolker et al., 1992). a_1 and a_2 mating-type clones contain non-homologous DNA segments which are flanked by similar nucleotide sequences. There is a single copy of either the a_1 or a_2 mating-type sequence within each haploid genome. Each allele is composed of a set of two genes, one encoding the precursor of a polypeptide which appears to be a mating factor (pheromone) and the other which is thought to code for the receptor of the pheromone secreted by cells of the opposite mating-type. The *a* mating-type alleles are proposed to provide the components involved in cell-to-cell signalling (Bolker et al., 1992).

The *b* locus is more complicated, with at least 25 naturally occurring alleles having been described (Rowell and DeVay, 1954; Puhalla, 1968). The *b* locus regulates hyphal growth and pathogenicity of the dikaryon formed by the fusion of two haploid sporidia. The *b*

alleles have to be heterozygous to form hyphae and to induce symptoms in the host.

A variety of molecular mechanisms have been proposed to explain recognition of identical versus non-identical alleles of *Basidiomycetes* incompatibility loci (Kuhn and Parag, 1972; Ullrich, 1978). One hypothesis is that the functional products of the *b* alleles are nucleic acids, either DNA or RNA, and recognition involves information found in the heteroduplex structures (Ullrich, 1978). According to another scheme the *b* locus codes for multimeric proteins (Parag, 1972). Recently four alleles from the *b* locus of *U. maydis* have been cloned (b_1, b_2, b_3 and b_4) (Kronstad and Leong, 1990; Schulz et al., 1990). They were found to contain a single open reading frame (ORF) which would encode a protein of 410 amino acids. The protein would have a variable N-terminal region and a highly conserved C-terminal region (60% and 93% identity respectively). The polypeptide appears to be a DNA binding protein containing a motif related to a homeodomain in their constant region (Schulz et al., 1990). It is suggested that *U. maydis b* alleles code for polypeptides whose association yields a regulatory protein that governs the developmental program and pathogenicity of this organism (Kronstad and Leong, 1990; Schulz et al., 1990).

In the rest of the *Ustilaginales*, including those that infect small-grain cereals, the mating-type loci are still an enigma in regard to their structure and mechanisms of control. However, it can be generalized that most of the small-grain infecting *Ustilago* species have a bipolar mating-type system (Thomas, 1991). *Ustilago nigra*

(Thomas, 1988), *Ustilago avenea*, *Ustilago kolleri* (Nielsen, 1988), *Ustilago nuda*, *Ustilago tritici*, *Ustilago bullata*, *Ustilago aegilopsis*, *Ustilago phragica* and *Ustilago turcomanica* all have a bipolar mating system, with one locus with two alleles, *A* and *a* (Nielsen, 1988; Thomas, 1991). In *Tilletia* spp. a single mating-type locus (bipolar system) has been reported. Multiple alleles apparently occur within *Tilletia controversa* (Hoffmann and Kendrick, 1969) and *Tilletia indica* (Duran and Cromarty, 1977; Royer and Rytter, 1985).

Ustilago hordei has one mating-type locus with two alleles which have been arbitrarily assigned the symbols *A* and *a* or "plus" and "minus". Fusion of compatible sporidia can be determined by the "Bauch" test which results in the production of aerial hyphae. Fusion between *A* and *a* sporidia results in the production of the dikaryotic hyphae that can infect the host (Fisher and Holton, 1957; Dinoor and Person, 1969; Thomas, 1988).

Recently, a survey of several cereal smut fungi was performed using the cloned *a* and *b* mating-type loci from *U. maydis* as probes (Bakkeren et al, 1992). Homology to these loci was found in *U. hordei*, indicating the presence of these loci in this fungus (Bakkeren et al., 1992).

CHAPTER 2

IMPROVED MEDIA FOR TESTING THE MATING REACTION AND GENETIC COMPLEMENTATION OF *USTILAGO HORDEI*

Introduction

Ustilago hordei is a basidiomycete which causes covered smut in barley (*Hordeum vulgare* L.) (Mathre, 1982). *U. hordei* grows asexually as budding, yeast-like sporidia on a variety of rich and minimal media. It is heterothallic, with mating controlled by a single mating-type locus with two alleles designated *A* and *a* (Thomas, 1988). When sporidia of opposite mating type are mixed, they are joined by a conjugation bridge. Conjugation initiates a number of developmental changes, including the transition from non-pathogenic, uninucleate sporidia to pathogenic, dikaryotic mycelium. While the asexual cycle can be completed on artificial media, the sexual cycle must be completed on the host. Infection occurs through the coleoptile after which the dikaryotic hyphae advance through the host tissue and become established in the plant meristem (Kozar, 1969b). During flowering, *U. hordei* ramifies throughout the ovarian tissue, forming a fungal mass which differentiates into sexual teliospores in place of the seed (Kozar, 1969b).

While teliospore formation and other late stages of the sexual cycle require growth on the host, the early stages can be monitored

ex planta (Thomas, 1988, and references cited therein). The ability to detect mating of *U. hordei* on artificial media is important for several reasons. First, the mating type of an unknown isolate can be determined without infecting the host and waiting several months until an infected head emerges. Secondly, as previously shown by Dinoor and Person (1969), complementation tests can be performed to determine allelism of mutations in cells of the opposite mating type; non-allelic mutations will complement each other and form dikaryotic mycelium. Finally, as shown with *U. maydis*, the ability to induce and detect dikaryotic mycelium on artificial media can be used to select mating-type mutants (Banuett and Herskowitz, 1988), and for cloning mating-type genes (Kronstad and Leong, 1989; Schulz et al., 1990).

Investigators studying *U. hordei* have long expressed dissatisfaction with the equivocal mating reaction which forms on a variety of agar media (Thomas and Person, 1965; Lade, 1967; Person et al., 1986; Henry et al., 1988; Thomas, 1988). Media containing activated charcoal have been used successfully to enhance the mating reaction of *U. maydis* (Day and Anagnostakis, 1971). I have evaluated the benefit of adding activated charcoal to several complete and minimal media and tested different incubation conditions in an attempt to enhance the *ex planta* mating reaction of *U. hordei*. The suitability of charcoal-supplemented minimal medium for genetic complementation studies is also described.

Materials and Methods

Ustilago hordei Strains

Ustilago hordei (Pers.) Lagerh. strains I4, mating-type *A* (ATCC #34037) and E3, mating-type *a*, (ATCC #34038) were both obtained independently from Dr. Barbara Christ, Pennsylvania State University and Dr. David Pope, University of Georgia. In keeping with guidelines proposed by Yoder et al. (1988), I am proposing that the mating-type locus of *U. hordei* be designated *MAT1*, and that the *A* allele be *MAT1-1* and the *a* allele be *MAT1-2*. Since *U. hordei* has only one known mating-type locus (Thomas, 1988), the allele designations can be abbreviated as *MAT-1* for the *A* allele and *MAT-2* for the *a* allele. Thus, I4 has the *MAT-1* allele and E3 has the *MAT-2* allele. Four sporidial cultures, MSU-1 through MSU-4, were used throughout this study. These were isolated as an unordered tetrad from a teliospore taken from a naturally infected barley head in Montana. In addition, random sporidial isolates of both mating types were isolated from teliospores of races 8 (strains 8A and 8a) and 14 (strains 14A and 14a). Teliospores of races 8 and 14 were kindly supplied by Dr. W. Pedersen, University of Illinois.

Auxotrophic Mutants

Sporidial strains 8A and 14a were grown in liquid Holliday's complete medium (HCM) (Holliday, 1974) to early log phase (Hood, 1968). The sporidia were adjusted to 1 x 10^3 cells/ml in sterile distilled water and plated on HCM agar. Uncovered petri plates were irradiated with UV light (234 nm wavelength, lamp "A" GE) 30 cm from the UV

source. Irradiation for 40 s (strain 8A) and 35 s (strain 14a) resulted in 90% killing of the cells. Following irradiation, the plates were covered and incubated for 24 h in complete darkness at 20°C, after which they were transferred to a 12 h cycle of light/darkness at 20°C. After 5-6 days of incubation, colonies were replica plated onto Vogel's minimal medium (VMM) (Vogel, 1956) and HCM agar. These plates were incubated at 20°C for two days. Cells that grew on HCM but not on VMM were picked from the master plate, transferred to HCM agar, and incubated as above. This plate was used to replica plate the colonies onto VMM supplemented with amino acids, vitamins, and nucleotides divided into 12 pools as described by Holliday (1956). Putative auxotrophs were purified and retested for auxotrophy and reversion rates.

In order to obtain sporidia of different mating type with a known allelic mutation, strains 8A-1 (*MAT-1 his*) and 14a-2 (*MAT-2 cys*) were crossed. To perform the cross, the sporidia were suspended in sterile distilled water, mixed, and inoculated onto susceptible barley seeds (Odessa, C.I. 934) using the partial-vacuum method described by Tapke and Bever (1942). All *U. hordei* strains and mutants used in this study are described in Table 1.

Media

Several media were tested for suitability in detecting mycelial dikaryons as an indication of successfully mated cultures. These included HCM, Holliday's minimal medium (HMM), double strength Holliday's complete medium (2xHCM) (Holliday, 1974), and VMM.

Table 1. *Ustilago hordei* strains used in this study.

Strain	Genotype	Source
I4	*MAT-1*[a]	B. Christ and D. Pope
E3	*MAT-2*	B. Christ and D. Pope
MSU-1	*MAT-1*	M. Bjarko[b]
MSU-2	*MAT-2*	M. Bjarko
MSU-3	*MAT-1*	M. Bjarko
MSU-4	*MAT-2*	M. Bjarko
8A	*MAT-1*	Random isolate from a race 8 teliospore
14a	*MAT-2*	Random isolate from a race 14 teliospore
8A-1	*MAT-1 his*	UV-induced His- auxotroph of 8A
14a-2	*MAT-2 cys*	UV-induced Cys- auxotroph of 14a
814-11	*MAT-2 his*	Segregant from 8A-1 x 14a-2
8A-6	*MAT-1 arg*	EMS-induced Arg- auxotroph of 8A; K. Dugan
14a-1	*MAT-2 arg*	UV-induced Arg- auxotroph of 14a
8A-3	*MAT-1 ilv*	UV-induced Ilv- auxotroph of 8A
14a-4	*MAT-2 ilv*	UV-induced Ilv- auxotroph of 14a
8A-2	*MAT-1 pro*	UV-induced Pro- auxotroph of 8A
14a-10	*MAT-2 pro*	EMS-induced Pro- auxotroph of 14a; K. Dugan

[a] *MAT-1* and *MAT-2* are the proposed gene designations for mating types *A* and *a*, respectively.
[b] MSU-1, -2, -3, and -4 were obtained as an unordered tetrad from a single teliospore isolate from Montana.

Activated charcoal (Sigma Chemical Co., St. Louis, MO, USA) was added to each medium at 1% or 5% (w/v). Mating reactions were tested on each medium at 6°C, 16°C, 20°C, and 25°C, using precision incubators (Sherer Dual Jet, Nor-Lake Inc., Hudson, WI, USA) with a fixed light/darkness cycle of 12 h.

The effect of pH on the mating reaction was tested on HMM and HCM with and without 1% activated charcoal. The pH of the media was adjusted to 5.5, 6.5, or 7.5 with 1 M NaOH or 1 M HCl. Duplicate plates were incubated at either 6°C or 20°C. An additional experiment was performed to evaluate the effect of the C:N ratio of the media.

The carbon level of HMM with 1% activated charcoal was adjusted by adding 0.1x, 1x, and 2x the normal concentration of dextrose (1x = 10 g/l). The same was done for the N source, ammonium nitrate (1x = 1.5 g/l). The C:N ratios used were 2xC:2xN, 2xC:1xN, 2xC:0.1xN, 1xC:2xN, 1xC:1xN, 1xC:0.1xN, 0.1xC:2xN, 0.1xC:1xN, 0.1xC:0.1xN. Duplicate plates of each medium were incubated at 6°C and 20°C.

Mating Reaction

Sporidial colonies from fresh cultures grown on HCM agar were suspended in 0.5 ml sterile distilled water at a concentration of $1x10^7$ cells/ml. For each "cross" (Thomas and Person, 1965), the mating reaction was initiated by placing 5 μl of the cell suspension of one strain onto the medium to be tested and then inoculating the same volume of a suspension of the second strain directly onto the first. Mating reactions were evaluated under a Wild M5A dissecting microscope at 18X magnification from day 1 to day 13 after inoculation. Five mating reaction scores, from 0 to 4, were assigned based on the amount of mycelium formed on the sporidial "button". The reactions were 0, with a complete lack of mycelium; 1, indicating a few aerial hyphae; 2, where the colony surface was covered with less than 50% mycelium; 3, where the colony surface was more than 50% mycelium but not completely covered; 4, where the surface was completely covered with mycelium. Mating of auxotrophic strains grown on minimal media containing the appropriate supplement was performed exactly as with the wild type strains. Conjugation bridges were observed by mixing sporidia of opposite mating-type on a flat square of 1.5% water agar

placed on a glass microscope slide. The slide was supported by glass rods in a petri plate containing a moist piece of filter paper and incubated at 16°C for 24 h (Henry et al., 1988).

Fluorescence Microscopy

The number of nuclei in sporidia and hyphae that developed on charcoal agar plates was determined in order to confirm that the hyphal cells were dikaryotic. Cells were placed on a glass microscope slide, fixed with 25% ethanol, and air dried. Five μl of a 5 μg/ml DAPI (4,6-diamidino-2-phenylindole) solution were added and allowed to dry. Calcofluor [disodium salt of 4,4,-bis(4-anilobisdiethylamino-S-triazin-2-ylaminol)2,2-stilbenedisulfonic acid] was then added at a concentration of 0.0125 to 0.1 μg/ml to stain the cell wall. Preparations were examined using a Zeiss fluorescence microscope.

Results and Discussion

Sporidial isolates MSU-1 through MSU-4, I4 and E3 (Table 1) were tested for mating genotype alone and in all possible combinations on each medium at each different temperature. The results of representative matings are shown in Table 2. Crosses of strains having opposite mating type resulted in the formation of white, aerial mycelium (Figure 1). This is distinct from the diffuse mycelium which forms on the agar surface around the periphery of sporidial colonies of some strains (e.g., 14a). However, no aerial mycelium was observed when any of the cultures were inoculated alone or when co-inoculated with a like-mating-type strain (i.e., MSU-1, MSU-2, MSU-3, or MSU-4

Table 2. Effect of medium and incubation temperature on the mating reaction[a] observed with *U. hordei* crosses.

		Media[b]								
Cross	T°C	HCM	HCM-1%	HCM-5%	2xHCM	2xHCM-1%	HMM	HMM-1%	VMM	VMM-1%
MSU-1	25	3	4	4	2-3	4	1	4	2-3	3-4
x	20	3	4	4	2-3	4	2	4	2	3-4
MSU-2	16	3	4	4	2-4	4	2-3	4	1-3	3-4
	6	3-4	4	4	3	4	3-4	4	2-3	4
MSU-3	25	3	4	4	2	4	1-2	3-4	2-3	3-4
x	20	3	4	4	2	4	2	4	2-3	3-4
MSU-4	16	2-3	4	3-4	2-4	4	3	4	1-3	3-4
	6	3-4	4	4	2-3	4	3-4	4	2-3	4
E3	25	1	1-3	2-3	0-3	0-3	1	1-3	0-3	1-3
x	20	1	1-3	2-3	0-3	1	1-2	2-3	0-3	1-3
I4	16	0-2	1-3	1-3	1	0	1	1-4	0-2	2-3
	6	0	1-2	1-2	0-1	0-1	1	1-4	0-1	2
I4	25	2-3	3-4	4	ND[c]	ND	ND	ND	ND	ND
x	20	2-3	4	3-4	ND	ND	ND	ND	ND	ND
MSU-2	16	1-2	3-4	3	ND	ND	ND	ND	ND	ND
	6	0-1	3-4	4	ND	ND	ND	ND	ND	ND
E3	25	2	2-4	3-4	ND	ND	ND	ND	ND	ND
x	20	1-2	3	3-4	ND	ND	ND	ND	ND	ND
MSU-1	16	1-2	2-4	2-3	ND	ND	ND	ND	ND	ND
	6	1	3-4	3-4	ND	ND	ND	ND	ND	ND

[a] The mating reactions were: 0, with a complete lack of mycelium; 1, indicating a few aerial hyphae; 2, where the colony surface was covered with less than 50% mycelium; 3, where the colony surface was more than 50% mycelium but not completely covered; 4, where the surface was completely covered with mycelium. The values represent the range of mating reactions observed for replicates of a given cross.

[b] Abbreviations for media are: HCM, Holliday's complete medium; HCM-1%, HCM supplemented with 1% activated charcoal; HCM-5%, HCM supplemented with 5% activated charcoal; 2xHCM, double-strength HCM; 2xHCM-1%, 2xHCM supplemented with 1% activated charcoal; HMM, Holliday's minimal medium; HMM-1%, HMM supplemented with 1% activated charcoal; VMM, Vogel's minimal medium; VMM-1%, VMM supplemented with 1% activated charcoal.

[c] ND = not determined.

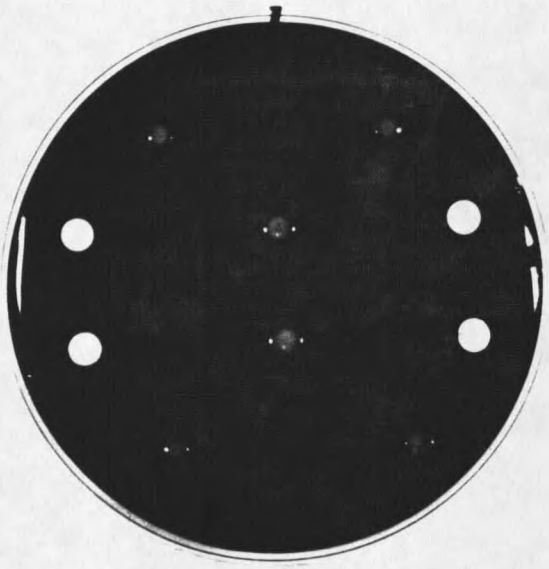

Figure 1. Mating reactions of sporidia isolated from a single teliospore on Holliday's complete medium supplemented with 1% activated charcoal. Top row from left to right: MSU-1 alone, MSU-2 alone; second row: MSU-1 x MSU-2, MSU-1 x MSU-3, MSU-1 x MSU-4; third row: MSU-2 x MSU-3, MSU-2 x MSU-4, MSU-3 x MSU-4; bottom row: MSU-3 alone and MSU-4 alone. Strains of opposite mating type show abundant, white, aerial mycelium, while strains inoculated alone or crossed with a like-mating-type strain have a yeast-like appearance.

alone, or MSU-1 x MSU-3 or MSU-2 x MSU-4) (Figure 1). These colonies retained a yeast-like appearance.

The addition of activated charcoal to any of the media tested resulted in an enhanced mating reaction when compared to the same media without charcoal (Table 2). The addition of 5%, instead of 1%, activated charcoal to HCM did not affect the mating reaction at any temperature. Compared to the reaction of newly isolated sporidia (MSU isolates 1-4), a weak mating reaction was observed when either of the independently obtained isolates of I4 and E3 were crossed with each other. The I4 x E3 cross displayed a weak mating reaction even when activated charcoal was added to the media, although charcoal usually strengthened the reaction to some degree. However, the mating reaction was improved when either E3 or I4 was crossed to a recently isolated strain (e.g., MSU-1 or MSU-2) (Table 2). Since crosses using any of the strains recently isolated from teliospores (including the MSU strains, strain 8A, and strain 14a) gave strong mating reactions, it is assumed that strains I4 and E3 display reduced fertility due to their long history of being maintained as sporidial cultures.

While the addition of activated charcoal enhanced the intensity of the mating reaction of *U. hordei*, incubation temperature also exerted a strong effect (Table 3). At higher temperatures (25°C and 20°C), the mating reaction was strongest at an earlier time, usually 2-3 days after inoculation. After that time the sporidia overgrew the mycelium, eventually resulting in a yeast-like colony. At lower temperatures (6°C), the strongest mating reaction was delayed until 6-10 days after inoculation, but the reaction remained stable indefinitely. Therefore,

Table 3. Effect of temperature and medium on the duration of the maximum mating reaction.

Media	Temperature			
	6°C	16°C	20°C	25°C
HCM[a]	7-13[b]	3-6	2-4	2-4
HCM-1%	6-13	2-9	1-6	1-4
HCM-5%	6-13	2-7	1-5	1-4
2xHCM	8-11	4-8	1-6	1-5
2xHCM-1%	6-11	3-11	3-5	1-4
HMM	8-13	3-9	1-6	1-4
HMM-1%	4-13	3-13	2-8	1-3
VMM	6-11	3-8	1-6	1-3
VMM-1%	6-12	2-12	1-9	1-6

[a] Abbreviations for media are the same as in Table 2.
[b] Days of incubation during which the strongest mating reaction was observed. Each value represents the range of the initial and final day of incubation, from 12 different crosses, during which the maximum reaction was observed.

if high temperatures are used, the plates must be observed during the 1-2 day window during which the mycelium is most apparent. Generally, the addition of activated charcoal to a medium resulted in a shorter incubation time before the maximum mating reaction was observed and increased the number of days that the mating reaction could be observed, regardless of incubation temperature. Thus, activated charcoal increased both the rate and stability of the mating reaction.

The aerial mycelium which developed on charcoal-containing media did not extend beyond the inoculation point. Mycelium transferred to a new plate containing a charcoal-containing medium remained stable, but

did not spread. However, if the mycelium was transferred to a complete or minimal medium without activated charcoal, it was soon overgrown by sporidia, resulting in a yeast-like colony. Therefore, the use of activated charcoal-containing media allows formation of stable dikaryotic mycelium but does not support growth of the mycelium.

The pH of the media had little or no effect on mating or stability of the reaction on any of the media (data not shown). Furthermore, the carbon:nitrogen ratio of the media had no effect on the mating reaction (data not shown).

The transition from sporidial to mycelial growth should result in a change in nuclear number from mononucleate to dikaryotic (Thomas, 1988). The number of nuclei in sporidial and hyphal cells obtained from the surface of charcoal-containing agar medium were examined by fluorescence microscopy (Figure 2). All sporidia showed one nucleus per cell (Figure 2A), while the hyphae were found to be dikaryotic (Figure 2B) as expected. Although some mycelial cells appeared to contain only one nucleus, the presence of 2 nuclei in most cells confirmed that the mycelium was dikaryotic and, therefore, resulted from mating.

In addition to being useful in identification of the mating type of unknown isolates, the ability to reliably form dikaryotic mycelium on an agar medium has utility as a method for assigning mutants to complementation groups (Dinoor and Person, 1969; Henry et al., 1988). When cells of opposite mating type with non-allelic mutations are crossed, the mutations should complement and permit mycelium formation. However, allelic mutations will not complement and mycelium will not