The a and b Loci of *Ustilago maydis*
Hybridize with DNA Sequences from Other Smut Fungi

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The smut fungi are obligately parasitic during the sexual phase of their life cycle, and the mating-type genes of these fungi play key roles in both sexual development and pathogenicity. Among species of smut fungi it is common to find a bipolar mating system in which one locus with two alternate alleles is believed to control cell fusion and establishment of the infectious cell type. Alternatively, several species have a tetrapolar mating system in which two different genetic loci, one of which has multiple alleles, controls fusion and subsequent development of the infection hyphae. Cloned sequences from the a and b mating-type loci of the tetrapolar smut fungus *Ustilago maydis* were used as hybridization probes to DNAs from 23 different fungal strains, including smut fungi with both tetrapolar and bipolar mating systems. In general, all of the smut fungi hybridized with the mating-type genes from *U. maydis*, suggesting conservation of the sequences involved in mating interactions. A selection of DNAs from other ascomycete and basidiomycete fungi failed to hybridize with the *U. maydis* mating-type sequences. Exceptions to this finding include hybridization of DNA from the *a1* idiomorph of *U. maydis* to DNA from one strain of *U. violacea* and hybridization of both *a1* idiomorphs to DNA from *Saccaromyces cerevisiae*.

Additional keywords: infectious dikaryon, multiallelic recognition.

The smut fungi are basidiomycete phytopathogens that attack a wide variety of monocotyledonous and dicotyledonous plants (Fischer 1953; Fischer and Holton 1957). Many of the best-characterized smut fungi cause infections on important crop species, in particular, small grain cereals and corn. One of the main hallmarks of a smut infection is the presence of large masses of sooty black teliospores within the host plant. In small grain cereals, these spores often replace the developing seeds, thereby reducing the yield and quality of the grain (Agrios 1988).

The smuts share many features of their life cycles including, in most species, a yeastlike vegetative cell type and an obligately parasitic, dikaryotic cell type arising from the fusion of compatible haploid cells. The infectious dikaryon, which has a filamentous morphology, grows systemically within host tissue and eventually sporulates—often within galls or floral tissue. The smut fungi differ in some important details of their life cycles, including the range of hosts that can be infected, the influence on host tissue (whether galls are induced), and the location in the host where sporulation occurs (Fischer and Holton 1957). For example, *Ustilago maydis* (DC.) Corda infects seedlings of corn (*Zea mays* L.) and induces galls on any above ground part of the plant; the fungus sporulates primarily within gall tissue. In contrast, *U. hordei* (Pers.) Lagerh. infects seedlings of small grain cereals, in particular barley (*Hordeum vulgare* L.), and sporulates primarily within floral tissue (without inducing galls).

One of the most interesting differences among the smuts is the presence of at least two different mating systems within the group (Holton et al. 1968). Many of the species, such as *U. hordei*, possess a bipolar mating system in which a single mating-type locus with two alternate alleles controls dikaryon formation. Other smuts, such as *U. maydis*, display tetrapolar mating in which two loci control dikaryon formation. The tetrapolar smut fungi generally have one locus with two alternate alleles that is thought to control cell fusion and a second locus with multiple alleles that controls the establishment of the dikaryon (Holton et al. 1968). If the bunt fungi of the genus *Tilletia* are included in the smut group, then a third mating system (multiple alleles at a single locus) has been reported for *T. controversa* Kühn in Rabenh. (Hoffman and Kendrick 1965).

Besides *U. maydis*, other smut fungi that display a tetrapolar mating system include *Sporisorium* species (smut of maize and sorghum), *U. striiformis* (Westend.) Niessl (smut of several grass species, e.g., *Agropyron subsecundum* (Link) Hitchc.), and *U. longissima* (Sowerby) Meyen (smut of *Glyceria sp.*) (Fischer 1953; Holton et al. 1968). Interestingly, there have been reports of interfertility between *U. maydis* and *Sporisorium* species (Rowell and DeVay 1954; A. Budde and S. A. Leong, unpublished results), and coinoculation of corn seedlings with haploid isolates of these fungi can give rise to an infection (Rowell and DeVay, 1954). Smut fungi with bipolar mating systems include those that infect grasses, such as *U. bulbata* Berk. and *U. aegilopsidis* Picbauer, and those that infect small grain cereals, such as *U. hordei* and *U. kolleri* Wille (covered smut of barley and oats), *U. nuda* (Jensen) Rostrup and

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U. tritici (Pers.) Rostrup (smut of barley and wheat), and U. nigra Tapke and U. avenae (Pers.) Rostrup (loose smut of barley and oats) (Holton et al. 1968). In many cases, these fungi are interfertile and apparently show only limited genetic differences (Huang and Nielsen 1984). In general, there have been few reports of interfertility between smuts containing a bipolar mating system and those containing a tetrapolar system. One exception is the report of Kniep (Kniep 1926; cited in Fischer and Holton 1957) of the fusion of U. longissima with U. nuda and with U. avenae. An important limitation to the demonstration of fusion or sexual compatibility between different smut species is the dependence on a common host to demonstrate true interfertility. This limitation has been overcome for some of the bipolar species with the discovery that two grasses will serve as common hosts for several smut species (Nielsen 1978).

Given the role of mating in establishing the infectious dikaryon (see Froeliger and Kronstad 1990 for a review), the characterization of the structure and function of the mating-type genes will be important for an understanding of pathogenicity. Molecular analysis of the mating systems in the smut fungi began with the isolation of two alleles of the multiallelic b locus of U. maydis (Kronstad and Leong 1989). Subsequently, at least five additional alleles have been isolated and characterized (Kronstad and Leong 1990; Schulz et al. 1990). The polypeptides predicted to be encoded by the b alleles contain a region with similarity to the homeodomain of regulatory proteins from Drosophila, and a comparison of different alleles indicated a variable amino terminal portion and a conserved carboxy terminal region (Kronstad and Leong 1990; Schulz et al. 1990). The sequences at the a loci (a1 and a2) of U. maydis have also been isolated and, unlike the b alleles, these regions contain large blocks of nonhomologous DNA (Froeliger and Leong 1991; Bolker et al. 1992). This organization is similar to that found for the mating-type idiomorphs of Neurospora crassa (Glass et al. 1988; Metzenberg and Glass 1990).

The availability of cloned DNAs from the a and b loci of U. maydis presented an opportunity to survey additional smut fungi with tetrapolar and bipolar mating systems for the presence of similar sequences. We report here that sequences hybridizing with the DNAs from the a and b loci of U. maydis were found in all of the smut fungi tested. In addition, sequences that hybridized with the a DNAs were found in Saccharomyces cerevisiae Hansen and, in the case of the at sequence, in U. violacea (Pers.) Roussel, but not in several other basidiomycete and ascomycete fungi.

| Table 1. Fungal strains and sources of DNA used in this study |
|------------|----------------|------------------|
| Species    | Strain and relevant markers | Source or reference |
| Ustilago maydis | 521 (a1 b1) | S. A. Leong |
|             | 518 (a2 b1) | S. A. Leong |
|             | 031 (a1 b2) | S. A. Leong |
|             | 032 (a2 b1) | S. A. Leong |
|             | ATCC 22906 (a2 b1) | ATCC |
|             | a1 bXo7 b1 W:HygB | This study |
|             | a2 bXo7 b1 W:HygB | This study |
|             | a1 bEl b1 W:HygB | Kronstad and Leong 1989 |
|             | a2 bEl b1 W:HygB | Kronstad and Leong 1989 |
| Ustilago longissima | a2 b | This study |
| Saccharomyces cerevisiae | 2R26-12C (MATa) | M. Smith³ |
| Sporotrichum reilianum | a112 | R. Frederiksen⁶ |
| U. hordei | A100 | C. Person⁷ |
| U. kolleri | | C. Person⁷ |
| U. avenae | | P. Thomas⁸ |
| U. angolensis | | P. Thomas⁸ |
| U. bullata | | P. Thomas⁸ |
| U. nigra | | P. Thomas⁸ |
| U. violacea | 01 | A. Day⁹ |
| U. violacea | 002 | A. Day⁹ |
| Puccinia graminis f. sp. tritici | SZA 2C-1 | L. Szabo⁹ |
| Tilletia controversa | B6-1 | D. Mills¹⁰ |
| T. controversa | H-1 | D. Mills¹⁰ |
| T. caries | T-9 | D. Mills¹⁰ |
| Cryptococcus neoformans | | U. Wisconsin Hospital |
| Neurospora crassa | 74A | M. A. Nelson¹¹ |
| N. crassa | ORSa | M. A. Nelson¹¹ |
| Schizosyphyllum commune | 4-40 | C. Novotny¹¹ |

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MATERIALS AND METHODS

Sources of strains or DNA. The fungi employed in this work are listed in Table I. *U. maydis* strains 518, 521, 031, and 032 are part of the collection maintained by S. A. Leong at the University of Wisconsin-Madison. *U. maydis* ATCC 22906 was obtained from the American Type Culture Collection, Rockland, MD. *U. maydis* strains carrying the hygromycin B resistance marker at the b locus were constructed as previously described (Kronstad and Leong 1989). It was later determined that the marker disrupted a second open reading frame (ORF) at the b locus (designated bW; see Results). A haploid isolate of *U. longissima* was obtained from germinated teliospores from the Herbarium in the Department of Plant Pathology, University of Wisconsin-Madison. The spores were isolated from a brown stripe smut infection on rattlesnake manna-grass (*Glyceria canadensis*). *S. cerevisiae* strain 2R26-12C was obtained from M. Smith, Biotechnology Laboratory, University of British Columbia. A haploid isolate of *S. reilianaum* (Kühn) Langdon Fullerton was obtained from teliospores provided by R. Frederiksen, Texas A & M University, College Station. *U. hordei* strains a112 and A100 were from the culture collection of the late C. Person, Department of Botany, University of British Columbia. Teliospores of *U. hordei*, *U. kolleri*, *U. avenae*, *U. aegilopsidis*, *U. bulbata*, and *U. nuda* were provided by P. Thomas, Agriculture Canada Research Station, Winnipeg. A strain of *Cryptococcus neoforms* was obtained from the University Hospital, University of Wisconsin-Madison. Purified DNAs from various fungi were provided by the following individuals: A. Day, Department of Plant Science, University of Western Ontario (*U. violacea*); L. J. Szabo, Cereal Rust Laboratory, University of Minnesota (*P. graminis* f. sp. tritici); D. Mills, Department of Botany and Plant Pathology, Oregon State University (*T. controversa* Kühn in Raben. and *T. caries* Tol & C. Tol); M. A. Nelson, Department of Biology, University of New Mexico (*N. crassa*); and C. Novotny, Department of Microbiology and Molecular Genetics, University of Vermont (*Schizosaccharomyces commune* Fr.;fr.).

Teliospore germination and cell growth. Teliospores of the *smut fungi* were germinated on potato-dextrose agar (PDA) at 30°C. Single haploid progeny were obtained by dispersing germinated teliospores in sterile water and plating them on PDA for single-colony isolates. Liquid cultures of the smut fungi and of *C. neoforms* were grown at 30°C in complete medium (Holliday 1974). Cells of *U. bulbata*, *U. aegilopsidis*, *U. kolleri*, *U. nuda*, and *U. avenae* were grown on PDA at 30°C.

DNA manipulations and hybridization conditions. DNA was isolated from the smut fungi and from *C. neoforms* by vortexing with glass beads (425-600 μm; Sigma Chemical Co.), as described by Elder et al. (1983). All DNA samples for hybridization analysis were digested with BamHI. Restriction digestion, agarose gel electrophoresis, and transfer of DNA to membranes were performed as described by Maniatis et al. (1982). For most of the experiments, nylon membranes were employed (Zetabind, Cuno Laboratory Products; Nytran, Schleicher & Schuell). The blots shown in Figs. 5A and 6A below were prepared on nitrocellulose [Schleicher & Schuell]. DNA fragments for use as hybridization probes were separated from vector sequences by electrophoresis on agarose gels (two separations). The fragments were isolated from agarose using the GeneClean Kit (Bio101) and labeled with 32P using an oligolabeling kit (Pharmacia). Hybridizations were performed as follows. Filters with bound DNA were prewashed at 65°C for 16 hr in 0.1X SSC (15 mM NaCl plus 1.5 mM sodium citrate) and 0.5% sodium dodecyl sulfate (SDS). Subsequently, the filters were incubated in prehybridization solution (30% formamide, 5X SSC, 0.1% SDS, 1 mM EDTA, 10 mM Tris pH 7.4, 1X Denhardt's solution, 100 μg/ml of calf thymus DNA [Maniatis et al. 1982]) for 2 hr at 42°C, the hybridization probe was added, and incubation was continued overnight. The filters were washed in 2X SSC, 0.1% SDS for 30 min at room temperature followed by a 20- to 60-min wash at 45°C in 0.5X SSC, 0.1% SDS. Additional washes at 65°C were performed as indicated in the figure legends. Autoradiography was performed with Kodak X-Omat AR film and one Cronex Lightning intensification screen at 70°C. For some experiments (not shown), the hybridization conditions of Amasino (1986) were employed.

RESULTS

Fungal isolates. The fungi chosen for this study are representative of both basidiomycetes and ascomycetes and include isolates of smut fungi with tetrapolar and bipolar mating systems (Table I). The smut fungi were chosen to include isolates that infect different monocotyledonous plants. Thus, species such as *S. reilianaum* and *U. maydis* infect corn, species such as *U. hordei* and *U. avenae* infect small grain cereals, and species such as *U. bulbata*, *U. longissima*, and *U. aegilopsidis* infect grass species. Two isolates among the smut fungi, *U. violacea* 001 and 002, infect dicotyledonous plants. These isolates have a similar life cycle compared with the smut fungi that infect monocotyledonous plants, but recent evidence (Deml and Oberwinkler 1982) indicates that *U. violacea* is not sufficiently related to other *Ustilago* spp. to be in the same genus. *U. violacea* is now placed in the genus *Microbotryum*. *C. neoforms* was chosen because this fungus is an important opportunistic pathogen of humans, and the perfect state of this fungus (genus *Filobasidiella*) is in the same order (*Ustilaginales*) as the smut and bunt fungi (Kwon-Chung 1975).

Existing information on the structure and organization of mating-type genes in fungi prompted the inclusion of strains of *N. crassa* and *S. commune*. *N. crassa* has been found to possess idiomorph sequences, i.e., large regions of nonhomologous DNA, at the mating-type locus (Glass et al. 1988). As mentioned above, a similar organization has been found for the a alleles of *U. maydis* (Froeliger and Leong 1991). *S. commune* is of interest because, unlike *U. maydis*, four different genetic loci are involved in sexual development and multiple alleles exist for all four loci (Raper 1966; Stankis et al. 1990).

Hybridization probes for the a and b loci. Maps depicting the region of the *U. maydis* genome containing the a locus are shown in Figure 1A. As described by Froeliger and
Leong (1991) and Bolker et al. (1992), the a alleles of *U. maydis* are actually idioms and contain large blocks of nonhomologous DNA (approximately 4.0-5.0 kb for the a1 region and approximately 7.5-8.5 kb for the a2 region). The sequences within the idioms, which have biological activity specific for each allele, have been identified (Froeliger and Leong 1991; Bolker et al. 1992) and are marked on the map. For the a1 allele, a 3.5-kb EcoRI fragment from within the nonhomologous region and including the region with biological activity was employed as a hybridization probe. A smaller fragment of 0.86 kb (*BamHI*-SstII) from within the region of the a2 idiomoph showing biological activity was chosen as a hybridization probe.

The b locus appears to comprise two multiallelic genes. The first ORF to be identified (Kronstad and Leong 1989, 1990; Schultz et al. 1990) encoded a 473-amino acid polypeptide and has been designated be (for bEast). A 1.3-kb cloned product of the polymerase chain reaction from within this ORF of the b7 allele (Kronstad and Leong 1990) was employed as a hybridization probe. The second probe is a 1.3-kb *BamHI*-HindIII restriction fragment from a region immediately upstream of the be ORF. This region contains a second ORF, designated bw (for bWest), believed to encode an approximately 626-amino acid polypeptide with a role in b allele function (Gillissen et al., 1992). The be and bw nomenclature will be used to designate the two different probes for the b locus.

We have confirmed the importance of the bw ORF in b mating-type function as shown in Figure 2. Strains constructed to contain the hygromycin B gene within the bw ORF (e.g., a2 bx70E/b1W::HygB; see Fig. 1B and Table 1) are capable of mating with wild-type strains (e.g., a1 b1E/b1W or a1 b2E/b2W) but are incapable of mating with other strains containing insertions of the marker at an identical position (e.g., a1 b1E/b1W::HygB'). This result indicates that at least one functional copy of the bw region (in either partner) must be present during mating inter-

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**Fig. 1.** Regions of the a and b loci used as hybridization probes. A. Maps of the a1 and a2 idiomorphs of *Ustilago maydis* are shown with the regions of nonhomology between the sequences depicted as open boxes (Froeliger and Leong 1991). The sequences outside the open boxes show conserved restriction sites (e.g., *BamHI*) and the regions of the idiomorphs with biological activity are indicated by cross-hatched boxes within the regions of nonhomology (Froeliger and Leong 1991). DNA fragments used as hybridization probes are shown above the maps; a 3.8-kb EcoRI fragment was used for the a1 idiomorph and an 0.86-kb *BamHI*-SstII fragment was used for the a2 region. Restriction sites are indicated as follows: B = *BamHI*, R = EcoRI, S = SstII. B. Map of the b locus of *U. maydis* showing the relative positions and orientations of the two open reading frames labeled bw and be. DNA fragments employed as hybridization probes are shown below the map; A 1.3-kb *BamHI*-HindIII fragment was used for bw and a 1.3-kb *BamHI* fragment from a polymerase chain reaction product was used for be (Kronstad and Leong 1990). The *BamHI* sites were incorporated as part of the primer sequences used for amplification. Restriction sites are indicated as follows: Bg = *BglII*, H = *HindIII*. The insertion of a 3.0-kb *BamHI*-BglII fragment carrying the gene for resistance to hygromycin B is shown at the *BglII* site (codon 49) in bw. The construction of strains containing this DNA in place of the wild-type b locus has been described by Kronstad and Leong (1989).

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**Fig. 2.** Disruption of the bw open reading frame in both mating partners blocks mating. A petri dish containing rich medium with 1% activated charcoal (Holliday 1974) was spotted with mixtures of each strain to be tested, and the plates were inoculated overnight at 30°C. A positive mating reaction is indicated by the formation of white aerial mycelium on the mixed colonies. Four tester strains with wild-type b alleles are shown on the right along with two strains containing a disruption in the bw open reading frame. The bE allele has both be and bw, similarly, b2 has both be and bw. Disruption of the bw ORF is designated b1W-HygB' and these strains were constructed as described in Kronstad and Leong (1989). As indicated, the spots on the left contain the bw disruption in a strain with the a1 mating type and the spots on the right contain the same disruption in an a2 strain. The b allele in these strains (bx70E) is a recombinant allele with a hybrid be/bE region containing parts of the be and b2E sequences (A. Yee and J. W. Kronstad, unpublished). Specifically, the bx70E allele contains codons 1-69 from the be gene fused to codons 70-410 of the b2E gene. This allele allows normal mating as shown by the reactions with the four tester strains.
actions. Sequence analysis of the \( bW \) region from the wild-type \( b/ \) allele also confirmed the presence of a 626-amino acid ORF (J. W. Kronstad, unpublished results).

Hybridization with the \( b7E \) probe. Initially, a DNA fragment (Fig. 1B) from within the ORF of the \( b7E \) gene from \( U. maydis \) was used as a hybridization probe to blot containing DNA from the 23 fungal isolates. As expected from earlier work (Kronstad and Leong 1989, 1990), the probe bound to an 8.5-kb fragment in each of the strains of \( U. maydis \) tested (lanes a and b, Fig. 3A and B). In fact, the other smut fungi with tetrapolar mating systems (\( S. reilianum \), lane d, Fig. 3A and \( U. longissima \), lane c, Fig. 3B) also possess an 8.5-kb \( BamHI \) fragment carrying sequences homologous to the probe. \( U. maydis \) is known to give a positive plate mating assay with both \( S. reilianum \) and \( U. longissima \) (E. H. Froeliger and S. A. Leong, unpublished results), and coinoculation of corn with \( U. maydis \) and \( S. reilianum \) results in infection (Rowell and Devay, 1954; A. Budde and S. A. Leong, unpublished results). It is interesting to note that the strain of \( S. reilianum \) tested also showed hybridization to a 1.0-kb DNA (Fig. 3A, lane d) fragment, raising the possibility of a second sequence related to \( bE \) in this smut fungus.

Hybridization of the \( b7E \) fragment to DNAs from the smut strains with bipolar mating systems revealed that one of two alternate fragments (2.8 or 1.5 kb) was detected in each isolate (Fig. 3A). The two different strains of \( U. hordiei \) tested were of opposite mating type and contained different size fragments (2.8 kb in a112, lane e and 1.5 kb in A100, lane f). To determine whether the bands represented opposite alleles at a single locus, DNA samples from random haploid progeny of germinated teliospores of \( U. hordiei \) were tested for hybridization. Of the 32 tested, 14 had the 2.8-kb band and 18 had the 1.5-kb band, indicating that the restriction fragment length polymorphism is present at the locus that hybridizes with the \( bE \) sequence (G. Bakkeren and J. W. Kronstad, unpublished results).

Similar results were also obtained upon analysis of random meiotic progeny from teliospores of \( U. bullata \), although the progeny containing the 1.5-kb band were underrepresented compared with those containing the 2.8-kb band. These results indicate that, for the \( bE \) sequence (and \( bW \), see below), the smut fungi with bipolar mating systems comprise a group with conserved restriction sites and sequences in the \( b \) region. Cosmid clones have been isolated that contain the 1.5- and 2.8-kb \( BamHI \) fragments from \( U. hordiei \); preliminary nucleotide sequence analysis indicates that these fragments contain homologs of \( bE \) and \( bW \) (G. Bakkeren and J. W. Kronstad, unpublished results).

One additional representative of the bipolar smut fungi, the flower-infecting species \( U. mada \), was tested for hybridization with the \( b7E \) probe. Because it is difficult to isolate haploid strains of this fungus (Nielsen 1968), DNA was prepared directly from cell material scraped from agar medium containing germinated teliospores. Therefore, the DNA tested was from a mixture of cells representing the progeny of meiosis. As for the other bipolar smut fungi, the \( b7E \) probe detected the 1.5- and 2.8-kb \( BamHI \) fragments (data not shown); thus it appears that these bands are conserved among all of the bipolar smut fungi tested.

As shown in Figure 3A and B, the \( b7E \) hybridization probe did not detect similar sequences in any of the other nonsmut fungi tested including the ascomycetes \( S. cerevisiae \) and \( N. crassa \) and the basidiomycetes, \( U. violacea \), \( C. neoformans \), \( S. commune \), \( T. caries \), and \( T. controversa \). The lack of hybridization to the \( Tilletia \) strains is noteworthy because these bunt fungi have very similar
life cycles to smuts and, in some cases, infect the same cereal hosts as the smuts.

Hybridization with the b1W probe. Results similar to those obtained with the b7E probe were found when a DNA fragment from the region containing the b1W ORF was used as a hybridization probe. As shown in Figure 4, cross-hybridizing sequences were found in all of the smut fungi tested but not in any of the other fungi. Again, the smuts with tetrapolar mating systems (U. maydis, U. longissima, and S. reilianum) showed hybridization to an apparently conserved 8.5-kb band. This is the same band detected with the b7E probe in Figure 3A. A polymorphism for BamHI was found among most of the smuts with bipolar mating systems; either an approximately 6.5- or an approximately 1.2-kb band was detected. The exception was U. bollata, where an approximately 2.9-kb band was found to hybridize. As mentioned above, preliminary restriction site and nucleotide sequence analyses of cosmic clones from U. hordei indicate that the organization of the bW and bE sequences is similar to that found in U. maydis (G. Bakkeren and J. W. Kronstad, unpublished results).

Hybridization with the a1 probe. As mentioned in the introduction, the a alleles of U. maydis are idiomorphs (Froeliger and Leong 1991; Bolker et al. 1992); i.e., large regions of nonhomology were discovered upon comparison of the alleles (Fig. 1A). Therefore, probes taken from within these regions of nonhomology should hybridize only to one of the two mating types of U. maydis. An example is shown in Figure 5A, in which the 3.5-kb EcoRI fragment probe from the a1 idiomorph hybridizes only to DNA from U. maydis a1 b1 (lane a; 10-kb fragment) but not to DNA from U. maydis a2 b1 (lane b). This probe detects sequences in some of the other smut fungi, e.g., S. reilianum, U. hordei a112, U. kollerii, U. avenae, and one strain of U. bullata. The presence of two bands in the DNA from these isolates may indicate that the probe overlaps a BamHI site or that two distinct regions hybridize. The lack of hybridization to the remaining smut strains, e.g., U. aegilopsidis and U. nigrum, may be due to a lack of sufficient sequence similarity or to the fact that the strains are of the opposite mating type. The finding of hybridization to only one of the two mating types of U. hordei supports the idea that some strains do not show hybridization because they are of the opposite mating type. In addition, only one of two isolates of opposite mating type of U. bullata was found to have the bands (compare lanes 1 and k in Fig. 5A) that hybridize with the a1 probe. Additional support for the idea that bands detected in Figure 5A are specific to the a1 mating locus comes from an analysis of random progeny of germinated telosporous of U. hordei. The bands that hybridize with a1 showed approximately a 1:1 segregation ratio, and there was an absolute correlation with mating type, i.e., the isolates with hybridizing sequences were all of the same mating type (G. Bakkeren and J. W. Kronstad, unpublished results). Thus, among the isolates surveyed in Figure 5A, the hybridization results suggest that the strains of S. reilianum, U. kollerii, and U. avenae tested, along with the a112 strain of U. hordei and one of the two strains of U. bullata, are of equivalent mating type to a1 of U. maydis.

It is interesting to note that one of the two mating strains of U. violacea (002) also shows weak hybridization to the a1 probe (Fig. 5B, lane c). This result was unexpected given the recent classification of U. violacea into a different genus. As mentioned below, no hybridization was detected to either of the U. violacea strains with the a2 probe. Hybridization was also detected between the a1 probe and the DNA from a MATa strain of S. cerevisiae (Fig. 5A, lane c), perhaps indicating a conservation of sequences with a role in mating (discussed below). In general, the other ascomycete or basidiomycete fungi (Fig. 5B) did not show hybridization to the a1 probe.
Hybridization with the a2 probe. As predicted from the analysis of the a idiomorphs of U. maydis, the a2 probe showed strong hybridization only to a fragment of approximately 6.0 kb (shown at 6.3 kb in Fig. 6) from the a2 strains of U. maydis (lane b, Fig. 6A and lane a, Fig. 6B). In addition, a band of the same size was detected in U. longissima (Fig. 6B, lane c). This result was anticipated because this strain of U. longissima gave a positive mating reaction when mixed on agar plates with strains of U. maydis carrying the a1 idiomorph (E. H. Froeliger and S. A. Leong, unpublished results). The same mating tests revealed that the b gene present in this strain appears to

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**Fig. 5.** Hybridization with a DNA fragment from the a1 idiomorph. Autoradiograms of Southern blots containing BamHI digested DNAs are shown. The DNAs were electrophoresed through a 0.7% gel, transferred to a membrane, and hybridized with a DNA fragment from the a1 idiomorph (Fig. 1A). A, The lanes are the same as in Figure 3A except that an additional lane containing DNA from a haploid isolate of U. ballota is in lane l. This isolate is of opposite mating type to the isolate analyzed in lane k (B. Gibbard and J. W. Kronstad, data not shown). The filter was exposed to film for 40 hr. B, The lanes are the same as in Figure 3B. The filter received an additional 20-min wash at 65° C in 1X SSC, 0.1% SDS and was exposed to film for 72 hr. The bands below 10 kb in lanes a and b probably represent nonspecific hybridization since they are in both mating types. Similar bands are seen in lanes a and b in A.

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**Fig. 6.** Hybridization with a DNA fragment from the a2 idiomorph. Autoradiograms of Southern blots containing BamHI digested DNAs are shown. The DNAs were electrophoresed through a 0.7% gel, transferred to a membrane and hybridized with a DNA fragment from the a2 idiomorph (Fig. 1B). A, The lanes contain DNA from the following fungal isolates: a, U. maydis S21 (a1 b1); U. maydis ATCC 22906 (a2 b2); c, Saccharomyces cerevisiae (2R26-12C); d, U. hordei a112; e, U. hordei A100; f, U. kollerii; g, U. avenae; h, U. aegilopsidis; i, U. ballota; j, U. niger; k, Sartoriotum reilianum. All lanes are from the same gel; distortion of the DNA samples in lanes e, f, and b occurred during electrophoresis. Subsequent analysis has shown that the bands in lanes d and e are of the same size. Several non-mating-type specific bands are present in lanes a and b, but restriction mapping (Froeliger and Leong, 1993) of the a2 idiomorphs indicates that, as shown, the 6.0-6.3 kb BamHI fragment is expected to hybridize. The exposure was 63 hr. B, The lanes are the same as in Figure 3B. The filter was exposed to film for 72 hr. Non-mating-type specific bands are present in lanes a, b and c; as in A the 6.0-6.3 kb band is expected to hybridize in strains with the a2 allele. Some of the nonspecific bands are similar in size to those seen in A.
have a different specificity than the b1 or b2 alleles of *U. maydis*. The *U. maydis* and *U. longissima* DNA samples gave the strongest signals with the a2 probe. Hybridization signals of lesser intensity were detected for bands of approximately 23 kb in *S. cerevisiae*, approximately 3.5 in *U. nigra* and bands of 5.6 and 3.8 kb in *S. reilianum*. It is interesting that a band in the 9.0–10.0 kb range was detected in *U. hordei* strains a112 and A100, *U. avenae*, *U. bullata*, *U. kollerii*, and *U. aegilopsidis*. Digestion of the DNAs from the two *U. hordei* strains with several different restriction enzymes and hybridization with the a2 probe revealed that the same fragments were hybridizing in both strains (data not shown). Therefore, it appears that a region with sequence similarity to the a2 probe is present in both mating-type genes. Alternatively, the hybridization detected may not be to a sequence with a role in mating (i.e., a true a2 homolog) but simply indicates the presence of another related sequence (detected because of the reduced stringency of hybridization) in strains of both mating-types. 

It is also possible that a2 sequences are present in a1 strains but that the sequences are non-functional. There is precedence for the presence of two different mating-type idiomorphs in one haploid genome. The homothallic species *N. terricola* has been found to have both the A and a sequences in one haploid genome (Metzenberg and Glass 1990). A functional test will be needed to determine whether the sequences detected in a1 strains have the activity expected of a idiomorphs.

The a2 probe did not detect hybridizing sequences in any of the ascomycete or basidiomycete fungi (Fig. 6B, tested with the exception of *S. cerevisiae* (Fig. 6A, lane c). It is interesting that both the a1 and a2 probes gave a reasonably good signal to DNA from *S. cerevisiae*. As discussed below, these results may be related to the finding of a homolog of the STE3 gene of *S. cerevisiae* in the a1 idiomorph of *U. maydis* (Bolker et al. 1992).

**DISCUSSION**

The most striking result obtained in this study is the finding that the smut fungi with bipolar mating systems contain sequences that hybridize with the DNAs from the a and b loci of *U. maydis*, a fungus with a tetratropic mating system. These results suggest that the sequences involved in mating are conserved among the bipolar and tetratropic smut fungi. Future work must focus on determining whether the sequences detected by hybridization actually play a role in mating in the bipolar species. Specifically, the sequences must be isolated and tested for activity. We are pursuing these goals with *U. hordei* as a representative of the smut fungi with bipolar mating systems. As mentioned earlier, cosmids containing the regions that cross-hybridize with bE and bW have been identified (G. Bakkkeren and J. W. Kronstad, unpublished results); preliminary analysis of the nucleotide sequences of the hybridizing regions suggests significant similarity to the b region of *U. maydis*. For the a locus, our results raise the possibility that some strains of the bipolar smuts may have both idiomorphs.

The four species that infect seedlings of small grains, *U. hordei*, *U. kollerii*, *U. avenae*, and *U. nigra*, are all inter-

fertile and apparently differ primarily in host range and spore morphology. Given these biological observations, it has been proposed that the four species be united into the single species, *U. segetum*, with two morphologically different varieties, *U. segetum* var. *avenae* (Pers.) Brid. and *U. segetum* var. *hordei* (Pers.) Brid. (Lindeberg and Nannfeldt 1959; cited in Huang and Nielsen 1984). Our finding that these fungi have conserved restriction fragments carrying sequences similar to the a and b genes supports this relationship.

The hybridization results indicate that more than one region with b sequences may be present in some species. The clearest example is *S. reilianum*, which showed hybridization of the bE fragment to two different BamHI fragments. Although this result could indicate a BamHI site within the *S. reilianum* sequence, the conservation in size of one of the bands (8.5 kb) compared with *U. maydis* strains suggests that this is not the case. Additional support for the presence of more than one b sequence comes from the finding that attempts to clone the b sequences from one strain of *U. hordei* yielded two different cosmids (G. Bakkkeren and J. W. Kronstad, unpublished results). As discussed below, Groth (1975) has presented evidence for two genes conditioning mycelial growth in *U. hordei*. It is conceivable that these genes are the b-like sequences detected during the isolation of the cosmids from *U. hordei*.

The hybridization results also revealed weak hybridization of the a1 probe to DNA from one strain of *U. violacea* and of the a1 and a2 probes to DNA from a *MATa* strain of *S. cerevisiae*. While potentially interesting, these results must be regarded with caution until the hybridizing sequences can be characterized further. It is relevant in this context to note that sequence analysis of the a1 idiomorphs of *U. maydis* has identified a region with sequence similarity to STE3 (encoding the receptor for the pheromone a-factor) of *S. cerevisiae* (Bolker et al. 1992).

The hybridization of the a1 probe to DNA from *U. violacea* strain 002 may suggest that genes related to pheromone production or perception are present in this strain or simply that some other sequence with similarity to the a1 idiomorph is present.

It is perhaps not surprising, given the similarities in life cycles, that sequences similar to the a idiomorphs of *U. maydis* are present in the smut fungi with bipolar and tetratropic mating systems. It is especially interesting, however, that the b sequences are present in the smut species with bipolar mating systems. In *U. maydis*, two different b alleles must be brought together by cell fusion (thought to be controlled by the a locus) to establish the infectious dikaryon. In addition, at least 25 different alleles are known for the b locus and any combination of two alleles will establish the infectious dikaryon. Despite many years of genetic analysis (Fischer and Holton 1957; Thomas 1991), a multiallelic locus with a role in dikaryon formation or pathogenicity has not been described for the bipolar smut fungi. A single genetic locus (the a locus) with two alternate alleles is thought to control both fusion and formation of the infectious dikaryon.

Although a b locus has not been genetically defined in the smut fungi that show bipolar mating, two genes that
condition mycelial growth in haploid isolates of *U. hordei* have been described by Groth (1975). It is conceivable that these genes have functions like *b* and that these are the genes that we detect by hybridization with the *b* probes. Thus, a *b* function may be present in the bipolar smut fungi but the genetic requirement for multiple alleles may be absent. It might be the case that the *b* function is constitutive in the bipolar species and that cell fusion is sufficient to trigger formation of the infectious dikaryon. In this situation, *b* function in haploid cells would not be sufficient to give the filamentous, pathogenic phenotype because haploid strains are yeastlike and nonpathogenic. It is possible, however, that the combination of two different *a* idiomorphs in the fusion product somehow triggers the activity of the *b* genes. An alternative explanation for the apparent absence of a multiallelic *b* locus in the bipolar species would be to postulate that the *a* and *b* loci are tightly linked to create a single complex mating-type locus. In this case, it would be sufficient to have two alternate alleles at the *b* locus, each linked to different *a* alleles. Fusion events between cells with opposite *a* idiomorphs would automatically bring together different *b* alleles and lead to establishment of the infectious dikaryon. Genetically, the *a* locus would be the only locus recognized in mating tests. The detailed analysis of the organization of the *a* and *b* sequences in progress for *U. hordei* should resolve these issues and provide considerable insight into the differences between bipolar and tetrapolar mating systems in the smut fungi. In addition, further analysis of the *a* and *b* genes will contribute to our understanding of how these genes control the pathogenic development of the fungus leading to the production of teliospores, the inoculum for new plant infections.

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LITERATURE CITED


