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Electrophoretic karyotypes have been determined for strains representing fourteen pathogenic races of Ustilago hordei, and for strains of U. aegilopsidis, U. avenae, U. bullata, U. kolleri, U. maydis, U. nigra, and U. tritici. The differences in the distribution of chromosome sizes suggests that U. aegilopsidis, U. bullata, U. hordei and U. nigra are fundamentally different from U. avenae, U. kolleri and U. tritici and should continue to be considered to be separate species. U. maydis is unique in not having any chromosomes larger than 2,000 kilobases. Chromosome-specific DNA clone mini-libraries and random genomic DNA clone libraries have been constructed to allow identification of DNA probes from each linkage group. Chromosome-length polymorphisms have been extensively mapped among the U. hordei strains using these probes, and have allowed identification of ten homologous chromosomes. The chromosome that has sequences homologous to the rDNA from Tilletia caries varies in length by as much as 2,000 kb. A probe homologous to sequences in the largest chromosome (3,150 kb in 13 strains) identifies a disomic chromosome or a translocation

in strain 9.1. Total DNA content is shown to vary by as much as 35% among the strains representing the fourteen races of U. hordei. One chromosome-length polymorphism has been identified from members of one meiotic tetrad of U. hordei, while several other tetrads of U. hordei have been analyzed and each yields four identical karyotypes. A technique for the preparation of high quality samples for pulsed-field gel electrophoresis (PFGE) that obviates the need for preparing protoplasts is described. Heat shock has been used to generate a chromosome-loss mutant that is altered in its cellular and colony morphology, and has an altered mating response. Transformation of U. hordei with the replicating plasmid pCM54 is described; the fate of the transforming DNA has been investigated using PFGE, and the plasmid is shown to be present as three different size classes that comigrate with different forms of the plasmid produced in Escherichia coli. In conclusion, a model that associates the electrophoretic karyotype of a fungus with the dominant stage in its life cycle is presented.

Molecular Karyotyping of *Ustilago hordei* and Related Smut Fungi

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Molecular Karyotyping of Ustilago hordei and Related Smut Fungi

INTRODUCTION

General introduction

Smut fungi cause important diseases of many crop plants throughout the world and the biology of these fungi has been studied at a variety of levels. Covered smut of barley is caused by the fungus Ustilago hordei, and is a continuing problem where barley is grown commercially (Thomas, 1988). This disease system is ideal for the study of interactions between a host plant and its parasites. Even though there are many different modes of disease production, the bulk of diseases are caused by fungi. This is in part due to the ability of fungi to reproduce rapidly and disperse efficiently (Agrios, 1988). Other important plant disease agents include bacteria, viruses, mycoplasmas, mollicutes, and abiotic agents such as nutritive deficiencies or environmental perturbations. Fungi cause billions of dollars of lost revenue annually and lead to a tremendous decrease in productivity (Agrios, 1988). Part of the cost is due to the expense of controlling diseases. Measures that are exercised in control of fungal plant pathogens include production of disease-free propagative material, seed treatment, soil treatment, biocontrol, fungicide sprays and product treatment (Agrios, 1988). Fungi can cause damage at any stage of the plant life cycle and can interact with environmental factors in causing disease. It is often these interactions that make fungi difficult to control.

The interactions of fungi and their host plants are seen in a variety of different forms. In some respects the development of technology has opened up new windows into the interactions between plant and pathogen (Bennett, 1985). The first view of fungal disease was as a spiritual malady of the crop that led the plant to misdirect its energies and produce a diseased state (Agrios, 1988). As it was not until 1807 that the fungal nature of many plant diseases was established by Prevost, this is a relatively new science. The elucidation of the interactions between the plant and fungus followed. The recognition that genes in both the host and the pathogen could condition the infection of an otherwise susceptible host was made by Flor in 1942. The genetic analysis of fungi began in earnest in the 1950's, and many fungi were studied intensively (Pontecorvo and Roper, 1956). Several important plant pathogenic fungi that had been the focus of the earliest description of fungal pathogens fell outside the scope of this investigation. Smuts, rusts, and many of the root parasitizing basidiomycetes were not amenable to the studies undertaken, either because they could not be cultured, or because their sexual stage was not easily manipulated. Other fungi were not studied because they have no known sexual stage (Fincham et al., 1979).

The importance of the sexual stage in the generation of variability has been clearly demonstrated in the Puccinia graminis fsp. tritici-wheat interaction. A significant degree of stabilization of the host-based resistance was achieved by eradicating the alternate host upon which sexual reproduction of the fungus occurred (Agrios, 1988). While this phenomenon has been known since the 1930's, there has been no clear demonstration of the mechanism by which it might occur. The demonstration that fungal karyotypes are highly variable (Mills and McCluskey, 1990) has suggested a mechanism by which fungi might be able to generate a high degree of genetic variability. Finally, the transfer of DNA from one fungus to another has been demonstrated for a variety of fungi (Boguslawski, 1985; Wang and Leong, 1991), and

has an increasing potential for studies of plant-microbe interactions. This technique helps in the identification of genes, as well as in the elucidation of gene function. Ultimately, the technique of DNA-mediated transformation may allow for the manipulation of fungal host range, improved myco-herbicides, and biocontrol agents (Wang and Leong, 1991).

Phylogenetic context

Many common fungi are basidiomycetes. The name basidiomycete describes the elongate shape of the cell that bears the sexual spores (Scagel et al., 1982). Most basidiomycete fungi have similar life cycles. Gametic basidiospores give rise to a monokaryotic mycelium that must find a compatible mycelium to progress further in the life cycle. Upon meeting, compatible mycelia anastamose and develop the vegetative dikaryon. This dikaryon is the main vegetative form and can produce many fruiting structures. The fusion of compatible nuclei, and subsequent meiotic divisions, take place in the basidia (Scagel et al., 1982). The ultrastructure of the basidium further divides the Basidiomycotina into two groups. The heterobasidiomycetes are characterized as having spores borne on a septate basidia. Many of the fungi in this group are pathogens of higher plants (Agrios, 1988), and among these, the basidium (often called a promycelium) is produced from a germinating teliospore. Among the smut fungi are species that have teliospores that germinate to produce an ordered tetrad of basidiospores (Fischer and Holton, 1957). These spores can often be isolated, cultured, and mated in culture, although completion of the life cycle requires infection of a compatible host plant. In many cases, however, the haploid spores fuse directly on the basidium, or are bypassed with the dikaryotic stage being produced directly from the promycelium. The rust fungi are similar to the smuts, in that their basidia are produced from teliospores, and they are also common pathogens of higher plants. In

most cases, however, the rusts are obligate pathogens, and cannot be cultured. The evolutionary origin of the heterobasidiomycetes remains unclear (Walker and Doolittle, 1982).

The majority of basidiomycetes are homobasidiomycetes whose spores are borne on one nonseptate cell (Scagel et al., 1982). The fungi that are commonly seen and recognized as mushrooms are almost exclusively from this group. Many basidiomycetes are saprophytes, and are important in degradation of organic material. Still others have developed symbiotic associations with the roots of higher plants (Scagel et al., 1982).

The second major subdivision of fungi is the Ascomycotina. The life cycle of ascomycetes differs from the basidiomycetes in that each individual anastamosis typically leads directly to the production of a fruiting structure (Scagel et al., 1982). The sexual spores produced by these fungi are formed within the cell wall of the mother cell, and the final structure is called an ascus, from the Latin for sac. Many ascomycetes are plant pathogens and reproduce freely by airborne or waterborne conidia. For some, the asexual stage is a primary means of reproduction and the sexual stage is only relevant when conditions deteriorate (Scagel et al., 1982). Finally, the imperfect fungi are those for whom a sexual stage is unknown (Fincham et al., 1979). Some fungi were previously classed as imperfect, and are known by their imperfect name, but recently have had sexual stages described and are now known by both names (Scagel et al., 1982).

Many fungi have evolved to have intimate interactions with plants (Agrios, 1988). These interactions are pathogenic, commensalist, or mutually beneficial. Smut fungi exist as pathogens on angiosperm plants (Fischer and Holton, 1957). That smuts do not form associations with plants other than angiosperms is interesting. The rusts which are taxonomically related to the smuts have associations with many plants,

including gymnosperms, and often have different stages in their life cycle on plants from different phyla (Agrios, 1988). Many smuts have as their hosts plants in the Graminae, or grass family and it is on these plants that the smuts cause the most important economic damage.

Mating and sexuality

Mating between compatible individuals is necessary for completion of the life cycle in most fungi. The genetics of this interaction are studied in a number of systems (Herskowitz, 1988; Kronstad and Leong, 1989; Raper, 1988). Mating type genes in fungi allow strains that differ in the ideomorph at the appropriate gene locus to mate. Fungi exhibit the most varied forms of mating limits and interactions (Bull and Pease, 1988). While many systems have simple +/- interactions, many fungi have hundreds of potential "genders" with any combination of different genders being compatible. U. hordei has a simple bipolar mating system (Thomas, 1988). Each meiosis produces four gametes, two of which being capable of mating with either of the other two, and not with one another. Other smuts that share this type of mating structure include U. nigra, U. kolleri, U. avenae, and U. nuda. While U. hordei and several of these other fungi clearly share mating type complementarity, they may fail to reproduce successfully even though they may establish a stable dikaryon. Within the family of the smut fungi, several genera have similar mating structures. Tilletia caries has a bipolar mating system. Conversely, there are fungi within the genus Ustilago that differ with respect to mating type structure. U. maydis, for example, has a tetrapolar mating system. This requires that each component of a given anastomosis differ at two different loci and limits the ability of basidiospores from one teliospore to interbreed.

Inbreeding is a mechanism that may serve to fix particular genotypes that are well adapted to a particular niche. It was shown by Flor in 1955 that genes in the

pathogen, Melampsora lini, the causal agent of flax rust, condition the virulence of the individual pathogen, and that these genes have counterparts in the host that condition the host's ability to withstand the pathogen's infection. In the pathogen, these genes are called avirulence genes, and the avirulent state is dominant. In the host, these genes are called resistance genes, and the resistant condition is dominant. A given fungal isolate will have the ability to infect only those host cultivars that have no dominant alleles at resistance loci that correspond to dominant avirulence genes in the pathogen. A population of pathogens with the ability to infect a certain range of host cultivars is called a physiologic or pathogenic race. Vanderplank pointed out in 1982 that a race designation was a shorthand way to distinguish between different pathogen genotypes.

Genetics of host-parasite interactions for Ustilago hordei

U. hordei has a described system of pathogenic races that dates to the 1920's. The ability to define the pathogenic races is dependant upon the availability of host cultivars that differ in their susceptibility to the pathogen. Faris (1923) described the relative importance of the fungal isolate versus a wide battery of environmental conditions in determining the pathogenicity of U. hordei upon a variety of barley cultivars. He was able to conclude that several different races of the fungus existed, and several of the host cultivars used in these pioneering studies are still employed. In another paper, published in 1924, Faris was able to describe five forms of the fungus, and was able to predict the existence of other races.

Building upon these studies, Tapke described eight races in 1937, and brought the number to thirteen in 1945. Over 400 isolates were utilized in these studies, including collections from 33 states. There was a clear bias in the distribution of races, with some races being found in only one state or region. Race 6 was found to be the most common, representing over 61 percent of the total collections, and three races, 1,

5, and 6 comprised 86 percent of the total collections. There was a clear correlation between the type of barley (winter versus summer) grown in a region and the prevalence of certain races.

Thomas and Person (1965) were able to distinguish between different pathogenic strains that had fallen into the "avirulent" category as described by Tapke (this included infections of 0-5%). To do so, they crossed sporidial strains derived from three teliospores to generate 36 dikaryons. These dikaryons were inoculated onto three barley cultivars, which allowed for the detection of two alleles that were segregating at one locus in the pathogen. In 1971, Sidhu and Person continued this work and elaborated upon the nature of higher levels of virulence (5-86% infection). These studies served to distinguish between two avirulence genes that controlled the interaction with the barley cultivars Excelsior or Hannchen and Vantage. Sidhu and Person were also able to clearly demonstrate that a gene-for-gene relationship existed between barley and U. hordei (1972). To establish the nature of the resistance genes in the host, they carried out crosses of the host plants, and studied the inheritance of susceptibility of the resulting progeny to describe the nature of the higher levels of virulence. Further studies by Ebba and Person (1975) allowed an understanding to be formed regarding the impact of varying environments and differing genetic backgrounds on the interaction between an avirulence gene and its corresponding resistance gene. The conclusion was made that certain pathogen strains might contain duplications of individual avirulence genes.

While all of the aforementioned studies were carried out without reference to the degree of infection of an individual plant, Groth et al. (1976) were able to distinguish between different measures of disease expression. The two infection types measured distinguished between the percent of plants showing at least one head smutted and the percent of smutted heads in those smutted plants. The suggestion was made that there

was genetic control of both levels of disease expression. The possibility of multiple infection, of parasexual recombination or of somatic mutation was not discussed.

Aggressiveness, a quantitative measure of pathogenicity, was studied by Caten et al. in 1984. These studies examined the possibility that there are genes that are not avirulence determinants that condition the ability of a given fungal dikaryon to produce disease on compatible host plants. This was carried out in response to the observation that the least avirulent pathogen strains rarely produce disease in excess of 50-60 percent infection. The conclusion was made that the factors affecting aggressiveness were fitness factors that may influence the evolution of new pathogenic races. Person and Cherewick demonstrated (1964) that more than one species of smut may infect an individual barley plant. In this context, the aggressiveness factors described by Caten et al. (1984) may be responsible for the dominance of one infecting dikaryon over another. Further studies by Cherewick (1967) demonstrated that different smuts, in this case U. hordei and U. nigra, could cross and contribute to the pathogenic capability of the offspring. Moreover, there was a suggestion that an avirulence gene derived from this (so called) interspecific cross was recessive. Building upon these studies, Fullerton and Nielsen (1973) demonstrated that identical avirulence genes could be detected in strains of U. hordei and U. nigra and suggested that one fungus could serve for both in host resistance testing programs.

Emara (1972) differentiated between virulence and aggressiveness, and demonstrated that there is a significant genetic contribution towards aggressiveness in dikaryons formed by mating all possible combinations of the thirteen described races. Aggressiveness was defined as the extent of infection of a susceptible cultivar. This study shows both that aggressiveness is variable among the races, and that inter-race crosses are not prohibited. Indeed, there appeared to be no barrier to sexual processes between representatives of different races. While virulence is controlled by individual

genes, this study was carried out using Odessa, the universally susceptible cultivar, and thus allows for an estimate to be made of the nature of control of aggressiveness. Emara concluded that aggressiveness was under the control of many genes. The uniformity of the response using sporidia of either mating type from a given race was used as evidence that the genes involved in conditioning aggressiveness are all borne chromosomally. While not stated by Emara, this also suggests that the strains used in this study were highly inbred. These results were expanded upon by Emara and Sidhu (1974) and the influence of environmental effects was incorporated by Emara and Freake (1981).

Thomas described the interaction of avirulence genes in 1976, and concluded that there was basis for identification of additional resistance genes in the host. Pedersen et al. (1977) described a recessive gene, ihi, that limits infection in a compatible reaction. The mechanism of this decrease is a reduction in the length and number of infection hyphae produced in a compatible mating reaction. The authors were careful to distinguish between the reduction of infection, presumably because the host outgrew the pathogen, and a true avirulence/resistance interaction.

Pedersen and Keisling (1979) examined the ability of U. hordei to adapt genetically to resistant host plants. They inoculated 84 sporidial matings from 21 teliospores onto nine differential cultivars. Only in two crosses did they find any increase in pathogenicity, and both resulted from crosses of progeny from one teliospore. One of the matings had lost avirulence on the cultivar Nepal, while the other lost avirulence on the cultivar Hannchen. Two tetrads derived from spores produced from the first mating (one from Odessa and one from Nepal) were tested for their avirulence phenotype, and were found to be avirulent on Nepal. Likewise, two tetrads from the second cross were selfed, and in this case, the spores produced on Odessa barley were still pathogenic only on Odessa, while spores produced on the cultivar

Hannchen had lost avirulence on the cultivars Hannchen, Lion and Trebi. The genes for avirulence on Lion and Trebi are 13 map units apart; the genes for avirulence on Hannchen, and Lion and Trebi are unlinked. While no obvious mechanism that could account for these changes was forthcoming, the hypothesis that a recessive regulator gene controlled these changes was strongly advanced.

U. hordei is convenient to study because it maintains its sporidial morphology well in culture. Groth (1975) has described two dominant genes that control the extent to which sporidial strains are converted to a mycelial habit. The distribution of mycelial strains among samples derived from Tapke's original thirteen races led Groth to conclude that these strains had been highly inbred. Conversely, Groth suggested that inbreeding was uncommon in wild isolates.

The close relationships of several Ustilago species were demonstrated by Huang and Nielsen in 1984. Their studies used a permissive host, Agropyron tsukushiense var. transiens, to cross U. hordei and U. nigra with the smuts of oat (Avena sativa) U. avenae and U. kolleri. Features of the spore wall morphology were discussed, and the nature of factors that limit the effectiveness of the interspecies hybridizations was explored. The conclusion that only one gene (encoding features expressed as spore wall echinulation) separates U. hordei from U. nigra was advanced with specific reference to the untenability of separating species by single gene differences.

While U. hordei and U. aegilopsidis will readily anastomose, completion of the life cycle only occurs on A. tsukushiense (Thomas and Huang, 1985). The ability to obtain F1 teliospores, and to isolate viable basidiospores upon germination of these teliospores suggests that the differentiation of these fungi into separate species may be excessive. As the major distinction is in host range, perhaps distinguishing these as pathovars would be more appropriate.

Pulsed-field gel electrophoresis and molecular karyotypes.

A recent development allows study of the fluidity and organization of the genomes of fungi and other organisms that have relatively small genomes without the need for phenotypic markers. The technique, called pulsed-field gel electrophoresis (PFGE), allows for the separation of fungal chromosomes in an agarose solidified gel in a reorienting electric field (Schwartz and Cantor, 1984). The separated chromosomes and the act of separating them are useful for asking questions about both genetic linkage (Magee et al., 1988) and chromosome structure (Game et al., 1989). The number of chromosomes, and their respective sizes are the most readily derived information, and these data are collectively referred to as the electrophoretic karyotype for an organism or strain (Carle and Olson, 1985). While yielding different information than a cytological karyotype, electrophoretic karyotypes fill a much needed niche because many fungi have been historically lacking any significant cytology (Fincham et al., 1979).

Those fungi that have been analyzed cytologically have been from among those where meiosis occurs concurrently in a large number of cells: the ascus of Schizophyllum commune (Radu,1974), or the basidium of Coprinus lagopus (Lu, 1794). Mitotic divisions are best studied in the mitosis that occurs at the termination of ascus formation in Neurospora and other ascomycetes (McClintock, 1945; Elliott, 1960; Carr and Olive, 1958), and although the cytology of fungal mycelia has been described as "notoriously difficult" (Fincham et al., 1979), good mitotic divisions have been seen in A. nidulans (Robinow and Caten, 1969) and N. crassa (Somers et al., 1960). For many fungi, cytology has been hampered by the small sizes of the chromosomes (Fincham et al., 1979), poor metaphase condensation, asynchronous separation of bivalents, and the persistence of the nuclear membrane (Scagel et al.,

1982). Four chromosomes were detected in U. hordei in 1942 (Kharbush) and similar numbers were detected in eleven other smut fungi (Wang, 1934).

In the context of modern molecular biology, PFGE allows a cloned gene or a cloned random DNA fragment to identify the physical location in a genome of a corresponding gene or homologous sequence (Kinscherf and Leong, 1988; Orbach et al., 1988; McCluskey and Mills, 1990). Genes and random DNA probes can be mapped relative to one another by taking advantage of the ability to cut DNA at specifically defined sites of varying frequency with bacterial restriction enzymes (Fan et al., 1989). A recent development utilizing short stretches of triple stranded DNA allows for introductions of single cuts into agarose-immobilized chromosomes (Strobel and Dervan, 1991). Electrophoretic karyotypes are useful in distinguishing between different fungi, and in several cases have been used as a taxonomic feature with a high degree of relevance (Steensma et al., 1988). The high degree of within-species variability does not always confound studies of species limits. The variability between species can be dramatically different and the ability of genomes from different species to recombine in interspecific hybrids appears somewhat restricted. Hybrid karyotypes appear to represent the sum of the parental karyotypes (Steensma et al., 1988) rather than a recombined karyotype as is seen in crosses of S. cerevisiae strains that are known to differ from one another in their karyotypes (Ono and Ishino-Arao, 1988).

That the DNA molecules resolved in PFGE represent whole intact chromosomes is surmised largely based on correlative data. For S. cerevisiae, the correlation between the number of genetically defined linkage groups suggests strongly that each band represents an intact chromosome. The mapping of a deletion by both genetic and electrophoretic means (Ono and Ishino-Arao, 1988) and the analysis of recombinant karyotypes shows that the molecules involved are chromosomes. Other fungi for which a good correlation between the number of electrophoretically-resolved

and genetically-defined chromosomes has been demonstrated include Aspergillus nidulans (Brody and Carbon, 1989; Elliott, 1960), Neurospora crassa (McClintock, 1945; Orbach et al., 1988), and Candida albicans (Magee et al., 1988).

For Ustilaginales and other phytopathogenic fungi that are recalcitrant to classsical genetic analysis, molecular karyotypes offer another approach to carrying out genetic mapping. While chromosomes separated in this manner yield a karyotype that contains different information than the information present in cytologically-determined karyotypes, there are several advantages to electrophoretically-defined karyotypes. The ability to define lengths to individual chromosomes implicitly defines the amount of information that can be stored on a chromosome. The ease of mapping genes to linkage groups by southern hybridization and examining recombined chromosomes makes possible genetic analysis without defined phenotypes. In systems where classical genetics can be combined with PFGE, polymorphisms that have no phenotypes associated with them can be mapped (eg. Ono and Ishino-Arao, 1988). Finally, the demonstration that DNA can be cloned in chromosome-specific mini-libraries promises to accelerate the pace of identification of linkage for new genes, and for organisms whose genetics are slow or non-existant (Marguet et al., 1988).

The development of electrophoretic karyotypes of phyto-pathogenic fungi has spawned new theories to describe the ability of these fungi to overcome host resistance genes. Pedersen and Keisling (1979) described changes in virulence of Ustilago hordei that can be best understood in terms of a breakdown of a disomic state. In this case, a recessive allele of an avirulence gene could persist in a population but never be expressed until the appropriate dominant allele was lost. While this was not the mechanism proposed, it is attractive for a variety of reasons including a simple probabilistic basis. Mundt (1991) has proposed that karyotypic reorganization in fungi is involved in the instability of major resistance genes in many crops.

There is good reason to believe that many, if not most, strains of wild fungi are aneuploid. This is borne out by studies by Kinscherf and Leong (1988) who worked with U. maydis, by Miao and VanEtten (1989) in Nectria, by Rollo et al. (1989) in Phoma tracheiphila and by Bakalinsky and Snow (1990) for wine strains of S. cerevisiae. Other fungi with variable karyotypes are described in Mills and McCluskey (1990). Aneuploidy could allow for the maintenance of a wealth of genetic information that is either not needed under the present conditions, or is masked by other genes (Tolmsoff, 1983). Day (1972; 1978) predicted that smut fungi would have a high frequency of aneuploidy. The extent of aneuploidy will only become known as the present techniques are applied to a large number of wild isolates of fungi.

Development of pulsed-field technology

PFGE was developed to allow separation of DNA molecules larger than 20 -25 kb. For molecules smaller than this, the sieving effect of agarose allows separation based on size; for molecules larger than 25 kb, velocity is independent of size (Schwartz and Cantor, 1984). The movement of large DNA molecules in a linear electric field is described as reptation, with one end leading as the molecule "snakes" through the pores of the agarose matrix (Olson, 1989). In PFGE, DNA moves by a yet undefined mechanism. The models proposed stipulate that molecules move by reptation, but that when the field reorients a molecule must reorient before it can make any progress in the new direction. According to this model, smaller molecules are able to reorient faster than larger molecules, and hence they move faster than the larger molecules (Olson, 1989; Southern et al., 1987). Schwartz and Cantor separated S. cerevisiae chromosomes in the first PFGE experiments, but their system did not allow linear separation, and hence size determination was not possible (1984). Carle and Olson (1985) applied a modification of this system, called orthogonal-field-alternation

gel electrophoresis (OFAGE), to describe a karyotype for S. cerevisiae that showed good correlation to the genetic map (Mortimer and Schild, 1985). This system could only maintain a limited electric field, and hence was useful for only a few lanes in one gel. Other systems have been developed that avoid this problem, but each has problems of its own. Field-inversion gel electrophoresis (Carle et al., 1986) allows separation of DNA molecules in many lanes, but does not have the same resolving power as OFAGE, and under some situations, size is not well correlated with migration (Ellis et al., 1987; Zimm, 1988). Transverse-alternating-field electrophoresis utilizes a vertical gel orientation and can separate DNA molecules in several lanes uniformly, but has a non-uniform reorientation angle, and hence migration is not linearly correlated with size (Gardiner et al., 1986).

The system developed by Chu et al. (1986) and designated contour-clamped homogeneous electric field (CHEF) allows for as many as forty lanes to be run in parallel without the distortion common to early systems. Since this is essentially OFAGE with a uniform field, size determinations are relatively simple and accurate. The most recent development builds upon the CHEF system, but allows for different reorientation angles and interrupts. This system, programmable, autonomously controlled electrodes (PACE), allows for faster separations of large DNA by using a steeper reorientation angle. The use of interupts, momentary field inversions or pauses, is said to allow separation of large DNAs without band broadening which is thought to be due to the inability of some molecules to reorient rapidly (Olson, 1989).

The applicability of PFGE to fungi was originally limited to species and strains from which high quality protoplasts could be reliably produced (Olson, 1989). Protoplasts are produced using one of many cell-wall degrading enzymes or preparations that are produced by mycopathogenic fungi or snails (Hamlyn et al., 1981). The appropriate enzyme, osmotic stabilizer and concentration, and reaction

conditions must be determined for each species, and sometimes differ for different strains of an individual species. Moreover, the enzymes used are expensive and often vary in activity among different manufacturing lots (Hamlyn et al., 1981). Once produced, protoplasts are embedded in agarose with osmotic stabilizer then treated at 50 °C in detergent, proteinase and a chelating agent (ethylene diamine tetraacetic acid, EDTA) to protect the DNA against the activity of DNA degrading enzymes. These samples are then stored under EDTA at 5° C. This method of sample preparation was used by Carle and Olson (1984) in their original description of PFGE. Bellis et al. later described a technique where the cells were embedded in the agarose matrix before treatment with enzymes (1987). Recently McCluskey et al. (1990) have described the ability to prepare high quality PFGE samples from non-protoplasted material. Apparently the DNA is able to migrate directly through the cell wall. This is useful for a variety of organisms, and even allows for colonies to be removed from the surface of agar-solidified medium and prepared directly for electrophoresis.

Transformation

The ability to transfer foreign DNA into a fungus and have it expressed either as DNA that has integrated into the host cell's genome, or as an autonomously replicating DNA molecule is an important capability in molecular biology (Bennett, 1985) . Transformation allows for the identification of genes by complementation of known mutations. For phytopathogenic fungi, transformation of a virulent strain to avirulence is a proposed mechanism for identification of avirulence genes (Wang and Leong, 1991). Transformation also allows for the elucidation of patterns of gene expression through the use of reporter genes such as the ß-glucuronidase (GUS) gene (Roberts et al., 1989). Finally, transformation promises to find broad application in industrially important fungi.

Transformation is accomplished by exposing competent cells to DNA that has a selectable phenotype. The transforming DNA can be linear or covalently closed. Linear DNA is apparently highly recombinogenic, and integrates into the host genome (Wang et al., 1988). Covalently closed DNA is most efficiently used when capable of autonomous replication (Tsukuda et al., 1989). The first step in accomplishing transformation is in generating competent cells. For many fungi, the best approach is to form protoplasts, and mix them with DNA in the presence of polyethylene glycol. For some fungi, the ability of protoplasts to regenerate is good, and the frequency of transformation by this approach is several thousand transformants per microgram of transforming DNA (Tsukuda et al., 1989). For some fungi, regeneration is a limiting step and the best frequencies are several hundred transformants per microgram of transforming DNA (Holden and Leong, 1989; Specht et al., 1988). Another technique takes advantage of the ability of lithium acetate to facilitate DNA uptake by intact cells (Bej and Perlin, 1989). The frequency of transformation achieved with this technique is relatively poor, but offers some advantages where other approaches are not practical or when having a high frequency is not important. Yet another technique, called electroporation, is thought to take advantage of the ability of electric pulses to transiently disturb the integrity of the cell membrane. This approach is commonly used to transform mammalian cells, and is useful both for fungal protoplasts, and for intact fungal cells (Delorme, 1989). One relevant example of a gene that was identified by transforming an appropriate recipient is the b mating type locus from U. maydis (Kronstad and Leong, 1989).

Goals of the present study

The present study has focused on identification of karyotypes of Ustilago species. The variability in the karyotypes of strains derived from the fourteen races of

U. hordei and the identification of linkage groups that vary among these strains has been studied in depth. The ability of the genome to change when presented with lethal environmental stress has been assessed using heat shock. The taxonomy of the cereal infecting smut fungi has been investigated, and electrophoretic karyotype has been supported as a meaningful and fundamental characteristic for distinguishing species. Other goals that have been addressed include the development of techniques for preparation of PFGE samples and chromosome specific DNA clone libraries. Finally, the present study addressed the ability to transform protoplasts of U. hordei with a replicating plasmid, and the fate and conformation of the transforming DNA.

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Identification and Characterization of Chromosome-Length Polymorphisms Among Strains Representing Fourteen Races of Ustilago hordei

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ABSTRACT

Electrophoretic karyotypes were determined for monosporidial strains that represent each of the 14 races of Ustilago hordei. A unique karyotype was observed for each strain and the number of chromosome-size DNA bands varied from a minimum of 15 to a maximum of 19, and their approximate size distribution ranged from 170 kilobases (kb) to 3,150 kb. Strains were also obtained from one ordered and six unordered meiotic tetrads representing four of the races. Karyotype was typically conserved among members of individual tetrads and between tetrads representing the same race. The strains from one tetrad from race 14, however, showed 2:2 segregation for one chromosome-size DNA band. Conversely, the total soluble protein from 14 of these strains fractionated by SDS-PAGE did not vary to the same extent as the karyotypes. Southern hybridization was used to identify chromosome-length polymorphisms and to map genomic fragments and heterologous conserved genes to the chromosomes.

Additional Keywords:. CHEF electrophoresis, karyotypic analysis, smut fungi, heterobasidiomycete, PFGE.

INTRODUCTION

Ustilago hordei Pers. (Lagerh) is the causal agent of covered smut of barley (Hordeum vulgare L.), a disease of continuing importance where barley is commercially grown (Thomas, 1984). The importance of this pathogen has prompted classical genetic investigations (Thomas, 1988) and, more recently, molecular genetic analysis (Holden et al., 1988). Heterokaryotic infectious hyphae produced by the fusion of compatible haploid basidiospores penetrate the coleoptile of a germinating barley seedling and reside in the developing meristem. The host plant remains essentially symptomless until heading, when the teliospores are formed and replace the kernels on diseased plants (Thomas, 1988). There is no vegetative or asexual reproduction of U. hordei and the proximity of compatible basidiospores on the probasidium of a germinated teliospore increases the probability that reproduction will occur by inbreeding (Caten, 1987). A well characterized physiologic race structure comprising at least fourteen races has been described for U. hordei (Tapke,1945; Pedersen and Kiesling, 1979 and Pedersen, personal communication), and the races are sexually compatible (Emara, 1972). Moreover, a gene-for-gene relationship (Flor, 1955) has been described for this host-parasite system by Sidhu and Person (1972).

The ability to fractionate chromosome-sized DNA molecules by pulsed-field agarose gel electrophoresis (PFGE, Schwartz and Cantor, 1984) has led to new approaches for karyotypic analysis of fungi, whose cytology is often intractable. More recently, the development of contour-clamped homogeneous electric field (CHEF) PFGE has made feasible the comparison of the chromosomes of numerous organisms or isolates in a single gel (Chu et al., 1986). Using PFGE, the haploid number of

chromosomes of laboratory strains of U. maydis (DeCandolle) Corda was determined to be at least 20 and ten highly conserved heterologous genes from fungi and animals were mapped by Southern hybridization (Southern, 1975) to chromosome-sized DNAs of this fungus (Kinscherf and Leong, 1988). Karyotypic variability was shown to correlate with pathogenic variation in Nectria haematococca Berk. & Broome (Miao and VanEtten, 1989), and has been used as a criterion for confirming the distinction between diploid and polyploid isolates of Phytophthora megasperma Drechs. (Howlett, in press). Other fungi in which genes have been mapped to chromosome-sized DNAs include Saccharomyces cerevisiae Hansen (Carle and Olson, 1985), Neurospora crassa Shear & Dodge (Orbach et al., 1988), and Candida albicans (Robin) Bekhout (Magee et al., 1988). Furthermore, chromosome length polymorphisms have been observed among different laboratory strains of S. cerevisiae (Ono and Ishino-Arao, 1988).

In this paper we report the electrophoretic karyotype of strains representing 14 races of U. hordei. A high degree of karyotypic variability was observed among individual strains representing each of the races but not among strains derived from individual meiotic tetrads, although the segregation of a chromosome-size polymorphism in one meiotic tetrad was observed. The strains show a high degree of similarity of the total soluble protein complement using SDS-PAGE. We have begun to define linkage groups using random DNA fragments.

MATERIALS AND METHODS

Fungal and bacterial strains, plasmids and media

Teliospores from collections representing the 14 races of Ustilago hordei (Table II.1) were kindly provided by W.L. Pedersen, and are derived from the original isolates described by Tapke (1945). Monosporidial strains of U. hordei were isolated from teliospores that were germinated on Potato Dextrose Agar (PDA, Difco Laboratories, Detroit, MI) at room temperature. Germinated teliospores containing four primary basidiospores were removed on a small block of agar with a fine glass needle and vortexed in 500 µl of Potato Dextrose Broth (PDB, Difco). The entire cell suspension was spread onto fresh PDA with a glass rod, and the individual colonies that emerged were transferred to PDA slants where they were maintained and transferred bimonthly. Alternatively, ordered tetrads were isolated with a Sensaur micromanipulator as described by Thomas (1988). Escherichia coli DH5α was used as host for plasmid transformations. Selections were carried out in LB medium with 50 or 100 µg/ml ampicillin, when appropriate. The plasmids used and constructed in this study are presented in Table II.1.

Chromosomal sample preparation.

Chromosome-sized DNA was prepared for electrophoresis by a modification of the method of Kinscherf and Leong (1988). Briefly, haploid sporidia grown for 18 hr in 100 ml of PDB at 28° C were harvested by centrifugation in a Sorvall SS34 rotor at 5000 X g for ten min. The cell pellet was suspended in 10 ml of a solution containing

Table II.1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Ustilago hordei		
1.3	Product of unordered tetrad of race 1	Pedersen[a], Tapke, (1945)
2.1	Product of unordered tetrad of race 2	Tapke, (1945)
3.1	Product of unordered tetrad of race 3	Tapke, (1945)
4.1	Product of unordered tetrad of race 4	Tapke, (1945)
5.1	Product of unordered tetrad of race 5	Tapke, (1945)
6.1	Product of unordered tetrad of race 6	Tapke, (1945)
7.1	Product of unordered tetrad of race 7	Tapke, (1945)
8.1	Product of unordered tetrad of race 8	Tapke, (1945)
9.1	Product of unordered tetrad of race 9	Tapke, (1945)
10.1	Product of unordered tetrad of race 10	Tapke, (1945)
11.1	Product of unordered tetrad of race 11	Tapke, (1945)
12.1	Product of unordered tetrad of race 12	Tapke, (1945)
13.1	Product of unordered tetrad of race 13	Tapke, (1945)
14.1a	Product of unordered tetrad of race 14	Pedersen and Kiesling, (1979)[b]
14.1b	Product of unordered tetrad of race 14	Pedersen and Kiesling, (1979)
14.1c	Product of unordered tetrad of race 14	Pedersen and Kiesling, (1979)
14.1d	Product of unordered tetrad of race 14	Pedersen and Kiesling, (1979)
U. maydis	Minnesota field isolate, 1987	K.B. Johnson
Escherichia coli	DH5α	Focus 8(2):9 (1986)
Plasmids		
pUC18	Ap^r cloning vector	Norrander et al. (1984)
pSF8	Aspergillus nidulans actin gene	Fidel et al. (1988)
pZHS3	Saccharomyces cerevisiae cox III	Muller et al. (1984)
pOSU4011	6 kb U. hordei EcoRI fragment in pUC18	This study
pOSU4012	1.2 kb U. hordei EcoRI fragment in pUC18	This study
pOSU4015	3.2 kb U. hordei EcoRI fragment in pUC18	This study
pOSU4117	6 kb U. hordei EcoRI fragment in pUC18	This study

[a]Ap^r, ampicillin-resistant; kb kilobase

[b]All U. hordei samples were provided by W.L. Pedersen as teliospores.

[c]Also listed as 14.1 in figures.

[d]Oregon State Univ., Corvallis

[e]Published by Bethesda Research Laboratories, Gaithersburg, MD.

10 mM ethylene diamine tetraacetic acid (EDTA) pH 8, 25 mM 2-mercaptoethanol and incubated with gentle shaking for 20 min at room temperature. The cells were centrifuged as before and the pellet was suspended in 4.5 ml of 0.8 M sorbitol, 50 mM sodium citrate pH 5.8 (SC50). A 0.5 ml aliquot of SC50 containing 50 Units/ml Novozyme 234 (Calbiochem, La Jolla, CA) and 200 mM 2-mercaptoethanol was added to each sample, and the cell suspension was incubated at room temperature for approximately two hr with gentle shaking. The protoplast mixture was centrifuged at 300 X g for ten min and the pellet was gently suspended in 100-200 µl of SC50 to a final concentration of approximately 5 X 10^8 protoplasts/ml. An equal volume of 2.5% low melting temperature agarose (FMC Bioproducts, Rockland, ME) dissolved in SC50 was added to each sample and the entire mixture was gently pipetted into a well of a precooled plug casting mold (Bio-Rad Laboratories, Richmond, CA). The plugs were incubated from 18 to 24 hr at 50° C in a solution containing 1 mg/ml protease (type XIV, Sigma St. Louis, MO), 0.45 M EDTA, pH 8 and 1% SDS. They were subsequently rinsed with 0.5 M EDTA (pH 8), and thereafter stored at 5° C in the same solution.

CHEF electrophoresis conditions

Electrophoresis was carried out in a Bio-Rad CHEF DR-II electrophoresis system. Bands in the 100 to 1,500 kilobase (kb) range were resolved by electrophoresis through 1% agarose (Sigma type II, medium electroendoosmosis) gels at 14° C in 0.5X TBE buffer (Maniatis et al., 1982) for 15 hr at 200 V with a 70 sec switch interval, followed by 11 hr at 200 V with a 120 sec switch interval. Resolution of bands in the 1,500 to 3,500 kb range was accomplished using a modification of the parameters described by Howlett (1989) to resolve Phytophthora chromosomes. The chromosome-sized DNA bands were visualized by staining with 0.5 mg/ml ethidium bromide in 0.5X TBE for 30 min and photographed after destaining overnight in 0.5X TBE. S. cerevisiae

and Schizosaccharomyces pombe Linder chromosome size standards were obtained from Bio-Rad Laboratories.

Source of DNA probes

Total genomic DNA was isolated from a haploid strain of race 8 using the method of Specht et al. (1982). The DNA was digested to completion with EcoRI, ligated into pUC18, and the entire mixture was used to transform E. coli DH5α according to the methods of Maniatis et al. (1982). Plasmid DNA mini-preps were obtained by alkaline lysis according to the method of Birnboim and Doly (1979). The inserts in randomly selected recombinant plasmids were released by digestion with EcoRI, and purified from low-melting temperature agarose gels using Elutip-d (Schleicher & Schuell, Keene, NH) according to the manufacturers' instructions. Radiolabelled DNA was prepared using the random priming technique of Feinberg and Vogelstein (1983) and labelled to a specific activity of 10^8 to 10^9 cpm per microgram of DNA.

DNA hybridizations

Gels were blotted to nylon membranes (Genetran, Plasco, Woburn, MA) according to the methods of Orbach et al. (1988). Blots were prehybridized overnight in 20 ml of 3X SSPE (Maniatis et al., 1982), 50% formamide, 5% Dextran sulfate, 5X Denhardt's solution, 0.1% SDS, 100 μg/ml salmon DNA at 42° C. Hybridization reactions were carried out in 10 ml of the same solution without the salmon DNA, but containing 10^7 to 10^8 dpm of labelled probe DNA at 42 ° C for 16 to 36 hr. Blots were washed in 120 ml of 3X SSPE containing 50% formamide and 0.1% SDS for 15 min at 42° C, followed by two successive washes of 30 min at 60° C in 2X SSPE and 1X SSPE, each also containing 0.1% SDS. Membranes were blotted dry and placed on a clean sheet of 3MM paper and wrapped in plastic film. X-ray film (X-Omat AR, Kodak,

Rochester, NY) was exposed overnight at -70° C in Kodak cassettes containing regular intensifying screens.

Protein sample preparation

Monosporidial cultures grown to stationary phase in PDB were harvested by centrifugation at 6000 X g for 10 min. Each cell pellet was washed once in 40 ml 10 mM Tris-HCl, 1 mM EDTA, 25 mM NaCl, pH 7.5 and the final pellet was frozen at -20° C. A 250 µl aliquot of the same buffer was added to each sample which was kept on ice, and the entire mixture was sonicated for 30 sec at one half maximum power in a "sonifier cell disruptor" (Model W185, Heat System Ultrasonics, Farmingdale, NY). Cellular debris was removed by centrifugation at 10,000 X g for 10 min and the supernatant was heated to 100° C for 10 min and then frozen at -20° C. The proteins were boiled in sample buffer containing 1% SDS and separated by SDS-PAGE (Laemmli, 1970) and visualized with Coomassie brilliant blue stain (type R, Sigma).

RESULTS

Protoplast preparation

Protoplasts were observed after incubation of secondary sporidia of U. hordei in Novozyme for 1-2 hr using a Wild EB-11 light microscope. Protoplasts were typically observed to emerge from one end of a cell, and complete digestion of the sporidial walls was not observed even after several hours of digestion. Approximately 80 to 95% of the sporidia had formed protoplasts under these conditions as determined by plating samples diluted either in water or an osmotically buffered solution (SC50). Approximately 4 % of these protoplasts were regenerable.

Molecular karyotypic analysis

The electrophoretic karyotypes of one of the meiotic products of a germinated teliospore from collections of each of the 14 races of U. hordei are presented in Figure II.1. Visual inspection of ethidium bromide stained gels revealed a dissimilar banding pattern for each strain. The chromosome-sized DNA bands resolved with these parameters range in size from approximately 170 kb for the smallest band in strain 1.3, to a broad high molecular weight band that is present in all strains. A preliminary report of this work (McCluskey and Mills, 1989) did not include resolution of the higher molecular weight bands. The diffuse band at approximately 2,200 kb was shown to include three to five bands ranging in size from approximately 2,000 kb to 3,150 kb (Figure II.2 and Table II.2).

All of the strains had at least three bands of approximately 2,700, 2,850 and 3,150 kb, and all strains except 1.3, 2.1, 4.1 and 5.1 had an additional band that was

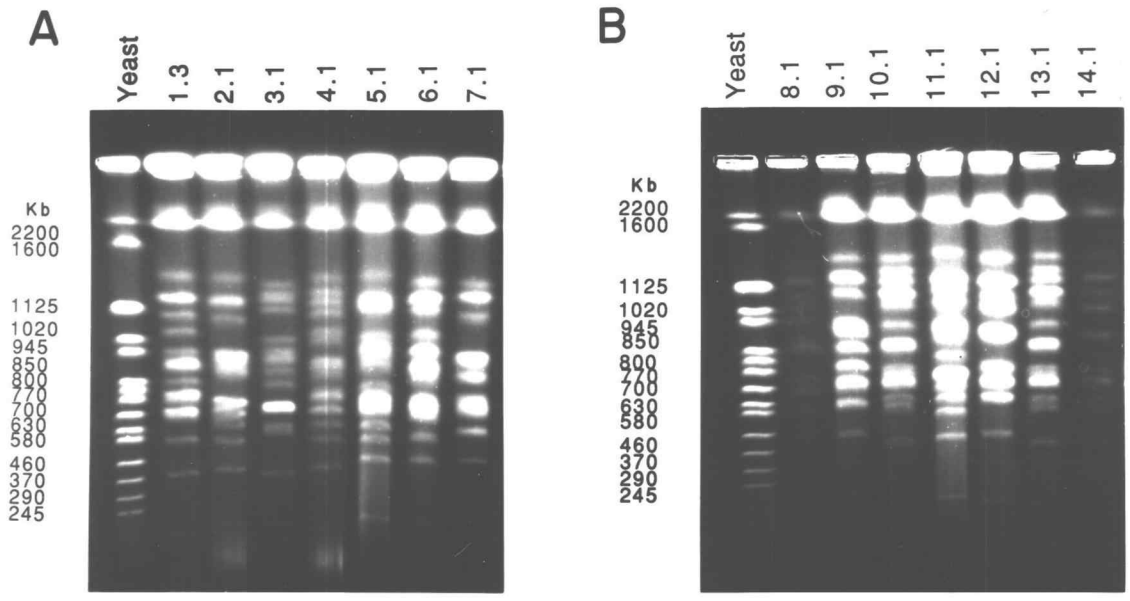

Figure II.1. CHEF pulsed-field gel electrophoresis of chromosome-sized DNA. molecules of Ustilago hordei. A. Strains obtained from races 1-7; B. Strains obtained from races 8-14. Left lane in each panel contains S. cerevisiae chromosomes for size standards.

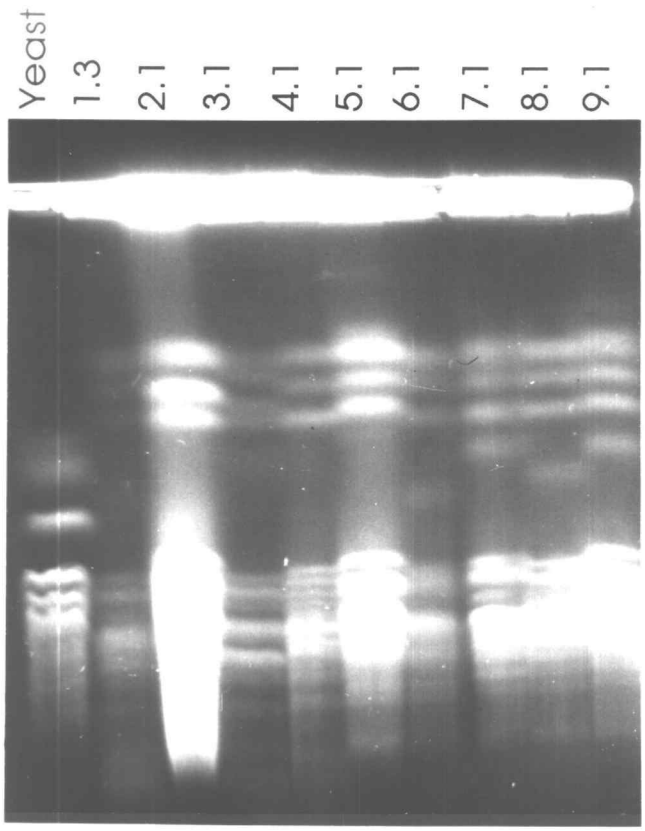

Figure II.2. CHEF pulsed-field gel electrophoretic resolution of largest chromosome-sized DNA molecules of Ustilago hordei. The broad band at 2,200 kb in all strains (Figure II.1) was resolved into 3 to 5 bands ranging in size from 2,000 to 3,150 kb (top bands in gel) shown here for strains from races 1-9. S. cerevisiae and S. pombe (not shown) chromosomes were used as standards. Conditions of electrophoresis: 0.8% agarose; (1) initial 24 hr, 60 min pulse at 50 V; (2) then 48 hr ramped 1,900-900 sec with the initial 20 hr at 60 V, followed by 28 hr at 85 V: (3) followed by 24 hr ramped 900-480 sec at 90 V; and finally, (4) 23 hr with a 420 sec pulse at 100 V.

Table II.2. Chromosome-sized DNAs of fourteen strains of Ustilago hordei

1.3	2.1	3.1	4.1	5.1	6.1	7.1	8.1	9.1	10.1	11.1	12.1	13.1	14.1a
3,150[a]	3,150	3,100	3,150	3,150	3,150	3,150	3,150	3,150	3,150	3,150	3,150	3,150	3,150
2,850	2,850	2,900	2,850	2,850	2,850	2,850	2,850	2,850	2,850	2,850	2,850	2,850	2,850
2,700	2,700	2,750	2,700	2,700	2,700	2,700	2,700	2,700	2,700	2,700	2,700	2,700	2,700
1,290	1,290	2,700	1,310	1,290	2,000	2,450	2,200	2,450	2,200	2,000	2,200	2,200	2,200
1,190[b]	1,190[b]	2,400	1,230	1,200[b]	1,300	1,290	1,200	1,260	1,260	1,280	1,260	1,260	1,260
1,110	1,110	1,260	1,190	1,180	1,210[b]	1,230[b]	1,150[b]	1,180[b]	1,210	1,180	1,150[b]	1,210	1,170[b]
1,050	960	1,210	1,140	1,040	1,160	1,140	1,090	1,100	1,150	1,130	1,100	1,150	1,100
960	940	1,200	1,060	1,000	1,060	975	1,040	980	1,100[b]	1,075	1,075	1,100[b]	1,040
910	890	1,150	1,025	950	990[b]	900	980	925	1,050	970	1,020	1,060	920[b]
900	760	1,025	940[b]	910	960[b]	850[b]	920	850[b]	960	920	960	970	830
840	700	975	860	840	875	800	850[b]	780[b]	890	890	890	890	760
770	650	940	790[b]	800	810	710	720	720	850	830	800	850	720[b]
750	570[c]	890	710[b]	700	700	650[b,c]	650	700	770	770	760	770	630
700	460	840	650	650	610[c]	510	590[b,c]	620[b,c]	735	750	720	735	590[c]
685	220	720[d]	585[c]	585[c]	510	245	450	470	710[b]	700	660	710[b]	460
570[c]		650	460	500	245		200	200	640	630	640[c]	640	190
430		620[c]	210	245					590[c]	580[c]	490	590[c]	
170		450							450	460	190	450	
		220							170	200		190	

[a]Lengths more than 2,200 kilobases are estimates. Lengths less than 2,200 kilobases were calculated by Cricket Graph (Cricket Graphics, Inc., Philadelphia PA).

[b]Bands interpreted as doublets based on intensity of ethidium bromide staining.

[c]Identified by pSF8 as containing swquences homologous to an actin gene from Aspergillus nidulans.

[d]Band interpreted as a triplet based on intensity of ethidium bromide staining.

at least 2,000 kb in size. Strain 3.1 was unusual in that it had five bands that were 2,400 kb or larger. A faint band of approximately 5,000 kb was also detected in strain 5.1 (Figure II.2). Because this band is not present in all strains, nor in all preparations of strain 5.1, it is not included in the summary of the estimates of the numbers and sizes of chromosome-sized DNA bands of each race (Table II.2).

Although the banding pattern revealed certain bands whose sizes were unique to a particular strain (e.g. the 2,750 kb band of strain 1.3, Table II.2) other bands of similar size recurred in several strains (e.g. the 1,060 kb band in strains 4.1, 6.1 and 13.1). The minimum number of bands detected was 15 in strains 2.1 and 7.1, although a band of 1,190 kb in strain 2.1 and three bands in strain 7.1 are assumed to be doublets. The maximum number of bands detected was 19 in strains 10.1, 11.1 and 13.1, although 2 of the bands in strain 10.1 and 13.1 were presumed to be doublets.

To ascertain whether the molecular karyotype determined for these strains varied from preparation to preparation, or was affected by maintenance in storage, samples were prepared from strain 11.1 over the course of a year and run in parallel. The banding pattern was identical for all of these samples (data not shown).

Molecular karyotypic analysis of unordered and ordered tetrads

Teliospores from collections of several races were germinated and meiotic tetrads were isolated. The strains derived from these tetrads were analyzed with CHEF PFGE to ascertain whether the karyotype for strains from one teliospore were variable or conserved. The molecular karyotype was determined for three unordered tetrads from teliospores of the race 8 collection, one tetrad from a teliospore of the race 2 collection, two tetrads from teliospores of the race 14 collection, and for an ordered tetrad from a teliospore of the race 11 collection. All twelve monosporidial strains derived from the race 8 collection had a karyotype that was identical to the karyotype of strain 8.1 (Figure

II.1B). The karyotype of the four monosporidial strains from the race 2 and the race 11 collections were identical to strains 2.1 and 11.1, respectively (Figure II.1 A, B). Six of the eight monosporidial strains from the race 14 collection had identical karyotypes, but among the four monosporidial strains from one tetrad, a chromosome-length polymorphism segregated 2:2 (Figure II.3).

Analysis of total soluble proteins

Because the molecular karyotypes of the representative strains for 14 races were dissimilar, it was of interest to determine whether another technique frequently used to distinguish different fungal strains might resolve the races into common groups. The electrophoretic pattern of total soluble proteins, which was previously used to distinguish between phytopathogenic species of Phytophthora (Hamm and Hansen, 1983), was also used in this study. However, the protein banding pattern was very similar for each strain (data not shown), excluding the use of this technique for differentiating strains of U. hordei.

Southern hybridization analysis

Evidence for the presence of chromosome-length polymorphisms was verified by Southern blot hybridization of CHEF PFGE-fractionated chromosomes with randomly selected homologous DNA probes. Radiolabelled probe made of a 1.2 kb EcoRI fragment (pOSU4012) obtained from strain 8.1 (Table II.1) had homology with a single band in each of the other strains which ranged in size from approximately 1,200 to 1,300 kb (Figure II.4). Although the mobility of this band differs among the strains, it clearly represents the same linkage group and provides evidence for chromosome length polymorphisms of as much as 100 kb.

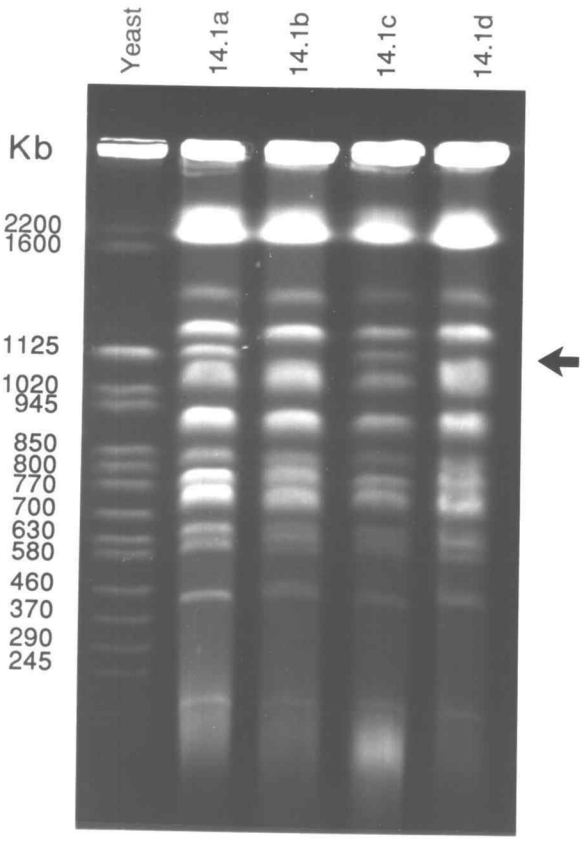

Figure II.3. Electrophoretic karyotype of members of an unordered tetrad of race 14 of Ustilago hordei. The arrow identifies a chromosome-size DNA band (ca. 1,100 kb) that segregates 2:2 among the progeny. Electrophoresis was carried out using the conditions that are described in Figure II.1.

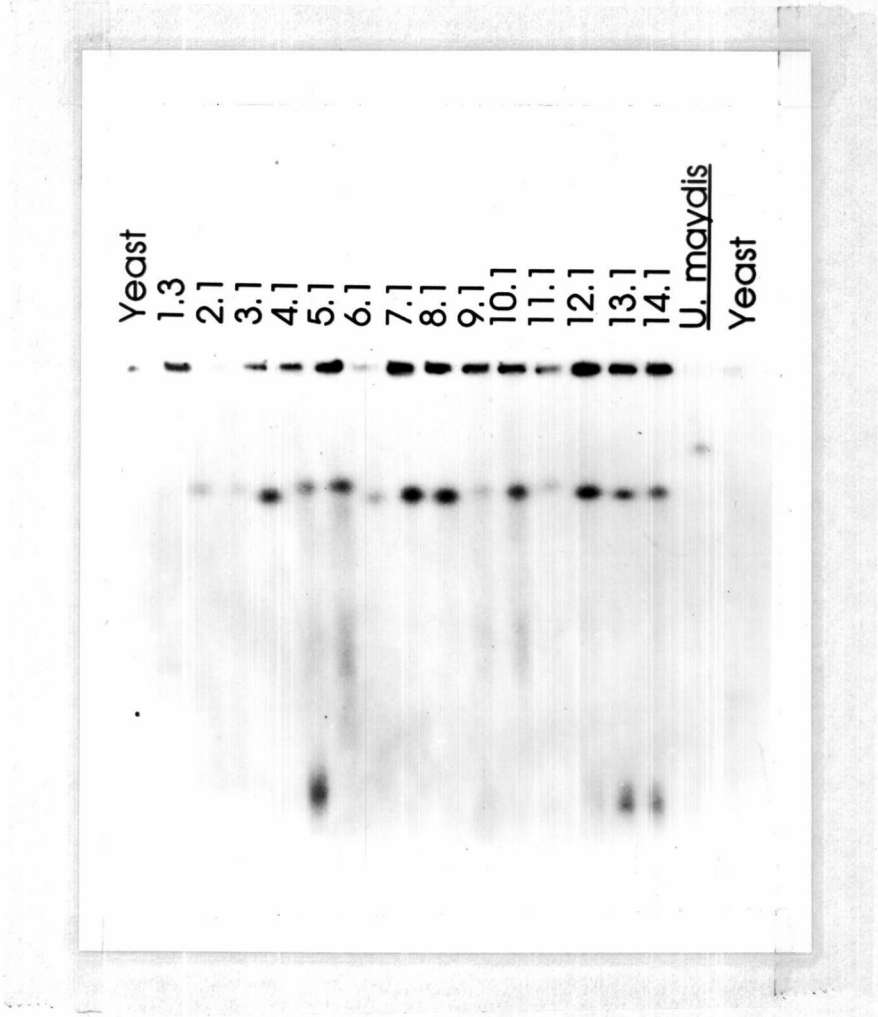

Figure II.4. Detection of chromosome-length polymorphisms in Ustilago hordei. A gel containing chromosome-sized DNA molecules from a strain of each race was Southern-blotted and probed with a 1.2 kb EcoRI fragment (pOSU4012) from U. hordei.

This approach was also used to map fragments to other linkage groups and to develop a better understanding of length polymorphisms. The inserts in pOSU4015 and pOSU4117 (Table II.1) do not cross-hybridize, but hybridize to the same chromosome-sized DNA band in strains 9.1 and 13.1 (Figure II.5). However, the 3.2 kb insert (pOSU4015) and the 6.0 kb insert (pOSU4117) respectively hybridized to a 750 kb band and a 700 kb band of strain 11.1 (Figure II.5). These probes also hybridized to bands of slightly different mobility in strains 10.1 and 12.1, suggesting that the probes are either identifying different bands in a doublet or hybridizing to sequences that are linked in strains 9.1 and 13.1, and unlinked in strains 10.1, 11.1, and 12.1.

The 6 kb insert of pOSU4011 showed intense hybridization to sequences in the sample well, and faintly to small, diffuse DNA near the bottom of the blot (data not shown). The cox III mitochondrial gene from S. cerevisiae (Table II.1) hybridized with yeast and U. hordei DNA in the sample well, and to a diffuse band of DNA near the bottom of the blot only in the lane containing yeast chromosomes. Relaxed forms of circular DNA molecules, such as the mitochondrial genome, do not readily enter the gel under these conditions (Hightower and Santi, 1989), which suggests that the insert in pOSU4011 may be of mitochondrial origin. Moreover, these results indicate that the smallest band in each strain is not the mitochondrial genome because it did not hybridize with the yeast mitochondrial DNA probe nor with the insert in pOSU4011 (data not shown).

To show the feasibility of mapping heterologous conserved genes to electrophoretically separated chromosomes of U. hordei, a probe made of the Aspergillus nidulans (Eidam) Winter actin gene (Table II.1) hybridized to a band in each strain that migrates between 570 and 650 kb (Table II.2), indicating a chromosome-length polymorphism of approximately 80 kb for this chromosome.

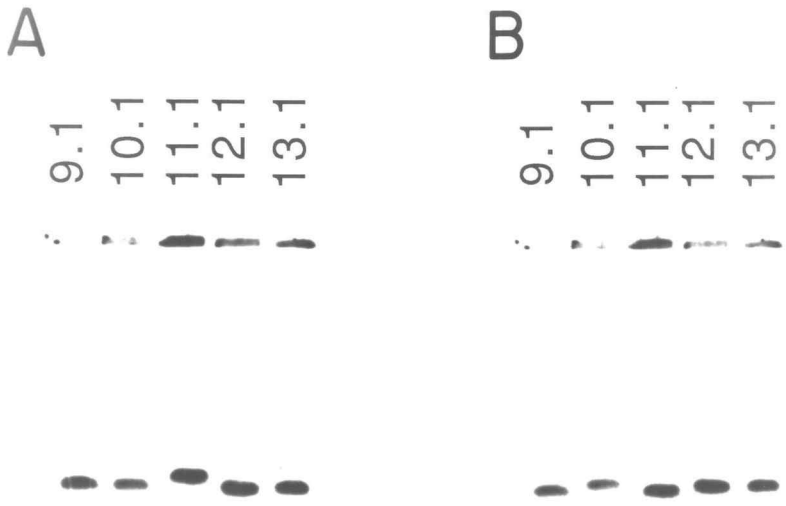

Figure II.5. A Southern blot of CHEF-fractionated chromosome-sized DNA molecules of Ustilago hordei probed with two genomic EcoRI fragments. The gel presented in Figure II.1B was blotted to a nylon membrane. A. Blot probed with a 3.2 kb fragment (pOSU4015). B. Same blot stripped and probed with a 6.0 kb fragment (pOSU4117).

DISCUSSION

CHEF pulsed-field gel electrophoresis was used to examine the karyotypes of strains representing 14 races of U. hordei. The karyotype of strains from each race varied with respect to both the number and size of chromosome-sized DNA bands. The karyotypic variability could not be attributed to artifacts of the the extraction procedure, although the possibility that some bands arise by specific cleavage at fragile sites on the chromosomes could not be ruled out (Sutherland and Hecht, 1985). The similarity of total soluble proteins extracted from these strains and separated by SDS-PAGE precluded the use of this technique for distinguishing isolates of U. hordei.

The number of chromosome-sized DNA bands varied from a minimum of 15 for strains 2.1 and 7.1, with one or more bands being interpreted as a doublet, to a maximum of 19 in strains 10.1 and 11.1, with 3 bands in strain 10.1 being interpreted as doublets (Table II.2). These results indicated that CHEF PFGE analysis alone cannot provide an accurate number of the haploid complement of chromosomes for two reasons. First, a doublet band could be comprised either of disomic chromosomes or of heterologous chromosomes of similar size. Secondly, Southern blot analysis, revealed chromosome length polymorphisms for some chromosomes (Figure II.4). Consequently, homologous disomic chromosomes that differ in size would be indistinguishable from heterologous chromosomes by CHEF PFGE analysis. In the absence of corroborative Southern blot linkage data for all bands, the occurrence of chromosome-length polymorphisms among disomic chromosomes would lead to an inflated base number of the haploid complement of chromosomes. The highly variable number of chromosome-sized DNA bands reported here is not unique to this smut

fungus, as similar results have been reported for laboratory strains of U. maydis (Kinscherf and Leong, 1988).

Although a larger number of DNA probes will be required to establish the precise number of linkage groups, the CHEF PFGE analysis of these strains (Figures. II.1 and II.2) allows an estimate of the maximum number of chromosomes and the size of the haploid genome of U. hordei. Strain 2.1 with the fewest bands should be most representative of a strain with the haploid complement of chromosomes. If each band represents an intact chromosome, and the lone doublet (Table II.1) is arbitrarily assumed to be comprised of different chromosomes, the maximum number of chromosomes per haploid cell would not exceed 16. The combined size of these bands is approximately 19,600 kb, which would make the haploid genome of U. hordei approximately 1.6 times larger than S. cerevisiae (Mortimer and Schild, 1985). Strain 10.1 has the largest number of bands including doublets, which have a combined length of approximately. 26,100 kb, and a DNA content 1.35 fold greater than strain 2.1. The possibility that the variable bands presented here are B chromosomes is impossible to exclude without further study. B chromosomes are unknown in basidiomycetous fungi, and are unstable in inbreeding plant species (Jones and Rees, 1982), although a preliminary report of unstable chromosomes in an ascomycetous fungus has appeared (Miao and VanEtten, 1989).

Chromosome-length polymorphisms have been described for sexually compatible strains of S. cerevisiae (Ono and Ishino-Arao, 1988), for strains of Candida albicans (Magee et al., 1988) and for laboratory strains of U. maydis (Kinscherf and Leong, 1988). Moreover, in crosses of S. cerevisiae strains having chromosome length polymorphisms for specific homologous chromosomes, a high percentage of viable offspring were obtained and both parental and variable-sized recombinant chromosomes were recovered (Ono and Ishino-Arao, 1988). These results are consistent with

observations presented here, which clearly indicate that the karyotypes of strains representing each race of U. hordei are highly variable, although individual strains from different races are sexually compatible (Emara, 1972).

Several bands in each strain were consistently present. The uniformity in size among the highest molecular weight bands (Figure II.2) may be either a function of the resolution of DNA molecules in this size range by the CHEF system, or a manifestation of an ability of smaller bands to tolerate a higher degree of variability. The band at 1,100 to 1,175 kb is present in every strain (Figure II.1), but caution is warranted in assuming that bands of identical size represent homologous DNA molecules. In several strains, only some of the similar-sized bands had homology with a DNA probe (Figure II.5). Moreover, two U. hordei genomic EcoRI fragments used as probes appear to belong to the same linkage group in strains 9.1 and 13.1 because they have homology to a common band, but they have homology with different bands in strains 10.1 and 11.1. These results cannot be definitively interpreted. The two fragments could have become linked or unlinked by reciprocal translocation, or they have homology with dissimilar DNA molecules of identical size. The development of specific chromosome libraries as sources of DNA probes (K. McCluskey and D. Mills, unpublished data) will be useful in resolving these questions.

Heterologous DNA probes made of highly conserved genes were used in Southern blot analysis to map homologous genes to chromosomes of U. maydis, and to demonstrate that most of the mitochondrial DNA remains in the sample well (Kinscherf and Leong, 1988). Using a radiolabelled probe of the actin gene of A. nidulans we have determined that a homologous gene resides on a single band in each strain that ranges in size from 570-620 kb (Table II.2). Furthermore, using cox III (oxi 2), a mitochondrial gene from S. cerevisiae (Muller, et al., 1984), we have determined that homologous sequences remain in the sample well and that the smallest band in each strain is

apparently not of mitochondrial origin. Heterologous probes will allow both the rapid development of genetic linkage maps and the cloning and description of important genes from U. hordei.

The upper limit of size of U. hordei chromosomes appears to be in the range of 3,000 kb (Figure II.2). The size limit of these chromosomes is similar to that seen for U. maydis (Kinscherf and Leong, 1988), but somewhat smaller than observed for P. megasperma (Howlett, 1989) and for Tilletia spp. (B. W. Russell and D. Mills, unpublished data). Moreover, the variability in size and number of bands among these strains is of the magnitude observed in U. maydis (Kinscherf and Leong, 1988).

A valuable feature of U. hordei as an experimental system is the production of ordered tetrads of basidiospores. This feature will facilitate studies of the distribution of genes on individual chromosomes, and analyses of polymorphisms associated with specific chromosomes. Although the karyotypes appeared to be essentially invariant for strains derived from one or more tetrads of a particular race, a chromosome-size polymorphism segregated 2:2 among the four members of a tetrad from race 14 (Figure II.3). The apparent variability in chromosome number suggests that there has been a need to maintain a disomic state for some chromosomes. The variability in size of individual bands may be a physical manifestation of genome rearrangements that require the maintenance of disomic chromosomes. Work is currently in progress to determine whether this hypothesis accurately describes genetic mechanisms acting in the genesis of races of U. hordei.

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Mapping Linkage Groups Among Chromosomes Separated by PFGE from Strains Representing Fourteen Races of Ustilago hordei

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ABSTRACT

Ten electrophoretically separated chromosomes of strains representing the fourteen physiologic races of Ustilago hordei have been mapped. Chromosome-specific DNA clone mini-libraries have been constructed from two strains to allow identification of DNA probes from each chromosome. Anonymous, random genomic and heterologous gene probes have been also utilized. This approach has allowed the unequivocal identification of ten chromosomes. The ribosomal RNA genes reside on a single chromosome that varies from 1,190 kilobases (kb) to greater than 3,150 kb among the strains and identifies a putative disome or a translocation in strain 5.1. Other probes identified polymorphisms of several hundred kb, and demonstrate that two chromosomes of similar length may not hybridize to the same probe. Similarly, chromosomes that share the same relative position in the gel, for example, the fourth smallest, may not share homology to individual probes.

INTRODUCTION

The development of electrophoretic karyotypes for fungi has allowed a new insight into mechanisms of genetic change (Miao and VanEtten, 1989), karyotypic variability (Mills and McCluskey, 1990) and genome structure (Fan et al., 1989). The demonstration that polymorphisms are present in isolates from localized populations (McDonald and Martinez, 1991), as well as among strains isolated at various geographic locations (McCluskey and Mills, 1990) suggests that fungi have a high ability to tolerate karyotypic variability. The observation of B chromosomes in fungi has been made by several workers (Miao et al., 1991; discussed in Mills and McCluskey, 1990), and contradicts early statements made denying that B chromosomes exist in fungi (Jones and Rees, 1982). There has not been a clear demonstration of the underlying nature of the variability in chromosome numbers, however, and further work needs to be done to define whether this variability is due to aneuploidy, to the presence of fragile sites on specific chromosomes, or to the presence of B chromosomes. Jones and Rees (1982) point out that B chromosomes are unstable in inbreeding plant species; this should be readily testable for fungi, as karyotypes are being developed for species that are inbreeding, asexual, and freely breeding.

The ability to define the number of chromosomes is independent of the identification of individual linkage groups, and a number of workers has gone to great lengths to associate electrophoretically resolved bands to linkage groups (Brody and Carbon, 1989; Kinscherf and Leong, 1988; Orbach et al., 1988). This work has drawn upon the availability of cloned genes homologous to markers that are known to reside on specific chromosomes. Cloning DNA from specific chromosomes was first reported by

Marguet et al.(1988), and promises to accelerate the pace of research into genetics of organisms whose chromosomes can be separated by pulsed-field gel electrophoresis (PFGE). For some organisms this is a critical breakthrough as there is a dearth of genetic information, either because sexual stages are unknown, or because crosses are difficult for a variety of reasons. To overcome the slow nature of carrying out crosses in Ustilago hordei we have attempted to identify linkage groups in PFGE separated chromosomes from strains representing fourteen physiologic races using heterologous gene probes, cloned random anonymous genomic DNA probes, and cloned chromosome-specific DNA probes.

MATERIALS AND METHODS

Strains and plasmids

The strains representing the fourteen physiologic races of U. hordei have been previously described (McCluskey and Mills, 1990). Plasmid DNA manipulations were carried out in the Escherichia coli strain DH5α (Bethesda Research Laboratories, Gaithersburg, MD). Saccharomyces cerevisiae chromosomes were prepared from strain X164, a haploid, adenine-requiring laboratory strain. Chromosome-specific DNA was cloned into the high copy cloning vector pUC19 (Norrander et al., 1984). Tilletia caries DNA homologous to the complete ribosomal RNA repeat from Neurospora crassa and cloned into the cloning vector pUC19 by B. W. Russell (personal communication) is designated pOSU1101. The U. hordei random homologous genomic fragment cloned in pUC18 and given the designation pOSU4012 was described in McCluskey and Mills, (1990). The cloned actin gene from Aspergillus nidulans was kindly provided by Dr. N. R. Morris (Rutgers University, NJ.) and is described in Fidel et al. (1988).

Source and cloning of DNA for probes

Chromosome-specific DNA was extracted from preparative CHEF gels that were run as described (McCluskey and Mills, 1990) and modified by the use of low gelling temperature agarose (FMC, Rockland, ME.). Bands were excised under UV illumination, taking care to minimize the UV exposure. The excised bands were transferred to 15 ml polypropylene tubes, and were melted at 68°C for 10-20 min in 5 ml of 1M NaCl, 25 mM Tris pH 7.5, 1 mM EDTA. Each sample was mixed gently, then frozen at -20° C for 1 to 16 hr. The samples were thawed for 30 min at 37° C and again

mixed gently. The freezing and warming were repeated once, then the samples were subjected to centrifugation at 10,000 g for 30 minutes at 5° C. The supernatant was transferred to a clean tube and incubated at -20° C for 1 to several hr with the addition of an equal volume of isopropyl alcohol to precipitate the DNA. The DNA was collected by centrifugation for 30 minutes at 10,000 g, the supernatant decanted and the pellet suspended in 400 µl of 10 mM tris HCl, pH 7.5, 1 mM EDTA (TE). This solution was transferred to a 1.5 ml microfuge tube and mixed with an equal volume of phenol saturated with TE. The sample was subjected to vigorous inversions, and the phases were separated by centrifugation for 5 min at 12,000 g in a micro-centrifuge. The aqueous phase was removed and the phenol phase was back-extracted twice with 100 µl TE by mixing and centrifuging as above. The pooled aqueous phases were mixed with 500 µl $CHCl_3$:isoamyl alcohol (24:1) and the phases separated by centrifugation as above. The aqueous phase was transferred to a fresh 1.5 ml microfuge tube, and 50 µl 3 M NaAcetate (pH 5.2) and 2 vol 95% ethanol were added and the DNA was precipitated at -20 °C overnight. The DNA was collected by centrifugation at 12,000 x g at 5° C for 20 minutes, washed briefly in 70% ethanol and dried in a vacuum dessicator. The phenol and chloroform extractions were repeated and the DNA was collected as described above. This DNA was twice digested with either EcoRI or BamHI using buffers supplied by the manufacturer and extracted with phenol and chloroform as described above between digestions and after digestion. The status of the digestion was assessed by running an aliquot on an agarose mini-gel as described by Maniatis et al. (1982). The cloning vector pUC19 was prepared by cleavage with the appropriate restriction enzyme followed by treatment with calf intestinal alkaline phosphatase (Maniatis et al., 1982). One half of each sample of the digested chromosome-specific DNA was ligated into pUC19 using T4 DNA ligase (BRL) according to the manufacturer's instructions. The entire ligation mixture was used to transform

competent E. coli cells (Maniatis et al., 1982). Insert-containing clones were identified by their inability to produce a blue color on medium amended with the chromogenic substrate X-gal . Plasmid DNA was extracted from E. coli using a modified alkaline lysis procedure (Birnboim and Doly, 1979).

Pulsed-field gel electrophoresis and Southern hybridizations

Samples for pulsed-field gel electrophoresis (PFGE) were prepared as described in McCluskey et al. (1990). PFGE was carried out in a Bio-Rad Laboratories (Richmond, CA) CHEF-DR II electrophoresis apparatus. Conditions for electrophoresis were as described in McCluskey and Mills, (1990). DNA bands were visualized by staining in 0.5 μg/ml ethidium bromide and illuminating with UV light. DNA from CHEF gels was transferred to Genetran nylon membranes (Plasco, Woburn, MA) after denaturation in 0.4 N NaOH using 0.4 N NaOH as the solvent. DNA from bands larger than 2,000 kilobases (kb) was transferred to Genetran nylon membranes as described in McCluskey and Mills, (1990). DNA radiolabelling and Southern hybridizations (Southern, 1975) were carried out as described in McCluskey and Mills (1990).

RESULTS

Cloning chromosome-specific DNA

As much as 1 μg of chromosome-specific DNA could be obtained from individual bands when 5 or 6 lanes of material were used. The DNA obtained had an approximate size of 100 kb (data not shown). Digestion was monitored by running an aliquot on an agarose mini-gel, and for best results, each sample was digested twice. Digested DNA was distributed from the lower limit of detection (approximately 0.5 kb) to approximately 9 kb. Among random E. coli clones selected for analysis, two plasmids contained inserts with an internal EcoRI site (8.1a-18 and 8.1a17). The supposition was made that there was incomplete digestion of the chromosome-specific DNA. As many as 50% of the E. coli transformants that produce no color on X-Gal amended medium were seen to contain inserts in extracted plasmids. In other preparations, as few as 10% of the colorless E. coli transformants yielded insert-containing plasmids. In total, 38 inserts have been identified from ten different chromosomes. Six of the inserts were determined to have resulted from either incomplete digestion of the chromosome-specific DNA or from ligation of non-contiguous chromosome-specific DNA. A total of approximately 112 kb of chromosome-specific DNA has been cloned.

Mapping linkage groups

Hybridizations with a variety of DNA probes have allowed unambiguous identification of ten linkage groups among the fourteen strains studied (Figures III.1, III.2 and III.3). The random genomic insert of pOSU4012 hybridized to a chromosome

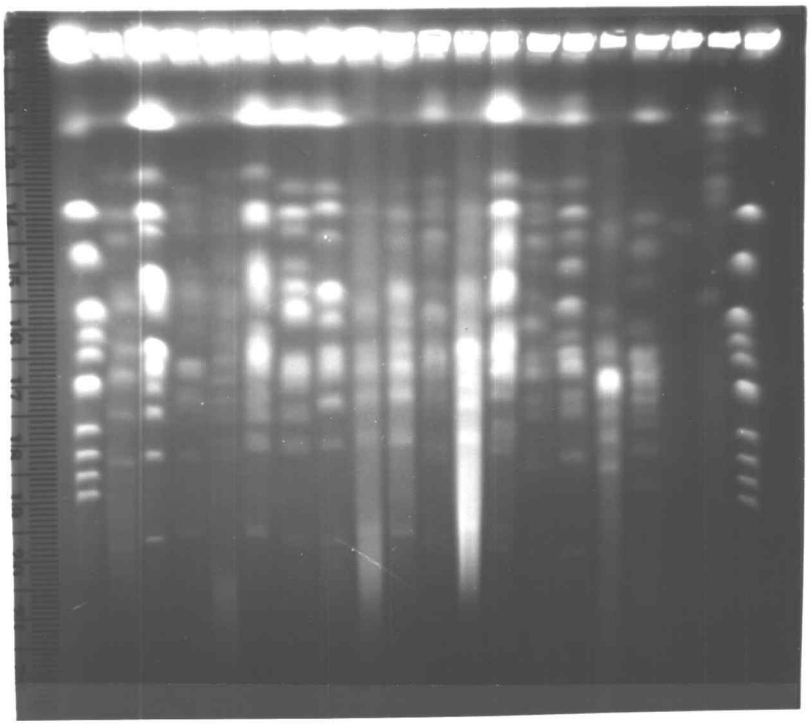

Figure III.1. CHEF PFGE of fourteen strains of Ustilago hordei. Gel V-82. Left to right, S. cerevisiae strain X164, U. hordei strains 1.3, 2.1a, 3.1, 4.1, 5.1, 6.2a, 7.1a, 8.1a, 9.1, 10.1a, 11.2a, 12.1, 13.1, 14.1a, U. maydis, U. bulatta, U. tritici, T. controversa, S. cerevisiae strain X164.

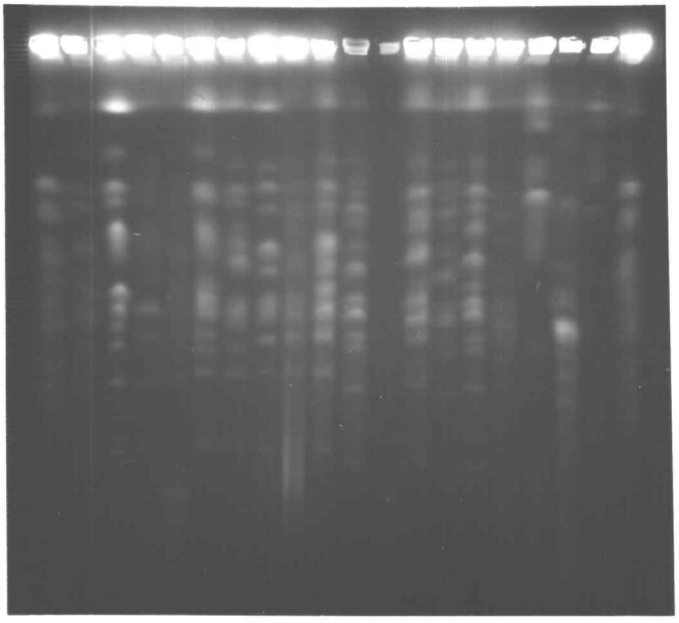

Figure III.1. (continued) Gel V-86.

Left to right, S. cerevisiae strain X164, U. hordei strains 1.3, 2.1a, 3.1, 4.1, 5.1, 6.2a, 7.1a, 8.1a, 9.1, 10.1a, 11.2a, 12.1, 13.1, 14.1a, U. bulatta, U. tritici, U. maydis, T. controversa, S. cerevisiae strain X164.

Figure III.2. Hybridization with a variety of cloned homologous DNA probes allows mapping of linkage groups among fourteen strains of Ustilago hordei. A) Gel II-56 (not shown) probed with the insert from pOSU4012 (also Figure II.4). B) Gel V-86 probed with 8.1a-88. C) Gel V-86 probed with 8.1a-18.2. D) Gel V-82 probed with 8.1a-62. E) Gel V-82 probed with 8.1a-611. F) Gel II-59 (not shown) probed with 8.1a-43. G).Gel III-85 (Figure II.2) probed with the insert from pOSU1101. H) Gel III-85 probed with 8.1a-112.

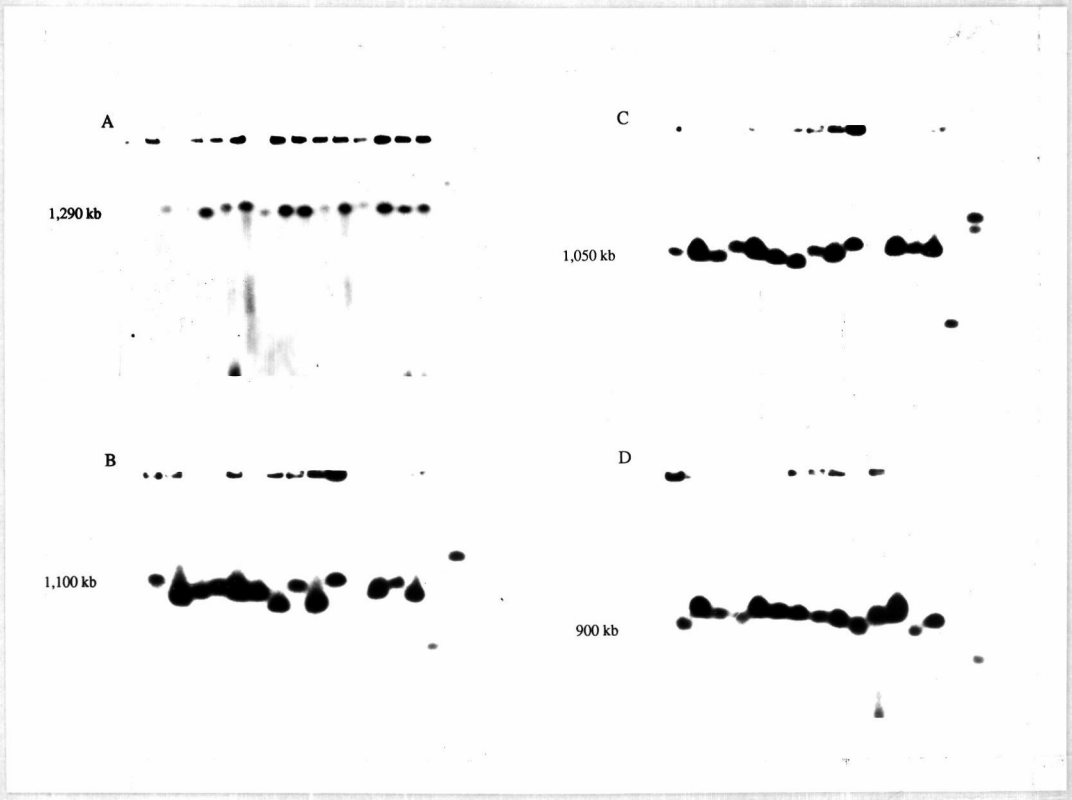

Figure III.2.(continued)

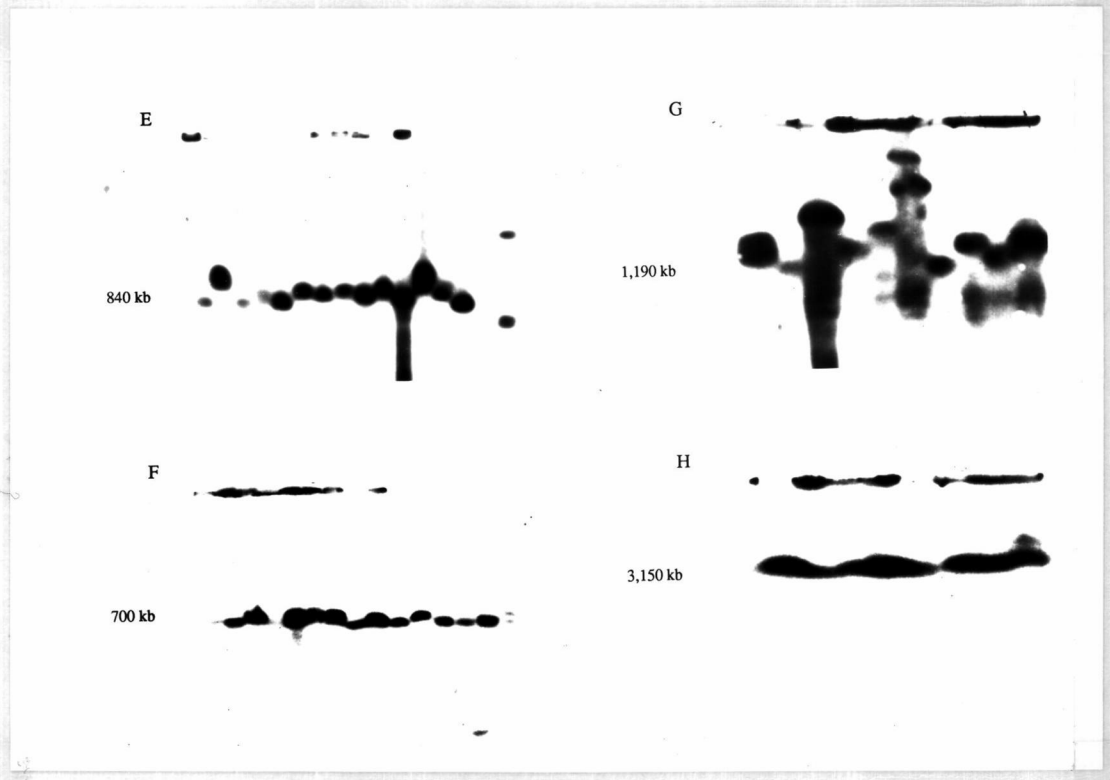

Figure III.2. (continued).

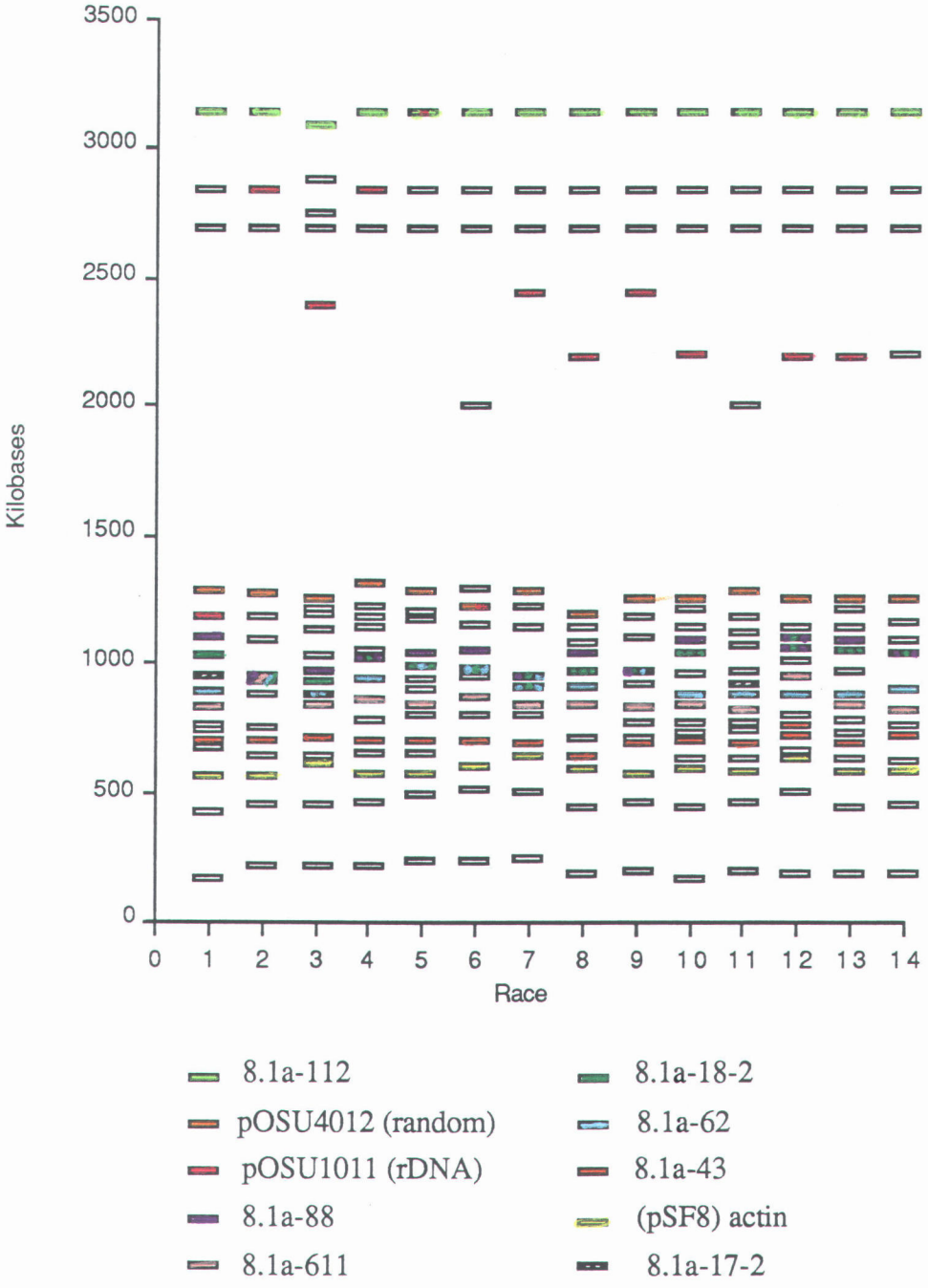

Figure III.3. A graphical representation of the linkage data assembled for fourteen strains of Ustilago hordei.

that varied from 1,200 kb in strain 8.1a to 1,310 kb in strain 4.1. This probe hybridized to a chromosome of 1,260 kb in six strains, and to a chromosome of 1,290 kb in three strains (McCluskey and Mills, 1990 and Figure III.2). The chromosome specific probe 8.1a-88 hybridized to the 1,040 kb chromosome in strain 8.1a, the chromosome from which it was cloned. This probe detected a high degree of interstrain variability, hybridizing to chromosomes of 1,100 kb, 1,060 kb, 1,040 kb 1,025 kb, 980 kb, and to an unresolved cluster at 940-950 kb.

The insert from clone number 8.1a-18.2 was one of two EcoRI fragments cloned as one insert from the same preparation as 8.1a-112 (above). This insert hybridizes to unique chromosomes of 1,050 (strain 1.3), and 975 kb (strain 3.1). In ten other strains this probe hybridizes to chromosomes that are either poorly separated from similarly sized chromosomes (Figure III.1), or that hybridize to other probes in addition to 8.1a-18.2 (Figure III.2). In strain 2.1a, probe 8.1a-18.2 hybridizes to the same unresolved cluster of 940-950 kb as probe 8.1a-88. In strain 7.1a, 8.1a-18.2 hybridizes to a cluster of 900-975 kb, even though probe 8.1a-88 was able to distinguish between two bands. In strain 14.1a, 8.1a-18.2 hybridizes to a band of 1,040 kb, as does probe 8.1a-88. Interestingly, in strain 8.1a which represents the presumptive parent of strains from race 14, these two probes do not hybridize to the same chromosome but rather 8.1a-18.2 hybridizes to a chromosome of 980 kb, wheras 8.1a-88 hybridizes to a chromosome of 1,040 kb (Figure III.3).

Probe 8.1a-62 was cloned from the unresolved bands of 850 and 920 kb (band 6) from strain 8.1a (resolved in other experiments) and hybridizes to the 920 kb chromosome in that strain (Figures III.2 and III.3). This probe hybridizes to chromosomes that vary in size from 890 kb to 1,025 kb. The 890 kb chromosome is identified in strains 10.1a, 11.1, 12.1a, and 13.1a. Although there are chromosomes of 890 kb in strains 2.1a and 3.1a they do not show homology to this probe. Rather this

probe hybridizes to a cluster from 940 to 960 kb in strain 2.1a, and to a 940 kb chromosome in strain 3.1a. The 1,025 kb chromosome is uniquely identified by this probe in strain 4.1 and a 920 kb chromosome has homology to this probe in strains 8.1a and 14.1a (Figure III.3). Probe 8.1a-611 is also derived from the unresolved cluster designated band six, and hybridizes to a chromosome of 850 kb in strain 8.1a. This probe identifies a single band in twelve strains (Figure III.3). The two strains in which it is apparently linked to other probes are strain 2.1a, where it identifies a chromosome of 940 kb that is part of an unresolved band that has homology to probes 8.1a-88, 8.1a-18.2, and 8.1a-62, and strain 12.1, where it identifies unresolved chromosomes of 960 and 890 kb. In strain 12.1, the 890 kb chromosome has homology with probe 8.1a-62.

The chromosome specific probe 8.1a-43 was cloned from the 650 kb chromosome in strain 8.1a and hybridizes to this chromosome (Figure III.2). This probe identifies relatively little variability, hybridizing to chromosomes of 650, 700, 710, 720 and in one unresolved case, apparently to a 760 kb chromosome. Strains 2.1a, 3.1, 4.1, 5.1, and 7.1 have 650 kb chromosomes that do not hybridize with this probe. Strains 10.1, 11.1, 12.1, and 13.1a have chromosomes that are apparently only 10 kb larger or smaller than this, and do not show homology to this probe. Moreover, probe 8.1a-43 identified the chromosome that is the fourth smallest in five strains, the fifth smallest in eight strains, and the sixth smallest in one strain (Figure III.3), further demonstrating the risk of assuming that bands of the same size of relative position represent homologous chromosomes.

The ribosomal RNA gene probe identified the greatest chromosome length polymorphism. The insert from pOSU1101 hybridized to a band of approximately 1,190 kb in strains 1.3 and 6.1, and to two bands that are greater than 3,150 in strain 5.1 suggesting that this chromosome is disomic in this strain. The rDNA probe hybridized to a band of 2,200 kb in four strains and to unique bands of 2,850, 2,700, 2,400, and

2,000 kb. The insert from clone number 8.1a-112 was cloned from a region of a gel containing the smallest chromosome and degraded genomic DNA. 8.1a-112 hybridized to the largest chromosome (3,150 kb) in each strain (Figure III.3).

The heterologous probe for actin (pSF8) identified a chromosome that is the third smallest in every strain. This chromosome varies from 570 to 650 kb, and is 585-590 kb in strains 4.1, 5.1, 8.1a, 10.1a, 13.1a, and 14.1a (Figure III.3). Other heterologous gene probes have been employed, but have met with little success, largely due to the utilization of inappropriate stringency conditions.

DISCUSSION

Cloning DNA from pulsed-field gels has allowed for the rapid identification of DNA probes from ten different chromosomes in U. hordei. The yield of DNA from the gels is sufficient to allow cloning on a larger scale but the DNA extracted from the gel using this approach is sheared to approximately 100-200 kb. The possibility that more efficient extraction of chromosomal DNA could be achieved using electro-elution (Marguet et al., 1988) can not be discounted. Although no effort was made to maximize the efficiency of cloning from gels, the cloning of over 100 kb of DNA in several experiments suggests that this approach could be used to facilitate cloning of DNA that is known to be associated with a particular chromosome or large restriction fragment. The sizes of the fragments cloned was dependent on the restriction enzyme employed. For inserts derived from BamHI digested DNA, the average size was approximately 1.2 kb, while for inserts derived from EcoRI digested DNA, the average insert size was approximately 3.5 kb. The BamHI derived inserts varied in size from approximately 0.3 kb to 1.5 kb while those derived from EcoRI digested DNA ranged in size from approximately 0.5 kb to as much as 9 kb. Of the probes utilized, two from the BamHI digested DNA are repeated DNA elements, while only one from the EcoRI digested DNA hybridized to multiple bands. These data suggest that the genome of U. hordei is higher in guanine and cytosine bases than in adenine and thymine.

Mapping linkage groups with chromosome specific DNA probes has allowed the determination of the identities of different chromosomes among the fourteen strains studied. The hybridization of a particular probe to two or more different size bands can be attributed to several different causes. The bulk of the bands could be homologous,

representing homologous chromosomes, and the difference in size could be a manifestation of differences in structural components of the chromosome. Telomeres are known to vary in size in yeast, and can account for chromosome-length polymorphisms by containing differing numbers of a 6.75 kb repeated sequence (Walmsley, 1987). Conversely, the bands could represent different linkage groups and the probe DNA may be hybridizing to small regions that have translocated from one chromosome to another. Regardless of the mechanism, the finding that chromosomes identified by the same probe may have different sizes, and different relative positions among the entire complement of chromosomes is startling.

The demonstration of variability of almost 2,000 kb for the chromosome that contain the ribosomal RNA genes is remarkable. This represents almost two thirds of the DNA on this chromosome, and suggests that these fungi are able to tolerate reductions in the number of repeats of the ribosomal RNA genes. Emara (1972) reported that the different races of U. hordei are sexually compatible but did not report on the viability of the teliospores produced in crosses between races. In light of the results presented here, it becomes particularly relevant to test whether strains from races 1 and 5 which exhibit the greatest difference in the size of the chromosome that hybridizes to the rDNA probe can produce viable progeny when mated. Given the presence of a length polymorphism of 2,000 kb for homologous chromosomes, the viability of the basidiospores that would arise from germinating teliospores from such a cross would likely be reduced.

The assumption that the 3,150 kb band represents the same chromosome in each strain was supported by the hybridization to this band by probe 8.1a-112. This probe also hybridized to a band that is larger than the 3,150 kb band in strain 9.1 even though there was no clear indication that such a band could be reliably detected in an ethidium bromide stained gel. The possibility that the culture that was used to prepare the sample

for this experiment consisted of two different populations that differ in karyotype cannot be eliminated. Probes 8.1a-88, 8.1a-18.2, 8.1a-62, 8.1a-17.2, and pOSU4012, all identified chromosome-length polymorphisms of 100 kb or more. These probes also defined variability in the relative position of different bands, and in some cases (eg. 8.1a-88 vs 8.1a-62 in strain 7.1) have been able to distinguish bands that are not clearly resolved on the ethidium bromide stained gel. Probe 8.1a-43 hybridizes to a band of 650 kb in strain 8.1a (the band from which it was cloned), but does not hybridize to a band of the same size in five other strains. Moreover, the relative position of the band that has homology to this probe varies from the fourth smallest in several strains to the fifth smallest in eight other strains and even the sixth smallest in one strain. Clearly one cannot assume that bands of the same size represent homologs without carrying out mapping experiments using cloned genes, or cloned anonymous DNA.

The ability to clone DNA from specific chromosomes has facilitated the mapping of linkage groups among fourteen strains of U. hordei that differ in their electrophoretic karyotype. The identification of bands of differing size as the same linkage group will benefit from the placement of greater numbers of markers on each chromosome, but the present technology will allow for DNA of unknown function to replace phenotypic markers in this role. The observation of chromosome length polymorphisms of almost 2,000 kb for the chromosome that contains the ribosomal RNA genes demonstrates the potential of this technique for assessing the status of genomes in flux.

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Electrophoretic Karyotypic Variability Among Eight Species of Ustilago: Redefining Species Limits

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ABSTRACT

Electrophoretic karyotype has been determined for eight species in the genus Ustilago. Monosporidial strains from each species were used to produce high-quality samples without first generating protoplasts. The karyotypes were found to vary with respect to the number of bands, and the sizes of the specific bands. Three distinct groups were distinguished by the distributions of the band sizes. Ustilago avenae, U. kolleri and U. tritici were found to have the majority of their bands larger than 1,000 kilobasepairs (kb). U. aegilopsidis, U. bullata, U. hordei and U. nigra were found to have the broadest size distribution, with bands as small as 200 kb and as large as 3,200 kb. U. maydis was alone in having no bands larger than 2, 000 kb. The conclusion that U. avenae is synonymous with U. kolleri , and that U. hordei is synonymous with U. nigra is supported by these data. Karyotype does not support grouping all of these species together as U. segetum.

INTRODUCTION

Many fungi in the genus Ustilago are parasitic on cereal crop plants (Fischer and Holton, 1957), and among these there are several that are both economically important and well characterized with respect to their physiology, genetics, and host relations. Features that serve to distinguish these species include host selection, infection cycle, mating systems, physiology, morphology, and development. For the species that are distinguished on the basis of the host or infection cycle, mating can be accomplished, and sometimes brought to fruition on an appropriate host. U. hordei and U. nigra have been shown to hybridize and segregate genes for avirulence (Cherewick, 1967). U. hordei can hybridize with the smuts of oat, U. kolleri and U. avenae, and with the grass smut U. aegilopsidis on the permissive host, Agropyron tsukushiense , suggesting a close relationship for these organisms (Huang and Nielsen, 1984; Thomas and Huang, 1985).

A study of the 5S ribosomal RNA nucleotide sequences of a variety of smut fungi found too little variability even to distinguish between U. hordei and Tilletia controversa, but enough to distinguish between U. hordei and U. maydis (Blanz and Gottschalk, 1984). U. hordei differs in many respects from U. maydis, including infection cycle and mating system, but has been shown to have an electrophoretic karyotype that has similar numbers of bands with a similar size distribution (Kinscherf and Leong, 1988; McCluskey and Mills, 1990). Four chromosomes were detected in U. hordei in 1927 (Kharbush) using classical cytological techniques, and similar numbers were detected in eleven other smut fungi (Wang, 1934).

The focus of the present study was to determine if electrophoretic karyotype varied among eight different smut fungi that parasitize plants from the graminae. Further, samples were prepared from intact cells to determine if the rapid protocol for chromosome sample preparation described in McCluskey et al., (1990) was applicable to related smut fungi. Represented among these are species that have bipolar or tetrapolar mating systems, and species that parasitize crops or wild grasses. Infection cycle also varies among the species studied.

MATERIALS AND METHODS

Strains

U. hordei strain 8.1a and U. maydis have been previously described (McCluskey and Mills, 1990). U. tritici and U. bullata are sporidial strains from teliospores provided by Dr. P. A. Koepsell of the Oregon State University Agriculture Experiment Station Plant Clinic. Teliospores of U. aegilopsidis (strain 4327), U. avenae (strain H60), U. kolleri (strain K45), U. nigra (strain 83-138) and U. nuda (strain 72-66) were kindly provided by Dr. J. Kronstad (University of British Columbia, Vancouver BC). Teliospores were germinated on Potato Dextrose Agar (PDA; Difco Laboratories, Detroit, MI) at room temperature and removed individually to 250 µl Potato Dextrose Broth (PDB, Difco) when they reached the four spore stage. This mixture was vortexed vigorously, and then spread on the surface of PDA with a sterile glass rod. Mucoid colonies were transferred to PDA slants, where they were maintained at room temperature and transferred bimonthly. Saccharomyces cerevisiae chromosomes used as molecular weight markers were prepared from strain X164.

Preparation of samples for pulsed-field gel electrophoresis

U. bullata, U. hordei, U. maydis, and S. cerevisiae chromosome samples were prepared from overnight liquid cultures in PDB as described in McCluskey et al. (1990). The remaining samples were prepared from cells that were removed from the surface of PDA medium with a rubber scraper. The cells were suspended in 10 ml of 10 mM Tris HCl, pH 7.5, 1 mM ethylene diamine tetraacetic acid (EDTA) and collected by

centrifugation at 5,000 g for 10 min. This procedure was repeated twice to minimize the amount of viscous material in the cell pellet. The final cell pellet was cast into agarose plugs and treated with protease and sodium dodecyl sulfate in EDTA as described (McCluskey et al., 1990).

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis was carried out in a Bio-Rad Laboratories CHEF-DR II system. For resolution of bands in the 100 to 2,000 kilobasepair (kb) range, gels of 1% agarose (Sigma type II medium EEO) were run at 14° C in 0.5X TBE buffer (Maniatis et al., 1982) for 15 hr with a 70 sec switch interval at 200 V, followed by 11 hr at 200 V with a 120 sec switch interval. For resolution of larger bands, gels of 0.85% agarose (International Biotechnologies, Inc. New Haven CT., UltraPure Electrophoresis grade) were run at 14°C for 68 hr with an 1,800 sec switch interval at 50 V, followed by 24 hr with a 480 sec switch interval at 100 V. DNA was visualized by staining with 0.5 μg/ml ethidium bromide and illuminating with ultraviolet light.

RESULTS

Rapid mini-preparation of chromosome samples

It has been previously shown that U. hordei, S. cerevisiae, Tilletia caries and T. controversa are amenable to production of chromosome samples without first preparing protoplasts (McCluskey et al., 1990). As the limiting step in determining electrophoretic karyotypes is often the production of high quality protoplasts, this technique was applied to eight Ustilago species. U. hordei, U. maydis, and U. bullata chromosomes were prepared from cells grown in liquid culture. U. avenae, U. aegilopsidis, U. hordei, U. kolleri, U. maydis and U. nigra were prepared from cells removed from the surface of agar-solidified medium. One 150 mm petri dish yielded enough material for 10 to 15 samples. The karyotypes for samples prepared in either manner were comparable (Figure IV.1). Neither cells from liquid or plate culture yielded informative karyotypes for U. nuda.

Electrophoretic karyotypes of Ustilago species

The electrophoretic karyotypes for eight smut fungi have been determined (Figure IV.1 and Table IV.1), and differ in several respects. With 16 bands, U. hordei strain 8.1a has the greatest number of resolved bands. U. nigra has the second largest number of resolved bands, with 15. U. aegilopsidis and U. avenae each have 13 bands. U. bullata has 12 bands, and U. maydis and U. kolleri both have 11. U. tritici has only ten bands, the fewest among the species studied. An estimate of the number of chromosomes in each strain is outside the scope of the present study. U. avenae, U. kolleri, and U. tritici have similar distribution of sizes, with most of the bands larger than

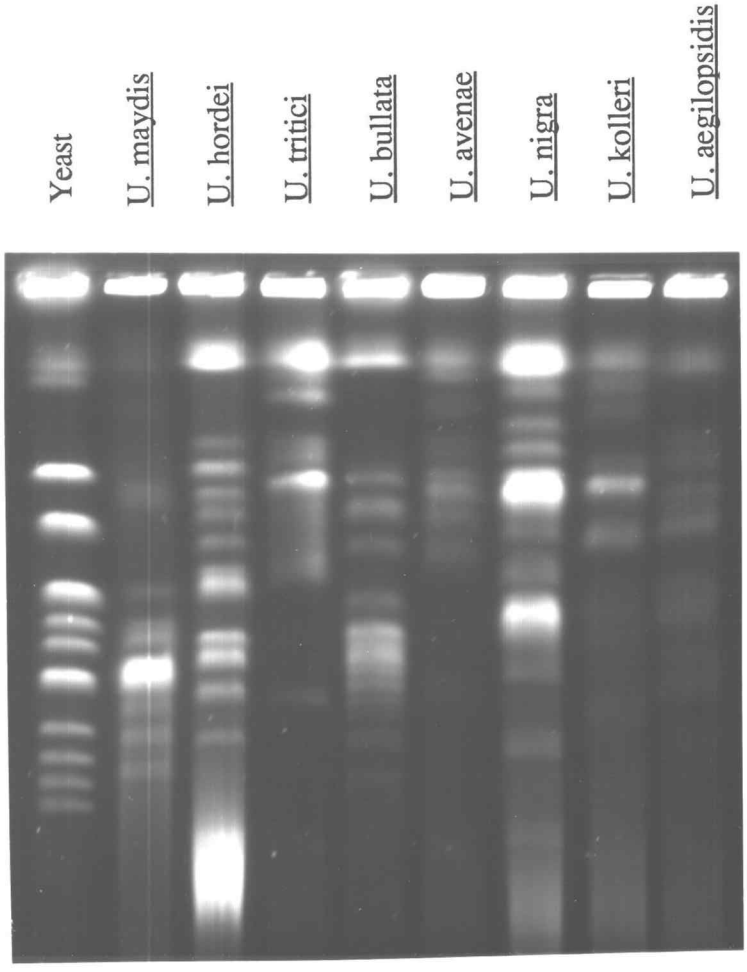

Figure IV.1. Electrophoretic karyotypes of eight Ustilago spp.

Table IV.1. Estimates of band sizes for electrophoretically separated chromosomes of several smut fungi. (Kilobases)

U. aegilopsidis	U. avenae	U. bullata	U. hordei	U. kolleri	U. maydis	U. nigra	U. tritici
2 > 3Mb	2 > 3 Mb	1 > 2 Mb	3,150	2 > 3 Mb	2,000	3,200	2 > 3 Mb
2,500	2,000	1,150	2,850	2,000	1,600	3,000	1,700
2,000	1,600	1,100	2,700	1,700	1,400	2,500	1,500
1,400	1,500	1,025	2,200	1,500	1,100	2,250	1,275
1,300	1,400	850	1,200	1,300	900	1,500	1,200
1,125	1,250	800	1,150	1,150	800	1,350	1,100
1,025	1,175	750	1,090	1,000	775	1,250	1,000
900	1,150	700	1,040	925	700	1,125	900
850	1,100	650	980	800	550	1,075	600
700	1,000	550	920	550	475	1,000	
650	900	450	850		350	900	
450	600	350	720			875	
			650			725	
			590			500	
			450			250	
			200				

1,000 kb. This is in contrast to the karyotypes for the remaining five species which have as many as half of their bands smaller than 1,000 kb. U. maydis has no bands larger than 2,000 kb, and is unique among the smut fungi in this respect. U. maydis has a size distribution that is most similar to that of S. cerevisiae (Figure IV.1). The smear of DNA in the smallest size class of the U. hordei sample has been shown to consist of degraded genomic DNA and mitochondrial DNA (data not shown). The bulk of the mitochondrial genome from S. cerevisiae, U. hordei and Tilletia (B. W. Russell and D. Mills, unpublished data) have been shown to remain in the sample well and this is assumed to be the case for the seven other smut fungi whose karyotypes are presented here.

DISCUSSION

The differences in the electrophoretic karyotypes presented for different species of smut fungi occur both in the numbers of the bands and in the overall distribution of the sizes of the bands. This is contrasted with the cytological detection of four chromosomes in several smut fungi (Kharbush, 1927; Wang, 1934). Several strains of U. hordei have four chromosomes larger than 2,000 kb, and these may represent the limit of detection of chromosomes by cytological techniques. The demonstration that U. maydis is unique in having no bands larger than 2,000 kb substantiates the claim of Blanz and Gottschalk (1984) that U. maydis is evolutionarily separated from the other smut fungi (as determined by its 5S rRNA sequences). Several DNA probes that are derived from U. hordei and that have homology with U. bullata and U. tritici do not hybridize to U. maydis chromosomes (Figure III.2 D, E). The insert in pOSU4012, a random genomic EcoRI fragment from U. hordei has homology to a single U. maydis chromosome (Figures II.4 and III.2 A). DNA probes that hybridize to different species do not hybridize to chromosomes of the same or similar sizes (Figure III.2 B) or to the same number of bands (Figure III.2 C, F) in different species.

U. tritici, U. avenae and U. kolleri have a distribution of sizes that separates them from the other smut fungi studied. The similarity of the sizes and numbers of chromosomes supports the synonymy proposed for U. avenae and U. kolleri (Huang and Neilsen, 1984). The similarity in the karyotypes of U. aegilopsidis, U. hordei strain 8.1a, and the U. nigra strain examined is similar in magnitude to differences seen between different strains of U. hordei (McCluskey and Mills, 1990), and suggests that the synonymy proposed for these species is valid (Huang and Neilsen, 1984; Thomas

and Huang, 1985). The proposal that the four seedling infecting smuts of oat and barley were all variants of one species to be called U. segetum (Lindeberg and Nannfeldt, 1959) is not supported by these data. The criterion upon which this synonymy is based, spore wall morphology, is controlled by only one or two genes (Huang and Neilsen, 1984), and is insignificant as a taxonomic feature when compared to the divergence in the karyotypes seen among these fungi.

Electrophoretic karyotype is valuable for separating groups that differ in the overall organization of their genomes, and for distinguishing among individual strains within a particular species. The variability in chromosome sizes within a species make it difficult to distinguish between closely related species. Steensma et al. (1988) used electrophoretic karyotype to distinguish between closely related species of Kluyveromyces, and determined that, while the variability between species was comparable to that seen among strains of one species, the inability of the parental karyotypes to recombine in inter-species crosses allowed for distinction between species. Recombined karyotypes are typically found in crosses of S. cerevisiae strains that contain chromosome-length polymorphisms (Ono and Ishina-arao, 1988). The demonstration that different species of Ustilago have chromosomes in different size ranges is useful in distinguishing species.

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Electrophoretic Karyotyping Without the Need for Generating Protoplasts

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PREFACE

The portions of the following work that describes the karyotypes of Tilletia spp, and the production of samples for PFGE from Tilletia spp was carried out by B. W. Russell. The initial observation of the ability to produce samples for PFGE without generating protoplasts, the preparation of samples from other fungi, and the preparation of samples from algae were by K. McCluskey.

ABSTRACT

Chromosome samples for pulsed-field electrophoresis have been prepared without first generating protoplasts. The technique employed involves treatment of intact, agarose-solidified cell material with protease in the presence of EDTA and SDS. Saccharomyces cerevisiae, Ustilago hordei, Tilletia caries, and T. controversa karyotypes are clearly resolved with this technique. Colonies of U. hordei and S. cerevisiae removed from the surface of agar-solidified media and prepared for PFGE by this abbreviated method yield well resolved karyotypes.

Key words: PFGE - Phytopathogenic fungi - Molecular karyotype -Intact cells

INTRODUCTION

Electrophoretic karyotyping is emerging as a useful and informative technique for studying the genetics of fungi and other eukaryotes. The nearly constant relationship between the physical and genetic maps of Saccharomyces cerevisiae suggest that the bands resolved electrophoretically represent intact chromosomes rather than fragments (Carle and Olson, 1985; Schwartz and Cantor, 1984; Mortimer and Schild, 1985) indicating the validity of this method. By inference, electrophoretic karyotypes derived for many other organisms lacking extensive genetics may prove equally valid. Pulsed-field gel electrophoresis (PFGE) is being used as a tool to study genetics at a level never before possible, and is finding application in many fields including clinical pathology (Magee et al., 1988), molecular genetics (Game et al., 1989), plant pathology (Kinscherf and Leong, 1988; McCluskey and Mills, 1990), and genome mapping of higher organisms (Vollrath et al., 1988). However, this approach has been limited to organisms from which high quality protoplasts can be prepared in large numbers. This limitation has, unfortunately, eliminated many plant pathogenic fungi from consideration. Moreover, variability in efficiency of protoplast formation has limited the usefulness of this method even among strains of the same species. Finally, degradation of DNA in the course of protoplast preparation introduces potential artifacts and further limits the applicability of this technique.

We report here the ability to reliably produce high quality chromosome samples for pulsed-field electrophoresis without the need for first generating protoplasts. This protocol has allowed equal or better resolution of electrophoretic karyotypes for a variety of organisms including Ustilago hordei, U. maydis, Tilletia caries, T. controversa, and

S. cerevisiae. Other fungi that have shown positive results in preliminary experiments include strains of Rhizopus, Botrytis, Pseudocercosporella herpotrichoides, and Cephalosporium graminieum, as well as the green alga, Chlorella. Finally, yeast and U. hordei colonies removed from the surface of agar-based media and prepared directly in a mini-scale preparation yielded high quality chromosome samples, making feasible the karyotypic analysis of large numbers of isolates or strains.

MATERIALS AND METHODS

U. hordei and U. maydis have been previously described (McCluskey and Mills, 1990). Ustilago strains were grown in potato dextrose broth (PDB, Difco, Detroit, MI) and maintained on potato dextrose agar (PDA) slants. The haploid S. cerevisiae strain X164 was cultured in 1% yeast extract, 2% peptone and 2% glucose with 1.5% agar when appropriate. T. caries and T. controversa strains were cultured in modified T19 medium (Trione, 1964). Protoplasts of U. hordei and U. maydis were prepared as described in McCluskey and Mills (1990). T. caries and T. controversa protoplasts were prepared from log phase mycelial cultures. A 1.5 ml mycelial pellet was suspended in 10 ml 1M sorbitol, 50 mM NaH_2PO_4, 50 mM Na_2HPO_4 pH 5.8 with 4,000 U/ml b-glucuronidase (Sigma, St. Louis, MO, type H-2) and 32 U/ml Novozym 234 (Calbiochem, San Diego, CA) and incubated at room temperature with constant shaking (100 RPM) for 1-2 hr. Protoplasts were separated from mycelial fragments by filtration through a 20 μm mesh nylon screen and collected by centrifugation (800 g for 15 min). The pellet was suspended in one volume of 25 mM tris pH 7.5, 1 M sorbitol, 25 mM EDTA. Protoplasts were mixed with an equal volume of warm 2.5% low gelling temperature (LGT) agarose in the same buffer (approximate concentration, 4×10^8 protoplasts/ ml) and pipetted into a well of a plug forming stand and allowed to solidify for 2-5 minutes. The plugs were transferred to 10 ml of a solution containing 1 mg/ ml Protease (Sigma, type XIV) in 0.45 M EDTA, pH 8.0 and 1% SDS and incubated for 16 to 24 hr at 50° C. Following a rinse with 0.5 M EDTA, pH 8, the plugs were stored in this solution at 5°C. In our simplified method for preparation of chromosome samples, intact S. cerevisiae cells and yeast-like cells of U. hordei and U. maydis, and haploid

sporidia of T. caries were cast directly into plugs and treated with protease in EDTA and SDS as above. T. controversa and T. caries mycelia were collected from 35 ml of a log phase culture by centrifugation (2,000 g, 10 minutes). The resulting pellet was suspended in 10 ml buffer (1M sorbitol, 25 mM Tris pH 8, 25 mM EDTA) and ground using a Pyrex tissue grinder. The ground mycelia were pelleted at 5,000 g, resuspended in grinding buffer and pelleted as above. The final pellet was suspended in 2 volumes of 1.5% LGT agarose in 125 mM EDTA,500 mM sorbitol, then cast into plugs and treated with 20 ml of protease, EDTA and SDS as above. Electrophoresis was performed with a Bio-Rad CHEF DR-II system using 0.5 X TBE (Maniatis et al., 1982) cooled to 12-15°C. Gels were stained with 0.5 µg/ml ethidium bromide and destained in used 0.5 X TBE.

RESULTS AND DISCUSSION

The electrophoretic karyotype determined for U. hordei, U. maydis, T. caries, T. controversa, and S. cerevisiae using intact cell material were resolved as well as, or better than protoplasted material (Figure V.1). U. hordei low, and high molecular weight DNA bands were equally resolved using either protoplasts or intact cells. For T. caries and T. controversa, equal or better resolution of all size-range chromosome-size DNA was seen for non-protoplasted sporidial and mycelial preparations than for protoplasts (Figure V.1). For both U. hordei and S. cerevisiae, colonies were removed from solid medium and prepared directly for PFGE. The chromosomes from these samples were clearly resolved and produced karyotypes that were indistinguishable from larger scale preparations. The chromosomes from mycelial cultures of other fungi including strains of Rhizopus, Botrytis, Fusarium, Pseudocercosporella herpotrichoides, Cephalosporium graminieum and the unicellular green alga Chlorella have proven amenable to this simplified technique. The mechanism whereby chromosome-sized DNA escapes from un-protoplasted cells is unknown, although our current working hypothesis assumes that the DNA is electrophoresed directly through the remaining wall material. The possibility that the integrity of wall has been disturbed by the protease treatment cannot, however, be discounted. The applicability of this rapid method for a variety of organisms, its suitability for analysis of chromosomes of morphologically distinct cell types (fungal sporidia, mycelia and algal cells), as well as its usefulness for mini-preps from individual colonies will greatly increase the ease of studying genome flux among different groups in natural populations. A further benefit of this technique rests upon its application to organisms whose genetics and cytology are intractable.

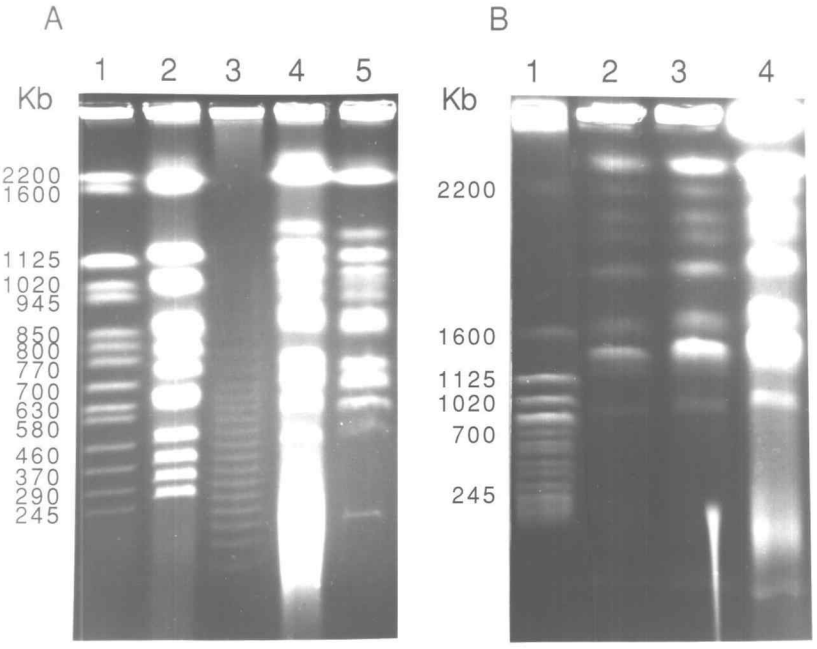

Figure V.1. PFGE comparison of intact cells versus protoplasts

A) 1% agarose gel run 25 hrs in 0.5 X TBE at 200 V, first 15 hr with 70 sec switch duration, then 10 hr with 120 sec switch duration. Lane 1, yeast molecular weight standards (Bio-Rad Laboratories), lane 2, intact yeast cells, lane 3, Lambda concatamers (Bio-Rad Laboratories), lane 4 U. hordei protoplasts, lane 5, intact U. hordei cells. B) 1% agarose gel run 72 hr in 0.5 X TBE at 105 V with a 480 sec switch duration. lane 1, Yeast (Bio-Rad Laboratories), lane 2, T. caries mycelia, lane 3, T. caries sporidia, lane 4, T. caries protoplasts.

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Heat Shock Generates a Mutant of Ustilago hordei That has Lost a Chromosome and is Altered in Morphology and Mating Response

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ABSTRACT

Heat shock has been used to derive a mutant of a Ustilago hordei that has lost a chromosome of 960 kilobases. This mutant has an altered cellular morphology, growing as filaments rather than sporidia, and has a dry, mycelial colony morphology. While the wild-type progenitor will mate with a tester strain of opposite mating type, the mutant fails to mate under the same conditions. The progenitor strain was obtained as a presumptive haploid basidiospore but was previously shown to have the greatest number of chromosomes, amounting to 35% more DNA than the strain with the fewest chromosomes, also a presumptive haploid basidiospore isolate. The fate of the chromosome involved was investigated by constructing chromosome-specific DNA clone mini-libraries. One probe derived from the 960 kb band hybridizes to ten bands in the wild-type progenitor strain, but only nine in the mutant.

INTRODUCTION

Heat shock has been shown to induce chromosome loss in genetically marked strains of Candida albicans (Hilton et al., 1985). The strains of C. albicans involved in these studies were diploid, aneuploid or tetraploid, and loss of several linked markers showed that an entire chromosome was lost. Colony morphology mutants of C. albicans arose at a frequency of 1.4% and were shown to have altered electrophoretic karyotypes with several strains exhibting more than one change (Rustchenko-Bulgac et al., 1989). One-third of the mutans strains examined were unstable, and showed sectoring in culture.

As Candida is an asexual fungus, the suggestion that these chromosome-length polymorphisms are important in generating variability is particularly relevant. Many phytopathogenic fungi have been shown to have polymorphic karyotypes (reviewed in Mills and McCluskey, 1990), and speculation as to the role of this variability in plant-fungi interactions is beginning (Mundt, 1991).

Chromosome length and number has been shown to be variable in presumptive haploid strains of the phytopathogenic fungus Ustilago hordei (McCluskey and Mills, 1990). The possibility that at least some of these strains were disomic for at least one chromosome suggested that it might be feasible to induce chromosome loss using heat shock. The strains selected for the initial heat shock experiments differ in their total DNA content by as much as 35%, as determined by electrophoretic karyotyping. Strain 10.1a has the greatest number of chromosomes; strain 2.1a has the fewest chromosomes.

In this study heat shock has been used to generate a mutant that has a filamentous cellular morphology, and is missing an entire chromosome band, as determined by PFGE. The fate of the missing band has been asessed by hybridization with chromosome-specific DNA probes, and the ability of the mutant to mate with a compatible tester strain has been assayed.

MATERIALS AND METHODS

Strains and plasmids

U. hordei strains 2.1a and 10.1a were described in McCluskey and Mills (1990). Strain 2.1a was shown to have the fewest chromosomes, and strain 10.1a was shown to have the greatest number of chromosomes. U. hordei strain 11.2a is derived from an ordered tetrad, and mates with strain 10.1a. The high copy Escherichia coli cloning vector pUC19 (Norrander et al., 1984) as well as recombinant plasmids containing chromosome-specific inserts were prepared from the E. coli strain DH5α (Bethesda Research Laboratories, Gaithersburg, MD) by an alkaline lysis procedure (Birnboim and Doly, 1979). DNA probes were liberated by digestion with the appropriate restriction enzyme and were separated from the vector in low gelling-temperature agarose gels according to the methods of Maniatis et al. (1982). Radiolabelled DNA probes were prepared according to the random priming technique of Feinberg and Vogelstein (1983).

Heat shock

U. hordei strains 2.1a and 10.1a were cultured in Potato Dextrose Broth (PDB, Difco Laboratories, Detroit, MI) to mid log phase (ca.10^6 cells per ml). A 3 ml aliquot of each culture was transferred to a 5 ml polypropylene tube and incubated at 52° C for two to ten minutes. Heat shock was terminated by transferring 100 µl of the heat treated sample to 900 µl PDB at room temperature, and dilutions were spread on the surface of Potato Dextrose Agar (PDA) medium and incubated at room temperature for two to five days until colonies were visible. An aliquot of the starting material was diluted and plated on PDA to determine the concentration of the starting culture, and this value was

compared to the average of several platings of the heat treated sample to determine the percent of surviving cells.

Pulsed-field gel electrophoresis

Samples for CHEF PFGE were prepared from liquid culture for strains 2.1a, 10.1a and 10.1a-1, and from PDA plate culture for 10.1a-1 and other strains that were screened for the presence of chromosome-length polymorphisisms as described in McCluskey et al. (1990). CHEF PFGE was carried out as described in McCluskey and Mills (1990), using pulse parameters designed to allow separation of bands in the 100 to 2,200 kilobase range. Chromosomes greater than two megabases were resolved using 480 sec pulse for 48 hr at 200 V in 0.85% Ultrapure agarose (International Biotechnologies, Inc. New Haven, CT). DNA probes were generated by excising a 960 kilobase band from a CHEF gel run with low-melting temperature agarose (FMC Bioproducts, Rockland, ME), and extracting the DNA using a freeze/thaw protocol. Briefly, the excised band was melted at 68° C for 15-20 minutes in 5 ml 1M NaCl, 25 mM Tris pH 7.5, 1 mM EDTA, gently mixed, then frozen at -20° C and thawed at 37° C, twice. The agarose was collected by centrifugation for 30 minutes at 10,000 g, and the DNA was precipitated with an equal volume of isopropanol at -20° C overnight. The DNA was collected by centrifugation for 30 minutes at 10,000 g and the resulting pellet was suspended in 400 µl 10 mM Tris pH 7.5, 1 mM EDTA (TE). The DNA was subsequently extracted twice with an equal volume of TE-saturated phenol then with chloroform, and finally precipitated with ethanol (Maniatis et al., 1982). The final DNA preparation was digested to completion with EcoRI or BamHI according to the manufactures' instructions, and ligated into the cloning vector pUC19 using standard cloning techniques (Maniatis et al., 1982). DNA probes were prepared as described by McCluskey and Mills (1990). UV-nicked DNA was transferred to Genetran nylon

membranes (Plasco, Woburn, MA) using 0.4 N NaOH as the solvent without neutralizing the gel following denaturation. Hybridizations were carried out as described by McCluskey and Mills (1990).

Microscopy and mating of U. hordei strains

Fluorescence microscopy was carried out on a Zeiss standard microscope using epifluorescent illumination. Samples were stained for one hr at room temperature in 10 mM tris, 1 mM EDTA pH 7.5 using 0.0025 % calcofluor (Sigma, St Louis, MO) and 20 µg\ml Hoechst 33258 (Sigma) in the dark. Matings were carried out with cells from fresh colonies grown on PDA as described by Lade and Jensen (1967) but modified by the use of PDA supplemented with 1% activated charcoal.

RESULTS

Heat shock and morphology

Heat shock at 52° C produced approximately 99.9% lethality after seven minutes for both strains 2.1a and 10.1a (data not shown). Several of the surviving colonies had altered colony morphologies, and of 25 selected for electrophoretic karyotyping, strain 10.1a-1 was found to have lost a chromosome band of 960 kilobases (Figure. VI.1). The ability to associate chromosome loss with heat shock is based on the analysis of one mutant derived from a heat shocking experiment. The mutant strain 10.1a-1 was cultured successfully, and after samples had been prepared in a liquid culture, the stock culture was found to have a reduced viability. The entire culture was plated on fresh medium, and the altered karyotype was present in the rescued strain. While the 960 kb chromosome was completely missing in the rescued strain, the original strain seems to have had a faint band in this size range suggesting that the original culture was not pure. The rescued mutant has a completely mycelial growth habit, and the production of chromosome samples is hampered by an apparent paucity of nuclei. While highly concentrated samples (with respect to cellular material) are readily prepared, the intensity of staining of separated chromosomes with ethidium bromide is low. No surviving strains derived from strain 2.1a were found to have altered karyotypes. Bands not resolved in the gel shown in Figure VI.1 were resolved by long-term electrophoresis and determined to be the same in both strains 10.1a and 10.1a-1 (data not shown). Strain 10.1a has a mucoid, convex colony (Figure. VI.2 A) while strain 10.1a-1 has a dry, mycelial colony, whose overall appearance was of being crusty (Figure. VI.2 C). Microscopic observation (Figure. VI.3) showed that cells of the mutant strain 10.1a-1

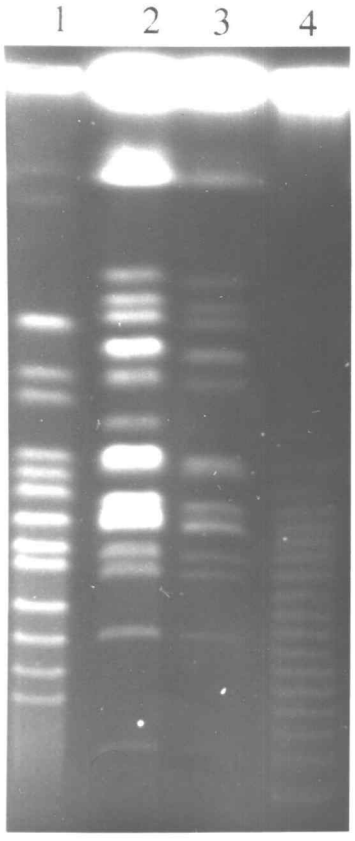

Figure VI.1. Heat induced karyotypic variability in Ustilago hordei.

Lane 1. Saccharomyces cerevisiae chromosome size standards (BioRad). Lane 2 U. hordei strain 10.1a chromosomes. Lane 3. U. hordei heat shock mutant 10.1a-1 chromosomes. Lane 4. Concatamers of the phage lambda as a size standard (BioRad).

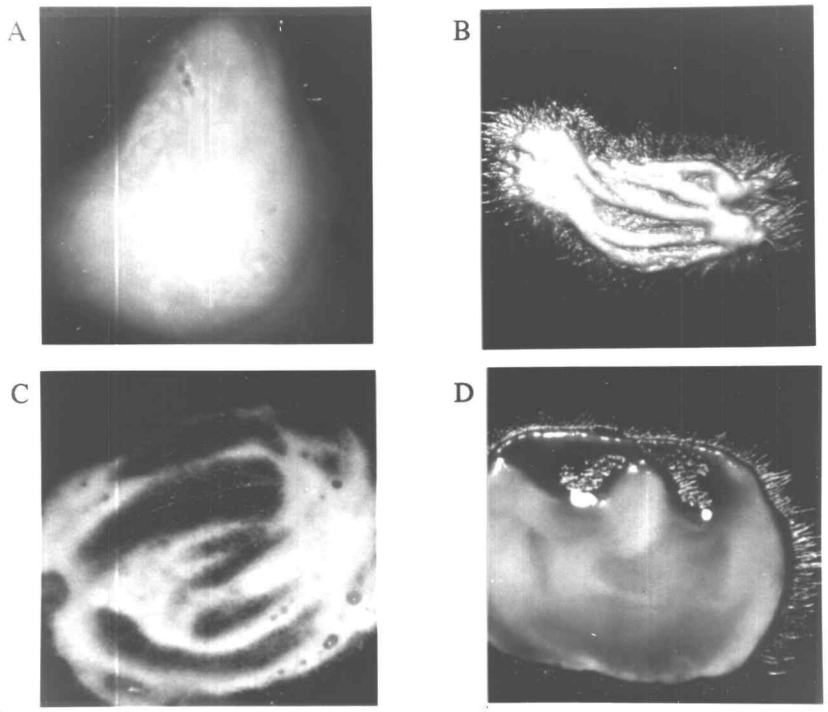

Figure VI.2. Colony morphology and mating response of Ustilago hordei strains 10.1a and 10.1a-1. A) Strain 10.1a. B) Strain 10.1a X strain 11.2a. C) Strain 10.1a-1. D) Strain 10.1a-1 X strain 11.2a.

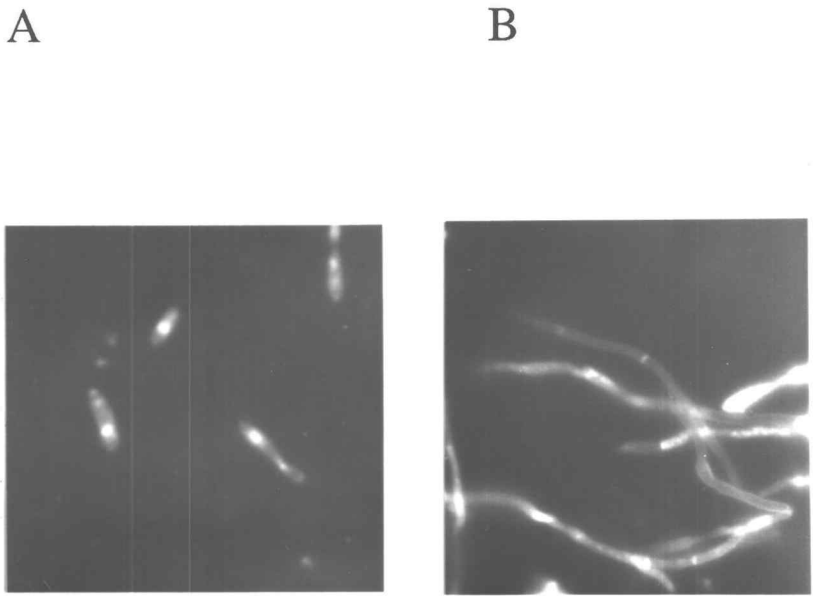

Figure VI.3. Microscopic analysis of cell morphology of Ustilago hordei strains 10.1a and 10.1a-1. A) Strain 10.1a. B) Strain 10.1a-1.

are filamentous while the wild-type strain 10.1a has a yeast-like, sporidial morphology. Clamp connections were not observed in strain 10.1a-1, nor were multiple nuclei visible in individual cells (Figure VI.3), although intense fluorescence was seen in hyphal tips. Mating tests between strain 10.1a and tester strain 11.2a showed the fuzzy aerial hyphae typical of the compatible interaction (Figure VI.2B). The mutant strain 10.1a-1 failed to mate in several tests with testers of both mating types, and the response with strain 11.2a is shown in Figure VI.2D. This strain was observed to produce sparse aerial hyphae in matings carried out on double strength complete medium amended with 1% activated charcoal (J. W. Kronstad, personal communication).

Hybridization analysis

Hybridization with DNA probes derived from the 960 kb band from strain 10.1a has not allowed an unequivocal determination of the fate of the missing band. Other bands in the same size range hybridized with other probes, none of which hybridized to the 960 kb band in strain 10.1a (Figure III.3). The mutant strain 10.1a-1 was not included in many of these other hybridizations. One probe (chromosome specific probe 10.1a-8.6) was derived from the 960 kb band in a gel that had a considerable amount of degradation and hybridized to bands of 1,260 and 1,150 kb, and to unseparated bands of 890 and 850 kb (data not shown) in both strains. Another probe (10.1a-620) from the same preparation hybridized to unresolved bands of >2,000 kb. Probe 10.1a-812 that was cloned from BamHI digested chromosome-specific DNA from the 960 kb band in strain 10.1a hybridized to ten bands in strain 10.1a, and to nine band(s) in strain 10.1a-1 with the only difference being the 960 kb band (Figure VI.4).

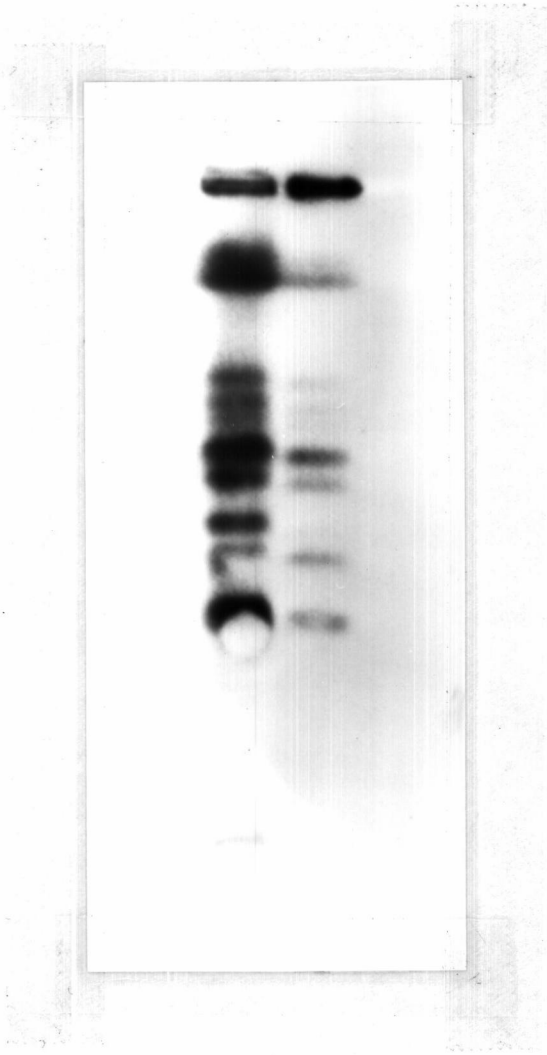

Figure VI.4. Hybridization to Ustilago hordei strain 10.1a and the chromosome-loss mutant 10.1a-1 with a probe derived from the 960 kb chromosome in strain 10.1a. The gel corresponding to this autoradiogram is shown in Figure VI.1.

DISCUSSION

A systematic screening of colonies of U. hordei that survived varying heat stresses revealed one colony that has an altered karyotype. The mutant strain is designated 10.1a-1, and is derived from strain 10.1a, the strain with the greatest number of chromosomes among strains representing the fourteen physiologic races. Twenty-four other colonies derived from strain 10.1a that survived heat shock for varying amounts of time were not seen to have altered electrophoretic karyotypes. Karyotypic variability was not detected in colonies of strain 2.1a that had survived heat shock. This result is relevant as strain 2.1a has the fewest chromosomes among strains representing the fourteen physiologic races. Strains that have an altered colony morphology but were not heat shocked have been found to have normal karyotypes (P. Wong, K. McCluskey and D. Mills, unpublished data). The karyotypic anomalies reported by Rustchenko-Bulgac et al. (1989) were found in strains of C. albicans that were naturally occurring colony morphology mutants. U. hordei strains regenerated from protoplasts during transformation experiments have the same karyotype as the progenitor strain (Chapter VII). Other stresses, such as treatment with compounds that destabilize mitotic spindle, have not been evaluated for their effect on karyotype. The possibility that the chromosome lost has no coding function and that the colony morphology mutation is unrelated to the loss of this chromosome cannot be evaluated at present. Moreover, the inability to mate may be a manifestation of the slower growth rate exhibited by the mutant or of difficulties in anastomosis related to the altered cell morphology. The filamentous growth habit displayed by the mutant 10.1a-1 is similar to the morphology of the infective dikaryon for U. hordei. This morphology is typically produced when a strain

contains two different ideomorphs of the mating type locus. The production of a filamentous morphology suggests that this strain has become de-repressed for filamentous growth, suggesting that a gene that is normally active in the monokaryon, but inactivated in the dikaryon is missing.

The DNA probe derived from the 960 kb chromosome in the wild-type progenitor strain 10.1a hybridized to ten bands in that strain, suggesting that it is a repeated DNA element. Other inserts derived from the 960 kb chromosome have been identified but have not been hybridized to separated chromosomes. These experiments will serve to define whether this band is supernumerary or disomic in strain 10.1a, and whether it has undergone recombination in the mutant rather than complete deletion.

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Transformation of Ustilago hordei with the Replicating Plasmid pCM54 and Detection of Putative Plasmid Concatamers

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ABSTRACT

The monokaryotic Ustilago hordei strain 8.1a was transformed to hygromycin B resistance with the replicating plasmid pCM54 at a frequency of 70 to 250 transformants per microgram of DNA per 10^6 recipient protoplasts. Protoplasts were observed to regenerate at approximately 4% efficiency in the absence of selection. Spontaneously resistant colonies were not detected with 150 µg/ml hygromycin B. Chromosomes from several transformants were separated by pulsed-field gel electrophoresis and three DNA bands that migrated with yeast chromosomes of 800 and 945 kilobases (kb) and two that migrated below 100 kb but that were vot visible in the ethidium bromide stained gel were detected by homology to the hygromycin B phosphotransferase (HPT) gene coding region. The number of bands detected by hybridization with HPT varies from two to five among different transformants studied. Plasmid DNA prepared from Escherichia coli was shown to comigrate with these different size classes of DNA, and it is possible that the high molecular weight forms are concatamers.

INTRODUCTION

To understand the influence of avirulence genes, genes that confer aggressiveness, inhibitors to hyphal development, and mating functions on the interaction between Ustilago hordei and Hordeum vulgare, it will be necessary to express cloned genes in defined pathogen strains. Moreover, to determine the function of cloned DNA, it is desirable to complement mutations of known phenotype. Genes cloned in smut fungi by complementing mutations include URA3, LEU2, and TPI1 from U. maydis (Kronstad et al., 1989). Kronstad and Leong (1989) reported identification of the b mating type allele from U. maydis by screening for a change in colony morphology following transformation. Bej and Perlin (1989) reported on a transformation system for the fungus U. violacea that yielded 60 - 80 transformants per microgram of pUCH1 DNA using lithium acetate and polyethylene glycol treatment of intact sporidia. Plasmid DNA extracted from the transformed strains and used to transform Escherichia coli contained deletions and the authors suggested that the plasmid was recombining out of the fungal chromosome at low frequency. Another simple method of transformation that has been employed for Saccharomyces cerevisiae and may be useful for transferring replicative plasmids into phytopathogenic fungi takes advantage of the ability of E. coli to transfer plasmid DNA from one cell to another by conjugation (Heinemann and Sprague, 1989).

Holden and Leong (1989) transformed U. hordei and U. nigra using a modified protocol developed for U. maydis. They concluded that the vector pHL1 had undergone reorganization associated with integration into the host genome. The transformation frequency reported for U. hordei was 10 to 50 transformants per microgram of

transforming DNA per 2 x 10^7 protoplasts. This is an order of magnitude lower than that seen for U. maydis (Wang et al., 1988). The differences between U. hordei and U. maydis have been more prominent than any similarities, and this is true for transformation as well. Protoplasts of U. hordei are produced by budding (McCluskey and Mills, 1990) rather than by complete digestion of the walls, as is seen in U. maydis (Wang et al., 1988). This difference is further exemplified by the difficulty of regenerating protoplasts of U. hordei. While regeneration frequencies of 60% are readily achievable for U. maydis (Wang et al., 1988; Tsukuda et al., 1989), U. hordei protoplasts regenerate at a frequency of approximately 1% (Holden and Leong, 1989) to 4% (McCluskey and Mills, 1990). The protocol developed for high frequency transformation of U. maydis by Tsukuda et al. (1989) has proven more reliable for transformation of U. hordei, although this protocol had to be modified for application to U. hordei. The transformation of U. hordei, as well as the fate of the transforming DNA, will be described.

MATERIALS AND METHODS

Strains and plasmids

U. hordei strain 8.1a, as described in McCluskey and Mills (1990), is a monokaryotic strain with a mucoid convex colony morphology. S. cerevisiae chromosomes used as molecular weight markers were prepared from strain X164. The plasmid pCM54 was kindly provided by Dr. W. M. Holloman (New York, NY), and is described in Tsukuda et al. (1989). The plasmid pLG90 was kindly provided by Dr. S. A. Leong (Madison, WI) and is described in Gritz and Davis (1983). Plasmid DNA prepared from the E. coli strain DH5α (Bethesda Research Laboratories, Gaithersburg, MD) by a modified alkaline lysis procedure (Birnboim and Doly, 1979) was further purified by bouyant density centrifugation in CsCl gradients (Maniatis et al., 1982).

Protoplast preparation

Protoplasts of U. hordei prepared as described in McCluskey and Mills (1990) were observed with a Wild EB-11 microscope. To separate protoplasts from cellular debris, protoplasts were further treated by three successive washes achieved in 5 ml of 0.8 M sorbitol 100 mM $CaCl_2$ and 10 mM tris, pH 7.5 (STC) followed by centrifugation at 1500 x g for 10 minutes. Protoplasts were ultimately suspended in STC at a concentration of 2×10^7 per ml . Protoplast numbers were determined by counting diluted suspensions on a haemacytometer. Protoplasting efficiency was determined by diluting the protoplast suspension in either STC or water and plating on either regeneration medium or on Potato Dextrose Agar (PDA; Difco Laboratories, Detroit, MI). Regeneration frequency was determined by comparing the number of cells plated

(determined by counting on a hemacytometer) and the observed number of colonies after eight to ten days incubation without selection.

Transformation

The transformation protocol employed was essentially that of Tsukuda et al., 1988). Briefly, 50 µl of the protoplast suspension was mixed with 100 or 400 ng of the plasmid pCM54 and 15 µg heparin. The entire mixture was placed on ice for 10 minutes, after which 0.5 ml of 40 % Poly Ethylene Glycol (average molecular weight 4000) in STC was added and mixed gently. The suspension was incubated on ice for an additional 15 minutes after which it was mixed with 5 ml 50° C Holliday's complete medium (Holliday, 1974) prepared with the addition of 1% yeast extract as described (Holliday, 1974), omitting the salts and adding 0.8 M sorbitol. This mixture was spread on the surface of 20 ml of agar solidified complete medium amended with 0.8 M sorbitol in a 150 mm petri dish and the plates were overlain with filter sterilized hygromycin B in double distilled water at a final concentration of 150 µg/ml after six hours at room temperature. These plates were maintained at room temperature and colonies became visible after 4-5 days incubation. Transformants were maintained on PDA supplemented with 150 µg/ml hygromycin B.

Pulsed-field gel electrophoresis and hybridizations

Samples for CHEF PFGE were prepared as described in McCluskey et al. (1990) from colonies removed from the surface of PDA containing 150 µg per ml hygromycin B. CHEF PFGE was carried out as described in McCluskey and Mills (1990), using pulse parameters designed to allow separation of bands in the 100 to 2,200 kilobase (kb) range. UV nicked DNA was transferred to Genetran nylon membranes (Plasco, Woburn MA) using 0.4 N NaOH as the solvent without neutralizing the gel following

denaturation. Hybridizations were carried out and DNA labelled as described in McCluskey and Mills (1990).

The 1.05 kb BamHI restriction fragment containing the hygromycin B phosphotransferase (HPT) gene coding region was liberated from the plasmid pLG90 (Gritz and Davis, 1983) by digestion with BamHI according to the instructions supplied with the enzyme and separated from remaining vector DNA by electrophoresis in low gelling temperature agarose (FMC, Rockland ME). DNA was extracted from the melted gel by extraction with phenol (Maniatis et al., 1982).

RESULTS

Transformation and regeneration

Transformation efficiency varied from approximately 70 to 250 transformants per microgram of DNA per 10^6 protoplasts. These results are based upon observation of 106 transformants from one experiment. The protoplasting efficiency in this experiment was greater than 99% and the regeneration efficiency was approximately 4%. Other experiments gave comparable results, but were confounded by errors in spreading the protoplast mixture on the regeneration medium. Regeneration of 4% has been seen in several experiments (data not shown).

Fate of transforming DNA

The fate of transforming DNA was determined by hybridizing ^{32}P labelled HPT coding region DNA to chromosomes separated in an agarose gel (Figure VII.1). The plasmid pCM54 is apparently present as an autonomously replicating molecule that is present in several different size classes. The replicative forms were not visible on the ethidium bromide stained gels in two separate experiments, but were shown to comigrate with plasmid pCM54 that was derived from E. coli (Figure. VII.1). The higher molecular weight form is present in two bands that comigrate with linear S. cerevisiae chromosomal DNA of 950 and 800 kb in several transformants (Figure VII.1). The E. coli derived plasmid DNA also migrated at the same positions (Figure VII.1) and is visible on the ethidium bromide stained gel. Total genomic DNA from several transformed U. hordei strains has been used to transform E. coli to ampicillin resistance,

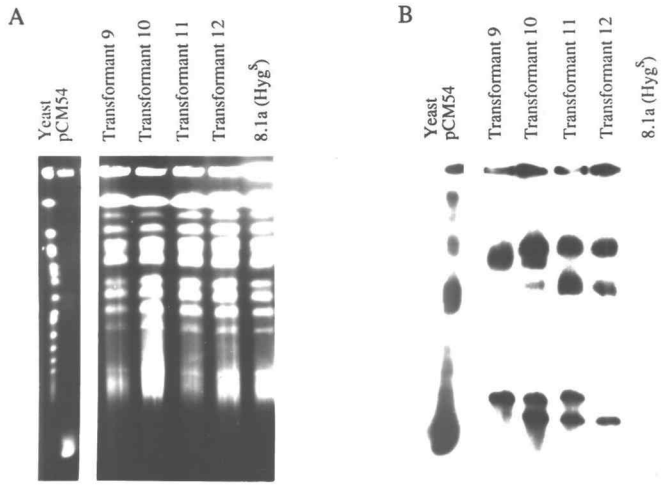

Figure VII.1. Detection of pCM54 among separated chromosomes in transformed strains of Ustilago hordei. A) CHEF pulsed field gel showing four transformed strains, the Hygromycin B sensitive progenitor strain, the transforming plasmid, and Yeast chromosomes as a size marker. B) Hybridization of the HPT gene from pLG90 to the gel in panel A.

and the plasmid pCM54 is recoverable from these E. coli transformants (K. Hoffman and D. Mills, unpublished data).

In transformant 9 the HPT homologous DNA that migrated with the 800 and 950 kb chromosomes was present as one band of intermediate size. The smallest band, presumably a monomer was also present in only one size. In transformant 10, there were three HPT homologous bands in the 800 to 950 kb size range, and two that migrated in the size range of the presumptive monomer. Transformant 11 had two bands in each size range that hybridized to the HPT probe DNA, while transformant 12 had two bands in the high molecular weight range and only the smaller of the two bands that migrate in the size range of the presumptive monomer. There was no hybridization between the HPT coding region and the hygromycin B sensitive progenitor strain 8.1a, or to yeast chromosomes used as molecular weight markers (Figure VII.1).

DISCUSSION

The efficiency of transformation that has been achieved in the course of this study is approximately five fold higher than that achieved by Holden and Leong (1988), but is still ten-fold less than that seen by Tsukuda et al. (1988) in their studies of U. maydis. This difference may represent difficulties in regeneration of U. hordei protoplasts, as 60% regeneration is not uncommon for U. maydis while the best regeneration seen for U. hordei in the course of the present study was 4%.

The presence of multiple sizes of the plasmid pCM54 suggests that it has undergone structural recombination associated with transformation or replication. The detection of replicative forms in U. hordei that seem to coincide in size with putative concatamers from E. coli suggests that similar mechanisms of replication occur. The recovery of pCM54 from U. hordei by transformation of E. coli strongly supports the conclusion that the molecules detected by homology to HPT are replicative forms. The replication in E. coli is presumed to be under the control of Ori C, while the replication in U. hordei is presumed to be due to the presence of the Ustilago Autonomously Replicating Sequence (UARS) described by Tsukuda et al. (1990). While the size correspondence between the E. coli derived DNA and the U. hordei DNA was not exact, the samples from U. hordei have much more DNA per lane, which could affect the migration of the plasmid DNA. It is possible that the mechanism of concatamerization is similar in U. hordei under the control of the UARS as in E. coli, although other mechanisms that could give rise to the same result are not ruled out. One plausible mechanism that could lead to similar results would require that the plasmid integrate into the same chromosome in several strains. The data presented above do not support this

theory because the hybridization to the HPT probe occurs in a region of the gel where there are no bands visible on the ethidium bromide stained gel (Figure VII.1). The fate of transforming DNA, and the presence of different sizes of replicative forms is a problem that warrants further study. Replicating plasmids are ideal for the study of genetics of fungi where they can be selected for. The construction of mitotically stable vectors, or artificial chromosomes will require replicative plasmids. Finally, replicating plasmids give a higher frequency of transformation and will allow for the rapid recovery of transforming DNA.

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A Predictive Model that Associates Fungal Karyotypes with Fungal Life-Cycles

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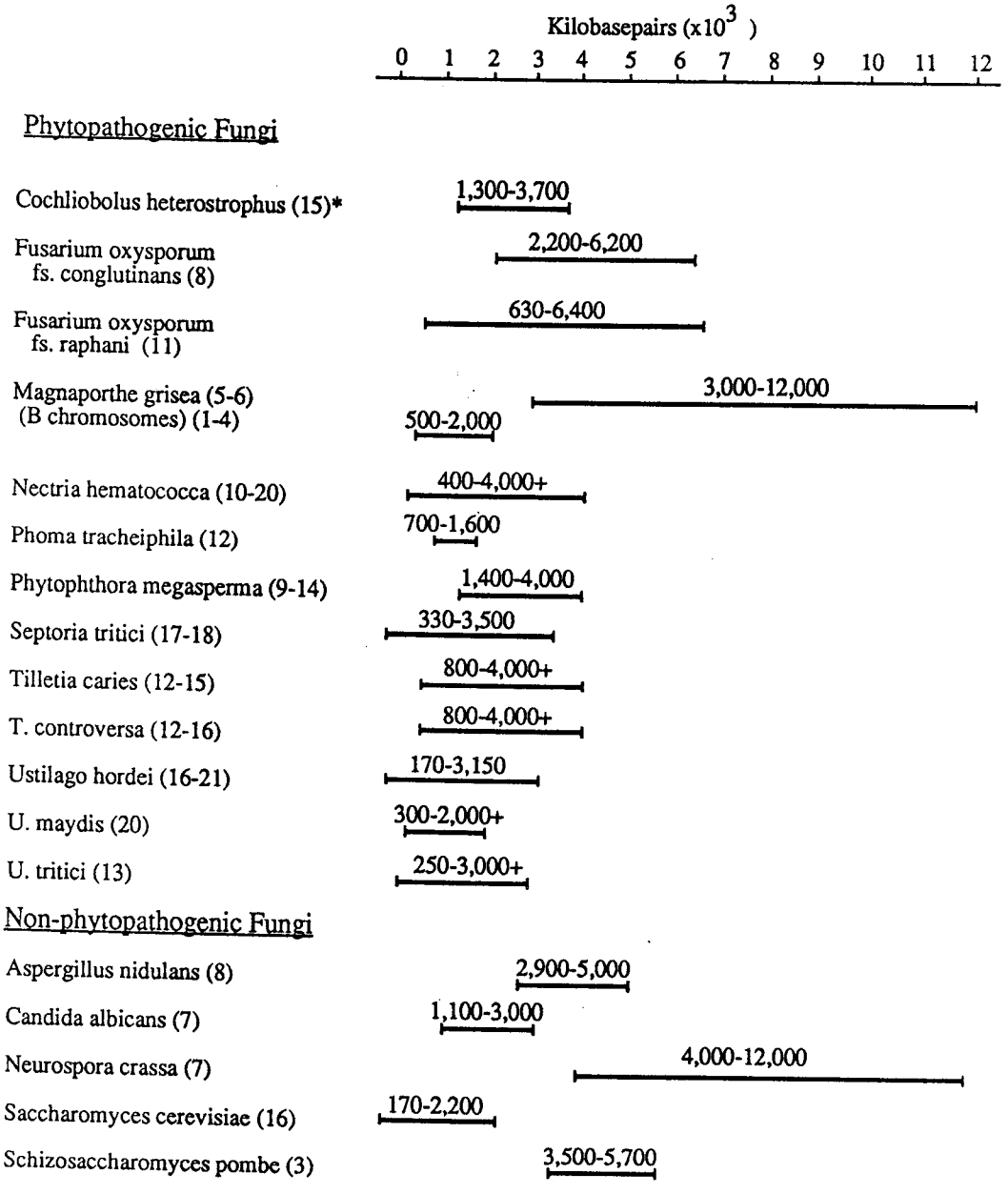
THE MODEL

The recent development of electrophoretic karyotyping has allowed a rapid expansion of information regarding the structure of genomes of many different fungi. There appear to be two different categories of fungi that are defined by having either a few large chromosomes or many smaller chromosomes. There is no obvious reason to predict whether a given fungus would fall into either of the two groups. , However, a thorough examination of the fungal electrophoretic karyotypes compiled by Mills and McCluskey (1990) (depicted in Figure VIII.1) allows for a hypothesis to be formulated that utilizes the present data to associate karyotype with lifecycle. In this hypothesis, fungi that are primarily haploid and asexual, or for whom asexual reproduction plays a primary role in their life cycle, are seen to have a small number of relatively large chromosomes; conversely, fungi that are primarily, or obligately sexual or are diploid or dikaryotic have a larger number of smaller chromosomes. This hypothesis is supported by the available data, and by theoretical considerations. Let us first address the data, and then the theoretical support for the proposed association.

Schizosaccharomyces pombe has a normally haploid vegetative thallus and is typically heterothallic. The karyotype of S. pombe consists of three chromosomes of 3.5, 4.6 and 5.7 megabases (Mb) respectively (Smith et al., 1987). In this extreme case, each chromosome comprises from 25 to 41 percent of the entire genome. Similarly, this correlation between chromosome number and the life cycle follows for other fungi. Neurospora crassa has seven chromosomes of 4 to 12 Mb (Orbach et al., 1988), is typically haploid, and reproduces freely by asexual conidiospores. Aspergillus nidulans has eight chromosomes ranging from ca. 2.9 to at least 5 Mb (Brody and Carbon, 1989)

Chromosome Lengths Among Fungi



* Number of chromosome bands detected in various strains

Figure VIII.1. A graphical representation of the distribution of chromosome sizes for several different fungi.

and, although it has a sexual stage, many other members of the genus do not, and the sexual stage of A. nidulans is difficult to detect. Magnaporthe grisea has five to six chromosomes that are 3 to 12 Mb in length (discussed by Mills and McCluskey, 1990). The sexual stage for M. grisea was not known until recently. Nectria haematococca has been shown to have ten to twenty chromosomes ranging from 400 kb to greater than 4 Mb (Miao and VanEtten, 1991). The suggestion was made that many of these could be B chromosomes, and in this light, the lower estimate must be considered as representative of the haploid complement of chromosomes. Most of these fungi are able to build up rapidly in local populations due to the prolific nature of their asexual stages. It is this aspect of their life cycle that seems to associate them.

At the other end of the scale, there are a number of yeasts and phytopathogenic fungi with sexual reproduction being of greater importance, or that are diploid or dikaryotic for a large proportion of their lifecycles. Most of these fungi have complicated karyotypes with ten or more chromosomes and a high degree of variability among isolates. First among these is Saccharomyces cerevisiae (Carle and Olson, 1985) which has sixteen chromosomes ranging from approximately 150 kilobases (kb) to 2,200 kb. S. cerevisiae has an important vegetative phase, but exists primarily as a diploid, due largely to its normally homothallic state. Other yeasts have fewer chromosomes with similar size distributions (DeJonge et al., 1986). Ustilago maydis was shown to have as many as 20 chromosomes varying in length from 300 to greater than 2,000 kb (Kinscherf and Leong, 1988). Strains from fourteen races of U. hordei have been shown to have from 16 to 21 chromosomes that vary in length from approximately 170 to 3,150 kb (McCluskey and Mills, 1990). This fungus is obligately sexual, as is U. maydis. Five other species of Ustilago have been shown to have from thirteen to twenty chromosomes (McCluskey and Mills, unpublished data). In a recent report, Boehm et al. describe the karyotype of Puccinia graminis f. sp. tritici as consisting of eighteen

chromosomes (Fourth International Mycological Congress, abstract # ID-172-4). In this fungus, each chromosome comprises three to nine percent of the entire genome. Among these sexual fungi are several that exploit limited or evanescent niches, or who are opportunistic and can readily adapt to restrictive (or resistant) hosts.

The theoretical support for this theory may be based on the genetic mechanisms leading to variability through sexual reproduction, and in the frequency of chromosome nondisjunction at mitosis. From a simplistic perspective, it is obvious that greater variability can be generated by random assortment of parental chromosomes at meiosis in organisms that contain greater numbers of chromosomes. While longer chromosomes may be envisioned to have a greater frequency of crossing over at meiosis, physical length may not always correlate well with recombination frequency. Moreover, two strand double crossovers will cause markers distal to both crosses to appear as if no crossover had occurred. Crossingover involving three or more chromatids will complicate this situation, but the variability contributed by these events is of greater importance in outcrossing systems. Therefore, it is envisioned that selection for a higher number of chromosomes may be favored in sexual, diploid or dikaryotic fungi.

For haploid, asexual fungi this is not as viable a scenario. Selective forces may be acting to limit the number of chromosomes. Chromosome nondisjunction at mitosis in haploid fungi would lead to one strain that was disomic and another, a nullo, that is missing a particular chromosome. The individual that failed to receive a chromosome would, in all likelihood, be non-viable. The probability that any particular chromosome will be involved in a nondisjunctional event may be the same whether an organism has many or few chromosomes. Conversely, the probability that any chromosomes may be involved in a nondisjunctional event is proportional to the number of chromosomes. Thus, barring any effect on nondisjunction rate, an organism with only one chromosome has only a finite probability of being involved in a nondisjunctional event at any

individual mitosis. If the same organism had ten chromosomes, it would theoretically have ten times as many opportunities, and hence ten times the probability of having a single nondisjunction at an individual mitosis. By having fewer chromosomes, a haploid fungus that reproduces asexually would have a lower probability of producing non-viable offspring. Fungi that are sexual only when faced with deteriorating environmental conditions may benefit by preserving genotypes that were beneficial during asexual growth. Having a limited number of chromosomes may allow for maintenance of co-adapted genes that function synergistically. Asexually reproducing fungi that are predominantly diploid or dikaryotic may not subject to the same level of selection for fidelity at mitosis. These fungi would be predicted to be subject to selection for other traits which fall outside the scope of the present discussion.

If this prediction is correct, the prediction of the lifecycle of an unknown fungus could be made given an electrophoretic karyotype. If confronted with a pattern of one to ten chromosomes with several of the chromosomes representing greater than ten percent of the entire genome each, the prediction would be that this was an asexual haploid fungus. Conversely, if confronted with a karyotype that contained more than ten chromosomes each representing one to fifteen percent of the entire genome, the prediction is that the organism reproduces primarily sexually and is diploid or dikaryotic during a considerable portion of its life cycle. The prediction of the karyotype of a fungus based on its lifecycle is simply the reverse of this argument. The recognition that many of the fungi that fall into the asexual category are ascomycetes is relevant because the differences between the ascomycetes and basidiomycetes are related to the life cycles of the different subdivisions. Ascomycetes typically have a haploid phase as the vegetative stage, and immediately undergo sexual reproduction when a suitable dikaryon is formed with each mating giving rise to a single fruiting structure. Basidiomycetes,

conversely, can have a persistant dikaryotic thallus, and a single mating can give rise to many fruiting structures.

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