Selection and Culture of Auxotrophic and Drug-Resistant Mutants of *Tilletia caries*

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ABSTRACT

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Mutagenesis and selection procedures for isolation of auxotrophic mutants of *Tilletia caries* race T-1 were developed. Mutants with requirements for adenine, uracil, glycine, or a general nitrogen source were identified. Only spontaneous mutation to cycloheximide resistance was observed in tests with 12 growth-inhibiting drugs. These latter mutants had restricted colony growth and generally failed to produce secondary sporidia in the presence of cycloheximide. Paired, sexually compatible, auxotrophic, haploid strains produced mycelial growth on minimal medium, presumably because of heterokaryosis. Secondary sporidia derived from these matings exhibited the nutrient requirement of either parent, indicating that the frequency of reverse mutation and stable diploid formation was low.

Additional key words: common bunt, complementation, smut.

The bunt fungi of the genus *Tilletia* are among the most destructive cereal pathogens (7); however, studies concerning the genetics of pathogenicity of these smut fungi have not kept pace with similar studies of other plant pathogens (8). The slow progress in understanding the genetics of the common wheat bunt pathogen, *Tilletia caries* (DC.) Tul., and related fungi can be attributed in part to the absence of experimental strains with suitable genetic markers.

Previous interspecific and intraspecific hybridization studies have examined the inheritance of diploid parental traits of *Tilletia* spp. (6,11–13,23,24). These studies used properties such as pathogenicity, teliospore morphology, teliospore germination, and sorus shape as characters for segregation analysis. Expression of these factors was highly variable, and progeny often showed a continuous range of characteristics rather than discrete parental phenotypes. Genetic studies of other plant-pathogenic fungi have commonly used biochemical mutations controlled by single genes as nuclear genetic markers, in addition to general physiological or morphological traits (1,4,10,15,18,22). Similar mutants of *T. caries* and related bunt fungi would greatly facilitate genetic studies of these pathogens.

This paper reports the development of mutagenesis and selection procedures for isolation of biochemical and drug-resistant mutants of *T. caries*. Sexually compatible auxotrophic strains were used in complementation tests, and the resulting prototrophic growth was examined for heterokaryosis. *T. caries* was selected for use in this investigation over other wheat bunt fungi because of the availability of monosporidial haploid strains with favorable laboratory culture characteristics.

MATERIALS AND METHODS

Strains and media. Monosporidial haploid strains of *T. caries* (race T-1) no. 18 (+ mating type) and nos. 24 and 26 (− mating type) (14) were maintained on noncommercial potato-sucrose agar (PSA) at 4 C and subcultured at 2-mo intervals. Abundant filiform and lunate secondary sporidia were produced for mutagenesis experiments in liquid T-19 medium (27), referred to in this report as medium A. The cultures were maintained by transfer of an aliquot (0.5 ml) of inoculum to 50 ml medium A at 5- to 7-day intervals. The generation time for each strain has been described previously (3).

Two additional media were used to isolate and characterize auxotrophic strains. A minimal medium, medium B, differed from medium A in that KNO_3 (4 g/L) was substituted for asparagine, and Noble agar (1.5%) was used as a solidifying agent when required. A complete medium, medium C, contained the constituents of medium A plus nucleic acid bases (10 mg/L), Edamin amino acids (1 g/L) (Sheffield Chemical Co., Norwich, NY 13815), and an aliquot (10 ml/L) of 100 × vitamin solution. The vitamin solution contained riboflavin, pyridoxine-HCl, and *p*-aminobenzoic acid (each 50 mg/L); calcium pantothenate, nicotinic acid, and choline chloride (each 200 mg/L); inositol (400 mg/L); and biotin (2.6 mg/L). All cultures were incubated in the dark at 19 C. Liquid cultures were grown on a rotary shaker (175 rpm).

Mutagenesis and selection of auxotrophs. Log-phase 5- to 7-day old liquid cultures of secondary sporidia were harvested from medium A for mutagenesis experiments by filtration through sterile 20-μm nylon mesh. The concentration of sporidia in the filtrate was estimated with a Neubauer hemacytometer, and the cells were collected by centrifugation at 6,000 *g* for 10 min., then washed and resuspended in medium A. Sporidia (2×10^7 cells per milliliter) were exposed to 100 μg/ml *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG) (Aldrich Chemical Co., Milwaukee, WI 53233) in 0.2 M acetate buffer, pH 5.6, for 20 min to 3 hr on a rotary shaker. After the exposure period, the NTG suspension was diluted with 15 volumes of medium C, and the sporidia were collected by centrifugation and washed. They were then transferred to 50 ml liquid medium C in flasks and incubated on a rotary shaker for 5 days to permit the cells to recover from the mutagen treatment (17,26). Secondary sporidia from these cultures were harvested by filtration, washed twice in medium B, and either transferred directly to medium C plates (total isolation procedure) or subjected to filtration enrichment (29).

Filtration enrichment of mutant cells consisted of two consecutive incubation periods in medium B, which prohibited growth of auxotrophs. Ungerminated sporidia, including putative

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auxotrophs, were separated from hyphal growth by filtration through 20-μm nylon mesh. After an initial 24-hr period in medium B, sporidia were harvested and collected by centrifugation, then washed and resuspended in fresh medium B for a second 24-hr incubation period. Ungerminated sporidia from the second harvest filtrate were concentrated on a 25-mm sterile polycarbonate filter (1-μm pore size). Sporidia were suspended in liquid medium C and transferred to agar plates of the same medium at a concentration of 50–75 sporidia per plate. The plates were incubated for 1.5–2 wk to allow colony diameter to reach about 3 mm.

Colonies arising on medium C plates were replica-plated to medium B to identify potential auxotrophs. Colonies that failed to develop within 1–1.5 wk were retested on complete and minimal media at least three additional times at weekly intervals. Putative auxotrophic mutants were tested for nutritional requirements by the methods of Holliday (9). A sample of each colony was homogenized in sterile distilled water in a tissue grinder, and test plates were inoculated with a 5-μl suspension of each auxotrophic strain and examined for growth after 1.5–2 wk. All mutant strains were subsequently maintained on PSA, medium C, or medium B supplemented with the appropriate growth factor.

Isolation of drug-resistant mutants. Late-log-phase secondary sporidia were harvested from liquid medium A and tested for their growth response to 12 drugs. Medium A plates containing various concentrations (0.01–300 μg/ml) of growth inhibitors were inoculated with 10^7–10^8 sporidia per plate and incubated for 2 wk. Presumptive drug-resistant mutants were transferred to medium A and permitted to grow in the absence of an inhibitor for 2 wk before retesting for drug resistance. Colonies that showed growth within 4 days were considered spontaneous drug-resistant mutants and maintained on drug-containing PSA plates.

Nuclear complementation and nutrient diffusion tests. Inocula were prepared either by homogenizing 5-mm plugs of each mutant strain in sterile distilled water in a tissue grinder or by dislodging sporidia and mycelial fragments from the surface of a plate using a moistened glass rod. Aliquots (5 μl) of two compatible strains were superimposed on a medium B plate and incubated at 19 C. Unpaired strains placed on the same plate served as controls.

To determine whether dissimilar compatible auxotrophs were able to grow on a minimal medium by cross-feeding of nutrients, a mycelial plug (3 mm^2) of one auxotroph was placed face down on a sterile nitrocellulose filter (0.2-μm pore size) on solid medium B, medium C, or on a growing culture of a dissimilar auxotroph. Each auxotrophic culture had been grown previously on medium B supplemented with a single required growth factor. The plates were incubated for 6 days before each growth response was recorded.

Nuclear staining. The nuclear condition of complementing auxotrophic strains was examined by staining hyphae and secondary sporidia with a DNA-specific fluorochrome, 4'6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co., St. Louis, MO 63172) as described previously (3).

RESULTS

Mutagenesis and mutant selection. A survival curve established in response to NTG exposure over a 12-hr period indicated that more than 95% of the cells were killed with treatments in excess of 5 hr. Mutant selection was successful with exposures of 20 min to 3 hr (about 85–15% survival, respectively) (Table 1), but a period of growth in complete medium was essential for recovery of auxotrophs. Although the growth period in medium C was omitted from initial experiments, one auxotroph was isolated from cells exposed to NTG for 3 hr (Table 1). Sporidia from this experiment were stored at 4 C in medium C for 12 days; such conditions permitted slow growth of these haploid strains. Both filtration enrichment and total isolation were satisfactory procedures for isolating auxotrophs, although the rate of mutant isolation was very low in all cases (0.04–0.16%).

Characterization of auxotrophic mutants. Adenine, uracil and glycine auxotrophs, and four organic nitrogen-requiring mutants were obtained after NTG mutagenesis (Table 2). These mutants grew slower on medium C than their wild-type parent strains. The ade^- strain also sporulated more profusely than the wild-type parent and usually produced tan or light amber-colored colonies rather than the white colonies of wild-type strains. The mutant strains requiring organic nitrogen grew very poorly on medium B compared with the wild-type parent strain. When this medium was supplemented with one or more amino acids as the nitrogen source, growth was comparable to the wild-type strain. Asparagine and glutamine provided the best source of organic nitrogen, although enhanced growth was observed in the presence of proline, arginine, phenylalanine, and tyrosine.

Isolation of drug-resistant mutants. The response of *T. caries* secondary sporidia to several growth-inhibiting drugs is given in Table 3. Only mutants resistant to cycloheximide were recovered. Antimycin A and oligomycin initially appeared useful for selecting spontaneous drug-resistant mutants, but colonies that initially seemed resistant failed to grow when retested. The other drugs failed to inhibit growth or caused only a delayed growth response.

Spontaneous cycloheximide-resistant strains were isolated at a drug concentration of 20 μg/ml. These strains grew equally well on liquid or agar medium containing 50 μg/ml cycloheximide; however, they showed more restricted growth than wild-type strains and generally failed to produce secondary sporidia in the presence of the drug. In the absence of cycloheximide, sporulation was initiated but failed to reach concentrations observed in wild-type cultures. Cycloheximide-resistant strains often produced a dark brown or rust-colored pigment in minimal medium; this characteristic proved to be a useful additional selective marker for these strains.

Complementation and nuclear condition of paired auxotrophic strains. The pairing of two sexually compatible auxotrophs (eg, $ade^- \times ura^-$, $ade^- \times gly^-$) on medium B produced colonial growth that continued as long as 3 mo, whereas the individual auxotrophs

TABLE 1. Summary of experimental conditions for *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) mutagenesis of secondary sporidia of *Tilletia caries*[a]

Experiment number	Strain mutagenized	Exposure time	Selection method	Number of colonies tested (replica-plated)	Number of colonies retested (toothpick-streaked)	Number of auxotrophs isolated	Mutant isolation rate (%)
NTG I[b]	18	1 Hr	Total isolation	267	11	0	0
		3 Hr	Total isolation	59	1	0	0
		6 Hr	Total isolation	10	2	0	0
		1 Hr[c]	Total isolation	693	99	0	0
		3 Hr[d]	Total isolation	620	102	1	0.16
NTG II	18	30 Min	Filtration enrichment	3,990	464	5	0.13
NTG III	24	20 Min	Filtration enrichment	2,613	578	1	0.04
Total				8,252	1,257	7	0.09

[a] Data reported here are only from experiments in which mutant strains were isolated successfully. In many initial mutagenesis experiments, no auxotrophs were isolated and the experimental data have not been included.
[b] NTG-treated sporidia were not allowed a growth period in medium C at 19 C.
[c] NTG-treated sporidia were stored in liquid medium C at 4 C for 8 days before transfer to medium C plates.
[d] NTG-treated sporidia were stored in liquid medium C at 4 C for 12 days before transfer to medium C plates.

TABLE 2. Growth and sporulation characteristics of *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG)-induced auxotrophic mutants of *Tilletia caries*

Mutant strain	Parent strain	Mating type	Mutation	Growth habit on medium B	Sporulation properties	Experiment number[a]
78	18	+	Uracil	Nonleaky	Poor	NTG I
577	24	−	Adenine	Nonleaky	Profuse	NTG III
430	18	+	Glycine	Slightly leaky	Good	NTG II
112	18	+	N-metabolism[b]	Leaky	Good	NTG II
280	18	+	N-metabolism[b]	Leaky	Good	NTG II
369	18	+	N-metabolism[b]	Leaky	Good	NTG II
441	18	+	N-metabolism[b]	Leaky	Good	NTG II

[a]Experimental conditions outlined in Table 1.
[b]Mutant strain has requirement for organic nitrogen. All N-metabolism mutants were isolated independently.

TABLE 3. Growth response of *Tilletia caries* secondary sporidia in the presence of various growth-inhibiting compounds

Drug	Range of concentrations tested (μg/ml)	No. cells per plate	Growth response (average no. colonies/plate)[a]
Acriflavin · HCl	10.00–300.00	$3–4 \times 10^7$	Lawn[b]
Actinomycin D	5.00–15.00	4×10^7	Lawn
Amphotericin B	10.00–50.00	1.3×10^8	Lawn
Antimycin A	0.01–0.05	1×10^8	Lawn
	0.10	1×10^8	4 (1.2)
	0.50–50.00	$4–13 \times 10^7$	No growth
5-Bromouracil	10.00–300.00	2×10^7	Lawn
Canavanine sulfate	10.00–100.00	$1–2 \times 10^8$	Lawn
Chloramphenicol	10.00–300.00	2.4×10^7	Lawn
Cycloheximide	0.30–5.00	1×10^8	Lawn
	7.00	1×10^8	379 (40.5)
	10.00	1×10^8	196 (17.0)
	15.00	1×10^8	38 (11.6)
	20.00	2×10^8	2 (1.5)
Ethionine	10.00–300.00	2×10^7	Lawn
p-Fluoro-phenylalanine	10.00–300.00	$3–4 \times 10^7$	Lawn
Oligomycin	0.10–0.50	1×10^8	Lawn
	1.00	1.3×10^7	135 (35.2)
	5.00–35.00	$2–13 \times 10^7$	No growth
Proflavine hemisulfate	10.00–300.00	2.4×10^7	Lawn

[a]Each test included three to 10 replicates at each drug concentration. The standard deviation is listed within parentheses.
[b]Lawn of growth on agar surface of all plates.

failed to grow or grew very poorly. Syntrophic growth through cross-feeding was not a factor in the growth of paired strains on minimal medium. Neither the ade$^-$ nor the ura$^-$ mutant grew across a membrane filter when it was superimposed on medium B or the mycelial mat of an auxotrophic culture that could theoretically allow growth through cross-feeding. Either strain grew across a filter when superimposed on medium C plates.

Paired nuclei typical of a stable dikaryon were not observed in a consistent pattern throughout the mycelium. Many of the hyphal cells contained single nuclei although, occasionally, two or three nuclei were observed together or at a distance from one another in what appeared to be a single cell. Individual hyphal cells were not easily distinguished within the mycelial mat, but in a limited sample of cells that were examined, the number of nuclei varied from zero to three.

Secondary sporidia produced by prototrophic colonies on medium B (ie, putative heterokaryons) were mononucleate and auxotrophic. All attempts to isolate viable hyphal tips of *T. caries* to test for heterokaryosis were unsuccessful. None of 175 hyphal tips transferred to medium C grew. Because the narrow hyphae (about 0.5–3 μm) typically grew as an intermingled mass of mycelium, dissociation of a hyphal tip from the proximal chain of evacuated cells was extremely difficult. Additionally, hyphal tips were easily damaged on contact with a metal or glass transfer tool.

DISCUSSION

The procedure developed for NTG mutagenesis of *T. caries* haploid strains included several steps, of which a period of growth in complete medium after mutagen treatment was crucial for successful mutant isolation. No mutants were obtained in initial experiments when this step was omitted. Expression of a mutant phenotype occurs only after the mutated DNA strand is replicated and packaged into a cell separate from that of the wild-type gene. If DNA replication and subsequent cytokinesis is not completed before transfer of cells to a complete agar medium, a "mosaic" colony consisting of both wild-type and mutant mycelia may arise and mutations to auxotrophy would go undetected. It is possible that our initial attempts at isolation of auxotrophic mutants were unsuccessful for this reason. Auxotrophs were detected only after we had established the generation time for these strains (3) and allowed sufficient time for nuclear division and cytokinesis to occur.

Filtration enrichment did not appear to increase our rate of mutant isolation compared with total isolation. We may have lost auxotrophs during filtration because filiform secondary sporidia are hyphalike in shape and, consequently, may be trapped with accompanying mycelia. However, our recovery of some auxotrophic mutants after filtration enrichment demonstrates its potential as an enrichment technique for future mutagenesis experiments with *Tilletia* spp.

The growth response of secondary sporidia after exposure to high concentrations (up to 300 μg/ml) of various drugs (Table 3) demonstrated the natural tolerance of *T. caries* to many growth-inhibiting compounds. Although spontaneous cycloheximide-resistant mutants were obtained without difficulty, we were unable to detect resistance to any of the other drugs tested. The growth of *T. caries* on medium containing antimycin A (0.1 μg/ml) or oligomycin (1.0 μg/ml) appeared to be due to nongenetic adaptation or incomplete solubility of the drugs in the culture medium. Low concentrations of antimycin A (0.5 μg/ml) and oligomycin (5.0 μg/ml) completely inhibited growth of wild-type strains, and it is possible that resistance to either drug could be induced. Leben et al (16) obtained well-defined resistance to antimycin A in *Venturia inaequalis* after ultraviolet irradiation of conidia.

Proof of heterokaryosis requires isolation of single cells from which the component nuclear types can be obtained (21). In the absence of viable hyphal tips or secondary sporidia from which both component nuclear types could be isolated, we were unable to provide definitive proof of heterokaryosis in matings of sexually compatible auxotrophs. However, nutrient cross-feeding between mutant strains played no role in allowing growth of the paired auxotrophs on a minimal medium. Neither reverse mutation nor diploidy were factors because secondary sporidia from prototrophic colonies exhibited the nutrient requirement of either auxotrophic parental type. In Basidiomycetes, nuclear migration results in formation of stable heterokaryons, and the capacity to dissociate into heterokaryotic components is not common although it has been reported (2,5,19–21). In our studies, the secondary sporidia arising from putative heterokaryons were mononucleate and auxotrophic, indicating that they contain a single haploid nucleus. These sporidia appear to have arisen by

migration of haploid nuclei into the emerging secondary sporidia produced by the heterokaryons. This indicates a mechanism by which the heterokaryons of *T. caries* may dissociate. It has been suggested that secondary sporidia were produced only by the haplophase of *T. caries* (25,28). In light of our observations, additional work will be required to obtain definitive proof of heterokaryosis in culture and to determine the ploidy of nuclei within hyphae and secondary sporidia.

We know of no previous report of the isolation of auxotrophic and cycloheximide-resistant mutants in any *Tilletia* spp. Several of these mutant strains have proven useful for *in planta* growth studies (A. C. L. Churchill and D. Mills, *unpublished*) because of their easily identifiable genetic markers. The ability to isolate and manipulate genetically marked strains of *T. caries* should also prove useful for genetic analysis of interactions among races of the common bunt fungus and other closely related species.

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