Production and Characterization of a Monoclonal Antibody Raised Against Surface Antigens from Mycelium of *Gaeumannomyces graminis* var. *tritici*: Evidence for an Extracellular Polyphenol Oxidase

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ABSTRACT

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A murine monoclonal antibody (MAb) of immunoglobulin class M (IgM) was raised against surface antigens from *Gaeumannomyces graminis* var. *tritici* and, by enzyme-linked immunosorbent assay, recognized isolates of *G. graminis* var. *tritici*, *G. graminis* var. *avenae* and *G. graminis* var. *graminis*. Characterization of the antigen by heat and protease treatments showed that the epitope recognized by the MAb was a protein. Antigen production was detected only in live mycelia. Immunofluorescence studies showed that the antigen was associated with both the broad melanized macrohyphae and hyaline mycelia of *G. graminis* var. *tritici*. Secretion of antigen into an aqueous minimal medium was promoted only by exposure of live mycelia to certain phenolic substrates, including monophenols *ortho*-, *para*-, and *meta*-cresol; 3,4,5-trihydroxybenzoic acid (gallic acid); and phenolic amino acid L-3-(3,4-dihydroxy-

phenyl) alanine (L-DOPA). Antigen secretion was not promoted by 3-(4-hydroxyphenyl) alanine (L-tyrosine). The MAb reacted strongly with purified enzyme laccase (polyphenol oxidase, EC 1.10.3.2) but did not recognize purified tyrosinase (monophenol oxidase, EC 1.14.18.1). Moreover, chemicals that bind to copper and inhibit copper-containing enzymes such as laccase completely inhibited antigen secretion in response to L-DOPA. The MAb was tested for specificity against a wide range of fungi, common yeast species, and gram positive and negative bacteria. It did not recognize antigens from a broad range of unrelated fungi, including *Gliocladium roseum*, *Fusarium* sp., *Phoma exigua*, *Phialophora fastigiata*, *Penicillium crustosum*, *Pythium ultimum*, *Rhizopus stolonifer*, *Rhizoctonia carotae*, *R. oryzae*, *R. tuliparum*, and *Trichoderma viride*, nor did it recognize surface antigens from yeasts or bacteria. The MAb cross-reacted with antigens from *Botrytis* spp., *Chaetomium globosum*, *R. cerealis*, and *R. solani*. However, secretion of antigen by *R. solani* and *R. cerealis* was not promoted by L-DOPA, and secretion by *C. globosum* in response to the phenolic amino acid was significantly less compared to *G. graminis* var. *tritici*.

The fungus *Gaeumannomyces graminis* (Sacc.) Von Arx & D. Olivier var. *tritici* J. Walker causes take-all, a major root disease of cultivated wheat that is distributed throughout the world in both northern and southern temperate zones. The soilborne fungal pathogen can exist saprophytically on plant debris in soil or parasitically on susceptible cereal crop or weed hosts (37). *G. graminis* var. *tritici* has been extensively investigated for many years, but control remains elusive. Because of this, take-all remains one of the most important factors limiting cereal production in many countries.

The search for effective disease control has been hampered, in part, by the absence of quick, sensitive, and specific assays for detection and quantification of *G. graminis* var. *tritici*. Conventional methods for detecting *G. graminis* var. *tritici*, involving pathogenicity tests and production of perithecia on host tissue, are notoriously slow and laborious. Furthermore, because *G. graminis* var. *tritici* typically does not sporulate in culture, correct identification relies on cultural characteristics, such as pigmentation, thick runner hyphae, and hyphal curling at the colony margins (2). Wheat roots infected with *G. graminis* var. *tritici* also commonly harbor fast-growing *Pythium*, *Fusarium*, and *Rhizoctonia* spp., which can interfere with recovery of the take-all pathogen. Several selective media have been developed to aid in recovery and diagnosis of *G. graminis* var. *tritici* from infected plant material (13,21),

and although these have undoubtedly improved the recovery rate of the target organism, their routine use is restrained by their labor-intensive nature.

Molecular techniques have considerable potential for detection and quantification of soilborne fungi. Nucleic acid-based techniques, such as DNA probes, polymerase chain reaction (PCR), and restriction length polymorphisms, have been used to detect and differentiate *G. graminis* var. *tritici* and related fungi from other fungal species in planta and to detect the pathogen in soil (18,19, 27), but these methods are unable to differentiate between live and dead fungal biomasses. Hybridoma technology allows the development of monoclonal antibodies (MAbs) that can be used to develop quick, sensitive, and specific assays that detect only live, actively growing fungal biomasses (32,33).

In this paper, we describe the production and characterization of a MAb raised against surface antigens of *G. graminis* var. *tritici*. We show that secretion of the antigen by live mycelia of *G. graminis* var. *tritici* is promoted only by certain phenolic compounds, including the phenolic amino acid L-3-(3,4-dihydroxyphenyl) alanine (L-DOPA). We present evidence showing that the antigen recognized by the MAb is the extracellular enzyme laccase and discuss how the MAb may be used to differentiate between *G. graminis* and other fungi.

MATERIALS AND METHODS

Fungal culture. MAbs were raised against an isolate of *G. graminis* var. *tritici* (referred to as GP20) obtained from G. L. Bateman, Institute of Arable Crop Research, Harpenden, England.

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Organisms used for screening antibodies (Table 1) were maintained on half-strength potato dextrose agar (PDA) (fungi and yeasts) or Luria agar (bacteria) either in petri dishes or on slants. *G. graminis* isolates were grown at 20°C, and *Rhizoctonia* isolates were grown at 17°C. All remaining organisms were grown at 23°C.

Development of MAbs, preparation of immunogen, and immunization regime. One-week-old PDA slant cultures of GP20 were washed with 2 ml of phosphate-buffered saline (PBS: 0.8% NaCl; 0.02% KCl; 0.115% Na₂HPO₄; 0.02% KH₂PO₄; pH 7.2). The surface washings were centrifuged in 1.5-ml microcentrifuge tubes at 12,000 × g for 10 min to remove cell debris, and the supernatant was transferred to a clean tube for use as immunogen.

Three 6-week-old BALB/c female white mice were each given three intraperitoneal injections (300 µl per injection) of fresh immunogen at 2-week intervals. The mice each received Quil A adjuvant (Superfos a/s, Vedbaek, Denmark) at a concentration of 33 µg per dose per injection.

Production and screening of hybridomas and determination of antibody specificities. Hybridoma cells were produced by the method described by Dewey et al. (10), and the supernatants were screened by enzyme-linked immunosorbent assay (ELISA). Microtiter wells (Labsystems 952107, Helsinki, Finland) arranged in strips were coated overnight at 4°C with cell-free PBS surface washings from 1-week-old PDA slant cultures of fungal isolates diluted 10-fold in PBS. After an overnight antigen immobilization step at 4°C the wells were washed four times with PBST (PBS and 0.05% [vol/vol] Tween 20) and once each with PBS and reverse osmosis water (R.O.H₂O) and air-dried at room temperature (RT; 23 ± 2°C) in a laminar flow hood. The strips were stored in sealed plastic bags at 4°C in preparation for screening of hybridoma supernatants by ELISA as described below.

ELISA formats. Preliminary screening. Preliminary screening of hybridoma supernatants was performed by ELISA, using microtiter wells coated with 10-fold dilutions of PBS surface washings

TABLE 1. Details of fungal cultures

Organism	Isolate no.	Source ^a	Organism	Isolate no.	Source ^a
Fungi			2-1	66154/21R01	ATCC/IPO
<i>Alternaria</i> sp.	3	FMD	2-1	21R61	IPO
<i>Botryosphaeria obtusa</i>	...	FMD	2-1	R5	RB
<i>Botrytis allii</i>	42078	FMD	2-2 IIIB	76124/22R01	ATCC/IPO
<i>B. cinerea</i>	1	FMD	2-2 IV	76125/22R02	ATCC/IPO
	2	CRT	2-2	22R28	IPO
	P69	FMD	2-2	22R20	IPO
<i>B. fabae</i>	225851	FMD	2-3	23R01	IPO
<i>Chaetomium globosum</i>	11	FMD	2T	2TR002	IPO
<i>Colletotrichum coccodes</i>	C3	CAG	2T	2TR144	IPO
<i>Fusarium graminearum</i>	F2	CAG	3	76167/03R01	ATCC/IPO
<i>F. oxysporum</i>	F01	CAG	3	303138	IMI
<i>F. roseum</i> f. sp. <i>cerealis</i> ('Culmorum')	C2087	CAG	4 HG-I	76126/04R02	ATCC/IPO
<i>Gaeumannomyces graminis</i> var. <i>avenae</i>	GP36	GLB ^b	4	04R22	IPO
<i>G. graminis</i> var. <i>graminis</i>	79/133	GLB ^b	4	303162	IMI
	85/20-3	GLB ^b	4	R3	RB
	89/5-1	GLB	5	76128/05R01	ATCC/IPO
<i>G. graminis</i> var. <i>tritici</i>	16/8	GLB ^b	5	303159	IMI
	GP20	GLB	6 HG-I	76129/06R01	ATCC/IPO
<i>Gliocladium roseum</i>	AB171	CAG	6 GV	76130/06R02	ATCC/IPO
<i>Hypocrea aureoviridis</i>	138258	IMI	6	303160	IMI
<i>Leptosphaeria lunderbergii</i>	20b)	FMD	7	303161	IMI
<i>Nectria galligena</i>	N9	CAG	8	76106/08R01	ATCC/IPO
<i>Penicillium crustosum</i>	P43	CAG	8	SH51	JC-S
<i>Phialophora fastigiata</i>	15	FMD	9	62804/09R01	ATCC/IPO
<i>Phoma exigua</i>	...	ADAS	BI	76132/BIR01	ATCC/IPO
<i>Pythium ultimum</i>	P38	CAG	<i>R. tuliparum</i>	236841	IMI
<i>Rhizoctonia carotae</i>	768256	IMI	<i>Rhizopus stolonifer</i>	...	CRT
<i>R. cerealis</i>	SL3/2	PN	<i>Sclerotinia fructigena</i>	S3	CAG
	SL3/3	PN	<i>Sclerotium cepivorum</i>	S4	CAG
	SL4/1	PN	<i>Stachybotrys atra</i>	AB182	CAG
	SL4/2	PN	<i>Trichoderma viride</i>	...	PM
	SL5/2	PN	<i>Verticillium</i> sp.	V6	CAG
	SL6/3	PN	Yeasts		
	SL8/2	PN	<i>Candida parapsilosis</i>	C12	KJ
	ES1/2	PN	<i>Geotrichum candida</i>	C35	KJ
	R82/261	PN	<i>Rhodotorula</i> sp.	C25	KJ
	R83/260	PN	<i>Saccharomyces cerevisiae</i>	S.C.EL1	KJ
	R84/280	PN	<i>Sporobolomyces</i> sp.	C31	KJ
	M41	PN	<i>Trichosporon</i> sp.	C51	KJ
	M42	PN	<i>Zygosaccharomyces bailli</i>	C21	KJ
	...	PN	Bacteria		
<i>R. oryzae</i>	62599	IMI	<i>Bacillus megaterium</i>	KM	KJ
<i>R. solani</i> anastomosis group			<i>B. subtilis</i>	1604	KJ
1	303154	IMI	<i>Escherichia coli</i>	DH5α	KJ
1-IA	76121/01R01	ATCC/IPO	<i>Pseudomonas corrugata</i> ^c	...	KSK
1-IC	76123/01R03	ATCC/IPO	<i>P. tolasii</i>	1116s	KJ

^a CRT: C. R. Thornton; FMD: F. M. Dewey; CAG: C. A. Gilligan; GLB: G. L. Bateman, AFRC Inst. Arable Crops Res. Exp. Stn., Harpenden, England; PN: P. Nicholson, Cambridge Lab., John Innes Centre Plant Sci. Res., Norwich, England; RB: R. Baker, Dep. Plant Pathol. Weed Sci., Colo. State Univ.; PM: P. Mills, Hortic. Res. Int., Wellesbourne, England; KJ: K. Johnstone, Dep. Plant Sci., Univ. Cambridge; KSK: K. S. Killham, Dep. Plant Soil Sci., Univ. Aberdeen, Scotland; IMI: Int. Mycol. Inst., Egham, England; ADAS: Agric. Devel. Advisory Serv., Cambridge; IPO: Inst. voor Plantenziekten. Onderzoek, Wageningen, the Netherlands.

^b Isolates supplied by C. Russell, Dep. Plant Soil Sci., Univ. Aberdeen, Scotland—originally obtained from G. L. Bateman, IACR, Rothamsted, England.

^c This isolate was transformed with the *lux* operon from *Vibrio fischeri*.

from 1-week-old PDA slant cultures of GP20. The assay procedure was as follows. Wells containing immobilized antigen were incubated successively with hybridoma supernatant for 1 h and then by commercial goat anti-mouse polyvalent (immunoglobulin classes IgG, IgA, and IgM) peroxidase conjugate (A0412; Sigma Chemical Co., Poole, Dorset, England) diluted 1:1,000 in PBST for another hour. Bound antibody was visualized by incubating wells with tetramethyl benzidine (TMB) substrate solution for 30 min, reactions were stopped by the addition of 3 M H₂SO₄, and absorbance values were determined with an EL311sx automated microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). Wells were given four 5-min rinses with PBST between incubations. Working volumes were 50 µl per well, and control wells were incubated with tissue culture medium (TCM) containing 10% (vol/vol) fetal calf serum. All incubation steps were performed at RT in sealed plastic bags.

Secondary screening. Hybridoma cell lines selected on the basis of recognition of GP20 antigens were tested further by ELISA for specificity against surface washings diluted 1:10 from 1-week-old PDA slant cultures of GP20 and other related and unrelated fungi and with soluble antigens from 2- and 1-day-old slant cultures of yeasts and bacteria respectively (Table 1). Soluble antigens from yeasts and bacteria were prepared according to the method for fungal surface washings. The ELISA format (format I) employed in this secondary screening procedure was as follows. Wells containing immobilized antigen were blocked for 30 min with 100 µl of Tris-buffered saline containing 0.3% (wt/vol) casein (TBS/c: 8 g of NaCl, 3 g of Tris, and 0.2 g of KCl per liter of R.O.H₂O, adjusted to pH 8.3 with 1 N HCl). Wells were incubated successively with MAb supernatant for 1 h, followed by commercial goat anti-mouse polyvalent (IgG, IgA, and IgM) peroxidase conjugate (A0412) diluted 1:1,000 in TBS/c for another hour. Bound antibody was visualized by incubating wells with TMB substrate solution for 30 min, reactions were stopped by the addition of 3 M H₂SO₄, and absorbance values were determined with an EL311sx automated microplate reader. Wells were given four 5-min rinses with TBS/c between incubations and a final rinse with R.O.H₂O prior to the addition of substrate. Working volumes were 50 µl per well, and control wells were incubated with TCM containing 10% (vol/vol) fetal calf serum. All incubation steps were performed at RT in sealed plastic bags. An additional ELISA format (format II) was employed as described above, but the incubation periods of primary antibody and secondary antibody-peroxidase conjugate were each reduced to 15 min, and substrate time was reduced to 5 min.

Determination of Ig subclass and cloning procedure. The Ig subclass of MAbs from selected cell lines was determined with a commercial mouse MAb isotyping kit according to the manufacturers instructions (ISO-1; Sigma). Hybridoma cell lines were cloned by limiting dilution, and selected cell lines were grown in bulk in a nonselective medium, preserved by slowly freezing in fetal bovine serum/dimethyl sulfoxide (92:8 [vol/vol]), and maintained in liquid nitrogen.

Antigen characterization and pronase and trypsin digestion of immobilized antigen. After washing, antigens (GP20 surface washings diluted 1:10) immobilized in microtiter wells (50 µl) were incubated with pronase (Protease XIV) (P5147; Sigma) (0.25 units per well) or trypsin (T4424; Sigma) solution (1 mg ml⁻¹ in PBS) at 37°C for the appropriate time period and washed three times with PBS. Wells incubated with trypsin were treated for 10 min with a 0.1 mg ml⁻¹ solution of trypsin inhibitor (T6522; Sigma) and given three more washes with PBS. Washed wells were blocked. Controls received PBS without pronase or trypsin and inhibitor but were otherwise treated similarly. The wells were assayed by ELISA format I with half-strength DD8 MAb.

Boiling. Cell-free surface washings from a 1-week-old PDA slant culture of GP20 washed with 2 ml of PBS were boiled for the appropriate period (up to 1 h), cooled, and centrifuged at

12,000 × g for 5 min. Samples were diluted with PBS to give 1/10-strength surface washings and transferred to microtiter wells. Control samples were incubated at RT but were otherwise treated similarly. After immobilization, wells were washed and assayed by ELISA format I with half-strength DD8 MAb.

Periodate oxidation of immobilized antigen. Immobilized antigens were treated with 50 µl of sodium metaperiodate (20 mM NaIO₄ in 50 mM sodium acetate buffer, pH 4.5) (S1878; Sigma), whereas control wells received only buffer. After incubation for the appropriate period in darkness at 4°C, wells were washed three times with PBS and assayed by ELISA format I with half-strength DD8 MAb.

Promotion of antigen secretion by phenolic substrates. Selected phenolic substrates (Table 2) were added separately to unsterilized minimal medium (5.0 g of NH₄NO₃, 14.0 g of KH₂PO₄, 0.5 g of MgSO₄·7H₂O, 1.5 mg of FeSO₄·7H₂O, 0.9 mg of ZnSO₄, 0.4 mg of MnSO₄, 0.4 mg of CuSO₄, 10.0 g of D-glucose, and 0.1 g of rifampicin [R3501; Sigma] per liter of R.O.H₂O) at 500 mg liter⁻¹ and mixed until fully dissolved. Aliquots (10 ml) of each solution were transferred to respective 25-ml polyvials and inoculated with single 7-cm-diameter plugs of mycelium taken from the leading edge of a 5-day-old PDA culture of *G. graminis* var. *tritici* grown at 20°C. Inoculated solutions were incubated without shaking at 23°C. At appropriate time intervals, 50-µl aliquots were removed from each tube, transferred to individual microtiter wells, and after immobilization (overnight at 4°C) and washing were assayed by ELISA format II with half-strength DD8 MAb. There were five replicate tubes for each phenolic compound studied and an equivalent number of replicate negative control samples that contained inoculated minimal medium only. The pH of solutions prior to study and after 8 h of incubation at 23°C are given in Table 2.

Inhibition of antigen production. The effects of inhibitors on antigen production were determined by the assay system described above. Selected inhibitors (Table 2) were added at a concentration

TABLE 2. List of substrates, inhibitors, and solution pHs used in the characterization of the *Gaeumannomyces graminis* var. *tritici* (isolate GP20) DD8 antigen

Substrate	Stock solution pH	
	Before incubation	After 8 h of incubation ^a
L-(+)-Ascorbic acid	3.80	3.66 ± 0.008
meta-Cresol	4.20	4.27 ± 0.005
ortho-Cresol	4.23	4.28 ± 0.007
para-Cresol	4.21	4.29 ± 0.015
L-3-(3,4-Dihydroxyphenyl) alanine (L-DOPA) (MM-LD)	4.17	4.27 ± 0.011
3,4,5-Trihydroxybenzoic acid (gallic acid)	3.76	3.83 ± 0.009
N,N'-Bis (3,5-dimethoxy-4-hydroxybenzylidene) hydrazine (syngaldazine)	4.22	4.29 ± 0.004
Tannic acid	4.06	4.13 ± 0.008
3-(4-Hydroxyphenyl) alanine (L-tyrosine)	4.18	4.26 ± 0.031
Negative control ^c	4.11	4.28 ± 0.005
Inhibitor		
Diethylthiocarbamate	4.31	4.41 ± 0.007
Ethylenediaminetetraacetic acid	4.06	4.08 ± 0.006
Fusaric acid	4.26	4.25 ± 0.005
2-Hydroxy-2,4,6-cycloheptatriene-1-one (tropolone)	4.18	4.24 ± 0.006
5-Hydroxy-2-(hydroxymethyl)-4H-pyran-4-one (kajic acid)	4.21	4.24 ± 0.008
2-Mercaptobenzothiazole	4.18	4.24 ± 0.004
Pyroquilon	4.19	4.25 ± 0.003
Negative control ^b	4.17	4.27 ± 0.011
Positive control ^c	4.14	4.28 ± 0.005

^a Mean pH of replicate samples ± standard error.

^b Minimal medium only.

^c Minimal medium containing only 500 mg of L-DOPA (MM-LD) per liter.

of 100 $\mu\text{g ml}^{-1}$ to unsterilized minimal medium that contained 500 mg of the phenolic amino acid L-DOPA per liter (MM-LD). Four replicate 10-ml samples of each mixture, contained in 25-ml polyvials, were inoculated, sampled, and tested by ELISA format II as described above. The negative control was as described previously. The positive control consisted of MM-LD only. The pH of solutions prior to study and after 8 h of incubation at 23°C are given in Table 2.

Secretion of antigen by other organisms. Three replicate 10-ml samples of MM-LD, contained in 25-ml polyvials, were inoculated separately with single 7-cm-diameter plugs of mycelium taken from the leading edge of 5-day-old PDA cultures of either *G. graminis* var. *tritici* (isolate GP20), *Botrytis cinerea* (isolate 2), *Chaetomium globosum* (isolate 11), *Rhizoctonia solani* (isolate 76124/22R01), or *R. cerealis* (isolate SL4/1). Each was sampled and tested by ELISA format II as described above.

Recognition of laccase. Microtiter wells were coated for 2 h at RT with 50 μl of poly-L-lysine (10 μg of PBS per ml) (P1274; Sigma). Wells were washed three times with PBS and incubated with commercial laccase (polyphenol oxidase, EC 1.10.3.2) (L5510; Sigma) solution double-diluted in PBS for another 2 h at RT. The initial concentration of the stock solution was 1 mg of solid per ml of PBS (1.5 μg of protein per ml) and was prepared by thorough mixing for 4 h at RT, followed by centrifugation at $12,000 \times g$ for 10 min. Control wells were prepared in the same way with a PBS solution of commercial tyrosinase (monophenol oxidase, EC 1.14.18.1) (T7755; Sigma) with an equivalent initial concentration of 1 mg ml^{-1} (105 μg of protein per ml). Wells containing immobilized protein were washed three more times with PBS and were blocked for 16 h at 4°C with TBS/c (pH 8.3). Blocking solution was removed from the wells, and the wells were assayed by ELISA format I with half-strength DD8 MAb. Protein concentrations were determined by the Bio-Rad (Richmond, CA) Bradford protein assay with bovine serum albumin (BSA) as the standard.

Fig. 1. Tissue culture supernatant from hybridoma cell line DD8 tested by enzyme-linked immunosorbent assay format II against surface washings from **A**, *Gaeumannomyces* spp. and unrelated, non-cross-reactive, fungal species (isolates are given in parentheses). 1, *Alternaria* sp. (3); 2, *Colletotrichum coccodes* (C3); 3, *Fusarium culmorum* (C2087); 4, *F. graminearum* (F2); 5, *F. oxysporum* (F01); 6, *G. graminis* var. *tritici* (16/8); 7, *G. graminis* var. *tritici* (GP20); 8, *G. graminis* var. *graminis* (79/133); 9, *G. graminis* var. *graminis* (85/20-3); 10, *G. graminis* var. *graminis* (89/5-1); 11, *G. graminis* var. *avenae* (GP36); 12, *Gliocladium roseum* (AB171); 13, *Hypocrea aureoviridis* (138258); 14, *Leptosphaeria lagerbergii* (20b); 15, *Nectria galligena* (N9); 16, *Penicillium crustosum* (P43); 17, *Phialophora fastigiata* (15); 18, *Phoma exigua*; 19, *Pythium ultimum* (P38); 20, *Rhizoctonia carotae* (768256); 21, *R. oryzae* (62599); 22, *R. tuliparum* (236841); 23, *Rhizopus stolonifer*; 24, *Sclerotinia fructigena* (S3); 25, *Sclerotium cepivorum* (S4); 26, *Stachybotrys atra* (AB182); 27, *Trichoderma viride*; 28, *Verticillium* sp. (V6). **B**, *Gaeumannomyces graminis* var. *tritici* (GP20) and unrelated, cross-reactive fungal species. 1, *Botryosphaeria obtusa*; 2, *Botrytis allii* (42078); 3, *B. cinerea* (1); 4, *B. cinerea* (2); 5, *B. cinerea* (P69); 6, *B. fabae* (225851); 7, *Chaetomium globosum* (11); 8, *G. graminis* var. *tritici* (GP20); 9 to 22, *Rhizoctonia cerealis*: 9, (SL3/2); 10, (SL3/3); 11, (SL4/1); 12, (SL4/2); 13, (SL5/2); 14, (SL6/3); 15, (SL8/2); 16, (ES1/2); 17, (R82/261); 18, (R83/260); 19, (R84/280); 20, (M41); 21, (M42); 22, (...); 23 to 51, *R. solani*: 23, (303154); 24, (76121/01R01); 25, (76123/01R03); 26, (66154/21R01); 27, (21R61); 28, (R5); 29, (76124/22R01); 30, (76125/22R02); 31, (22R28); 32, (22R20); 33, (23R01); 34, (2TR002); 35, (2TR144); 36, (76167/03R01); 37, (303138); 38, (76126/04R02); 39, (04R22); 40, (303162); 41, (R3); 42, (76128/05R01); 43, (303159); 44, (76129/06R01); 45, (76130/06R02); 46, (303160); 47, (303161); 48, (76106/08R01); 49, (SH51); 50, (62804/09R01); 51, (76132/BIR01). **C**, *Gaeumannomyces graminis* var. *tritici* (GP20) and yeasts and bacteria. 1, *G. graminis* var. *tritici* (GP20); 2, *Candida parapsilopsis* (C12); 3, *Geotrichum candida* (C35); 4, *Rhodotorula* (C25); 5, *Saccharomyces cerevisiae* (S.C.EL1); 6, *Sporobolomyces* (C31); 7, *Trichosporon* (C51); 8, *Zygosaccharomyces bailii* (C21); 9, *Bacillus megaterium* (KM); 10, *B. subtilis* (1604); 11, *Escherichia coli* (DH5 α); 12, *Pseudomonas corrugata*; 13, *P. tolasii* (1116s). Each bar (isolate/species against absorbance at 450 nm) represents the mean of duplicate samples.

Indirect immunofluorescence (IF). Cultures of GP20 were grown on multiwell slides (ICN 60-408-05; ICN Biochemical, Cleveland) and embedded in PDA (7) for 7 days at 20°C. The slides were air-dried and fixed according to Dewey (9) and blocked for 30 min with PBS containing 1.0% (wt/vol) BSA. The wells were incubated with half-strength DD8 MAb supernatant for 1 h, followed by goat anti-mouse IgM (μ -chain specific) fluorescein isothiocyanate conjugate (F9259; Sigma) diluted 1:40 in PBST containing 0.5% (wt/vol) BSA for another hour. All incubation steps were performed at RT in a moist chamber. Control wells were incubated with half-strength TCM. Slides were given three 5-min rinses with PBST between incubations and mounted in Citifluor (University of Kent, Canterbury, England) prior to examination with a Leica (Leica Cambridge Ltd., Cambridge) TCS 4D inverse confocal microscope with a $\times 20$ Ph2 dry 0.4 numerical aperture objective. The lens was corrected for a nominal cover-glass thickness of 170 μ m (No. 1). Line averaging was used to reduce electronic and mechanical noise.

RESULTS

Specificity of hybridoma cell lines. A single fusion was performed. From this, 320 hybridoma cell lines were screened for MAb production, and all tested positive for recognition of GP20 antigens using the preliminary screening procedure. Ten of these were selected for further testing on the basis of absorbance values ($A_{450} > 0.7$). In secondary specificity screening tests, 8 of the 10 cell lines selected produced MAbs that were nonspecific, reacting strongly in ELISA against surface washings from all of the fungal isolates tested (data not shown). Of the remaining two cell lines, one stopped producing antibodies, and the other, DD8, produced a MAb that exhibited specificity for antigens from *G. graminis* isolates. This cell line was subcloned three times.

In ELISA format II, the DD8 MAb strongly recognized surface antigens from *G. graminis* var. *tritici* (GP20) (Fig. 1A). It also recognized surface antigens from *G. graminis* var. *tritici* isolate 16/8 and *G. graminis* var. *graminis* and *avenae* isolates 79/133, 85/20-3, 89/5-1, and GP36. The MAb cross-reacted with surface antigens from three *Botrytis* species (5 isolates) and with antigens from a single isolate of *C. globosum* and multiple isolates of *R. cerealis* (14 isolates) and *R. solani* (28 isolates) (Fig. 1B). They did not recognize antigens from a broad range of other unrelated fungi, including *Gliocladium roseum*, *Fusarium* sp., *Phoma exigua*, *Phialophora fastigiata*, *Penicillium crustosum*, *Pythium ultimum*, *Rhizopus stolonifer*, *Rhizoctonia carotae*, *R. oryzae*, *R. tuliparum*, and *Trichoderma viride* (Fig. 1B) nor did they recognize surface antigens from a number of common yeast species or gram positive and negative bacteria (Fig. 1C).

Isotyping. The MAb from the subcloned cell line DD8 was from Ig class M.

Characterization of antigen and effect of heat, protease, and periodate. Surface antigens of *G. graminis* var. *tritici* (GP20) recognized by the DD8 MAb were heat labile. There was a sig-

nificant reduction in absorbance value, as determined by ELISA, when boiled, with a reduction in absorbance value experienced after only 5 min at 100°C (Fig. 2A).

Two protease treatments were employed, namely trypsin and pronase digestion of surface washings immobilized in microtiter wells. The protease trypsin reduced the binding of GP20 surface antigens to the DD8 MAb by over 50% after 5 h of exposure (Fig. 2B). Binding of the DD8 MAb to immobilized antigen was reduced by pronase treatment in a similar manner, except incubation of the antigen with pronase for more than 4 h had no additional effect (Fig. 2B). There was no effect of periodate oxidation on binding of the DD8 MAb to immobilized surface antigens in ELISA (Table 3).

Fig. 2. A, Effect of boiling on binding of the DD8 monoclonal antibody (MAb) to immobilized *Gaeumannomyces graminis* var. *tritici* isolate GP20 antigens. **●** = treated and **○** = control. **B,** Effect of protease treatment on binding of the DD8 MAb. **○** = pronase, **●** = trypsin and **■** = control. Each point (absorbance at 450 nm against incubation time in hours) represents the mean of duplicate samples.

TABLE 3. Absorbance values from enzyme-linked immunosorbent assay tests with periodate-treated antigens from *Gaeumannomyces graminis* var. *tritici* (isolate GP20), using DD8 immunoglobulin class M monoclonal antibodies^a

Time (h)	Absorbance (450 nm)	
	Periodate	Control
20	1.067	1.024
5	1.012	1.088
4	1.092	1.040
3	1.131	0.988
2	1.149	1.061
1	1.108	0.971
0.5	1.135	0.977

^a Each value represents the mean of replicated values. Least significant difference among means ($\alpha = 0.001$) = 0.104

Promotion of antigen secretion by phenolic substrates. Secretion of the extracellular antigen recognized by the DD8 MAb was promoted by a number of phenolic compounds (Table 2; Fig. 3). Secretion of the antigen into liquid minimal medium was promoted by phenolic compounds *meta*-, *ortho*-, and *para*-cresol and 3,4,5-trihydroxybenzoic acid (gallic acid) and by L-(+)-ascorbic acid and L-DOPA. No increase in secretion was realized upon exposure to N,N'-bis (3,5-dimethoxy-4-hydroxybenzylidene) hydrazine (syringaldazine), a cresol derivative, or to 3-(4-hydroxyphenyl) alanine (L-tyrosine) compared to the negative control (minimal medium only). Tannic acid had an inhibitory effect on antigen secretion.

Inhibition of antigen secretion. Production of the extracellular antigen by *G. graminis* var. *tritici* in response to MM-LD was completely eliminated by the addition of EDTA, fusaric acid, 2-mercaptobenzothiazole (MERCAP), and 2-hydroxy-2,4,6-cycloheptatriene-1-one (tropolone) to the incubating medium (Fig. 4). Reductions in antigen production also were realized when kojic acid, pyroquilon, or diethylthiocarbamic acid (DIECA) was added at a concentration of 100 mg ml⁻¹ to the external medium compared to the positive control (minimal medium plus 500 mg of L-DOPA per liter).

Secretion of antigen by other fungi. Promotion of antigen secretion by MM-LD, in fungi other than *G. graminis* var. *tritici*, was examined with single isolates of *B. cinerea*, *C. globosum*, *R. solani*, and *R. cerealis* (Fig. 5; Table 1). These represented the most cross-reactive of the species/isolates from secondary screening tests of antibody specificity (Fig. 1B). Maximum antigen secretion, as determined by ELISA, was exhibited by all of the isolates after 4 h of incubation in MM-LD. Figure 6 represents the relative absorbance of each isolate as a percentage of the control (*G. graminis* var. *tritici* isolate GP20). There was no promotion of antigen secretion in the *R. solani* and *R. cerealis* isolates. Although there was no significant difference in absorbance values between *G. graminis* var. *tritici* and *B. cinerea*, promotion of antigen secretion in *G. graminis* var. *tritici* was significantly greater than that of *C. globosum* after 4 h of exposure to MM-LD.

Fig. 3. Promotion of antigen secretion by phenolic substrates. Each bar (absorbance at 450 nm after 2 h of incubation) represents the mean of five replicate samples $\pm$ standard error.

Recognition of purified laccase. The DD8 MAb exhibited strong recognition of the purified enzyme laccase in ELISA, with a limit of detection of ~ 12 ng of protein per ml of buffer (Fig. 6). There was no recognition of the control (purified tyrosinase).

IF. The MAb from cell line DD8 recognized both the broad melanized runner hyphae and more delicate hyaline mycelia of *G. graminis* var. *tritici* (GP20) by IF (Fig. 7A). No fluorescence was exhibited by the control (Fig. 7B).

DISCUSSION

Using surface antigens from live mycelia of the soilborne plant pathogen *G. graminis* var. *tritici* as the immunogen, we raised a hybridoma cell line secreting an IgM MAb, DD8, that recognizes surface antigens from *G. graminis* var. *tritici*, *avenae*, and *graminis* but that does not recognize antigens from a broad range of unrelated fungi, including *Gliocladium roseum*, *Fusarium* sp., *Phoma exigua*, *Phialophora fastigiata*, *Penicillium crustosum*, *Pythium ultimum*, *Rhizopus stolonifer*, *Rhizoctonia carotae*, *R. oryzae*, *R. tuliparum*, and *T. viride*. The MAb from this cell line, therefore, represents a considerable improvement in specificity compared to polyclonal antisera raised against *G. graminis* var. *tritici* in previous studies (15). Furthermore, the MAb raised in this study does not cross-react with surface antigens from a number of yeast species or gram positive and negative bacteria. The ability to discriminate between *G. graminis* var. *tritici* and other fungi, particularly species of *Fusarium* and *Rhizopus*, is a major advantage, because these fast-growing organisms have often proved to be the leading cause of failure to isolate *G. graminis* var. *tritici* from infected plant tissue (1,21). The DD8 MAb cross-reacted with surface antigens from three *Botrytis* species, *C. globosum*, *R. cerealis*, and *R. solani* (Fig. 1B). Reasons for the cross-reactivity and procedures for the elimination of this nonspecific recognition are discussed below.

Characterization of the DD8 antigen by heat and protease treatments showed that the epitope recognized by the MAb was a protein. More specifically, sensitivity of the antigen to trypsin indicated that the MAb binds to a protein epitope that contains residues of

Fig. 4. Effect of inhibitors on promotion of antigen secretion by L-3-(3,4-dihydroxyphenyl) alanine. -▲- = positive control; -▽- = pyroquilon; -■- = DIECA; -◇- = kojic acid; -◆- = negative control; -●- = tropolone; -○- = fusaric acid; -△- = MERCAP; and -□- = EDTA. Each point represents the mean of four replicate samples. Curves were fitted with quadratic polynomials.

the amino acids serine and arginine. The lack of effect of periodate oxidation on MAb binding to immobilized antigen implies that the epitope bound by the MAb does not contain a carbohydrate component. However, this result does not exclude the possibility that the protein epitope forms part of a larger glycoprotein molecule.

Antigen production, as determined by ELISA, was detected only in live mycelia, not in moribund mycelia. Moreover, secretion of antigen from quiescent tissue was promoted only by exposure of live mycelia to a select number of substrates, including monophenols *o*-, *p*-, and *m*-cresol, benzoic acid gallic acid, phenolic amino acid L-DOPA, and antioxidant L-(+)-ascorbic acid. L-DOPA was used in the development of a semiselective medium for detection of *G. graminis* var. *tritici* (21) and is probably used by *G. graminis* var. *tritici* (8,35) and other fungi for melanin biosynthesis. Indeed, exposure of *G. graminis* var. *tritici* to both L-DOPA and gallic acid led to rapid blackening of mycelia. Promotion of secretion of the DD8 antigen by these substrates suggests the extracellular, water-soluble antigen is either a product of a melanin biosynthetic pathway, an intermediate in a pathway, or, more importantly, a phenol oxidase enzyme such as tyrosinase or laccase.

Many fungi secrete extracellular enzymes that oxidize foreign phenolic metabolites and, in so doing, convert them into new polymers, most notably melanins (16,25). Some fungi secrete peroxidase and hydrogen peroxide for this purpose (24), but this occurs only rarely in some species of the Basidiomycotina and not in the Ascomycotina to which *G. graminis* var. *tritici* belongs. Excluding a few cases of fungi in which tyrosinase is secreted extracellularly (7,23), tyrosinase is an intracellular enzyme. In contrast, many reports on laccase describe its secretion by mycelia at one stage or another of the life cycle of fungi (31,40). The DD8 MAb reacted strongly in ELISA with purified laccase from the rice blast fungus *Magnaporthe grisea* but did not recognize purified mushroom tyrosinase. Moreover, the antigen was not secreted into medium containing tyrosine. Laccases do not readily oxidize tyrosine. Interestingly, laccase secretion was not promoted by the cresol

derivative syringaldazine despite being considered uniquely a laccase substrate (17). However, this response also has been noted by other workers (20).

The evidence presented here strongly suggests the antigen recognized by the DD8 MAb is the enzyme laccase, and as such, the enzyme must represent an immunodominant molecule on the surface of *G. graminis* var. *tritici* hyphae. IF studies clearly showed that the enzyme is secreted by both the broad melanized runner hyphae (macrohyphae) and more delicate, hyaline mycelia of *G. graminis* var. *tritici*. Secretion of laccase by *G. graminis* var. *tritici* also was demonstrated by the Bavendamm reaction (data not shown), whereby the development of a dark-brown zone around a colony of *G. graminis* var. *tritici* grown on PDA containing 10^{-3} M gallic acid or L-DOPA indicates the presence of an extracellular laccase (3). Differentiation of *G. graminis* var. *tritici* from other soilborne fungi on the basis of melanin pigment formation in the presence of L-DOPA was used by Juhnke et al. (21) in the development of a selective medium for the detection of the take-all pathogen.

All laccases investigated so far are glycoproteins (28,29), and all contain copper. Consequently, reagents that interact with the copper in laccase can act as inhibitors of the enzyme (14). Such inhibitors include the specific copper chelators DIECA and MERCAP and other chelators less specific for copper, such as tropolone, EDTA, kojic acid, and fusaric acid. Fusaric acid, a toxin secreted by *Gibberella fujikuroi*, is a chelator of copper, cobalt, and iron and is an effective inhibitor of tyrosinase from various sources (6). Tropolone is one of the most potent inhibitors of mushroom tyrosinase (22). Tropolone, fusaric acid, EDTA, and MERCAP completely eliminated secretion of the DD8 antigen in response to MM-LD, whereas DIECA and kojic acid also significantly reduced secretion from the fungus (Fig. 4). However, the reduction due to DIECA was only evident during initial periods of incubation in minimal medium containing L-DOPA. Interestingly, tannic acid also was inhibitory to antigen secretion by *G. graminis* var. *tritici*. The phenolic hydroxyl groups of tannins form stable cross-links with proteins, and as a result of this cross-linking, enzymes may be inhibited (38). These results strengthen the inference that the DD8 antigen is the polyphenol oxidase enzyme laccase. The antipene-

Fig. 5. Promotion of antigen secretion in *Gaeumannomyces graminis* var. *tritici* and other organisms by phenolic amino acid L-3-(3,4-dihydroxyphenyl) alanine (L-DOPA). 1, *G. graminis* var. *tritici* (isolate GP20); 2, *Botrytis cinerea* (isolate 2); 3, *Chaetomium globosum* (isolate 11); 4, *Rhizoctonia solani* (isolate 76124/22R01); and 5, *R. cerealis* (isolate SL4/1). Each bar (mean of triplicate values) represents the relative absorbance as a percentage of the control (*G. graminis* var. *tritici* [GP20]) after 4 h of incubation in minimal medium containing L-DOPA.

Fig. 6. Recognition of the purified enzyme laccase by the DD8 monoclonal antibody: ●- laccase and ○- tyrosinase. Each point (absorbance at 450 nm against enzyme concentration in milligrams per liter) represents the mean of four replicate samples.

trant fungicide pyroquilon is another compound that reduced secretion of the DD8 antigen. No firm inference is drawn as to the mode of action of pyroquilon on antigen secretion, but it inhibits 1,8-dihydroxynaphthalene (DHN)-melanin biosynthesis in *Gaeumannomyces* species (14) and other fungi (39).

A study of the literature reveals that laccase is present in a large number of fungi, including Ascomycetes, Basidiomycetes, and Fungi Imperfecti (26,34). Secretion of laccases by wood-rot fungi and species of *Botrytis* and *Rhizoctonia* is well documented (5,12,30), as is the ability of the soft-rot fungus *C. globosum* to degrade phenol derivatives (16). Several studies describe the isolation and characterization of cellulolytic enzymes produced by *G. graminis* var. *tritici* both in vitro and in vivo (11), but with the exception of Caesar-Tonthat et al. (8), no mention could be found of production of laccase by this fungus. Our work supports the premise that the laccase produced by *G. graminis* is the wall-bound enzyme involved in the synthesis of DHN-melanin (4) and the oxidation of L-DOPA in the outer wall of the fungus (4).

Initial screening tests for specificity of the DD8 MAb showed that it cross-reacted with surface antigens from *C. globosum* and with antigens from isolates of *Botrytis* spp., *R. cerealis*, and *R. solani*. This cross-reactivity was not surprising in light of the ubiquity of polyphenol oxidases in these fungi. Moreover, recent work in this laboratory (C. R. Thornton, unpublished data), using *R. solani*-specific MAbs raised in a separate study (32), has shown that in addition to laccase (36) *R. solani* excretes the enzyme catechol oxidase (EC 1.10.3.1) and that secretion is promoted by the benzoic acid gallic acid (32). The similarity between these enzymes could explain the cross-reactivity of the DD8 MAb with *Rhizoctonia* surface antigens. Further work will aim to clarify this relationship.

Use of the DD8 MAb to detect and differentiate between the target organism *G. graminis*, other common root and basal culm pathogens of small grains, such as *R. cerealis* and *R. solani*, and

the soil-inhabiting fungi *C. globosum* and *B. cinerea* seemed, in the first instance, not feasible. However, further investigations clearly showed that although secretion of laccase by *B. cinerea* was promoted by L-DOPA, secretion in *R. solani* and *R. cerealis* was not promoted by this substrate, and secretion by *C. globosum* was significantly less compared to *G. graminis* var. *tritici*. Therefore, using a system of nutrient enrichment similar to that developed by Thornton et al. (32) for the detection of *Rhizoctonia solani* in soil, it may be possible to differentiate live mycelia of *G. graminis* from cross-reactive *Rhizoctonia* spp. Cross-reactivity of the DD8 MAb with *B. cinerea* and *C. globosum* remains a problem, and methods to prevent or minimize nonspecific recognition are being investigated. For example, preliminary evidence suggests that *B. cinerea* polyphenol oxidase is unable to oxidize *p*-cresol. Future work will examine the range of phenolic compounds oxidized preferentially by *G. graminis*.

The DD8 MAb is currently being used to develop enzyme immunoassays for specific detection, quantification, and visualization of *G. graminis* var. *tritici* in soil and in planta. These will be used to monitor the spatio-temporal dynamics of *G. graminis* var. *tritici* in media of increasing microbiological complexity under defined laboratory conditions. The use of the DD8 MAb in combination with nucleic acid-based techniques, such as PCR, has enormous potential for rapid, sensitive and accurate detection of *G. graminis* var. *tritici* in naturally infested plant and soil samples.

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Fig. 7. A, Photomicrograph of *Gaeumannomyces graminis* var. *tritici* (isolate GP20) mycelium immunostained with monoclonal antibodies from the cloned cell line DD8 and anti-mouse immunoglobulin class M (IgM) (μ -chain specific) fluorescein isothiocyanate (FITC) conjugate examined under epifluorescence (i) and differential phase contrast (ii) transmission microscopy, respectively. B, Control. Mycelium incubated with tissue culture medium followed by anti-mouse IgM (μ -chain specific) FITC conjugate. $\times 400$ magnification; frame size = 30 μ m.

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