Ornithine Decarboxylase Knockout in *Tapesia yellundae* Abolishes Infection Plaque Formation In Vitro but Does Not Reduce Virulence Toward Wheat

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A knockout strain of *Tapesia yellundae* lacking the single ornithine decarboxylase (ODC) allele has been created by targeted gene replacement. A central region of the ODC gene was isolated by polymerase chain reaction with degenerate oligonucleotides and used to probe a lambda genomic library. The gene was sequenced, and the encoded ODC protein sequence was shown to be similar to those from other fungi. The functionality of the *T. yellundae* ODC was confirmed by complementation of an *Aspergillus nidulans* mutant (*puA*) strain devoid of ODC activity, restoring growth in the absence of exogenous polyamines. Transformation-mediated gene replacement was used to create strains that were auxotrophic for putrescine and lack ODC coding sequences. ODC knockout strains were unable to differentiate infection structures after in vitro induction and showed an abnormal hyphal branching phenotype. Pathogenicity studies on these mutants showed that, surprisingly, they are not reduced in virulence compared with nondisrupted transformants. This suggests that the strains carrying an ODC disruption can obtain sufficient polyamines from the host plant for normal growth and differentiation and, therefore, that fungal ODC may not be a suitable target for fungicides.

Eyespot, caused by the ascomycete fungus *Tapesia yellundae* (previously *Pseudocercospora herpotrichoides*), is a major disease of winter wheat in temperate areas of the world. In surveys of U.K. crops, over 80% of wheat fields were affected each year, with losses equivalent to 4% of total production.

Polyamines are ubiquitous low-molecular-weight cationic organic compounds. A number of polyamines occur naturally. The most widely distributed ones are the diamine putrescine (1,4-diaminobutane), triamine spermidine (4-aza-octane-1,8-diamine), and tetraamine spermine (4,9-diazadodecane-1,12-diamine) (Tabor and Tabor 1985). These are the major polyamines in eukaryotes, including fungi. The enzymes involved in polyamine biosynthesis have been purified and character-

ized from a variety of sources. Ornithine decarboxylase (ODC) is of particular interest because it catalyses the rate-limiting step in the biosynthesis of polyamines (Tabor and Tabor 1976). There is a dramatic increase in ornithine decarboxylase activity at the onset of cell proliferation. This has been observed in fungi during germination of conidia of *Aspergillus nidulans* (Stevens et al. 1976), *Neurospora crassa* (Sikora and McDougall 1978), and cultured mitogenically activated cells (Abrahamsen and Morris 1991). Polyamines bind polyanionic macromolecules such as DNA, RNA, proteins, and phospholipids. The determination of the many and varied functions of polyamines has been made possible by the synthesis of specific inhibitors of polyamine biosynthesis. In general, active growth and cell division are associated with substantial polyamine biosynthesis in plants, animals, and microorganisms (Bagni et al. 1980; Palavan and Galston 1982; Pegg and McCann 1982). Polyamines also have been implicated in plant responses to osmotic stress (Slocum et al. 1984), chilling stress (Guye et al. 1986), and disease (Von Ropenack et al. 1998; Walters 1989), where it is likely that they interact with cell membranes. The synthesis of polyamines in eukaryotes begins with the decarboxylation of ornithine to form putrescine. This reaction is catalyzed by ODC. Higher plants and bacteria can form putrescine in a similar manner. In plants, putrescine also can be produced from agmatine, which itself is formed by the decarboxylation of arginine by the enzyme arginine decarboxylase (Pegg and McCann 1982; Smith 1985; Stevens and Winther 1979; Tabor and Tabor 1985; Walters 1989). Most fungi, including economically important plant pathogens (*Rhizoctonia solani*, *Botrytis cinerea*, *Fusarium oxysporum* f. sp. *lycopersici*, *Phytophthora infestans*, and *T. yellundae*) are prevented from growing by inhibiting ODC with the specific and highly potent inhibitor α -difluoromethylornithine (DFMO). The same concentration of DFMO does not inhibit plant growth because plants can synthesize polyamines from arginine. Thus ODC inhibitors have ideal characteristics for use as fungicides on diseased plants. Though DFMO itself is not toxic to plants per se, its toxicity to animals and high cost make it unsuitable for development as a fungicide. Walters (1995) has tested several inhibitors of ODC, S-adenosylmethionine decarboxylase (SAMDC), spermidine synthase,

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A recent study has shown that ornithine decarboxylase is required for virulence in the ascomycete plant pathogen *Stagonospora nodorum* (Bailey et al. 2000). Although this finding

Degenerate oligonucleotides designed against conserved amino acids (IF/YANPCK and NDGVYGS/N) within the ODC proteins of humans (Hickock et al. 1987), yeast (Fonzi and Sypherd 1987), *N. crassa* (Williams et al. 1992), and *Datura* spp. (Michael et al. 1996) were used as polymerase chain reaction (PCR) primers to amplify an internal fragment of the *T. yallundae* ODC gene (*ODC1*). A 609-bp product was obtained with *T. yallundae* DNA as template, cloned into phosphate-buffered saline-SK⁻, and sequenced to confirm its identity. The *ODC1* PCR fragment was used to screen a *T. yallundae* genomic DNA library. Four positive clones were identified after screening 160,000 recombinant phage and purifying their DNA. The DNA of clone 8 was digested with *Bam*HI, and a 5-kb fragment was subcloned into pUC19 (p8BamD19). Sequence analysis (DDBJ/EMBL/GenBank accession no. AF333773) indicated that the entire *ODC1* predicted open reading frame (ORF), including 650 bp of upstream sequence, was present. In order to obtain a longer upstream sequence, DNA from clone 10 was digested with *Hind*III, and a 4.5-kb fragment was subcloned into Litmus38 (p10HindD10). Sequence analysis indicated that part of the 5' end of the *ODC1* ORF and some additional 4-kb upstream sequence were present on this fragment (DDBJ/EMBL/Gen-Bank accession no. AF333773). The predicted amino acid sequence encoded by the ORF of *T. yallundae* genomic *ODC1* consists of 449 amino acids. The genomic *ODC1* sequence contains a 70-bp intron whose size and sequence is conserved between *T. yallundae*, *S. nodorum*, and *N. crassa* (Bailey et al. 2000; Williams et al. 1992). The highest degree of amino acid similarity was to the ODC sequence of *N. crassa* (77%) (Williams et al. 1992) and *S. nodorum* (76%) (Bailey et al. 2000). The ODCs of *T. yallundae* and *S. nodorum* were 62% identical, and the ODCs *T. yallundae* and *N. crassa* were 63% identical, whereas identity to the yeast ODC sequence (Fonzi and Sypherd 1987) was 44%, and identity to the *U. maydis* ODC sequence (Guevera-Olvera et al. 1997) was 38%. Southern analysis of *T. yallundae* genomic DNA restricted with *Eco*RI, *Pst*I, and *Bam*HI with the *ODC1* coding sequence as probe revealed only one hybridizing band for each of these enzymes. There were two hybridizing bands of 3.3 and 4.4 kb when genomic DNA was restricted with *Hind*III. Sequence analysis showed the presence of an internal *Hind*III site in the coding

Fig. 1. Gene disruption strategy and Southern blot analysis for ornithine decarboxylase (ODC) gene replacement. **A**, Strategy for disruption of the *Tapesia yallundae* *ODC1* gene, showing restriction maps of the disruption construct pODCHy2, *ODC1* genomic DNA, and expected gene replacement. The region used to probe Southern blots is indicated by a hatched bar. B = *Bam*HI; E = *Eco*RV; H = *Hind*III; S = *Sal*I. **B**, 5 μ g of genomic DNA from wild-type W22-433 (lane 1) and transformants 53 (lane 2), 69 (lane 3), 104 (lane 4), 109 (lane 5), 195 (lane 6), 228 (lane 7), and 235 (lane 8) were digested with *Eco*RV, electrophoresed on 0.8% agarose gel, and transferred to a nylon membrane. Membrane was then hybridized to a probe derived from the *ODC1* coding region, as shown.

sequence. This is consistent with the presence of a single ODC gene in the genome of *T. yallundae*.

ODC1 gene replacement in *T. yallundae*.

To determine the function of the *T. yallundae* ODC1 gene, homologous recombination was employed to replace the gene with a selectable marker. Linearized pODCHyg2, which contains a hygromycin resistance cassette flanked by the 5' and 3' regions of the ODC1 gene, was used to transform protoplasts of *T. yallundae*. Primary transformants were selected and purified on potato dextrose agar (PDA) containing 100 µg of hygromycin per and 60 µM putrescine. Hygromycin-resistant colonies (235) were tested subsequently for growth on medium that did not contain putrescine. Seven auxotrophic colonies, termed TyΔODC-53, -69, -104, -109, -195, -228, and -235, were identified after the plates had been incubated at 18°C for 1 week and, subsequently, at 22°C for an additional week. The loss of the ODC1 coding sequence in these mutants was verified by Southern analysis. Figure 1 shows a schematic of the gene disruption protocol and a Southern blot of genomic DNA from the seven mutants and wild-type fungus, restricted with *EcoRV* and probed with flanking sequences. Two bands of 1.8 and 4.5 kb were detected in wild-type DNA, whereas one larger band of 11 kb was seen in the lanes containing DNA from TyΔODC-53, -69, -104, -109, and -195, which is indicative of the loss of the two internal *EcoRV* sites by gene disruption. Two of the mutants, TyΔODC-228 and -235 showed a more complex integration pattern, arising from unknown integration events. The same Southern blot was subsequently stripped and hybridized with a probe comprising the coding region of ODC1. No hybridizing band was detected in the DNA of the seven mutants, which showed that all of them had lost the ODC1 coding sequence, whereas two bands of 1.8 and 4.5 kb were detected in wild-type DNA (data not shown).

Complementation of *Aspergillus nidulans* and *T. yallundae* ODC mutants.

To show the functionality of the *T. yallundae* clone and to confirm that the disruption phenotype arose from ODC disruption, the *A. nidulans* mutant *puA* and the *T. yallundae* ODC1 disruptant TyΔODC-53 were transformed with the ODC1 gene from *T. yallundae* and the *S. nodorum* ODC gene from a previous study (Bailey et al. 2000). Protoplasts from TyΔODC-53 were co-transformed with plasmids pAN8-1 (phleomycin resistance cassette) and either pODC or pXmn1 containing the complete ODC sequence of *T. yallundae* or *S. nodorum*, respectively. The transformants were selected on 500 µg of Zeocin per ml (Invitrogen, San Diego, CA, U.S.A.). Three Zeocin-resistant transformants, in the case of pODC, and nine transformants for pXmn1 were identified. These 12 transformants were tested subsequently for complementation of putrescine auxotrophy. One of the pODC transformants, TyΔODC53/eye, and two of the pXmn1 transformants, TyΔODC/snod-1 and /snod-2, showed putrescine-independent growth. The pODC transformants contained the *T. yallundae* ODC gene, as assessed by PCR, with primers ODC6 (5'-GTTGAATCTAGTAGCGGAGC-3') and ODC5 (5'-CTTTCTTCGTAGCTGATCTC-3'), which specifically amplified a 1,120-bp fragment of the *T. yallundae* ODC coding sequence. This diagnostic band was in PCR reactions with DNA isolated from wild-type *T. yallundae* and from TyΔODC53eye but not from

TyΔODC53 (Fig. 2A). Primers ODCsnod-1 and ODCsnod-2 (5'-GTGTTGAAAATCATGGCGCC-3' and 5'-TGCCCAGAGTAGCATCGGCG-3', respectively) specifically amplified a 932-bp fragment of the *S. nodorum* ODC coding sequence from DNA isolated from TyΔODC53/snod-2 but not from DNA isolated from TyΔODC53 (Fig. 2B). The *S. nodorum* ODC gene is thus able to complement the *T. yallundae* ODC mutant phenotype. The *A. nidulans* ODC mutant (*puA2-biA1*) (Sneath 1955) is auxotrophic for putrescine and biotin. Protoplasts from this mutant were isolated and transformed with pODC. The recovered transformants, termed *A. nidulans*/eye1-8, were selected directly for complementation of putrescine auxotrophy, demonstrating that the *T. yallundae* ODC1 gene was capable of complementing the *puA2* mutation. DNA was isolated from *A. nidulans*/eye2 and /eye5 and the insertion of the pODC into the fungal genome verified by PCR (Fig. 2C). Primers TYODC18 (5'-GTATTG-ATTCTGGTAGTACG-3') and TYODC3 (5'-ATCAGTTTG-AATCGGTAATC-3') specifically amplified the predicted 650-bp fragment from wild-type *T. yallundae* and *A. nidulans*/eye2 and /eye5 but not from the DNA isolated from the *A. nidulans* *puA2-biA1* mutant. These transformants were tested for their ability to germinate and sporulate in the absence of exogenous putrescine. All showed normal growth and differentiation.

ODC enzyme activity in wild-type and mutant fungus.

ODC enzyme activity was determined in wild-type and ODC⁻ *T. yallundae* TyΔODC-53. Because ODC activity was difficult to detect in mycelia, we determined activity in germinating spores where activity was expected to be high. Spores were harvested from TWA plates and washed with sterile distilled water. The spores were suspended in 100 ml of Czapek-Dox liquid media at 2×10^7 conidia per ml. It was observed that most of the *T. yallundae* conidia had already formed a germ tube when collected from TWA plates. Therefore, the conidia were conditioned only for 2 h in the Czapek-Dox liquid media in an orbital shaker at 220 rpm at 20°C. The conidia were harvested onto 47-mm nitrocellulose filters (0.45-µm pore size) and used in the ODC activity assay, as described. ODC activity for wild-type *T. yallundae* is 321.00 ± 34.64 pMol CO₂ per mg of protein, whereas ODC activity in

Fig. 2. Complementation analysis of ornithine decarboxylase (ODC) genes. **A**, Polymerase chain reaction (PCR) from *Tapesia yallundae* and TyΔODC53 transformed with pODC with primers ODC6 and ODC5. 1 = control without DNA; 2 = TyΔODC53-eye DNA; 3 = TyΔODC53 DNA; 4 = wild-type *T. yallundae* DNA. **B**, PCR from TyΔODC53 transformed with pXmn1 with primers ODCsnod1 and ODCsnod2. 1 = control without DNA; 2 = DNA from TyΔODC53/snod1; 3 = DNA from TyΔODC53/snod2; 4 = DNA from TyΔODC53. **C**, PCR from *Aspergillus nidulans* *puA2 biA1* transformed with pODC with primers TYODC18 and TYODC3. 1 = control without DNA; 2 = *A. nidulans*-eye2 DNA; 3 = *A. nidulans*-eye5 DNA; 4 = *A. nidulans* *puA2 biA1* mutant DNA; 5 = *T. yallundae* wild-type DNA.

Ty Δ ODC-53 is 44.48 ± 11.87 pMol of CO₂ per mg of protein. Peak wild-type activity is approximately 25-fold lower than peak activity in *A. nidulans* (biA1) (Stevens et al. 1976). Stevens et al. (1976) and Bailey et al. (2000), however, showed that ODC activity peaked approximately 8 to 10 h after the start of germination. The germination of *A. nidulans* and *S. nodorum* conidia was relatively synchronous. It was found that *T. yallundae* conidia harvested from TWA plates showed a range of different germination stages. This asynchronous germination could explain the lower ODC activity observed.

Fig. 3. Growth phenotype of wild-type and *ODC1*⁻ fungi in the presence or absence of polyamines. **A**, Rate of increase of hyphal biomass of *Tapesia yallundae* wild-type and *ODC1*⁻ mutant Ty Δ ODC53 at 18°C on minimal medium (MM) containing various concentrations of putrescine, spermidine, or spermine. **B**, Rate of hyphal extension of *T. yallundae* wild-type and *ODC1*⁻ mutant at 18°C on MM agar containing various concentrations of putrescine, spermidine or spermine. Shaded circle = wild-type during growth on spermine; open circle = wild-type during growth on spermidine; open square = wild-type during growth on putrescine; X = Ty Δ ODC53 during growth on spermine; open triangle = Ty Δ ODC53 during growth on spermidine; and open diamond = Ty Δ ODC53 during growth on putrescine. **C**, Frequency of hyphal branching for wild-type (open square) and *ODC1*⁻ mutant Ty Δ ODC53 (open circle) at various putrescine concentrations. **D**, Temperature-sensitive phenotype of ODC⁻ strains. Radial growth rates were measured for wild-type and mutant *T. yallundae* on MM agar in the presence or absence of 60 μ M putrescine at various temperatures. Growth rates were measured for 10 separate experiments then subjected to statistical analysis. Bars with lines sloping to the left = Ty Δ ODC53 without supplementary putrescine; bars with dots = Ty Δ ODC53 + 60 μ M putrescine; white bars = wild-type without supplementary putrescine; bars with lines sloping to the right = wild-type + 60 μ M putrescine. Error bars have been omitted from **A** and **B** for clarity. Dashed boxes enclose data points that are not significantly different from each other but show significant difference to other similarly enclosed boxes.

Growth phenotypes of *ODC1*⁻ *T. yallundae*.

To test the ability of different polyamines to restore growth of the *ODC1*⁻ mutant, strains were grown on minimal medium containing a range of polyamine concentrations. As measurement of hyphal growth rate on agar medium is not likely to reflect the true increase in fungal biomass, we also measured the rate of increase of hyphal biomass during growth in liquid medium. Growth was restored to near wild-type levels in liquid medium with the addition of 50 μ M putrescine, spermidine, or spermine (Fig. 3A), although higher concentrations of spermine decreased growth. The *ODC1*⁻ mutant was restored to wild-type growth rate on minimal medium (MM) agar with the addition of 0.5 μ M putrescine, spermine, or spermidine (Fig. 3B). The discrepancy between these results may arise from the fact that growth rate on solid media is measured as the rate of colony expansion and growth in liquid medium is measured as the rate of increase in biomass.

An alternative explanation may be that agar contains trace amounts of polyamines. If this were the case, however, increased levels of mutant growth would be expected on solid media. Visual observation of *ODC1*⁻ colonies grown on 0.5 to 50 μ M polyamine show that, although colony expansion is normal, hyphal growth is sparse. To reconcile these differences, we tested whether differences in hyphal growth rate and increase in biomass might arise from decreased hyphal branching at low polyamine concentrations. Wild-type W22-433 and Ty Δ ODC53 were inoculated into the center of 9-cm petri dishes containing various concentrations of polyamines, and hyphal branch distance was measured on a Axiovert 125 microscope (Carl Zeiss, Thornwood, NY, U.S.A.) with a calibrated reticule for comparison. Hyphal branch distance in wild-type *T. yallundae* is independent of exogenous polyamine concentration but the *ODC1*⁻ mutant produces hyphal branches less frequently at low polyamine levels (Fig. 3C). This difference may reflect a role for polyamines in the positioning or initiation of hyphal branching in fungi.

The *ODC1* null mutants behaved as polyamine auxotrophs when grown on minimal media at 28°C. Significant mycelial growth was observed at lower temperatures (Fig. 3D), although the hyphae were thin, less melanized, and growing sparsely. This suggests a requirement for polyamines in stabilizing cellular components at higher temperatures. It also was noted that the addition of 60 μ M putrescine to the medium at 25 and 28°C also caused a small but significant reduction in growth relative to unamended media.

Differentiation of infection structures by knockout mutants.

T. yallundae differentiates specialized multicellular infection structures, which are essential for its pathogenicity. They form by cell swelling that arises from hyphae by repeated apical branching and septation. These structures are induced in vitro by contact with solid surfaces such as the base of petri dishes or inert membranes (Deacon 1973). Infection plaque formation was induced in vitro under a hydrophobic polytetrafluorethylene membrane in the presence and absence of exogenous putrescine at 10°C. A plaque formation assay was set up with Ty Δ ODC53, Ty Δ ODC53/snod2, and Ty Δ ODC53/eye to investigate the requirement of polyamines and *ODC1* in the formation of these structures. Plaque formation was studied at 10°C to allow hyphal growth of unsupplemented *ODC1*

knockout strains. Ty Δ ODC53, Ty Δ ODC53/eye, and W22-433 conidia were harvested from unsupplemented TWA plates and plated onto TWA plates in the absence or presence of 60 μ M putrescine. The inoculum was covered by a layer of sterile Biofolie membrane (Heraeus, Hanau, Germany). When incubated at 10°C under constant white light, the conidia germinated and started to form infection plaques under the membrane within 4 to 10 days. The in vitro formation of infection plaques was assessed after 7 days. Wild-type W22-433, Ty Δ ODC53/eye, and Ty Δ ODC53/snod2 were able to form infection plaques under inductive conditions in the absence of polyamines. Ty Δ ODC53 conidia germinated and were able to form hyphae and grow as mycelia at the permissive temperature of 10°C but were unable to produce infection plaques under inductive conditions in the absence of putrescine. Supplementation of the medium with 60 μ M putrescine restored the ability of Ty Δ ODC53 to form highly melanized infection structures, identical to wild type infection plaques, under inductive conditions (Fig. 4).

Pathogenicity of *ODC1* knockout strains.

Conidia from the Ty Δ ODC53 and wild-type W22-433 were inoculated onto 2-week-old wheat seedlings. Symptoms were visible on the susceptible wheat cultivar Avalon at 6 weeks postinoculation. Distinct "eyespot" lesions, which penetrated several leaf sheaths, were observed after 8 weeks. Ty Δ ODC53 showed no apparent delay in symptom appearance or virulence compared with the wild type. Quantitative PCR was used to provide a measure of fungal biomass during infection. Infected stem bases were analyzed by competitive PCR. Fifty plants for each treatment were divided into pools of five plants and freeze dried. The dried material was sent to P. Nicholson at the John Innes Centre (Norwich, U.K.) where the DNA extraction and the quantitative competitive PCR were performed (Nicholson et al. 1997). The PCR was repeated twice, and each sample was replicated 10 times. The amounts of wild-type and Ty Δ ODC53 DNA present in the stem-base lesions were 0.034 ± 0.006 and 0.0295 ± 0.0065 μ g of sample per μ l, respectively. These values are not significantly different when compared with a Turkey's *t* test at 1% significance level (giving a *p* value of 0.771). Visual symptoms on these plants also were scored according to Scott and Hollins (1974). The disease index for the plants used in the quantitative DNA assessment is 76.9 for wild type and 57.8 for Ty Δ ODC53. This suggests that there is a small but significant decrease in symptom expression for Ty Δ ODC53 compared with wild type using a Student's *t* test (*p* = <0.01). In a repeat experiment, however, disease indexes were 65.3 for wild type and 59.2 for Ty Δ ODC53, which is not significant when compared as a Student's *t* test at 1% significance level (*p* = 0.143). This suggests that scoring disease by visual assessment may be subject to variability. The mean disease index and DNA levels, however, were lower for *ODC1* knockout mutants on all occasions tested. This may suggest a slight reduction in virulence, which might become statistically significant in a larger experiment.

DISCUSSION

Degenerate nested primers to sequences conserved in eukaryotic ODCs were used to clone the ODC gene of *T. yallundae* with a PCR-based approach. The 605-bp PCR fragment

was cloned, and its sequence showed characteristics typical of previously cloned ODCs. This fragment was then used to screen a genomic library made of *T. yallundae* DNA. Several positive clones were obtained, and a 5-kb hybridizing *Bam*HI fragment from one of the fragments was subcloned into pUC19. Sequence analysis revealed that it contained the whole *ODC1* ORF, and approximately 600 bp of upstream sequence. The deduced amino acid sequence shows high homology to the *N. crassa* ODC gene. Both have a 70-bp intron conserved at the same position in the coding sequence. A plasmid containing the complete *T. yallundae* *ODC1* gene was used to successfully complement an *A. nidulans* putrescine auxotroph by restoring vegetative growth and asexual sporulation.

Putrescine requiring mutants of *T. yallundae* were made by transformation-mediated targeted gene replacement of the *ODC1* gene. *ODC1* knockout mutants exhibited temperature-dependent growth with higher temperatures, restricting hyphal elongation. This observation suggests some role of polyamines in stabilization of cell components. The *ODC1* disruptants grow at a similar rate to the wild type when supplemented with sufficient putrescine. Biomass was reduced in the mutant strains, however, when exogenous polyamine levels were below 50 μ M. The discrepancy between biomass and hyphal growth rate seemed to result from decreased hyphal branching in the *ODC1* knockout strains. *ODC1* knockout strains were unable to form infection structures in the absence of exogenous polyamines, suggesting a role for polyamines in differentiation and hyphal branching in this organism.

Pathogenicity assays of the mutant strains with use of spore inoculum showed no reduction in virulence compared with the wild type. *ODC1* knockout mutants also developed the same biomass as wild type during infection. This suggests that the

Fig. 4. Infection structure formation of mutant and wild-type strains in the presence and absence of putrescine. *Tapesia yallundae* hyphae were grown on agar plates under inductive conditions at 10°C, as described. Micrographs show infection structure formation 7 days after induction. Bars = 20 μ m. **A**, Wild-type *T. yallundae* infection structures on unsupplemented minimal medium (MM). **B**, Ty Δ ODC53 hyphae on unsupplemented MM. **C**, Wild-type *T. yallundae* infection structures on MM + 60 μ M putrescine. **D**, Ty Δ ODC53 infection structures on MM + 60 μ M putrescine.

fungus can access up to $\geq 50 \mu\text{M}$ polyamines during infection. Visual inspection of lesions confirmed that all infection structures were formed normally by wild-type and knockout mutants. Analysis of symptom expression did not provide consistent results with repeats of the assay, giving statistically different results. In all cases, there was a minor reduction in lesion formation, which may arise from differences in hyphal morphology at low polyamine concentrations. This result is unexpected, given that the mutants were auxotrophic for polyamines and were unable to form infection structures in vitro under inductive conditions. The most plausible explanation for these findings is that the fungus is able to effectively scavenge for sufficient polyamines to support growth and differentiation from the plant during infection. It is intriguing to note that *ODC* gene disruption in the wheat pathogen *S. (Septoria) nodorum* causes major reduction in virulence toward wheat (Bailey et al. 2000). There may be several explanations for the difference in virulence caused by *ODC* disruption in these two pathogens. First, they may display different abilities to take up polyamines from the host plant. Second, the pathogens may have different requirements for polyamines, resulting from their different lifestyles, in that *S. nodorum* elaborates no infection structures and grows rapidly through plant tissue, whereas *T. yallundae* elaborates several different types of infection structure and ramifies slowly through plant tissue. Finally, the two pathogens attack different plant tissues, leaves, and stem bases, which may contain different concentrations of free polyamine. The observation that the disruption of homologous genes from different fungi can have widely different effects on pathogenicity and phenotype also must engender caution when extrapolating results obtained in one fungal pathogen to other pathogens. It would be interesting to see whether these strains are more sensitive to toxic polyamine analogs (Havis et al. 1994a; Havis et al. 1994b; Mackintosh and Walters 1997) than the wild type because they must utilize a polyamine uptake system to supply their requirement. It is noteworthy that a number of plant defense compounds are polyamine conjugates. Given the need for the uptake of exogenous polyamines, it is possible that the mutants would be more sensitive to such compounds.

MATERIALS AND METHODS

Strains and growth.

T. yallundae strain W-22-433 was used throughout the study. Cultures were maintained routinely as plug inocula from master plates or frozen mycelial stocks on 1.5% PDA (Oxoid, Basingstoke, U.K.). Plug inocula were put onto 1.5% tap water agar (TWA) for the production of conidia. *ODC1* mutants were grown on Czapek-Dox solid media (Oxoid) supplemented with trace elements (46 mg of $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 65.9 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 59 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 75 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 37.8 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ per liter), 59 μM thiamine, 81 nM biotin, 7.2 μM p-amino-benzoic acid (PABA), and 60 μM putrescine. For mycelial suspensions, plugs were inoculated into Czapek-Dox-V8 media and grown with shaking for 2 days at 18°C and 250 rpm. The grown mycelium was subsequently macerated and reinoculated into fresh Czapek-Dox-V8 media. Mycelial suspensions used for protoplast preparation were treated with 2.5 cm^2 of fungal plug and inoculated into 100 ml of previously centrifuged Czapek-Dox-

V8 in a 250-ml flask. The mycelial plug was macerated with a polytron in a 250-ml flask and incubated for 3 days with shaking at 250 rpm and 18°C. The fungal suspension was again macerated with the polytron, subcultured into Czapek-Dox-0.8% yeast extract, and grown for another 16 h at 18°C and 250 rpm. *A. nidulans* strain G0171 (*puA2-biA1*) was grown on complete media (20.0 g of Difco malt extract, 1.0 g of Bacto Peptone, 20.0 g of glucose, 20.0 g of Bacto agar, and appropriate supplements in 1,000 ml of H_2O).

Pathogenicity tests.

Pathogenicity tests were carried out in a growth chamber. Winter wheat (cv. Avalon), which is susceptible to eyespot, was grown in M3 compost (Levington Horticulture, Ipswich, U.K.) in 9-cm diameter pots. Ten seeds were sown into each pot and incubated for 10 days at 16°C under 12 h of white light. Ten days after sowing, seedlings were inoculated with 5×10^5 conidia harvested from TWA plates with 0.9% NaCl-0.5% Tween 40 solution. The relative humidity in the growth chamber was 95%, temperature was kept at 12°C, and day length was 12 h. The severity of stem lesions was assessed with the method used by Scott and Hollins (1974) on a scale of 0 to 3. A score of 0 indicates no visible stem symptoms, and a score of 3 indicates that the stem was girdled completely by lesions, resulting in the softening of stem tissue. Quantification of fungal biomass in infected stem tissue by quantitative PCR with the method of Nicholson et al. (1997) was used as an additional measure of infection.

Plasmids.

The plasmids used in the transformation of *T. yallundae* were pST28, which was constructed by ligating a 1.4-kb *HpaI* fragment from pCB1004 (Carroll et al 1994) containing the hygromycin resistance from *Escherichia coli* under control of the *TrpC* promoter from *A. nidulans* into pLitmus28 (New England Biolabs, Beverly, MA, U.S.A.). Plasmid pAN8-1 contains the phleomycin gene from *Streptococcus hindustanus* flanked by the highly expressed *A. nidulans* *gpd* gene and the terminator region of the *A. nidulans* *trpC* gene (Mattern and Punt 1988). Plasmid DNA was isolated by a modification of the alkaline lysis method (Birnboim and Doly 1979) and the use of miniprep columns (Qiagen, Crawley, U.K.). The knock-out plasmid pODCHyg2 was constructed as follows. The genomic *ODC1* clone p8BamD19, consisting of a 6-kb *BamHI* fragment containing the complete *ODC1* ORF in pBluescriptII KS, was cut with the restriction enzyme *SpeI*, and a 3-kb fragment was removed. The resulting 3' protruding ends were "filled in" with the Klenow fragment of *E. coli* DNA polymerase. A 1.4-kb *HpaI* fragment from plasmid pST38 containing the hygromycin resistance gene from *E. coli* under control of the *trpC* promoter from *A. nidulans* was then ligated into the *SpeI* cut and blunt-ended vector p8BamD19, creating plasmid pODCHyg. This plasmid contained flanking sequences 660-bp upstream and 1.9-kb downstream of the *ODC1* ORF. The resulting plasmid contained the hygromycin resistance cassette and 35 bp of the *ODC1* coding sequence. An 8-kb *BamHI* fragment from pODCHyg containing the hygromycin resistance cassette and the flanking sequences of the *ODC1* gene was ligated into *BamHI*-restricted p10HindD10, consisting of a 4.5-kb *HindIII* genomic region comprising the 5' section of the *ODC1* gene and upstream sequences, removing a

1.2-kb *Bam*HI fragment from p10HindD10 and creating pODCHyg2.

DNA isolation and Southern blotting.

DNA was purified by a modification of the method used by Specht et al. (1982). Liquid cultures were collected on Miracloth in a funnel and washed with TSE (150 mM NaCl; 100 mM EDTA; and 50 mM Tris HCl, pH 8.0). The washed mycelia subsequently were freeze dried, and 1 g of dry-weight mycelia was ground to a fine powder and transferred to a 250-ml conical flask. Twenty-five milliliters of extraction buffer (TSE + 2% sodium dodecyl sulfate [SDS]) and 0.2 (vol/vol) of toluene was added. The flask was shaken very slowly on a rotary shaker for 3 days. The DNA was separated from the toluene by spinning at $15,000 \times g$ for 15 min in a SS34 rotor. The DNA was concentrated in the interphase between the water and toluene phase. The DNA was removed and spun again to remove the toluene, 0.25 volume of 7.5 M NH_4Ac was added, and the polysaccharides were precipitated overnight at 4°C. The polysaccharides were then pelleted at $39,000 \times g$ for 30 min in a SS34 rotor. The DNA was precipitated with 0.7 volume of isopropanol. The precipitate was fished out with a sealed-off Pasteur pipette and resuspended in 750 μl Tris-EDTA (TE; 10 mM Tris-HCl, pH 7.5; and 1 mM EDTA) and 50 μg of RNase per ml. The RNase digestion was performed at 37°C for 30 min. Proteinase K was added to 100 μg of the solution per ml and left to incubate for another 2 h at 37°C. The DNA solution was extracted with phenol- CHCl_3 . The DNA was precipitated once more and resuspended in TE. For Southern analysis, 2 μg of genomic fungal DNA was digested with different restriction enzymes overnight at the appropriate temperature. The DNA was separated on 0.8% agarose gel and standard techniques (Sambrook et al. 1989) were employed to treat and blot the DNA onto a nylon membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). Prehybridization and hybridization solutions were 5 $\times$ Denhardt's (2% gelatin, 2% Ficoll-400, 2% polyvinylpyrrolidone [PVP]-360, and 10% SDS), 100 μg of denatured calf thymus DNA per ml, 0.2% SDS, and 1 $\times$ HSB (3 M NaCl; 0.1 M piperazine-*N,N'*-bis[21-ethanesulfonic acid]; and 20 mM EDTA, pH 6.8). Hybridization was performed overnight at 65°C. All washes were performed twice for 30 min at high stringency: 0.1 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.5% SDS at 65°C.

PCR of ornithine decarboxylase from *T. yallundae*.

For PCR, 100 ng of genomic DNA of *T. yallundae* was used in a "hot-start" PCR reaction. The amplification conditions were as follows: 94°C for 5 min and 75°C for 5 min, then 35 cycles of 94°C for 1 min, 52°C for 2 min, and 72°C for 3 min. The degenerate primers were used at a final concentration of 2.5 μM . One-hundredth of the first-round product was used in the second-round PCR with the inner pair of nested primers under identical amplification conditions. The outer pair of primers were the 5' primer [5'-CCNTT(C)/TA(T/C)GCNGTN-AA(A/G)TG(T/C)AA-3'], representing the amino acid sequence PFYAVKCN, and the 3' primer [5'-CC(A/G)TC(A/G)-CANGTNGGNCCCCA-3'], representing the sequence WGP-TCDG. The second-round internal pair of primers were the 5' primer [5'-TNATNT(T/A)(T/C)GCNAA(T/C)CCNTG-3'], representing the amino acid sequence I(F/Y)ANPC, and the 3' primer [5'-TNCC(A/G)TANACNCCA(A/G)TC(A/G)TT-3'],

representing the sequence NDGVYG(S/N). The products of several PCRs were pooled and size fractionated on a 1.5% (wt/vol) agarose gel. After the second round of PCR, only one distinct band of the right size was visible after ethidium bromide staining, and DNA from that band was isolated and cloned as a blunt-ended fragment into the *Eco*RV site of the vector pBluescript II SK+ (Stratagene, Cambridge, U.K.). The identity of the cloned fragment was determined by sequencing the PCR product.

Genomic library and screening.

A *T. yallundae* genomic library was prepared in Lambda-GEM12 (Promega, Southampton, U.K.). *T. yallundae* genomic DNA was partially restricted with *Sau*3A. The *Sau*3A sites were partially filled with Klenow polymerase and ligated into partial filled *Xho*I half-site arms of LambdaGEM12. The ligation reaction was packaged with the GigapackIII Gold Kit according to the manufacturer's protocol (Stratagene), and phage titers were determined in the bacterial host strain LE392. The *ODC1* PCR product was radioactively labeled with the High Prime Kit (Roche Diagnostics, Lewes, U.K.) and used to probe the genomic library. The positive plaques were purified by an additional three rounds of screening.

Sequencing.

All sequencing reactions were performed on double-stranded plasmid DNA with a ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Beaconsfield, U.K.). The sequence of both strands of the genomic *ODC1* clone was determined by a combination of oligonucleotide primer walking and subcloning into pUC18. Sequence determination was carried out with a Perkin-Elmer ABI377 automated sequencer and analyzed with the Genetics Computer Group's software package, version 8 (Madison, WI, U.S.A.).

Transformation of *T. yallundae*.

Mycelia were grown, as outlined previously, collected on sterile Miracloth (Calbiochem, Beeston, U.K.) by vacuum filtration, and washed with sterile water. A sterile spatula and forceps was used to place the mycelial pad in a sterile tube and weighed before resuspension in 10 ml per g of 1 M sorbitol. After two washes in 1 M sorbitol, 4 mg per ml of freshly prepared Novozyme 234 (InterSpex Products, Foster City, CA, U.S.A.) in 1 M sorbitol was added. Mycelia were digested by shaking gently at room temperature for 2 h. The digest was checked regularly for the release of protoplasts. The digested mycelia was filtered through four layers of sterile Miracloth and washed with 1 M sorbitol. Protoplasts were pelleted at $2,665 \times g$ in a tabletop centrifuge at 15°C. The pellet was resuspended in 1 M sorbitol. This wash was repeated twice. Protoplasts were resuspended at a concentration of 5×10^7 protoplasts per ml. Protoplast solution (200 μl) was mixed with 5 μg of linearized plasmid DNA, then incubated for 15 min at room temperature. One milliliter of polyethylene glycol (PEG) solution (25 mM Tris HCl, pH 7.5; 25 mM CaCl_2 ; and 66% PEG 8000) was added slowly over 30 min. Three milliliters of liquid TB3 (0.3% yeast extract, 0.3% Casamino Acids, and 20% sucrose) was added and mixed. TB3 containing 2% agarose was melted and kept at 50°C, then added to and mixed with the protoplast suspension, which was poured into

9-cm petri dishes. The plates were incubated overnight at 18°C and, the next day, overlaid with 1.5% PDA + 100 µg of Hygromycin B per ml, in the case of pST28, or 500 µg of Zeocin per ml, for pAN8-1. The plates were incubated at 18°C in the dark until the first microcolonies were visible. The microcolonies were transferred weekly to fresh selective media.

Transformation of *A. nidulans*.

A. nidulans was transformed, essentially as described by de Graaff (1989). Plates of transformed protoplasts were incubated at 37°C in the dark until the first microcolonies were visible. Spores from these microcolonies were removed and streaked out to a single colony on MM plates (minimal medium, 1.2% agar, and appropriate supplements). Spores from a single colony were transferred into 100 µl of water and subsequently propagated and tested by Southern blot for integration of the transforming plasmid.

Measurement of hyphal biomass in liquid culture.

T. yallundae conidia were collected from TWA plates and washed several times with sterile water. The conidia were diluted to 5×10^5 conidia per ml in liquid Czapek-Dox supplemented media. Polyamines were diluted to the appropriate concentration in Czapek-Dox supplemented liquid media, 80 µl was added to each well of a 96-well, flat-bottomed Nunc microtiter plate, and eight replicates were prepared for each concentration. Eighty microliters of conidial suspension was subsequently added to each well. The plates were incubated at 18°C with shaking at 200 rpm. The absorbance of each well of the microtiter plate was measured daily with a plate reader (Tecan, Weymouth, U.K.) at a wavelength of 600 nm. Protein was measured in samples with the Bio-Rad Protein Assay Kit (Hemel, Hempstead, U.K.) according to the manufacturer's instructions.

ODC enzyme assay.

Fungal ODC activity was measured, as described by Stevens et al. (1976), following the release of $^{14}\text{CO}_2$ from ($1\text{-}^{14}\text{C}$) ornithine. Spores were harvested from confluent sporulating Czapek-Dox-V8 plates and washed extensively with sterile distilled water. They were suspended in 100 ml of Czapek-Dox liquid media at 1×10^7 per ml and incubated at 25°C with shaking. Material was harvested by filtration through Miracloth. Samples were ground to a fine powder in liquid nitrogen with glass beads, then resuspended in 2 to 3 ml of extraction buffer (10 mM potassium phosphate; 2 mM dithiothreitol; 1 mM MgCl_2 ; 0.1 mM EDTA; and 0.1 mM pyridoxal phosphate, pH 7.6) and vortexed briefly. The resulting extract was centrifuged at $25,000 \times g$ to remove cell debris, then desalted to remove other decarboxylase substrates by buffer exchange in a Nap 10 column (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Reactions contained 50 mM Tris; 0.05 mM ornithine; 0.32 mM pyridoxal phosphate; 0.125 mCi ^{14}C -ornithine, pH 8.0; and 0.2 ml of extract in a final volume of 0.4 ml were carried out in 14-ml snap-top Falcon tubes. CO_2 was captured in suspended 0.5-ml Eppendorf tubes containing 50 µl 2-methoxyethanol-ethanolamine mixture (2:1 vol/vol). Reactions were incubated at 25°C for 30 min and stopped by the addition of 0.1 ml of 50% trichloroacetic acid, then left for an additional 60 min to trap released $^{14}\text{CO}_2$. Trapped $^{14}\text{CO}_2$ was counted in a scintillation counter. Protein

was measured, in 0.2-ml samples, with the Bio-Rad protein assay kit according to manufacturer's instructions.

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LITERATURE CITED

- Abrahamsen, M. S., and Morris, D. R. 1991. Regulation of expression of the ornithine decarboxylase gene by intracellular signal transduction pathways. Pages 107-119 in: *Perspectives on Cell Regulation: From Bacteria to Cancer*. M. Inouye, J. Campisi, D. D. Cunningham, and M. Riley, eds. Wiley-Liss, New York.
- Bagni, N., Malucelli, B., and Torrigiani, P. 1980. Polyamines, storage substances and abscisic acid-like inhibitors during dormancy and very early activation of *Helianthus tuberosus* tuber tissues. *Physiol. Planta*. 49:341-345.
- Bailey, A. N., Mueller, E., and Bowyer, P. 2000. Ornithine decarboxylase of *Stagonospora (Septoria) nodorum* is required for virulence towards wheat. *J. Biol. Chem.* 275:14242-14247.
- Birnboim, H. C., and Doly, J. 1979. A rapid alkaline extraction method for the isolation of plasmid DNA. *Nucleic Acids Res.* 7:1513-1522.
- Brawley, J. V., and Ferro, A. J. 1979. Polyamine biosynthesis during germination of yeast ascospores. *J. Bacteriol.* 140:649-654.
- Carroll, A. M., Sweigard, J. A., and Valent, B. Improved vectors for selecting resistance to hygromycin. 1994. *Fungal Genet. Newsl.* 41:23.
- Choi, S.-J., Ferro, A. J., and Shapiro, S. K. 1978. Relationship between polyamines and macromolecules in germinating yeast ascospores. *J. Bacteriol.* 133:424-426.
- Cohn, M. S., Tabor, C. W., and Tabor, H. 1978. Isolation and characterization of *Saccharomyces cerevisiae* mutants deficient in S-adenosylmethionine decarboxylase, spermidine and spermine. *J. Bacteriol.* 134:208-213.
- De Graaff, L. H. 1989. The structure and expression of the pyruvate kinase gene of *Aspergillus nidulans* and *Aspergillus niger*. Ph.D. thesis. Agricultural University, Wageningen, The Netherlands.
- Deacon, J. W. 1973. Pseudoparenchyma produced by *Cercospora herpotrichoides* in culture. *Trans. Br. Mycol. Soc.* 60:537-545.
- Fitt, B. D. L., Goulds, A., and Polley, R. W. 1988. Eyespot (*Pseudocercospora herpotrichoides*) epidemiology in relation to prediction of disease severity and yield loss in winter wheat: A review. *Plant Pathol.* 37:311-328.
- Fonzi, W. A., and Sypherd, P. S. 1987. The gene and the primary structure of ornithine decarboxylase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* 262:10127-10133.
- Guevara-Olvera, L., Xoonostle-Cazares, B., and Ruiz-Herrera, J. 1997. Cloning and disruption of the ornithine decarboxylase gene of *Ustilago maydis*: Evidence for a role of polyamines in its dimorphic transition. *Microbiology* 143:2237-2245.
- Guye, M. G., Vigh, L., and Wilson, J. M. 1986. Polyamine titer in relation to chill-sensitivity in *Phaseolus* sp. *J. Exp. Bot.* 37:1036-1043.
- Havis, N. D., Walters, D. R., Foster, S. A., Martin, W. P., Cook, F. M., and Robins, D. J. 1994a. Fungicidal activity of 3 putrescine analogs. *Pestic. Sci.* 41:61-69.
- Havis, N. D., Walters, D. R., Martin, W. P., Cook, F. M., and Robins, D. J. 1994b. Fungicidal activity of 3 putrescine analogs. *Pestic. Sci.* 41:71-76.
- Hickok, N. J., Seppanen, P. J., Gunsalus, G. L., and Janne, O. A. 1987. Complete amino-acid-sequence of human ornithine decarboxylase deduced from complementary DNA. *DNA* 6:179-187.
- Mackintosh, C. A., and Walters, D. R. 1997. Fungicidal activity of two spermidine analogues. *J. Phytopathol.* 145:43-45.
- Mattern, I. E., and Punt, P. J. 1988. A vector of *Aspergillus* transformation conferring phleomycin resistance. *Fungal Genet. Newsl.* 35:25.
- Michael, A. J., Furze, J. M., Rhodes, M. J. C., and Burtin, D. 1996. Molecular cloning and functional identification of a plant ornithine decarboxylase cDNA. *Biochem. J.* 314:241-248.
- Nicholson, P., Rezanoor, H. N., Simpson, D. R., and Joyce, D. 1997. Differentiation and quantification of the cereal eyespot fungi *Tapesia yallundae* and *Tapesia acuformis* using a PCR assay. *Plant Pathol.*

- 46:842-856.
- Palavan, N., and Galston, A. W. 1982. Polyamine biosynthesis and titer during various developmental stages of *Phaseolus vulgaris*. *Physiol. Planta*. 55:438-444.
- Paulus, T. J., Kiyono, P., and Davies, R. H. 1982. Polyamine-deficient *Neurospora crassa* mutants and synthesis of cadaverine. *J. Bacteriol.* 152:291-297.
- Pegg, A. E., and McCann, P. P. 1982. Polyamine metabolism and function. *Am. J. Physiol.* 243:212-221.
- Ruiz-Herrera, J. 1993. The role of polyamines in fungal cell differentiation. *Arch. Med. Res.* 24:263-265.
- Ruiz-Herrera, J. 1994. Polyamines, DNA methylation, and fungal differentiation. *Crit. Rev. Microbiol.* 20:143-150.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A.
- Scott, P. R., and Hollins, T. W. 1974. Pathogenic variation in *Pseudocercospora herpotrichoides*. *Ann. Appl. Biol.* 94:297-300.
- Sikora, L., and McDougall, K. J. 1978. The regulation of ornithine decarboxylase in *Neurospora crassa*. *Exp. Mycol.* 2:352-358.
- Slocum, R. D., Kaur-Sawhney, R., and Galston, A. W. 1984. The physiology and biochemistry of polyamines in plants. *Arch. Biochem. Biophys.* 235:283-303.
- Smith, T. A. 1985. Polyamines. *Ann. Rev. Plant Physiol.* 36:117-143.
- Sneath, P. H. A. 1955. Putrescine as an essential growth factor for a mutant of *Aspergillus nidulans*. *Nature* 175:818.
- Specht, C. A., DiRusso, C. C., Novotny, C. P., and Ullrich, R. C. 1982. A method for extracting high-molecular-weight deoxyribonucleic-acid from fungi. *Anal. Biochem.* 119:158-163.
- Stevens, L., and Winther, M. D. 1979. Spermine, spermidine and putrescine in fungal development. *Adv. Microbial Physiol.* 19:63-148.
- Stevens, L., McKinnon, I. M., and Winther, M. 1976. Polyamine and ornithine metabolism during the germination of conidia of *Aspergillus nidulans*. *Biochem. J.* 158:235-241.
- Tabor, C. W., and Tabor, H. 1976. Polyamine biosynthesis. *Ann. Rev. Biochem.* 45:285-306.
- Tabor, C. W., and Tabor, H. 1985. Polyamines in microorganisms. *Microbiol. Rev.* 49:81-99.
- Von Ropenack, E., Parr, A., and Schulze-Lefert, P. 1998. Structural analyses and dynamics of soluble and cell wall-bound phenolics in a broad spectrum resistance to the powdery mildew fungus in barley. *J. Biol. Chem.* 273:9013-9022.
- Walters, D. R. 1989. Polyamines and plant disease. *Plants Today* January-February:22-26.
- Walters, D. J. 1995. Inhibition of polyamine biosynthesis in fungi. *Mycol. Res.* 99:129-139.
- Williams, L. J., Barnett, G. R., Ristow, J. L., Pitkin, J., Perriere, M., and Davies, R. H. 1992. Ornithine decarboxylase gene of *Neurospora crassa*: Isolation, sequence, and polyamine-mediated regulation of its messenger RNA. *Mol. Cell. Biol.* 12:347-359.