Selection of Drug-Tolerant Strains of *Pythium sylvaticum* Using Sublethal Enrichment

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ABSTRACT

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Sublethal enrichment was used to generate mutants of *Pythium sylvaticum* tolerant to kanamycin and tetracycline. Kanamycin tolerance was readily generated, and mutants had growth rates similar to wild-type isolates at antibiotic concentrations lethal to wild-type isolates. Based on crosses between wild-type and mutant isolates, two types of inheritance of tolerance were identified. A high level of kanamycin tolerance was inherited in progeny only when the maternal parent was drug tolerant and

was correlated with the inheritance of maternal mitochondrial DNA. A lower level of tolerance was observed in some progeny when the paternal parent was tolerant to the antibiotic and, based on the lack of inheritance of paternal mitochondrial DNA, was presumably nuclear-encoded. Selection of mutants tolerant to tetracycline took longer to generate than kanamycin-tolerant mutants. Based on crosses between tolerant and wild-type parents, tolerance to tetracycline was nuclear-encoded. Tolerance to both antibiotics was stable, with cultures retaining tolerance following repeated transfers on nonamended medium and after storage for 7 years.

Additional keywords: drug mutants, mitochondrial inheritance.

Investigations on the soil ecology of *Pythium* spp. are often hampered by the inability to differentiate among species or identify an individual isolate of a species on culture media by morphological characteristics alone. This complicates research by requiring more extensive subculturing and analysis of the isolates to determine accurate identification. For similar reasons, investigations on the ecological interactions among different species in a soil sample also can present a daunting challenge to the investigator. Procedures that would streamline isolate identification would simplify this type of research.

Several aspects of the organism's biology have been examined in an attempt to simplify isolate identification including comparison of protein banding patterns (13,16), isozymes (1,13,15), serology (3,20,22,33,38,53,60-63), or the use of molecular markers (12, 14,23,31,32,35,41-43,47,49,50). While these approaches have their utility, they may be time consuming, expensive, or unamenable for rapid identification of specific isolates.

Selection for drug tolerance provides an alternative approach for identification of specific isolates of interest. While this approach would have limited utility for field investigations of undisturbed ecosystems, it could be useful in experimental systems in which specific isolates would be introduced and monitored. The use of drug-tolerant isolates would minimize the constraints mentioned above and allow rapid identification of a particular isolate by plating on selective medium. An example of this experimental approach is illustrated in a series of manuscripts by Stack and Millar (57-59) describing the selection of metalaxyl tolerance in *Phytophthora megasperma* f. sp. *medicaginis*, characterization of the mutation, and use of marker isolates for ecological investigations. An added benefit of using drug-tolerant isolates is that, once the mutation conferring tolerance has been characterized, it can be useful as a marker in investigations on Mendelian or population genetics.

Mutants of oomycetes tolerant to specific drugs have been selected using a variety of techniques. Isolates of *Phytophthora* spp. tolerant to different inhibitory compounds have been selected by screening zoospores for tolerance (18,30,55,57) or selecting for spontaneous mutants tolerant of the compounds derived by sublethal enrichment (2,8,18). Although these approaches for marker selection have been effective, more efficient selection against a broader range of toxic compounds has been obtained by treatment with general mutagens such as UV irradiation, ethylmethane sulphonate (EMS), or *N*-methyl-*N*-nitroso-*N*-nitroguanidine (MNNG) (2,6-8,10,18,28,30,34,36,37). Similar approaches have been used for selection of mutants in the genus *Pythium* (8,9,19,39,46,52).

Although treatment with general mutagens is an efficient method for induction of tolerance, a number of other mutations also may be generated by the nonspecific nature of the mutagenizing treatment. This was observed when selecting mutants of *P. oligandrum* tolerant to metalaxyl by treatment with UV light (46). While all progeny isolates of a selfed mutant were tolerant to metalaxyl, a wide range of abnormal growth rates and morphologies not found in selfed progeny of the wild-type isolate were observed. Five repeated selfings and selection of isolates with wild-type characteristics were required to eliminate these abnormal phenotypes in the progeny. A similar generation of nonparental phenotypes was observed by Bhat and Schmitthenner (6) when generating mutants of *Phytophthora sojae* by treatment with MNNG; 23% of the metalaxyl- and 33% of the *p*-fluorophenylalanine-tolerant mutants had altered virulence compared with the parental isolates.

The objective of this research was to evaluate the utility of using sublethal enrichment for generation of stable mutants of the heterothallic species *P. sylvaticum* tolerant of antibiotics and determine if the tolerance was nuclear- or cytoplasmically encoded. Portions of this research have been reported previously (44,48).

MATERIALS AND METHODS

Fungal strains. Opposite mating types of *P. sylvaticum* were used in this investigation; isolate 1987-14 was an oogonial and

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1987-73 an antheridial mating type. Both isolates are strictly heterothallic and did not self when grown on culture medium or grass blade culture (45). Additional information for both isolates was provided by Martin (45).

Selection of mutants. Isolates were grown on cornmeal agar (CMA) (Difco Laboratories, Detroit) amended with 0.01 M 2-[N-morpholino]ethanesulfonic acid buffer at pH 6.2. The buffer was added to the CMA to help ensure a stable medium pH for the prolonged periods of incubation that were necessary for generation of mutant sectors. The sensitivity of wild-type cultures to kanamycin, kasugamycin, or tetracycline was tested by amending the culture medium with increasing concentrations of the antibiotics and

incubating inoculated plates at 25°C in the dark. Mycelial growth was marked after 24 h and growth measured following additional incubation (Fig. 1).

Once the initial sensitivity of the wild-type isolates was determined, the isolates were transferred to CMA amended with antibiotic concentrations that inhibited approximately 90% of the linear growth. Plates were sealed with Parafilm and incubated at 25°C in the dark. Cultures were checked daily for growth and the presence of fast-growing sectors, which were transferred to medium amended with a slightly higher concentration of the antibiotic (for kanamycin and tetracycline, this initially was in increments of 5 to 10 µg/ml and 3 to 5 µg/ml, respectively). If isolates grew well following several serial transfers on CMA with increasing concentrations of antibiotics, they were evaluated for stability of drug tolerance. The isolates were cultured on CMA without antibiotics for 5 to 7 days, and then transferred back to CMA amended with the antibiotic. If cultures continued to grow at a rate similar to what was observed prior to the transfer to nonamended medium, tolerance was assumed to be due to a spontaneous mutation rather than a physiological adaptation to the antibiotic. Serial transfer to medium with increasing concentrations of the antibiotic was continued until isolates were derived that were tolerant to antibiotic concentrations that were significantly above levels that inhibited the wild-type isolate.

Crosses and progeny analysis. To evaluate inheritance of drug tolerance in crosses between mutant and wild-type parents, isolates were grown individually in half-strength V-8 cholesterol broth (4) for 3 days at 25°C. Mycelial mats of both mating types were aseptically removed, combined, and blended on low speed with fresh V-8 media for 5 s in a sterile blender prior to pouring into fresh plates. Cultures were incubated for 14 days at 25°C in the dark prior to harvesting oospores using procedures previously described (45). For some crosses, opposite mating types were coinoculated in a single culture plate and oospores were isolated from the contact zone between the cultures after 14 days of growth.

Randomly selected oospore progeny were evaluated for antibiotic tolerance by growing isolates at the same time on CMA either unamended or amended with the antibiotic (Table 1). For kanamycin tolerance, progeny were evaluated following 24 h of growth on medium amended with 200 µg of antibiotic/ml or 48 h of growth on medium amended with 75 µg of antibiotic/ml. For tetracycline tolerance, progeny were assessed after 48 h or 7 days of growth on media amended with 110 µg/ml of tetracycline. Progeny were classified as tolerant by several different criteria. In cases in which the antibiotic concentration tested completely inhibited the wild-type isolates, progeny were classified as tolerant if they grew at a rate of at least 1 mm in 24 h and they continued to grow at a similar rate following continued incubation. If the parental isolates were not completely inhibited by the antibiotic, progeny were classified as tolerant if they had a growth rate of at least 3 mm faster than the wild-type isolates.

Fig. 1. Sensitivity of wild-type isolates of *Pythium sylvaticum* (1987-14 and 1987-73) and mutants selected for tolerance to **A**, kanamycin and **B**, tetracycline on cornmeal agar (CMA) amended with increasing concentrations (µg/ml) of the antibiotic. Growth was measured for a 24-h time period beginning 24 h after transfer to CMA and incubating plates at 25°C.

TABLE 1. Inheritance of drug tolerance in crosses between tolerant mutant and wild-type isolates of *Pythium sylvaticum* assessed by measuring linear growth on cornmeal agar amended with antibiotics

Antibiotic	Cross	Parental mating type		Number of progeny	Antibiotic concentration µg/ml	Tolerant:sensitive
		Antheridial	Oogonial			
Kanamycin	1	1987-73	1988-45K	68	200	68:0
	2	17-25 ^a	1988-45K	40	200	40:0
	3	1988-46K	1987-14	55	200	44:11
	4	3-56K ^a	1987-14	149	75	62:87
	5	1-78K ^a	1987-14	60	75	47:13
Tetracycline	8	1987-73	1991-35T	31	75	25:6
	10	8-5T ^a	1987-14	42	110	35:7
				84	110	60:24

^a These isolates are progeny from a cross between a wild-type parent and one of the drug-tolerant isolates; the first digit of the isolate number corresponds to the cross number. Isolate 3-56K is tolerant of kanamycin at 75 µg/ml, but not at 200 µg/ml.

Since *P. sylvaticum* is a diploid organism, reassortment of alleles following outcrossing of wild-type isolates can generate significant variation in traits such as growth rates (also observed in *P. splendens*) (25). This is exemplified by examining the range of growth rates of progeny isolates derived from a cross between the wild-type parents (Fig. 2, y axis); these isolates were described previously (45), and the biparental inheritance of molecular markers for all progeny examined confirmed that they were the result of a sexual outcross. Because of the variable growth rates observed for progeny isolates, the data for evaluation of antibiotic tolerance are presented in a scatter plot with the growth rates on amended medium (x axis) plotted against growth on nonamended medium (y axis) (Figs. 3 to 6). If the antibiotic concentration evaluated had no effect on the growth rate of an isolate (tolerant of the antibiotic), the data point should align at a similar position relative to both the x and y axis. If the progeny isolates, in general, were tolerant of the antibiotic, the data points would cluster in a line that runs from the lower left corner to the upper right corner of the graph (Fig. 3). However, if the progeny were sensitive to the antibiotic, the data points should cluster more towards the y axis. Conversely, if the presence of the antibiotic has a stimulatory effect on growth rates, the data points should cluster towards the x axis.

Evaluation of mitochondrial DNA (mtDNA) background in progeny isolates. To clarify if there was a relationship between a specific parental mitochondrial type and inheritance of drug toler-

ance, molecular markers to differentiate parental mtDNA of the isolates used in this investigation were developed. The mitochondrial genome of isolate 1987-14 has a 1.5-kb insertion relative to isolate 1987-73 adjacent to a conserved *SalI* restriction site (43). This region and corresponding flanking sequences delimited by *Clal* restriction sites from both parental isolates were cloned from purified mtDNA (47) into pBluescript using standard techniques (51). The termini of double-stranded templates were sequenced using Sequinase (Amersham Life Science, Arlington Heights, IL) in accordance with the manufacturer's directions, and regions of sequence identity between the two isolates were selected for primer construction. Primers were selected that amplified different-sized DNA fragments diagnostic for the genomes in each isolate; a fragment of 2.0 kb was amplified for isolate 1987-14 and 0.75 kb for isolate 1987-73. The primers FM 18 (TAGGTGTAGTAATATC-GTCT) and FM 19 (TAGATGAGGATCATGTGCCA) were synthesized at the University of Florida Interdisciplinary Center for Biotechnological Research. Template DNA was extracted using a scaled-down version of a procedure previously reported (47), or it was isolated using a boiling miniprep procedure in which a small amount of hyphae was placed in a 500- μ l microfuge tube with sterile water and boiled for 5 min. A 1- to 3- μ l aliquot was removed and added to the amplification mixture. Occasionally, DNA from some tubes did not amplify, this generally was alleviated by dilution of the DNA sample.

Fig. 2. Sensitivity of 40 progeny from a cross between wild-type parental isolates (1987-14 $\times$ 1987-73) to 75 μ g/ml of medium. Growth was measured on cornmeal agar (CMA) for a 24-h time period beginning 24 h after transfer and incubating plates at 25°C.

Fig. 3. Sensitivity of single-oospore progeny from a cross between an antibiotic-sensitive antheridial parent (1987-73) and a kanamycin-tolerant oogonial isolate (1988-45K) to kanamycin (Table 1, cross 1). Growth on cornmeal agar (CMA) either unamended or amended with 200 μ g of kanamycin/ml of medium was measured for a 24-h time period beginning 24 h after transfer to the medium and incubating plates at 25°C.

Fig. 4. Sensitivity of 151 single-oospore progeny from a cross between a kanamycin-tolerant antheridial parent (1988-46K) and a wild-type oogonial isolate (1987-14) (Table 1, cross 3). Growth on cornmeal agar (CMA) either unamended or amended with **A**, 200 μ g or **B**, 75 μ g of kanamycin/ml of medium was measured for a 24-h time period beginning 24 h after transfer to the medium and incubating plates at 25°C.

Amplifications were conducted in a total volume of 25 μ l that contained 1 μ M of each primer; 2.5 μ l of 10 $\times$ buffer; 100 μ M dATP, dCTP, dGTP, and dTTP; 2 Mm MgCl₂; and approximately 100 ng of fungal DNA. One unit of *Taq* polymerase (Promega Corp., Madison, WI) was added to the reaction mixture in a volume of 5 μ l, and the tubes were layered with mineral oil prior to placing them in the thermocycler (Tempcycler II; Coy Laboratory Products, Inc., Ann Arbor, MI). The cycling parameters for amplification were 1 min of denaturation at 95°C followed by 35 cycles of 1 min of annealing at 56°C, 2 min of extension at 72°C, and 1 min of denaturation at 94.5°C. The final cycle had an extension time of 10 min at 72°C. Ramping times between all temperatures were set at the fastest settings for the machine. Amplified products were electrophoretically separated on 0.7% agarose for scoring of amplified bands. Parental isolates were included in all amplifications as positive controls, and amplifications were repeated if questionable results were observed.

To assess the limits of detection of paternal inheritance of mtDNA in progeny isolates, 100 ng of total DNA from the oogonial parent 1987-14 was amended with different concentrations of either purified mtDNA or total DNA from the antheridial parent

1987-73. Polymerase chain reaction amplification of the mtDNA markers was performed as described.

RESULTS

Antibiotic sensitivity of wild-type isolates. Linear growth of wild-type isolates was inhibited by approximately 95% at 50 μ g of kanamycin/ml of medium (Fig. 1A). Wild-type isolates also were sensitive to tetracycline, with approximately 96% inhibition of linear growth observed at 25 μ g of the antibiotic/ml (Fig. 1B). In subsequent trials, lower concentrations of tetracycline were found to inhibit linear growth, with approximately 90% inhibition at 5 μ g/ml of tetracycline (data not shown).

Selection of tolerant isolates. Isolates with significant levels of tolerance to kanamycin were obtained for each wild-type isolate after a series of 11 to 13 serial transfers on media with increasing concentrations of antibiotic (Fig. 1A). Isolate 1988-46K was derived from the wild-type antheridial isolate 1987-73, while 1988-45K was derived from the wild-type oogonial isolate 1987-14. Isolate 1988-44K also was derived from the wild-type antheridial isolate and had a level of kanamycin tolerance intermediate between 1988-45K and 1988-46K (data not shown). When selecting mutants tolerant of kanamycin, the starting antibiotic concentration was 45 μ g/ml, which was increased initially by 10 μ g/ml and later by as much as 30 μ g/ml over the previous concentration with each successive transfer. The elapsed time for selection of the tolerant isolates was approximately 4 months. Tolerant isolates were capable of growing at antibiotic concentrations lethal to wild-type isolates and were stable following culture on unamended medium. Tolerance also was found to be unaltered when isolates were retrieved

Fig. 5. Sensitivity of 42 single-oospore progeny from a cross between a tetracycline-tolerant oogonial parent (1991-35T) and a wild-type antheridial isolate (1987-73) (Table 1, cross 8). Growth on cornmeal agar (CMA) was measured for a 24-h time period beginning 24 h after transfer to the medium and incubating plates at 25°C, while growth on CMA amended with 110 μ g of tetracycline/ml of medium was measured for a 48-h time period beginning 24 h after transfer to the medium.

Fig. 6. Sensitivity of 84 single-oospore progeny from a cross between a tetracycline-tolerant antheridial parent (8-5T) and a wild-type oogonial parent (1987-14) (Table 1, cross 10). Growth on cornmeal agar (CMA) was measured for a 24-h time period beginning 24 h after transfer to the medium and incubating plates at 25°C, while growth on CMA amended with 110 μ g of tetracycline/ml of medium was measured for a 168-h time period 24 h after transfer to the medium.

Fig. 7. The use of mitochondrial molecular markers to examine the mitochondrial DNA (mtDNA) background present in isolates. Amplification of the mtDNA markers when target sequences of the antheridial parent were present in different amounts. Amplifications for the oogonial (O) and antheridial (A) parent were run with 100 ng of total DNA, while the dilution series in the right lanes reflect amplifications when different nanogram amounts of purified mtDNA from the antheridial parent were added to 100 ng of total DNA of the oogonial parent. The left lane is bacteriophage lambda-digested with *Pst*I; molecular sizes are in kilobases.

from long-term storage cultures in the absence of the antibiotic (potato dextrose agar slants covered with mineral oil or sterile water vials containing hemp seeds) after 7 years (data not shown). Similar results were obtained with selection of isolates tolerant to another aminoglycoside antibiotic, kasugamycin (data not shown). Cross-tolerance of mutants to kanamycin, kasugamycin, and gentamicin was observed.

A tetracycline-tolerant mutant was selected by initially growing isolates on CMA amended with 5 µg of the antibiotic/ml; with each successive transfer, the concentration was increased by 5 to 10 µg/ml. A stable spontaneous mutant (isolate 1991-35T) was derived from the wild-type oogonial isolate 1987-14 after nine serial transfers on amended medium over a period of 7 months and was tolerant to greater than 100 µg of tetracycline/ml (Fig. 1B). Similar results were obtained for a tolerant isolate (1992-35T) generated from the antheridial parent 1987-73 (data not shown). As noted above with the kanamycin-tolerant isolates, the tetracycline-tolerant isolates retained tolerance following long-term storage. Due to their slower growth, stable tetracycline-tolerant mutants were more difficult to obtain than kanamycin-tolerant mutants. Chlortetracycline was initially evaluated, but tests were discontinued due to an inability to generate mutants tolerant of greater than 40 µg of antibiotic/ml. Cross-tolerance between classes of antibiotics was not observed for either tetracycline- or kanamycin-tolerant isolates.

Inheritance of antibiotic tolerance. To determine if antibiotic tolerance was present in the wild-type parental isolates and expressed following reassortment of alleles during meiosis, a random subsample of 40 oospore progeny isolates from the above-mentioned cross between wild-type parents was tested for sensitivity to antibiotics. Only one isolate had a 24-h growth rate (7.3 mm) significantly greater than that of the parental isolates (antheridial and oogonial parents, 4.3 and 3.5 mm, respectively) on media amended with 75 µg of kanamycin/ml ($P = 0.05$). The remaining progeny had growth rates either equal to or less than the parental isolates (Fig. 2). In total, 22.5% of the progeny had growth rates of 1 mm or less on the kanamycin-amended medium, while 52% did

not grow on the amended medium. No growth was observed for isolates growing on medium amended with 200 µg of kanamycin/ml (data not shown). When growth rates were evaluated on medium amended with 110 µg of tetracycline/ml, no growth was observed for the parental isolates or any of the progeny (data not shown).

In crosses between an oogonial parent tolerant of kanamycin (1988-45K) and sensitive antheridial isolates, all of the progeny examined were highly tolerant of the antibiotic (Fig. 3; Table 1, crosses 1 and 2). Several isolates also were found for which greater growth was observed when the antibiotic was present (Fig. 3, lower right corner). In contrast, when an antheridial kanamycin-tolerant parent was crossed with a wild-type oogonial isolate, many progeny examined exhibited low growth rates on media amended with 200 µg of antibiotic/ml (Fig. 4A; Table 1, cross 3). However, when the antibiotic concentration was reduced to 75 µg of kanamycin/ml, a number of progeny were able to grow (Fig. 4B, Table 1), albeit to a lesser extent than observed in progeny when the oogonial parent was tolerant of kanamycin. Similar results were observed when kanamycin-tolerant antheridial progeny were backcrossed with the wild-type oogonial parent (Table 1, crosses 4 and 5) and when another antheridial kanamycin-tolerant mutant (1988-44K) was crossed with 1987-14 (data not shown).

In crosses between an oogonial tetracycline-tolerant isolate (1991-35T) and the wild-type antheridial parent (1987-73), all of the progeny were tolerant of tetracycline if growth was evaluated for only the initial 48 h after transfer to amended medium (Fig. 5 and Table 1, cross 8). However, if growth rates were assessed an additional 120 h later, only 35 of the 42 progeny continued to grow. When an antheridial progeny isolate exhibiting the greatest tolerance to tetracycline (8-5T) was backcrossed to the wild-type oogonial parent (1987-14), there was an approximate 2.5:1 segregation for antibiotic tolerance (Table 1, cross 10, 60:24 tolerant/sensitive; Fig. 6).

Inheritance of mtDNA. The patterns of inheritance for the high level of kanamycin tolerance (growth at antibiotic concentrations of 200 µg/ml) suggests that it is cytoplasmically inherited;

Fig 8. The mitochondrial DNA types in the antheridial (A) and F₁ progeny exhibiting low levels of kanamycin tolerance (right numbered lanes) from a cross between a kanamycin-tolerant antheridial (1988-46K) and a wild-type oogonial parent (1987-14) (Table 1, cross 3). The left lane is bacteriophage lambda-digested with *Pst*I; molecular sizes are in kilobases.

the greatest level of tolerance in the progeny was observed when the oogonial parent was drug tolerant. However, low levels of antibiotic tolerance (low growth at concentrations of 75 µg/ml) were observed for some progeny from crosses between a wild-type oogonial and drug-tolerant antheridial parent. To determine if this was due to leaky paternal inheritance of mitochondria conferring drug tolerance, molecular markers diagnostic for each parental mtDNA type were amplified. With primers FM 18 and FM 19, the paternal mtDNA would have a 0.75-kb fragment amplified, whereas the maternal mtDNA type would yield a 2.0-kb fragment (Fig. 7). To determine the limits of detection of the different mtDNA markers, experimentation with purified mtDNA from the antheridial parent mixed with total DNA from the oogonial parent was done. The results indicated that the antheridial mtDNA marker could be amplified if as little as 0.1 pg of mtDNA from the antheridial parent was added to 100 ng of total DNA from the oogonial parent (Fig. 7), suggesting that, due to its smaller size, the amplification of the 0.75-kb fragment would be more efficient than the 2.0-kb fragment. Similar results were observed when the experiment was repeated using a dilution series of total DNA from the antheridial parent instead of purified mtDNA; the antheridial mtDNA marker was amplified when 0.1 ng of antheridial total DNA was present (data not shown).

Progeny isolates exhibiting the highest level of kanamycin tolerance from a cross between a wild-type maternal isolate (1987-14) and kanamycin-tolerant antheridial parent (1988-46K) (Table 1, cross 3; Fig. 4B) were analyzed for their mtDNA type. All progeny had an amplified fragment of 2.0 kb, which is the size expected for the maternal parent mtDNA type (1987-14) (Fig. 8).

DISCUSSION

Sublethal enrichment was an effective means for selection of stable drug-tolerant mutants of *P. sylvaticum*. The procedure is relatively rapid and straight forward, generates stable mutants that are either nuclear- or cytoplasmically encoded without causing any noticeable nonspecific mutations, and mutants that are tolerant of different antibiotics can be selected. While it was easier to obtain tolerance to the aminoglycoside antibiotics (shorter time for selection, tolerance to greater antibiotic concentrations than tetracycline, and growth rates on antibiotic-amended medium more similar to wild-type isolates), due to cross-tolerance among mutants tolerant of kanamycin and kasugamycin, different classes of antibiotics will need to be used when doubly marked isolates are required. Preliminary studies evaluating the competitive saprophytic ability of isolates selected in this study (1988-45K and 1992-35T) in autoclaved and pasteurized soil indicate that they have a ecological competence similar to their wild-type progenitor isolates (F. N. Martin and C. R. Semer IV, *unpublished data*).

The use of sublethal enrichment for selection of stable marked isolates also has been effectively used for selection of kanamycin tolerance in *P. aphanidermatum* (L. Coelho and F. N. Martin, *unpublished data*) and streptomycin tolerance in *P. oligandrum* (F. N. Martin, *unpublished data*). While the selection procedure was not as effective as observed for kanamycin, kasugamycin, or tetracycline, limited success also has been obtained for selection of tolerance to hymexazol and phosphorus acid (F. N. Martin and C. R. Semer IV, *unpublished data*). All attempts for selection of tolerance to metalaxyl in *P. sylvaticum* have failed. The inability to generate stable metalaxyl-tolerant mutants by sublethal enrichment also has been reported for *P. aphanidermatum*, *P. arrhenomanes*, *P. graminicola*, and *P. vexans* (8).

It was interesting to note that several progeny isolates grew better in the presence of kanamycin (Fig. 3) and, for several isolates, very little growth was observed in the absence of the antibiotic. A similar type of enhanced growth or antibiotic dependence also was observed for streptomycin-tolerant mutants in the genus *Phytophthora*. Mutants tolerant of this aminoglycoside antibiotic

were selected by screening zoospores of *Phytophthora cactorum* (55) or by sublethal enrichment of *Phytophthora parasitica*, *Phytophthora palmivora*, *Phytophthora capsici*, and *Phytophthora cinnamomi* (2). In both reports, isolates were recovered that required streptomycin for growth. With *Phytophthora cactorum*, the antibiotic dependence was stable in single-zoospore and selfed-oospore cultures; however, if dependent strains were cultured on medium free of streptomycin for 20 days, sectors of the fungus were isolated that were tolerant of the antibiotic, but not dependent on it for growth (55).

When using sublethal enrichment for selection of marked isolates, it is important to confirm that isolates growing on amended medium are actually mutants tolerant of the antibiotic and have not adapted physiologically. An example of the importance of this was encountered when trying to select for tolerance to the fungicide ethazol. Rapidly growing sectors tolerant of the fungicide were readily isolated, but this tolerance was not stable following subculture on nonamended medium (F. N. Martin, *unpublished data*). This presumably was due to a physiological adaptation in the presence of the fungicide. The mode of action of ethazol is disruption of electron transport between cytochrome b and c, and an alternative pathway (utilizing ubiquinone) can be activated when the fungicide is present in sublethal concentrations (26,27).

Cross-tolerance was observed between mutants tolerant of kanamycin, kasugamycin, and geneticin, which are aminoglycoside antibiotics. It is unknown if these mutants are tolerant to other aminoglycoside antibiotics such as neomycin, gentamycin, hygromycin B, or streptomycin as well, but it does present a need for screening before experimentation involving generation of doubly marked isolates is attempted. This cross-tolerance, and the ease with which antibiotic tolerance was generated, could prove to be troublesome for experimentation with DNA transformation. A number of DNA transformation vectors use the aminoglycoside antibiotics hygromycin B or geneticin as a selectable marker. If the screening procedure for selection of transformants does not use an antibiotic concentration high enough to completely inhibit nontransformed cells, it is possible that spontaneous mutants may be generated. The observation that mutants tolerant of one drug are cross-tolerant of other members in the same class of compounds has been observed in other oomycetes. Mutants of *Phytophthora* spp. tolerant of metalaxyl were cross-tolerant of other acylalanine fungicides (7-9,28), as were metalaxyl-tolerant mutants of *Pseudoperonospora cubensis* (29) and *P. oligandrum* (F. N. Martin, *unpublished data*).

There have been several reports on the use of nonspecific mutagens for selecting drug markers in the genus *Pythium*. Dennett and Stanghellini (19) used EMS treatment of oospores of *P. aphanidermatum* to select for tolerance of chloramphenicol. Bruin and Edgington (9) and Madsen et al. (39) used UV irradiation of young mycelium for selection of metalaxyl tolerance in *P. ultimum* and *P. oligandrum*, respectively, while Martin and Hancock (46) used UV irradiation of oospores for selection of metalaxyl tolerance in *P. oligandrum*. Although UV irradiation was effective in generating mutants of *P. oligandrum* that were mitotically and meiotically stable for fungicide tolerance, this treatment caused a number of additional mutations that altered the phenotype of selfed progeny. The generation of phenotypic variants was not observed in this investigation with *P. sylvaticum* when sublethal enrichment was used to select for mutant isolates.

Inheritance of drug tolerance. The inheritance of a high level of kanamycin tolerance in progeny isolates was correlated with the oogonial isolate having the drug tolerance, which is consistent with cytoplasmic inheritance of the trait. A low level of tolerance was observed in some progeny when the antheridial isolate was tolerant of kanamycin, and, due to the patterns of inheritance of mtDNA, appears to be nuclear-encoded. Some of this low level of growth on amended medium was similar to what was observed for progeny from crosses between the wild-type parental isolates (Fig. 2)

and, therefore, may not reflect inheritance of tolerance from the drug-tolerant paternal parent; rather it may reflect indigenous tolerance present in the parental isolates similar to what was observed with *P. splendens* (25). However, the level of tolerance for some progeny was much higher than was observed for wild-type isolates, indicating that some level of nuclear-encoded tolerance was present in the mutant antheridial isolate.

In contrast to the findings with kanamycin, the inheritance of tetracycline tolerance was not correlated with cytoplasmic inheritance, but was consistent with what would be expected with the tolerance being nuclear-encoded. The instability and aberrant segregation ratios of inheritance of tetracycline in crosses could have several explanations. In the cross between 1987-73 and 1991-35T (Table 1, cross 8), the sensitive progeny initially grew for 2 days before ceasing growth; tolerance in these isolates may have been unstable and the sensitive isolates indicative of reversions. Alternatively, it is possible that these sensitive isolates are aneuploid for the chromosome(s) encoding drug tolerance or have lost the specific region of the chromosome encoding tolerance. Significant variation in ploidy and levels of chromosomal recombination were observed in outcrossed progeny from the wild-type parental isolates used in this investigation (45). In total, over 80% of chromosomal bands in the progeny, as detected by Southern analysis of pulsed field gel blots, were nonparental in size or clustering of specific coding regions. This also was reflected in the aberrant segregation ratios for several randomly amplified polymorphic DNA markers observed in some progeny. An alternative explanation for the observed segregation ratios could be that, instead of a mutation at a single locus conferring tolerance, mutations at multiple unlinked loci were responsible for the levels of tolerance observed. Similar explanations may be plausible for the non-Mendelian inheritance of chloramphenicol tolerance in a selfed mutant of *P. aphani-dermatum*, which had a 1:5.8 segregation of sensitive and tolerant progeny (19). Aberrant segregation of markers following meiosis also has been observed in *Phytophthora infestans* (24,54,56), *Phytophthora parasitica* (11,21), and *Phytophthora drechsleri* (30).

From studies done with other oomycetes, inheritance of drug resistance has been reported to be nuclear or cytoplasmic, depending on the marker evaluated. Tolerance to *p*-fluorophenylalanine was due to a single dominant nuclear allele for *Phytophthora drechsleri* (30) and *Phytophthora sojae* (5), as was tolerance to chloroneb in *Phytophthora parasitica* (11). In contrast, tolerance to metalaxyl exhibited incomplete dominance in *Phytophthora sojae* (5) and *Phytophthora infestans* (54); however, Chang and Ko (11) concluded that metalaxyl tolerance was dominant in *Phytophthora parasitica*. Crute and Harrison (17) also concluded that metalaxyl tolerance in *Bremia lactucae* was governed by a single locus with incomplete dominance. However, when the locus for tolerance was heterozygous, modifier genes could contribute to expression of tolerance. While tolerance to chloramphenicol was reported as nuclear-encoded in *Phytophthora drechsleri* (30), it was cytoplasmically inherited in *Phytophthora parasitica* (11). Cytoplasmic tolerance to another aminoglycoside antibiotic, streptomycin, also has been reported in *Phytophthora parasitica* (11).

Mitochondrial inheritance. From analysis of crosses between opposite mating types of *P. sylvaticum*, it was observed that progeny isolates contained only the mtDNA type of the oogonial parent (40,45); thus, the mitochondria are uniparentally inherited and contributed by the maternal parent only. The segregation of drug tolerance in progeny of crosses between wild-type isolates and kanamycin-tolerant mutants in this study supports this conclusion, as does the molecular data confirming the type of mtDNA present in the progeny. The data also suggest that stable leaky paternal inheritance of mitochondria did not occur. If it had, then more of the progeny from crosses between a tolerant antheridial parent and wild-type oogonial parent should have exhibited higher levels of kanamycin tolerance than were observed (assuming that growth on antibiotic-amended medium would selectively enrich the pro-

portion of mitochondria conferring drug tolerance). The molecular data also argue against leaky paternal inheritance; in all progeny examined from this cross, including those that exhibited low levels of drug tolerance, only the maternal mtDNA type (kanamycin sensitive) was observed. Since amplification of the mtDNA marker for the antheridial parent was more efficient than for the maternal parent, (the paternal marker would be amplified when antheridial mtDNA concentration was at least 1,000 times less than the oogonial mtDNA), low levels of leaky paternal mtDNA should have been detected if it was present.

In view of the above patterns for mitochondrial inheritance, the nature of sexual reproduction for *P. sylvaticum* is different than that observed for the genus *Phytophthora*. Opposite mating types in *Phytophthora* are bisexual, meaning that either parent can function as the maternal or the paternal parent. This bisexual nature of mating behavior is exemplified by analysis of crosses of *Phytophthora parasitica*. Forster and Coffey (21) observed that, while only a single type of mtDNA was present in an individual progeny isolate, both parental types of mtDNA could be found in progeny isolates, thereby indicating that each parental isolate behaved as a maternal parent. Similar results were observed with this species by Chang and Ko (11) when following the inheritance of two nuclear-encoded drug markers (metalaxyl and chloroneb) and two cytoplasmic markers (streptomycin and chloramphenicol). Hybrid progeny from outcrosses between the parental isolates contained either streptomycin or chloramphenicol tolerance inherited from their maternal parent.

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