Resistance of Selected Saprobiic and Zoopathogenic Fungi to Cycloheximide

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Spore germination was used as an assay to measure the sensitivities of selected fungi (*Achlya bisexualis*, *Cladosporium* sp., *Trichophyton mentagrophytes* and *Microsporum gypseum*) to cycloheximide and to determine their abilities to adapt to the drug. Two patterns of response were noted. The saprobes, *A. bisexualis* and *Cladosporium* sp., demonstrated acquired resistance. Spores from hyphae previously exposed to cycloheximide either germinated in the presence of concentrations of the drug that completely inhibited spores from unexposed hyphae (*Achlya*), or germinated with a shorter lag and to a greater extent in the presence of the antibiotic than did spores from unexposed hyphae (*Cladosporium*). Hyphae of *Achlya* adapted at concentrations of cycloheximide in which spores did not germinate and hyphae of *Cladosporium* adapted more rapidly than spores. *Achlya* adapted to only 12 μ M-cycloheximide whereas *Cladosporium* acquired resistance to 18 mM-cycloheximide. These fungi lost this acquired resistance after a single transfer to media lacking cycloheximide. The zoopathogens, *T. mentagrophytes* and *M. gypseum*, had a contrasting response, indicating constitutive resistance. Conidia from unexposed hyphae showed 90 to 100% germination on media containing up to 18 mM-cycloheximide; prior exposure to the drug did not affect their response.

INTRODUCTION

Cycloheximide, an antibiotic produced by several strains of *Streptomyces griseus* (Krain-sky) Waksman and Henrici (Ford & Leach, 1948), inhibits the development of many eukaryotic organisms. Whiffen (1948, 1950) noted considerable variation in the sensitivity of fungi to the drug, but zoopathogens consistently grew at higher concentrations of antibiotic than saprobes and phytopathogens. This response led to the widespread use of cycloheximide in selective isolation and diagnostic media (Georg, Ajello & Gordon, 1951; Taplin, Zaia & Blank, 1969; Tsao, 1970). However, characterization of a fungus as either sensitive or resistant may be misleading, for response to cycloheximide depends upon a number of factors, particularly antibiotic concentration, duration of exposure and ability of the strain to acquire resistance (McDonough *et al.*, 1960; Grover & Moore, 1961; Gundersen & Wadstein, 1962; Salkin & Hurd, 1972; Salkin, 1975).

Methods of assessing sensitivities of fungi to cycloheximide have varied from simple observations of growth/no growth (Whiffen, 1948, 1950) and visual estimations of develop-

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Table 1. *Sources of cultures studied*

Organism	Perfect stage	Strain(s)	Source*
Saprobies			
<i>Achlya bisexualis</i>	<i>Achlya bisexualis</i>	65-1	D. H. Griffin
<i>Cladosporium</i> sp.	—	242B	Laboratories for Mycology
Zoopathogens			
<i>Trichophyton mentagrophytes</i>	<i>Arthroderma benhamiae</i>	354, 362B, 360A	Laboratories for Mycology
<i>Microsporium gypseum</i>	<i>Nannizzia gypsea</i>	270B, 270F, 271L	Laboratories for Mycology
	<i>Nannizzia incurvata</i>	271, 271Z, 271J	Laboratories for Mycology
	Not determined	R87 (ATCC 28249)	J. J. Stock

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ment in six graded categories (McDonough *et al.*, 1960) to measurements of the radial extension of colonies (Salkin & Hurd, 1972).

We have determined percentage spore germination to compare the abilities of selected saprobic and zoopathogenic fungi to adapt to cycloheximide.

METHODS

Stock cultures. Sources of the cultures studied are given in Table 1. Stock cultures of *Achlya bisexualis* were maintained on slants of glutamate defined medium (Griffin, Timberlake & Cheney, 1974) under paraffin oil; those of the other fungi were maintained on half-strength Sabouraud dextrose agar (Difco) supplemented with Difco Bacto-agar to 2% (w/v). All stock cultures were incubated at 10 °C.

Preparation of spore suspensions. Encysted zoospores of *A. bisexualis* were obtained by a modification of the technique of Griffin & Breuker (1969), in which 40 ml glucose/casein hydrolysate broth medium (CAA; Griffin *et al.*, 1974) was inoculated with 10^5 spores and incubated at 28 °C on a New Brunswick rotary shaker operating at 100 rev. min⁻¹ for 24 h. The resulting mycelium was harvested on a sterile glass Millipore filter holder and washed with sporulation medium (0.25 mM-KCl and 0.25 mM-CaCl₂ in glass-distilled water) using gentle suction. It was then suspended in 500 ml sporulation medium in a 1 litre Florence flask and incubated overnight at 28 °C on a reciprocal shaker with a 5 cm throw at 60 oscillations min⁻¹. The suspension was then gravity filtered through a sterile Kimwipe (Kimberly-Clark) to separate the spores from the parent mycelium.

Cladosporium conidia, *Trichophyton* microconidia and *Microsporium* macroconidia were obtained by scraping the surface of 7-d-old cultures on Difco Cantino PYG agar with a wire loop and suspending the adherent fungus in a small volume of sterile distilled water. It was not necessary to separate the selected spore type from the other fungal elements.

Spore concentrations were determined with a Neubauer-Levy Ultra Plane haemocytometer (C. A. Hausser, Philadelphia, Pennsylvania, U.S.A.), and the suspension volumes for each strain of fungus were adjusted to give the following spore inocula: *Achlya bisexualis*, 3.5×10^4 encysted spores; *Cladosporium* sp., 1×10^5 conidia; *Microsporium gypseum*, 7×10^8 macroconidia; *Trichophyton mentagrophytes*, 4×10^8 microconidia. These inocula, in 50 to 100 µl water, were streaked evenly over the surface of Difco Cantino PYG agar and incubated at 28 °C (all four species) and 37 °C (*Microsporium* and *Trichophyton*).

Effect of cycloheximide on germination. Cycloheximide (Upjohn Co.) was sterilized by Millipore filtration (0.45 µm porosity) and added aseptically to cooled molten agar before pouring. The concentrations tested were in 10-fold increments from 0.012 to 12 µM with *Achlya*, 1.8 µM to 18 mM with *Cladosporium* and 1.8 and 18 mM with *Microsporium* and *Trichophyton*.

The standard error of the mean (S.E.M.) for the germination of *Achlya* spores (Table 2) was determined by counting 10 samples of 20 spores in fields selected at random using a compound microscope at 100× magnification. For the experiments showing the time course of germination, 100 spores were counted for each data point in fields selected at random using a compound microscope at 100× magnification for *Achlya* and *Microsporium* and at 240× magnification for *Cladosporium* and *Trichophyton*.

Measurement of acquired resistance to cycloheximide. Spores or hyphae formed on mycelium grown in the absence of cycloheximide are described as 'unexposed', while those spores and inocula produced from mycelium grown in media containing cycloheximide are described as 'exposed'. Exposed *Achlya* spores

were produced with cycloheximide incorporated into both the medium for growth of mycelium and the sporulation medium at the concentrations given in Table 2. Concentrations of cycloheximide incorporated into PYG media to produce exposed *Cladosporium* conidia were 180 μM , 1.8 mM and 18 mM. Only the data for conidia exposed to 180 μM -cycloheximide are shown as the others gave substantially identical results.

The experiment presented in Fig. 1(b) was designed to ascertain how long it took for *Achlya* mycelium to acquire resistance to 12 μM -cycloheximide. CAA medium (50 ml) inoculated with 3000 spores ml^{-1} was dispensed aseptically into sterile 250 ml Erlenmeyer flasks and incubated on a New Brunswick rotary shaker (120 rev. min^{-1}) at 28 °C. Cycloheximide (12 μM) was added to some of the flasks 14 h after inoculation, the remainder served as untreated controls. The cultures from duplicates of control and treated flasks were harvested at the times indicated and dry weights were determined as described by Griffin *et al.* (1974).

The experiment presented in Fig. 1(c) was designed to ascertain when, in the course of germination of *Achlya* spores, resistance to 12 μM -cycloheximide was acquired. Cultures in CAA medium were prepared and incubated as described above and 12 μM -cycloheximide was added to triplicate flasks at the times indicated in Fig. 1(c). Incubation was then continued until 72 h when the cultures were harvested and their dry weights determined.

Effect of cycloheximide on growth rates. *Cladosporium* 242b mycelial macerate was prepared by first growing the organism on plates of modified Sabouraud dextrose agar [MSDA; 2% (w/v) dextrose, 1% (w/v) Difco Neopeptone and 2% (w/v) agar] for 14 d at 27 °C. Four mm diameter plugs were then removed from the periphery of the culture with a stainless-steel cork borer, transferred to 50 ml modified Sabouraud dextrose (MSD) broth in 125 ml Erlenmeyer flasks, and incubated at 27 °C in a Labline Environ shaker at 170 rev. min^{-1} for 14 d. The mycelium/agar suspensions from two flasks were then aseptically suction-filtered through a specially prepared 5 cm square stainless-steel wire basket held within a porcelain Buchner funnel. The suspension was washed twice with sterile saline, resuspended in 80 ml MSD broth in a 1 litre monomel Waring blender cup, and homogenized for 25 s at high speed. The resulting brei was inoculated at 1 ml per 50 ml MSD in 125 ml flasks and incubated as described above for 7 d. This process, with incubation for 7 d, was repeated twice to dilute out the remaining agar. Inocula for the growth experiments, prepared by this washing-blending procedure from 7-d-old macerate cultures descended from this initial process, amounted to 7 to 14 mg dry wt per flask. Dry weights were determined as described by Griffin *et al.* (1974).

The growth curves for *Achlya* were determined as previously described (Griffin *et al.*, 1974); cultures were incubated on a New Brunswick rotary shaker (120 rev. min^{-1} at 28 °C) using 50 ml CAA per 250 ml Erlenmeyer flask. Cultures from triplicate flasks were harvested at 45 to 90 min intervals and five mean data points were obtained during exponential-phase growth for each curve. To determine the growth curves for *Cladosporium*, cultures were incubated in a Labline Environ rotary shaker (170 rev. min^{-1} at 27 °C) using 50 ml MSD broth per 125 ml Erlenmeyer flask. Duplicate cultures were harvested at 12 h intervals and five mean data points were obtained during exponential-phase growth for each curve.

The specific growth rates (α) were determined by the least squares method and the statistical analysis of these data was according to Miller & Freund (1965). The equation for α used in these calculations was:

$$\ln (M_2/M_1) = \alpha(t_2 - t_1) \quad (1)$$

where M_1 , M_2 are the dry weights at times t_1 , t_2 . Lag phases (see Results) were determined by calculating the time on the log growth lines described by equation (1) when the dry weight M_1 equalled the dry weight of the inoculum as measured at the time of inoculation.

RESULTS

Achlya bisexualis

Cycloheximide at 0.012 and 0.12 μM had no effect on germination whereas 1.2 μM -cycloheximide delayed the germination of unexposed spores of *Achlya* and 12 μM -cycloheximide caused total inhibition (Fig. 1a). Germination of unexposed spores on 12 μM -cycloheximide did not occur even after 21 d incubation. In contrast, unexposed hyphae grew, after a 45 h delay, in the presence of 12 μM -cycloheximide added 14 h after inoculation (Fig. 1b). The specific growth rates (α) of hyphae were 0.40 h^{-1} (s.e.m. = 0.058) in the presence and 0.59 h^{-1} (s.e.m. = 0.053) in the absence of the drug. The shift from sensitive spores to adaptable hyphae correlated with the appearance of germ tubes, which occurred 5 h after inoculation. Little growth was obtained when cycloheximide was added to the

Fig. 1. Response of *Achlya bisexualis* to cycloheximide. (a) Percentage germination of unexposed spores in the presence of cycloheximide at (μ M): ∇ , 0.012; $\square$, 0.12; $\blacksquare$, 1.2; $\blacktriangle$, 12; $\circ$, control (no cycloheximide added). (b) Development of unexposed hyphae in the presence of 12 μ M-cycloheximide added 14 h after inoculation ($\blacktriangle$). Note the change in time scale after 20 h. $\circ$, Control (no cycloheximide added). (c) Growth of unexposed spores in the presence of 12 μ M-cycloheximide added at different times after inoculation. (d) Percentage germination of exposed spores in the presence of 12 μ M-cycloheximide ($\blacktriangle$) or in its absence ($\circ$), and of unexposed spores in the absence of cycloheximide ($\bullet$). Unexposed spores did not germinate in the presence of 12 μ M-cycloheximide even after 21 d incubation.

germination medium up to 4.5 h after inoculation; maximum growth was obtained when the drug was added 5.75 h after inoculation and later (Fig. 1c).

No sporulation was observed when unexposed hyphae were transferred to sporulation medium containing 12 μ M-cycloheximide. However, normal sporulation occurred when hyphae grown in the presence of 12 μ M-cycloheximide were transferred to sporulation medium also containing 12 μ M-cycloheximide. Spores derived from the latter germinated, after an 8 h delay, in medium containing 12 μ M-cycloheximide (Fig. 1d) but spores from unexposed hyphae did not germinate even after 21 d incubation on this medium.

Germination of spores exposed to 0.012 to 1.2 μ M-cycloheximide was greater than that of

Table 2. Germination of *Achlya* spores exposed to cycloheximide

Germination was scored 6.5 h after inoculation on PYG medium.
Results are expressed as percentage germination $\pm$ S.E.M.

Cycloheximide in growth and sporulation media (μ M)	Cycloheximide in germination medium (μ M)				
	0	0.012	0.12	1.2	12*
0	97.5 ($\pm$ 3.5)	98.5 ($\pm$ 2.4)	98.5 ($\pm$ 2.4)	4.0 ($\pm$ 2.1)	0.0 ($\pm$ 0.0)
0.012	99.0 ($\pm$ 2.1)	98.5 ($\pm$ 2.4)	98.5 ($\pm$ 2.4)	45.0 ($\pm$ 13.3)	0.0 ($\pm$ 0.0)
0.12	99.5 ($\pm$ 1.6)	98.0 ($\pm$ 2.6)	99.5 ($\pm$ 1.6)	91.0 ($\pm$ 7.4)	0.0 ($\pm$ 0.0)
1.2	96.5 ($\pm$ 4.1)	98.0 ($\pm$ 2.6)	95.0 ($\pm$ 6.2)	91.0 ($\pm$ 7.4)	89.5 ($\pm$ 7.8)

* Germination scored 20 h after inoculation on PYG medium.

Fig. 2. Response of *Cladosporium* sp. to cycloheximide. (a) Percentage germination of unexposed conidia in the presence of cycloheximide at: Δ , 1.8 μ M; $\blacktriangle$, 18 μ M; ∇ , 180 μ M; $\square$, 1.8 mM; $\blacksquare$, 18 mM; $\circ$, control (no cycloheximide added). (b) Percentage germination of conidia exposed to 180 μ M-cycloheximide on germination medium containing cycloheximide at: $\square$, 1.8 mM; $\blacksquare$, 18 mM; $\circ$, control (no cycloheximide added).

unexposed spores in medium containing 1.2 μ M-cycloheximide when scored 6.5 h after inoculation (Table 2). Resistance to 12 μ M-cycloheximide was acquired only after exposure to 1.2 or 12 μ M-cycloheximide. However, this acquired resistance was lost when hyphae exposed to cycloheximide were sporulated in the absence of the drug. Mycelium grown in 12 μ M-cycloheximide was completely inhibited at 120 μ M.

Cladosporium sp.

When *Cladosporium* conidia from hyphae unexposed to cycloheximide were germinated on media containing up to 180 μ M-cycloheximide, germination was inhibited by only 10%, but there was a marked progressive increase in the lag from 8 to 44 h as measured by the time taken for 50% germination. Higher concentrations of cycloheximide caused progressively greater inhibition of total germination as well as further increases in the lag (up to 70 h) (Fig. 2a). However, conidia derived from hyphae exposed to 180 μ M-cycloheximide germinated completely after only a slight lag on media containing up to 18 mM-cycloheximide (Fig. 2b). This resistance was lost after a single transfer in the absence of cycloheximide.

Cycloheximide caused a small decrease in the growth rate of hyphae. The specific growth rates (α) of unexposed hyphae were 0.0621 h⁻¹ (S.E.M. = 0.0024) and 0.0471 h⁻¹ (S.E.M. =

Fig. 3. Response of *Microsporum gypseum* r87 to cycloheximide at 37 °C (a, b, c) and 28 °C (d, e, f). Percentage germination in the presence of 1.8 mM (b, e) or 18 mM (c, f) cycloheximide in the germination medium, or in its absence (a, d). Cycloheximide concentrations in the sporulation media were: ○, 0; □, 1.8 mM; ■, 18 mM.

0.0021) in the absence and presence of 18 mM-cycloheximide respectively, while exposed hyphae gave corresponding values of 0.0508 h^{-1} (S.E.M. = 0.0038) and 0.0449 h^{-1} (S.E.M. = 0.0024). However, cycloheximide did cause a significantly longer lag with unexposed hyphal inocula. Lag phases (calculated as the time on the log dry weight growth line when the dry weight of the cultures equalled that of the inoculum) for unexposed hyphae were 11 and 32 h in the absence and presence of cycloheximide respectively, and those for exposed hyphae were 6 and 11 h respectively. These latter values are probably not significantly different from each other.

Microsporum gypseum

Macroconidia of *M. gypseum* derived from hyphae unexposed to cycloheximide were completely resistant, at both 28 and 37 °C, to antibiotic concentrations up to and including 1.8 mM (Fig. 3). Data are shown for strain r87 only; the other six strains produced similar results. Unlike *Cladosporium*, prior exposure of the macroconidia to the antibiotic did not significantly influence spore germination. When incubated at 28 °C on medium containing 18 mM-cycloheximide, exposed and unexposed spores behaved similarly, but at 37 °C germination of spores of strain r87 exposed to 18 mM-cycloheximide was slightly delayed. Those exposed to 1.8 mM or unexposed showed a greater delay and decrease in the percentage germination. This pattern was not observed with the other strains used.

Fig. 4. Response of *Trichophyton mentagrophytes* 360A microconidia to cycloheximide at 37 °C (a, b) and at 28 °C (c, d). Percentage germination in the presence of 18 mM-cycloheximide in the germination medium (b, d) or in its absence (a, c). Cycloheximide concentrations in the sporulation media were: ○, 0; ■, 18 mM.

Trichophyton mentagrophytes

Microconidia of *T. mentagrophytes* from hyphae unexposed to cycloheximide were completely resistant, at both 28 and 37 °C, to concentrations up to 18 mM (Fig. 4). Results obtained with strain 360A are shown; other strains behaved similarly. Prior exposure of microconidia to 18 mM-cycloheximide did not alter these responses.

DISCUSSION

Two patterns of resistance to cycloheximide are apparent from our data. The first requires prior exposure to the antibiotic, suggesting an acquired resistance or adaptation, and is exhibited by the saprobes *Achlya* and *Cladosporium*. *Achlya bisexualis* showed acquired resistance by hyphal growth after a lag period in 12 μ M-cycloheximide, by sporulation and germination of the resulting spores at 12 μ M-cycloheximide and by the more rapid spore germination at 1.2 μ M-cycloheximide after exposure to cycloheximide. Similarly, the complete resistance of exposed conidia of *Cladosporium* sp. and the decreased lag in the development of the hyphal fragments after exposure suggest an adaptation to cycloheximide. The mycelia of both genera acquire resistance more rapidly than spores. Only the mycelia of *Achlya* were able to acquire resistance in 12 μ M-cycloheximide, although this resistance could be transferred to the spores produced. The unstable nature of the acquired resistance of spores or mycelia suggests that it was a transitory physiological phenomenon rather than a genetic process involving the induction or selection of mutants by exposure to cycloheximide.

The second pattern of resistance to cycloheximide, which appears to be constitutive rather than acquired, is demonstrated by the zoopathogens *M. gypseum* and *T. mentagrophytes*. The conidia and hyphae of all strains of both organisms exhibited an initial high resistance to cycloheximide which was unaltered by prior exposure to the drug.

The results obtained with *M. gypseum* strains, in particular R87, differ from those reported previously. Leighton & Stock (1969) found that incubation at 37 °C was required for maximum germination at 24 h, with only poor germination at temperatures below 30 °C, but we found maximum germination at both 28 and 37 °C within 24 h. Dill, Leighton & Stock (1972) reported 100% inhibition of germination at 180 μ M-cycloheximide (50 μ g ml⁻¹) while we found the antibiotic had no measurable effect upon germination at concentrations

as high as 1.8 mm (500 $\mu\text{g ml}^{-1}$). We have no explanation for these differences. We have repeated our tests using their methods and obtained results identical to those presented here.

These studies show that scoring spore germination is an extremely useful system for assessing the responses of fungi to cycloheximide. It is more sensitive than measurement of hyphal growth as spores may be more susceptible. It is quicker than the colony diameter measurements utilized by Salkin (1975), and adaptation to cycloheximide is more readily detected than with longer term measurements such as maximum dry weights. While growth-rate measurements by dry weight are accurate, they are also far more laborious and time-consuming than spore germination measurements.

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