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Abstract—We have isolated several mutants of *Schizophyllum commune* Fr. able to grow on media containing cycloheximide in concentrations up to 30 mg/L. Genetic analyses of the resistant phenotypes show them to be due to the action of a single gene located on the first linkage group between the A-mating type factor and a gene for an adenine requirement (*ade-11*). We have analyzed the growth and development of dikaryons made homoallelic for either the resistant or sensitive alleles as well as the heteroallelic dikaryon. These dikaryons showed different abilities to fruit when placed on media containing various concentrations of cycloheximide. Homoallelic sensitive strains failed to grow on any concentration of cycloheximide tested (>1 mg/L). Homoallelic resistant strains grew and fruited on all concentrations of cycloheximide up to 30 mg/L. Heteroallelic dikaryons showed a response that was dose-dependent for fruiting, but not growth. As the concentration of cycloheximide was increased, these heteroallelic colonies showed a progressive loss of ability to form fruiting bodies and at concentrations greater than 30 mg/L, they gave rise to homokaryons as outgrowths from the colony edge. These homokaryons were all of the nuclear genotype associated with the original resistant component of the dikaryon.

Introduction

The study of the development of basidiomycetes has been focused quite naturally on the aspects of these organisms which are unique to them, especially the establishment of dikaryosis after a mating between compatible strains and the development of a multi-cellular structure, the basidiocarp, from a colony of almost independent cells, the mycelium. Genetic analysis of these phenomena has concentrated on dikaryosis (1) since the production of basidiocarps is necessary for genetic analysis and phenotypes that lack basidiocarps or have altered structures are thus not available for genetic analysis. Physiological analysis has concentrated on the larger, more amenable basidiocarp as an experimentally accessible structure (2). We have chosen drug resistance as a possible means of studying both the genetics and physiology of basidiocarp formation.

Strains of *Schizophyllum commune* Fr. resistant to the antibiotic cycloheximide have been described elsewhere (3,4,5). Although an exact mechanism of action for cycloheximide in *Schizophyllum* has not been described, several sources attribute its primary action to a blockage of protein synthesis (5). Other mechanisms have been proposed for the anti-fungal action of cycloheximide, including inhibition of DNA synthesis, RNA synthesis, respiration, ion absorption and amino acid uptake (6). Work with a related basidiomycete, *Coprinus cinereus,* has demonstrated that at least two genes that can confer resistance to cycloheximide exist in that organism. These genes have alleles which confer resistance as either a dominant trait (*cy-1*) or a recessive trait (*cy-2*)(7). Resistant alleles of *cy-1* are common in nature.

Materials and Methods

Culture media. A complete yeast medium (CYM) was prepared according to the method of Snider and Raper (8) and used for growth and maintenance of cultures. Experimental media were CYM supplemented with cycloheximide (Sigma Chemical Co.) in various concentrations ranging from 0 mg/L (control) to 30 mg/L final media concentration. Plates to be used for transfer experiments and growth rate determinations were overlaid with cellophane membranes (DuPont) which had been sterilized separately and allowed to equilibrate with the medium for 24 hours before inoculation. Nutritional requirements were determined on minimal medium (MIN) (9). MIN was supplemented with the appropriate nutrient for use in mapping crosses.

Strains and nomenclature. Strains used were derived from dikaryotic stocks resulting from a mating of strains 699 and 701 of J.R. Raper. Mutant alleles used were: *ade-11,* which confers an adenine requirement; chx^r/chx^s which confer cycloheximide resistance/sensitivity, respectively. A-mating type factor and B-mating type factor alleles (which determine mating activity) were A41/A43 and B41/B43, respectively. Strains used were: H12- chx^s, A41/B41; H13- chx^s, A43/B43; C6C- chx^r, *ade-11,* A41/B42; H11- chx^r, A43/B43; and Hl4- chx^r.

Dikaryons were made by mating the following: H11 x H12, H11 x H14, H12 x H13, and H13 x H14. These were used in dominance testing and to examine the breakdown of dikaryosis. Phenotypes were scored for growth/no growth on the appropriate media. Dedikaryotization was accomplished by transfer of dikaryons grown atop cellophane

membranes on CYM to medium containing cycloheximide. The resultant homokaryons were isolated as sectors growing out from the edge of the colony.

Growth conditions. Growth vs. non-growth was determined after seven days at 20°C. Morphology and fruiting competence were also scored at this time. Growth rates were determined by the method of Anderson and Deppe (9), modified by growing the colonies at 30°C and determining the rate of growth on medium containing 10 mg/L cycloheximide in addition to CYM.

Microscopy. Observations were made on material which had been grown on cellophane membranes (DuPont P120, uncoated) and washed free of the membrane onto a slide for staining and examination. Staining was with lactophenol cotton blue (10) for 1 minute, followed by permanent mounting for later observation. Septa were scored as "normal" when the clamp connections were complete, with fused hook cells and complete septa between cells, and as "abnormal" if the hook cells were unfused or if there were no hook cell or clamp connection present (simple septa) in accordance with the observations of Parag (11,12).

Results

A number of strains in our possession showed an ability to grow on media containing cycloheximide. From these we chose H11 and H14 as they were sibling strains isolated from a tenth generation backcross of strains 699x701 of J.R. Raper. For comparison we selected the sensitive strains H12 and H13 which were also progeny of the same backcross. The sensitive strains are unable to grow on media containing 2 mg/L cycloheximide. Crosses of resistant x sensitive strains yielded progeny that segregated 1:1 for resistance:sensitivity. Early observations showed that resistance was loosely linked to the A-factor mating type locus. A mapping cross confirmed this and placed the locus of resistance on linkage group I (13) 15.6 cM from A and 17.2 cM from *ade-11* and between *ade-11* and A (Table 1).

Table 1. Linkage data for *chx*^{r}.

Progeny type	Genotype of progeny	Observed no.
Parental	*ade-11*$^-$, *chx*^{r}, A41	38
	ade-11$^+$, *chx*^{s}, A42	49
Recombinant	*ade-11*$^-$, *chx*^{s}, A42	15
	ade-11$^+$, *chx*^{r}, A41	6
	ade-11$^-$, *chx*^{r}, A42	2
	ade-11$^+$, *chx*^{s}, A41	17
	ade-11$^-$, *chx*^{s}, A41	0
	ade-11$^+$, *chx*^{r}, A42	1

Inferred gene order and distances: ade-11-17.2cM-chx-15.6cM-A
Coefficient of coincidence =0.291

Dominance of cycloheximide resistance was determined for the heteroallelic dikaryon grown on media with concentrations of cycloheximide up to 30 mg/L. When scored for growth alone, the resistant allele appeared to be completely dominant since growth comparable to the homoallelic resistant dikaryon occurred. This growth was also comparable in extent and rate to the resistant homokaryon. Doubling times based on wet weight of membrane grown colonies at 30°C show no significant difference (Student's *t* test) among resistant dikaryons and heteroallelic dikaryons grown on cycloheximide medium or among any of the strains grown on CYM (Table 2). Growth rates for controls grown on CYM and colonies grown on 10 mg/L cycloheximide as representative of cycloheximide-grown colonies are given. (Data for growth rates at other concentrations not shown.) Growth determinations at very high concentrations of cycloheximide (>30 mg/L) were not done due to the breakdown of the dikaryon. Neither the sensitive homokaryons nor the homoallelic sensitive dikaryon grew on any of the media containing cycloheximide at any of the concentrations used.

The determination of dominance in the ability of the heteroallelic dikaryon to produce fruiting bodies is less certain. As can be seen in Figure 1, the heteroallelic dikaryon

Table 2. Doubling times (± std. dev.) at 30°C, log-phase growth.

Strain	CYM	CYM + 10 mg/L cycloheximide
Homokaryons:		
H11 (*chx*^{r})	3.8 ± 0.5 hr.	4.1 ± 0.9 hr.
H12 (*chx*^{s})	4.5 ± 0.7 hr.	NO GROWTH
H13 (*chx*^{s})	4.4 ± 0.2 hr.	NO GROWTH
H14 (*chx*^{r})	4.1 ± 0.9 hr.	4.8 ± 0.3 hr.
Dikaryons:		
H11 x H14 (*chx*^{r}/*chx*^{r})	4.3 ± 0.5 hr.	4.4 ± 2.0
H14 x H13 (*chx*^{r}/*chx*^{s})	3.8 ± 0.5 hr.	5.1 ± 1.3
H11 x H12 (*chx*^{r}/*chx*^{s})	4.3 ± 0.0 hr.	4.6 ± 0.9
H12 x H13 (*chx*^{s}/*chx*^{s})	3.7 ± 0.4 hr.	NO GROWTH

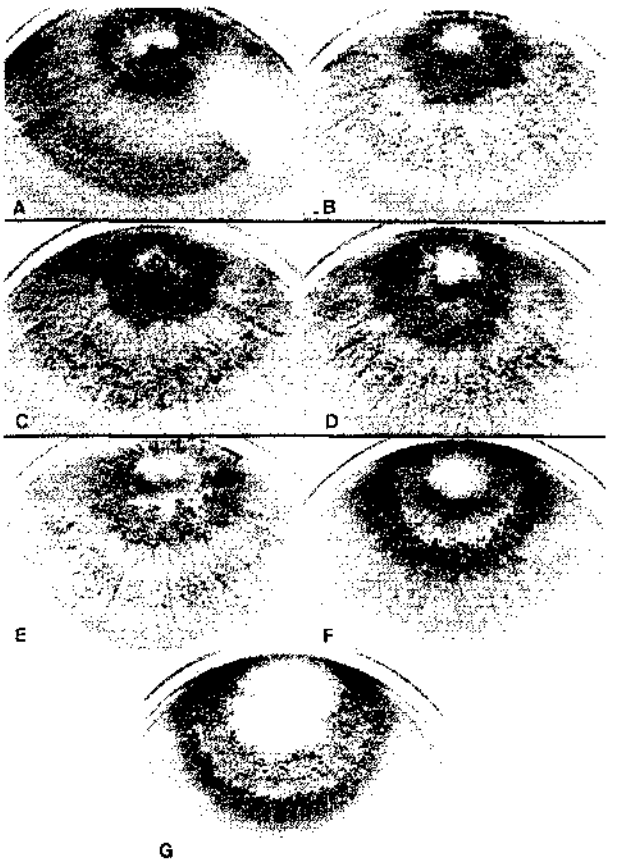

Figure 1. Ten day cultures of H13 x H14 *chx*^{r}/*chx*^{s} heterozygous dikaryons grown on CYM supplemented with: A) 0 mg/L cycloheximide, B) 2 mg/L, C) 4 mg/L, D) 7 mg/L, E) 10 mg/L, F) 15 mg/L, and G) 20 mg/L.

produced fruiting bodies that were altered in their morphology when grown on medium containing cycloheximide, increasingly so as the concentration of cycloheximide was increased. There was also a noticeable trend toward later development of the fruiting body primordia as the concentration was increased. This can be determined from the figures by noting the distance from the site of the inoculum that fruiting bodies and/or primordia appear. Such fruiting bodies as do appear have few gill structures, which appear only at lower concentrations. These fruiting bodies are stunted and very poorly developed, especially at the higher concentrations. At 30 mg/L no structure that can be called a fruiting structure appeared at all.

At concentrations of cycloheximide greater than 30 mg/L, the heteroallelic dikaryon undergoes a change we do not fully understand. The mycelial growth appears to be fully inhibited for a time which can vary from experiment to experiment before the colony shows outgrowths of vigorous sectors from the colony edge. Upon subculturing these sectors are found to be homokaryotic, both morphologically and genetically. Furthermore, the alleles carried by the homokaryotic sectors are the alleles of the resistant nuclear component of the original heteroallelic dikaryon even where those alleles are not linked to the resistant locus (as with the unlinked B-mating type factor).

The micro-structure of the mycelia at high concentrations of cycloheximide also shows changes that vary with the genetic constitution of the colony and with time. At 15 mg/L cycloheximide, heteroallelic dikaryons display abnormal septa in a proportion rising from 10 ± 2 percent at the time of transfer from CYM to 50 ± 4 percent after 24 hours of incubation. Neither of the two homoallelic dikaryons shows any increase in abnormal septa even after prolonged incubation although the homoallelic sensitive strains cease any active growth upon transfer. At 30 mg/L all dikaryotic mycelia regardless of genetic constitution show an increase in abnormal septa and a great variability in the percentages and timing of the increases.

Discussion

The resistant phenotype is attributable to an allele at a single locus which we designate *chx*. It is not clear whether resistance or sensitivity to cycloheximide is the mutant trait. A large number of the "wild type" strains that we have examined are resistant to cycloheximide and several have resistant alleles of the *chx* gene (data not shown). In this sense we find the present case analogous to the *cy-1* locus of *Coprinus cinereus*, which also has a large number of resistant alleles in the general population and similar behavior with respect to dominance interactions when scored for growth of the mycelium (7). The data from growth studies support an interpretation of the resistant allele as fully dominant with respect to growth. Caution should be exercised in the interpretation of any growth curve generated with membrane-grown cultures, however. The colonies enter logarithmic growth phase at times ranging from 24 to 72 hours after inoculation and do not remain in log growth for more than two or three doublings. The weight of the colonies when they begin log growth is about 1 mg, at which level the amount of error in individual weighings can be significant due to variance in the speed with which colonies are weighed and the rate of water loss from the membrane. Nonetheless, the data are reproducible, which gives us some confidence in the hypothesis of complete dominance for growth. A thorough discussion of the difficulties of the wet weight method of growth determination is presented elsewhere (9). Most importantly, it should be noted that the number of cells in a colony is unknown and not determinable due to the filamentous growth habit of the fungus. Fruiting competence of heteroallelic dikaryons grown on media containing cycloheximide is restricted at concentrations which do not inhibit growth. Thus, for the fruiting phenotype, the resistant allele is not dominant to the sensitive allele.

We find that the inheritance of resistance seen in these strains is different from the resistance described elsewhere (3,4) Those reports describe a polygenic form of resistance that was based on the ability of *Schizophyllum* to detoxify cycloheximide in the medium. We suspect that the difference between polygenic resistance and the single gene resistance presented here is due to differences in experimental technique for describing resistance and to differences in the strains employed to examine resistance. The earlier work employed concentrations of cycloheximide which were well above those used here (50 mg/L vs. 2 mg/L). At those high concentrations of cycloheximide, our resistant strains are inhibited. We would attribute the effects observed by those workers to be due to polygenically-based detoxification by strains carrying a resistant allele of the *chx* gene. We have tested the strains described in the earlier work at low concentrations of cycloheximide and find them to carry chx^r alleles. Our own genetically marked stocks are derived from the same collection as in the earlier work with the difference that a sensitive allele of *chx* was present in one of our strains (a spontaneous mutation, perhaps?).

Consideration of the cycloheximide detoxification process might help explain the results we see when scoring our dominance tests for fruiting ability. The increase in radial distance between the site of the inoculum and the formation of fruiting primordia that is seen with increasing cycloheximide concentration could well be due to the length of time it takes the colony to bring about detoxification of the media below some critical value. In tests of residual activity of cycloheximide in media under and around a growing colony, activity decreases with time and is found to be uniform in all areas under the colony, regardless of distance from the growing edge (4). If the generally accepted model for cycloheximide toxicity is invoked (14), it is difficult to reconcile the lack of significant effects on growth with the effects on fruiting. The later appearance of fruit-body primordia on media with greater concentrations of the drug can be seen as an indication that fruiting body formation is more sensitive than growth to the effects of the drug. When half of the available ribosomes are inhibited by the drug, as in cycloheximide-created heteroallelic dikaryons, these ribosomes are not available for protein synthesis. It may well be the case that fruiting, a developmental process, requires a greater rate or amount of protein synthesis than growth, which is dependent on the "housekeeping" functions of the cell.

The breakdown of the dikaryon to yield only resistant homokaryons is more problematic. It has been demonstrated in other organisms that cycloheximide does not always act by inhibiting protein synthesis, but rather can inhibit a wide variety of cellular functions (6). We hypothesize that the mechanism of breakdown could involve an inability of the hook cell to fuse with the subterminal cell during clamp connection formation due to the death of either cell because of cycloheximide sensitivity and subsequent outgrowth of the surviving cell.

As a test of this hypothesis we examined stained material from the growing edge of the three dikaryon types (chx^s/chx^s, chx^s/chx^r, chx^r/chx^r) that had been transferred from CYM to media containing cycloheximide. At 15 mg/L cycloheximide, the heteroallelic dikaryon showed an increase in abnormal septa scored as unfused hook cells or simple septa while the homozygotes showed no such increase (the homoallelic resistant dikaryon continued to grow and the homoallelic sensitive dikaryon died). However, at 3 mg/L cycloheximide there was no difference among any of the tested dikaryons, which may be an indication of toxic effects beyond the inhibition of protein synthesis. From these results it remains unclear whether or not inhibition of clamp formation plays a significant role in dedikaryotization.

The ability to recover a mycelium with a particular nuclear type by cycloheximide dedikaryotizaton is dependent upon the presence of a resistant allele in the desired nucleus. Treatment of such a dikaryon with the drug then offers an alternative to the use of protoplasts as a means of recovering homokaryons from dikaryons while remaining outside the usual channel of mieosis and sporulation. This method could prove useful when the study of cells of a particular nuclear type is desired rather than a mixture of cells with different nuclei. The technique also has possible advantages over the protoplast method since recovery of particular nuclear type should not be biased by the B-mating type factor (15). However, we have not yet tested enough strains to determine this for a significant number of B factors. We find no difference in our ability to recover nuclei containing either B41 or B43 when the B-factor allele is in the same parental nucleus with the chx^r allele. The mechanism of dikaryotic breakdown remains obscure. We have not been able to determine if our observations of breakdown follow the pattern shown by the spontaneous breakdown of the common-B heterokaryon, described elsewhere (12).

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