

Pisatin resistance in *Dictyostelium discoideum* and *Neurospora crassa*: comparison of mutant phenotypes

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The pea phytoalexin pisatin, at its inhibitory concentration, was shown to have two distinct inhibitory effects on amoebae of the cellular slime mould *Dictyostelium discoideum*. One effect was cytolytic and was demonstrable even in non-growing cells whereas the second effect was observed only under conditions favourable to growth. Pretreatment with a sublethal concentration of pisatin induced the amoebae to acquire resistance to both these effects. Mutations in *nysC* that alter membrane sterols and confer resistance to the polyene antibiotics nystatin and pimarinin blocked resistance to the growth-associated inhibitory effect but did not affect acquisition of resistance to the cytolytic effect. The *nysB sunD* double mutant HK412 displayed a partially constitutive resistance to the cytolytic effect but, like the *nysC* mutants, was blocked in the acquisition of resistance to the growth-associated inhibitory effect. Pisatin-treated cells incubated in pisatin-free medium lost their ability to grow on pisatin-containing medium much more rapidly than they lost resistance to the cytolytic effect of pisatin. These results suggest that the induction of pisatin resistance may involve the turning-on of independent resistance mechanisms against the two inhibitory effects of pisatin. This could account for our inability to isolate pisatin-resistant mutants in a single step. The *Neurospora crassa erg1* and *erg3* mutants that have altered membrane sterols and are nystatin resistant displayed sensitivity to pisatin. The pisatin-sensitivity phenotype of the *erg* mutants was used in selections to identify complementing plasmids from an ordered *Neurospora* genomic library. The association of pisatin sensitivity with membrane sterol alterations in both *D. discoideum* and *N. crassa* supports the hypothesis that mechanisms underlying nondegradative pisatin resistance are evolutionarily conserved.

Introduction

Phytoalexins are antimicrobial compounds produced by plants, possibly to prevent microbial infection. Pisatin (6a-hydroxy-3-methoxy-8,9-methylenedioxy pterocarpin) is the major isoflavonoid phytoalexin of pea (*Pisum sativum* L.). We have previously reported that wild-type amoebae of the cellular slime mould *Dictyostelium discoideum* can be induced to acquire a nondegradative resistance to an inhibitory concentration of pisatin when grown on a medium containing a sublethal concentration (50 µg ml⁻¹) (Kasbekar & Papavinasundaram, 1992). When plated on medium containing 150 µg pisatin ml⁻¹, the induced amoebae displayed a greater than 10⁴-fold increase in plating efficiency compared to uninduced amoebae derived from pisatin-free medium. Maintenance of resistance required the continued presence of pisatin in the medium because pisatin-resistant cells

reverted rapidly during passage on pisatin-free medium. Inducible nondegradative pisatin resistance has previously been reported for the pea pathogenic fungus *Nectria haematococca* Berk. and Br. mating population VI (imperfect form: *Fusarium solani*) (Denny & Van-etten, 1983) but efforts to elucidate its mechanism have not been very successful (Denny *et al.*, 1987). Since *D. discoideum* is a more tractable system for genetic and molecular studies than most phytopathogenic fungi, we have used it as a model system to study nondegradative pisatin resistance in the expectation that cellular slime moulds and fungi may share the same resistance mechanism.

We found that an alteration of membrane sterol composition, either with the azasterol A25822B or by mutations in *nysC* that confer resistance to the polyene antibiotics nystatin and pimarinin, suppressed the induction of pisatin resistance in *D. discoideum* (Kasbekar & Papavinasundaram, 1992). Induction was also suppressed in the *nysB sunD* double mutant strain HK412 (Kasbekar & Bhavani Prasanna, 1992). HK412

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was isolated in screens for suppressors of *nysB* (K. Lionetti & E. R. Katz, unpublished). Mutations in *nysB* also alter membrane sterols and confer nystatin resistance (Scandella *et al.*, 1980) but unlike the *nysC* mutations, they display the wild-type phenotype on pisatin (Kasbekar & Papavinasundaram, 1992). The *nysB sunD* double mutant has the same sterol composition as the *nysB* parent (K. Lionetti & E. R. Katz, unpublished) which suggests that the *sunD* mutation defines an additional requirement, perhaps another membrane component, for pisatin resistance.

In spite of the ease with which pisatin resistance can be induced in the wild-type, and in contrast to the readiness with which multiple mutations have been identified that suppress the induction phenomenon, we have not been able to isolate constitutive pisatin-resistant mutants by direct selection on inhibitory concentrations. One possible explanation for this result is that pisatin has more than one inhibitory effect at its inhibitory concentration and that the induction of resistance involves the turning-on of distinct and independent resistance mechanisms. Consequently, a single gene mutation would not simultaneously turn on all the mechanisms required for growth on pisatin medium and would thereby account for our inability to obtain pisatin-resistant mutants in a single step. If this model is correct, we might expect the *nysC* and the *nysB sunD* mutants to be blocked in some, but not all, of the pisatin-induced resistance mechanisms, that is, they might show a partial induction of resistance during growth on the sublethal concentration. In this paper, we present results of experiments designed to test this possibility. We interpret our results to suggest that pisatin inhibits amoebae by two distinct inhibitory effects; a cytolytic effect and an additional inhibitory effect that is expressed only during growth. Further, we demonstrate that the mutations block the acquisition of resistance to only the latter effect but do not affect the acquisition of resistance to the cytolytic effect.

Additionally, we have found that sterol mutations in *Neurospora crassa* also confer sensitivity to pisatin but there is a significant difference in the pisatin-sensitivity phenotype of the sterol mutants in *Neurospora* from that in *Dictyostelium*. The model for the involvement of independent mechanisms induced against the inhibitory effects of pisatin also provides a simple explanation for the differences in the pisatin-sensitivity phenotypes in the two systems.

Methods

Strains. All the *D. discoideum* strains used were derivatives of *D. discoideum* NC4 (Raper, 1935) and have been described previously (Scandella *et al.*, 1980; Kasbekar & Bhavani Prasanna, 1992). *N. crassa* strains 988 (ORa), 2721 (*erg1a*) and 2725 (*erg3a*) were obtained from the Fungal Genetics Stocks Center (FGSC), Department of Micro-

biology, University of Kansas Medical Center, Kansas City, KS, USA, and also from Dr Morris Grindle, University of Sheffield, UK.

Pisatin extraction. Pisatin was extracted from germinated pea seeds (*Pisum sativum* L.) following published procedures (Sweigard & VanEtten, 1987).

Media and growth conditions. *D. discoideum* amoebae were grown on lawns of *Enterobacter aerogenes* at 22 °C on SM agar plates (Sussman, 1966). A stock solution (10 mg ml⁻¹) of pisatin (pis) in dimethyl sulphoxide (DMSO) was used to supplement SM agar plates just before the latter were poured. The concentrations used are indicated in the abbreviations SM-pis50 (50 µg ml⁻¹) and SM-pis150 (150 µg ml⁻¹).

Bonner's salt solution (BSS) (Bonner, 1947) was used for suspending *D. discoideum* amoebae to make serial dilutions for determining the efficiencies of plating (EOP), and to incubate the amoebae for investigating the induction and the loss of pisatin resistance. Wherever pisatin was used in the treatments, an equal volume of DMSO was included in the controls. At the concentrations used, DMSO had no effect on cell viability. Routinely, more than 95% of the cells estimated from a haemocytometer count produced plaques when plated on SM medium.

Mutagenesis of *D. discoideum* amoebae was carried out following the procedure of Loomis (1987), with the modification that the amoebae were treated with 500 µg *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) ml⁻¹ for 5 min. In control experiments, we obtained a 30-fold increase in the frequency of mutants resistant to growth on medium containing 3% (v/v) methanol (frequency of methanol-resistant mutants before and after MNNG treatment was 8 × 10⁻⁶ and 2.4 × 10⁻⁴ per viable cell, respectively). This indicated that the mutagen treatment was effective.

The *N. crassa* strains were maintained at room temperature on Vogel's minimal medium (Davis & de Serres, 1970) supplemented with 1.5% (w/v) sucrose, or on glucose-peptone (GP) medium (10 g glucose, 5 g Oxoid peptone, 1 g KH₂PO₄, 0.5 g MgSO₄ · 7H₂O, in 1 litre distilled water).

Inhibition of protein synthesis. *D. discoideum* amoebae were suspended (approx. density 10⁷ ml⁻¹) in BSS, and in BSS containing 200 µg cycloheximide (Sigma) ml⁻¹, and incubated on a shaker at 22 °C. After 30 min, 10 µCi ml⁻¹ (370 kBq ml⁻¹) [³⁵S]methionine [1094 Ci mmol⁻¹ (4.048 × 10¹⁰ kBq mmol⁻¹)] and 0.3 µM carrier-methionine were added and incubated for a further 4 h. Incorporation of the label was stopped by the addition of excess (10⁴-fold) carrier-methionine, and the amoebae were lysed in BSS containing 0.1% SDS. An aliquot of cell lysate was precipitated onto Whatman GF/C discs with 10% (w/v) trichloroacetic acid (TCA) containing 0.1 M carrier-methionine. TCA-soluble material was removed by washing the filters twice at 25 °C and once at 70 °C using 5% (w/v) TCA containing 0.05 M carrier-methionine. The filters were then soaked briefly in ethanol, dried, and the incorporation of label in the TCA-insoluble fraction was determined by counting in a toluene-based scintillation fluid.

Cloning of the *Neurospora erg1* and *erg3* genes. Twenty-three pools of plasmid DNA were prepared, each representing two or three 96-well microtitre plates of the 50-plate Orbach/Sachs *Neurospora* genome library (obtained from the FGSC). The plasmid pools were used to transform protoplasts prepared from *erg1* and *erg3* mutant strains. Published protocols were followed to prepare the protoplasts (Vollmer & Yanofsky, 1986) and for transformation (Sellitrennikoff & Sachs, 1991). Double selection on Vogel's medium supplemented with hygromycin and pisatin was used to identify the pools that harboured plasmid DNA capable of yielding pisatin-resistant transformants. These plasmids presumably contained the corresponding wild-type alleles. The identified pools were subdivided into subpools and the

Table 1. Induction of pisatin resistance in wild-type and mutant *D. discoideum*

The EOPs on SM agar were calculated as the ratio of the number of plaques on SM agar following the BSS-pis150 treatment to the number following the BSS-DMSO control treatment. The EOPs on SM-pis150 were calculated as the ratio of the number of plaques on SM-pis150 agar to that on pisatin-free SM agar.

Strain	Relevant genotype	EOP on SM after incubation in BSS-pis150*			EOP on SM-pis150 after incubation in BSS-pis50†
		Uninduced	Induced on SM-pis50	Induced in BSS-pis50†	
DdB	+	$< 5 \times 10^{-6}$	1.0	0.37	0.52
M28	+	4×10^{-5}	0.30	0.5	0.02
TS12M	+	3.6×10^{-5}	0.83	0.5	0.05
HK10	<i>nysC208</i>	2×10^{-5}	0.40	1.0	$< 10^{-5}$
HK12	<i>nysC210</i>	8×10^{-5}	0.68	1.0	9×10^{-6}
HK412	<i>nysB sunD</i>	3.7×10^{-3}	1.0	1.0	$< 1.4 \times 10^{-5}$

* Amoebae (approx. 5×10^6 ml⁻¹) were incubated in BSS-pis150 and in BSS-DMSO. After 5 h incubation, the amoebae were plated on SM agar to determine the viable counts.

† Amoebae (approx. 5×10^6 ml⁻¹) were incubated in BSS-pis50 for 4 h.

process was iterated until subpools representing single clones were identified. This approach is known as cloning by sib selection (Vollmer & Yanofsky, 1986). The selection conditions were 40 µg pisatin ml⁻¹ in the top and bottom agar and 200 µg hygromycin ml⁻¹ in the bottom agar.

Results

Incubation in BSS-pis150 is cytotoxic

To determine whether pisatin has a cytotoxic effect on the amoebae, we examined whether the amoebae lost viability during incubation in BSS containing 150 µg pisatin ml⁻¹ (BSS-pis150). BSS is a non-nutrient medium which ordinarily allows amoebae to maintain viability for more than 24 h. If pisatin induces cell death, the EOP upon transfer of the amoebae from BSS-pis150 to SM medium should decrease compared to that of amoebae incubated in a BSS-DMSO control. We found that after a 5 h incubation in BSS-pis150, the EOP of the amoebae had dropped to approximately 10^{-5} (Table 1). Examination under a microscope revealed that incubation in BSS-pis150 induced the amoebae to swell and lyse. The addition of 0.5 M-sucrose to the BSS-pis150 protected against the pisatin-induced lysis and loss of viability (EOP after a 5 h incubation of DdB amoebae in BSS-pis150 with 0.5 M-sucrose was 0.1). As anticipated, wild-type amoebae induced to become pisatin-resistant by growth on SM medium containing a sublethal concentration of pisatin (SM-pis50) (Kasbekar & Papavinasundaram, 1992) were resistant in this assay (Table 1). These experiments demonstrated that at its inhibitory concentration (150 µg ml⁻¹), pisatin has a cytotoxic effect that is mediated perhaps by a loss in osmotic control due to a permeability change of the cytoplasmic membrane,

and that the amoebae acquired resistance to this effect during growth on medium containing a sublethal concentration of pisatin (SM-pis50).

Evidence for an additional inhibitory effect of *pis150*

We examined whether the block in the acquisition of pisatin resistance in *nysC* and the *nysB sunD* mutants could be explained by their inability to acquire resistance to the cytotoxic effect. We found, however, that growth on SM-pis50 medium (or pretreatment in BSS-pis50, see below) induced the mutant amoebae to acquire resistance to the cytotoxic effect of pisatin as effectively as the wild-type (Table 1). These results indicated that the acquisition of resistance to lysis by pisatin in the assay above was not sufficient to support growth on SM-pis150. One model suggested by this result is that in addition to exerting a cytotoxic effect, *pis150* also inhibits amoebae by an additional effect. Consequently, for growth on SM-pis150, the amoebae have to acquire resistance to both the inhibitory effects of pisatin. In this model, the *nysC* and the *nysB sunD* mutants can acquire resistance to the cytotoxic effect as effectively as the wild-type but are blocked in the acquisition of resistance to the second inhibitory effect.

An alternative model that is compatible with the above results is that pisatin has only one inhibitory effect (i.e. cytotoxicity), and that the acquisition of resistance to this effect is indeed sufficient for pisatin resistance in the wild-type, but that in the mutants the combination of two different membrane alterations, one pisatin-induced and the other mutation-induced, in some way inhibits growth. To distinguish between these models, we did experiments to determine whether the resistance to the cytotoxic effect

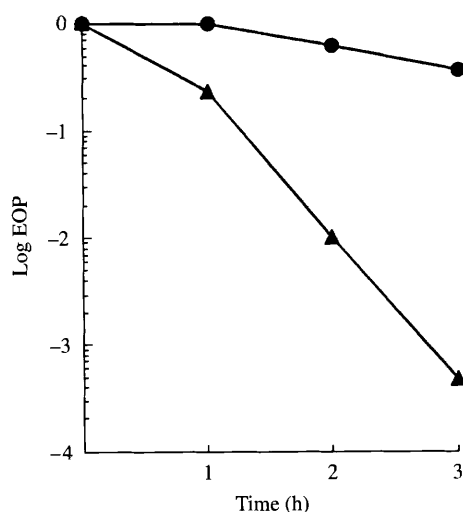


Fig. 1. Kinetics of loss of pisatin resistance in wild-type amoebae. DdB amoebae ($1.6 \times 10^6 \text{ ml}^{-1}$) derived from SM-pis150 medium were incubated in BSS for 3 h. At different time-points during this incubation, aliquots of the suspension were assayed for EOP on SM-pis150 (▲) by determining the ratio of the number of plaques on SM-pis150 agar to that on pisatin-free SM agar. At the same time-points, the amoebae were also assayed for resistance to cytolysis during a 4 h incubation in BSS-pis150 (●). In this experiment, the EOP refers to the ratio of the number of plaques on SM agar following the BSS-pis150 treatment to the number following the BSS-DMSO control treatment. Data are plotted on a log scale relative to the 0 h value.

could be dissociated from competence to grow on SM-pis150, even in the wild-type. We found that wild-type amoebae that were induced to become pisatin-resistant lost their resistance phenotype after a 4 h incubation in pisatin-free BSS. However, this loss in resistance appeared to occur in a stepwise manner; the ability to grow on SM-pis150 was lost considerably faster than the resistance to lysis by BSS-pis150. After a 3 h incubation in BSS, the EOP of pisatin-resistant wild-type amoebae on SM-pis150 had dropped by four orders of magnitude but there was only a 63% reduction in their resistance to the cytolytic effect (Fig. 1). This demonstrated that resistance to the cytolytic effect is not sufficient for growth on SM-pis150, even in the wild-type. This result argues against the second model (that acquisition of resistance to the cytolytic effect is sufficient for the growth of the wild-type on SM-pis150), and therefore supports the first model (that amoebae have to acquire resistance to the cytolytic and the additional inhibitory effect of pisatin in order to be able to grow on SM-pis150).

It is noteworthy that SM-grown (that is, uninduced) HK412 amoebae were generally more resistant to the cytolytic effect of pisatin than similarly grown wild-type amoebae (Table 1). However, the two populations of amoebae are equally sensitive to growth on SM-pis150

(Kasbekar & Bhavani Prasanna, 1992). These results rule out the possibility that resistance to the two inhibitory effects reflects two thresholds of a single mechanism and suggest instead that the mechanisms that confer resistance to the two inhibitory effects are independently induced.

To determine the nature of the second inhibitory effect of pisatin we examined the fate of SM-pis50-derived *nysC* mutant cells following their plating on SM-pis150 medium. At various time-points following the transfer, the cell viability was determined by harvesting cells and bacteria from approximately equal areas of the SM-pis150 agar surface and replating on drug-free SM medium. Even after 24 h on SM-pis150, 20% of the HK10 cells remained viable, that is they were capable of forming plaques on SM medium. Comparable results were obtained for the strain HK12. Thus, in contrast to the cytolytic effect, growth inhibition of induced *nysC* cells on SM-pis150 is not due to rapid cell death. This result suggests that inhibition by pisatin of induced *nysC* cells represents a cytostatic effect. The fivefold decrease in cell viability that was observed over the duration of the experiment may reflect a secondary toxic effect of rapidly growing bacteria on non-dividing amoebae. In order to distinguish this inhibitory effect of pisatin from the cytolytic effect, we refer to it as the growth-associated inhibition.

Induction of pisatin resistance in BSS-pis50

In a previous report (Kasbekar & Papavinasundaram, 1992), wild-type amoebae were shown to acquire pisatin resistance during growth on SM-pis50. We have now tested whether amoebae could acquire the pisatin-resistance phenotype during incubation in BSS-pis50. Table 1 summarizes the results of these experiments and shows that a 4 h incubation in BSS-pis50 induced the wild-type amoebae, but not the *nysC* mutants nor the *nysB sunD* mutant, to acquire the pisatin-resistance phenotype (i.e. the ability to grow on SM-pis150).

Since a 4 h incubation in BSS-pis50 can induce pisatin resistance, and pisatin-resistant amoebae are not killed by a 5 h incubation in BSS-pis150, we subjected amoebae to both the treatments in a sequential manner (i.e. a 4 h incubation in BSS-pis50 followed by a 5 h incubation in BSS-pis150). Following the sequential treatment, the amoebae were plated on SM medium to determine the EOP. The result (Table 1) shows that the wild-type, as well as the *nysC* and the *nysB sunD* mutants, acquired resistance to the cytolytic effect of pisatin. The amoebae were also plated on SM-pis150 following the BSS-pis150 incubation and, as expected, the wild-type strains were resistant whereas the mutant strains were sensitive to growth on the phytoalexin-containing medium (data not

Table 2. Induction of pisatin resistance involves active metabolic responses requiring protein synthesis

SM-derived DdB amoebae (approx. 10^6 ml⁻¹) were pretreated in BSS for 4 h at 22 °C unless otherwise indicated. The EOPs were calculated as the ratio of the number of plaques on SM-pis150 agar to that on pisatin-free SM agar.

Pretreatment	EOP on SM-pis150
DMSO	< 10 ⁻⁶
pis50	0.52
pis50, 4 °C	< 10 ⁻⁵
pis50 + cycloheximide*	< 10 ⁻⁶

*Cycloheximide was used at 200 µg ml⁻¹.

Table 3. Loss of pisatin resistance involves active metabolic responses requiring protein synthesis

SM-pis50 derived DdB amoebae (approx. 10^6 ml⁻¹) were incubated in BSS for 4 h as indicated. The EOPs were calculated as the ratio of the number of plaques on SM-pis150 agar to that on pisatin-free SM agar.

Pretreatment	EOP on SM-pis150
22 °C	9×10^{-6}
+ Cycloheximide*	0.54
4 °C	0.7

*Cycloheximide was used at 200 µg ml⁻¹.

shown). These results support our conclusion that *nysC* and the *nysB sunD* mutations do not impair the acquisition of resistance to the cytolytic effect of pisatin even though they block the acquisition of resistance to

growth on SM-pis150 (and therefore to its growth-associated inhibition).

Acquisition and loss of pisatin resistance involve active metabolic responses requiring protein synthesis

The induction of pisatin resistance in the wild-type DdB amoebae in BSS-pis50 was blocked if the incubation temperature was lowered to 4 °C or by the addition of the cytoplasmic-translation-inhibitor cycloheximide (200 µg ml⁻¹; Table 2). Likewise, the loss of pisatin resistance during incubation of resistant amoebae in BSS was blocked by incubation at 4 °C or by the addition of cycloheximide at 200 µg ml⁻¹ (Table 3). These results indicate that both the induction and the loss of pisatin resistance involve active metabolic responses requiring protein synthesis. At the concentration used, cycloheximide blocked protein synthesis by 50% (as measured by the incorporation of [³⁵S]methionine into the TCA-precipitable material) but this treatment had no effect on cell viability. Post-treatment viability was determined by plating the amoebae on drug-free medium (EOP on SM was 1.0).

To determine whether cycloheximide blocked the acquisition of resistance to the cytolytic effect of pisatin, we incubated the wild-type DdB amoebae in BSS-pis50 together with cycloheximide (200 µg ml⁻¹) for 4 h; the amoebae were then washed in BSS and subjected to an additional 5 h incubation in BSS-pis150. The EOP on SM of DdB amoebae after the second treatment was 2.5×10^{-5} . This value is about four orders of magnitude lower than that obtained for amoebae pretreated in BSS-pis50 without cycloheximide. This result suggests that protein synthesis is required for the acquisition of resistance to the cytolytic effect.

Table 4. Complementation in the *erg1* and *erg3* transformants of *N. crassa*

Strain	Relevant genotype	Growth period (h)	Mycelial diameter (mm)*			Percentage inhibition by:	
			GP	GP-pis40	GP-nys2	Pisatin (40 µg ml ⁻¹)	Nystatin (2 µg ml ⁻¹)
ORa	+	13	50	19	0	62	100
2721	<i>erg1</i>	20	41	2.3	34	94	17
2721/G17F12†	<i>erg1/erg</i> ⁺	14	48	11	0	77	100
2725	<i>erg3</i>	31	45	3	25	93	44
2725/G18A10†	<i>erg3/erg</i> ⁺	28	48	15	4	69	92

*Growth was calculated by measuring the mycelial diameter and subtracting the diameter of the inoculating plug (4 mm). As the growth rate varied between the wild-type and mutant strains, the measurements were taken when the colony diameter reached 50 mm on GP medium. Data represent means of four replicates. Symbols: GP-pis40, GP medium supplemented with 40 µg pisatin ml⁻¹; GP-nys2, GP medium supplemented with 2 µg nystatin ml⁻¹.

† Mutant protoplasts transformed with the corresponding complementing plasmids: 2721 with G17F12 and 2725 with G18A10.

Sterol mutants of N. crassa are pisatin-sensitive

If the mechanisms underlying the nondegradative pisatin resistance were conserved, we would predict that sterol mutations in other fungi would also confer pisatin-sensitivity. We tested this prediction using the *erg* mutants of *N. crassa*. The *Neurospora erg* mutations alter the composition of membrane sterols and confer nystatin resistance (Grindle, 1973) by blocking specific steps in ergosterol biosynthesis: *erg1* mutations affect $\Delta^{8,7}$ isomerization (Grindle & Farrow, 1978), *erg3* mutations affect the $\Delta^{14,15}$ reductase activity (Ellis *et al.*, 1991). We found that, compared to the wild-type, the *erg1* and *erg3* mutants were much more sensitive to growth inhibition on medium containing 40 μg pisatin ml^{-1} (Table 4).

We used the pisatin-sensitive phenotype of the *erg1* and *erg3* mutants to clone the respective genes by transforming mutant protoplasts with pooled plasmid DNA from the Orbach/Sachs ordered *Neurospora* genomic library (obtained from the FGSC) and selecting for pisatin-resistant transformants. The clones G17F12 and G18A10 were found to complement *erg1* and *erg3*, respectively. The pisatin-resistant transformants had become nystatin-sensitive (Table 4), thereby suggesting that the complementing plasmids contained the corresponding wild-type alleles. A detailed molecular characterization of the cloned genes will be published elsewhere.

Discussion

Our results suggest that pisatin inhibits *D. discoideum* amoebae by two distinct inhibitory effects; one cytolytic and demonstrable even in non-growing cells and the other apparently cytostatic and demonstrable only under conditions favourable to cell growth. The *nysC* and the *nysB sunD* mutants can acquire resistance to the cytolytic effect of pisatin, but they do not become resistant to the growth-associated inhibitory effect. Pisatin-resistant amoebae lose their resistance phenotype during incubation in BSS by first losing their resistance to the growth-associated inhibitory effect and then losing resistance to the cytolytic effect. The phenotype of the HK412 strain (*viz.* a partially constitutive resistance to the cytolytic effect but incapable of acquiring resistance to the growth-associated inhibitory effect) argues against the possibility that resistance to the two effects represents two thresholds in the continuum of a single mechanism. We propose that the induction of pisatin resistance involves the turning-on of independent mechanisms against the two inhibitory effects.

One of our original goals was to select pisatin-resistant mutants. We were unable to obtain such mutants by direct selections on SM-pis150 medium, even after mutagenesis with MNNG (frequency $< 10^{-8}$). We can

now account for our inability to obtain such mutants by our model that independent resistance mechanisms have to be turned-on for growth on SM-pis150, in which case it would be unlikely that a single gene mutation would confer the pisatin-resistance phenotype.

Mutants affected in nondegradative pisatin resistance have been reported, thus far, only in *D. discoideum* (Kasbekar & Bhavani Prasanna, 1992; Kasbekar & Papavinasundaram, 1992). We have now demonstrated that in *N. crassa*, the *erg1* and *erg3* mutations that alter sterol composition also conferred pisatin sensitivity. There is an important difference, however, between the pisatin-sensitivity phenotype of the *erg* mutants of *N. crassa* and that of the *nysC* mutants of *D. discoideum*. The *erg* mutants are constitutively sensitive to a concentration of pisatin (40 μg ml^{-1}) that is not inhibitory to the wild-type. In contrast, the pisatin-sensitivity phenotype of the uninduced *nysC* mutants is the same as that of the uninduced wild-type (Kasbekar & Papavinasundaram, 1992). Both grow equally well on SM-pis50 (the subinhibitory concentration of pisatin), and are equally sensitive on SM-pis150 (the inhibitory concentration). The only difference between them was in their ability to acquire pisatin resistance during pretreatment with the sublethal concentration of pisatin; the wild-type was inducible whereas the *nysC* mutants were blocked (Kasbekar & Papavinasundaram, 1992).

Our model that independent mechanisms are induced against the different inhibitory effects of pisatin can account for this difference in phenotype of *N. crassa* and *D. discoideum* sterol mutants on the assumption that, in *N. crassa*, a much higher concentration of pisatin is required for the cytolytic effect (possibly due to the presence of a cell wall) than for its growth-associated inhibitory effect. Consequently, at 40 μg ml^{-1} the wild-type remains viable, acquires resistance to the second effect, and only then resumes growth. The *erg* mutants cannot grow because they are blocked in the acquisition of resistance to the growth-associated inhibitory effect. In contrast, we suggest that in *D. discoideum* both the inhibitory effects occur at approximately the same pisatin concentration. Therefore, uninduced wild-type and *nysC* amoebae are equally sensitive to lysis at the inhibitory concentration (150 μg ml^{-1}) and both are rapidly killed. After induction, the wild-type acquires resistance to both inhibitory effects but the mutants can acquire resistance to only the cytolytic effect. Hence the wild-type can grow after transfer to SM-pis150 but the mutant cannot. Thus in both systems, sterol alterations suppress the same mechanism, namely the growth-associated inhibition.

Preliminary studies (D. P. Kasbekar, unpublished) show that treatment with pisatin, together with the sterol biosynthesis inhibitor azasterol A25822B, completely blocked mycelial growth in the pea pathogen *Nectria*

haematococca. At the concentrations used, pisatin alone was uninhibitory whereas the azasterol alone inhibited conidial development and retarded growth to some extent. This synergism of the action of pisatin with sterol change suggests that nondegradative pisatin resistance will be suppressed in *erg*-like mutants of *Nectria*. By examining the virulence of such mutants on pea, it should be possible to assess the role of this mechanism in pathogenesis.

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