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Nondegradative pisatin-resistance in *Dictyostelium discoideum, Neurospora crassa* and *Nectria haematococca:* Similarities and differences

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Abstract. Paradoxically, on pisatin-medium (150 µg/ml) the cellular slime mould *Dictyostelium discoideum* grows only when plated as spores but not when plated as amoebae. The recent discovery of inducible nondegradativc pisatin resistance in amoebae has allowed us to formulate a model that resolves this paradox. In this model, the germinating amoeba is postulated to acquire a pisatin-resistance phenotype while ensconced within the spore wall. This article reviews the findings on which this model is based and extends it to also account for the differences in pisatin sensitivity phenotype that result from sterol alteration in cellular slime mould[s] and fungi.

Keywords. Phytoalexin tolerance; sterol mutants; spore walls; azasterol A25822B.

1. Introduction

1.1 A *paradox and its resolution*

Pisatin (6a-hydroxy-3-methoxy-8,9 methylene dioxypterocarpan) is the major phytoalexin (plant antibiotic) produced by the garden pea *Pisuin sativum* in response to microbial infection. We have been studying the interaction of pisatin with a wide variety of microorganisms, including the cellular slime mould *Dictyostelium discoideum*, the filamentous fungus *Neurospora crassa* and the phytopathogenic fungus *Nectria haematococca*. One remarkable result was that on nutrient (SM) medium supplemented with 150 µg/ml pisatin (SM-pis150), the wild type *D. discoideum* strain, DdB, could form plaques when plated as spores but not when plated as amoebae. This is a paradox because cellular slime moulds form plaques only *via* the growth and division of amoebae; spores have to first germinate and release amoebae in order to form plaques. Thus, whereas on the one hand amoebae are ordinarily pisatin-sensitive, on the other, we have to explain how the amoebae that emerge from spores germinated on pisatin medium (and subsequent generations thereon) become pisatin-resistant. Recent findings on the induction of pisatin-resistance in *Dictyostelium* amoebae (Papavinasasundaram and Kasbekar 1993; Kasbekar and Papavinasasundaram 1992) have served to resolve this paradox. Based on these findings, I propose that whereas naked amoebae are rapidly killed by the cytolytic effect of pisatin, the spores survive long enough on the pisatin medium to be induced for, and thus secondarily acquire, a nondegradative pisatin-resistance phenotype. In other words, the germinated amoeba is postulated to have acquired pisatin-resistance while still ensconsced within the spore wall. This article reviews

the findings on which this model is based and relates this model to results from *Neurospora* and *Nectria.*

1.2 *Background on inducible, nondegradative pisatin-resistance in D. discoideum amoebae and N. haematococca mycelia*

D. discoideum amoebae are ordinarily incapable of growth on SM-pis 150 medium; however, pretreatment with a sublethal concentration (50 kg/m1) was shown to induce a resistance phenotype that enabled the cells to now grow on SM-pis150 (figure 1). Pretreatment could be effected either by growth on SM-medium supplemented with 50 p.g/m1 pisatin (SM-pis50) or alternatively, by incubating amoebae for 3 h in a non-nutrient salts solution (SS) supplemented with 50 µg/m1 pisatin (SS-pis50). The resistant phenotype was nondegradative, in that it did not involve the production of pisatin-detoxifying enzymes (Kasbekar and Papavinasasundaram 1992). Resistant cells rapidly reverted to pisatin-sensitivity during incubation in pisatin-free salts solution (SS) (or, alternatively, after transfer to pisatin-free SM-medium), thereby indicating that pisatin was required for the continued maintenance of the resistant phenotype. Induction as well as loss of resistance were blocked by chilling to 4°C or by the addition of cycloheximide, which suggested that both these processes involved active metabolic responses by the cells (Papavinasasundaram and Kasbekar 1993).

Uninduced amoebae were rapidly lysed during incubation in SS containing 150 µg/ml pisatin (SS + pis150) whereas, not unexpectedly, induced amoebae were resistant to such pisatin-mediated cytolysis. Lysis of uninduced cells could be prevented by the addition of 0·5 M sucrose to the SS + pis150. These results led us to conclude that pisatin-induced cytolysis is mediated perhaps by a loss of osmotic control and that induction allows the amoebae to acquire resistance to the loss of such osmotic control.

However, the mere acquisition of resistance to pisatin-induced cytolysis was not sufficient to support cell growth on SM-pis150; pisatin was found also to exert a distinct cytostatic effect. Thus, in order to grow on SM-pis150, it was necessary for the induced amoebae to have acquired resistance to this second inhibitory effect as well. These conclusions were based on the following observations. Mutations in the *nysC* gene that alter membrane sterol composition (and thereby confer resistance to the polyene antibiotics nystatin and pimaricin) (Scandella *et al* 1980; Kasbekar *et al* 1988) suppressed induction of resistance to pisatin's cytostatic effect (figure 1), but did not affect the ability of cells to acquire resistance to pisatin's cytolytic effect. The *nysC* phenotype could be mimicked in the wild-type by treatment with the sterol biosynthesis inhibitor azasterol A25822B (figure 2). The *nysB sunD* mutant strain, HK412, displayed a partially constitutive resistance to the cytolytic effect but, like the *nysC* mutants, was blocked in the acquisition of resistance to the cytostatic effect (Kasbekar and Bhavani Prasanna 1992). These results suggested that the induction of pisatin-resistance may involve the turning on of independent resistance mechanisms against the cytocidal and cytostatic effects of pisatin, and that the sterol alteration interfered with the acquisition of resistance only to the cytostatic effect. The requirement for induction of independent mechanisms for the resistance phenotype might account for our inability to obtain pisatin-resistant mutants in a single step selection (i.e., by plating uninduced amoebae on SM-pis 150).

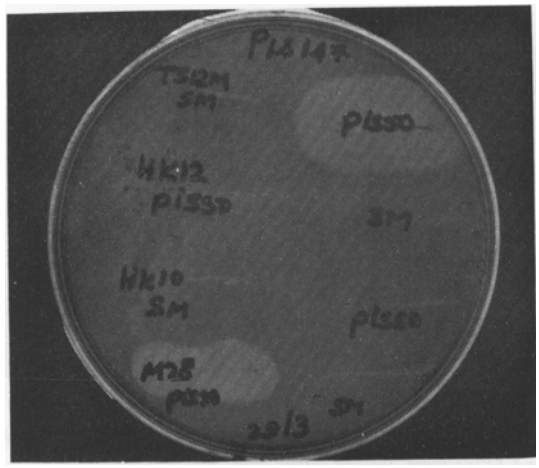

Figure 1. Growth on sublethal pisatin concentration induces resistance to higher concentrations in the wild-type but not in *nysC* mutants. Wild-type (TS12M and M28) and *nysC* (HK10 and HK12) amoebae were derived either form pis50 medium or from SM. Only pis50-derived wild-type cells could grow on the SM plate supplemented with pisatin (147 ug/m1). Growth of all other streaks is inhibited.

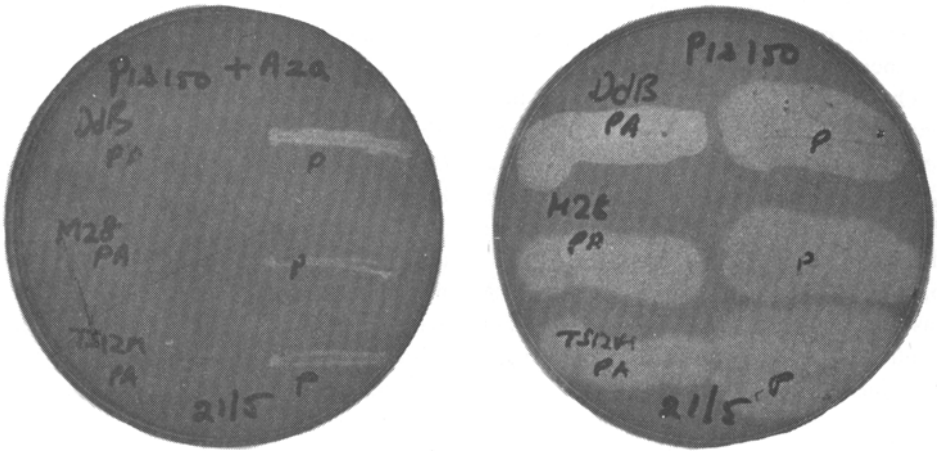

Figure 2. Effect of the azasterol A25822B on pis50 induction of pisl50-resistance. The SM agar plate on the left is supplemented with 150 µg/m1 pisatin and 15 µg/ml azasterol (pis150 + aza) and the plate on the right is supplemented with only 150 µg/m1 pisatin (pis150). Wild-type cells derived from pis50 + azal5 plates (PA) or only pis50 (P) were streaked with toothpicks. All streaks (PA and P) grew on pis150 but PA streaks did not grow on pis + aza plates. The growth of P streaks was also severely retarded on pis150+ aza. (The little growth that did occur may be due to the residual wild-type sterol being carried over.)

Figure 3 shows that amoebae grown on SM-pis 150 acquire cross-resistance to nystatin (but not to pi maricin). However, after transfer to nystatin medium, the amoebae lost their pisatin-resistance phenotype but remained nystatin-resistant. To

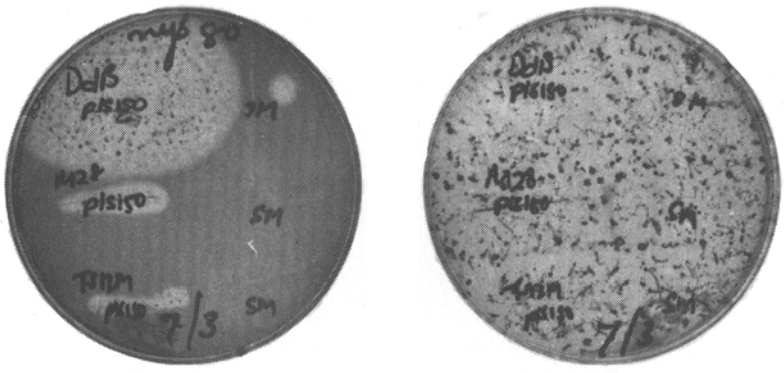

Figure 3. Growth on pisatin medium induces cross resistance to nystatin. Wild-type amoebae (DdB, TS12M and M28) were derived either from pis150 medium or from SM. Only pis150 derived cells could grow on the SM plate supplemented with nystatin (801.tg/m1). SM-derived cells are inhabited. The plate on the right is a drug-free SM plate on which all the streaks have grown, merged and proceeded to develop fruiting bodies.

account for this asymmetry in the induction and maintenance of cross-resistance after growth on pisatin- and nystatin-media it was proposed that the nystatin-resistance phenotype may be governed by yet another mechanism. This model presumes that growth on pisatin induces membrane alteration that predisposes cells to acquire nystatin- resistance but that the pisatin-induced alteration is not maintained in the absence of pisatin.

Our studies on pisatin-resistance in *D. discoideum* were prompted by an earlier demonstration by Denny and VanEtten (1983) of inducible, nondegradative pisatin-tolerance in the pea pathogenic fungus *Nectria haematococca* mating population (MP) VI. Most isolates of *N. haematococca* MPVI detoxify pisatin but they also possess an inducible pisatin-tolerance mechanism that does not depend on pisatin-degradation. This latter mechanism was studied using a strain that does not degrade pisatin. Mycelia pretreated with 0·1 mM pisatin resumed growth sooner and at a faster rate when challenged subsequently with an additional 0·6 mM pisatin than controls that had not been so pretreated. Without the pisatin pretreatment, growth was inhibited for at least 24 h by 0·7 mM pisatin. The adaptation was lost within 4 h after transfer of the mycelium to pisatin-free buffer. Chilling to 1°C prevented both the induction and the loss of enhanced pisatin-tolerance. Induced mycelia also showed cross-resistance to the polyene antibiotic amphotericin B. In short, inducible nondegradative pisatin-tolerance in both *Nectria* and *Dictyostelium* shared many characteristics most notably, pisatin-dependence and cross-tolerance of polyene antibiotics. In light of these similarities, I also examined the effect of membrane sterol alterations on the acquisition of nondegradative pisatin resistance in *Nectria*.

2. Materials and methods

2.1 *Strains*

The *D. discoideum* strains used (DdB, TS12M and M28) were derivatives of *D.*

discoideum NC4 and have been previously described (Scandella *et al* 1980). *N. haematococca* MPIV strain 272-26-1 (Pda) was from Hans D VanEtten (University of Arizona, USA).

2.2 *Growth conditions*

D. discoideum amoebae were grown on lawns of Enterobacter aerogenes at 22°C on SM agar plates (Sussman 1966). Stock solutions (10 mg/m1) of the azasterol A25822B (Eli Lilly and Co.) and nystatin (Sigma) in dimethyl formamide and of pisatin in dimethyl sulphoxide were used to supplement SM agar plates just before the latter were poured. Nystatin agar plates were shielded from light by being wrapped with aluminium foil. At the concentrations used, dimethyl formamide and dimethyl sulphoxide themselves had no detectable effects on the growth, development or drug resistance phenotypes of the slime moulds. Salt solution (Bonner 1947) was used to suspend the amoebae and to make serial dilution. The *N. haematococca* strain was maintained on V-8 agar.

2.3 *Pisatin extraction*

Pisatin was extracted from germinated pea seed (*P. sativum*) by the procedure of Sweigard and VanEtten (1987).

2.4 *Isolation of M28 × TS12 M diploid*

The diploid was isolated as described by Katz and Susman (1972).

3. **Results and discussion**

3.1 *Induction of pisatin resistance in D. discoideum spores*

Table 1 compares the plating efficiencies of spores and amoebae of different *D. discoideum* strains on SM-pis150. The plating efficiency is defined as the ratio of number of plaques on pisatin medium to that on pisatin-free medium. As mentioned above and quantitated in the table, the wild-type strain DdB displays pisatin-sensitivity when plated as amoebae and pisatin-resistance when plated as spores.

Table 1 also compares the viabilities of spores and amoebae during incubation in SS + pis150. Whereas the amoebae of all the strains were lysed within 5 h, the spores retained 100% viability even after 24 h incubation in SS + pis150. This demonstrated that spores are indeed resistant to pisatin-induced cytolysis.

Interestingly, spores of the TS12M and M28 strains did not form plaques on SM-pis 150 despite the facts that their spores were resistant to pisatin-induced cytolysis and that their amoebae are quite competent to acquire pisatin-resistance *via* the pretreatment procedure (Kasbekar and Papavinasasundaram 1992). However, diploids constructed between these two strains showed complementation of the parental strains' spore-sensitivity phenotype; that is, spores of the diploid derivative were able to plaque on SM-pis150. Thus, we are led to conclude that the two

Table 1. Comparison of plating efficiencies and viabilities of *D. discoideum* amoebae and spores.

	Plating efficiency on SM-pis150		Viability after incubation in BSS-pis150*	
Strain	Amoebae	Spores	Amoebae	Spores
DdB	$< 10^{-5}$	0·15	$< 10^{-5}$	1
TS12M	$< 10^{-5}$	$< 10^{-5}$	$< 10^{-5}$	1
M28	$< 10^{-5}$	$< 10^{-5}$	$< 10^{-5}$	1
TS12M × M28	$< 10^{-5}$	0·01	ND	ND
DdBaza	ND	$< 10^{-5}$	ND	ND

ND, Not determined.
*Incubation time for amoebae, 5 h; spores 24 h.

strains carry mutations in different genes that specifically block induction of pisatin-resistance in spores. Further genetic analysis of these strains will allow us to identify the genes that affect this phenomenon.

Spores derived from DdB amoebae grown on medium containing 15 ug/m1 of the azasterol A25822B were pisatin-sensitive. This reinforces earlier observations that azasterol-grown cells are phenocopies of *nysC* mutants (Kasbekar *et al* 1985; Kasbekar and Papavinasasundaram 1992).

3.2 *Synergistic effects of pisatin and sterol alterations in Nectria*

In view of the similarities in nondegradative pisatin-tolerance induction in *Dictyostelitun* and *Nectria,* we wanted to examine the effects of altering membrane sterol composition on pisatin-tolerance in *Nectria.*

The germination of *Nectria* macroconidia and the subsequent growth and development of the mycelium were examined in glucose-asparagine (GA) liquid medium and in GA medium supplemented with pisatin (280 p.g/ml), azasterol A25822B (10 tg/ml), or pisatin (280 µg/m1) + azasterol (10 µg/m1).

In GA medium a homogeneous mycelial suspension with macroconidia was obtained after overnight incubation. In GA + pisatin, the inoculated macroconidia germinated after a delay of approximately 8 h but subsequently grew as rapidly as the mycelium in GA medium. These mycelia developed microconidia rather than macroconidia. It is not clear what triggers the development into one or the other type of conidium.

In GA + azasterol, the macroconidia germinated as rapidly as in GA medium but subsequent growth of the mycelium was slower and it formed a clumpy rather than a homogeneous suspension. The mycelia did not form any conidia. Instead, a reddish-brown pigment accumulated and eventually the mycelium stopped growing. Comparison of the sterols from GA and GA + azasterol-grown mycelia by thin-layer chromatography confirmed that the azasterol-grown mycelia accumulated a different (but as yet unidentified) sterol. This is consistent with the action of azasterol as a sterol biosynthesis blocker. In GA + pisatin + azasterol, the macroconidia germinated after a delay of approximately 6 h but growth was arrested shortly after germination at which time the germlings had elongated approximately four-fold. No conidia developed nor did 'the medium accumulate the reddish-brown pigment.

These results suggest that although pisatin alone has relatively little effect on growth and differentiation, it arrests growth in the presence of azasterol-induced sterol alterations. In other words, the two antibiotics interact as they do in *Dictyostelium,* supporting our contention that nysC-like mutations may suppress nondegradative pisatin resistance in *Nectria.* However, this conclusion must be tempered somewhat by our observation that at the concentrations used, the azasterol itself inhibited conidial development and significantly retarded growth.

3.3 *Prospectus*

In the context of the above results, it is encouraging that the *erg* mutants that alter membrane sterol composition in *N. crassa* also display pisatin sensitivity (Papavinasasundaram and Kasbekar 1993). There is an important difference, however, between the pisatin-sensitivity phenotype of the *Neurospora* and *Dictyostelium* sterol mutants. The *Neurospora erg* mutants were constitutively sensitive to a pisatin concentration that was not inhibitory to the wild-type. In contrast, the *Dictyostelium nysC* mutants were indistinguishable from the wild-type on both SM-pis50 and SM-pis 150 (resistant to the former and sensitive to the latter). The only difference was that the wild-type was inducible (i.e., could acquire resistance to SM-pis150) whereas the *nysC* mutants were not inducible (figure 1). We can account for this difference in phenotypes in the two systems on the assumption that in *Neurospora* the cell wall protects the mycelial cells against pisatin's cytolytic effect, and that consequently the wild-type remains viable and resumes growth after acquiring resistance to the cytostatic effect. The *erg* mutants are blocked in the acquisition of resistance to the cytostatic effect and therefore do not grow on the inhibitory concentration. In contrast, both uninduced wild-type and uninduced *nysC* cells are equally sensitive to pisatin-induced lysis and are rapidly killed on SM-pis150. After induction, however, the wild-type acquires resistance to both the cytolytic and the cytostatic effects (and is therefore pisatin-resistant) whereas the *nysC* cells, despite acquiring resistance to the lytic effect, remain sensitive to the cytostatic effect and do not grow after transfer to SM-pis150. The *Neurospora* phenotype is in fact similar to that displayed by *Dictyostelium* spores presumably because both *Neurospora* mycelia and *Dictyostelium* spores are protected from the cytolytic effect by their cell walls. In this view, the *Neurospora erg* mutants are analogous to azasterol-grown DdB spores in that they are resistant to the cytolytic effect but are unable to overcome the cytostatic effect.

I imagine that the *Nectria erg-like* mutants will show a phenotype like that of the *Neurospora erg* mutants rather than the *Dictyostelium nysC* amoebae. Attempts to identify *Nectria erg-like* mutants by selection on nystatin-medium are currently being pursued in our laboratory (S Sengupta and D P Kasbekar, unpublished). As an alternative strategy we have cloned the *Neurospora erg]* and *erg3* genes by selection for complementation or their pisatin sensitivity phenotype (Kasbekar and Orbach 1993), and the cloned genes are being used as probes for identification of their *Nectria* homologs (K G Papavinasasundaram and D P Kasbekar, unpublished). Obtaining the *Nectria* genes will . enable us to construct erg-like (i.e., nys*C*-like) mutants by gene disruption and then to test the virulence properties of these mutants on pea.

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