Dictyostelium caveatum mutants selected for a nystatin-resistant phenotype are cross-resistant to pisatin and other isoflavonoid phytoalexins

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Abstract

Cellular slime mould amoebae can be induced to become resistant to an otherwise inhibitory concentration of pisatin, an isoflavonoid phytoalexin of pea, if they are first treated with a subinhibitory concentration. We report here the serendipitous isolation of pisatin-resistant mutants in the cellular slime mould *Dictyostelium caveatum*. However, the pisatin resistance phenotype of the mutants appears to have a different basis than the inducible pisatin resistance phenotype of the wild type.

[Kasbekar D.P., Prasanna B.T. and Vairamani M. 1998 *Dictyostelium caveatum* mutants selected for a nystatin-resistant phenotype are cross-resistant to pisatin and other isoflavonoid phytoalexins. *J. Genet.* 77, 37–40]

Introduction

Dictyostelium caveatum and *Dictyostelium discoideum* are species of cellular slime moulds, which are free-living soil amoebae that feed on bacteria. Our laboratory is studying the responses of *Dictyostelium* amoebae to isoflavonoid phytoalexins. Phytoalexins are antimicrobial compounds that plants produce in response to injury and microbial infection, and isoflavonoids are the major phytoalexins of the leguminous plants (Smith and Banks 1986). The rhizosphere of leguminous plants is particularly rich in bacterial flora and we anticipated that dictyostelids might show novel responses to isoflavonoid compounds that increased their access to the rhizobial bacteria. We have proposed that leguminous plants might employ isoflavonoids to manipulate the foraging behaviour of *Dictyostelium* amoebae so as to rid the plant of potentially pathogenic rhizosphere bacteria from the vicinity of root lesions (Bhavani Prasanna *et al.*, manuscript submitted for publication).

For the most part these studies used *D. discoideum*. Amoebae of this species are normally quite sensitive to pisatin (6a-hydroxy-3-methoxy-8,9-dioxypterocarpan), an isoflavonoid phytoalexin of the garden pea (*Pisum sativum* L.). But under certain conditions (e.g. growth in the presence of a sublethal concentration), they can be induced to acquire a pisatin-resistant phenotype (Kasbekar and Papavinasasundaram 1992; Papavinasasundaram and Kasbekar 1993; Kasbekar 1994). The inducible 'pisatin resistance' phenotype actually represents a general response to isoflavonoid compounds (Bhavani Prasanna *et al.*, manuscript submitted for publication). Isoflavonoids inhibit the growth of *D. discoideum* amoebae by two different effects, one cytostatic and the other cytolytic (Papavinasasundaram and Kasbekar 1993). Additionally, isoflavonoids inhibit the aggregation of amoebae that is normally triggered by starvation (Bhavani Prasanna *et al.*, manuscript submitted for publication). Despite persistent efforts we never obtained pisatin-resistant mutants of *D. discoideum* in a single-step selection. The inability to obtain such mutants was taken as evidence that the inducible resistance required independent mechanisms to overcome the cytocidal and cytostatic effects. In contrast, it was possible to identify mutations that block the inducible pisatin resistance phenotype, specifically mutations in the *nysC* gene that block wild-type sterol biosynthesis and can be isolated by selection for resistance to the polyene antibiotic nystatin (Kasbekar and Papavinasasundaram 1992).

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Keywords. pisatin; maackiain; biochanin A; nystatin; pimaricin; tomatine.

In this paper we report extension of our studies to *D. caveatum*. Like *D. discoideum*, *D. caveatum* amoebae also can display an inducible resistance to isoflavonoid phytoalexins. But we made the serendipitous discovery that, unlike in *D. discoideum*, pisatin-resistant mutants can indeed be obtained in a single-step selection in *D. caveatum*. This latter result appeared to flagrantly contradict the carefully mustered arguments of Papavinasasundaram and Kasbekar (1993) purporting to explain why the inducible pisatin-resistant phenotype cannot be constitutively turned on by a single-gene mutation. We offer a possible resolution of this dilemma.

Materials and methods

Strain and growth conditions: The *D. caveatum* strain was obtained from ATCC (catalog number 66413) and is described by Waddell and Duffy (1986). It was grown on *Enterobacter aerogenes* lawns on LP agar plates (Waddell and Duffy 1986). *D. caveatum* is a unique cellular slime mould species in that, in addition to bacteria, it can feed upon amoebae of the other cellular slime moulds (Waddell 1982). Our original objective in using this species was to determine whether it scavenged sterols from phagocytosed cells of other cellular slime moulds. Our strategy depended on isolation of *nysC* mutants of *D. caveatum*, in the expectation that they would be blocked in acquisition of inducible pisatin resistance, and then attempt to complement this phenotype by having the mutant amoebae feed upon wild-type *D. discoideum* cells. Complementation would provide evidence that the host cell can scavenge wild-type sterol from the phagocytosed cells. Unfortunately this original objective was not achieved because none of the nystatin-resistant mutants isolated showed the *nysC* phenotype. Instead, they were all highly cross-resistant to pisatin.

Stock solutions (10 mg/ml) of nystatin (Sigma) in dimethyl formamide, and pisatin, maackiain and biochanin A in DMSO were used to supplement the LP agar just before the plates were prepared. Pisatin was extracted from germinated pea seeds by the procedure of Sweigard and VanEtten (1987). Maackiain and biochanin A were gifts from Dr Scott Soby and Dr Hans D. VanEtten (University of Arizona, USA). Pimaricin (Sigma) was purchased as a 2.5% aqueous suspension that was directly added to the LP medium. The concentration of pisatin (μg/ml) in the LP agar is indicated by the abbreviations LP-pis25, LP-pis70 and LP-pis130 and of nystatin by LP-nys30. Nystatin and pimaricin agar plates were shielded from light by wrapping with aluminium foil. At the concentrations used, dimethyl formamide and DMSO themselves had no detectable effects on growth or drug resistance phenotype of the cellular slime moulds.

A balanced salt solution (Bonner 1947) was used to suspend the amoebae and to make serial dilutions for determining the efficiency of plating (EOP) and also to incubate the amoebae whenever necessary. Routinely, more than 95% of the cells, estimated from a haemocytometer count, produced plaques when plated on the agar medium. The EOP was determined by dividing the number of plaques obtained on the drug plates by the number obtained on drug-free SM plates.

Mutagenesis: Amoebae were mutagenized by the procedure of Loomis (1987), with the modification that the amoebae were treated with 55 μg/ml NTG for 5 minutes.

Sterol analysis: Sterols were extracted from amoebae that were washed in water to remove bacteria. Each gram (wet weight) of amoebae was homogenized first with 10 ml reagent grade methanol (Qualigens) and then again with the addition of 20 ml reagent grade chloroform (Merck). The homogenate was allowed to stand for 20–30 minutes and then vacuum-filtered. The filtrate was washed with one-fourth the volume of 0.88% aqueous KCl and the bottom layer was washed again with one-fourth the volume methanol–water (1 : 1 v/v). The bottom layer was separated with a separatory funnel and dehydrated in an anhydrous $MgSO_4$ flash column. It was then evaporated to dryness in a rotovaporator and dissolved in HPLC-grade hexane.

The sterol preparations were analysed on a Hewlett Packard HP 5890 gas chromatograph using an HP Ultra 1 fused silica capillary column (50 m length, 0.33 mm internal diameter). The injector temperature was set at 300°C and the detector at 325°C. Helium was used as carrier gas at a flow rate of 3 ml/min. Cholesterol was used as an internal standard. Relative retention times (RRT) were calculated by dividing the retention time of the peak of interest by the retention time for cholesterol.

Gas chromatography – mass spectrometry (GC–MS) analysis was carried out on a Micromass Auto Spec M mass spectrometer (Manchester, UK) using an OPUS V3.1 X data system. All samples were introduced using an HP 5890 Series II gas chromatograph through an OV-1 capillary column (30 m length, 0.25 mm internal diameter, 0.25 μm film thickness). The injector and interface temperatures were set at 275°C and the column oven temperature was 230°C. The spectra were acquired by scanning the mass range 40–600 Da with a scan time of 1 second per decade and 0.5 second interscan delay.

Results

Inducible pisatin resistance in D. caveatum

Like *D. discoideum*, *D. caveatum* amoebae also are ordinarily sensitive to pisatin but can acquire a pisatin-resistant phenotype if they are first grown on a sublethal pisatin concentration (figure 1). Also, growth on pisatin could induce resistance to nystatin just as it does in *D. discoideum* (figure 2). These results show that barring differences in the effective drug concentrations, the amoebae of these two species respond to pisatin in the same way. In both species, wild-type cells induced for the pisatin resistance phenotype did not become cross-resistant to pimaricin and tomatine

Figure 1. Pisatin resistance phenotype of induced wild-type *D. caveatum* and a nystatin-resistant mutant (DCN5). Uninduced amoebae of the wild-type strain (66413) are sensitive to LP-pis 70 (left plate, top streak). In contrast, 66413 amoebae that were grown on medium containing a sublethal pisatin concentration (66413 pis25) are able to grow on LP-pis70 (left plate, lower streak). However, both induced and uninduced amoebae are equally sensitive to LP-pis130 (top and bottom streaks, middle plate). Significantly, the DCN5 mutant is able to grow on LP-pis130 (right plate). The boundary between the zone of growing amoebae and the bacterial lawn is faintly visible across the lower half of the plate.

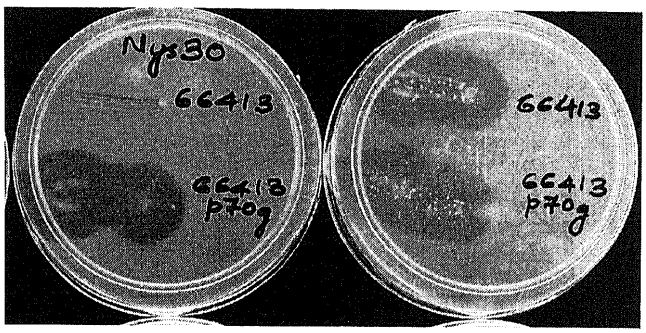

Figure 2. Growth on pisatin medium induces nystatin resistance in *D. caveatum*. Uninduced amoebae of the wild-type strain (66413) are sensitive to LP-nys30 (left plate, top streak). In contrast, 66413 amoebae that were grown on pisatin medium (66413 pis70g) are nystatin-resistant (left plate, lower streak). Both induced and uninduced amoebae grow on the control LP plate (right).

(data not shown). Pimaricin is another polyene antibiotic and α-tomatine is a steroidal glycoside from tomato whose proposed mode of action is similar to that of polyene antibiotics. These results indicate that the induction of nystatin resistance by pisatin is relatively specific and does not represent a general resistance to polyene antibiotics.

Pisatin-resistant mutants

We selected five nystatin-resistant mutants on medium containing 60 μg nystatin per ml following NTG mutagenesis. All five mutants had apparently identical phenotypes and were highly cross-resistant to pisatin. Significantly, the mutants could grow on considerably higher pisatin concentrations (130 μg/ml) than the parental wild-type amoebae induced for the pisatin resistance phenotype (typically 70 μg/ml).

The mutants made very diffuse plaques on the bacterial lawn thereby rendering difficult determination of EOPs, but

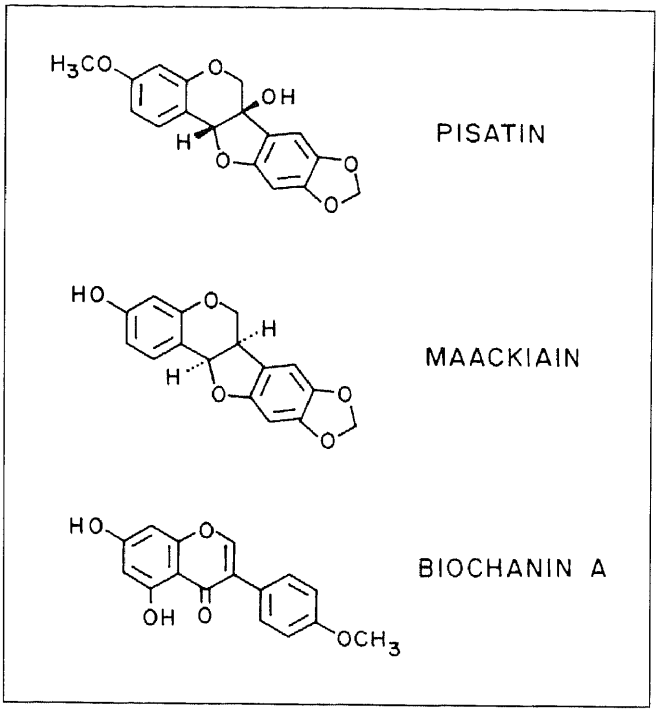

Figure 3. Structures of pisatin, maackiain and biochanin A.

we were able to ascertain their drug resistance phenotypes by streaking on drug plates (data not shown). The mutants were resistant to maackiain (50 μg/ml), biochanin A (50 μg/ml), pimaricin (20 μg/ml) and α-tomatine (120 μg/ml) whereas the wild type is sensitive to all these compounds. Maackiain is an isoflavonoid phytoalexin of chickpea (*Cicer arietinum*) and has the opposite stereochemistry to pisatin at positions 6a and 11a, and biochanin A is an isoflavone from alfalfa (*Medicago sativa*) (figure 3).

The mutations do not block wild-type sterol biosynthesis

The sterols of the mutant and the parental wild-type strains were compared by GC. The GC trace of the wild type had a single sterol peak with an RRT of 1.18. The GC traces of all five mutants had four peaks of approximately equal sizes with RRTs of 1.09, 1.18, 1.23 and 1.27. We confirmed by GC-MS that the peak with RRT 1.18 in both the mutant and the wild type represented the same sterol (MW 412). This demonstrated that the mutants can synthesize the wild-type sterol. We have not determined the chemical structures corresponding to the other peaks. One possibility is that they represent precursor sterols that are accumulated in the mutant but not in the wild type.

Discussion

The pisatin resistance phenotype of the *D. caveatum* mutants was most unexpected because mutants with this phenotype could never be obtained in *D. discoideum*. This result

demonstrates that single-gene mutations can confer pisatin resistance in at least some dictyostelids and therefore appears to challenge the hypothesis of Papavinasasundaram and Kasbekar (1993) that the inducible pisatin resistance involves independent mechanisms. However, the pisatin resistance phenotype of the *D. caveatum* mutants differed in two ways from the inducible resistance phenotype of the parental wild type. First, the mutants could grow on much higher pisatin concentrations than the induced wild type. Second, the mutants were cross-resistant to pimaricin and tomatine whereas the inducible resistance did not extend to these antibiotics. These differences suggest that the pisatin resistance phenotype of the mutants probably has a different basis than the inducible pisatin resistance of the wild type and the hypothesis that inducible pisatin resistance requires independent mechanisms remains valid.

In *D. discoideum* nystatin-resistant mutants define three complementation groups, *nysA*, *nysB* and *nysC*. The *nysA* mutants have the same sterol composition as the wild type whereas the *nysB* and *nysC* mutants have altered sterols (Scandella *et al.* 1980). The nystatin-resistant mutants of *D. caveatum* synthesized the wild-type sterol like the *D. discoideum nysA* mutants but they differed from *nysA* in that they were highly resistant to isoflavonoids, tomatine and pimaricin. We do not understand why mutants with *nysA*, *nysB* and *nysC* phenotypes were not obtained in *D. caveatum*. Efforts are on in our laboratory to clone the *nys* genes of *D. discoideum* via REMI mutagenesis (D.P. Kasbekar, T. Bhavani Prasanna, K. Aparna and H. Adachi, unpublished results). This might enable us to isolate their *D. caveatum* homologues and thereby generate *nysA*, *nysB* and *nysC* mutants in this species by targeted gene disruption. Likewise, cloning the gene(s) defined by the *D. caveatum* mutants might enable us to ask why comparable mutations have not yet been identified in their *D. discoideum* homologues.

Acknowledgements

Scott Soby and Hans VanEtten generously gifted the maackiain and biochanin A. J. Gowrishankar commented on an earlier version of the manuscript. Sherlock Holmes inspired us with the maxim (in *A Study in Scarlet*) that when a fact appears to be opposed to a long train of deductions, it invariably proves to be capable of bearing some other interpretation. This work was supported in part by a Rajiv Gandhi Research Grant for Innovative Ideas in Science and Technology to D.P.K.

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Received 13 March 1998