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Rapid identification of non-allelic nystatin resistance mutations in *Dictyostelium discoideum*

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Abstract. Spontaneous nystatin resistance mutations in *Dictyostelium discoideum* fall into three complementation groups; *nys A*, *nys B* and *nys C*. We demonstrate three methods for rapidly distinguishing mutations in the three complementation groups. In the first method *nys B* and *nys C* mutations are identified by their sensitivity to the sterol biosynthesis inhibitors azasterol A25822B and fenarimol respectively. The second method exploits the differential sensitivities of the *nys* mutations to the polyene antibiotic pimaricin. In the last method we show that *nys C* and non-*nys C* mutants can be distinguished on the basis of the Lieberman-Burchard color reaction for sterols.

Keywords. *Dictyostelium discoideum*; nystatin; pimaricin; azasterol; fenarimol.

1. Introduction

Spontaneous nystatin resistance mutations have been described in *Dictyostelium discoideum* and have been shown to fall into three complementation groups *nys A*, *nys B* and *nys C* (Scandella *et al* 1980). Mutations in *nys B* and *nys C* are associated with sterol alterations whereas *nys A* mutants have the wild-type sterol. Kasbekar *et al* (1983) showed that nystatin resistant mutants became sensitive to growth in the presence of coumarin and this was found to be useful in parasexual genetic analysis in *Dictyostelium*. Nystatin resistant mutants could be selected without mutagenesis and diploids could be selected between haploid strains bearing non-allelic nystatin mutations by complementation on coumarin-containing plates. The use of nystatin resistance mutations in parasexual genetics thus depends on distinguishing between the different *nys* complementation groups. This required crossing newly selected nystatin mutants with standard *nys A*, *nys B* and *nys C* strains and looking for non-complementation with one of them. Since this method requires three crosses to identify each unknown nystatin mutation it is quite time consuming. In an effort to simplify the method we have looked for other ways to distinguish between the complementation groups. In this work we describe three methods for rapidly identifying the complementation group of newly isolated *nys* mutations.

In the first method we show that mutations in the different *nys* genes have different patterns of cross sensitivities to the sterol biosynthesis inhibitors; azasterol

A25822B and fenarimol. In the second, we show that the different mutations can be identified by their different sensitivities to the polyene antibiotic pimaricin. The third method is based on distinguishing *nys C* mutations from *nys A* and *nys B* on the basis of the Lieberman-Burchard colour reaction for sterols.

2. Materials and methods

2.1 *Strains and growth conditions*

The origin and genotypes of most of the *nys* strains used has been described previously (Scandella *et al* 1980). Strain HK17 (*nys B 215*) was derived from the wild-type strain DdB which is itself a derivative of NC-4 (Raper 1935). All the strains were grown in association with *Enterobacter aerogenes* at 22°C on SM agar plates (Sussman 1966). The azasterol and fenarimol supplemented plates were made by adding 20 mg/ml stock solutions of the drugs in dimethyl formamide (Sigma) to the SM agar prior to pouring. The azasterol A25822B and fenarimol were gifts from the Eli Lilly Co., Indianapolis, Indiana. Pimaricin was purchased from the Sigma Chemical Co. as an aqueous suspension and added to the SM agar prior to pouring.

2.2 *Lieberman-Burchard reaction*

Amoebae were harvested (one 100 × 15 mm plate was sufficient) from a confluent plate of amoebae. After washing, sterol was prepared as previously described (Scandella *et al* 1980). The Lieberman-Burchard (LB) reagent (Cook 1961) was prepared by mixing on ice, chilled, reagent grade acetic anhydride with chilled reagent grade sulfuric acid (4:1). The dried sterol was brought up in 5 ml chloroform and 2 ml of LB reagent added at room temperature. If the reaction was positive some color was seen immediately after the mixture was vortexed.

3. Results

3.1 *Azasterol and fenarimol*

Azasterol A25822B and fenarimol are drugs known to interfere with sterol biosynthesis (Bailey *et al* 1976; Hays *et al* 1977; Woloshuk *et al* 1979; Buchenauer 1977). Since *nys B* and *nys C* mutants have sterol alterations we examined the *nys* mutations for cross resistance or sensitivity to the azasterol and fenarimol. In table 1 it can be seen that all *nys B* mutants exhibit sensitivity to growth in the presence of 15 μg/ml of the azasterol whereas all *nys C* mutants exhibit sensitivity to fenarimol at 2 μg/ml. The wild type can grow on both drugs. Of the two *nys A* mutants, *nys A201* can grow on both drugs but *nys A212* is sensitive to the azasterol and resistant to fenarimol. We have shown previously that these two alleles also differ in their coumarin sensitivity (Kasbekar *et al* 1983). Although Kasbekar (unpublished) has shown that *nys A201* and *nys A212* do not complement genetically, the anomalous semi-dominant behavior of *nys A212* forces us to leave

Table 1. Plating efficiencies of wild-type and nystatin-resistant mutant amoebae on azasterol A25822B and fenarimol containing agar

Strain (*nys* allele)	E.o.P. on 15 μg/ml azasterol	E.o.P.* on 2 μg/ml fenarimol
DdB (*nys*$^+$)	0·4	1.0
M28 (*nys*$^+$)	0·71	1·0
ts12M (*nys*$^+$)	0·35	$7{\cdot}1 \times 10^{-3}$
HK4 (*nys A201*)	1·0	1·0
HK14 (*nys A212*)	$2{\cdot}36 \times 10^{-5}$	0·93
HK17 (*nys B215*)	$< 10^{-6}$	1·0
HK7 (*nys B205*)	$1{\cdot}04 \times 10^{-6}$	0·9
HK8 (*nys B206*)	$< 10^{-6}$	1·0
HK5 (*nys B203*)	$< 10^{-6}$	$7{\cdot}1 \times 10^{-3}$
HK13 (*nys B211*)	$< 10^{-6}$	0·2
HK10 (*nys C208*)	1·0	$< 1{\cdot}9 \times 10^{-7}$
HK15 (*nys C213*)	0·18	$4{\cdot}5 \times 10^{-5}$

*Efficiency of plating (E.o.P) was calculated as the ratio of the number of plaques appearing on the drug plates divided by the number of plaques of the same culture that appeared on a non-drug plate.

the question of *nys A* allelism unresolved. Since spontaneous *nys A* alleles arise very infrequently (only two have been identified so far), this ambiguity does not present a serious problem.

Although the strain ts12M is wild-type at all three *nys* loci, it grows rather poorly on 2 μg/ml fenarimol, hence growth inhibition by fenarimol-agar is not as reliable a test to identify *nys C* mutations derived in this strain. These results show that streak testing on azasterol-agar is a simple means of distinguishing *nys B* from non-*nys B* mutations and if the parental strain is not already fenarimol-sensitive, sensitivity to fenarimol can be used to distinguish *nys C* from non-*nys C*.

3.2 *Azasterol-fenarimol synergism*

Since *nys B* mutants are sensitive to the azasterol and *nys C* mutants are sensitive to fenarimol, we examined the possibility of selecting complementing diploids on azasterol-fenarimol plates. This could not be accomplished, however, since quite surprisingly wild-type cells were found to have an extremely low plating efficiency ($< 3 \times 10^{-5}$) on the double drug plates. That is, whereas wild-type cells are resistant to either drug alone, they are sensitive to the two drugs together. It is possible to explain this result on the basis of an earlier finding that wild-type cells grown on the azasterol accumulated a sterol different from stigmastenol (Kasbekar *et al* 1985). We propose that the presence of this new sterol confers sensitivity to fenarimol in a manner similar to the fenarimol sensitivity conferred by *nys C* sterol. This model is supported by the observation that azasterol grown amoebae are nystatin resistant and spores derived from such amoebae can germinate on nystatin containing plates just like *nys C* mutants (Kasbekar *et al* 1985). Furthermore, azasterol grown amoebae show temperature sensitive developmental abnormalities similar to those shown by *nys C* amoebae (Kasbekar 1984).

3.3 *Pimaricin*

Pimaricin, like nystatin is a member of the polyene family of antibiotics. We first determined the minimum inhibitory concentration of pimaricin for the wild-type strain (DdB) and found it to be 17 μg/ml (table 2). At this concentration the *nys A* and *nys B* mutants were also sensitive but the *nys C* mutants were highly resistant (table 2). Since the *nys B* mutants showed a two-order of magnitude lower plating efficiency at 17 μg/ml compared with the wild-type, we examined lower pimaricin concentrations. At 10 μg/ml *nys B* mutants are still sensitive to the drug but the wild-type, *nys A* and *nys C* mutants are still resistant. Thus by screening the nystatin resistant mutants on 10 μg/ml and 17 μg/ml pimaricin we can rapidly distinguish the different *nys* complementation groups.

3.4 *Lieberman-Burchard reaction*

The Lieberman-Burchard reaction (Cook 1961) is a colorimetric assay for sterols. In order to produce a color, a sterol must have a 3′ OH and a double bond in the B ring. Since the wild-type sterol, stigmastenol, is saturated in the B ring it gives no color. Since *nys B* and *nys C* mutants have altered sterols (Scandella *et al* 1980) it was of interest to test them. The *nys B* mutants gave no color but all the *nys C* mutants produced a very dark purple color which changed to blue and then blue-gray with time. The positive color reaction of *nys C* sterols suggests that they contain a double bond in the B ring. The lack of color in the *nys B* mutants suggests that the principle sterol in this mutant, although different from the wild-type, is still saturated in the B ring. As expected, the *nys A* mutants which possess the wild-type sterol (Scandella *et al* 1980), also gave no color. Thus the Lieberman-Burchard reaction provides a rapid way of distinguishing *nys C* from non-*nys C* nystatin resistant mutants.

Table 2. Plating efficiencies of wild-type and nystatin-resistant mutant amoebae on pimarin containing agar

Strain (*nys* allele)	E.o.P. on 10 μg/ml pimaricin	E.o.P. on 17 μg/ml pimaricin
DdB (*nys*$^+$)	0·43	$9{\cdot}23 \times 10^{-6}$
M28 (*nys*$^+$)	1·0	$1{\cdot}9 \times 10^{-5}$
ts12M (*nys*$^+$)	0·47	$2{\cdot}33 \times 10^{-6}$
HK4 (*nys A201*)	0·68	$3{\cdot}85 \times 10^{-4}$
HK14 (*nys A212*)	0·12	$2{\cdot}33 \times 10^{-4}$
HK17 (*nys B215*)	$1{\cdot}3 \times 10^{-5}$	$< 1{\cdot}67 \times 10^{-7}$
HK7 (*nys B205*)	$< 1{\cdot}85 \times 10^{-7}$	$< 1{\cdot}85 \times 10^{-7}$
HK8 (*nys B206*)	$< 2{\cdot}53 \times 10^{-7}$	$< 2{\cdot}53 \times 10^{-7}$
HK5 (*nys B203*)	$1{\cdot}93 \times 10^{-5}$	$1{\cdot}89 \times 10^{-6}$
HK13 (*nys B211*)	$< 2{\cdot}22 \times 10^{-7}$	$< 2{\cdot}22 \times 10^{-7}$
HK10 (*nys C208*)	0·78	$3{\cdot}4 \times 10^{-2}$
HK15 (*nys C213*)	0·77	0·23

Efficiency of plating (E.o.P) was calculated as the ratio of the number of plaques appearing on the drug plates divided by the number of plaques of the same culture that appeared on a non-drug plate.

Discussion

We have discribed three ways to distinguish non-allelic nystatin resistance mutations. In the first, we have used the sterol biosynthesis inhibitors azasterol A25822B and fenarimol. Azasterol has been shown to block the reduction of the Δ^{14} unsaturation that follows the C-14 demethylation of lanosterol (Bailey *et al* 1976; Hays *et al* 1977; Woloshuk *et al* 1979). If the same mechanisms of action occur in *D. discoideum* we would expect amoebae grown in the presence of azasterol to contain different sterols from amoebae grown on normal medium. This is indeed the case (Kasbekar *et al* 1985). Since *nys B* mutations affect sterol biosynthesis (Scandella *et al* 1980), we propose that the azasterol induced alteration superimposed on the defective *nys B* pathway results in the production of a sterol that does not support growth.

Fenarimol is known to block the C-14 demethylation step (Buchenauer 1977). Therefore it appears that sterols methylated at C-14 are unable to function in *Dictyostelium* membranes. It is possible that the fenarimol-sensitivity of *nys C* mutants results from an increase in membrane permeability causing the drug to accumulate to toxic levels. Scandella *et al* (1980) have shown that *nys C* mutants that are obtained in a cycloheximide resistant strain are more sensitive to cycloheximide than the parental strain.

In the second method of distinguishing non-allelic *nys* mutations we used the polyene antibiotic pimaricin and were able to demonstrate differential sensitivity of mutations in the three nystatin resistance genes. Although pimaricin, like the other polyenes, depends on binding to sterol for its effectiveness (Medoff and Kobayashi 1980), it is not unusual to find different patterns of cross resistance among different polyene resistant mutants (Scholer and Polak 1984).

The third way of distinguishing the mutants used the Lieberman-Burchard reaction. Since both *nys B* and *nys C* mutations have previously been shown to produce sterol alterations, the Lieberman-Burchard reaction, in addition to providing a way to distinguishing *nys C* from non-*nys C* nystatin resistance mutations, also indicates that *nys C* sterol is unsaturated in the B ring.

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