The Differential Effects of Nystatin on Growth of Auxotrophic and Prototrophic Strains of *Aspergillus nidulans*

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**Summary**

Nystatin affects the transfer of nutrients into fungal cells. Auxotrophs of *Aspergillus nidulans* vary in their susceptibility to nystatin, hence it is possible that they could be used to diagnose the effects of this antibiotic on cellular transport mechanisms for the uptake of different metabolites. Nystatin treatment can select auxotrophs from a largely prototrophic population in *A. nidulans* because newly germinated conidia are more sensitive to nystatin than ungerminated conidia. As the mycelium of germinated conidia ages it becomes less sensitive, so that critical conditions for maximum selection are a suitable period on minimal medium agar, sufficient only for germination of prototrophs, followed by the addition of nystatin in complete medium agar at a level which preferentially kills germinated conidia, thus allowing enrichment for ungerminated auxotrophic conidia. After treatment of prototrophic conidia of *A. nidulans* with ultraviolet irradiation auxotrophic selection was achieved by the nystatin method.

**Introduction**

The polyene antifungal antibiotic, nystatin, is effective in selecting auxotrophs, when these are present in mixed populations with prototrophs, both in *Saccharomyces cerevisiae* (Snow, 1966) and in *Penicillium chrysogenum* (Macdonald, 1968). The methods are similar to those for the enrichment of auxotrophic bacteria using penicillin (Davis, 1948; Lederberg & Zinder, 1948). In the technique devised for *P. chrysogenum* conidia from a prototroph were treated with a mutagenic agent and plated on minimal medium agar for a period sufficient to allow prototrophic conidia to germinate. When nystatin, incorporated in complete medium agar, was then added as an overlay, germinated conidia were apparently more sensitive to the effects of the antibiotic than ungerminated conidia so that auxotrophs which had arisen following mutagenesis survived preferentially. Nystatin gradually lost its activity under the experimental conditions used (Eisenberg, Weiss & Flippin, 1956; Kubista & Derse, 1959; Macdonald, 1968) so eventually auxotrophic conidia could germinate and grow and thus were enriched relative to prototrophs.

In the present studies the nystatin method for the selection of auxotrophs of *Penicillium chrysogenum* was applied successfully to *Aspergillus nidulans*. Experiments to examine suppositions made by Macdonald (1968) to explain the selection of auxotrophs by nystatin showed that in *A. nidulans* differential sensitivity to the antibiotic between germinated and ungerminated conidia existed for only a short period after germination but sufficiently long to allow selection to operate when a suitable concentration of nystatin was used.

Auxotrophs with different growth factor requirements differed in their sensitivity to nystatin suggesting that their behaviour could prove useful in studies on the mode of action of the antibiotic.
METHODS

Organisms. Two wild-type strains of Aspergillus nidulans were obtained from the Department of Genetics of the University of Birmingham. Their Birmingham numbers were 33 and 66. From strain no. 66 a series of mutants were produced by treatment with ultraviolet (u.v.) irradiation (see below). The strain numbers of these mutants, their growth factor requirements and mutant spore colour (where applicable) are shown below with their abbreviations in parentheses: 66/2, adenine (ade-01); 66/4, proline (pro); 66/7, riboflavin (rib); 66/9, yellow spores, proline (yel pro); 66/18, adenine (ade-02); 66/21, p-aminobenzoic acid (pab); 66/22, methionine (met). Strains 66/2 and 66/18 were different isolates.

Media. The minimal medium agar (MM) was that of Pontecorvo et al. (1953) with the following composition: NaNO₃, 6 g.; KCl, 0.52 g.; MgSO₄·7H₂O, 0.52 g.; KH₂PO₄, 1.52 g.; FeSO₄·7H₂O, 0.01 g.; ZnSO₄·7H₂O, 0.01 g.; glucose, 10 g.; biotin, 0.001 μg.; agar (Oxoid no. 3), 12 g.; distilled water to 1 l. The pH was adjusted to 6.5 and the media autoclaved at 115° for 10 min. The complete medium agar (CM) was as described by Macdonald, Hutchinson & Gillett (1963). It contained KCl, 0.5 g.; MgSO₄·7H₂O, 0.5 g.; FeSO₄·7H₂O, 0.01 g.; KH₂PO₄, 1 g.; corn-steep liquor (Garton’s), 10 g.; 3 ml. each of thymus and yeast nucleic acid hydrolysates (Pontecorvo et al. 1953); DL-methionine, 0.05 g.; phenylacetylthanolamine, 1 g.; riboflavin, 2.6 mg.; sucrose, 30 g.; agar (Oxoid no. 3), 12 g.; distilled water to 1 l. The medium was adjusted to pH 6 and autoclaved at 115° for 10 min.

Plating procedures. In all experiments replication was done ten times and each Petri dish was plated with approximately 100 conidia except after mutagenic treatment when allowance was made for the reduction in viability. All incubations were at 37°.

Mutagenic treatment. A conidial suspension (10⁶/ml.) in distilled water was irradiated with u.v. from a Hanovia lamp type 11 for 8 min. (survival after irradiation 1 to 5%). The suspension (10 ml.) in an uncovered Petri dish (9 cm. in diam.) was mechanically rocked 30 cm. underneath the lamp.

Nystatin stock suspension. Nystatin (Mycostatin; E. R. Squibb & Sons, New York) was supplied in vials containing 500,000 units, 1 mg. of the solid material being approximately equal to 3000 units. The contents of a vial were suspended in 100 ml. of sterile distilled water to form a stock suspension of 5000 units/ml. and stored in the dark at 4° for not longer than 4 weeks. Immediately before use the contents were shaken to ensure even suspension of the insoluble nystatin.

Nystatin treatment. Twenty ml. of melted MM, cooled to 50°, were poured into a sterile plastic Petri dish (9 cm. in diam.). After solidification 0.05 ml. of spore suspension was spread on the surface of the medium. An overlay of CM containing nystatin was added immediately or after a period of incubation. The CM was melted, cooled to 50° and the nystatin added just before dispensing. The concentrations of nystatin referred to in the text are those incorporated in CM. Petri dishes were then incubated for 1 week and colonies counted.

Isolation of auxotrophs. Colonies which developed from conidia surviving treatment were isolated by the total isolation method and tested and characterized as described by Pontecorvo et al. (1953).
RESULTS

Conidia of *Aspergillus nidulans* strain no. 66 and of several auxotrophic mutants were plated on MM and overlayered immediately with CM containing increasing levels of nystatin (Fig. 1). Auxotrophs differed in their sensitivity to the effects of nystatin. The behaviour of the two strains requiring amino acids, which survived least well in the presence of low concentrations of nystatin was in agreement with the results of Stachiewicz & Quastel (1963), who reported that transport mechanisms for amino acids are among the most sensitive to the action of nystatin in yeast.

![Fig. 1. Survival of conidia from prototrophic strain no. 66 of *Aspergillus nidulans* and from its auxotrophic derivatives after treatment with various concentrations of nystatin. Conidia plated on MM and overlayered immediately with CM containing the antibiotic. ×—×, Prototrophic conidia; —,— auxotrophic conidia; Δ, ade-01; ○, pro; □, rib; ▲, ade-02; ■, pab; ○, met.](image-url)

The survival of conidia from the prototroph and an auxotroph were then compared after increasing periods of incubation on MM before the addition of CM without and with nystatin. Strain *yal pro* was chosen as the auxotroph since this isolate had yellow conidia and could be distinguished visibly from the green sporing prototroph. The survival of conidia from the *yal pro* strain was similar to that of the conidia from the *pro* strain under the experimental conditions illustrated in Fig. 1.

Conidia of strains no. 66 and *yal pro* were plated on MM followed, after increasing
lengths of time, by the addition of a layer of CM without nystatin (Fig. 2). The viability of the prototroph gradually fell when the period without CM was prolonged; after 16 h. on MM it was reduced to about 80% (Fig. 2). When conidia of the prototroph were plated on MM and on CM separately their viabilities were similar so that this fall cannot be attributed

![Graph](image)

Fig. 2. Survival of conidia from prototrophic strain no. 66 of *Aspergillus nidulans* and from its auxotrophic derivative, *yel pro*, when plated on MM followed after increasing time by an overlay of CM without (broken line) or with (continuous line) nystatin (80 units/ml); ×, Prototroph; ○, auxotroph.

![Graph](image)

Fig. 3. Survival of conidia from prototrophic strain no. 66 of *Aspergillus nidulans* and from its auxotrophic derivative, *yel pro*, when plated on MM followed after increasing time by an overlay of CM containing various concentrations of nystatin. Continuous line (×), prototroph; broken line ○, auxotroph. The number of hours on MM before adding CM with nystatin are shown above each graph.
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to different survival levels on the two media. It is possible that enzyme systems can become unbalanced to some extent if CM is added after metabolism has become adapted to MM and lead to loss of viability. Auxotrophic conidia survived even less well than prototrophic conidia as the period of MM was extended (Fig. 2). Pontecorvo et al. (1953) have shown that when conidia of monoauxotrophs are plated on MM they die off because of starvation for their specific growth factor. The increased death rate of auxotrophic conidia relative to prototrophic conidia may therefore be explained as a consequence of the lack of proline in MM.

Conidia from the prototroph and the yel pro strains were then spread on MM and CM containing nystatin as an overlay. Conidial viability was measured after varying both the concentration of nystatin in the overlay and the period at which the overlay was added; nystatin concentrations of 0, 20, 40, 60, 80, 100 and 120 units/ml. were used after periods on MM of 0, 1, 3, 5, 7, 9, 11 and 16 h. The results after 0, 5, 7 and 16 h. incubation on MM before overlaying with CM and nystatin are shown in Fig. 3. After 1, 3, 9 and 11 h. on MM the results were similar to those after 0 and 16 h. on MM; the prototroph survived better than the auxotroph at all concentrations of nystatin. However, after 5 and 7 h. on MM before the addition of nystatin at concentrations of 80, 100 and 120 units/ml., the auxotroph survived better than the prototroph (Fig. 3). This is more clearly illustrated in Fig. 2 where the conidial viabilities of both strains are shown after different periods on MM prior to the addition of nystatin in CM at a concentration of 80 units/ml. When CM with the antibiotic was added immediately after plating the conidia on MM, this amount of nystatin reduced the initial viability of both prototrophic and auxotrophic conidia, but the latter more than the former.

For the yel pro strain, when the period on MM was increased to 16 h. before the addition of CM with 80 units of nystatin/ml., conidial viability fell to about 50%, of that found when CM with nystatin was added at 0 h.; this was similar to the fall shown after 16 h. on MM in the experiment when CM without the antibiotic was added and was attributed to the effects of starvation for proline, as mentioned earlier (Fig. 2). The prototrophic conidia behaved differently. At the shorter periods of incubation on MM only survival was similar to that of the control when CM with nystatin was added immediately after plating conidia on MM. But after 5 h. there was a sudden drop in viability which remained low until about 8 h., when it began to increase. These results were interpreted as follows: most of the prototrophic conidia had germinated after 4 or 5 h. on MM, and for the next 2 or 3 h. the young mycelia were particularly sensitive to the effect of nystatin; later, as the mycelia became older, they became less susceptible, and eventually became as resistant as ungerminated conidia (cf. survivals at 0 and 16 h., Fig. 2). Considering the loss of viability among prototrophic conidia when the period of MM was extended to 16 h., before overlaying with CM not containing nystatin, this latter result suggested in fact that old mycelium might be more resistant to the effects of nystatin than ungerminated conidia.

An attempt was then made to select auxotrophs from a population of prototrophic conidia of Aspergillus nidulans which had received u.v. irradiation. Birmingham strain no. 33 was chosen because auxotrophs of this strain were required for other work, and difficulty had been experienced in isolating them by non-selective total isolation methods. In preliminary experiments it was discovered that the period of incubation on MM alone had to be extended from 5 to 7 h. to 7 to 9 h. in order that selection could operate. It was also found that higher concentrations of nystatin were necessary than in reconstruction experiments; 120 to 160 units/ml. were more suitable than 80 to 120 units/ml. It may be that differences between strain no. 33 and strain no. 66 were responsible; the latter and a derivative were used in
reconstruction experiments. However, the increased period on MM might have been required because u.v. treatment delays germination. It is also possible to suggest why an increase in the amount of nystatin was necessary without invoking differences between strains as an explanation. Because of the loss of viability due to u.v. irradiation, 100 times as many conidia were plated per Petri dish than in reconstruction experiments. Nystatin is thought to act on fungal cells by becoming bound to a sterol in the cell membrane (Lampen, 1966) and, although most of the conidia in the sample would be inviable after u.v. irradiation, the greater number plated probably increased the number of binding sites available, thus necessitating the increase in nystatin concentration. The results of an experiment shown in Table I indicated that the nystatin technique could be used successfully to select auxotrophs of *A. nidulans*.

**Table 1. Auxotrophic yield when conidia of prototrophic strain Birmingham no. 33 of Aspergillus nidulans were treated with nystatin after u.v. irradiation**

Conidia (irradiated to 1% survival) were plated on MM and incubated for 7 and 9 h. before addition of CM containing different concentrations of nystatin. Colonies which arose from surviving conidia were tested for auxotrophy. The broad groups to which auxotrophs belonged are indicated by their growth responses to vitamins (V), amino acids (A), nucleic acid derivatives (D), reduced inorganic sulphur (S), and reduced inorganic nitrogen (N) in the final column of the table.

<table>
<thead>
<tr>
<th>Period on MM agar (h.)</th>
<th>Concentration of nystatin in CM agar (u./ml)</th>
<th>Survival after nystatin treatment (%)</th>
<th>No. of colonies isolated</th>
<th>Auxotrophs</th>
<th>Growth responses of auxotrophs*</th>
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<td></td>
<td></td>
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<td>%</td>
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* Additions to MM as follows:

V = Vitamins (% w/v): riboflavin, 0.001; nicotinamide, 0.001; p-aminobenzoic acid, 0.0001; pyridoxin HCl, 0.0005; thiamine HCl, 0.0005; biotin, 0.0001; Ca pantothenate, 0.002; choline HCl, 0.002; inositol, 0.004.

A = Casamino acids (Difco), 2.5% (w/v).

D = 1.5% yeast and 1.5% thymus nucleic acid hydrolysates (v/v) prepared according to Pontecorvo *et al.* (1953).

S = Na$_2$S$_2$O$_3$, 0.25% (w/v).

N = (NH$_4$)$_2$SO$_4$, 0.25% (w/v).

**DISCUSSION**

The differing sensitivities of auxotrophs to nystatin might prove a valuable tool in biochemical studies on the mode of action of this antibiotic. The susceptibilities of particular auxotrophs to nystatin could be a more precise method of diagnosing the effects of the antibiotic on the transport mechanisms for individual metabolites than biochemical studies with prototrophic cultures. Although Stachiewicz & Quastel (1963) have reported that amino acid transport into yeast cells is particularly sensitive to nystatin, their work has been criticized by Lampen (1966) on the grounds that they used unbuffered cells at pH 5 and this could have led to irreversible changes in the cells not related to nystatin treatment. However, the limited results we have obtained agree with those of the former authors.
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In *Penicillium chrysogenum*, after u.v. treatment, the number of auxotrophs isolated following selection with nystatin suggested that all auxotrophic conidia survived antibiotic treatment (Macdonald, 1968). However, at the concentrations of nystatin necessary for selection in *Aspergillus nidulans* there was a loss of viability among prototrophic and auxotrophic conidia. With nystatin selection in *P. chrysogenum* there was no apparent selection for any particular sorts of auxotrophs (Macdonald, 1968). In *A. nidulans*, since different auxotrophs differed in their susceptibility to nystatin, it could be expected that this would be reflected in the proportions of auxotrophic types found after selection. Although in reconstruction experiments strains requiring amino acids, for example, survived relatively poorly in the presence of nystatin (Fig. 1), there was no evidence of a shortage of these types when the antibiotic was used to enrich for auxotrophs after u.v. treatment of a prototroph (Table 1). The quantitative and qualitative spectrum of auxotrophic types found after nystatin treatment (Table 1) can be compared with the results obtained by Pontecorvo et al. (1953) after total isolation. With the provisos that the latters’ data were not homogeneous since different strains of *A. nidulans* were used and also that the strain employed in the present work differed from the strains used by Pontecorvo et al. (1953), the comparison suggests that after nystatin selection there is a shortage of types requiring reduced inorganic nitrogen and reduced inorganic sulphur. In the reconstruction experiment, when the survival of conidia from different auxotrophs were compared, the conidia were plated on MM and overlaid immediately with CM containing nystatin (Fig. 1). On the other hand, when auxotrophs were selected following nystatin treatment of prototrophic conidia which had been u.v. irradiated, the latter were incubated for a period on MM alone before CM with nystatin was added. It may be that after a few hours on MM auxotrophic conidia were in a different physiological state from dormant conidia exposed immediately to the effects of nystatin so that their spectrum of susceptibility to the antibiotic was different.

Suggestions put forward to account for the selection of auxotrophs with nystatin from a largely prototrophic population in *Penicillium chrysogenum* (Macdonald, 1968) have been supported by the present studies with *Aspergillus nidulans*. It is clear that the length of the incubation period on MM alone and the concentration of nystatin to be added afterwards are both critical factors in deriving optimum conditions for the selection of auxotrophs.

We are grateful to Professor J. L. Jinks, F.R.S., of the University of Birmingham for the gift of the two cultures of *Aspergillus nidulans*.

REFERENCES


