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SHORT PAPER

The selection of auxotrophs of *Penicillium chrysogenum* with nystatin

By K. D. MACDONALD

Microbiological Research Establishment, Porton Down, Salisbury, Wiltshire

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1. INTRODUCTION

The discovery of antifungal antibiotics led to the hope of devising a technique of auxotrophic selection in fungi similar to the penicillin method so widely used in bacteria (Davis, 1948; Lederberg & Zinder, 1948). In yeast, several antifungal antibiotics have been examined in reconstruction experiments where artificial mixtures of auxotrophs and prototrophs were treated with antibiotic under conditions allowing growth of prototrophs but not of auxotrophs. Among those antibiotics found to favour the survival of auxotrophs was nystatin (Moat & Srb, 1957; Moat, Barnes & McCurley, 1966).

This polyene antibiotic is believed to kill fungal cells by becoming bound to a sterol in the cell membrane (Lampen, 1966). Sterol molecules probably impart mechanical strength to the membrane by aligning phospholipids and proteins. Nystatin affects the membrane structure in such a way as to damage transport systems for small molecules so that the organism is unable to concentrate essential metabolites; an added consequence is the leakage of small ions and sugars (Lampen, 1966).

Although, in initial studies with yeast, it was found possible to enrich auxotrophs in reconstruction experiments, a technique for auxotrophic selection after mutagenic treatment was not confirmed (Moat & Srb, 1957; Moat *et al.* 1966). A problem in yeast is that respiratory deficient mutants survive nystatin treatment and when the initial proportion of auxotrophs is low their enrichment is largely masked by the survival of respiratory deficient prototrophs. Recently, Snow (1966) has described a technique where prototrophic yeast cells were treated with nystatin after mutation had been induced by ethyl methane sulphonate. He found that about 10% of the cells surviving antibiosis were auxotrophs. However, since a period of growth was allowed on a complete medium between mutagenic and nystatin treatment, the auxotrophs could have included clones of mutants.

The auxotrophic selection method reported here has been developed with the filamentous fungus, *Penicillium chrysogenum*, and use has been made of two properties of nystatin. First, young mycelium is more sensitive to the effects of nystatin than old mycelium or spores (Stanley & English, 1965) and secondly, nystatin gradually loses its activity on complex media at the incubation temperature employed in the work to be described (see discussion on unpublished results in pamphlet issued by Squibb with each vial of nystatin). It was planned that the technique would operate as follows. After mutagenic treatment, conidia would be plated on minimal medium agar to allow germination of prototrophs, followed, after a suitable interval, by the addition of complete medium agar containing sufficient nystatin to kill germinated spores but not to harm ungerminated spores. It was hoped that the sequence of events would then be: (*a*) death of prototrophs; (*b*) loss of nystatin activity to a level below that which was lethal to germinated spores; (*c*) germination and growth of auxotrophs.

2. MATERIALS AND METHODS

Organism. P. chrysogenum strain NRRL 1951 was used (Raper & Alexander, 1945). This is a prototroph and was the original parent of industrial strains in current use for penicillin production.

Incubation. This was at 25 °C.

Media. Minimal medium (MM) agar and complete medium (CM) agar were as described by Macdonald, Hutchinson & Gillett (1963).

Mutagenic treatment. Ten ml. of a conidial suspension of strain NRRL 1951 in distilled water (10^6 conidia/ml.) were irradiated with ultraviolet (u.v.) light from a Hanovia lamp Type 11 for 10 min. The suspension was contained in a $3\frac{1}{2}$ in. diameter Petri dish, with lid removed at a distance of 30 cm from the u.v. source and was mechanically rocked during treatment.

Nystatin treatment. The nystatin preparation used was manufactured by Squibb (Trade name: Mycostatin, 500000 units/vial; 1 mg of solid is approximately equal to 3000 units). Plastic 'Sterilin' Petri dishes of $3\frac{1}{2}$ in. diameter containing 20 ml of MM agar were spread with 0·1 ml amounts of conidial suspensions of required density and then incubated. After allowing a period of time for prototrophs to germinate 15 ml of CM agar containing nystatin was added as an overlay. The nystatin was made up freshly as a suspension in distilled water and added to the molten CM agar cooled to 50 °C. The CM was shaken to disperse the nystatin before pouring. Plates were then incubated for about 3 weeks and colonies which developed were isolated by total isolation methods and characterized as described by Pontecorvo *et al.* (1953).

Table 1. *The effect on auxotrophic yield when conidia of Penicillium chrysogenum treated with ultraviolet light were plated on minimal medium (MM) agar for varying times followed by the addition of complete medium (CM) agar containing different concentrations of nystatin*

Period of u.v. treatment (min)	Period on MM agar (h)	Concentration of nystatin in CM agar (units/ml)	% viability relative to unirradiated control	% viability after nystatin treatment	% auxotrophs*
0	0	0	100	—	0
10	0	0	1·270	100	1
10	4	50	0·113	10·00	8·7
		75	0·800	7·05	6·7
		100	0·063	5·57	2·9
10	6	50	0·092	8·11	4·8
		75	0·049	4·37	14·4
		100	0·027	2·39	5·8
10	8	50	0·063	5·57	17·3
		75	0·032	2·86	14·4
		100	0·020	1·72	9·6
10	16	50	0·064	5·67	11·5
		75	0·039	3·49	4·8
		100	0·011	0·99	12·5

* One hundred and four isolates were tested for auxotrophy in each series.

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3. RESULTS

Reconstruction experiments with strain NRRL 1951 and a yellow-spored auxotroph of this strain showed that when the method of nystatin treatment described above was employed there was selection for the auxotroph. The nest step was to attempt auxotrophic enrichment after mutagenesis. Mutagenic and nystatin treatment were carried out as described; two variables examined were time on MM agar before nystatin application and amount of nystatin added. The results in Table 1 show clear evidence that there was selective enrichment of auxotrophs. The treatment giving the highest percentage of auxotrophs was 8 hours on MM agar followed by 50 u./ml of nystatin in CM agar.

After u.v. treatment, 1 % of the isolates tested were auxotrophs; this is a slightly higher frequency than found previously (Macdonald *et al.* 1963). In the series yielding most mutants, where there was a drop in viability to 5·57 % due to the effects of nystatin, 17·3 % of the isolates were auxotrophs. In this series the number of auxotrophs per 100 conidia surviving u.v. treatment can be calculated as 0·96 (i.e. $17{\cdot}3 \times 0{\cdot}057$) which is hardly different from the 1 % found without nystatin selection. The qualitative and quantitative spectra of mutants discovered after nystatin treatment should not differ therefore from those in untreated material. Auxotrophs isolated from the above nystatin series giving 17·3 % mutants were grouped as amino acid dependent, vitamin dependent, etc., and compared with those obtained in previous work with *P. chrysogenum* where mutation was induced by u.v. light and isolates made without nystatin selection (Macdonald *et al.* 1963). The results in Table 2 show no obvious selection for any particular groups of mutants isolated by the nystatin method.

Table 2. *A comparison of the frequency of different types of auxotrophs obtained after ultraviolet light treatment with and without nystatin selection*

	Auxotrophs with growth factor requirements for											
	Amino acid		Vitamin		Adenine		$(NH_4)_2SO_4$		$Na_2S_2O_3$		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
With nystatin selection	7	38·8	7	38·8	1	5·6	2	11·2	1	5·6	18	100
Without nystatin selection	13	43·3	10	33·3	1	3·3	3	10	3	10	30	100

4. DISCUSSION

In the method reported here the experimental manipulations have been greatly simplified in comparison with those used in yeast (Snow, 1966) and it has been found possible to select auxotrophs directly after mutagenic treatment. Methods for the enrichment of auxotrophs can lead to the selection of particular types (Macdonald & Pontecorvo, 1953; Woodward, De Zeeuw & Srb, 1954), but there was no evidence that this occurred with the present technique since auxotrophs survived the concentration of nystatin used. It is believed that the technique could be applied to other moulds, particularly filamentous fungi. Adjustments of the time on MM agar and of the level of nystatin in CM agar might be necessary, especially if the incubation temperature was different from 25 °C, since the inactivation of nystatin is temperature dependent.

Certain potent mutagens (see, for example, Adelberg, Mandel & Chein Chung Chein, 1965) which give high yields of auxotrophs, may cause multiple genetic damage which would not always be desirable, particularly if labelled strains with single gene changes are required; the selection method described here offers an alternative way of augmenting

auxotrophic yield using relatively mild mutagens which lessen the possibility of excessive genetic impairment. However, if only a large stock of auxotrophs is required, without regard to the genetic mechanisms involved, since there are chemical mutagens more effective than u.v. light, their use could further increase the proportion of auxotrophs isolated after nystatin treatment.

Multi replication rather than total isolation and the chemical restriction of colony size, allowing more colonies per plate, both could increase the efficiency of the technique (Roberts, 1959; Macintosh & Pritchard, 1963). The pH of the medium influences absorption of nystatin by fungal cells so that the selective efficiency of the method might also be improved by attention to this detail.

SUMMARY

A simple technique for the selection of auxotrophs of *Penicillium chrysogenum* after mutagenic treatment has been devised using nystatin. It relies on the differential sensitivity of germinated and dormant conidia to nystatin and on the gradual loss of antibiotic activity which occurs on complex media at laboratory incubation temperatures. After mutagenic treatment, conidia were plated on MM agar to allow germination of prototrophs and then overlaid with CM agar containing enough nystatin to kill germinated conidia but not ungerminated auxotrophic conidia. When nystatin activity had decreased sufficiently the latter germinated and grew and thus were enriched relative to prototrophs.

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REFERENCES

Adelberg, E. A., Mandel, M. & Chien Chung Chein, G. (1965). Optimal conditions for mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in *Escherichia coli* K12. *Biochem. Biophys. Res. Commun.* **18**, 788–795.

Davis, B. D. (1948). Isolation of biochemically deficient mutants of bacteria by penicillin. *J. Am. Chem. Soc.* **70**, 4267.

Lampen, J. O. (1966). Interference by polyenic antifungal antibiotics (especially nystatin and filipin) with specific membrane functions. *Symp. Soc. gen. Microbiol.* **16**, 111–130.

Lederberg, J. & Zinder, N. (1948). Concentration of biochemical mutants of bacteria with penicillin. *J. Am. Chem. Soc.* **70**, 4267–4268.

Macdonald, K. D. & Pontecorvo, G. (1953). Starvation technique. *Adv. Genet.* **5**, 159–170.

Macdonald, K. D., Hutchinson, J. M. & Gillett, W. A. (1963). Isolation of auxotrophs of *Penicillium chrysogenum* and their penicillin yields. *J. gen. Microbiol.* **33**, 365–374.

Macintosh, M. E. & Pritchard, R. H. (1963). The production and replica plating of microcolonies of *Aspergillus nidulans*. *Genet. Res. Camb.* **4**, 320–322.

Moat, A. G. & Srb, A. M. (1957). Selection and isolation of yeast mutants using antibiotics. *Bact. Proc.* p. 53.

Moat, A. G., Barnes, I. J. & McCurley, E. H. (1966). Factors affecting the survival of auxotrophs of *Saccharomyces cerevisiae* in mixed populations. *J. Bact.* **92**, 297–301.

Pontecorvo, G., Roper, J. A., Hemmons, L. M., Macdonald, K. D. & Bufton, A. W. J. (1953). The genetics of *Aspergillus nidulans*. *Adv. Genet.* **5**, 141–238.

Raper, K. B. & Alexander, D. F. (1945). Penicillin. V. Mycological aspects of penicillin production. *J. Elisha Mitch. Sci. Soc.* **61**, 74–113.

Roberts, C. F. (1959). A replica plating technique for the isolation of nutritionally exacting mutants of a filamentous fungus (*Aspergillus nidulans*). *J. gen. Microbiol.* **20**, 540–548.

Snow, R. (1966). An enrichment method for auxotrophic yeast mutants using the antibiotic 'Nystatin'. *Nature, Lond.* **211**, 206–207.

Stanley, V. C. & English, M. P. (1965). Some effects of nystatin on the growth of four *Aspergillus* species. *J. gen. Microbiol.* **40**, 107–118.

Woodward, V. H., De Zeeuw, J. R. & Srb, A. M. (1954). The separation and isolation of particular biochemical mutants of *Neurospora* by differential germination of conidia, followed by filtration and selective plating. *Proc. natn. Acad. Sci. U.S.A.*, **40**, 192–200.