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ENRICHMENT METHOD FOR THE ISOLATION OF AUXOTROPHIC MUTANTS OF MUCOR USING THE POLYENE ANTIBIOTIC N-GLYCOSYL-POLIFUNGIN

by

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The polyene antibiotic N-glycosyl-polifungin has been used as an effective counterselective agent for biochemical mutants in Mucor. Treatment with this antibiotic has resulted in at least 40-fold enrichment of auxotrophic mutants in a mutagenised spore population of M. circinelloides. The mutants obtained from this species belong to at least six different phenotypic classes, most of them not previously described. This is the first report of the isolation of amino acid auxotrophs from M. miehei.

1. INTRODUCTION

Many genetic investigations and DNA transformation are made possible by the existence of mutants. Mutations occur spontaneously in all cell populations but usually at a very low frequency. The development of efficient methods of induction, isolation and characterisation of mutants is therefore of major importance. Mutagenic treatments increase the mutation rate, but generally this is not sufficient to produce a diverse collection of mutants.

In recent years total isolation techniques, in which mutants have to be identified from the entire surviving cell population, have often been replaced by other methods aiming at the selective elimination of the prototrophic survivors of the mutagenic treatment and the retention of the desired auxotrophs. Most of these methods are based on the fact that metabolically active cells are more sensitive to antibiotics and physical agents than inactive cells, such as ungerminated spores. This results in preferential killing of prototrophs when mutagenised cultures growing in minimal medium are exposed to a counterselective agent.

Penicillin, the standard agent for isolating auxotrophs in Escherichia coli and other bacteria (3, 7), cannot be used in fungi since they are insensitive to this antibiotic. However, other procedures have been developed, exploiting similar principles.

The enrichment in auxotrophic mutants by differential freeze-killing of germinating versus non-germinating spores has been described in Neurospora crassa (8) and in Mucor racemosus (syn. Mucor circinelloides (12))(10).

The selection of drug-resistant mutants and auxotrophs by differential heat sensitivity of germinated prototrophic spores as opposed to ungerminated auxotrophic spores, has been re-

Abbreviation: NPG = N-glycosyl-polifungin.

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ported in Phycomyces blakesleeanus by BRUNKE et al. (2).

A different approach is based on the use of polyene antibiotics which induce considerable changes in permeability by interaction with the sterols in the cell walls (9) of metabolically active cells (14). The polyene antibiotic N-glycosyl-polifungin (NGP) has been applied successfully to select auxotrophic and temperature-sensitive mutants in Aspergillus nidulans (1) and in Saccharomyces cerevisiae (11). It has the advantage of being water-soluble, unlike most polyene antibiotics previously described e.g., nystatin (4, 11).

The present communication reports the isolation and characterisation of phenotypically different auxotrophic mutants of Mucor circinelloides and of lysine-requiring auxotrophs of Mucor miehei, by selective enrichment using N-glycosyl-polifungin (NGP). As part of a project to establish a genetic transformation system for Mucor species, it was considered desirable to have different auxotrophic mutants. These are useful as recipient strains in transformation experiments, since Mucor shows a low intrinsic sensitivity to most of the antibiotics for which genes conferring resistance from other organisms are available. In an accompanying paper the transformation to prototrophy of one of the M. circinelloides leucine auxotrophs obtained is described (5).

2. MATERIALS AND METHODS

2.1. Strains

Mucor circinelloides f.lusitanicus CBS 277.49 (syn. Mucor racemosus ATCC 1216b) (12) and Mucor miehei CBS 370.65 (13) were used as parental strains for the isolation of auxotrophic mutants and as wild type strains in the control experiments.

The auxotrophic mutant, M. circinelloides leu-2A was obtained from J. PETERS (10). The lysine auxotroph R10 was derived from M. miehei CBS 370.65 by the procedure described in this paper.

2.2. Media and growth conditions

The minimal medium used was YNB (0.5 g Difco yeast nitrogen base without amino acids and ammonium sulphate, 1.5 g ammonium sulphate and 1.5 g glutamic acid per litre of H_2O with 1% glucose and 1 $\mu g \cdot ml^{-1}$ thiamine and niacin added post sterilisation). For growth of auxotrophs, the minimal medium was supplemented with different amino acids at a final concentration of 100 $\mu g \cdot ml^{-1}$ or with casamino acids at 2 $mg \cdot ml^{-1}$. To produce restricted colony growth, the media were adjusted to pH 3.0 with 1 M-HCl (added post sterilisation), in which case 20 $g \cdot l^{-1}$ of agar was included. Liquid media were adjusted to pH 4.5 as described above.

Cultures of M. circinelloides were incubated at 28 °C and those of M. miehei at 40 °C.

2.3. Chemicals

N-glycosyl-polifungin was synthesised by Professor BOROWSKI and collaborators at the Department of Drug Technology and Biochemistry of the Gdańsk Institute of Technology, Poland (German patent 2239891: Antibiotics containing polyene macrolide groups; L. FALKOWSKI et al., 1973). It was kindly supplied by Professor ENRIQUE CERDÁ OLMEDO, Departamento de Genética, Sevilla, Spain.

2.4. Mutagenesis

About 10^8 spores from the wild type strains, CBS 277.49 and CBS 370.65, were spread separately on YNB medium supplemented with casamino acids. The spores were immediately irradiated with a dose of ultraviolet light sufficient to give a survival between 0.5% to 2%, and then incubated to allow them to complete a full vegetative cycle. After 7 days, the next generation of spores produced by each plate were independently collected and kept frozen at -20 °C until further use.

Survival titres were determined from colony counts of diluted samples before and after the mutagenic treatment.

2.5. Conditions for the treatment with N-glycosyl-polifungin

Cultures inoculated with 10^8 spores, were grown in 25 ml of liquid minimal medium, and incubated with shaking at the appropriate tem-

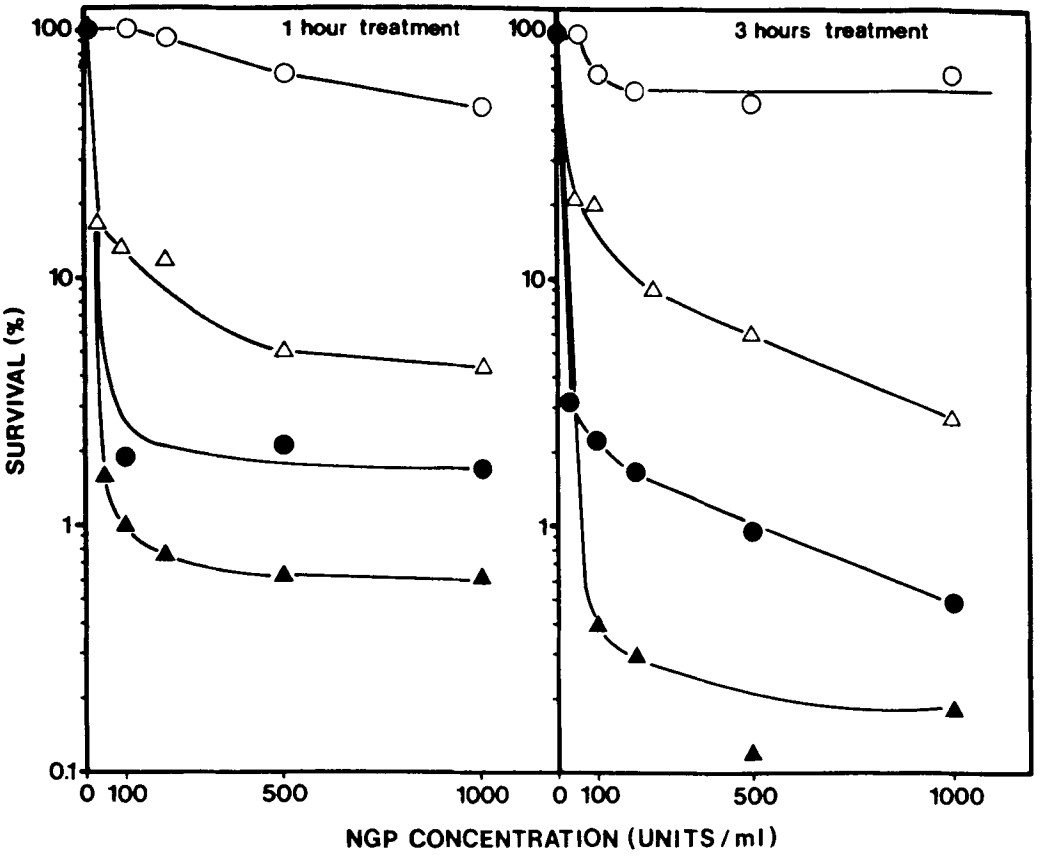

Figure 1. The effect of different concentrations of NGP on the survival of Mucor cells for 1 or 3 hours of treatment. The symbols correspond to the following strains: M. circinelloides CBS 277.49 (wild type ●); M. circinelloides leu-2A (leucine auxotroph ○); M. miehei CBS 370.65 (wild type ▲); M. miehei R10 (lysine auxotroph △).

perature for several hours until about 90% of the spore population had started to germinate (determined by light microscopy). The germlings were collected by centrifugation, resuspended in an equal volume of YNB lacking ammonium sulphate (to avoid precipitation of NGP) and dispensed into tubes before adding different aliquots of an aqueous solution of NGP. After incubation in the presence of this antibiotic for 1 to 3 hours, depending on the experiment, the germlings were washed three times with H_2O and plated on YNB medium at a dilution subsequently yielding 50 to 100 colonies per plate. Plates were incubated for 3 to 5 days before colony counting or replica plating.

2.6. Isolation and characterisation of auxotrophic mutants

The colonies surviving the antibiotic treatment were allowed to sporulate whereafter duplicates were plated on YNB and on YNB-containing casamino acids in order to detect auxotrophic mutants. Those colonies failing to grow on selective minimal medium but capable of growing in the presence of casamino acids were classified as auxotrophic mutants and their phenotype was further characterised. The putative auxotrophs were transferred to various supplemented minimal media, each one containing a different combination of 4-5 different amino acids as described by HOLLIDAY (6). The nutri-

tional requirement of a particular mutant was identified by detection of growth on one or more of the above combinations.

3. RESULTS

3.1. Determination of the optimal conditions for the treatment with N-glycosyl-polifungin

Experiments were performed to determine the optimal concentration and time of exposure to NGP, so that auxotroph survival would be high and the survival of the prototrophs would be very low. Since this antibiotic is most effective on metabolically active cells, the spores were incubated in minimal medium for a period of 4-5 hours to ensure a 90% germination of the prototrophic population before addition of the NGP.

About 10^8 viable cells of the two strains of M. circinelloides, CBS 277.49 (wild type) and the auxotrophic mutant leu-2A, and of the two strains of M. miehei, CBS 370.65 (wild type) and R10 (lysine auxotroph), were treated with the following concentrations of NGP: 0, 50, 100, 500 and 1,000 units per ml, for 1-3 hours. Viabilities, expressed as percent of survival, are shown in Figure 1. The survival of the wild type strain of M. circinelloides, after incubation for 3 hours in the presence of NGP (1,000 units ml^{-1}), was 0.5%, while that of the Leu auxotroph was 68%. Under the same conditions, the wild type strain of M. miehei showed a survival of 0.18% whereas that of the Lys auxotroph was 2.8%. The ratio between the survival of one auxotrophic mutant to that of the wild type from the same species, at a given concentration of NGP, provides an estimate of the effectiveness of the treatment. From these results it was possible to calculate the theoretical enrichment factors that could be obtained in the isolation of auxotrophic mutants from a mixed population of mutagenised cells, and therefore to determine the optimal conditions for the treatments.

3.2. Isolation and characterisation of auxotrophic mutants

With the enrichment factors deduced from the above experiments (section 3.1), a treatment with 1,000 units of NGP per ml for 3 hours was predicted to produce the best mutant yield in M. circinelloides. The results obtained for M. miehei did not so clearly indicate optimal treatment conditions, so, 3 different concentrations of NGP were used.

Germinated spores of M. circinelloides CBS 277.49 and of M. miehei CBS 370.65, previously mutagenised (section 2.4), were treated with 0 and 1,000 units, and with 0, 50, 100 and 1,000 units of NGP, respectively for 3 hours, washed

Table I.
Isolation of auxotrophic mutants from Mucor

Species	Induction	NGP (units/ml)	Number of colonies screened	Number of auxotrophs obtained	Mutant yield(%)	Enrichment factor
M.circinelloides						
	U.V.	0	952	0	<0.1	1
	"	1,000	478	21	4.39	>44
	spontaneous	1,000	2,521	0	<0.04	
M.miehei						
	U.V.	0	1,428	2	0.14	1
	"	50	867	0	<0.11	
	"	100	1,736	0	<0.06	
	"	1,000	2,726	10	0.36	2.5

Spores were germinated in minimal medium (YNB) for about 5-6 hours, then incubated for 3 hours in the presence of different concentrations of NGP and plated on YNB supplemented with casamino acids. After sporulation the colonies were replicated to YNB and to YNB with casamino acids, to identify the auxotrophic mutants.

repeatedly and plated onto YNB medium supplemented with casamino acids. After incubation for 7 days, the sporulated colonies were replicated to YNB with and without casamino acids and auxotrophic mutants were identified. Table I shows the results of the total mutant yield obtained in independent experiments. Characterisation of their auxotrophic requirements was by the method described by HOLLIDAY (6).

All 12 mutants obtained from M. miehei appeared to be lysine-requiring auxotrophs. The 21 mutants obtained from M. circinelloides were characterised as belonging to the following phenotypic classes: 3 requiring leucine, 1 requiring either cysteine or methionine, 8 requiring isoleucine, 2 requiring methionine, 1 requiring proline, 1 requiring isoleucine and valine and 5 undetermined (probably requiring more than one amino acid).

4. DISCUSSION

Treatment of mutagenised spore populations with the polyene antibiotic N-glycosyl-polifungin has provided a successful method for the isolation of auxotrophic mutants in Mucor species.

The enrichment factor obtained, at least 40-fold, in the isolation of M. circinelloides auxotrophs is in good agreement with the expected estimations deduced from the results of initial experiments, in which a Leu$^-$ mutant was shown to be 50 to 100 times more resistant to NGP than the wild type strain. Twenty-one auxotrophs were obtained from M. circinelloides, which represents a mutant yield of 5%. This high frequency allowed the isolation of at least 6 different phenotypic classes. PETERS and SYPHERD reported a cryobiological method in which 2-3% of auxotrophs were obtained in M. racemosus (syn M. circinelloides (12)), after the first cycle of selection, and 17-75% after the second cycle (10). Although the method described in this paper only involves one round of treatment with NGP, it is possible that successive rounds would lead to a further increase in the frequency of mutants obtained. Additional cycles of counterselection might be advisable when specific and rare phenotypes of mutants are needed.

The low yield of mutants found after treatment of mutagenised spores of M. miehei with NGP, can be explained by the relatively high sensitivity of these auxotrophs. This conclusion is supported by the results shown in Figure 1. The fact that all 12 mutants appeared to be lysine auxotrophs might be due to aneuploidy or partial heterozygosity of the parental strain, CBS 370.65, used in these experiments; but not to a relatively higher resistance of the Lys$^-$ mutants to NGP since two such auxotrophs were isolated without any exposure to this agent.

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