Method for Isolating Auxotrophic Mutants in *Aspergillus nidulans* Using *N*-glycosyl-polifungin

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SUMMARY

A method for the selection of auxotrophic mutants in *Aspergillus nidulans* using *N*-glycosyl-polifungin has been developed. The method results in about 9000-fold enrichment. All types of auxotrophs were obtained. Under certain conditions preferential selection of nucleic acid hydrolysate-requiring auxotrophs was observed. The frequency of occurrence of these mutants was about 50% of all colonies tested. Cysteine auxotrophs of *A. nidulans* were obtained for the first time by means of this method.

INTRODUCTION

There are many methods of selecting auxotrophic mutants. The penicillin method is effective and widely used for bacteria (Davis, 1948; Lederberg & Zinder, 1948). No such universal technique is available for fungi. In 1959 Moat, Peters & Srb made the first attempts to use the antifungal effects of polyene antibiotics for selection of auxotrophic mutants in yeast. Polyene antibiotics form complexes with the sterols of cell walls (Lampen, Arnow, Borowska & Laskin, 1962; Norman *et al.* 1972) causing considerable changes in permeability (Lampen *et al.* 1962; Stachiewicz & Quostel, 1963; Kinsky, Luse & van Deenen, 1966). It has also been suggested that polyenes have an effect on the general conformation of the cell wall (Chuen-Chin & Feingold, 1973). The selective action of polyene antibiotics is especially strong during increased metabolic activity of the cell (Snow, 1966). This allows their application as agents for selecting auxotrophs.

Polyene antibiotics yielded positive results when used for selection of auxotrophic mutants in yeast (Snow, 1966; Thouvenot & Bourgeois, 1971) when a method similar to the penicillin method was applied. In the selection of auxotrophic mutants in bacteria and yeast the cells after mutagenesis were placed in complete liquid medium to allow the phenotypic expression of the induced mutations and were subsequently transferred to selective medium. On this medium the cells were exposed to the antibiotic. In the case of filamentous fungi, the time of the incubation in liquid medium is strictly limited because they form a mycelium, so the method described above is inadequate for these organisms. The attempts which were made hitherto to bypass this difficulty (Ditchburn & MacDonald, 1971) were not completely satisfactory, see Discussion.

In this paper a method for isolating auxotrophic mutants in *A. nidulans* using *N*-glycosyl-polifungin as the selective agent is described. This agent is a derivative of polifungin (Kotiuszko, Wituch, Morawska & Siejko, 1971). *N*-glycosyl-polifungin has been synthesized by Professor Borowski. It is at present the only water-soluble polyene antibiotic – a property that makes it more convenient to use.

METHODS

Strains. In this work the strains *mecA1*, *biA1*; *pabaA2*, *biA1* and *y*, *biA1*, *argA1* from the Department of Genetics of the University of Warsaw were used. The symbols represent appropriate nutrient requirements, *mec* representing the inability to catabolize methionine (Pieniążek, Stępień & Paszewski, 1973) which does not lead to auxotrophy.

The genetic analysis was performed by inoculating 26 colonies per plate with complete medium; after incubation they were replicated using a replicator with 26 steel needles on selective media.

Media. All media as well as nutrient supplements necessary for growth were standard (Pontecorvo *et al.* 1953).

Chemicals. The tris salt of *N*-glycosyl-polifungin (NGP) obtained at the Department of Drug Technology and Biochemistry of the Gdańsk Institute of Technology, Poland (Patent Application P149994: Method for obtaining derivatives of the macrolide polyene antibiotic; L. Falkowski, J. Pawlak, E. Bylec, J. Golik, P. Kołodziejczyk, T. Zieliński, A. Rudkowski & E. Borowski, 1971) was used. Three different samples from the experimental production with activities of 1100, 960 and 1300 units/mg respectively were used. The activity of the preparations was determined by the cylinder flat method in *Saccharomyces cerevisiae* ATCC9763 against a standard of insoluble polifungin (POLFA).

The antibiotic was stored at -10°C . Solutions were prepared immediately before use by dissolving NGP in liquid medium to the required final concentration. The solutions were not sterilized before use.

The choice of NGP as the most effective antibiotic was made on the basis of comparisons between mycostatin (SQUIBB), nystatin (POLFA), insoluble polifungin (POLFA) and NGP (unpublished data).

RESULTS

Determination of the optimal time of action and concentration of NGP

As the polyene antibiotics are most effective during germination of the conidia, the time of germination was determined in the *mecA1*, *biA1* strain. In biotin-supplemented minimal medium conidia of this strain germinate between the fourth and sixth hour of incubation. Therefore the antibiotic in subsequent experiments was added 4.5 h after starting the incubation in experiments in which 1 h incubation with the antibiotic was applied, and 3 h after starting the incubation in experiments in which 3 h incubation with the antibiotic was applied.

It was necessary to determine an antibiotic treatment in which the survival of auxotrophs was high and the survival of prototrophs very low. The method proposed by Moat *et al.* (1959) was used. The sensitivities of strains *mecA1*, *biA1* and *y*, *biA1*, *argA1* to various concentrations of the antibiotic were compared. On biotin-supplemented minimal medium the former strain behaves as a prototroph while the later is an arginine auxotroph. The ratio of survival of the auxotroph to that of the prototroph at a given antibiotic concentration was a measure of the effectiveness of the NGP. This value, described as the enrichment factor, equals 1 in the control sample. The antibiotic concentrations used were 50, 100, 200, 500 and 1000 units/ml. The experiments were performed using both 1 and 3 h incubation with the antibiotic.

The analysis of the results obtained indicates that NGP is very effective (Fig. 1). The sensitivity of the prototroph is much greater than that of the auxotroph. The enrichment factors were 1658 and 8882 for 1 and 3 h incubation with the antibiotic respectively. Both values were obtained when the final concentration of NGP was 500 units/ml.

Fig. 1. The effect of different concentrations of NGP for (a) 1 h, (b) 3 h, on the survival of conidia of *mecA1, biA1* and *γ, biA1, argA1* strains of *Aspergillus nidulans* in biotin-containing minimal medium. ■, *mecA1, biA1*; ▲, *γ, biA1, argA1*; ●, enrichment factor.

The action of the NGP in the selection of new mutants

Induction of mutants. The mutations were induced by irradiating a conidial suspension with u.v. (254 nm wavelength). An u.v. CAMAG (Switzerland) lamp was used. Survival of the conidia was in the range 0.5 to 2 %.

Phenotypic expression of induced mutations. Snow (1966) showed that selection for auxotrophs immediately after mutagenesis did not yield positive results. Induced mutations were not expressed phenotypically. To determine if this were also true for u.v.-induced mutations in *A. nidulans*, the number of auxotrophic mutants in a sample of u.v.-irradiated conidia and a sample exposed to NGP after mutagenesis were compared. The NGP was added after 4.5 h incubation in liquid minimal medium supplemented with biotin. Conidia were incubated for 1 h with NGP at a final concentration of 100 units/ml. In both experiments the frequency of auxotrophic mutants was about 0.1 %. In subsequent experiments the conidia after mutagenesis were plated on solid medium to allow the phenotypic expression of the mutations.

Selection of the mutants. Ultra-violet-irradiated conidia from the *mecA1, biA1* strain were plated on to complete solid medium. After 3 days' incubation the conidia were collected into water containing Tween 20 (0.1 % solution) and centrifuged to remove the remains of the solid medium. Biotin-supplemented liquid minimal medium was inoculated with these conidia. The density of this suspension never exceeded 10^7 conidia/ml. The suspension was incubated in an orbital shaker at 34 °C. After the appropriate time the solution of the antibiotic was added. Both 1 and 3 h incubation with the NGP were used. Conidia were separated from the antibiotic by centrifugation and plated on complete solid medium. The number and type of mutants in samples of conidia subjected to various concentrations of NGP were examined (see Methods). A considerable increase in the number of mutants was observed as compared with the untreated sample (Table 1). All kinds of mutants were obtained. The greatest enrichment was observed after 3 h incubation with the antibiotic at 200 units/ml. At higher concentrations smaller numbers of auxotrophs were obtained. A similar dependence was observed for 1 h incubation with NGP, though the number of mutants was smaller and the maximal enrichment was given by a concentration of 500 units/ml (Table 1).

Preferential selection of nucleic acid hydrolysate auxotrophs. In all experiments the

Table 1. *Comparison of frequency and types of auxotrophs of Aspergillus nidulans mecA1, biA1 with respect to the time of action and concentration of NGP*

Time of NGP action (h)	Concn of NGP (units/ml)	No. colonies tested	Auxotrophs					Enrichment factor
			No.	Percentage	Type*			
					A	V	N	
—	—	2500	1	0.04	—	1	—	1.0
1	50	308	5	1.60	—	1	4	40.0
	100	382	9	2.40	1	1	7	60.0
	200	389	11	2.80	2	2	7	70.0
	500	388	35	9.00	1	8	26	225.3
	1000	311	14	4.20	1	3	10	112.5
3	50	115	4	3.50	1	—	3	87.5
	100	182	19	10.40	—	2	17	260.0
	200	323	52	16.10	4	4	46	402.5
	500	228	23	10.10	2	1	20	252.5

* A, amino acid auxotrophs; V, vitamin auxotrophs; N, nucleic acid hydrolysate-requiring auxotrophs.

majority of the mutants required a hydrolysate of nucleic acids for growth. The proportion of nucleic acid hydrolysate requiring auxotrophs was dependent on the degree of antibiotic treatment (Table 1). Experiments were performed to determine whether this were a property of the *mecA1, biA1* strain. Conidia of the *pabaA2, biA1* strain after mutagenesis and phenotypic expression of mutations were incubated in minimal liquid medium supplemented with biotin and *p*-aminobenzoic acid and treated with NGP at 100 units/ml for 1 h. Among 16 auxotrophs, 15 required nucleic acid hydrolysate for growth (Table 2).

Selection of specific mutant classes

Obtaining specified classes of mutants. The preferential selection of nucleic acid hydrolysate-requiring auxotrophs does not exclude the possibility of obtaining other types of mutants. If conidia after mutagenesis are plated on medium lacking hydrolysate of nucleic acids all nucleic acid hydrolysate-requiring auxotrophs will be eliminated. To isolate amino acid-requiring mutants in the *mecA1, biA1* and *pabaA2, biA1* strains the conidia after mutagenesis were plated on minimal medium supplemented with biotin, or biotin and *p*-aminobenzoic acid, and casein hydrolysate. To isolate vitamin auxotrophs, conidia were plated on minimal medium supplemented with vitamins. In both cases (Table 2) more mutants were obtained than by total isolation. When conidia after mutagenesis were plated on minimal medium with a hydrolysate of nucleic acid and appropriate vitamins, 62.5 % of nucleic acid hydrolysate-requiring auxotrophs were obtained in the *mecA1, biA1* strain and 25.7 % in the *pabaA2, biA1* strain (Table 2), which confirms the preferential selection of this class of mutants. The lower number of auxotrophs in the *pabaA2, biA1* strain than in the *mecA1, biA1* strain could be due to differences of composition of sterols in the cell wall (see Introduction).

It is possible to repeat the NGP treatment before mutant isolation. In an experiment with the *mecA1, biA1* strain the percentage of mutants was lower after the second than after the first incubation with NGP. This was probably due to the more efficient formation of conidia by prototrophs than by auxotrophs.

Obtaining cysteine auxotrophs. An attempt was made to obtain cysteine auxotrophs since these mutants had not been obtained by velvet replication even though approximately 50 000 colonies had been screened. The mutations were induced in the *mecA1, biA1* strain

Table 2. Comparison of the frequency and types of auxotrophs of *mecA1*, *biA1* and *pabaA2*, *biA1* strains of *Aspergillus nidulans* obtained by total isolation and the polifungin method

Isolation method	Strain	Medium on which conidia were plated after mutagenesis	No. colonies tested	Auxotrophs				
				No.	Per-centage	Type*		
						A	V	N
Total isolation	<i>mecA1</i> , <i>biA1</i>	CM	2500	1	0.04	—	1	—
	<i>pabaA2</i> , <i>biA1</i>		1040	1	0.10	—	1	—
Polifungin method	<i>mecA1</i> , <i>biA1</i>	CM	274	122	44.50	2	—	120
	<i>pabaA2</i> , <i>biA1</i>		516	16	3.10	—	1	15
	<i>mecA1</i> , <i>biA1</i>	MM + bi + amino acids	277	15	5.40	15	—	—
	<i>pabaA2</i> , <i>biA1</i>	MM + bi + paba + amino acids	458	8	1.70	8	—	—
	<i>mecA1</i> , <i>biA1</i>	MM + vitamins	234	23	9.80	—	23	—
	<i>pabaA2</i> , <i>biA1</i>		494	6	1.20	—	6	—
	<i>mecA1</i> , <i>biA1</i>	MM + bi + nucleic acids	260	162	62.30	—	—	162
	<i>pabaA2</i> , <i>biA1</i>	MM + bi + paba + nucleic acids	494	127	25.40	—	—	127

* For abbreviations see Table 1.

in which methionine catabolism is blocked (Pieniążek *et al.* 1973). After mutagenesis the conidia were plated on minimal medium supplemented with biotin and cysteine. The conidia were harvested and inoculated onto biotin-supplemented liquid minimal medium and incubated for 4.5 h. Subsequently NGP (100 units/ml) was added for 1 h. After incubation, the antibiotic was removed and the conidia plated on minimal medium supplemented with biotin and cysteine. Colonies obtained on this medium were inoculated in the same medium (26 colonies/plate) and replicated on to minimal medium with biotin. In four independent experiments 2073 colonies altogether were screened and 13 cysteine auxotrophs obtained. It is possible that most of these mutants are members of a clone; this has not been further investigated. A detailed biochemical study of cysteine auxotrophs has been made by N. J. Pieniążek, J. Bal, E. Balbin & P. P. Stępień (1974).

DISCUSSION

The method of selection of auxotrophic mutants described is not completely analogous to the penicillin method since *A. nidulans* forms a mycelium and therefore cannot be incubated for long in liquid media. However, plating of conidia on solid medium allows the expression of the induced mutations and leads to cloning of mutants. This cloning is especially useful with auxotrophs which occur with very low frequency.

The method described in this paper differs basically from the method proposed for *A. nidulans* by Ditchburn & MacDonald (1971). These authors plated conidia after mutagenesis on minimal medium, then poured melted complete medium containing an antibiotic – nystatin – over the plates. They claimed the prototrophs were killed immediately and the antibiotic decomposed before the auxotrophs began to germinate. However, Snow (1966) has shown that exposure to nystatin for several hours also caused non-specific killing of auxotrophs. This has been confirmed in our experiments (unpublished data) in which we used nystatin. In optimal conditions Ditchburn and MacDonald obtained 8.8 % of auxotrophs; we obtained 44.5 %, a 1000-fold enrichment. From the data of Ditchburn and MacDonald it is difficult to estimate the degree of enrichment.

In our investigations on *A. nidulans* using various types of polyenes (unpublished data)

we observed preferential selection of nucleotide auxotrophs. The preferential selection of nucleic acid hydrolysate-requiring auxotrophs may be because germination of conidia is preceded by replication of DNA. A cell incapable of synthesizing nucleic acids cannot germinate and should be insensitive to NGP.

Cysteine auxotrophs were obtained for the first time using this method, which suggests it may also be effective in the search for other types of auxotrophs.

Of the colonies initially characterized as auxotrophs, 20 % reverted to prototrophy in subsequent tests. These 'revertants' were not taken into consideration in this paper. A similar phenomenon has been observed with both the nystatin and penicillin methods. The reasons are unknown.

Our method of selecting *A. nidulans* auxotrophs is more effective and easier to apply than previously described methods. It should be applicable to other filamentous fungi.

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