

GENETIC STUDY OF AUXOTROPHIC AND RESISTANT MUTANTS OF *Aspergillus niger* AND THEIR GLUCOAMYLASE PRODUCTION

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ABSTRACT

Auxotrophic and resistant mutants have been isolated from an *Aspergillus niger* glucoamylase-producing strain. A resistant mutant, *mgrA*₂ (malachite green), and the auxotrophic mutants, *metA*₁ (methionine) and *mysA*₁ (methionine or cysteine) were the only ones not to show significantly lower yields compared to the parental strain. Production levels of heterozygous diploids indicated that lower yields observed in the other mutants might be due to pleiotropic effects. Four linkage groups have been identified by means of heterozygous diploid segregation. The gene *mgrA*₂ was shown to be recessive and *etbA*₅ (resistance to ethidium bromide), semi-dominant. The latter could be used for gene order determination in linkage group I.

INTRODUCTION

The filamentous fungus *Aspergillus niger* is widely used in industry to produce many types of metabolites, including glucoamylase (E.C.3.2.1.3); this is of economic interest for its capacity to turn starch into glucose (Banks *et al.*, 1976) and because of the large amount produced by industrial strains (Brunt, 1986).

Although it is known already that one structural gene produces the two forms of the enzyme (Boel *et al.*, 1984a,b), there are few genetic studies on its production (Ball *et al.*, 1978; Bonatelli Jr. *et al.*, 1984).

To develop a genetic study, mutants with little or no pleiotropic activity are desirable along with a good knowledge of the recombination mechanism available to the species; in the case of *A. niger*, the latter is the parasexual cycle (Pontecorvo *et al.*, 1953a).

Several genetic studies have been carried out in *A. niger* (Lhoas, 1967; Tuyll, 1977; Bos, 1985), all giving the same results concerning the number of linkage groups. Nevertheless, the strains used were of different origins and this fact can lead to problems in genetic analysis due to incompatibility between strains and/or to nonisogenic backgrounds (Bos *et al.*, 1988; Bonatelli Jr., data not shown).

In the present work we describe the isolation of auxotrophic and resistant mutants from a glucoamylase producing strain of *A. niger*; the effects of these mutations on enzyme yields and their location based on studies through the parasexual cycle.

MATERIAL AND METHODS

Organism

The following *A. niger* strains were used: *pabA*₁ *fwnA*₁; *nicA*₁ *obvA*₃ and *pabA*₁ *fwnA*₁ *argA*₁ (Bonatelli Jr. *et al.*, 1982; Calil, 1988). These mutant alleles determine: *fwnA*₁, fawn conidia; *obvA*₃, olive conidia; *argA*₁, *nicA*₁, *pabA*₁, requirement, respectively, for arginine, nicotinic acid and p-aminobenzoic acid.

Media

The growth media utilized were the complete medium (CM) and minimal medium (MM) described by Pontecorvo *et al.* (1953b), and the medium to assay glucoamylase production (MAC) as described in Bonatelli Jr. *et al.* (1984).

GENERAL TECHNIQUES

Mutant isolation

Two mutagens were used: ethyl methanesulfonate (EMS, SIGMA) 8% solution (v/v) in distilled water, and ultraviolet light (UV, 254 nm). Treatments with EMS lasted 2 hours and with UV 7.5 minutes, in order to give a survival rate of 2-4%. The UV treated conidia were transferred to 250 ml Erlenmeyer flasks containing 50 ml of MM, and 5 µg/ml of p-aminobenzoic acid. These flasks were incubated with agitation at room temperature from 1 to 3 days and the medium was then filtered through gauze (Fries, 1947). After these procedures, and also after EMS treatment, conidia were plated in Petri dishes with CM and incubated from 3 to 5 days. Auxotrophic mutants were classified by an auxanographic method (Pontecorvo *et al.*, 1953b). The mutants resistant to malachite green (MERCK) and ethidium bromide (SIGMA) were isolated after plating about 10⁴ conidia, previously exposed to UV, onto CM containing

0.5 $\mu\text{g/ml}$ and 15 $\mu\text{g/ml}$ of the drugs respectively. The allelic interaction of resistant mutants was determined by inoculation of parental, mutant and heterozygous diploid strains on Petri dishes containing CM plus increasing concentrations of the respective drug. Resistance to malachite green was tested by percentage germination after 7 days and to ethidium bromide by size of the colonies after 2 days' incubation. Incubation was at 28°C for all experiments, unless otherwise stated.

Enzyme production

Conidia of the strains to be tested were collected in tween-80 solution (0.1% v/v) at a final concentration of $10^6/\text{ml}$. One ml samples were inoculated into 125 ml Erlenmeyer flasks containing 25 ml of MAC. At least five replicates were set up for each strain. For auxotrophic strains, MAC was supplemented with 80 $\mu\text{g/ml}$ of nitrogen bases, sodium thiosulfate and aminoacids, and 5 $\mu\text{g/ml}$ of vitamins. The flasks were incubated for 4 days. Glucoamylase was estimated after incubation of 0.5 ml diluted filtrate with 0.5 ml of a 1% soluble starch solution in 0.05 M citrate buffer pH 4.0, for 60 minutes in a water bath at 60°C (Park and Papini, 1970; Banks *et al.*, 1976). The amount of reducing sugars is expressed in mg/100 ml and 10 mg/ml is equivalent to one unit (U) of glucoamylase (GA).

Isolation of diploids and segregants

Diploids were obtained using Roper's method (1952) with minor modifications (Bonatelli Jr. *et al.*, 1983). Their characterization was based on Pontecorvo *et al.* (1953a). Isolation of haploid segregants was done by point inoculation of diploid conidia onto Petri dishes with CM or MM with nutritional requirements plus Benlate (fungicide) at a concentration of 1 to 1.5 $\mu\text{g/ml}$ (Hastie, 1970). Segregants were characterized for conidia colour and auxotrophic markers in a way identical to mutants (see above).

RESULTS AND DISCUSSION

Mutant isolations

Table I shows the auxotrophic and resistant mutants obtained. The frequency of auxotrophic mutants using UV plus enrichment by filtration ($1.6 \cdot 10^{-3}$) and EMS ($8 \cdot 10^{-4}$) was low compared to that reported by Bonatelli Jr. *et al.* (1982) using total isolation. This could be attributed to the frequency of binucleate conidia (24%) produced by the strain *pabA*₁ *fwnA*₁ (Bonatelli Jr., 1981) which could have hampered the isolation of auxotrophic mutants, since they are usually recessive. Nevertheless,

Table I - Mutants isolated from the parental strain *pabA*₁ *fwnA*₁ after UV and enrichment by filtration or EMS treatment.

Mutants	Nutritional requirements or resistance	Mutagen
<i>tioA</i> ₁	thiosulfate	UV*
<i>mysA</i> ₁	methionine or cysteine	UV
<i>metA</i> ₁	methionine	UV
<i>nicA</i> ₃	nicotinic acid	UV
<i>lysA</i> ₁	lysine	UV
<i>purA</i> ₁	Purines (guanine or adenine)	EMS*
<i>mgrA</i> ₂	malachite green resistance	UV
<i>etbA</i> ₅	ethidium bromide resistance	UV

* UV - Ultraviolet light

EMS - Ethyl methanesulfonate

new mutants requiring aminoacids were isolated through these procedures (Bonatelli Jr. *et al.*, 1982). The mutant *mysA*₁ differs from *tioA*₁ by not growing on MM plus thiosulfate.

The frequency of malachite green and ethidium bromide mutants were $1.1 \cdot 10^{-2}$ and $1.4 \cdot 10^{-4}$ respectively. All resistant mutants selected on both drugs showed higher resistance levels than the ones reported in the literature for *A. nidulans* (Warr and Roper, 1965; Scarazzatti *et al.*, 1979). Our results with ethidium bromide are similar to the ones reported for *A. awamori* NRRL 3112 (Vialta, 1987; Vialta and Bonatelli Jr., 1988) and this probably reflects the taxonomical closeness of these species (Raper and Fennell, 1965).

Allelic interaction of the resistant mutants

The malachite green resistance mutation, *mgrA*₂, seems to be recessive (Figure 1) as is observed in *A. nidulans* (Warr and Roper, 1965). Resistance level to malachite green by percentage of germinated conidia was used because of a haploidization effect observed with heterozygous diploid strains during construction of a dose-response curve using point inoculation. Haploidization was also observed with diploids which were not heterozygous for *mgrA2* gene, and it seems to exclude resistant sectors selection (data not shown).

The ethidium bromide resistant mutation *etbA*₅ seems to be semi-dominant (Figure 2). Similar results were observed for *A. nidulans* by Scarazzatti *et al.* (1979), who also mentioned that homozygous resistant segregants are favoured by this drug.

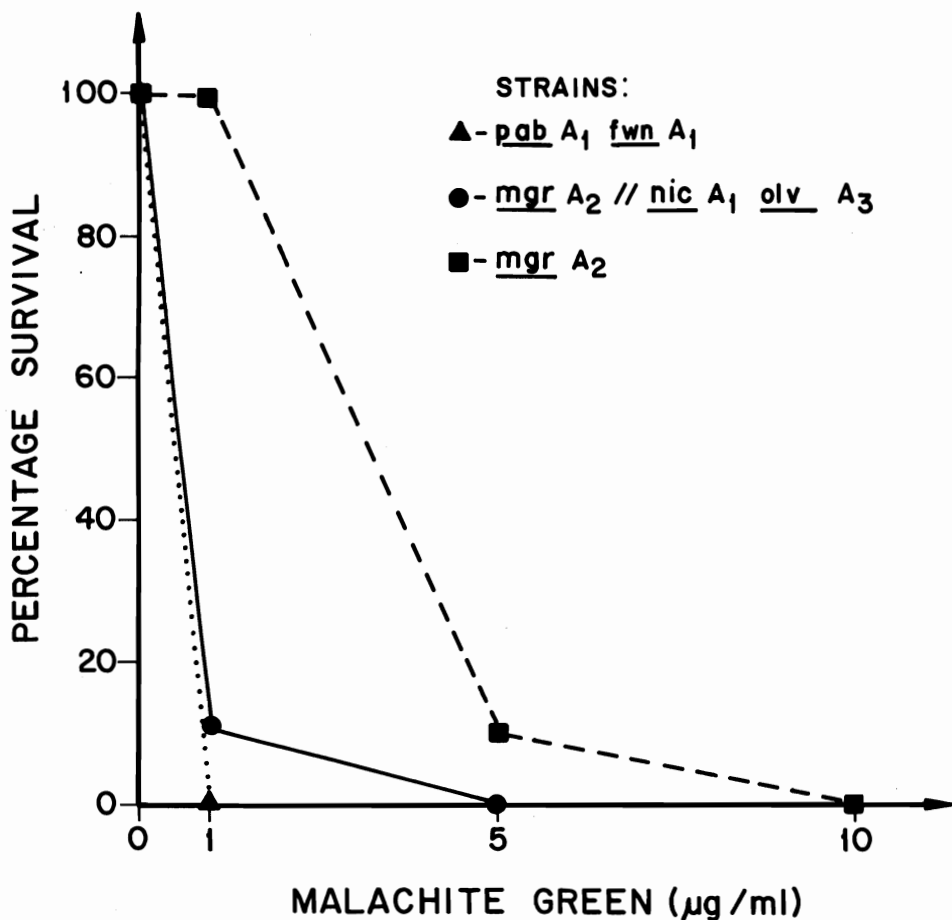


Figure 1 - Survival of conidia from parental sensitive (▲), resistant mutant (■) and heterozygous diploid strains (●) exposed to malachite green.

So, we suggest that the *etbA₅* gene could be used for segregant selection through the parasexual cycle, as is true for *acrA₁* in *A. nidulans*, in order to determine gene order on linkage group I (Roper and Käfer, 1957).

Linkage Map

Table II shows the mitotic analysis of all markers studied, comparing the number of parental and recombinant type segregants.

The *purA₁* mutation was not recovered from any of the heterozygous diploids

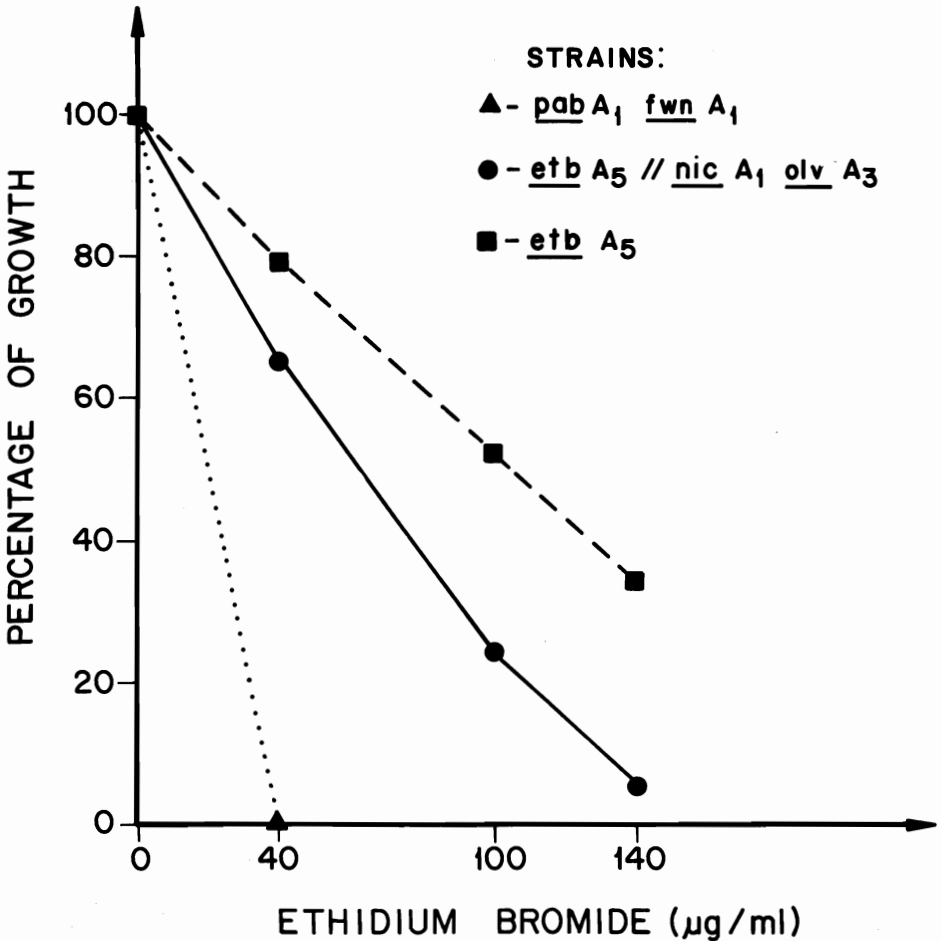


Figure 2 - Percentage of growth of parental sensitive (▲), resistant mutant (■) and heterozygous diploid strain (●) on ethidium bromide.

isolated, a fact that did not occur with any other mutant or wild allele in these or other diploids. That observation could be explained by homozygosis for *pur*⁺ or selection against the mutant allele due to physiological or genetical causes. The latter hypothesis seems to be favoured since diploids, presumably heterozygous for *pur*A₁, had been isolated from different heterokaryons synthesized on different occasions. It was not possible to isolate heterokaryons or diploids when strains with *nic*A₃ and *nic*A₁ markers were crossed, which suggests that these mutations are allelic.

Another observation is concerned with the difficulty of isolating segregants carrying the *olv*A₃ allele, which agrees with previous reports (Pontecorvo *et al.*, 1953a;

Table II - Segregants of parental and recombinant types isolated from heterozygous diploids.

	<i>nicA</i> ₁		<i>metA</i> ₁		<i>argA</i> ₁		<i>mgrA</i> ₁		<i>etpA</i> ₅		<i>tioA</i> ₁		<i>mysA</i> ₁		<i>lysA</i> ₁		<i>fwnA</i> ₁		<i>olvA</i> ₃	
	P*	R**	P	R	P	R	P	R	P	R	P	R	P	R	P	R	P	R	P	R
<i>pabA</i> ₁	230	220	58	42	72	78	25	25	29	21	19	31	23	27	65	85	231	219	231	219
<i>olvA</i> ₃	449	1	60	40	91	59	50	0	50	0	45	5	47	3	65	85	***			
<i>fwnA</i> ₁	449	1	60	40	91	59	50	0	50	0	45	5	47	3	57	93				
<i>lysA</i> ₁	85	65	26	24	62	88	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>mysA</i> ₁	47	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>tioA</i> ₁	44	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>etbA</i> ₅	50	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>mgrA</i> ₂	50	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>argA</i> ₁	91	59	45	4																
<i>metA</i> ₁	60	40																		

* Parental type segregants.

** Recombinant type segregants.

*** Epistatic genes.

- Crosses not done.

Ball *et al.*, 1978). A probable explanation is the lower growth rate of strains with that allele, compared to those with the wild type allele.

From the overall results the following linkage groups could be suggested:

Group I: *nicA*_{1,3}; *fwnA*₁; *olvA*₃; *tioA*₁; *mgrA*₂; *etbA*₅; *mysA*₁

Group II: *pabA*₁

Group III: *lysA*₁

Group IV: *argA*₁; *metA*₁

The gene order in linkage group I was not established but, because of the semi-dominant *etbA*₁, and the mutations affecting conidial colour, it could be done if at least one of them is located distal to the centromere (Pontecorvo and Kafer, 1958).

According to Lhoas (1967), Tuyll (1977) and Bos *et al.* (1988), *A. niger* has at least six linkage groups. This was not confirmed by the present work probably because of the number of genetic markers studied. As the strains used by Lhoas (1967) were not compatible with those available in our laboratory (data not shown), it would be of interest to use strains isolated by Tuyll (1977) or Bos *et al.* (1988), and attempt to establish a pattern of genetic analysis in that species. Even though it is reasonable to suppose that these strains are not isogenic, it would contribute to a better genetic knowledge of a species which is so important from the industrial point of view.

Glucoamylase production

Table III shows the glucoamylase production of some mutants, diploids and parental strains. Three groups are observed: I) strains showing a highly significant reduction compared with the parental *pabA*₁ *fwnA*₁, i.e. mutants *lysA*₁; *argA*₁ and diploid *pabA*₁ *fwnA*₁/*nicA*₁ *olvA*₃ I; II) strains showing reduction, but not significant, compared to the parental, i.e. mutants *mysA*₁ and *mgrA*₂ and strains showing little, but significant reduction, i.e. mutants *etbA*₅; *tioA*₁; *purA*₁ and diploid *lysA*₁/*nicA*₁ *olvA*₃; III) strains showing unaltered production.

Significant reduction in glucoamylase production could be due to a pleiotropic effect, which is often observed in industrial strains (MacDonald *et al.*, 1963, 1972; Chang and Terry, 1973; Ball *et al.*, 1978; Bonatelli Jr. *et al.*, 1982; Ilczuk and Fiedurek, 1985, 1986). However, our results cannot exclude the possibility of a mutation in another gene.

Although it seems more reasonable to use mutants with little or no effect on glucoamylase production to study genetic elements specifically involved with enzyme

Table III - Glucoamylase (GA) production from parental, mutant and diploid strains.

Strains	GA production (U/ml)	Number of replicates
<i>metA</i> ₁	15.96 ± 2.16	5
<i>metA</i> ₁ // <i>nicA</i> ₁ <i>olvA</i> ₃	15.09 ± 1.99	5
<i>mysA</i> ₁ // <i>nicA</i> ₁ <i>olvA</i> ₃	14.75 ± 2.00	5
<i>pabA</i> ₁ <i>fwnA</i> ₁ *	14.16 ± 3.04	35
<i>argA</i> ₁ // <i>nicA</i> ₁ <i>olvA</i> ₃	13.49 ± 3.66	10
<i>tioA</i> ₁ // <i>nicA</i> ₁ <i>olvA</i> ₃	13.28 ± 1.45	10
<i>pabA</i> ₁ <i>fwnA</i> ₁ // <i>nicA</i> ₁ <i>olvA</i> ₃ II	12.99 ± 4.38	25
<i>purA</i> ₁ // <i>nicA</i> ₁ <i>olvA</i> ₃	12.38 ± 2.35	10
<i>nicA</i> ₁ <i>olvA</i> ₃	12.18 ± 3.27	35
<i>etbA</i> ₅ // <i>nicA</i> ₁ <i>olvA</i> ₃	11.95 ± 1.71	10
<i>mgrA</i> ₂ // <i>nicA</i> ₁ <i>olvA</i> ₃	11.75 ± 1.93	10
<i>purA</i> ₁	10.97 ± 4.19	10
<i>lysA</i> ₁ // <i>nicA</i> ₁ <i>olvA</i> ₃	10.27 ± 2.09	10
<i>etbA</i> ₅	9.98 ± 2.85	10
<i>mysA</i> ₁	9.46 ± 7.74	5
<i>mgrA</i> ₂	9.45 ± 1.81	10
<i>tioA</i> ₁	9.19 ± 2.64	10
<i>pabA</i> ₁ <i>fwnA</i> ₁ // <i>nicA</i> ₁ <i>olvA</i> ₃ I	5.46 ± 1.55	20
<i>argA</i> ₁	4.76 ± 2.67	15
<i>lysA</i> ₁	0.92 ± 0.35	10

* Parental strain (see Material and Methods).

yields, problems of pleiotropic effects can be overcome by careful genetical analysis. Detection of pleiotropic effects can be used, if confirmed, at least to determine other rate-limiting steps in glucoamylase production than those directly involved in polypeptide production.

None of the diploids studied showed significantly higher production than the parental strain, *pabA*₁ *fwnA*₁. Increases in metabolite production by diploids have been reported (MacDonald *et al.*, 1972; Das and Roy, 1978), but it is more common that production is similar to those of haploid strains, indicating intergenic complementation (Sermoniti, 1956; Chang and Terry, 1973; Bennett, 1979; Bonatelli Jr. and Azevedo, 1982).

A very important aspect observed with diploids is the significant difference in production of the control diploids *pabA*₁ *fwnA*₁//*nicA*₁ *olvA*₃ I and II (Table III). They were synthesized from the same parental strains, by the same technique. Another

difference between them is that diploid I was obtained about six years before diploid II; at that time, and during almost five years, it showed unchanged yields compared with the parental *pabA*₁ *fwnA*₁ (Valent, 1985; Calil, 1988), as did diploid II in the present work. Research is now in progress trying to determine cause(s) for the difference between these diploids. One of the possibilities considered is unintentional selection for lower production because of instability of the diploid nucleus; this was shown to be higher than that observed in other species of *Aspergillus* (Lhoas, 1967; Bonatelli Jr. *et al.*, 1983). It is necessary to mention that there are no morphological characters of diploid I that would be useful to distinguish it from diploid II. In any case, one should consider this type of phenomenon when studies are made with diploids in order to determine genic complementation. To determine whether this is a common occurrence, it seems reasonable to propose the isolation of more than one diploid, especially in *A. niger*. The other obvious recommendations are to determine whether it is necessary to work with freshly isolated diploids (Lhoas, 1967) or to preserve diploids through lyophylization or another method that would minimize selection.

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RESUMO

Mutantes auxotróficos e resistentes foram isolados de uma linhagem de *A. niger* produtora de glucoamilase. Somente um mutante resistente, *mgrA*₂ (verde malaquita) e os auxotróficos, *metA*₁ (metionina) e *mysA*₁ (metionina ou cisteína) não mostraram diminuição significativa de produção comparados com a linhagem parental. A produção dos diplóides heterozigotos indicou que a baixa produção observada nos outros mutantes poderia ser devido a efeito pleiotrópico. Quatro grupos de ligação foram identificados através de segregação de diplóides heterozigotos. O gene *mgrA*₂ se mostrou recessivo e o *etbA*₅ (resistência a brometo de etídio), semi-dominante. O segundo poderia ser usado para determinação da ordem dos genes no grupo de ligação I.

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