

## CHARACTERIZATION OF BENOMYL RESISTANT MUTANTS OF *Aspergillus nidulans*

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### ABSTRACT

Three colonies of *Aspergillus nidulans*, BEN-36, BEN-37, BEN-38, selected for resistance to thiophanate-methyl were also resistant to benomyl. The level of resistance to benomyl shown by these mutant strains was at least 45 and 4.5 times higher than those shown by the wild type strain and a known benomyl-resistant strain (FGSC 524), respectively. The resistance marker was due to mutation in a single gene at the *benA* locus. The dosage resistance curves of the heterozygous diploids to benomyl and thiophanate-methyl indicated that the *benA36* and *benA37* alleles are semi-dominants, and the *benA38* allele is apparently fully dominant. Unexpectedly, the presence of benomyl or thiophanate methyl in the culture medium did not inhibit the conidiation of these mutants, a fact possibly indicating the presence of a second mutation responsible for this phenomenon.

### INTRODUCTION

Benzimidazole fungicides (thiophanates, benomyl, thiabendazole, etc.) are metabolically transformed by animals, plants and fungi into methyl-2-benzimidazole carbamate (MBC) (Tuyl, 1977). For this reason, it has been suggested that MBC has a key role in the mechanism of fungitoxic action of these structurally related fungicides (Seiler, 1975). This is supported also by the cross-resistance shown by some benomyl-selected mutants of *A. nidulans* and *Neurospora crassa* to thiophanate and thiabendazole (Hastie and Georgopoulos, 1971; Bork and Braymer, 1974).

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Benomyl resistance in *A. nidulans* has been shown to be determined by at least three genes (Tuyl, 1977; Morris, 1986). One of them (*benA*) confers a high level of resistance and codes for the beta-1 and beta-2 tubulin polypeptides (Hastie and Georgopoulos, 1971; Tuyl, 1977; Sheir-Neiss *et al.*, 1978). The other two (*benB* and *benC*) confer less pronounced resistance to this fungicide (Tuyl, 1977). A mutant was selected recently as a suppressor of the *benA22* mutation, that is able to conidiate in the presence of benomyl and lacks beta-3 tubulin, both characteristics being the result of the same mutation. However, it has not been determined whether this mutation is in *tubC*, the structural gene for beta-3 tubulin, or in some other gene that affects the level of beta-3 tubulin (Morris *et al.*, 1986).

In the present paper we describe three mutations, at the *benA* locus of *A. nidulans*, conferring higher levels of resistance to benomyl and thiophanate-methyl fungicides than those described previously. These strains also show good conidiation in the presence of these fungicides.

## MATERIAL AND METHODS

### Strains

The following strains were used: a) The haploid, *proA1 pabaA6 yA2*, used to select resistant mutants and b) Master strain E, MSE (*suA1 adE20, adE20, yA2, wA3, galA1, pyroA4, facA303, sB3, nicB8, riboB2*) (McCully and Forbes, 1965). Both strains were derived from the Glasgow stocks. c) FGSC 524 (*biA1, benA10, fwA1*), obtained from the Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City, USA. The mutant alleles used in the present study were: *fwA1, wA3* and *yA2*, fawn, white and yellow conidia respectively; *galA1* and *facA303*, inability to grow on galactose and acetate as sole carbon source respectively; *adE20, biA1, nicB8, pabaA6, proA1, pyroA4, riboB2* and *sB3*, growth requirements for adenine, biotin, nicotinic acid, *p*-aminobenzoic acid, proline, pyridoxin, riboflavine and thiosulphate, respectively; *suA1 adE20*, a suppressor of *adE20*; and *benA10* a marker for resistance to benomyl.

### Fungicides and media

Benomyl (methyl-1-butylcarbamoyl-2-benzimidazole carbamate) and thiophanate-methyl (1,2-bis-(3-methoxy-carbonyl-2-thioureido)-benzene) were purchased from E.I. Du Pont Nemours & Co., Delaware, USA and Nippon Soda Co. Ltd, Tokyo, Japan, respectively. Minimal medium (MM) and complete medium (CM) were those described by Pontecorvo *et al.* (1953). The incubation temperature was 37°C throughout.

### *Isolation of resistant mutants and dosage response*

Conidia of the strain *proA1 pabaA6  $\gamma$ A2*, treated with UV light, were inoculated on CM dishes containing thiophanate-methyl fungicide at a concentration that inhibited growth (40  $\mu\text{g/ml}$ ). Resistant colonies were isolated by the 7th day of incubation. Strains were characterized by measuring the diameters of colonies grown for 72 hours in CM dishes with various concentrations of thiophanate-methyl or benomyl. Fungicides were mixed with minimal volumes of sterile distilled water and immediately added to molten agar medium. Relative toxicity was expressed as  $\text{ED}_{50}$ , i.e., the concentration reducing the colony radial growth by 50%.

### *Genetic analysis*

Genetic analysis was performed as described by Pontecorvo *et al.* (1953). Mutants were assigned to their linkage groups by mitotic analysis (Forbes, 1959). Diploids were constructed as described by Roper (1952) and haploidization was facilitated by the use of *p*-fluorophenylalanine (Lhoas, 1961; Morpurgo, 1961). Crosses between the resistant strains were performed to carry out the allelism test.

## RESULTS AND DISCUSSION

Three thiophanate methyl-resistant colonies were randomly selected from the survivors of the mutagen treatment. These were cross-resistant to benomyl and were designated BEN-36, BEN-37 and BEN-38. Cross-resistance to thiophanates and benomyl has been described already (Hastie and Georgopoulos, 1971; Borck and Braymer, 1974), and the present study emphasizes the central role of MBC in the toxicity of benzimidazole compounds.

The toxicity of thiophanate methyl and benomyl, as measured by reduction in colony size, was tested on sensitive and resistant haploids and heterozygous diploids. The benomyl  $\text{ED}_{50}$  values, for sensitive and FGSC 524 strains, were 0.7 and 7.1  $\mu\text{g/ml}$  respectively (Table I). Similar results were obtained by Tuyl (1977). However, the benomyl  $\text{ED}_{50}$  values for the BEN-36, BEN-37 and BEN-38 strains were 32.0, 36.7 and more than 160.0  $\mu\text{g/ml}$ , respectively. The  $\text{ED}_{50}$  values of thiophanate methyl for sensitive and FGSC 524 strains were about 10 times the  $\text{ED}_{50}$  values of benomyl. The ratio of  $\text{ED}_{50}$  values between the two fungicides for BEN-36 and BEN-37 was over 60, indicating a higher level of resistance to thiophanate methyl in both mutants (Table I).

Each resistant strain was crossed with the MSE strain, which is sensitive to benzimidazole compounds. In all crosses, resistance and sensitivity segregated at a ratio of about 1:1 (Table II) indicating inheritance of a single gene mutation located

Table I - Relative toxicity ( $ED_{50}$ ) of sensitive and resistant haploids and of heterozygous diploids to benomyl and thiophanate-methyl fungicides.

Strains	Relevant genotype	$ED_{50}$ ( $\mu\text{g/ml}$ )	
		Benomyl	Thiophanate-methyl
MSE		0.7	7.0
<i>proA1 pabaA6 yA2</i>		0.7	7.0
FGSC 524	<i>benA10</i>	7.1	80.0
BEN-36	<i>benA36</i>	32.0	>2000.0
BEN-37	<i>benA37</i>	36.7	>2000.0
BEN-38	<i>benA38</i>	>160.0	>2000.0
BEN-36//MSE	<i>benA36//benA<sup>+</sup></i>	18.6	457.1
BEN-37//MSE	<i>benA37//benA<sup>+</sup></i>	17.7	871.0
BEN-38//MSE	<i>benA38//benA<sup>+</sup></i>	>160.0	>2000.0

BEN-36, BEN-37 and BEN-38 were isolated on thiophanate-methyl (40  $\mu\text{g/ml}$ ). The // sign indicates the diploid between 2 strains.

in linkage group VIII. Crosses between the resistant segregants showed allelism of *benA36*, *benA37*, *benA38* and *benA10* mutations, since the ascospores from hybrid cleistothecia produced only resistant colonies. At least 150 meiotic segregants from each cross were analysed. Benomyl-resistant mutations located in linkage group VIII were designated *ben-1* and *ben-2* by Hastie and Georgopoulos (1971), *benA3* to *benA30* (including the *benA10* from FGSC 524) by Tuyl (1977), and *benA31* to *benA33* by Oakley and Morris (1981) and Weil *et al.* (1986), all of them being allelic to each other.

Table II - Meiotic analysis of the benomyl-resistant mutants.

Cross	Ascospore segregation		
	Sensitive	Resistant	
		Normal conidiation	Nonconidiation
BEN-36 x MSE	114	55	63
BEN-37 x MSE	127	70	60
BEN-38 x MSE	91	39	44

The dosage response curves of the heterozygous diploids to benomyl and thiophanate methyl (not shown) indicated that *benA36* and *benA37* are semi-dominants (see ED<sub>50</sub> values in Table I). The diploid *BEN-38/MSE* gave a dose response curve (not shown) similar to that of *BEN-38*, suggesting that *benA38* is dominant for benomyl resistance (see ED<sub>50</sub> values in Table I). Mutations to resistance are often semi-dominant (Tuyl, 1977), though Hastie and Georgopolous (1971) described their mutations to benomyl resistance as recessive. Tuyl (1977) showed that there is considerable variation in the degree of dominance depending on the mutations, the fungicide and the concentrations tested. Nevertheless, this author described two benomyl resistant mutants at the *benA* locus as recessive (Tuyl, 1977). Thus, the benomyl-resistant mutants described here differ from those described previously. These differences concern: first, the degree of resistance to both fungicides tested; second, the dominance relationship presented; and third, conidiation. Characteristically the *benA* mutants do not conidiate at a benomyl concentration exceeding 5 µg/ml (Tuyl, 1977; May *et al.*, 1985; Weatherbee *et al.*, 1985). This was observed in the present study for FGSC 524, but not for the new strains isolated. *BEN-36* and *BEN-37* showed normal conidiation at up to 10 µg/ml benomyl, while the *BEN-38* strain conidiated at up to 100 µg/ml benomyl. This difference in conidiation capability was more marked when the thiophanate methyl fungicide was used. Presence of this fungicide in the culture medium, at concentrations of 10 µg/ml or more, did not permit FGSC 524 to conidiate or to have normal morphology, whereas the *BEN-36* and *BEN-37* mutants conidiated normally at thiophanate methyl concentrations of 50 µg/ml, and the *BEN-38* mutant did so even at a concentration of 1000 µg/ml. The *benA* mutation codes for the beta-1 and beta-2 tubulin polypeptide in *A. nidulans* (Sheir-Neiss *et al.*, 1978; Tuyl, 1977). The beta-3 tubulin polypeptide is the product of another gene (*tubC*) which is involved in fungal conidiation (Weatherbee *et al.*, 1985) and which is developmentally regulated in its expression (May and Morris, 1988). It has also been observed that the absence of normal beta-3 tubulin (*tubC* mutation) permits the *benA* mutant to conidiate in the presence of benomyl (Weatherbee *et al.*, 1985). Even though the mutants isolated here were not tested for the presence of beta-3 tubulin, they behaved as if they were double mutants (*benA tubC*). Indeed, when these strains were crossed with the *MSE* strain, approximately 50% of the meiotic segregants were sensitive to benomyl and 50% were resistant, as indicated earlier. Among these resistant segregants, approximately 50% conidiated normally and 50% did not conidiate in the presence of benomyl Table II. Conidiation was recovered in CM without fungicide. A possible explanation for this phenomenon is the presence of a second mutation, not linked to *benA* in the strains isolated here, that would permit them to conidiate in the presence of benomyl. In this case, if these strains were crossed with a wild strain for both traits, such as *MSE*, 25% of the segregants would have the genic combination of benomyl-resistance and nonconidia-

tion, as was the case in the present study. This putative mutation, that permits benomyl-resistant strains to conidiate in the presence of the fungicide, can not be located in linkage group VIII since some mitotic segregants also showed benomyl resistance but without conidiation. The use of medium containing thiophanate methyl to select benomyl-resistant mutants may have permitted, or even selected, the development of these double mutants and may also be responsible for high resistance to the fungicides.

On the basis of their characteristics, at least two of the benomyl-resistant mutations described here, and located at the *benA* locus, are probably mutations at different sites in the same locus, giving origin to different patterns of resistance and dominance. This hypothesis can be tested by molecular analysis of these mutants, as was shown for three herbicide-resistant mutants in *Chlamydomonas reinhardtii*. Analysis of these mutants showed that three different amino acid residues in a specific membrane protein (herbicide-binding protein) can be altered independently to produce three different patterns of resistance to s-triazine and urea-type herbicides (Erickson *et al.*, 1985).

In *A. nidulans*, benomyl acts directly on beta-tubulin by inhibiting microtubule polymerization, and benomyl resistance is caused by mutation in tubulin (Morris, 1986). In fact, any mutation altering binding sites for this fungicide (or MBC) in microtubular proteins, without affecting their functionality would lead to resistance (Seiler, 1975). Since the benomyl binding site is not known (Morris, 1986), the sequencing of the *benA36*, *benA37* and *benA38* genes could be very useful to define this site and to learn more about the structure of tubulins.

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### RESUMO

Três colônias de *Aspergillus nidulans* selecionadas para resistência ao tiofanato metil mostraram-se também resistentes ao fungicida benomil (BEN-36, BEN-37 e BEN-38). O nível de resistência ao benomil apresentado por estes mutantes foi pelo menos 45 e 4,5 vezes maior que os apresentados respectivamente pela linhagem selvagem e por uma conhecida linhagem resistente a benomil (FGSC 524). A marca de resistência a estes fungicidas era devido a uma única mutação localizada no locus *benA*. As curvas de dose-resposta obtidas com diplóides heterozigotos em presença de tiofanato metil e benomil indicou que os genes *benA36* e *benA37* são semidominantes enquanto que o *benA38* é provavelmente dominante.

Contrariamente ao esperado, a presença de benomil ou tiofanato metil no meio de cultura não inibiu a conidiação destes mutantes, o que pode indicar a presença de uma segunda mutação responsável por este fenômeno.

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