Resistance to ethidium bromide in *Aspergillus nidulans*[1]

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Summary. A mutant of *Aspergillus nidulans* resistant to ethidium bromide was isolated and the semi-dominant gene responsible for this resistance was allocated on linkage group II at 17.42±3.05 units of recombination from the wA_3 gene. The gene also confers cross-resistance to acriflavin, malachite green and crystal violet. It was also shown that riboflavin is antagonistic to the toxic effect of ethidium bromide, at certain concentrations. The mechanisms which could be responsible for the toxic effect of this drug are discussed and compared with those of acriflavin. The use of the Etb_1 gene in genetical analysis through the parasexual cycle is suggested.

Ethidium Bromide (EB), a phenanthridinium dye, originally of interest as a trypanocidal agent[2], has recently been used extensively as a molecular probe because of its interaction with extrachromosomal and chromosomal DNA and its effects on the macromolecular biochemistry of the cell (for a review see Levy and Ashry[3] and Macgregor and Johnson[4]). In *A. nidulans,* this drug reduces the number of sectors produced by diploid and duplication strains[5]. In the present work, we report the isolation of a resistant mutant, its cross-resistances and the effects of riboflavin on the inhibitory action of EB.

Material and methods. Minimal medium (MM) was Czapek-Dox with 1% glucose. Complete medium (CM) was a complex medium-containing yeast extract, casein hydrolysate, hydrolyzed nucleic acid, vitamins, etc.[6]. Solid media contained 2% agar. Strains of *A. nidulans* used, all derived from Glasgow stocks, were: Duplication strain A of Nga and Roper[7] (figure 1); strain MSE of McCully and Forbes[8]: suA_1adE_{20}, yA_2, adE_{20}; wA_3; $galA_1$; $pyroA_4$; $facA_{303}$; sB_3; $nicB_8$; $riboB_2$; and strain $AcraA_1$: suA_1adE_{20}, $pabaA_1$, yA_2, adE_{20}; $AcrA_1$; $lysB_5$; $chaA_1$. Those mutant alleles determined: yA_2, yellow conidia; $chaA_1$, chartreuse conidia; wA_3 (epistatic to y/y^+), white conidia; adE_{20}, biA_1, $lysB_5$, $nicB_8$, $pabaA_1$, $proA_1$, $pyroA_4$, $riboB_2$, sB_3, requirements respectively for adenine, biotin, lysine, nicotinic acid, p-aminobenzoic acid, proline, pyridoxine, riboflavine, thiosulfate; suA_1adE_{20}, suppressor of adenine requirement caused by adE_{20}; $galA_1$ and $facA_{303}$, inability to grow on medium with galactose and sodium acetate as the only source of carbon; and $AcrA_1$, which confers resistance to acriflavin. The resistant mutant to EB was obtained by plating about 10^8 conidia from the duplication strain A on solid CM dishes containing 10 µg/ml of EB (Sigma). Diploids MSE//A and MSE//A Etb_1 were isolated by Roper's technique[9]. The general techniques of genetical analysis used were those described by Pontecorvo et al.[6]. The dose response of the strains was estimated by the reduction of the colony size after inoculation on solid CM dishes containing different concentrations of EB or acriflavin hydrocloride (Baker) or malachite green (E. Merck) or crystal violet (Gurr). The effects of riboflavin (E. Merck) were estimated by the addition of 10, 20, 100 and 200 µg/ml of this drug to CM containing different concentrations of EB or acriflavin. Estimation of dose response and effects of riboflavin was made in triplicate and scored after 48 h of incubation. The incubation temperature was 37 °C in all cases.

Results and discussion. The determinant of resistance to EB in the mutant strain behaved as a single nuclear gene. A

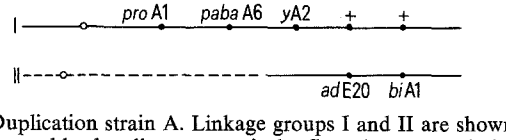

Fig. 1. Duplication strain A. Linkage groups I and II are shown by unbroken and broken lines, respectively. Centromeres are designated by open circles. AdE_{20}, biA_1, $pabaA_6$, $peoA_1$ and yA_1 are genes for adenine, biotin, p-aminobenzoic acid, proline requirements and yellow conidia, respectively.

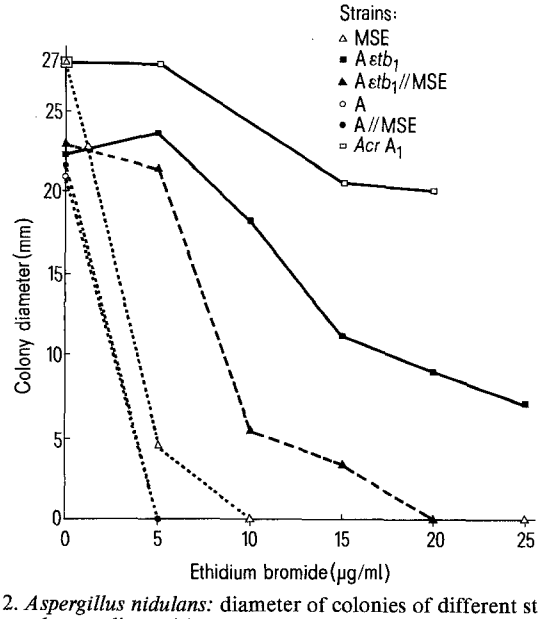

Fig. 2. *Aspergillus nidulans:* diameter of colonies of different strains on complete medium with or without ethidium bromide (0–25 µg/ml). The curves represent sensitive strains (.....), resistant strains (——) and the diploid strain heterozygous to Etb_1 gene (---).

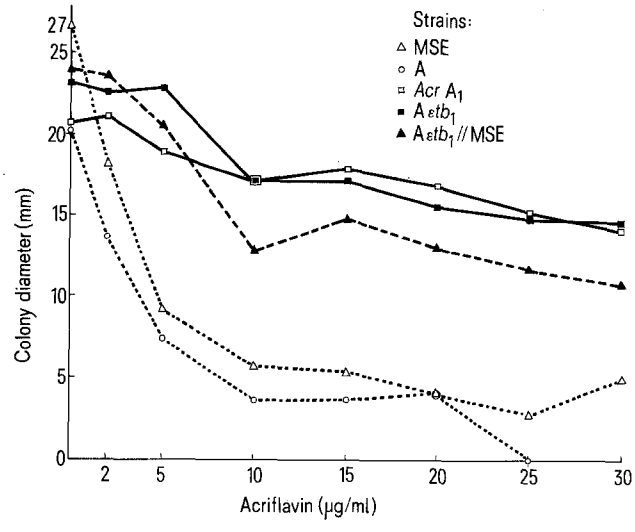

Fig. 3. *Aspergillus nidulans:* diameter of colonies of different strains on complete medium with or without acriflavin (0–30 µg/ml). The curves represent sensitive strains (.....), resistant strains (——) and the diploid strain heterozygous for Etb_1 gene (---).

Effects of riboflavin added to CM with different concentrations of EB or acriflavin

Inhibitor (μg/ml)	Riboflavine (μg/ml)	MSE	A	Strains A*Etb*$_1$	*Acr*A$_1$	MSE/A	MSE/A*Etb*$_1$
Ethidium bromide							
5	none	17.2	0.0	100.0	nd*	0.0	100.0
	10	36.7	30.4	100.0		58.6	100.0
	20	66.7	100.0	100.0		81.5	100.0
	100	86.2	100.0	100.0		75.0	100.0
	200	72.4	50.0	100.0		96.0	100.0
15	none	-**	-	86.4	nd	-	50.0
	10	-	-	83.4		-	48.2
	20	-	-	90.3		-	52.0
	100	-	-	100.0		-	76.9
	200	-	-	100.0		-	95.8
Acriflavine							
5	none	43.7	36.8	118.4	105.0	nd	100.0
	10	40.0	41.0	104.8	92.9		87.2
	20	40.1	42.8	107.5	97.6		86.3
	100	43.1	46.1	112.5	90.9		89.8
	200	38.9	48.7	106.8	85.1		92.0
20	none	18.7	18.4	76.3	85.0	nd	50.0
	10	14.0	17.9	73.8	78.6		48.9
	20	20.4	16.7	75.0	87.8		35.3
	100	25.5	20.5	62.5	77.3		42.8
	200	18.5	15.4	63.6	68.1		46.0

The values represent percent of growth in relation to control. * Not done, ** total inhibition.

cross between the resistant strain and a sensitive strain (MSE) gave 75 nonresistant and 81 resistant segregants. The gene which confers resistance to EB, now designated *Etb*$_1$, was allocated by mitotic analysis using Benlate as a haploidizing agent[10] on linkage group II at 17.42±3.05 units of recombination from the *w*A$_3$ gene also located in the left arm of the same linkage group. The dose responses to EB and acriflavin are summarized in figures 2 and 3, respectively.

The strain A *Etb*$_1$ had cross-resistance to acriflavin, and a similar response was observed for the strain which bears the gene *Acr*A$_1$. Strain A *Etb*$_1$ also showed cross-resistance to malachite green and crystal violet, and the gene *Etb*$_1$ behaved as a semi-dominant gene regarding its resistance both to EB and acriflavin. Similar behavior was found for the *Acr*A$_1$ gene and this characteristic makes *Etb*$_1$ useful for the selection of segregants through the parasexual cycle as is true for *Acr*A$_1$[11]. However the 2 genes are not alleles. A cross between strains carrying *Etb*$_1$ and *Acr*A$_1$ in repulsion has shown that the two genes are linked (6.8±0.12 units of recombination). They also differ in relation to their resistance to EB; at least at the concentrations used, the *Acr*A$_1$ strain was more resistant than the *Etb*$_1$ strain. The lower susceptibility to the effects of EB was related in resistant mutants class 2 to trialkyl tin salts[12] and in petites of *Saccharomyces cerevisiae* in relation to mitochondrial DNA replication[13]. The effects of riboflavin on the inhibitory action of EB and acriflavin are shown in the table. We observed an antagonistic effect of the vitamin regarding the toxicity of EB even in strains carrying the gene for resistance. Similar results were found for acriflavin[14] and malachite green[15]. We also found a remarkable reduction in the number of resistance sectors selected by EB from the heterozygous diploid MSE//A *Etb*$_1$ in the presence of riboflavin. In the strains A and MSE, not carrying the *Etb*$_1$ gene, the highest concentrations of riboflavin used (200 μg/ml) potentiates the action of EB. The same is not found in resistant strains. EB is a potent respiration inhibitor[16,17] in which are implicated enzymes which have riboflavin as a coenzyme[18]. The effects of riboflavin can then favour the hypothesis of an intervention of EB at the level of energy production to explain the increased stability of diploid and duplications strains of *A. nidulans*[5].

The effects of riboflavin are less pronounced when compared with the mode of action of acriflavin. At least at the concentrations of riboflavin used, we found a potentiation of the toxic action of acriflavin in the resistant strains and a decrease in the susceptible strains. The results are the opposite of those found with EB where potentiation occurs in susceptible strains. This suggests different modes or sites of action for the 2 drugs.

The isolation of more resistant mutants and studies in progress at the biochemical level could provide a better understanding of the mechanism of action of EB and acriflavin in *A. nidulans*.

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2 G. Brownlee, M.D. Goss, L.G. Goodwin, M. Woodbine and L.P. Walls, Br. J. Pharmac. *5*, 261 (1950).
3 A. Levy and A. Ashri, Mutation Res. *28*, 397 (1975).
4 J.T. Macgregor and I.J. Johnson, Mutation Res. *48*, 103 (1977).
5 R. Bonatelli, Jr, and J.L. Azevedo, Experientia *33*, 311 (1977).
6 G. Pontecorvo, J.A. Roper, L.M. Hemmons, K.D. Macdonald and A.W.J. Bufton, Adv. Genet. *5*, 141 (1953).
7 B.H. Nga and J.A. Roper, Genetics *58*, 193 (1968).
8 K.S. McCully and E. Forbes, Genet. Res., Camb. *6*, 352 (1965).
9 J.A. Roper, Experientia *8*, 14 (1952).
10 A.C. Hastie, Nature *226*, 771 (1970).
11 J.A. Roper and E. Käfer, J. gen. Microbiol. *16*, 660 (1957).
12 W.E. Lancashire and D.E. Griffiths, Eur. J. Biochem. *51*, 377 (1975).
13 P. Nagley and J.S. Mattick, Molec. gen. Genet. *152*, 277 (1977).
14 M.G. Sewag and J.S. Gotts, J. Bact. *56*, 723 (1948).
15 J.R. Warr and J.A. Roper, J. gen. Microbiol. *40*, 273 (1965).
16 L. Hanssens and H. Verachtert, J. Bact. *125*, 829 (1976).
17 B.G. Grinwood and R.P. Wagner, Arch. Biochem. Biophys. *176*, 43 (1976).
18 A.L. Lehninger, Biochemistry, 2nd ed. Worth Publishers, USA 1975.