The Genetic Analysis of Resistance to Benomyl in *Neurospora crassa*

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SUMMARY

Mutants of *Neurospora crassa* resistant to the fungicide, methyl-1-(butylcarbamoyl)-benzimidazolecarbamate, were isolated following u.v. irradiation of conidia. Genetic analysis of 15 of the mutant strains revealed that resistance was due to a single allele averaging 2.9 map units right of *ylo-1*, 17.9 units right of *tryp-2* and 32.1 units left of *chol-2* in linkage group VI. Tests of heterokaryons containing both resistant and sensitive alleles indicated that resistance is dominant. The mutants did not possess the same degree of tolerance to the fungicide.

INTRODUCTION

Within the last decade there has been increasing interest, both public and scientific, in the use of systemic agricultural fungicides. Methyl-1-(butylcarbamoyl)-benzimidazolecarbamate (benomyl) has shown particular promise due to its broad-spectrum antifungal activity and low mammalian toxicity. Benomyl is fungitoxic at very low concentrations and has therefore become the subject of several investigations concerning its mode of action (Clemons & Sisler, 1971; Hammerschlag & Sisler, 1972, 1973; Davidse, 1973).

One consideration in the understanding of fungicidal action is the development of resistant organisms. Spontaneous resistance to benomyl and related benzimidazole fungicides occurs in a number of phytopathogenic fungi (Dekker, 1972), and laboratory-induced mutations to benomyl resistance may be obtained with relative ease (Bartels-Schooley & MacNeill, 1970; Hastie & Georgopoulos, 1971; Fourcade & Laville, 1973). The frequency with which resistant strains appear is undoubtedly a reflection of the number of mutations necessary to promote tolerance and the mutability of the genes responsible (Dekker, 1972). Resistance arising through mutations at a number of genetic loci may well be an indication that the toxicant interferes with cell biochemistry at more than one metabolic step.

Our studies (unpublished) on the inhibitory mechanisms of benomyl in a representative Ascomycete, *Neurospora crassa*, have included a genetic analysis of benomyl-resistant mutants with the idea that such investigations might provide additional information as to whether single- or multi-site inhibition is involved. It was also of interest to determine whether resistance is dominant or recessive and what levels of benomyl tolerance could be produced. *Neurospora* was particularly good experimental material, since the wild-type strain is so sensitive to benomyl that growth is completely inhibited by concentrations as low as 0.25 µg/ml and the organism has been well characterized both biochemically and genetically.

METHODS

Strains. All strains of *N. crassa* utilized in this study are designated according to the gene symbols used by the Fungal Genetics Stock Center, (FGSC), Humboldt State College

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Foundation, Arcata, California, U.S.A. Benomyl-resistant mutants were induced in a histidine auxotroph, *hist-1A* (FGSC681), and wild-type strain 74-OR8-1a (FGSC988) was used in the initial cross with isolated mutants. Standard mating type testers were *flA* (FGSC1838) and *fla* (FGSC1690). Strains employed in mapping procedures included both mating types of *alcoy* (FGSC997 and FGSC998), *tryp-1*; *ylo-1* (FGSC1207 and FGSC1208) and *tryp-2*; *ylo-1*; *chol-2* (FGSC2091 and FGSC2092). All stock cultures were obtained from the FGSC with the exception of *flA* and *fla* which were provided by Dr D. R. Parker, Stanford University, U.S.A. Conidia were preserved at 5 °C by the silica-gel technique of Perkins (1962).

Media. Stock cultures were maintained on slants containing Vogel's (1964) citrate minimal salts solution, 1.5 % sucrose and 1.5 % agar. When auxotrophic mutants were used, growth supplements were added as follows ($\mu\text{g/ml}$): histidine HCl, 100; choline chloride, 30; indole, 20; and tryptophan, 150. Medium for *tryp-1* mutants was enriched with indole, and *tryp-2* mutants were grown on minimal medium plus tryptophan. All crosses were carried out on the medium recommended by Westergaard & Mitchell (1947) with 2 % sucrose and the required growth factors. Colonial growth of *Neurospora* was induced by the addition of 1 % (w/v) sorbose, 0.025 % glucose and 0.025 % fructose to minimal medium containing histidine. Benomyl-resistant mutants were scored on minimal slants containing appropriate growth requirements and either 0.5 or 1.0 μg benomyl/ml. Technical grade benomyl (E. I. duPont de Nemours and Co.) was dissolved in 100 % ethanol immediately before use and added to the autoclaved medium using standard aseptic procedures, the volume of benomyl added never exceeding 0.1 % (v/v) of the medium.

Isolation, selection and mapping of benomyl-resistant mutants. An aqueous suspension of conidia from 5-day-old cultures of *hist-1A* was filtered through cheese cloth and the concentration adjusted to approximately 10^7 conidia/ml. A thin layer of the suspension was poured into a sterile Petri dish and conidia were irradiated at 50 % kill (125 s) under a 30 W General Electric G30T8 germicidal lamp at a distance of 15 cm from the light source. Benomyl-resistant colonies were selected after plating treated conidia on sorbose medium containing either 0.5 or 1.0 μg benomyl/ml. To ensure that benomyl-resistant strains were homokaryotic for the mutant allele, resistant ascospores were selected after crossing resistant colonies to strain 74A. Methods for crossing and random ascospore isolation have been described by Perkins (1959). Fifteen out of a total of 41 resistant isolates were used for mapping (Perkins, Newmeyer, Taylor & Bennett, 1969).

Resistance levels of mutants. Tolerance to various concentrations of benomyl was examined for all mutants used in mapping studies. Growth rates of each resistant strain were measured on benomyl medium by the method of Ryan, Beadle & Tatum (1943), and average growth rates at each concentration of benomyl were computed from a plot of growth (mm) versus time (days).

Formation of heterokaryons. From crosses between benomyl-resistant mutants and strains 2091 and 2092, recombinants were selected which exhibited either resistance or sensitivity and only one auxotrophic phenotype (e.g. either *Tryp*⁻ or *Chol*⁻, but not both). Both cultures forming the heterokaryon were tested on minimal medium to ensure that *tryp* and *chol* mutations were non-leaky. Heterokaryons between recombinants were produced (Beadle & Coonradt, 1944) possessing the benomyl-sensitive (*ben-s*) and benomyl-resistant (*ben-r*) alleles. They were tested for resistance by transfer to benomyl slants. Specific tolerance levels were determined for two of the heterokaryons using the procedures described above.

Fig. 1 (a). Partial map of linkage group VI showing only the markers utilized in mapping of the *ben-r* allele. Regions between markers are arbitrarily numbered and refer to those regions designated in Table 1. (b) Map distances derived by calculation of mean values presented in Table 1.

RESULTS

Genetic analysis of benomyl-resistant mutants. The 15 resistant isolates chosen for complete mapping procedures represented both mating types as well as Hist⁺ or Hist⁻ phenotypes. Eight of these mutants were crossed to suitable mating types of *alcoy* for preliminary linkage tests. In *alcoy* there are three reciprocal translocations with easily scorable markers near the interchange points, and the gene in question may therefore be placed in either of the linkage groups involved in the translocation according to its linkage to the marker. The marker for translocation between linkage groups I and II is *al* (albino); *cot-1* (colonial, temperature-sensitive) serves as a marker for IV:V translocation and *ylo-1* (yellow) for a III:VI translocation. The data (not shown) from these crosses clearly demonstrated that benomyl resistance was linked to *ylo-1*, e.g. all isolates having orange pigmentation were resistant and all yellow were sensitive. The resistant allele(s) could therefore be placed in linkage groups III, VI or both. Segregation of benomyl resistance relative to *cot-1* and *al* was approximately 50% in all cases.

The assignment of benomyl resistance to a site on linkage group VI was accomplished via a cross of mutants to strains 1207 and 1208, which carry the marker *tryp-1* on linkage group III and *ylo-1* on linkage group VI. Phenotypic scoring of ascospores revealed close linkage of resistance to *ylo-1*, and in no instance was segregation of resistance paired with that of *tryp-1*. The localization of resistance on a single linkage group suggested that benomyl tolerance might be conferred by mutation at a single site. Therefore, the resistant allele in all 15 mutants was mapped with respect to markers *tryp-2*, *ylo-1* and *chol-2* in a cross with the triple mutant strains 2091 and 2092.

Ascospores from each cross were scored for recombinations in regions 1 to 5, which are illustrated in Fig. 1 (a). The results are presented in Table 1 and in Fig. 1 (b), which gives the map units of each region as calculated by averaging the recombination frequencies given in the Table. The correlation of all map unit values for each region strongly implies that benomyl resistance is conferred by a single allelic mutation which maps approximately 2.9 units to the right of *ylo-1*, 32.1 units left of *chol-2* and 17.9 units from *tryp-2*. Although the mean recombination frequencies in regions 4 and 5 were similar, the difference between frequencies in regions 3 and 1 suggests that *ben-r* is to the right of *ylo-1*.

Tolerance levels. Linear growth on solid medium in the presence of various concentrations

Table 1. *Recombination frequencies in regions 1 to 5 (see Fig. 1a) as determined from crosses of benomyl-resistant strains with triple mutant strains 2091 and 2092*

Resistant strains	Recombination (%) in region:					No. ascospores analysed
	1	2	3	4	5	
511	15	4.8	18	39	39	124
519	12	2.9	15	34	33	103
526	17	3.1	20	32	35	130
5210	11	4.8	16	31	31	124
557	19	3.3	22	26	27	90
572	15	3.2	18	32	35	124
586	25	1.8	24	33	35	114
5810	12	3.5	15	29	32	113
5102	21	2.4	23	34	35	127
5107	14	3.0	18	26	27	132
5128	19	3.0	18	32	31	134
111	14	2.5	15	30	31	119
123	15	3.8	18	39	36	105
128	10	0.0	10	32	32	114
159	17	1.8	19	32	34	112
Mean*	15.7	2.9	17.9	32.1	32.9	
Standard error*	1.08	0.24	0.95	0.99	0.87	

* Calculated from the sum total of the data listed in each column.

Table 2. *Growth rates of wild-type Neurospora (74A) and benomyl-resistant mutants on solid medium containing various concentrations of benomyl*

Strain	Growth rate (mm/day) at concentrations of benomyl of (μ g/ml medium):											
	0	0.1	0.15	0.2	0.25	0.5	1.0	2.0	3.0	5.0	7.0	10.0
74A	93	75	62	35	0	0	—	—	—	—	—	—
511	92	—	—	100	—	95	95	—	80	62	42	30
519	82	—	—	—	—	—	78	—	68	40	35	20
526	100	—	—	96	—	96	85	—	55	38	0	—
5210	30	—	—	—	—	—	25	—	10	3	2	0
557	65	—	—	60	—	55	35	—	0	0	—	—
572	50	—	—	—	—	41	40	31	0	0	0	0
586	40	—	—	—	—	35	30	23	0	0	0	0
5810	95	—	—	95	—	95	95	—	50	45	7	0
5102	66	—	—	—	—	—	62	45	15	0	0	0
5107	70	—	—	—	—	85	80	55	—	—	—	—
5128	85	—	—	—	—	—	65	—	40	30	11	0
111	55	—	—	—	—	—	48	38	10	0	—	—
128	42	—	—	—	—	—	32	—	—	—	—	22
159	90	—	—	—	—	—	70	50	40	35	0	0

of benomyl was used to estimate the sensitivities of mutants which had been mapped. This procedure was more desirable than measurements of dry weight from liquid cultures, primarily because our observations (unpublished) have shown cell wall synthesis continues in the presence of benomyl such that dry weight as an index of growth becomes inaccurate. Data presented in Table 2 demonstrates that some of the mutants were capable of growth in concentrations of benomyl nearly 100 times the levels inhibiting growth of wild-type *Neurospora* (74A). In all cases the growth rate on benomyl medium remained linear over a period of 96 h, indicating that mutants were unable to recover from or adapt to the presence

Table 3. *Relative levels of resistance expressed by ben-r/ben-s heterokaryons of N. crassa*

Strains paired in heterokaryon	Characteristics of each strain		Relative growth in benomyl medium*	
	Mating type	Phenotype		
			0.5 µg/ml	1.0 µg/ml
526,38	a	Ben-s, Tryp ⁺ , Chol ⁻	+++	+
5128,80	a	Ben-r, Tryp ⁻ , Chol ⁺		
511,70	A	Ben-s, Tryp ⁻ , Chol ⁺	+++	++
572,97	A	Ben-r, Tryp ⁺ , Chol ⁻		
511,44	A	Ben-s, Tryp ⁻ , Chol ⁺	+++	+
511,10	A	Ben-r, Tryp ⁺ , Chol ⁻		
586,11	a	Ben-s, Tryp ⁺ , Chol ⁻	+++	+++
5128,80	a	Ben-r, Tryp ⁻ , Chol ⁺		
159,97	A	Ben-s, Tryp ⁺ , Chol ⁻	+++	+
5107,11	A	Ben-r, Tryp ⁻ , Chol ⁺		
5107,5	A	Ben-s, Tryp ⁻ , Chol ⁺	+++	++
128,9	A	Ben-r, Tryp ⁺ , Chol ⁻		

* Qualitatively scored as sparse (+) to heavy, luxuriant (+++) growth.

of the toxicant. The resistant strains did not exhibit the same growth rates or the same degree of tolerance to increases of benomyl concentration.

Heterokaryons. Tests for benomyl resistance among heterokaryons (Table 3) revealed that all seven were able to grow in the presence of the fungicide, so the mutations are dominant. Preliminary estimations of the growth rates exhibited by two of the heterokaryons at various levels of benomyl indicated complete dominance, i.e. although the growth rates of heterokaryons on benomyl medium were much slower than those of individual strains, the maximum tolerance levels were equal to those of the dominant member of the heterokaryon.

DISCUSSION

Genetic analysis of a number of benomyl-resistant mutants of *N. crassa* suggest that dominant mutations in a single gene are responsible for tolerance. The results are in contrast to those of Hastie & Georgopoulos (1971) who concluded that benomyl resistance in *Aspergillus nidulans* was due to mutation at either of two unlinked loci, *ben-1* or *ben-2*. Multiple-gene resistance to actidione has been reported in *Saccharomyces cerevisiae* (Wilkie & Lee, 1965; Brusick, 1972) and in *N. crassa* (Hsu, 1963); therefore it is conceivable that more than one gene for benomyl resistance could be found in *Neurospora*. Our selection and mapping procedures exclude the possibilities that additional dominant mutations at another site have taken place or that the isolates under investigation have all arisen from a single mutation early in the selection procedure.

It is possible that recessive mutations to benomyl resistance occur. In a multinucleate conidium, the presence of a recessive allele for resistance would go unnoticed. In our system, the isolation of a recessive allele for benomyl resistance would require that only the nucleus carrying the mutation survived the u.v. irradiation or that the mutation occurred in a mononucleate conidium. It is also conceivable that modifier genes exist, similar to those found for actidione resistance in *S. cerevisiae* (Wilkie & Lee, 1965), which promote varying degrees of resistance, and the difference in tolerance levels observed among the mutants suggests that this may be so.

The apparent lack of a multi-genic system for resistance and the extremely low levels of fungicide necessary to inhibit growth of wild-type *Neurospora* may indicate that benomyl has only a single enzymic site of inhibition. Biochemical studies of benomyl and related compounds, such as 2-methyl benzimidazolecarbamate and thiabendazole suggest that they have similar modes of action (Clemons & Sisler, 1971; Hammerschlag & Sisler, 1972, 1973; Davidse, 1973) and cross-resistance to these fungicides has been reported in some organisms (Bartels-Schooley & MacNeill, 1971; Hastie & Georgopoulos, 1971). Thus multiple resistance to benzimidazoles might result from mutation at a single locus. Such a finding would suggest these toxicants inhibit fungal growth by similar means.

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