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Three genes determine the carboxin sensitivity of mitochondrial succinate oxidation in *Aspergillus nidulans*

BY I. A. U. N. GUNATILLEKE,* H. N. ARST, JR.†
AND C. SCAZZOCCHIO‡

Department of Genetics, University of Cambridge, Milton Road, Cambridge CB4 1XH, England

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SUMMARY

Partially dominant mutations to carboxin resistance occur in three, freely recombining, nuclear genes in *Aspergillus nidulans*. Mutations at all three loci reduce carboxin inhibition of succinate dehydrogenase (EC 1.3.99.1), succinate-cytochrome *c* reductase (EC 1.3.99.1) and succinate oxidase (EC 1.3.99.1) in mitochondrial preparations. It is therefore probable that the ability of carboxin to prevent growth of *A. nidulans* is a direct consequence of its ability to prevent succinate oxidation.

1. INTRODUCTION

There is considerable evidence that the systemic fungicide carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) inhibits succinate dehydrogenase (EC 1.3.99.1), at least in intact mitochondria (White, 1971; Ulrich & Mathre, 1972; Georgopoulos, Alexandri & Chrysayi, 1972; White & Thorn, 1975). The fungitoxicity of carboxin to the basidiomycete *Ustilago maydis* probably results from inhibition of succinate dehydrogenase because mutational alteration of this enzyme confers resistance *in vivo* (Georgopoulos *et al.* 1972). Here we report that mutations in at least three nuclear genes confer *in vivo* resistance to carboxin in the ascomycete fungus *Aspergillus nidulans*. Mutations in all three genes lead to considerable carboxin resistance in isolated mitochondrial preparations of succinate dehydrogenase and the related activities succinate-cytochrome *c* reductase (EC 1.3.99.1) and succinate oxidase (EC 1.3.99.1).

2. MATERIALS AND METHODS

(i) *Strains, genetical techniques, and growth tests*

The *A. nidulans* strains used carried markers in general use (Clutterbuck, 1974). All strains used for biochemical analysis carry the biotin auxotrophy *bi*A-1 and

* Present address: Department of Botany, University of Sri Lanka, Vidyalankara Campus, Kelaniya, Sri Lanka.

† Address correspondence to this author.

‡ Present address: Department of Biology, University of Essex, Wivenhoe Park, Colchester, CO4 3SQ, England.

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are otherwise isogenic apart from the specified genotypes. Genetical techniques were modified after Pontecorvo *et al.* (1953) and McCully & Forbes (1965). Growth testing of *A. nidulans* has been described previously (Arst & Cove, 1969). Five mM urea was used as nitrogen source, and carbon sources were used at 1% (w/v) except for acetate, used as a mixture of sodium acetate and acetic acid at pH 6·5 at a final concentration of 144 mM. Carboxin (Vitavax 97%, Uniroyal Ltd, Bromsgrove, Worcestershire) was added as a methanolic solution such that the concentration of methanol in the medium never exceeded 1% (v/v). Methanol is not a sole carbon source for *A. nidulans*, and this concentration is well below the toxic level.

(ii) *Isolation of carboxin resistant mutants*

Thirteen carboxin resistant mutants were isolated after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis (Alderson & Hartley, 1969) of a strain of genotype *bi*A-1 *lu*A-1 *cnx*H-5 (biotin-requiring, L-leucine-requiring, unable to utilize nitrate or hypoxanthine as nitrogen source) as able to grow on appropriately supplemented minimal medium (Cove, 1966) containing acetate as sole carbon source, urea as nitrogen source, and 12·5 mg/l carboxin.

(iii) *Culture of mycelium for mitochondrial preparations*

Mycelium of wild type and mutant strains was grown 22–24 h at 25 °C in shaken liquid minimal medium (Cove, 1966) containing 1% (w/v) D-glucose as carbon source, 5 mM urea as nitrogen source, and 10 μg/l D-biotin. Freshly grown mycelium was used directly after harvesting for mitochondrial preparations.

(iv) *Preparation of mitochondria*

Mitochondria were prepared by a method similar to that described by Rowlands & Turner (1974). All procedures were carried out at 5 °C. The extraction medium (Lambowitz, Smith & Slayman, 1972) contained 440 mM sucrose, 10 mM tris, 2 mM EDTA (disodium salt), and 0·08% bovine serum albumin (fraction V, Sigma) and was adjusted to pH 7·2 with HCl. Mycelium was suspended in approximately 2·5 times the amount (v/w) of precooled extraction medium. 150–200 ml of this mycelial suspension was ground twice in a grind-mill (Weiss *et al.* 1970) and further diluted with extraction medium to a final ratio of 1:10 wet weight to extraction medium volume. The homogenate was then shaken vigorously in an MSE Atomix blender for 30 sec and filtered through muslin. The filtrate was centrifuged at 850 *g* for 8 min to remove mycelial debris and ungerminated conidia. The resulting supernatant was centrifuged at 23000 *g* for 40 min. The thick brown pellet obtained was resuspended in 30 ml of extraction medium using a ground glass homogenizer. After a second differential centrifugation, the pellet was resuspended in cooled extraction medium to give a mitochondrial preparation with a final protein concentration of 20–30 mg/ml.

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(v) *Respiration measurements*

Oxygen uptake was measured polarographically at 25 °C with a Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambridge). The reaction chamber contained 3·0 ml of oxygen-saturated respiration medium (300 mM sucrose, 6·67 mM succinate (disodium salt) or 1 mM NADH, 8 mM NaH_2PO_4, 700 μM EDTA (disodium salt), 8 mM tris, 0·1 % bovine serum albumin (fraction V, Sigma), pH 7·0) with sufficient mitochondrial preparation to give a final protein concentration of 1–2·5 mg/ml.

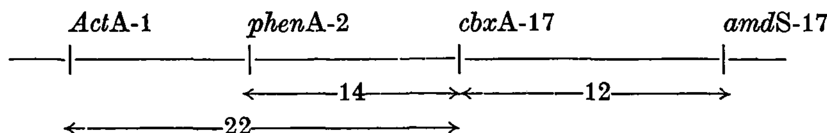
(vi) *Enzyme assays and protein determination*

Succinate-cytochrome *c* reductase was assayed as described by King (1967). Succinate dehydrogenase was assayed as described by Veeger, DerVartanian & Zeylemaker (1969). NADH-cytochrome *c* reductase was assayed as for succinate-cytochrome *c* reductase except that 33 mM NADH replaced 20 mM succinate in the assay mixture. Cytochrome oxidase was assayed by the method of Wharton and Tzagoloff (1967). Protein was determined by the biuret method (Layne, 1957).

3. RESULTS AND DISCUSSION

(i) *Genetic analysis and dominance relationships*

None of the 13 carboxin resistance mutations showed extranuclear inheritance when subjected to the version of the heterokaryon test described by Gunatilleke, Scazzocchio & Arst (1975). Meiotic analysis showed that mutations to carboxin resistance map in at least three, freely recombining, nuclear genes, designated *cbx*A (mutant allele nos. 7, 13, 16, 17 and 30), *cbx*B (mutant allele nos. 5, 8, 10, 12 and 28) and *cbx*C (mutant allele nos. 34, 38 and 41). Haploidization analysis (McCully & Forbes, 1965) located *cbx*A-17 and *cbx*B-28 to linkage group III and *cbx*C-34 to linkage group VIII. The following map position for *cbx*A-17 was obtained relative to other linkage group III markers (Clutterbuck, 1974):



(Distances are expressed in cM and were determined from crosses in which 125 progeny were analysed.) *cbx*A-17, *cbx*B-28 and *cbx*C-34 are all partially dominant in both heterokaryons and diploids.

(ii) *Growth responses of wild type and mutants*

Carboxin is approximately ten times more toxic to *Ustilago maydis*, *Neurospora crassa* and *Saccharomyces pastorianus* when acetate serves as carbon source than when glucose serves as carbon source (Ragsdale & Sisler, 1970). A similar situation

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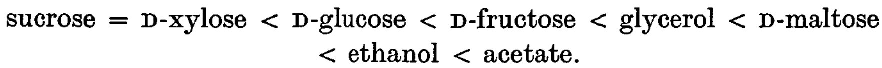
exists in *A. nidulans* where carboxin toxicity to the wild type increases with a change of carbon source in the following order:



sucrose = D-xylose < D-glucose < D-fructose < glycerol < D-maltose < ethanol < acetate.

Representative toxicity data on three carbon sources are given in Table 1. The positions of maltose and acetate in this series show that carboxin toxicity does not increase with carbon catabolite derepression (Arst & Cove, 1973; Bailey & Arst, 1975) at least in a straightforward fashion. Moreover, the greater toxicity on maltose as compared to glycerol suggests that this order does not simply reflect a greater toxicity on non-fermentable than on fermentable carbon sources. Because *A. nidulans* is an obligate aerobe, inhibitors of mitochondrial functions are not necessarily more toxic on non-fermentable carbon sources (Gunatilleke *et al.* 1975). In *N. crassa*, succinate dehydrogenase levels are several-fold higher in acetate-grown mycelia than in glucose-grown mycelia (Flavell & Woodward, 1970). Therefore the above order may represent the resultant of the relative amount of succinate dehydrogenase present during growth on different carbon sources and the essential substrate flux through succinate dehydrogenase on each of these carbon sources. Carboxin inhibition is not relieved by supplying a source of Krebs cycle intermediate beyond succinate such as 10 mM fumarate, 10 mM malate, or 5 mM L-aspartate to glucose medium.

Table 1. *Growth of wild type and* cbx *strains on different carbon sources with carboxin*

Carbon source	Carboxin concentration (μg/ml)	Growth score*			
		Wild type	*cbx*A-17	*cbx*B-28	*cbx*C-34
D-glucose	0	5	5	5	5
	50	2	5	5	5
	100	1	5	5	5
	150	0	4	5	4
Ethanol	0	5	5	5	5
	12·5	0	5	5	5
	25	0	4	5	5
	50	0	2	5	4
Acetate	0	5	5	5	5
	12·5	0	4	5	5
	25	0	3	5	4
	50	0	1	5	3

* Scored after 2 days' incubation at 37 °C. Scores range from 0 (no growth) to 5 (normal growth). Growth scores on different carbon sources are not necessarily equivalent.

The *cbx* mutants are resistant to carboxin on all carbon sources. Their growth on inhibitor-free media is indistinguishable from that of the wild type on a wide range of carbon sources including 1% (w/v) succinic acid.

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(iii) *The nature of carboxin inhibition*

Carboxin inhibits succinate-cytochrome *c* reductase non-competitively with respect to succinate (Fig. 1). Carboxin is also a non-competitive inhibitor of succinate oxidation in *U. maydis* and *Cryptococcus laurentii* (White, 1971). Concentrations of carboxin which strongly inhibit succinate oxidation in *A. nidulans* have little or no effect on NADH oxidase (EC 1.6.99.3), NADH-cytochrome *c* reductase (EC 1.6.99.3), or cytochrome oxidase (EC 1.9.3.1) (Table 2).

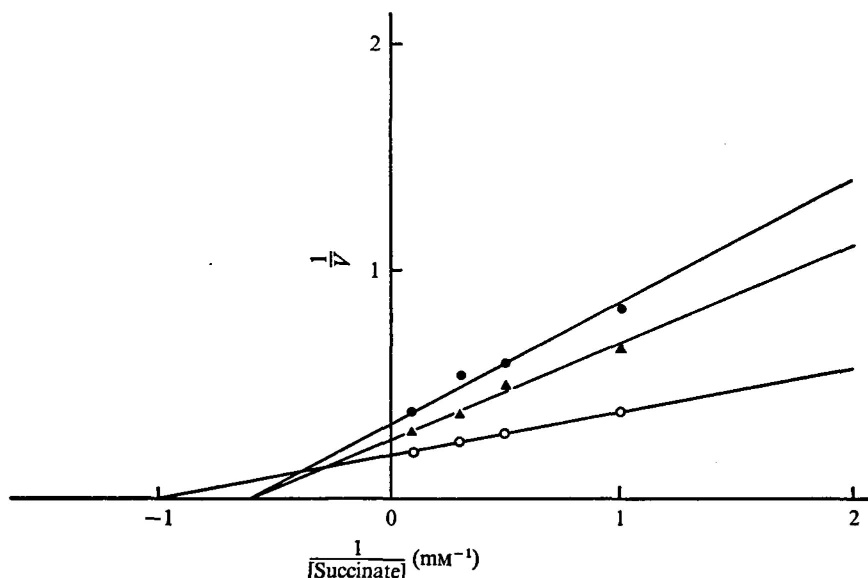

Fig. 1. Double reciprocal plot showing inhibition of wild type succinate-cytochrome *c* reductase by carboxin. *V* is expressed in μmoles of ferricytochrome *c* reduced per min per mg protein. ○—○, No carboxin added; ▲—▲, 33 ng/ml carboxin; ●—●, 83 ng/ml carboxin.

Table 2. *Effect of 8·3 μg/ml carboxin on various activities of the electron transport system of wild type and* cbx *strains*

Activity	Inhibition (%) Wild type	*cbx*A-17	*cbx*B-28	*cbx*C-34
Succinate oxidase	92	75	52	64
NADH oxidase	4	5	5	5
Succinate-cytochrome *c* reductase	97	83	64	77
NADH-cytochrome *c* reductase	4	5	5	5
Succinate dehydrogenase	93	52	37	45
Cytochrome oxidase	8	8	6	7

(iv) *The basis of carboxin resistance*

*cbx*A-17, *cbx*B-28 and *cbx*C-34 all reduce carboxin inhibition of succinate oxidase, succinate-cytochrome *c* reductase, and succinate dehydrogenase in mitochondrial preparations (Figs. 2–4). The relatively highest carboxin resistance

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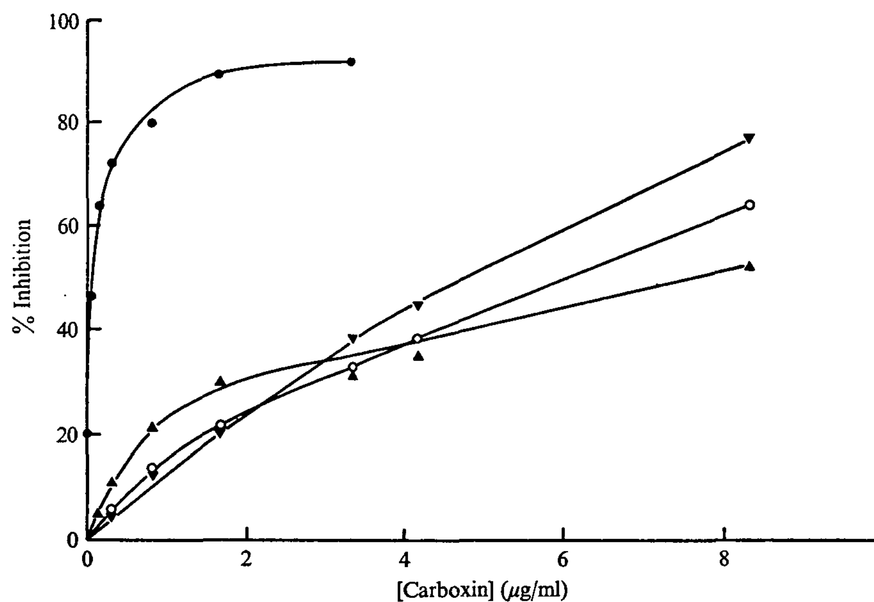

Fig. 2. Effect of carboxin on rate of oxygen uptake by mitochondrial preparations of wild type and carboxin resistant mutants with succinate as substrate. ●—●, Wild type; ▼—▼, *cbx*A-17; ▲—▲, *cbx*B-28; ○—○, *cbx*C-34.

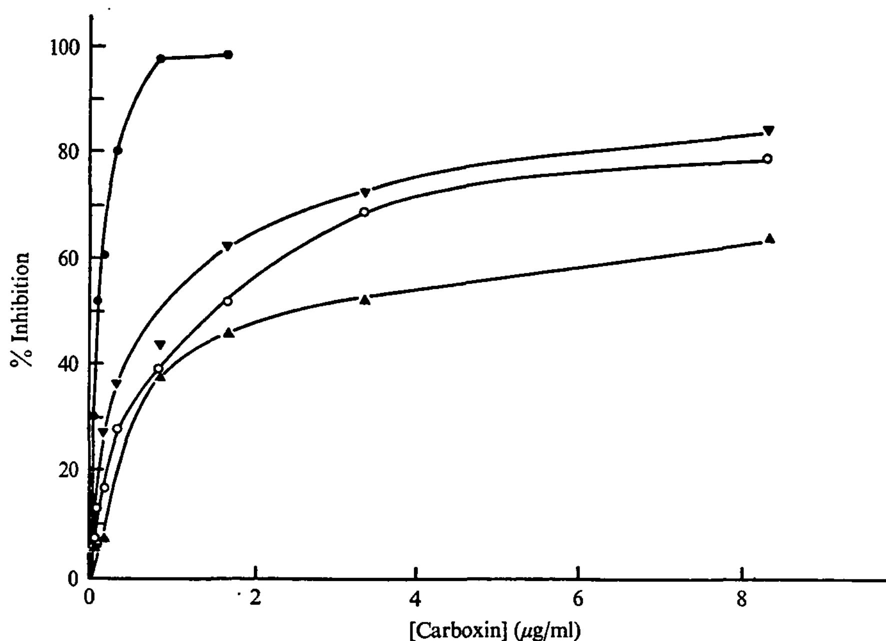

Fig. 3. Effect of carboxin on succinate-cytochrome *c* reductase in mitochondrial preparations of wild type and carboxin resistant mutants. For symbols, see legend to Fig. 2.

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in vitro of *cbx*B-28 strains is mirrored *in vivo* where *cbx*B-28 strains are demonstrably more resistant to carboxin toxicity than *cbx*A-17 or *cbx*C-34 strains (Table 1). However, the resistance of all three mutants *in vitro* shows that none of these three genes is involved (at least exclusively) in transport of carboxin across the cell membrane. Further work will be necessary to establish which, if any, of these three genes specify structural components of succinate dehydrogenase and which might code for components of the mitochondrial membrane interacting with succinate dehydrogenase or involved in carboxin transport across the mitochondrial membrane.

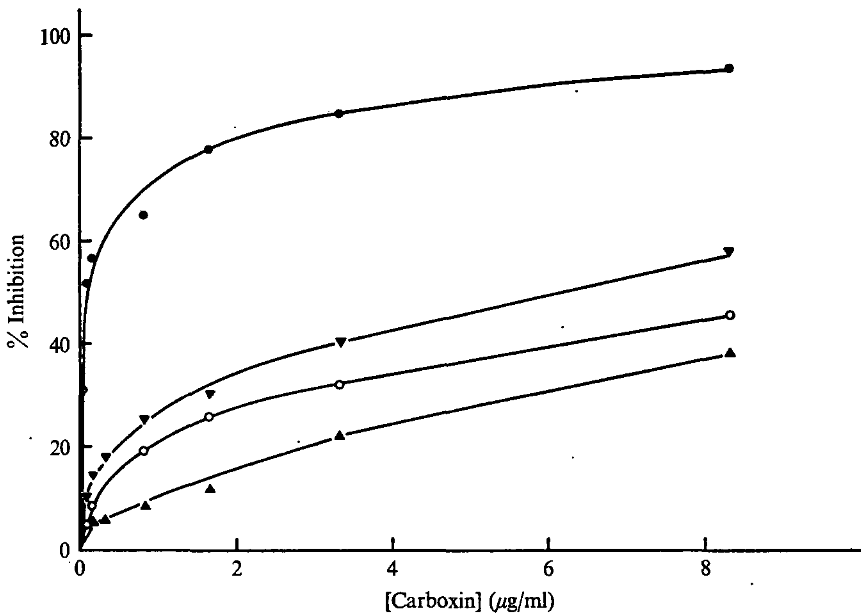

Fig. 4. *Effect of carboxin on succinate dehydrogenase in mitochondrial preparations of wild type and carboxin resistant mutants. For symbols, see legend to Fig.* 2.

Unlike the *ants* mutation of *Ustilago maydis*, which confers some resistance to carboxin (Georgopoulos & Sisler, 1970), *cbx*A-17, *cbx*B-28 and *cbx*C-34 do not diminish the level of antimycin A-insensitive, cyanide-insensitive respiration of isolated mitochondrial preparations using either succinate or NADH as substrate (unpublished data).

In any case, our results strongly suggest that in *Aspergillus nidulans*, as in *Ustilago maydis* (Georgopoulos *et al.*, 1972), the ability of carboxin to inhibit fungal growth is a direct consequence of its ability to prevent succinate oxidation. Moreover, it is especially interesting that mutations in at least three genes can affect carboxin inhibition *in vitro* in *A. nidulans*. Two such genes have been identified in *U. maydis* (Georgopoulos *et al.* 1972; Georgopoulos, Chrysayi & White, 1975; Georgopoulos, personal communication), whilst polygenic inheritance of carboxin tolerance in *Ustilago hordei* has been suggested (Ben-Yephet, Henis & Dinoor, 1975).

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Note added in proof. After submission of this paper, a brief report by J. M. van Tuyl (*Neth. J. Pl. Path.* **81**, 122–123 (1975)) came to our attention. He identified three genes where mutation can lead to carboxin resistance in *Aspergillus nidulans*. His *carB* gene is in linkage group VIII and might therefore be equivalent to our *cbxC*. His *carC* gene is linked to *phenA* in linkage group III and is probably therefore equivalent to our *cbxA*. However, his *CarA* gene is in linkage group VII. Therefore we retested our *cbxB*-28 and found that it also is in linkage group VII, and not in linkage group III as reported in the text of this paper. Our *cbxB*-28 is, however, only partially dominant, unlike his apparently fully dominant *CarA* mutations. If the *CarA* and *cbxB*-28 mutations be allelic, this difference of dominance reflects an interesting heterogeneity of phenotype. Alternatively, it could merely be an artefact in consequence of differences in test media (malt agar in his case) or of his testing at carboxin concentrations too low to permit distinction between diploids heterozygous and homozygous for a *CarA* mutation.

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