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The *ftr* Cistron of *Coprinus cinereus* is the Structural Gene for a Multifunctional Transport Carrier Molecule

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Summary. Mutants of the basidiomycete *Coprinus cinereus* which were selected for their resistance to growth inhibitions caused by hexose analogues are all alleles of the *ftr* cistron. They are shown to have approximately normal levels of activity of enzymes involved in intracellular sugar metabolism and to accumulate normal levels of sugar phosphates. However, the mutants show greatly depressed rates of sugar uptake. Uptake rates from 0.01 mM solutions of 2-deoxy-D-glucose were only 1 to 4% of the wild type rate, and from 15 mM solutions the mutant rates were between 16 and 40% of normal. Kinetic analysis showed that the mutant V_{max} values were reduced to a few per-cent of normal while K_m values were relatively little changed and in some cases the mutants had an increased affinity for the substrate. Reverse mutations restored the V_{max} value *and* the K_m to about the wild type level. Previous data had shown that position of mutants within the allele map depended on selection conditions in a way that implied some interaction between the *ftr* gene product and the substrate. Since the mutants are defective in transport from both high and low sugar concentrations, and since they exhibit coordinated alterations in K_m and V_{max}, it is concluded that the *ftr* cistron is the structural gene for a product involved in sugar translocation (both as carrier and energisation link) in both high and low affinity glucose transport systems.

Key words: Sugar transport – Transport mutants – *Coprinus cinereus*

Introduction

Evidence has been presented which suggests that glucose transport in the basidiomycete *Coprinus cinereus* is an active transport process involving a substrate-concentration-dependent transition between two kinetic states which differ in substrate affinities, specificities and modes of energisation [1]. Further analysis of the system would be greatly assisted if appropriate mutants were available and in this paper we show that mutants isolated originally for their resistance to the growth inhibitory effects of sugar analogues [2] are alleles of the structural gene for a transport carrier molecule.

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Materials and Methods

Organism. The organism used was *Coprinus cinereus* (Schaeff. ex Fr.) S. F. Gray. Two prototrophs, BC9/6,6 (mating type A_6B_6) and ZBw601/40, 40 (mating type $A_{40}B_{40}$) and three sugar analogue resistant mutants, Z154/6,6, Z492/6,6 and ZR215/40, 40 were employed. Isolation of the mutants and the methods and media used for growth tests have been described [2].

Sugar Uptake. Kinetics of sugar accumulation by germinated oidia were established using methods described by Moore and Devadatham [1]. In some experiments the amount of tissue taken in each sample for sugar accumulation measurement was determined by scintillation counting; the tissue being labelled prior to exposure to the experimental sugar with ^{14}C or ^{3}H-labelled leucine. In these cases oidia were germinated in medium supplemented with 0.01 μCi/ml L-[U-^{14}C] leucine or L-[4,5-^{3}H] leucine, this isotopic label being chosen to be complementary to that used in the sugar being studied. Dual-labelled samples were measured in a Packard A300CD LS-spectrometer. The fate of accumulated sugars was determined by paper chromatography of cell-free extracts. Vegetative mycelia grown for five days in a complete medium were starved by incubation for 2 h in carbon-free medium before being exposed for 20 min to 10 μCi/ml of isotopically labelled sugar. The mycelia were filtered onto Whatman GF/A filters, washed and immersed directly in liquid nitrogen. The frozen samples (about 1 g fresh weight) were extracted by grinding with 10% (w/v) trichloroacetic acid in 30% (v/v) methanol. The homogenate was kept at 4°C for 1 h then centrifuged at 2 °C for 10 min at 6,000 rpm. The pellet was washed

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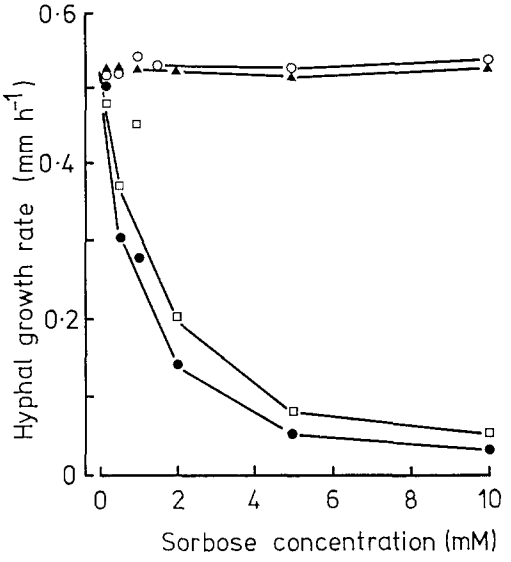

Fig. 1. Growth of wild type and mutants on solid medium containing L-sorbose. Hyphal growth rates were measured of colonies growing on the basal medium listed in Table 1 supplemented with 5 mM Na acetate and various concentrations of L-sorbose. *Strains tested:* wild type BC9/6,6 (•); *ftr* mutant strain Z492/6,6 (○); *ftr* mutant strain Z154/6,6 (▲); revertant strain R1, which is a revertant of Z492/6,6 (□)

Table 1. Growth yield of *Coprinus cinereus* wild type and mutants on media containing glucose or fructose as sole carbon sources. Mycelia were grown for 7 d at 37 °C as still cultures in quadruplicate sets. The basal medium contained 10 mM Na_2HPO_4, 10 mM KH_2PO_4, 2 mM Na_2SO_4, 0.5 mM $MgSO_4$, 3 μM thiamine hydrochloride, 30 mM NH_4Cl, plus the indicated carbon source

Strain	Mycelial dry weight (mg per 100 ml)	
	Fructose (10 mM)	Glucose (10 mM)
BC9/6,6 (wild type)	54	48
Z492/6,6 (*ftr* mutant)	3	21
Z154/6,6 (*ftr* mutant)	9	50
R10 (revertant of Z492/6,6)	38	41
RV3 (revertant of Z154/6,6)	73	58

once and the supernatants pooled. Trichloroacetic acid was removed with ice-cold ether and the resultant extract reduced to 1 ml by desiccation over P_2O_5. Quantities of 20 μl were chromatographed on acid-washed Whatman no. 1 paper in a solvent containing propan-1-ol, ammonia and water (16:3:1). In this system all sugar phosphates remain at the origin while free sugars migrate. Sugars were located with the alkaline silver oxide reagent of Trevelyan et al. [3], and were identified by co-chromatography of authentic compounds. The spots were excised and radioactivity determined by scintillation counting.

Enzyme Assays. Activities of various enzymes involved in sugar metabolism were determined using published methods modified so as to optimise them to *Coprinus* cell-free extracts.

Isolation of Revertants. Revertants of the mutants Z154/6,6 and Z492/6,6 were isolating by selecting for the prototrophic ability to grow well with fructose as sole carbon source. The mutants grow weakly on fructose so by plating large numbers of spores in fructose medium revertants with wild type phenotype could be distinguished as densely-growing colonies against a background of weak mutant growth. Isolated revertants were purified by oidial streaking and retested thoroughly before being put into stock culture.

Results and Discussion

Mutants have been selected for resistance to inhibitions caused by 2-deoxy-D-glucose [2], L-sorbose and D-glucosamine [4]. All of the mutants obtained so far have proved to be alleles of a single gene called, because of the lack of allelic complementation, the *ftr* cistron [5]. Irrespective of the analogue used for selection, all *ftr* alleles are cross resistant to all three sugar analogues and all grow weakly with fructose as sole carbon source though growth on glucose is variable. The major phenotypic expressions of *ftr* mutants are illustrated in Fig. 1 which shows response to growth with sorbose included in the medium, and Table 1 which depicts growth yield with either fructose or glucose as sole carbon source.

These are the mutational effects which need to be accounted for: inability to utilise fructose and resistance to sugar analogue inhibitions.

Assay of representative enzymes involved in sugar metabolism revealed no substantial differences in activity between wild type and *ftr* mutants (Table 2) so there is no obvious enzyme defect which could account for the inability to use fructose. The ratio of sugar phosphate to free sugar in the intracellular pool was also similar in both genotypes, showing that no change in phosphatase enzymes can account for sugar analogue resistance although this has been recorded as an explanation of this phenotype in other organisms [6]. The accumulation of sugar from the medium was grossly impaired in the mutants. Figure 2 shows that at both high (15 mM) and low (0.01 mM) external concentrations of 2-deoxy-D-glucose the mutants accumulated the sugar much more slowly than did the wild type; yet in all strains the internal concentration eventually came to exceed the external. We conclude that the mutants still possess an active transport system but that their rate of accumulation is impaired, The *ftr* mutants retain only 1 to 4% of the wild type rate of uptake from 0.01 mM solutions (the uptake rate of wild type BC9/6,6 was 97.6 pmol (mg dry weight)$^{-1}$ min^{-1}, compared with 3.9 and 1.2 pmol (mg dry weight)$^{-1}$ min^{-1} in Z492/6,6 and Z154/6,6 respectively). These two *ftr* strains retained 16.4%

Table 2. Activities of some enzymes involved in carbohydrate metabolism in wild types and mutants of *Coprinus cinereus*. Mycelia were grown for 5 d on an orbital incubator operating at 37 °C in a medium consisting of the salts solution listed in Table 1 supplemented with 25 mM asparagine and 50 mM glucose. Prior to assay the mycelia were harvested by centrifugation and transferred to a medium consisting of the salts mixture plus 50 mM glucose for a further 20 h incubation. They were finally harvested by filtration and frozen at −40 °C to await assay. Units are nmole substrate min^{-1} (mg protein)$^{-1}$

Strain	Gluco-kinase	Fructo-kinase	Phospho-glucose isomerase	Phospho-fructo-kinase	Aldolase	Pyruvate kinase	Glucose 6-phosphate dehydro-genase	6-phospho gluconate dehydro-genase
BC9/6,6	223	135	225	163	90	234	47	79
Z154/6,6	213	97	244	128	207	255	149	124
Z492/6,6	253	165	250	129	188	306	154	170
ZBw601/40,40	345	150	282	116	184	333	120	33
ZR215/40,40	316	187	265	182	168	271	210	273

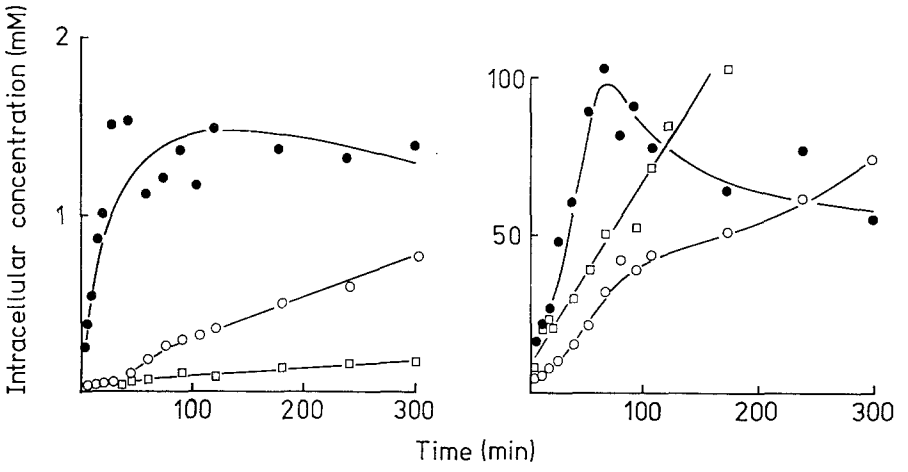

Fig. 2. Accumulation of 2-deoxy-D-glucose from solution with an initial concentration of 0.02 mM (left hand plot) or 15 mM (right hand plot). In each case the accumulation is expressed as the internal (mM) concentration. *Strains used:* wild type BC9/6,6 (•); *ftr* mutant Z492/6,6 (○); *ftr* mutant Z154/6,6 (□)

(Z492/6,6) and 43.1% (Z154/6,6) of the wild type rate of uptake from 15 mM solutions. The two concentrations of 2-deoxy-D-glucose used in these experiments enable tests to be concentrated on one or other of the glucose uptake systems. Glucose uptake by the wild type is characterised by two kinetic systems – the high affinity system which has broad substrate specificity but high affinity for aldohexoses (glucose K_m = 27μM), and the low affinity system which has a greater specificity but lower affinity (glucose K_m = 3.3 mM) [1]. Substrate concentration effectively distinguishes between the two systems and since *ftr* mutants showed an accumulation defect at both 15 mM and 0.01 mM substrate concentrations we conclude that the mutation affects both uptake systems. Each of these mutations have been mapped [5] and they behave genetically like point mutations within a single cistron. The implication, therefore, is that this *ftr* cistron is the structural gene for a product which contributes to both transport systems.

Revertants, especially temperature sensitive ones, have often been used to provide support for identifications of structural genes. We carried out 35 separate experiments with spores of Z154/6,6 and Z492/6,6 to select for wild type revertants. A total of 7.4×10^9 viable oidia were screened in numbers varying from 1.6×10^7 to 1.8×10^9 per experiment. Selection was carried out at both 37 °C and 27 °C and with and without treatment with the chemical mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. A total of 30 revertants were isolated. All revertants were able to use glucose, fructose and acetate as well as the wild type, and they were sensitive to inhibition by L-sorbose at both 27 °C and 37 °C. No temperature sensitive conditional revertants were isolated although one failed completely to grow at 37 °C, behaving like the irreparable 'temperature extremely sensitive' mutant described by Munkres [7]. Genetic analysis of the revertants showed that 9 of them carried a suppressor gene mutation. All the suppressors were linked to *ftr* and they fell into two groups, one of which showed about 2% recombination with *ftr* and the other about 8%. Revertant numbered R1 is here shown as representative of the majority class which did not carry suppressors

Table 3. Ratios of intracellular sugar phosphate to free sugar in wild type and mutants of *Coprinus cinereus*

Strain	ratio sugar-phosphate / free sugar	
	2-deoxy-D-glucose	glucosamine
BC9/66	0.05	0.08
Z154/6,6	0.13	0.10
Z492/6,6	0.05	0.20

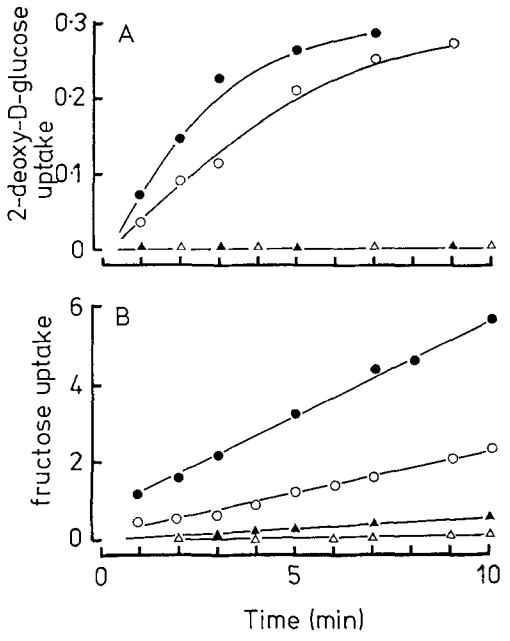

Fig. 3. A. Initial uptake kinetics of 2-deoxy D-glucose from 0.02 mM solutions (scale = pmole sugar accumulated (dpm ^{14}C-leucine)$^{-1}$; **B.** initial uptake kinetics from 1 mM solutions of fructose (scale = pmole sugar accumulated (dpm ^{3}H-leucine)$^{-1}$. *Strains used:* wild type BC9/6,6, (●); *ftr* mutant Z492/6,6 (△); *ftr* mutant Z154/6,6 (▲); revertant R1 which is a revertant of Z492/6,6 (○)

and were therefore judged to result from mutation within the *ftr* gene. Phenotypically, R1 was sensitive to growth inhibition by L-sorbose (Fig. 1) and like other revertants (Table 1) grew well with fructose as sole carbon source. The initial rate of accumulation of 2-deoxy-D-glucose from 0.01 mM solutions was restored to about 90% of the wild type rate in revertant R1 (Fig. 3A). Fructose uptake rate, however, although about 20 times greater than in *ftr* mutants was only restored to about 50% of the wild type rate (Fig. 3B). The kinetic constants for uptake of these two sugars have been determined (Table 4). These data show firstly that the greatest kinetic alteration observed in *ftr* mutants is in the V_{max} which is reduced to less than 2% of the wild type value. The K_m, on the other hand is more variably altered; affinity for fructose is some 4 times greater in the mutants, while affinity for 2-deoxy-D-glucose is unchanged in one mutant and decreased by a factor of five in the other. The reversion event, however, has restored both of these factors to their wild type value. Strain R1 was derived by reversion of Z492/6,6 and not only is the V_{max} increased to a value close to that of the wild type, but the K_m for fructose is also changed, the revertant having an affinity which is less than that of its parental mutant and more similar to that of the wild type.

Kinetic analyses of transport systems in other organisms have led to the belief that two components might be involved; one affecting affinity (the K_m factor) and another affecting the translocation process (the V_{max} factor). Change in affinity may arise by mutation in the gene specifying the carrier molecule [8–12]. On the other hand the V_{max} factor is considered to be involved in energy coupling [13, 14]. Analysis of *mtr* mutants of *Neurospora crassa* showing a change in V_{max} without alteration in K_m values was concluded to imply that the *mtr* gene product may participate in the translocation step of amino acid transport [15]. Mutation in the *ftr* locus of *Coprinus* obviously grossly affects the V_{max} but

Table 4. Kinetic constants of sugar transport in wild type and mutants of *Coprinus cinereus*. The values presented are for the high affinity transport system [1]

Strain	Substrates			
	2-deoxy-D-glucose		Fructose	
	K_m(μM)	V_{max}	K_m	V_{max}
BC9/6,6 (wild, type)	105	385[a]	802	380[b]
Z154/6,6 (*ftr* mutant)	553	7	228	4
Z492/6,6 (*ftr* mutant)	103	5	208	3
R1 (revertant of Z492/6,6)	65	222	1,108	233

[a] Units are pmol 2-deoxy-D-glucose per 10^3 dpm ^{14}C-leucine per min
[b] Units are pmol fructose per 10^3 dpm ^{3}H-leucine per min

causes only a slight change to the K_m. The alteration in K_m may be seen as being so small as to be negligible, suggesting that the *ftr* gene product is specifically involved in sugar translocation as opposed to sugar binding. However, we consider it significant that the revertant not only showed restoration of V_{max} towards the wild type value, but restoration of the K_m as well (Table 4). Thus we conclude that the *ftr* gene product participates in both binding and translocation. The kinetic analysis reported here strongly indicates a role in translocation and previously published data support the conclusion that the *ftr* product is involved in binding to substrate. A great many *ftr* mutations have been isolated and a detailed allele map prepared from allelic recombination values. It has been demonstrated that the position of an allele within the map depends on the conditions used for its selection [16]. Mutants selected for resistance to sorbose tended to be localised at one end of the gene, alleles selected on medium containing acetate and 2-deoxy-D-glucose were localised at the other end of the gene. Clearly selective pressure varied with the identity of the sugar in the medium, but since selection is imposed against the gene product rather than the gene the implication is that the *ftr* gene product interacts with the substrates and the only transport component which must necessarily do that is the carrier or binding protein. We consequently conclude that the *ftr* cistron is the structural gene for a product which participates in transport via both the high affinity and low affinity systems and is involved in both substrate binding and translocation.

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