Germination triggers of *Metarhizium anisopliae* conidia are related to host species

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The role of selectable strain variations in the development of pathogen strategies was examined using lines of *Metarhizium anisopliae* isolated from homopteran (isolate 549) or coleopteran (isolate 808) hosts. Conidia of strain 549 germinated in either alanine, glucose, cyclic AMP or the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX). The non-metabolizable glucose analogues, 3-O-methylglucose and 6-deoxyglucose, did not allow germination by themselves but stimulated germination when added to IBMX. By contrast, 2-deoxyglucose (dGlc) blocked germination on glucose or IBMX and inhibited hyphal growth on other carbon sources including alanine and glycerol. Conidia of strain 808 germinated rapidly in alanine but responded slowly to glucose or IBMX in the medium and were resistant to the growth inhibitory effects of dGlc. Radioactive dGlc was taken up by conidia of strains 549 and 808 at similar rates and was recovered mainly as 2-deoxyglucose 6-phosphate. Competition experiments utilizing both strains demonstrated that glucose, dGlc and 3-O-methylglucose were transported by the same system. Fructose was much less able than glucose to inhibit uptake of dGlc indicating that fructose is taken up by a different transport system than that for glucose. It is unlikely, therefore, that the resistance of strain 808 to dGlc is explained by reduced sugar transport compared with strain 549 but that strains 549 and 808 differ in the regulation of carbon metabolism with some systems in strain 808 showing resistance to the catabolite-repressing effects of glucose. Apparently, catabolite repression is subdivided into different segments as glucose inhibited the derepression of a number of catabolite repressible enzymes in strain 808, including the pathogenicity determinant protease Pr1. The same effect was produced by dGlc but not by 3-O-methylglucose, indicating that the trigger for catabolite repression occurs at the level of transport-associated glucose phosphorylation. A comparative study of 26 isolates indicated that most lines from coleopteran hosts were dGlc resistant and germinated poorly on glucose. Conversely, isolates germinating well on glucose (mostly from hemipteran and lepidopteran hosts) were dGlc susceptible.

Keywords: *Metarhizium anisopliae*, glucose analogues, catabolite repression, conidial germination

INTRODUCTION

The penetration of insect cuticle by entomopathogenic fungi often requires, in addition to spore germination, the precise differentiation of an organized series of structures.

Of these, the appressorium is developmentally the first and most important structure formed in preparation for host colonization.

Entomopathogenic fungi have not been studied systematically to search for the molecular events leading to spore germination and differentiation of appressoria, but processes that influence penetration include perception of stimuli, transmission of the differentiation signals, activation of macromolecular biosynthesis, and differential

Abbreviations: CzD, Czapek Dox agar; dGlc, 2-deoxyglucose; dGlcPO₆, 2-deoxyglucose 6-phosphate; IBMX, 3-isobutyl-1-methylxanthine; PDA, potato dextrose agar; YEM, yeast extract medium.

Table 1. Effects of glucose, glucose derivatives and second messengers on the growth of *M. anisopliae* strains 549 and 808

Addition	Strain 549						Strain 808					
	Percentage germination		Mean length ($\pm$ SE) of germ-tube (μ m)		Percentage differentiation of appressoria		Percentage germination		Mean length ($\pm$ SE) of germ-tube (μ m)		Percentage differentiation of appressoria	
	18 h	24 h	18 h	24 h	18 h	24 h	18 h	24 h	18 h	24 h	18 h	24 h
None		0	-	-	-	-	0	-	-	-	-	-
cAMP (5 mM)	34	76	14 $\pm$ 6	22 $\pm$ 9	16	32	12	32	12 $\pm$ 5	19 $\pm$ 5	0	2
Adenosine (5 mM)	22	83	17 $\pm$ 3	28 $\pm$ 7	5	16	24	59	16 $\pm$ 5	34 $\pm$ 8	0	0
IBMX (5 mM)	9	17	4 $\pm$ 1	6 $\pm$ 2	0	0	0	4	0	4 $\pm$ 0.5	0	0
Alanine (0.5%)	45	87	60 $\pm$ 12	93 $\pm$ 7	24	62	28	> 90	53 $\pm$ 9	78 $\pm$ 11	8	31
Glucose (0.1%)	40	78	36 $\pm$ 9	66 $\pm$ 8	6	27	3	13	12 $\pm$ 4	32 $\pm$ 8	0	0
+ cAMP (5 mM)	> 90	> 90	87 $\pm$ 16	116 $\pm$ 27	52	74	48	73	27 $\pm$ 5	54 $\pm$ 14	0	11
+ Adenosine (5 mM)	72	> 90	63 $\pm$ 7	82 $\pm$ 17	22	34	27	62	47 $\pm$ 8	59 $\pm$ 11	0	8
+ 2-Deoxyglucose (0.5%)	< 1	< 1	0	0	0	0	5	34	9 $\pm$ 3	19 $\pm$ 4	0	0
+ 6-Deoxyglucose (0.5%)	23	47	29 $\pm$ 8	47 $\pm$ 13	3	7	2	2	6 $\pm$ 1	8 $\pm$ 3	0	0
+ Alanine (0.5%)	> 90%	> 90%	64 $\pm$ 12	110 $\pm$ 17	62	> 90	> 90%	> 90%	62 $\pm$ 10	87 $\pm$ 14	22	73
2-Deoxyglucose (0.5%)		0	0	0	0	0	0	2	0	6 $\pm$ 2	0	0
+ cAMP (5 mM)	< 1	< 1	0	0	0	0	0	2	0	11 $\pm$ 5	0	0
+ Adenosine (5 mM)	< 1	< 1	0	0	0	0	17	43	6 $\pm$ 2	13 $\pm$ 5	0	0
+ IBMX (5 mM)	< 1	< 1	0	0	0	0	7	15	14 $\pm$ 3	18 $\pm$ 5	0	0
+ Alanine (0.5%)	72	84	22 $\pm$ 11	48 $\pm$ 8	18	23	64	81	43 $\pm$ 9	82 $\pm$ 12	13	28
6-Deoxyglucose (0.5%)	< 1	2	0	6 $\pm$ 1	0	0	0	0	0	0	0	0
+ cAMP (5 mM)	50	72	7 $\pm$ 1	16 $\pm$ 2	0	8	12	37	9 $\pm$ 2	15 $\pm$ 2	0	0
+ IBMX (5 mM)	12	3	8 $\pm$ 1	15 $\pm$ 2	0	0	0	5	0	6 $\pm$ 0.5	0	0
3-O-Methylglucose (0.5%)	2	4	0	4 $\pm$ 0.5	0	0	0	0	0	0	0	0
+ cAMP (5 mM)	45	71	6 $\pm$ 2	17 $\pm$ 3	0	3	0	0	0	0	0	0
+ IBMX (5 mM)	15	36	7 $\pm$ 1	19 $\pm$ 4	0	6	0	0	0	0	0	0
Glycerol (1%)	78	> 90	42 $\pm$ 6	95 $\pm$ 7	18	64	74	> 90	34 $\pm$ 8	53 $\pm$ 13	9	51
+ Glucose (0.5%)	> 90	> 90	74 $\pm$ 18	118 $\pm$ 23	17	30	> 90	> 90	67 $\pm$ 9	104 $\pm$ 16	11	47
+ 2-Deoxyglucose (0.5%)	81	> 90	27 $\pm$ 7	38 $\pm$ 7	7	14	71	82	37 $\pm$ 10	54 $\pm$ 15	11	42
+ 6-Deoxyglucose (0.5%)	85	> 90	33 $\pm$ 8	61 $\pm$ 9	6	11	60	73	25 $\pm$ 4	56 $\pm$ 12	8	40

gene expression. Germlings of the *Metarhizium anisopliae* isolate ME1 (now given the ARSEF number 2575) used in our previous studies possess many membrane-bound cell receptor systems [e.g. a GTP-regulated adenylate cyclase, tyrosine protein kinases, serine and threonine protein kinases, phosphoprotein phosphatase, and an ATPase (St Leger *et al.*, 1989a, b, 1990a, b, c)] that act to change second messenger levels (cAMP or Ca^{2+}) or are themselves activated by second messengers to trigger differentiation. Fluctuations in the relative levels of cAMP and Ca^{2+} control the morphology of *M. anisopliae* ARSEF 2575 via changes in nuclear division, the cytoskeleton and the cell wall (St Leger *et al.*, 1990a). A model for appressorium formation has been proposed in which a localized change in transmembrane potential produced by deformation of the plastic hyphal tip, i.e. the thigmotropic induction signal, affects the levels of Ca^{2+} and cAMP disrupting apically localized wall synthesis. Subsequent differential Ca^{2+} redistribution in the cell enlargement zone causes germ-tube expansion by cytoplasmic pressure against an expanding area of primary wall produced by uniformly dispersed wall synthesis (St Leger *et al.*, 1991).

To date, our model does not encompass the role of nutrients in stimulating differentiation – an important omission since patterns of growth and differentiation among 114 isolates of *M. anisopliae* were frequently related to the nutrient conditions pertaining to their hosts (St

Leger *et al.*, 1992). For example, in the case of Homoptera, endogenous nutrients are likely to be supplemented by insect secretions rich in sugars, and it is interesting that in contrast to coleopteran-derived isolates some lines isolated from Homoptera demonstrated good germination and production of appressoria in glucose media. These observations suggest that host recognition mechanisms are keyed to nutrient levels available on appropriate host cuticles (St Leger *et al.*, 1991). Genetic similarities determined by allozyme analysis provided evidence that Brazil and Colombia contain a common, widely dispersed genotypic class (class 14). The lines of class 14, isolated from several insect orders including Homoptera (e.g. ARSEF 549) demonstrated similar growth patterns (good germination and high levels of differentiation) in yeast extract media or glucose media. The exception was ARSEF 808, which germinated poorly in glucose media, and was the only line from class 14 derived from a coleopteran host.

Evidently, being in the same genotypic class, defined by a series of electrophoretic phenotypes, does not rule out pathogenic diversity in patterns of growth and differentiation. This provides an opportunity for comparative studies on differences in perception mechanisms, transduction chains, and physiological responses, against a similar genetic background. We have initiated such a study to determine whether unifying themes exist between

Fig. 1. Micrographs of *M. anisopliae* (ARSEF 549) conidia germinated in basal medium (20 mM potassium phosphate buffer, pH 6.7, 0.2% NaNO_3 , 0.05% MgSO_4) on polystyrene for 22 h (a–h). Basal medium was supplemented with (a) 0.1% glucose, (b, c, d) 0.1% glucose + 5 mM cAMP (note terminal blastospore-like cell indicated by arrowhead), (e) 0.5% alanine, (f, g) 0.5% alanine + 0.5% dGlc [note swelling of the tip cells (indicated by arrowheads) delineated from hyphae by septa]. For both strain 549 (h) and strain 808 (i) anastomosis was frequently observed between hyphae (indicated by arrowheads). app, Appressorium; c, conidium. Bars, 10 μm .

the different ways in which two isolates in class 14 (549 and 808) respond to environmental signals.

METHODS

Organisms and growth. The 26 isolates of *Metarhizium anisopliae* used in this study were described by St Leger *et al.* (1992). Conidia were obtained from 11 d PDA cultures incubated at 27 °C. Conidia were washed from the agar surface, centrifuged and resuspended twice in distilled water. To test the isolates' nutritional requirements for germination and differentiation, conidia were germinated in 5.5 cm polystyrene dishes (St Leger *et al.*, 1989a) containing basal medium (20 mM potassium phosphate buffer, pH 6.7, 0.2% NaNO_3 , 0.05% MgSO_4) and carbon source as specified. One hundred spores from each of four replicates were scored to assess germination and differentiation frequency. Where solid medium was required, 1.5% (w/v) noble agar (Difco) was added to the basal medium. Supplements were filter sterilized (0.22 μm) and added to the medium at 50 °C.

Preparation and analysis of culture filters. Standardized mycelial inocula (5 g wet wt) from 48 h Sabouraud dextrose

broth cultures (St Leger *et al.*, 1991) were incubated with shaking (100 r.p.m.) for 5 h in 100 ml chitin medium (1% (w/v) chitin, 0.02% KH_2PO_4 , 0.01% MgSO_4 , pH 6) supplemented with carbon and/or nitrogen sources at 0.2–2% (w/v). Chymoelastase activity (Pr1) in culture filtrates (Whatman No. 1 filter paper) was determined using succinyl-alanine-alanine-proline-phenylalanine *p*-nitroanilide as substrate (St Leger *et al.*, 1988). Filtrates were also characterized using the 19 enzyme activities in the semi-quantitative API ZYM system as described previously (St Leger *et al.*, 1986b).

Quantitative measurements of resistance to dGlc were made by inoculating 50 ml standing cultures (basal medium with fructose or glucose at 1% (w/v) and various concentrations of dGlc) with 10^6 conidia, incubating the cultures for 3 d at 25 °C, harvesting the mycelial mat and determining dry weight.

Uptake of [^{14}C]dGlc. Aliquots (2.5 ml) of a conidial suspension (5×10^6 spores ml^{-1}) in basal medium were shaken for 7 h at 25 °C and supplemented with [^{14}C]dGlc [final concn 20 $\mu\text{Ci ml}^{-1}$ (740 kBq ml^{-1})]. At intervals, a 1 ml sample of each conidial suspension was spotted directly onto a glass-fibre (Whatman GF/A) disc. The conidia were washed (five $\times$ 20 ml) with 0.2 M potassium phosphate buffer containing KCl (0.1 M) and counted

with their filters in 6 ml of Cytoscent (ICN) cocktail using a Beckmann LS 5801 scintillation counter. Adsorption of radioactivity to conidia and filters was also determined in cells incubated on ice. These values were subtracted from experimental samples to give the total uptake of [14 C]dGlc by conidia. In competition studies, conidia were exposed to glucose (1–10 mM), 3-O-methylglucose (10 mM) or fructose (10 mM), and labelled dGlc simultaneously, and the rate of uptake determined.

The ability of mycelia to transport [14 C]dGlc or methyl[14 C]glucose was assayed using agar cultures grown for 3 d with or without unlabelled dGlc and glucose or fructose as carbon sources. Mycelia were scraped from media with a razor blade, weighed and suspended in distilled water. The cells were harvested by filtration, washed thoroughly with water, and suspended at a density of 1–3 mg (dry wt) ml $^{-1}$ in basal medium supplemented with methyl[14 C]glucose or [14 C]dGlc (20 μ Ci ml $^{-1}$). At intervals, samples were collected and washed as described for conidia, dried overnight, weighed and counted with Cytoscent.

Cell-free extracts of conidia or mycelia exposed to labelled glucose analogues for 0.5–2 h were obtained by harvesting cells on a HAWP Millipore filter, washing as described above, boiling cells for 10 min in 0.5 ml distilled water and centrifugation (20000 g, 15 min at 4 °C). Extracts were chromatographed on Whatman silica gel thin layer plates in n-propanol/ammonia/water (6:1:3, by vol.). Spots of methyl[14 C]glucose, [14 C]dGlc and its metabolites were localized with ammonium molybdate/perchloric acid spray (Bourret, 1987) or by placing dried chromatograms adjacent to X-Omat-AR film at room temperature for 2–3 d.

Chemicals. 3-O-Methyl-D-[U- 14 C]glucose was purchased from Amersham. 2-[U- 14 C]deoxy-D-glucose was from NEN. Yeast extract was from Difco. All other reagents, chemicals and nutrients were from Sigma.

RESULTS

Growth characteristics

Data from a comparative study of germination and appressorium formation of *M. anisopliae* strains 549 and 808 under different nutrient conditions are shown in Table 1. Strain 549 germinated well on glucose and subsequently produced moderate levels of appressoria. By contrast, strain 808 germinated poorly in glucose media and did not produce appressoria. cAMP allowed germination of both isolates but only strain 549 produced appressoria. Adenosine also activated spores of both isolates indicating that the effect was not specific to cyclic nucleotides. Significant growth on cAMP and adenosine indicates that they may function as a nutrient source. However, the cyclic nucleotide phosphodiesterase inhibitor IBMX allowed significant germination of strain 549 (but not 808) without extensive hyphal growth suggestive of a specific cAMP activation event. The effects of glucose on germination and differentiation were greatly potentiated by the presence of cAMP or adenosine ($P < 0.01$). The appearance of appressoria produced by each strain also varied with growth medium. Strain 549, which germinated on both glucose and glucose supplemented with cAMP produced larger, often branching,

appressoria on the more complex medium (compare Fig. 1a with Fig. 1b, c and d). Both strains germinated well and produced appressoria with alanine, a nitrogenous nutrient, and the non-fermentable carbon source glycerol.

The glucose analogues dGlc, 6-deoxyglucose, and 3-O-methylglucose did not allow germination of either strain alone. However, supplementing IBMX with 6-deoxyglucose or 3-O-methylglucose stimulated germination compared with IBMX alone ($P < 0.01$ in both instances). Unlike 6-deoxyglucose, dGlc blocked germination of strain 549 on glucose, cAMP and adenosine. dGlc was not inherently toxic because it did not inhibit germination on alanine or glycerol. However, in the presence of dGlc and alanine, many conidia of strain 549 produced terminal blastospore-like cells which were delineated from hyphae by a septum (Fig. 1f, g). These were occasionally seen in other media (Fig. 1d) but rarely with alanine alone (Fig. 1e). By contrast, strain 808 appeared to be unaffected by dGlc in combination with any of the other carbon sources tested (Table 1).

For both strains 549 and 808, anastomoses were frequently observed between hyphae (Fig. 1h, i).

Effects of dGlc on *M. anisopliae* cultures in liquid or solid media

Fig. 2 shows that the presence or absence of dGlc in the liquid culture medium did not exert a major influence on total mycelial growth of line 808. By contrast, dGlc (16 mM) inhibited growth of strain 549 on fructose and glucose by 77 % and 33 %, respectively.

As liquid cultures do not allow sporulation, further observations were performed on agar plates containing different carbon sources and dGlc (Table 2 and Fig. 3). The addition of dGlc to cultures of strain 808 decreased

Fig. 2. Inhibition of growth by 2-deoxyglucose. Conidia (10^6) of ARSEF 549 (open symbols) or ARSEF 808 (closed symbols) were inoculated into standing cultures [basal medium with fructose (□, ■) or glucose (○, ●) at 1% (w/v) and various concentrations of dGlc] and incubated for 3 d at 25 °C. Mycelial mats were harvested and weighed. Each point is the mean of three replicates. Variation around the mean was less than 7.5 % in each instance.

Table 2. Growth and sporulation by *M. anisopliae* strains 549 and 808 on agar medium containing various carbon sources with or without dGlc, 4 d after inoculationResults are means $\pm$ SE for eight replicates per test.

Carbon source (1 %, w/v)	Strain 549				Strain 808			
	- dGlc		+ dGlc		- dGlc		+ dGlc	
	Growth (mm)	Conidial density*	Growth (mm)	Conidial density*	Growth (mm)	Conidial density*	Growth (mm)	Conidial density*
Glucose	13.3 $\pm$ 0.36	++	3.4 $\pm$ 0.2	—	13.6 $\pm$ 0.45	++	11.5 $\pm$ 0.30	+
Fructose	11.2 $\pm$ 0.51	+	< 0.5	—	10.92 $\pm$ 0.27	++	9.2 $\pm$ 0.21	—
Galactose	10.5 $\pm$ 0.37	+	1.1 $\pm$ 0.02	—	11.3 $\pm$ 0.61	+	11.8 $\pm$ 0.18	—
Mannose	12 $\pm$ 0.32	+++	1.2 $\pm$ 0.01	—	12.7 $\pm$ 0.45	+++	11.7 $\pm$ 0.24	—
Xylose	11.7 $\pm$ 0.34	+	< 0.5	—	10.3 $\pm$ 0.25	+	9.5 $\pm$ 0.22	—
Raffinose	11.5 $\pm$ 0.41	+	1.1 $\pm$ 0.02	—	11.2 $\pm$ 0.34	++	7.5 $\pm$ 0.04	—
Ribose	11.1 $\pm$ 0.47	+++	< 0.5	—	8.5 $\pm$ 0.21	+++	9.2 $\pm$ 0.08	—
Sorbose	2.5 $\pm$ 0.04	—	< 0.5	—	3.7 $\pm$ 0.1	—	7.0 $\pm$ 0.04	—
Alanine	10.5 $\pm$ 0.33	++	1.0 $\pm$ 0.02	—	11.8 $\pm$ 0.25	++	9.2 $\pm$ 0.05	—
Sodium acetate	9.7 $\pm$ 0.38	+	2.3 $\pm$ 0.05	—	9.5 $\pm$ 0.14	—	9.6 $\pm$ 0.06	—
Glycerol	8.2 $\pm$ 0.05	+++	1.5 $\pm$ 0.04	—	13.7 $\pm$ 0.18	++	11.5 $\pm$ 0.10	+

* An arbitrary scale of increasing conidial density: —, none; +, poor; ++, fair; +++, good.

Fig. 3. Growth of *M. anisopliae*, strains 549 and 808 on YEM ('yeast'), mannose and sorbose with or without dGlc, 4 d after inoculation. dGlc reduces growth of strain 549 and stimulates production of aerial hyphae by strain 808.

the accumulation of conidia and stimulated the elongation of aerial hyphae. Otherwise, strain 808 grew well with dGlc on fermentable sugars, glycerol and sodium acetate (non-fermentable substrates) and alanine. By contrast, dGlc greatly reduced growth of strain 549 on all of these substrates. In addition, dGlc caused production of black pigment (melanin?) on glycerol and, to a lesser extent, on sodium acetate.

Uptake of dGlc

We measured the uptake of dGlc by strains 549 and 808 to test whether the resistance of strain 808 to toxic analogues

is caused by a reduction in sugar transport. Conidia of strain 808 took up $\sim 30\%$ less [^{14}C]dGlc compared with strain 549 over a 30 min period ($P < 0.05$) (Table 3). The uptake of labelled dGlc by both strains was progressively reduced by the addition of increasing concentrations of glucose or 10 mM 6-deoxyglucose indicating that glucose, 6-deoxyglucose and dGlc probably share a common entry system. Fructose did not repress uptake.

To test whether growth in the presence of dGlc alters transport systems, mycelia of strain 808 were harvested from agar plates containing fructose (derepressing conditions) or glucose (repressing conditions) with or with-

out dGlc (Table 4). Uptake of labelled substrate by fructose-grown mycelia was 40 % higher than by glucose-grown cultures. Supplementing glucose cultures with dGlc further lowered mycelial uptake of substrate by ca. 40 % compared to glucose alone ($P < 0.01$ in both instances).

To determine if dGlc is metabolized by *M. anisopliae*, as by other organisms, to 2-deoxyglucose phosphate (Moore, 1981), extracts of conidia or mycelia exposed to labelled dGlc for 2 h were chromatographed (Fig. 4). Heating conidia of strains 808 or 549 in water extracted more than 95 % of the label; extraction from mycelia was ≥ 83 % (Table 4). Much of the radioactivity in extracts from spores that had been fed labelled dGlc appeared on TLC along with authentic 2-deoxyglucose phosphate. This was

also a major product of mycelia of strain 808 grown in the presence of dGlc but some higher molecular mass products were also apparent (Fig. 4, E). By contrast, though mycelia took up labelled 3-O-methylglucose at levels similar to [14 C]dGlc, chromatographs indicated that

Table 3. Effects of glucose, 6-deoxyglucose and fructose levels on [14 C]dGlc uptake by conidia of *M. anisopliae* strains 549 and 808

Conidia were exposed to labelled dGlc ($20 \mu\text{Ci ml}^{-1}$) for 30 min, then aliquots of conidia were analysed for dGlc uptake. Percentage values are given in parentheses.

Extracellular sugar concn (mM)	$10^{-3} \times [\text{dGlc uptake}]$ [c.p.m. $\pm$ SE; $n = 5$]	
	Strain 549	Strain 808
Glucose		
0	265 ± 33 (100)	195 ± 26 (100)
2.5	127 ± 21 (48)	82 ± 11 (48)
10	48 ± 6 (18)	23 ± 5 (12)
50	6 ± 2 (2)	0
6-Deoxyglucose		
10	56 ± 9 (21)	39 ± 4 (20)
Fructose		
50	245 ± 28 (92)	174 ± 17 (89)

Fig. 4. Metabolism of dGlc by *M. anisopliae*. Chromatograms of hot water extracts from cells that have taken up labelled dGlc for 2 h (A–D) or 4 h (E) were developed as described in Methods. Extracts were from (A) strain 549 conidia, (B) strain 808 conidia, (C+E) mycelia of strain 808 grown on 0.5 % fructose + 0.5 % dGlc, (D) mycelia of strain 808 grown on 0.5 % glucose + 0.5 % dGlc. Each experiment was repeated three times. In each case, two bands corresponding to authentic dGlc and dGlcPO₄ predominated. However, their relative contribution to total radioactivity and the presence or absence of other bands was variable between replicates. O, origin.

Table 4. [14 C]dGlc uptake by mycelia of *M. anisopliae* strain 808 grown on glucose or fructose in the presence of dGlc

Mycelia of strain 808 were harvested from 3 d agar cultures containing 1 % (w/v) glucose or fructose in the presence or absence of dGlc, suspended in basal medium and exposed to [14 C]dGlc ($20 \mu\text{Ci ml}^{-1}$) for 1 h. Cell-free extracts were obtained by boiling mycelia and centrifuging. The percentage of label released from cells is given in parentheses. Results are expressed as c.p.m. (mg dry wt cells) $^{-1} \pm$ SE; $n = 5$.

	Glucose-grown mycelia		Fructose-grown mycelia	
	Glucose	Glucose + dGlc (1 %)	Fructose	Fructose + dGlc (1 %)
[14 C]dGlc uptake	641 ± 73	374 ± 61	1123 ± 163	972 ± 46
Hot-water-soluble fraction	625 (98)	310 (83)	1018 (90)	954 (98)

Table 5. Effects of glucose and glucose analogues on enzyme production by *M. anisopliae* strains 549 and 808

Carbon source	Isolate	API ZYM tests*																				Pr1 ($\mu\text{mol pNA}$ $\text{min}^{-1} \text{ml}^{-1}$)
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
1% Chitin	549	0	<1	3	1	<1	4	5	1	2	0	4	4	<1	<1	<1	1	2	1	0	<1	0.37
	808	0	0	3	2	0	3	5	<1	1	0	5	5	<1	3	0	0	2	1	0	0	0.41
1% Chitin + 2% glucose	549	0	0	1	1	0	1	2	<1	<1	0	4	4	0	0	0	0	<1	1	0	0	0.02
	808	0	0	1	<1	0	0	<1	0	0	0	2	2	0	1	0	0	0	<1	0	0	0.05
1% Chitin + 0.2% dGlc	549	0	0	2	1	<1	1	2	<1	0	0	4	4	0	0	0	0	<1	<1	0	0	0.014
	808	0	0	2	1	0	<1	2	<1	<1	0	4	5	0	2	0	0	1	<1	0	0	0.054
1% Chitin + 2% dGlc	549	0	0	2	1	0	1	2	<1	0	0	4	4	0	0	0	0	<1	0	0	0	0.014
	808	0	0	2	<1	0	1	2	<1	<1	0	3	2	0	1	0	0	1	0	0	0	0.014
1% Chitin + 2% 3-O-methylglucose	549	0	0	<1	1	<1	2	4	<1	2	0	4	4	<1	0	<1	<1	1	1	0	0	0.33
	808	0	0	2	1	0	2	4	1	1	0	5	5	0	0	0	0	1	1	0	<1	0.32

* API ZYM tests. 1, Control; 2, phosphatase alkaline; 3, esterase (C_4); 4, esterase lipase (C_8); 5, lipase (C_{14}); 6, leucine aminopeptidase; 7, valine aminopeptidase; 8, cysteine aminopeptidase; 9, trypsin; 10, chymotrypsin; 11, phosphatase acid; 12, phosphoamidase; 13, α -galactosidase; 14, β -galactosidase; 15, β -glucuronidase; 16, α -glucosidase; 17, β -glucosidase; 18, β -glucosaminidase; 19, α -mannosidase; 20, α -fucosidase. Number values 1–5 indicate increasing colour intensity. Assays against the Pr1 substrate Su-(Ala)₂-Pro-Phe-pNA, were performed at 23 °C in 10 mM Tris/HCl, pH 8, 4% (by vol.) dimethylsulfoxide and 0.06 mM substrate. Activities were determined in 5 h chitin culture filtrates. Phosphoamidase production by *M. anisopliae* strain 549 varied from moderate to strongly positive in different culture flasks. This was the only reaction which varied substantially (by more than one grade) on repeated testing with any of the isolates.

it accumulated in cells without being metabolized (not shown).

Glucose-repressible enzymes

The results in the previous section suggest that the resistance of strain 808 to dGlc is not simply explained by reduced sugar transport compared with strain 549. To test whether strains 808 and 549 differ in some aspect of carbon regulation, we assayed a variety of enzymes, including the Pr1 protease known to be glucose repressible in *M. anisopliae* (St Leger *et al.*, 1988). Both strains produced high levels of Pr1 in chitin cultures (Table 5). Levels of Pr1 were greatly reduced in chitin cultures supplemented with glucose or dGlc. By contrast, 3-O-methylglucose allowed derepression of Pr1 by strains 549 and 808. Glucose repression was also observed for both strains in esterase, aminopeptidase, and β -glucosidase activities assayed with the API-ZYM system (Table 5). Acid phosphatase and phosphoamidase production by strain 549 were resistant to glucose repression.

Strain differences in *M. anisopliae*

Data from a comparative study of germination and growth of 26 isolates on different carbon sources are shown in Table 6. As reported previously (St Leger *et al.*, 1992), lines isolated from Coleoptera, particularly scarabaeids, are frequently less adaptable than other isolates with little germination on glucose, and sometimes YEM. Of the 18 lines showing less than 30% germination on glucose media, 13 were isolated from coleopterans. Growth of these isolates on fructose media was reduced by an average of 33% by incorporation of 1% dGlc. Five of the eight lines showing good (> 30%) germination on glucose were isolated from Lepidoptera and Homoptera. Mean growth of these lines was reduced by 70% by dGlc.

A second glucose analogue, L-sorbose, also reduced growth of most dGlc-susceptible isolates; exceptionally strains 549 and 1066 were resistant to sorbose. Conversely, the dGlc resistant isolate 760 was susceptible to sorbose. 3-O-Methylglucose did not affect the growth characteristics of any of the isolates in fructose media (data not shown).

DISCUSSION

As strains 549 and 808 differed in their ability to germinate rapidly on glucose, we tested well-characterized glucose analogues (Eraso & Gancedo, 1985; Moore, 1981) that are phosphorylated but not extensively metabolized (e.g. dGlc) and sugars that can only be transported into the cell (6-deoxyglucose and 3-O-methylglucose) to determine to what extent a sugar should be metabolized to allow germination and catabolite repression. The conidia of *M. anisopliae* strain 549 germinate rapidly in response to exogenous glucose. In conidia of several other fungal systems, glucose acts as a positive effector of germination without metabolism by causing an increase in cAMP content, which in turn initiates an enzyme cascade (Van Mulders & Laere, 1984). This apparently requires a functional glucose transport system (Beullens *et al.*, 1988). Other factors must also be necessary in strain 549 as non-metabolizable analogues were transported but did not trigger germination. Also, uptake of dGlc was competed for by glucose and 6-deoxyglucose which therefore probably utilized the same transport system. Glucose and fructose are transported through the same carrier in *Saccharomyces cerevisiae* (Schuddemat *et al.*, 1988). By contrast, in *M. anisopliae*, fructose was much less able than glucose to inhibit uptake of dGlc indicating that fructose may be taken up by a different transport system than that for glucose. Consequently, dGlc inhibited growth of *M. anisopliae* to a greater extent on fructose-based media than

Table 6. Frequency (%) of germination of each of 26 isolates of *M. anisopliae* (incubated 24 h) in yeast extract medium (YEM) or glucose and their ability to grow on Czapek Dox agar or fructose agar medium with or without dGlc or sorbose, 4 d after inoculation

The growth results are means $\pm$ SE of four replicates per test. The numbers in parentheses are the percentage growth in the presence of sorbose or dGlc compared with unsupplemented Czapek Dox or fructose agar media.

Strain	Host	Germination (%)		Growth (mm)				
		YEM (0.02%)	Glucose (0.5)	CzD			Fructose (1%)	
				+1% sorbose		+1% dGlc	+1% dGlc	
1946	Coleoptera	< 1	0	5.5 $\pm$ 0.1	1.0 $\pm$ 0 (18.2)	1.0 $\pm$ 0 (19.2)	6.3 $\pm$ 0.1	2.5 $\pm$ 0 (39.7)
346	Coleoptera	1	0	9.1 $\pm$ 0.2	3.4 $\pm$ 0.05 (37.4)	3.2 $\pm$ 0.05 (35.2)	7.5 $\pm$ 0.3	6.5 $\pm$ 0.2 (86.7)
797	Coleoptera	1	< 1	11.3 $\pm$ 0.3	2.0 $\pm$ 0 (18.2)	7.4 $\pm$ 0.3 (66.7)	9.4 $\pm$ 0.3	7.0 $\pm$ 0.3 (74.5)
1946	Coleoptera	< 1	< 1	5.5 $\pm$ 0.05	1.0 $\pm$ 0 (18.2)	1.0 $\pm$ 0 (18.2)	6.0 $\pm$ 0.2	2.5 $\pm$ 0 (41.7)
2165	Coleoptera	2	< 1	7.5 $\pm$ 0.2	0.5 $\pm$ 0 (6.7)	2.0 $\pm$ 0 (36.4)	6.5 $\pm$ 0.2	2.0 $\pm$ 0 (30.8)
297	Coleoptera	1	0	4.7 $\pm$ 0.2	0.5 (10.6)	0.5 $\pm$ 0 (10.6)	7.5 $\pm$ 0.2	2.0 $\pm$ 0 (42.3)
1015	Lepidoptera	1	1	12.8 $\pm$ 0.4	7.2 $\pm$ 0.2 (56.3)	4.3 $\pm$ 0.1 (33.6)	12.6 $\pm$ 0.5	6.5 $\pm$ 0.4 (51.6)
1066	Coleoptera	4	1	6 $\pm$ 0.2	5.5 $\pm$ 0.05 (91.7)	2.0 $\pm$ 0 (33.3)	5.5 $\pm$ 0.1	1.2 $\pm$ 0 (21.8)
324	Orthoptera	7	4	5.4 $\pm$ 0.15	1.0 $\pm$ 0 (18.5)	0.5 $\pm$ 0 (9.3)	5.5 $\pm$ 0.1	0.5 $\pm$ 0 (9.1)
932	Coleoptera	9	7	8.5 $\pm$ 0.2	6.2 $\pm$ 0.2 (72.9)	7.4 $\pm$ 0.2 (87.1)	9.5 $\pm$ 0.4	7.7 $\pm$ 0.2 (81.0)
724	Coleoptera	33	8	10.0 $\pm$ 0.2	1.0 $\pm$ 0 (10.0)	5.5 $\pm$ 0.1 (55.0)	9.5 $\pm$ 0.2	6.2 $\pm$ 0.1 (65.3)
820	Coleoptera	27	1	8.6 $\pm$ 0.15	7.8 $\pm$ 0.15 (90.7)	7.5 $\pm$ 0.2 (87.2)	8.5 $\pm$ 0.3	7.4 $\pm$ 0.2 (92.9)
760	Coleoptera	23	1	11.8 $\pm$ 0.3	2.5 $\pm$ 0 (21.2)	9.3 $\pm$ 0.2 (78.8)	11.1 $\pm$ 0.4	9.2 $\pm$ 0.4 (82.9)
1080	Lepidoptera	24	1	11.4 $\pm$ 0.2	3.3 $\pm$ 0.05 (4.3)	10.5 $\pm$ 0.2 (4.4)	11.5 $\pm$ 0.4	7.3 $\pm$ 0.2 (63.5)
1187	Lepidoptera	22	6	7.1 $\pm$ 0.3	3.2 $\pm$ 0.05 (45.1)	3.2 $\pm$ 0.05 (45.1)	7.0 $\pm$ 0.3	5.6 $\pm$ 0.3 (80.0)
755	Hemiptera	18	8	10.5 $\pm$ 0.2	2.5 $\pm$ 0 (23.8)	8.1 $\pm$ 0.2 (77.1)	9.4 $\pm$ 0.3	9.4 $\pm$ 0.2 (100)
808	Coleoptera	75	4	9.3 $\pm$ 0.3	9.0 $\pm$ 0.3 (96.8)	7.3 $\pm$ 0.2 (78.5)	8.5 $\pm$ 0.4	8.3 $\pm$ 0.3 (97.6)
2575	Coleoptera	80	14	11.7 $\pm$ 0.2	8.5 $\pm$ 0.3 (72.6)	1.3 $\pm$ 0.5 (11.1)	10.3 $\pm$ 0.4	9.5 $\pm$ 0.4 (92.2)
939	Coleoptera	28	36	14.3 $\pm$ 0.3	6.4 $\pm$ 0.2 (44.8)	0.5 $\pm$ 0 (3.5)	13.4 $\pm$ 0.6	0.5 $\pm$ 0 (3.7)
1892	Homoptera	74	63	9.5 $\pm$ 0.1	4.5 $\pm$ 0.1 (47.4)	10.5 $\pm$ 0 (5.3)	9.5 $\pm$ 0.5	0.5 $\pm$ 0 (5.3)
23	Coleoptera	72	47	8.3 $\pm$ 0.2	2.5 $\pm$ 0 (30.1)	4.2 $\pm$ 0.2 (50.6)	5.5 $\pm$ 0.4	4 $\pm$ 0.1 (72.7)
988	Lepidoptera	> 80	75	7.7 $\pm$ 0.2	10.5 $\pm$ 0 (7.0)	4.2 $\pm$ 0.1 (59.2)	6.3 $\pm$ 0.3	0.5 $\pm$ 0 (7.9)
552	Lepidoptera	> 80	> 80	10.4 $\pm$ 0.2	3.0 $\pm$ 0.05 (28.8)	0.5 $\pm$ 0 (4.8)	6.1 $\pm$ 0.2	0.5 $\pm$ 0 (8.2)
549	Homoptera	> 80	76	10.5 $\pm$ 0.3	8.4 $\pm$ 0.4 (80.0)	1 $\pm$ 0 (9.5)	9.2 $\pm$ 0.4	1.2 $\pm$ 0 (13.0)
798	Homoptera	> 80	79	4.0 $\pm$ 0.1	1.5 $\pm$ 0 (37.5)	2 $\pm$ 0 (50.0)	3.0 $\pm$ 0	2 $\pm$ 0 (66.7)
1878	Coleoptera	> 80	53	9.9 $\pm$ 0.4	7.1 $\pm$ 0.3 (71.7)	6.5 $\pm$ 0.2 (65.7)	7.5 $\pm$ 0.3	4 $\pm$ 0.2 (53.3)

on those containing glucose. Dillon & Charnley (1990) found that methylglucose promotes protein synthesis in swollen (water-soaked) spores of *M. anisopliae* but high levels of germination required a utilizable carbon source. This suggests that, though glucose may act as a trigger, its metabolism is also important for germination.

Cyclic AMP itself is usually ineffective when applied exogenously to fungi, probably because of low penetration by this hydrophilic and negatively charged compound (Pall, 1981). Where cAMP or its analogues are effective at influencing fungal growth, it is implicitly assumed that this is due to activation of cAMP-dependent kinases (Pall, 1981). However, cAMP and the non-cyclic nucleotide adenosine supported growth of strain 549 indicating both uptake and utilization of the nucleotides as carbon sources. By contrast, only short germ-tubes were produced in the presence of IBMX, indicating that lowering the phosphodiesterase activity triggers germi-

nation by affecting intracellular levels of cAMP. 6-Deoxyglucose increased germination when applied with IBMX. The same combination increased intracellular cAMP levels when applied to *Pilobolus longipes* spores (Bourret, 1986).

In a previous study utilizing an *M. anisopliae* line (ARSEF 2575) isolated from a beetle we found cAMP in ungerminated conidia and the pre-germination synthesis of cAMP target proteins (St Leger *et al.*, 1990a, b). However, their role appeared to be minor compared with a Ca^{2+} /calmodulin-dependent kinase which serves to modulate the effects of Ca^{2+} on the system and triggers germination (St Leger *et al.*, 1990a, b, 1991). In contrast to glucose, nitrogen-source-induced trehalase activation is not mediated by cAMP in the yeast *S. cerevisiae* (Thevelein & Beullens, 1985) and we suggested there may be a connection between the regulatory mechanisms observed in line 2575 conidia and their poor germination on

glucose as compared with germination on nitrogen sources (St Leger *et al.*, 1990b, 1992). Line 808 resembles strain 2575 in being isolated from a beetle and showing slow germination on glucose as compared with alanine or YEM. Unlike strain 2575, it is in the same genotypic class as line 549 which allows us to assume a similar genetic background.

The two lines, 808 and 549, most clearly resemble each other in the ability of glucose to inhibit the derepression of a number of catabolite repressible enzymes including the pathogenicity determinant Pr1. The same effect was produced by dGlc indicating that metabolism of the sugar beyond the sugar phosphate step is not necessary. By contrast, 3-O-methylglucose did not affect enzyme production, demonstrating a need for hexokinase phosphorylation of the sugar after transport. Up to now, no clear evidence exists on the nature of the signal(s) produced by glucose to cause catabolite repression in yeasts (Gancedo, 1992). However, genetic studies have shown that the primary trigger of the cAMP signal in *S. cerevisiae* is at the level of transport and/or phosphorylation of the sugar (Beullens *et al.*, 1988). They suggest that a glucose transport protein/kinase complex interacts with adenylate cyclase. Recent data have shown that a GTP binding protein regulates the cAMP signal in *M. anisopliae* (St Leger *et al.*, 1991) and presumably would mediate this interaction. However, cAMP does not by itself affect Pr1 production in *M. anisopliae* (2575) (St Leger *et al.*, 1988) so it is possible that the carrier/kinase complex interacts with several regulatory proteins to trigger specific cellular events. These results suggest that dGlc will be useful in subsequent studies on the phenomenon of catabolite repression in *M. anisopliae*, particularly in those isolates such as 808 where it is not toxic.

The biochemical basis of dGlc resistance in strain 808 is not yet clear. Conidia of strain 808 transport dGlc at a 30% reduced rate compared to strain 549. However, the resistance of strain 808 cannot be explained simply by a reduction in sugar transport given that both isolates were efficient sugar fermenters using many sugars as sole carbon sources indicative of several intact transport systems and metabolic pathways. Phosphorylation of dGlc can deplete intracellular ATP, an effect which may contribute to the toxic effects of dGlc (Bourret, 1987), but strains 549 and 808 appear to metabolize dGlc similarly to 2-deoxyglucose phosphate indicating that neither is altered in phosphorylation or phosphatase activities. This indicates that energy must be expended continuously during growth of strain 808 to maintain an intracellular pool of dGlcPO₄.

Many of the strains of *M. anisopliae* which germinated well on glucose were particularly susceptible to dGlc. It is doubtful whether any inhibition of sugar uptake by dGlc could be a major component of the growth inhibition since dGlc causes inhibition of growth of strain 549 on media containing alanine or glycerol. It is tempting, therefore, to speculate that the toxic effect of dGlc on strain 549 and its ability to germinate rapidly on glucose are linked, presumably because dGlc is being used in

metabolism in place of glucose. It is well established that dGlcPO₄ weakens the cell wall of yeasts by adversely affecting the incorporation of glucose into wall polysaccharides (Moore, 1981). Microscopic examination of strain 549 revealed no evidence for lysis of hyphal tip cells. Also, analysis of hot water cell extracts revealed that very little dGlc or its metabolites were trapped by being incorporated directly into the cell walls of *M. anisopliae* as occurs in *S. cerevisiae* (Krátky *et al.*, 1975). Any dGlc-induced effects on the cell wall of *M. anisopliae* are likely to be indirect by interference of phosphorylated metabolites with metabolic processes involved in cell wall synthesis. Morphological changes in *M. anisopliae* (e.g. formation of appressoria) result from changes in cell wall synthesis and possibly turgor pressure (St Leger *et al.*, 1991). It is possible that dGlc (in alanine media) induced swelling of the tip cells of strain 549 hyphae and production of blastospore-like structures by weakening the cell walls resulting in osmotic fragility. It is also possible that the change in cell shape results from alterations in wall synthesis directed by endogenous respiratory events. In any event, dGlc may be useful in determining the metabolic functions associated with these morphological changes.

dGlc has proved useful for the isolation of catabolite repression mutants of *S. cerevisiae* (Zimmermann & Scheel, 1977) and filamentous fungi (Moore, 1981; Allen *et al.*, 1989; Kirimura *et al.*, 1992). These mutants display pleiotropic alterations in enzymes that are normally glucose-repressible. Similarly, dGlc-resistant mutants of *Neurospora crassa* were also resistant to sorbose (Allen *et al.*, 1989). Some, but not all, dGlc-resistant strains of *M. anisopliae* were cross-resistant to sorbose as well, suggesting possible allelism. It is likely that dGlc would appear toxic to strain 549 if it interfered with the derepression of the enzymes needed for utilization of fermentable carbon sources. Also, strain 808, but not strain 549, grew well with dGlc in glycerol medium, a non-fermentable substrate, the utilization of which requires glucose derepression of enzymes of the TCA cycle (Johnson & Carlson, 1992). It is noteworthy that dGlc altered morphogenetic events of strain 808 on solid media including the formation of aerial hyphae and conidiation. Fluoroacetate (a Krebs cycle inhibitor) causes similar alterations (unpublished data). It is possible, therefore, that dGlc affects the balance between fermentation and oxidative pathways for carbohydrate utilization in strain 808.

The fact that glucose and dGlc repress Pr1 production by strain 808 suggests that catabolite repression can be divided into different segments. The slow response of line 808 conidia to glucose in the medium is presumably due to one of the components in the chain from glucose to germination being dormant or less active. This assumes a relatively small difference at the molecular level. We are currently investigating glucose-repressible proteins involved in the germination of strains 549 and 808. In particular, the physiological effects of IBMX on germination are causing us to focus our attention on phosphodiesterase activities in spores.

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