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Glucose-initiated Germination of *Mucor racemosus* Sporangiospores

By MATTHEW L. TRIPP[1]† AND JOHN L. PAZNOKAS[1,2]*

Departments of Bacteriology and Public Health[1] and Basic Medical Sciences[2], Washington State University, Pullman, Washington 99164, U.S.A.

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Treatments leading to the initiation of germination of *Mucor racemosus* sporangiospores were examined. The results support the hypothesis that glucose is a specific trigger molecule for the initiation of *Mucor racemosus* sporangiospores. Glucose and some of the glucose analogues tested could initiate germination, mannose, 3-*O*-methyl-D-glucose, 5-thio-D-glucose and 6-deoxy-D-glucose being the most effective. The initiation event appeared to depend on the concentration of the initiator, with glucose and 3-*O*-methyl-D-glucose exhibiting nearly identical kinetic constants. Spores accumulated not only glucose and 3-*O*-methyl-D-glucose, but also the 1-*O*-methyl-D-glucose analogue, which did not initiate germination. The accumulated 3-*O*-methyl-D-glucose was not metabolized. The initiation sequence appeared to require the continued presence of the initiator as well as protein synthesis.

INTRODUCTION

Asexual sporangiospores of *Mucor racemosus* treated with a germinant undergo a morphological transition from an elliptical spore of 20 μm^3 to a spherical cell of 130 μm^3 with subsequent germ tube formation. The ability of a sporangiospore of *M. racemosus* to be triggered to germinate by a compound depends on how the spore is grown (Tripp & Paznokas, 1981). Sporangiospores produced on a nutritionally complex medium, denoted C-spores, germinate in a defined medium containing yeast nitrogen base, $(NH_4)_2SO_4$ and any one of several carbon sources including glucose, cellobiose, xylose or glutamate. In contrast, spores produced on a minimal sporulation medium, denoted M-spores, do not germinate on any of several carbon sources that were tested except glucose or mannose. The structural similarity between glucose and mannose suggests the existence of a common, but specific triggering mechanism. Such specificity in compounds that trigger germination has been reported for both bacterial spores (Levinson & Hyatt, 1964; Vary, 1978) and fungal spores (Hashimoto *et al.*, 1972; Hobot & Gull, 1977; Marchant & White, 1966; Ekundayo & Carlile, 1964). In this study we present data which support the hypothesis that *M. racemosus* sporangiospores are triggered to germinate by a glucose-dependent pathway that does not require metabolism of the glucose.

METHODS

Organism and culture conditions. Mucor racemosus (*M. lusitanicus*) ATCC 1216B was used in all experiments. Asexual sporangiospores were produced on two types of media as described previously (Tripp & Paznokas, 1981). Sporangiospores designated C-spores were produced on a complex sporulation medium (CSM) consisting of 1% (w/v) peptone (Difco), 0·3% (w/v) yeast extract (Difco), 2% (w/v) agar (Difco) and 2% (w/v) D-glucose. Sporangiospores designated M-spores were produced on a minimal sporulation medium (MSM) consisting of 0·05% (w/v) yeast nitrogen base without amino acids (YNB; Difco), 10 mM-$(NH_4)_2SO_4$, 10 mM-L-glutamate, 2% (w/v) agar (Difco) and 2% (w/v) D-glucose. The glucose was autoclaved separately and added aseptically. The pH

† Present address: Department of Biology, University of California-San Diego, San Diego, California, U.S.A.

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of all media was adjusted to 5·0 with H_2SO_4 except where noted. The sporulation media were spread-inoculated with 0·05 ml of a stationary phase yeast culture (grown in 0·3% yeast extract, 1% peptone, 1% glucose, under 100% CO_2) and incubated at 22 °C. Sporangiospores were harvested 5 d after inoculation, washed twice in distilled water and counted with either a haemocytometer or a Coulter counter, and used as the inoculum.

Germination test media. The minimal germination medium (MGM) was a modification of the MSM in which L-glutamate and agar were omitted and D-glucose was replaced by other substrates, at the various concentrations noted in the Table legends. Portions (10 ml) of MGM contained in 50 ml flasks were inoculated to a final concentration of 1×10^6 sporangiospores ml^{-1} and incubated at 25 °C in a gyrorotatory water bath incubator shaking at 150 rev. min^{-1}.

Initiation of spore germination. Sporangiospore volume was monitored with a Coulter counter (model ZB1) and Channelyzer (model II) as previously described (Tripp & Paznokas, 1981). Greater than 90% of either M-spores or C-spores have a volume <45 μm^3 at the time of harvesting. Spores that had achieved a volume >45 μm^3 were considered to have initiated germination.

Accumulation of glucose or 3-O-methyl-D-glucose (3-OMDG). C-spores (1×10^7 ml^{-1}) were incubated in MGM containing 3-*O*-methyl-D-[U-^{14}C]glucose or [U-^{14}C]glucose at a final concentration of 1 mM (1 μCi μmol^{-1}, 37 kBq μmol^{-1}) and with or without 100 mM-L-glutamate. Samples of the reaction mixture (0·2 ml) were removed at indicated times, collected by vacuum filtration on a Millipore membrane filter (0·45 μm, HAWP) and washed three times with 2 ml of the incubation medium containing an equivalent concentration of unlabelled 3-OMDG. For the uptake experiments, the filters were placed directly into scintillation vials containing 10 ml of 3a70b cocktail (Research Products International Corp., Elk Grove Village, Ill., U.S.A.) and counted in a Beckman LS-3133T series liquid scintillation counter.

Metabolism of 3-O-methyl-D-glucose. To determine if 3-OMDG was metabolized or incorporated into cellular products, spores treated with ^{14}C-labelled 3-OMDG were collected on Millipore filters (0·45 μm), placed into 3 ml of 0·1 M-HCl and subjected to three cycles of freezing and thawing using liquid nitrogen. These samples were then centrifuged at 3000 *g* for 20 min and the top 2·5 ml of the supernatant was removed and lyophilized. The lyophilized samples were redissolved in 0·25 ml distilled water. Portions (10 μl) were counted in the scintillation counter. Thin-layer chromatography was performed on silica gel (Eastman 1381) with a solvent system containing *n*-butanol/pyridine/water (1 : 1 : 1, by vol.). The dried chromatogram was placed adjacent to Kodak no-screen X-ray film NS-ST at room temperature for 14 d.

Chemicals. 3-*O*-Methyl-D-[U-^{14}C]glucose was purchased from Amersham/Searle, [U-^{14}C]glucose was purchased from New England Nuclear. Unlabelled 3-OMDG was purchased from Aldrich Chemical Co. The 6-deoxy-D-glucose was kindly donated by Dr J. Vary, UIMC, Chicago, Illinois. All other chemicals were reagent grade.

RESULTS

Effects of glucose analogues on germination

Previous studies (Tripp & Paznokas, 1981) have shown that *Mucor racemosus* sporangiospores are initiated to germinate synchronously and completely by glucose or the glucose analogue 3-*O*-methyl-D-glucose. Both C- and M-spores were treated with a number of compounds that are structurally related to glucose (Table 1) in a medium which also contained yeast nitrogen base, ammonium sulphate and glutamate. Both M- and C-spores initiated germination when treated with mannose, 3-*O*-methyl-D-glucose, 5-thio-D-glucose and 6-deoxy-D-glucose. However, only C-spores were triggered by treatment with arabinose, galactose, glucosamine, glutamate or xylose.

We considered the possibility that some of these analogues might resemble glucose but be toxic to the cell. This was tested by treating spores with both glucose and the glucose analogues. Of those tested, only 2-deoxy-D-glucose inhibited germination; 3-*O*-methyl-D-glucose, α-methyl-D-mannose, 1-*O*-methyl-D-glucose and fucose had no effect.

Effect of concentration of glucose and 3-O-methyl-D-glucose on germination rate

In the absence of a metabolizable carbon source M-spores do not germinate even when treated with 3-OMDG; upon the addition of 100 mM-L-glutamate these spores will germinate (Tripp & Paznokas, 1981). The rate of germination was dependent upon the concentration of either glucose or 3-OMDG in the germination medium (Fig. 1). Very little germination was observed at concentrations below 50 μM. The difference in germination rate between spores

Table 1. *Germination of dormant sporangiospores of M. racemosus induced by chemicals structurally related to glucose*

Germination medium for M-spores contained YNB, $(NH_4)_2SO_4$, glutamate (100 mM) and the indicated test compound (100 mM). Germination medium for C-spores contained YNB, $(NH_4)_2SO_4$ and the indicated test compound (100 mM).

	Percentage of spores >45 μm³ by 8 h	
Compound	M-spores	C-spores
D(+)-Glucose	>95	>95
Mannose	>95	>95
Galactose	<5	56
L(−)-Sorbose	<5	<5
L-Rhamnose	<5	<5
Fucose	<5	<5
β-Glucuronic acid	<5	<5
Glucosamine	7	24
Mannosamine	<5	<5
Galactosamine	<5	<5
Mannitol	<5	<5
Sorbitol	<5	<5
3-*O*-Methyl-D-glucose	>95	>95*
5-Thio-D-glucose	>95	>95*
6-Deoxy-D-glucose	>95	>95*
2-Deoxy-D-glucose	<5	<5
1-*O*-Methyl-α-D-glucose	<5	<5
1-*O*-Methyl-β-D-glucose	<5	<5
α-Methyl-D-mannose	<5	<5
α-Methyl-D-xylose	<5	<5
Xylose	<5	63
Arabinose	<5	18
Ribose	<5	<5
Lyxose	<5	<5
Glutamate	<5	58

* Swelling stopped short of germ tube formation (88 μm³).

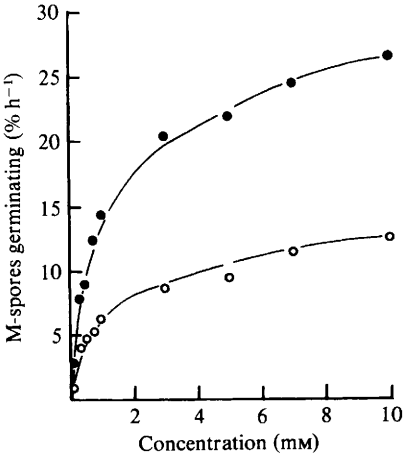

Fig. 1. Germination rate for M-spores in MGM/glutamate (100 mM) containing glucose (●) or 3-OMDG (○).

treated with glucose and 3-OMDG probably reflects the difference between glucose and glutamate as the primary carbon and energy source. The $K_{1/2}$ for glucose and 3-OMDG was calculated to be the same (1·0 mM).

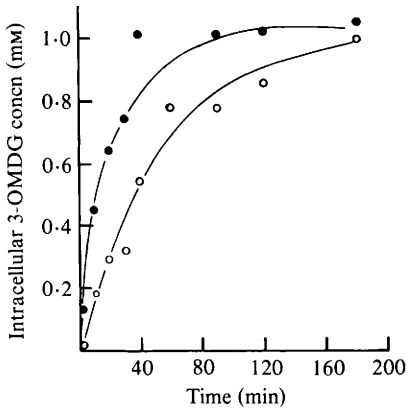

Fig. 2

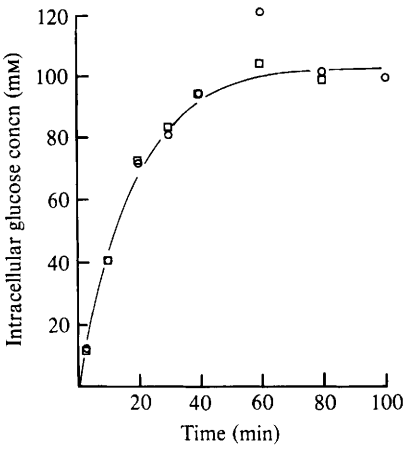

Fig. 3

Fig. 2. Intracellular concentrations of 3-OMDG in C-spores (●) and M-spores (○) calculated from the accumulation of ^{14}C-labelled 3-OMDG (1 mM extracellular concentration).

Fig. 3. Intracellular concentrations of D-glucose in C-spores (○) and M-spores (□) calculated from the accumulation of ^{14}C-labelled D-glucose (1 mM extracellular concentration).

On the other hand, C-spores will initiate germination with 3-OMDG in the absence of a carbon source and swell to 90 μm^3. The concentration dependence of these spores was similar to that for M-spores. Spore types were tested to see if they were able to accumulate 3-OMDG or glucose. Spores were treated with 1 mM ^{14}C-labelled 3-OMDG for the indicated times in MGM without a carbon source and the samples were collected on filters. Both M- and C-spores accumulated 3-OMDG to approximately the same concentration as that in the external medium (Fig. 2). Glucose was also accumulated, but to a much higher concentration (Fig. 3). This might be expected if glucose is metabolized while 3-OMDG is not metabolized.

Triggering by 3-O-methyl-D-glucose *under* CO_2

Mucor requires a fermentable hexose for anaerobic growth (Bartnicki-Garcia, 1968). If 3-OMDG was metabolized then some growth might be expected under anaerobic conditions. No C-spores germinated under 100% CO_2 in a medium containing 3-OMDG (50 mM), peptone (1%) and yeast extract (0·3%) even after 48 h incubation; greater than 95% germinated by 24 h in the same medium where 3-OMDG was replaced by glucose. Shifting the yeast extract/peptone/3-OMDG culture to aerobic conditions resulted in complete germination by 6 h.

Metabolism of 3-O-methyl-D-*glucose*

The compound 3-OMDG is reported to be a non-metabolizable glucose analogue (Kletzien *et al.*, 1975). The observation that M-spores are triggered to germinate by 3-OMDG only when a carbon source is also present suggests that the former is not utilized as an energy source by *M. racemosus* spores, but is mimicking glucose as an effector molecule. Conceivably, however, *M. racemosus* spores may be metabolizing 3-OMDG to glucose, or another trigger molecule. To test this hypothesis, M-spores were incubated in the presence of ^{14}C-labelled 3-OMDG under conditions in which germination is dependent on the presence of 3-OMDG or glucose. After 3 h incubation the M-spores had synchronously initiated germination and increased in volume to 34 μm^3. The ^{14}C-labelled 3-OMDG was extracted at 3 h, concentrated and subjected to thin-layer chromatography. The material migrating with the 3-OMDG standard contained 98·7% of the total counts in the 3 h sample. The remaining

Table 2. *Chromatographic analysis of accumulated 3-O-methyl-*D*-[U-^{14}C]glucose from germinating M. racemosus sporangiospores*

Material extracted from M-spores after 3 h incubation with ^{14}C-labelled 3-OMDG was subjected to thin-layer chromatography, together with standard 3-OMDG. The chromatograms were cut into 2 cm sections and the radioactivity was eluted and counted as described in Methods.

Section		Standard (c.p.m.)	Material from M-spores (c.p.m.)	R_F value*
Top	14–16 cm	47	48	
	12–14 cm	15992	3866	0·89
	10–12 cm	51	47	
	8–10 cm	54	51	
	6–8 cm	108	106	0·52
	4–6 cm	50	51	
	2–4 cm	53	39	
Origin	0–2 cm	55	58	
Blank		58		
Recovery		97·4%	95·6%	

* From the autoradiogram.

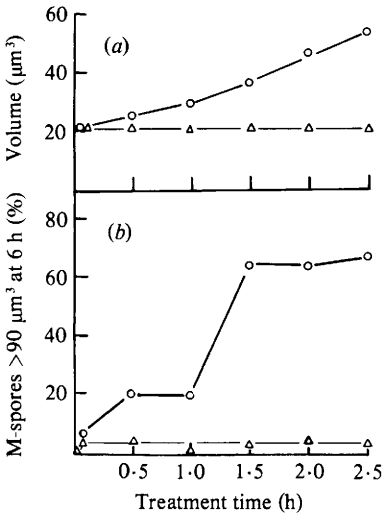

Fig. 4

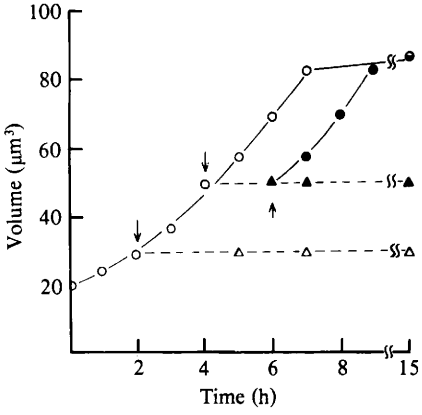

Fig. 5

Fig. 4. Effect of glucose (○) or glucose/cycloheximide (△) treatment for different times on the development of competence of M-spores to germinate in MGM/glutamate: (*a*) average volume of M-spore culture immediately after treatment, prior to being placed into MGM/glutamate; (*b*) percentage of M-spore population that initiated germination in MGM/glutamate.

Fig. 5. Effect of the removal (↓) and the re-addition (↑) of 3-OMDG from C-spores which had initiated germination in MGM/3-OMDG (no carbon source).

counts were present in a single spot (R_F 0·52) which contained 0·9% of the counts (Table 2). The starting material also contained the R_F 0·52 compound (0·3%). In this solvent system glucose has an R_F of 0·8. No counts were detected in this position on any of the chromatograms.

Effect of time course of glucose or 3-O-methyl-D-glucose treatment

Glucose triggers *Bacillus megaterium* endospores to germinate (Vary, 1978). Exposure to glucose for 5 min results in greater than 95% germination. Once germinated, however, these cells require an exogenous carbon and energy source for continued growth. *Mucor racemosus* spores responded similarly in that short periods of exposure to glucose resulted in initiation, but not continued outgrowth. In these experiments M-spores treated with 100 mM-glucose for different periods of time were harvested by filtration, washed and placed into MGM with or without 100 mM-glutamate. Those spores placed into MGM without a carbon source arrested at the volume attained while in the glucose medium (Fig. 4). Those placed into glutamate media following a 90 min glucose treatment continued to germinate synchronously.

Similarly, C-spores treated with 3-OMDG in MGM without an exogenous carbon source initiate germination and begin to swell. When 3-OMDG was removed the spores arrested at that point in their development (Fig. 5). Swelling continued upon re-addition of 3-OMDG. Addition of a metabolizable carbon source resulted in synchronous and complete germination. This process is sensitive to the protein synthesis inhibitor cycloheximide, which blocks development during the 3-OMDG treatment phase as well as during outgrowth (Fig. 4). These spores were not inactivated by cycloheximide since 95% initiated germination after being washed free of the cycloheximide and placed into MGM/glucose.

DISCUSSION

The data presented here support the hypothesis that glucose is acting as a positive effector molecule triggering *M. racemosus* sporangiospore germination. Of the stereoisomers of glucose tested, only mannose (not galactose) triggered M-spore germination. Glucosamine, mannosamine and 2-deoxy-D-glucose did not trigger germination, and 2-deoxy-D-glucose was inhibitory to both germination and glucose uptake. This suggests that the stereoconfiguration at the C-2 position of glucose may be important. Analogues methylated in the C-1 position (1-*O*-methyl-D-glucose or 1-*O*-methyl-D-mannose) were inactive as triggers; however, 3-*O*-methyl-D-glucose, methylated at the C-3 hydroxyl group, was as active as glucose in triggering germination. The glucose analogue 6-deoxy-D-glucose also triggered germination, but the 6-deoxy-D-glucose stereoisomers rhamnose and fucose did not. These data demonstrate that only a limited number of compounds with specific structural arrangements trigger *M. racemosus* M-spore germination.

C-spores, on the other hand, were triggered (<65%) by a few additional compounds including L-glutamate. All of these compounds support aerobic growth of vegetative mycelia. C-spores were not triggered by a variety of compounds which do not support vegetative growth such as sorbose or fucose (data not shown).

Since both M- and C-spores are triggered to germinate almost completely by the same glucose analogues it is possible that both spore types possess the same glucose triggering mechanism. The ability of certain glucose analogues to trigger germination may be related to their ability (i) to be transported, whereas analogues which do not trigger germination may not be transported, or (ii) to be metabolized to a trigger molecule such as glucose or glucose-6-phosphate, or (iii) to interact stereospecifically with a hypothetical dormancy-regulatory protein.

Uptake studies demonstrated that 1-OMDG and 3-OMDG were accumulated to similar concentrations; however, of these two compounds only 3-OMDG triggered germination. Hence, both triggers and non-triggers get into the spores. This does not rule out the possible existence of glucose-activated surface receptors but instead opens up the possibility that the effect is intracellular.

In some bacteria, glucose affects cyclic nucleotide concentrations in the cell and therefore the expression of catabolite repressible systems. This control appears to require a functional glucose transport system. This mechanism does not appear to be important here since

1-OMDG was transported but did not trigger germination. Also, uptake of 1-OMDG was competed for by glucose (data not shown) and therefore probably utilizes the same transport system.

Studies of the metabolism of ^{14}C-labelled 3-OMDG by M-spores have shown that after a 3 h 3-OMDG treatment (under conditions where >95% of the spores have initiated germination) spores contained only two labelled compounds. The major fraction was authentic 3-OMDG (96%), the minor fraction (<1%) was unknown. This compound, however, was also found in the starting material (0·3%) and could represent either breakdown products of 3-OMDG (but not glucose) or material used in the synthesis of 3-OMDG. It can therefore be concluded that no significant metabolite of 3-OMDG is present in germinating spores initiated by 3-OMDG. That 3-OMDG is non-metabolizable has been reported for several other systems (Kletzien *et al.*, 1975). Concentrations as high as 50 mM did not trigger M-spore germination in the absence of a carbon source (Tripp & Paznokas, 1981), and 3-OMDG did not trigger C-spore germination under conditions requiring a fermentable hexose (i.e. anaerobiosis). Aerobically, 3-OMDG (and also 5-thio-D-glucose and 6-deoxy-D-glucose) triggered the initiation of germination of C-spores in the absence of an exogenous carbon source. These data suggest that glucose, and not a metabolite of glucose triggers *M. racemosus* spore germination.

It is possible that glucose is acting as a positive effector molecule interacting stereospecifically with some regulatory protein(s). The number of specific events that a glucose-regulatory protein interaction might trigger are numerous and include substrate transport, energy metabolism, carbon and nitrogen balance, and RNA and protein synthesis. Some of these possibilities are currently being investigated.

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REFERENCES

BARTNICKI-GARCIA, S. (1968). Control of dimorphism in *Mucor* by hexoses: inhibition of hyphal morphogenesis. *Journal of Bacteriology* **96**, 1586–1594.

EKUNDAYO, J. A. & CARLILE, M. J. (1964). The germination of sporangiospores of *Rhizopus arrhizus*: spore swelling and germ tube emergence. *Journal of General Microbiology* **35**, 261–269.

HASHIMOTO, T., WU, C. D. R. & BLUMENTHAL, H. J. (1972). Characterization of L-leucine-induced germination of *Trichophyton mentagrophytes* microconidia. *Journal of Bacteriology* **112**, 967–976.

HOBOT, J. A. & GULL, K. (1977). The influence of glucose availability on events during germination of *Syncephalastrum racemosum* sporangiospores. *Journal of General Microbiology* **98**, 431–437.

KLETZIEN, R. F., PARIZA, M. W., BECKER, J. E. & POTTER, V. R. (1975). A method using 3-*O*-methyl-D-glucose and phloretin for the determination of intracellular water space of cells in monolayer culture. *Analytical Biochemistry* **68**, 537–544.

LEVINSON, H. S. & HYATT, M. T. (1964). Effect of sugars and other carbon compounds on germination and postgerminative development of *Bacillus megaterium* spores. *Journal of Bacteriology* **88**, 1403–1415.

MARCHANT, R. & WHITE, M. F. (1966). Spore swelling and germination in *Fusarium culmorum*. *Journal of General Microbiology* **42**, 237–244.

TRIPP, M. L. & PAZNOKAS, J. L. (1981). Relationship between sporulation medium and germination ability of *Mucor racemosus* sporangiospores. *Journal of General Microbiology* **127**, 35–43.

VARY, J. C. (1978). Glucose-initiated germination in *Bacillus megaterium* spores. In *Spores VII*, pp. 104–108. Edited by G. Chambliss & J. C. Vary. Washington, D.C.: American Society for Microbiology.