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Structural Features of Sugars That Trigger or Support Conidial Germination in the Filamentous Fungus *Aspergillus niger*

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The asexual spores (conidia) of *Aspergillus niger* germinate to produce hyphae under appropriate conditions. Germination is initiated by conidial swelling and mobilization of internal carbon and energy stores, followed by polarization and emergence of a hyphal germ tube. The effects of different pyranose sugars, all analogues of D-glucose, on the germination of *A. niger* conidia were explored, and we define germination as the transition from a dormant conidium into a germling. Within germination, we distinguish two distinct stages, the initial swelling of the conidium and subsequent polarized growth. The stage of conidial swelling requires a germination trigger, which we define as a compound that is sensed by the conidium and which leads to catabolism of D-trehalose and isotropic growth. Sugars that triggered germination and outgrowth included D-glucose, D-mannose, and D-xylose. Sugars that triggered germination but did not support subsequent outgrowth included D-tagatose, D-lyxose, and 2-deoxy-D-glucose. Nontriggering sugars included D-galactose, L-glucose, and D-arabinose. Certain nontriggering sugars, including D-galactose, supported outgrowth if added in the presence of a complementary triggering sugar. This division of functions indicates that sugars are involved in two separate events in germination, triggering and subsequent outgrowth, and the structural features of sugars that support each, both, or none of these events are discussed. We also present data on the uptake of sugars during the germination process and discuss possible mechanisms of triggering in the absence of apparent sugar uptake during the initial swelling of conidia.

Aspergillus niger is a filamentous fungus that is widely distributed (1) and produces large numbers of black asexual spores, termed conidia, to facilitate survival and dispersal (2). Conidia from some fungal species are responsible for causing food spoilage and the propagation of infection in plants and animals (3, 4).

In nature, most conidia probably germinate in response to lignocellulose (2) or the sugars associated with fruits, but many environments have very low levels of available sugars (5, 6). The germination of conidia on lignocellulosic material requires the sensing of the material and induction of genes encoding degradative enzymes, including those that lead to the synthesis of inducing compounds (7). Although the responses of *A. niger* mycelia to lignocellulose are documented (8), the responses of *A. niger* conidia to lignocellulose are less well described. Irrespective of this, there will be events that lead to and support the breaking of dormancy and germination. The development of polarity will then afford the secretion of enzymes that degrade the polymeric materials available in natural environments.

Environmental conditions must be favorable for the germination of fungal conidia, and water alone is insufficient to initiate germination. Exogenous carbon and nitrogen sources must also be present (9–11). A combination of D-glucose and water is sufficient to break dormancy and initiate germination of conidia in *A. nidulans* (12, 13). An early visual indicator of conidial germination involves the isotropic swelling of conidia (14, 15) before switching to polarized growth, which results in the formation of a germ tube and further mycelial growth. The breaking of dormancy is associated with uptake of water and the initiation of metabolic activity and protein synthesis (13). Conidial carbon and energy storage compounds, especially D-trehalose and D-mannitol, are known to be mobilized early in germination (16–18), and it is thought that the metabolism of D-glucose derived from D-trehalose provides the energy for conidial germination (19).

Onset of germination of fungal conidia (*Metarhizium anisopliae*) in the presence of various sugars and amino acids has been previously reported (20), but a systematic analysis of the structural features of D-glucose analogues that either trigger conidial germination or support the production of hyphae has not yet been reported. The effect of different sugars on the metabolism of D-trehalose has not been reported for *A. niger*, although in *A. nidulans*, it was reported that D-glucose and 6-deoxy-D-glucose were able to activate D-trehalose breakdown (21). D-Glucose will support mycelial growth and 6-deoxy-D-glucose will not, so it can be concluded that the triggering of D-trehalose degradation at an early stage of conidial germination differs from the provision of metabolic energy needed to support mycelial development. Here, we have studied the effects of different sugar molecules, all analogues of D-glucose, on the germination of *A. niger* conidia, with an emphasis on determining which analogues can be sensed and affect the different stages of germination. A "germination trigger" is defined here as a particular sugar molecule being detected, which leads to the catabolism of D-trehalose and the swelling of conidia. We hypothesize that there is a succession of events associated with germination. The initial trigger event is followed by the uptake and metabolism of specific sugars to generate metabolic energy and carbon skeletons for conidial outgrowth. The different sugars that facilitate both events, neither of the events, or one of the events are identified.

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MATERIALS AND METHODS

Sugars. The majority of sugars were obtained from Sigma-Aldrich (Poole, Dorset, United Kingdom). D-Idose and D-gulose were purchased from Carbosynth Ltd., Berkshire, United Kingdom; D-tagatose from VWR, Leicestershire, United Kingdom; D-glucose from Fisher Scientific, Loughborough, United Kingdom; and D-talose from MP Biomedicals, LLC, Cambridge, United Kingdom. All sugars were in the D form and ≥98% pure. L-Glucose and L-arabinose were also used in this study and were of ≥99% purity. The chemical structures of the 16 sugars in their pyranose ring conformation are given in Table S1 in the supplemental material (CS ChemOffice Ultra 2002, version 7.0; CambridgeSoft). The D-trehalose used as a standard in HPLC was obtained from Sigma-Aldrich and was ≥99% pure.

Strains and growth conditions. *A. niger* strain N402, a *cspA* derivative of a wild-type strain (22), was used throughout, and it was grown on *Aspergillus* minimal medium (AMM) (8). For provision of conidia from agar slopes, AMM was supplemented with 10 g D-glucose per liter and 20 g agar per liter, and the inoculated medium was incubated at 28°C for 6 days, ensuring a consistent supply of young conidia of the same age. For inoculation of liquid batch cultures (100 ml of AMM containing 100 μM or 55.5 mM 1% [wt/vol] D-glucose analogues), dormant conidia were harvested by washing agar slopes with 3 ml Tween 80 (0.01%, vol/vol). The conidial suspensions were then vortexed and filtered through sterile synthetic wool, and the numbers of conidia were counted using a hemocytometer. Experimental cultures were inoculated with 10^6 conidia/ml, shaken at 150 rpm, and incubated at 28°C.

Microscopy. *A. niger* conidial development was followed using an HBO 50 microscope (Carl Zeiss, Welwyn Garden City, Hertfordshire, United Kingdom) with a ×40 magnification objective lens and a final magnification of ×640. Microscope images were recorded after either 14 h or 24 h of incubation of conidia at 28°C, with specified amounts of the 16 sugars investigated. Conidial development was also followed in the absence of a carbon source (No-C-MM). The developing conidia were sedimented using an Eppendorf 5810 R centrifuge (Eppendorf, United Kingdom; A-4-62 rotor, 4°C, 3 min, 1,600 × *g*). The supernatant was removed and the pellet washed 3 times with 1 ml Tween 80 (0.01%, vol/vol) using a Biofuge Pico microcentrifuge (Heraeus Instruments, Germany; 3328 rotor, room temperature, 1 min, 9,000 × *g*). After the final wash, the pellets were resuspended in 1 ml No-C-MM. Five microliters of this sample was then placed onto a microscope slide (Thermo Scientific; 76 by 26 mm), with a coverslip (VWR International; 22 × 22 mm and 0.13 to 0.17 mm thick) on top to be viewed under the microscope using the Axiovision version 3.0 software (Carl Zeiss, United Kingdom).

Flow cytometry. The development of *A. niger* conidia was analyzed using a BD FACSCanto flow cytometer (Becton, Dickinson [BD] Biosciences, San Jose, CA, USA). Experimental cultures were prepared as mentioned before, but there was an additional step of vortexing (1 min) prior to centrifugation, and the resuspension was in 0.5 ml Tween 80 (0.01% vol/vol). The samples were then transferred to 5-ml round-bottom BD Falcon tubes (BD Biosciences, Bedford, MA, USA). The BD FACSDiva software (BD Biosciences) was used to measure the size of 1×10^5 conidia after 1 h of incubation at 28°C in No-C-MM and in the presence of a range of 100 μM D-glucose analogues. Dormant (0-h) conidia (1×10^5) were also analyzed. They were harvested from agar slopes as described above, and 1 ml of the conidial suspension was centrifuged using the Biofuge Pico centrifuge, the supernatant removed, and the pellet vortexed and washed 4 times with 1 ml Tween 80 (0.01%, vol/vol) and finally resuspended ready for analysis. The forward scatter (FSC) parameter, a measure of conidial size (23, 24) was determined for each sample. Experiments were duplicated, and average values were calculated and plotted.

High-pressure liquid chromatography (HPLC) analysis of D-trehalose. D-Trehalose content was quantified as described elsewhere (25). Duplicate samples of dormant conidia and 1-h conidia germinating either in No-C-MM or in the presence of a 100 μM concentration of a given D-glucose analogue were analyzed.

Measurement of uptake of D-[U-^{14}C]glucose and D-[1-^{14}C]galactose. The uptake medium consisted of 10 ml of 55.5 mM D-glucose minimal medium and 10 μCi D-[U-^{14}C]glucose (Perkin-Elmer). Uptake was initiated by the addition of 10^6 conidia. Air was drawn through the culture by vacuum and bubbled through hyamine hydroxide (Perkin-Elmer) to absorb $^{14}CO_2$. One-milliliter conidial suspension samples were removed over a period of 0 h to 1 h 30 min and rapidly filtered through 28-mm cellulose nitrate filters (0.45-μm pore size). Filters were prewashed with 5 ml of 55.5 mM D-glucose minimal medium (with no ^{14}C). After sample filtration, filters were again washed three times with 5 ml D-glucose (55.5 mM) minimal medium (with no ^{14}C). Filters were placed into 5-ml ScintiSafe 3 liquid scintillation cocktail (Fisher Scientific, United Kingdom), and radioactive emissions were counted using a Packard Tri-Carb 2100 TR liquid scintillation analyzer. The same methodology was used for measuring the uptake of D-galactose; however, the uptake medium consisted of 10 ml of 55.5 mM D-galactose minimal medium containing 1 mM D-glucose and 10 μCi D-[1-^{14}C]galactose (Perkin-Elmer), and the wash steps were done using this medium (with no ^{14}C). Also, the uptake of D-[1-^{14}C]galactose was measured in the absence of D-glucose or with D-glucose replaced by 100 μM 2-deoxy-D-glucose (trigger only). At each time interval studied, 0.5 ml hyamine hydroxide was removed and added directly to 5 ml scintillation cocktail for measurement of radiolabel to determine the amount of $^{14}CO_2$ produced. The experiments were done in duplicate at 28°C. The amount of ^{14}C present in each experiment was determined by direct sampling of 100 μl into scintillation fluid, and all the dpm values obtained from the scintillation analyzer were converted to uptake and $^{14}CO_2$ output from D-[^{14}C]glucose or D-[^{14}C]galactose (pmol/100,000 conidia) and plotted.

RESULTS

Determination of the minimum D-glucose concentration for germination. Since most D-glucose analogues are available at only 99% purity, it is possible that false germination results may be caused by the 1% impurities unless the overall sugar concentrations are reduced to the minimum concentrations. Experiments were therefore carried out using a series of log-diminishing concentrations of D-glucose in AMM to determine the minimum concentration required for germination. Germination was measured microscopically, where any conidial swelling and formation of germ tubes was recorded, and by using flow cytometry, where the FSC parameter gave quantifiable data on conidial swelling (Fig. 1).

Observations by microscopy confirmed the flow cytometry data. They both showed that without D-glucose present, conidia did not swell and remained unchanged (their size was similar to that of dormant, 0-h conidia). D-Glucose at 55.5 mM caused rapid conidial swelling, which was easily discernible at 1 h, while diminishing concentrations of D-glucose caused a progressive lowering of the rate of conidial swelling, as evidenced by flow cytometry. Observations by microscopy at 14 h of incubation showed that hyphae were formed with D-glucose concentrations ranging from 10 μM to 55.5 mM. Conidial swelling without subsequent formation of germ tubes was observed over D-glucose concentrations ranging from 10 nM to >1 μM. At 1 nM D-glucose, conidial swelling did not occur. If conidial swelling is regarded as an indication of conidial germination, the results indicate that a very low concentration of D-glucose (10 nM) is required to initiate conidial germination and that higher concentrations are required for hyphal outgrowth (Fig. 1).

Analysis of D-glucose analogues inducing germination of *A. niger* conidia. From the experiments described above, 100 μM was selected as a minimal concentration that induced conidial swelling and also led to germ tube formation and hyphal out-

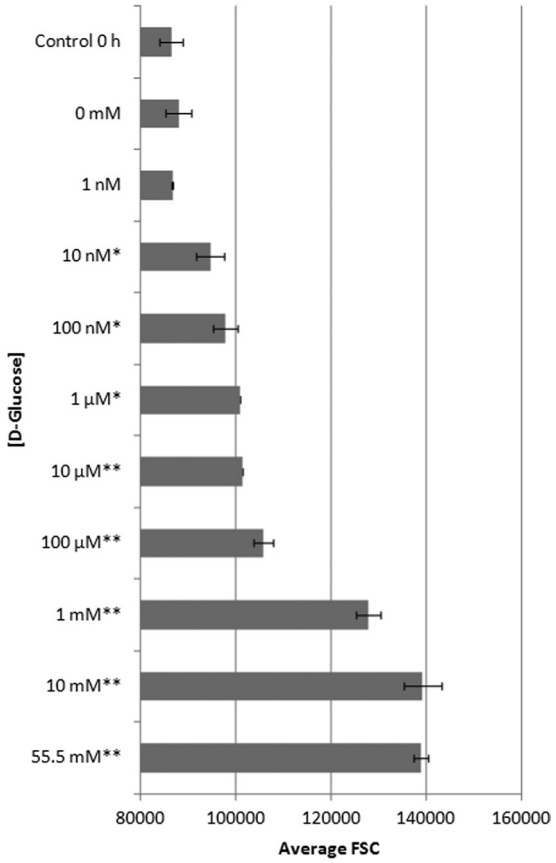

FIG 1 Average FSC (forward scatter, a measure of conidial size using the flow cytometer) detection of conidial swelling of *A. niger* incubated for 1 h at 28°C in AMM containing different concentrations of D-glucose (0 to 55.5 mM). Control 0-h FSC represents the dormant conidial size. Standard deviations for duplicate samples are shown. Asterisks indicate that conidial swelling only (*) or hypha formation (**) occurred at these concentrations (determined by microscopy at 14 h; microscopy images are not shown).

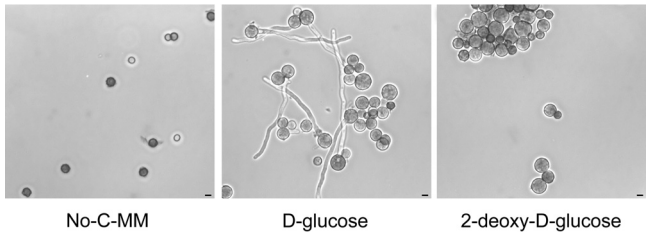

FIG 2 Microscopy images (with 5-μm scale bars) of *A. niger* conidia incubated for 14 h in no-carbon minimal medium (No-C-MM) and in the presence of D-glucose and the D-glucose analogue 2-deoxy-D-glucose at 100 μM. These images represent the three outcomes identified. In No-C-MM (left image), conidia did not swell or germinate to hyphae, which also resembled the phenotype of conidia developing in the presence of D-allose, D-altrose, D-idose, D-gulose, D-talose, D-galactose, D-arabinose, L-arabinose, or L-glucose. In the presence of D-glucose (middle image), there was evident conidial swelling and formation of hyphae, which also resembled the phenotype of conidia developing in the presence of D-xylose, D-mannose, or D-glucosamine. In the presence of 2-deoxy-D-glucose (right image), conidial swelling only was evident, with no formation of hyphae, which also resembled the phenotype of conidia developing in the presence of D-tagatose or D-lyxose.

growth (processes associated with germination). Sixteen analogues of D-glucose were then added to AMM at 100 μM to determine which were able to induce conidial germination. Unexpectedly, three outcomes were identified. First, a number of analogues did not induce germination. Over a period of 14 h of incubation, conidia did not swell or germinate to hyphae in the presence of D-allose, D-altrose, D-idose, D-gulose, D-talose, D-galactose, D-arabinose, L-arabinose, or L-glucose. Control tests also showed no swelling or germination in the absence of a carbon source (No-C-MM) (Fig. 2). Second, a series of D-glucose analogues was found to induce conidial swelling and formation of hyphae in a similar manner to that of the D-glucose control. These were D-xylose, D-mannose and D-glucosamine. Third, a few D-glucose analogues, i.e., D-tagatose, D-lyxose, and 2-deoxy-D-glucose, were found to induce conidial swelling at a slightly reduced rate compared to D-glucose. However, after the period of conidial swelling, no hyphae were formed over 14 h of incubation (Fig. 2). *A. niger* conidia were then left to incubate for 24 h in the presence of these three substrates to see if germ tubes would eventually be produced. However, even after such prolonged incubation, the conidia still remained swollen with no germ tubes formed (see Fig. S1 in the supplemental material, and data not shown).

Test results on conidial swelling were quantified in large populations (1×10^5) of germinating conidia using the FSC parameter in flow cytometry. The data are presented in Fig. 3. The data for conidial swelling at 1 h confirmed the earlier findings. Therefore, germination, as measured by conidial swelling, was induced only by D-glucose, D-xylose, D-mannose, D-glucosamine, D-tagatose, D-lyxose, and 2-deoxy-D-glucose.

It may be possible that D-tagatose, D-lyxose, and 2-deoxy-D-glucose at 100 μM caused conidial swelling but failed to produce germ tubes because the applied concentration was too low. A repeat experiment was therefore set up using 2-deoxy-D-glucose (with D-galactose and D-glucose controls) at 55.5 mM, using microscopy to follow the development of conidia, but there were no differences between the two sugar concentrations (data not shown). Swelling of conidia occurred in the presence of D-glucose and 2-deoxy-D-glucose within 1 h of inoculation, though the isotropic expansion occurred to a greater extent in D-glucose. There was no evident swelling or germ tube formation in the presence of D-galactose or in the absence of a carbon source. It was only in the presence of D-glucose, among this selection of sugars, that full germination was observed. Following prolonged incubation for 24 h, only very few conidia in the presence of 2-deoxy-D-glucose established polarity. At this stage, however, further conidial germination ceased. This experiment at 55.5 mM confirmed that 2-deoxy-D-glucose induced conidial swelling but did not allow hyphal development.

***A. niger* conidial germination determined by D-trehalose breakdown.** D-Trehalose breakdown is an early event in conidial germination (18). D-Trehalose degradation, determined by HPLC, was therefore used as an independent measure of early germination in the presence of analogues of D-glucose, each at 100 μM. Dormant conidia (0 h) contained 110 ± 2 pmol of D-trehalose/100,000 spores (Fig. 4). The same 7 sugars causing conidial swelling (D-glucose, D-xylose, D-mannose, D-glucosamine, D-tagatose, D-lyxose, and 2-deoxy-D-glucose) also caused a reduction in the conidial D-trehalose content over the first hour of germination. The reduction in D-trehalose content was marginally greater at 1 h in the presence of D-glucose than with the other 6 sugars. In the presence of the other 9 remaining sugars (D-allose, D-altrose, D-idose, D-gulose, D-talose, D-

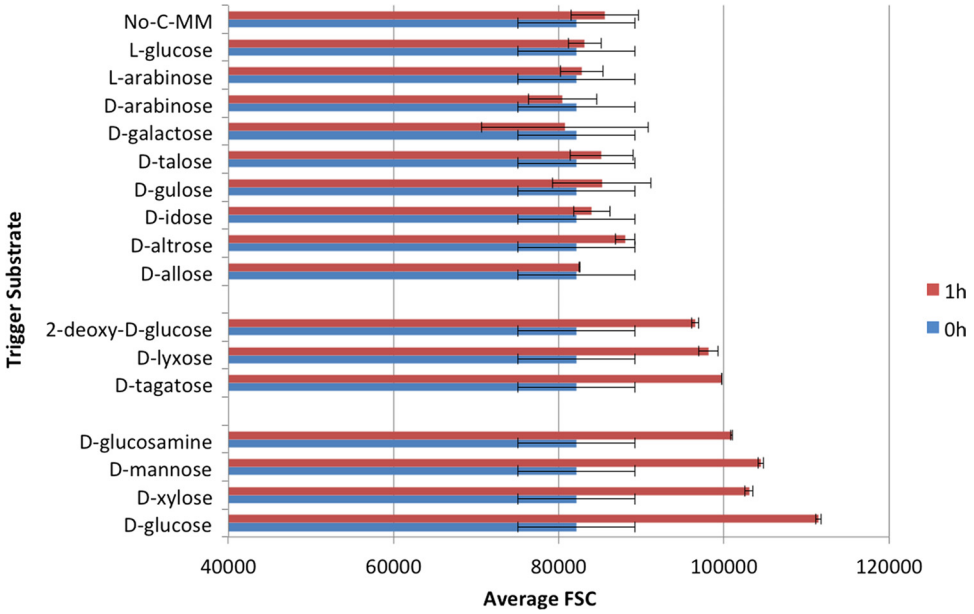

FIG 3 *A. niger* conidial swelling at 1 h of germination, as determined by flow cytometry, in no-carbon minimal media (No-C-MM) and in the presence of a range of D-glucose analogues at 100 μM. Potential trigger substrates are grouped into nongerminators (e.g., L-glucose), conidial swelling inducers (e.g., 2-deoxy-D-glucose), and full germination (conidial swelling and hypha formation) inducers (e.g., D-glucose). Standard deviations are shown for duplicates samples. *t* tests at 0 to 1 h showed no significant differences for nongerminators (e.g., L-glucose [$P = 0.43$]) but significant differences for the conidial swelling (e.g., 2-deoxy-D-glucose [$P = 0.04$]) and full germination (e.g., D-glucose [$P = 0.01$]) inducers.

galactose, D-arabinose, L-arabinose, and L-glucose) or in the absence of a carbon source, there was no alteration in the conidial D-trehalose content (Fig. 4).

Germination triggering and outgrowth are two distinct events. The data presented so far demonstrate that triggering of germination and germ tube outgrowth into hyphae are two distinct events. They differ (i) in the time of occurrence, (ii) in the D-glucose concentration required, and (iii) in the identity of sugars affecting each event. In theory, an external carbon source can act as an inducer for germination and as an energy source and can form carbon skeletons for metabolism. 2-Deoxy-D-glucose triggers germination (conidial swelling and D-trehalose breakdown)

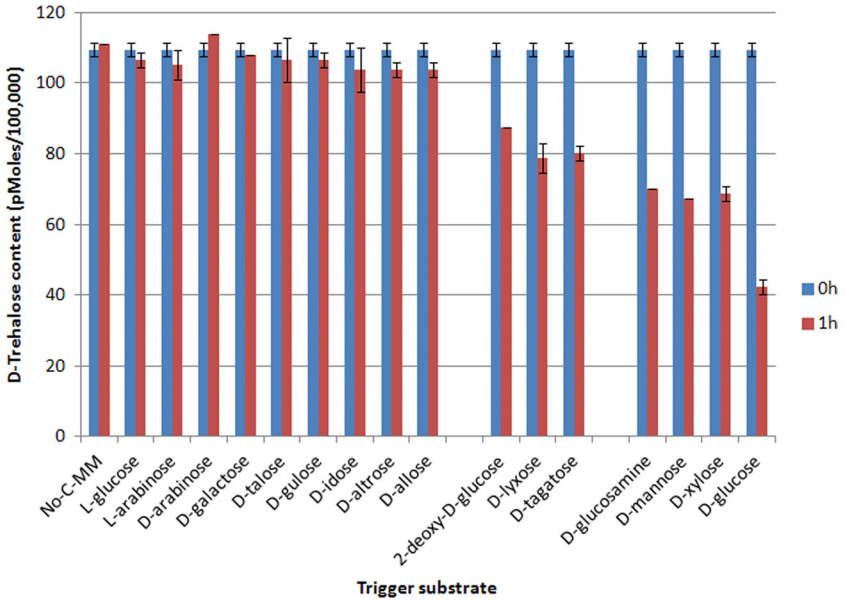

FIG 4 D-Trehalose degradation over the first hour of *A. niger* conidial germination, as determined by HPLC, in no-carbon minimal medium (No-C-MM) and the presence of a range of D-glucose analogues at 100 μM. Results are presented as pmol/100,000 conidia. Trigger substrates are grouped into nongerminators (e.g., L-glucose), conidial swelling inducers (e.g., 2-deoxy-D-glucose), and full germination (conidial swelling and hypha formation) inducers (e.g., D-glucose). Standard deviations for duplicate samples are shown.

TABLE 1 Division of D-glucose analogues into categories based on their effects on germination of *A. niger* conidia and ability to be metabolized

Category or subcategory[a]	Analogues[b]
Trigger and metabolic substrates	D-Glucose, D-xylose (5), D-mannose (2), D-glucosamine (2)
Trigger-only substrates	D-Tagatose (1, 2, 5), D-lyxose (2, 5), 2-deoxy-D-glucose (2)
Nontriggering (potentially metabolizable) substrates	
Metabolizable substrates	D-Galactose (**4**), D-arabinose (2, **3**, 5), L-arabinose (**4**, 5)
Nonmetabolizable substrates	D-Allose (**3**), D-altrose (2, **3**), D-idose (2, **3**, **4**), D-gulose (**3**, **4**), D-talose (2, **4**), L-glucose (2, **3**, **4**, 5)

[a] Sugars inducing germination and hyphal outgrowth are listed as "trigger and metabolic substrates," those initiating germination only as "trigger-only substrates," and those not affecting germination as "nontriggering substrates." Nontriggering analogues of D-glucose are subdivided into those able to be metabolized by *A. niger* in combination with a germination trigger, either D-tagatose, D-lyxose, or 2-deoxy-D-glucose, and those neither triggering germination nor being metabolized by *A. niger*.
[b] The carbons on which there are changes from the D-glucose pyranose ring structure are given (the essential carbon atoms, 3 and 4, are highlighted in bold, while nonessential atoms are given in normal font).

but does not allow hyphal growth. It is known that 2-deoxy-D-glucose is not an energy source, since it is nonmetabolizable (26–28). This probably indicates that 2-deoxy-D-glucose is sensed by the conidia and used to trigger germination, but because no energy can be liberated from such a substrate, hyphal formation cannot occur. We therefore conclude that germination of conidia has two parts and that the trigger can be distinguished from the generation of metabolic energy. It appears that only the triggering event occurs in the presence of 2-deoxy-D-glucose but that both events occur in sequence in the presence of sufficient D-glucose, as an example.

Testing the two-event hypothesis. From the results presented thus far, the list of D-glucose analogues can be divided into those triggering germination and allowing hyphal growth, those triggering germination only, and those not triggering germination (Table 1). It is possible that some of the nontriggering analogues can be metabolized to provide energy and carbon, after germination has been initiated (by another sugar, a triggering compound), acting to complement the triggering event to achieve full germination. To test this proposal, *A. niger* conidia were incubated for 24 h with a combination of a trigger-only substrate (Table 1) at 100 μM and a potential metabolic substrate (Table 1) at 55.5 mM. Microscopy was used to visualize the development of conidia.

The potential metabolic substrates were divided into the two categories of metabolizable or nonmetabolizable substrates. The former group includes D-galactose, D-arabinose, and L-arabinose, any one of which can enable the full germination of conidia into hyphae when also in the presence of any of the trigger-only substrates. The latter group includes the analogues that did not complement the triggering substrates: D-allose, D-altrose, D-idose, D-gulose, D-talose, and L-glucose (Table 1). The results thus confirmed that combinations of trigger-only substrates and certain metabolizable substrates can together result in full conidial germination. Only D-galactose, D-arabinose, and L-arabinose, of this group of sugars, can be metabolized.

Further confirmation that the triggering event occurs first during the germination of *A. niger* conidia, followed by the provision of energy and carbon from external sources, and that complementation between triggering and metabolizable substrates can be achieved, was obtained through a time course. Microscopy demonstrated that conidia developing in the presence of 2-deoxy-D-glucose remained swollen at 14 h and 24 h. Transfer of conidia into No-C-MM for 10 h after they had been triggered by 2-deoxy-D-glucose failed to lead to the formation of hyphae. *A. niger* conidia that were incubated with 2-deoxy-D-glucose for 14 h and then washed and transferred to D-galactose for a further 10 h showed a swollen morphology and formation of hyphae (see Fig. S1 in the supplemental material).

Chemical structures of D-glucose analogues triggering germination. Examination of the chemical structures of the 7 sugars triggering germination of *A. niger* conidia and those 9 sugars not triggering germination (Table 1), revealed a number of structural characteristics. Alterations in the positioning of hydroxyl groups on carbons 2 and 5 on D-glucose showed no differential effect on germination triggering. Carbon 2 analogues of D-glucose, D-mannose, 2-deoxy-D-glucose, or D-glucosamine and the carbon 5 analogue D-xylose all triggered conidial germination. However, any alteration in the orientation of hydroxyl groups on carbon 3 (D-allose) or carbon 4 (D-galactose) prevented the triggering of germination. This pattern was followed in all other analogues having multiple alterations in structure. Sugar structures are listed in Table S1 in the supplemental material. These data suggest that either a specific receptor site is present within or on the surface of conidia to initiate germination or the shape of the sugar determines transport into the conidia (29). To test this suggestion, uptake of D-[U-^{14}C]glucose and D-[1-^{14}C]galactose was compared in *A. niger* dormant conidia.

Investigation of the location and nature of the triggering sensor. The uptake of radiolabeled D-glucose and D-galactose and any labeled-CO_2 output were measured. Initially, samples were taken every 20 min over the first hour of germination, and the data are presented in Fig. S2 in the supplemental material. The results show that there was no detectable uptake of either sugar and no $^{14}CO_2$ output over the 1-h incubation period (see Fig. S2A and B in the supplemental material). Previous data had shown that uptake of D-glucose from the external environment occurs between 1 and 2 h (data not shown). Thus, to determine when the uptake occurs, samples were taken every 5 min after 1 h of incubation for 30 min (Fig. 5). The results show that rapid uptake of both D-glucose and D-galactose was detectable at 1 h 20 min, which was reinforced by the increased $^{14}CO_2$ output at this time (Fig. 5A and B). Approximately 800 pmol of D-glucose was taken up over the period of 1 h 30 min by 10^5 conidia, with approximately 150 pmol of $^{14}CO_2$ being formed (Fig. 5A), thus giving a ratio of ~8:2 (from the actual data, ca. 84% of the carbon is being used for biosynthesis and ca. 16% for energy generation). In comparison, 400 pmol of D-galactose was taken up over the same period, with approximately 100 pmol $^{14}CO_2$ being formed (Fig. 5B), resulting in a similar ratio of carbon allocation to biosynthesis and energy generation. The data in Fig. 5 also show that the uptake of D-glucose is

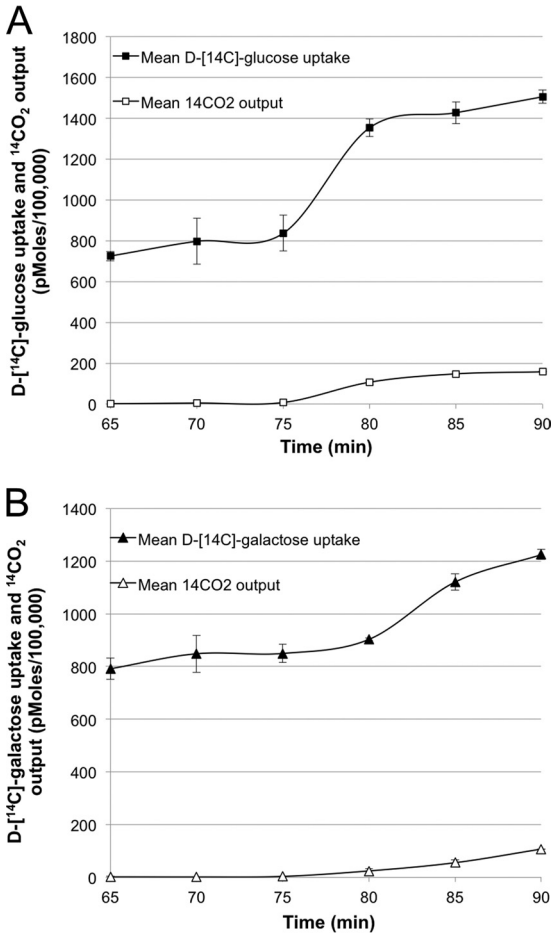

FIG 5 Uptake of D-[^{14}C]glucose (A) and D-[^{14}C]galactose (B) and the formation of $^{14}CO_2$ by 100,000 *A. niger* conidia taken every 5 min after 1 h of incubation for 30 min (pmol/100,000). The means and standard deviations for duplicate samples are shown.

faster than that of D-galactose. Uptake of D-galactose did not occur unless a trigger sugar was included, and for the data shown, 1 mM D-glucose was used. However, the D-glucose could be replaced by 100 μM 2-deoxy-D-glucose without altering the kinetics of D-galactose uptake (data not shown).

Further experiments showed that D-glucose and D-galactose competed for uptake into conidia between 1 h and 1 h 30 min (data not shown). The presence of 10 mM D-galactose reduced the uptake of 1 mM D-glucose by approximately 50%, with uptake being initiated as described for Fig. 5. Output of $^{14}CO_2$ from D-[U-^{14}C]glucose was similarly reduced by the presence of D-galactose.

DISCUSSION

After dispersal from the parent fungus, each conidium must continually interact with its environment, and, if conditions are favorable, the first stage of development will involve the germination of the dormant conidium (30). The model of germination accepts that there is a series of morphological and biochemical changes that are associated with conidial development. These involve the initial increase in conidial size and the catabolism of internal storage compounds, followed by the formation of hyphae (15, 18, 31), all of which have been experimentally monitored in this study. From our data, we believe that this model can be extended to include the initial sensing of correctly shaped sugars that trigger the germination process. This study has a focus on the roles of individual sugars in conidial germination and does not extend to the roles of other molecules, including L-amino acids. Neither does the study include detailed measurements of DNA synthesis and nuclear division. However, on that note, many of the conidia used in the study were already binucleate at 0 h, and the first round of nuclear division was complete at 6 to 8 h of germination (unpublished observations).

Previous studies have commonly used D-glucose to induce the germination of fungal conidia (10, 31), and to date, no study has investigated the functionality of D-glucose analogues in triggering and supporting conidial germination. Furthermore, it is only the proportion of germination (percent germination) that is often reported, with the initial swelling of conidia often not being distinguished as a separate event (12). Thus, the use of D-glucose analogues in this study has enabled us to make the distinction between these two events and the sugars which support them. We wanted to understand how *A. niger* conidia sense, respond to, and utilize different soluble sugars for germination. Confirming previous studies (10, 11, 32), we show that no swelling or germination of conidia occurred in the absence of a carbon source. Our data also showed that only low concentrations of specific sugars were required to trigger the breaking of dormancy to initiate germination; 10 nM was sufficient for conidial swelling, and 10 μM supported the formation of hyphae.

Detailed examination of the initial spore germination-triggering event showed that the arrangement of hydroxyl groups on carbons 3 and 4 on the pyranose ring structure of D-glucose is essential for recognition as a trigger to initiate the germination process. In comparison to D-glucose, if the —OH group on carbon 3 is below the ring (e.g., in D-allose) and/or the —OH group on carbon 4 is above the ring (e.g., in D-galactose), the sugars are undetectable to the conidia and do not trigger germination. The other carbon atoms making up the ring structure of D-glucose can have substitutions, which still results in the onset of germination, and they are therefore thought to be nonessential for recognition. This implies that the trigger interaction is highly specific and that only certain sugars (D-glucose, D-xylose, D-mannose, D-glucosamine, D-tagatose, D-lyxose, and 2-deoxy-D-glucose) of those tested serve as triggering compounds and have the correct shape to fit the triggering sensor. Osherov and May (12), however, speculated that it was a metabolite and not the D-glucose molecule itself that initiated germination, and they could not rule out the possibility that 2- or 6-deoxy-D-glucose was not recognized as D-glucose by the sensor. We provide detailed evidence in the present study that 2-deoxy-D-glucose has the structure to trigger germination, as it can be recognized by the triggering sensor. However, there is a limit to the amount of development that can occur due to its nonmetabolizable nature (26), and conidia which have been triggered by 2-deoxy-D-glucose will remain swollen, and very few will start to establish a site of polarity.

We found that L-arabinose did not trigger conidial germination, and yet, L-arabinose is commonly used to support the growth of filamentous fungi, including *A. niger*, and when growth is initiated with conidia (33–35). None of those studies was performed using defined minimal medium, so it is likely that other compounds included in the media used served as triggers, allowing L-arabinose to be metabolized subsequently. We explored that

possibility and showed that although L-arabinose alone in minimal medium did not trigger conidial germination, the addition of a low level (0.1 g/liter) of either yeast extract or peptone was able to trigger and allow mycelial growth on L-arabinose (unpublished data).

The trigger interaction induces the mobilization of D-trehalose that leads to conidial swelling and the activation of metabolic pathways required to sustain conidial outgrowth. Previous estimates of the D-trehalose content of *A. niger* spores show that approximately 5% of the dry weight is comprised of D-trehalose (18, 31), which could possibly contribute toward the energy for the early stages of germination (19). Since the internal stores are limited in amount, a transition must take place in metabolism of the stores to the carbon source present in the conidial environment. External carbon sources will need to be transported into the conidium for the purpose of biosynthesis and energy generation, in order to support the continued growth of the fungi.

The germination responses of different fungal species may vary; D-galactose induces germination in *A. nidulans* (12), whereas our data show that D-galactose does not trigger germination in *A. niger*, but it does have relevance as a later, metabolizable substrate. Fekete et al. (29) showed that D-galactose was not taken up by *A. niger* conidiospores, but D-glucose uptake was not measured. We show that over the first hour of germination, the conidia do not take up or use either D-glucose or D-galactose. It can therefore be hypothesized that D-trehalose degradation, and not the catabolism of external sugar molecules, provides the initial metabolic carbon and energy for germination, especially over the first hour. Since D-trehalose breakdown does occur within the first hour, this demonstrates that germination triggering must occur before this time. If sugar uptake was easily detectable over this period, it would suggest that the sensor was internal. Similarly if D-glucose, but not D-galactose, had been found to be transported into the conidia, it would have suggested that the transporter itself is the sensor (29). However, this was not the case, suggesting the possibility of an external sensor to trigger germination. Although the actual identity of the triggering sensor in *A. niger* is as yet unknown, our data suggest that the location of the sensor is most likely on the surface of the conidium, so it is possible that it may be a G-protein-coupled receptor (36). However, we certainly cannot rule out the possibility that very low levels of sugars (experimentally undetectable) could penetrate the spore and trigger germination via an internal sensor. Signaling was not explored further here, although we and others (12, 25, 31, 36) have reported changes in levels of relevant transcripts during conidial germination. Germination in the presence of D-glucose showed, for example, that transcript levels for RgsA and RasA were higher at 1 h than in 0-h conidia, whereas transcript levels for adenylate cyclase (An11g01520) and the cyclic AMP (cAMP)-dependent protein kinase regulatory (An16g03740) and catalytic (An02g04270) subunits were higher in the 0-h conidia.

The uptake of exogenous sugars (D-glucose and D-galactose) occurs shortly after 1 h, and it seems from the data that the rate and extent of uptake of D-glucose are higher than those of D-galactose. The simultaneous onset of uptake may imply that the transport of both sugars occurs using a shared transport system, which has a higher affinity for D-glucose than for D-galactose. The data therefore also suggest that there is competition between sugars for transport (this has been shown experimentally [data not shown]).

To conclude, we present evidence of a two-step germination process in *A. niger* conidia: a trigger step that precedes and is separate from the uptake and metabolism of certain external sugars that support the continued outgrowth of the spore. Recent studies have suggested that fermentative metabolism present at the early stages of spore germination is rapidly replaced by respiratory metabolism (25, 32). We speculate that it is possible that D-trehalose is utilized by fermentation and the external sugars are metabolized by respiration.

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