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Germination of *Aspergillus niger* Conidia Is Triggered by Nitrogen Compounds Related to L-Amino Acids

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Conidial germination is fundamentally important to the growth and dissemination of most fungi. It has been previously shown (K. Hayer, M. Stratford, and D. B. Archer, Appl. Environ. Microbiol. 79:6924–6931, 2013, http://dx.doi.org/10.1128/AEM.02061-13), using sugar analogs, that germination is a 2-stage process involving triggering of germination and then nutrient uptake for hyphal outgrowth. In the present study, we tested this 2-stage germination process using a series of nitrogen-containing compounds for the ability to trigger the breaking of dormancy of *Aspergillus niger* conidia and then to support the formation of hyphae by acting as nitrogen sources. Triggering and germination were also compared between *A. niger* and *Aspergillus nidulans* using 2-deoxy-D-glucose (trigger), D-galactose (nontrigger in *A. niger* but trigger in *A. nidulans*), and an N source (required in *A. niger* but not in *A. nidulans*). Although most of the nitrogen compounds studied served as nitrogen sources for growth, only some nitrogen compounds could trigger germination of *A. niger* conidia, and all were related to L-amino acids. Using L-amino acid analogs without either the amine or the carboxylic acid group revealed that both the amine and carboxylic acid groups were essential for an L-amino acid to serve as a trigger molecule. Generally, conidia were able to sense and recognize nitrogen compounds that fitted into a specific size range. There was no evidence of uptake of either triggering or nontriggering compounds over the first 90 min of *A. niger* conidial germination, suggesting that the germination trigger sensors are not located within the spore.

Asexual fungal spores (conidia) are designed for airborne dispersal and to germinate and grow on arrival in a suitable environment. Understanding the mechanisms of conidial germination is important, since the outgrowth of conidia plays a role in fungal dispersal and growth, food spoilage, and pathogenic fungal infections of plants and animals. Conidia break dormancy when conditions are favorable. Morphological changes characterize the germination as conidia swell isotropically and then develop germ tubes and hyphae in a polarized manner (1–3).

Nitrogen is an essential element for the synthesis of many macromolecules, including proteins, nucleic acids, and chitin, in the fungal cell (4). Thus, the availability in the environment of nitrogen for assimilation is crucial for the growth of fungi. Recent transcriptome studies in *Aspergillus niger* have indicated that the synthesis of proteins occurs at an early stage of conidial germination (5, 6) which supports earlier work in which Shepherd (7) reported that *Aspergillus nidulans* exhibits an increase in the nitrogen content of spores (within proteins) during the transition of resting spores into mycelium. Studies using inhibitors have also shown that protein synthesis is an essential metabolic process associated with the germination of fungal spores (8, 9).

Many types of molecules can serve as a source of nitrogen, e.g., ammonium ions, nitrate and nitrite ions, and L-amino acids. Nitrate ions are highly abundant in the soil (10), and L-amino acids are provided as a result of protein recycling. Ammonium and L-glutamate are thought to be the preferred nitrogen sources for fungi (4, 11, 12), and the two compounds can be readily interconverted by L-glutamate dehydrogenases (10). In the absence of the preferred nitrogen sources but in the presence of nitrate, a cluster of genes involved in the assimilation/utilization of nitrate are expressed through the activities of AreA and NirA, transcriptional nitrogen-responsive activators (13, 14). This enables nitrate ions to be intracellularly converted, via nitrite, into ammonium ions (4, 10). This process is well established in fungi, and the genetic regulation of nitrate assimilation is well reported (12, 13, 15–17).

Fungal conidia require nutrients to be present in their environment for germination to occur and for outgrowth into hyphae (18–20). Although studies have shed light on the biochemical requirements for spore germination, they were not done in a minimal medium and were therefore complicated by the inclusion of a known triggering compound, D-glucose. Germination of conidia occurred in a medium containing exogenous carbon and nitrogen sources, and it was shown (19, 20) that certain L-amino acids supported germination better than others, e.g., L-glutamic acid, L-proline, and L-alanine were better than L-leucine, L-arginine, and L-cysteine.

In a recent study (21), it was found that germination of *A. niger* conidia occurred in two distinct stages. First, germination was triggered, leading to spore swelling by specific sugar structures that were not necessarily related to the carbon sources required for growth. Second, the swollen spores grew out to hyphae in the presence of suitable carbon and nitrogen sources. A minimal medium containing water, salts, and D-glucose (or specific D-glucose analogs) and nitrate for the provision of carbon and nitrogen, respectively, enabled the full germination of *A. niger* conidia, i.e., the initial swelling followed by the outgrowth of hyphae (21). That

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study also reported that complex nitrogenous substrates, such as yeast extract or peptone alone in *Aspergillus* minimal medium (AMM), were able to trigger germination but that nitrate present in the AMM was not a trigger for germination. This raised interest in the role of nitrogenous compounds in the process of germination.

Here, we aimed to identify nitrogenous compounds that can serve as germination triggers.

MATERIALS AND METHODS

Substrates. All the substrates utilized, including the L-amino acid derivatives, were purchased from Sigma-Aldrich, with a few exceptions. Urea was from Fisher Scientific (Loughborough, United Kingdom); sodium nitrite was from FSA Laboratory Supplies (Loughborough, United Kingdom); Casamino Acids were from Becton Dickinson (BD) and Company (USA); and yeast extract, peptone, and tryptone were from Oxoid (Hampshire, United Kingdom). The purities of all the substrates were ≥98%, and ultrapure L-serine, L-cysteine, and L-valine were also assessed, as were D-serine and D-valine. Two sources of 2-deoxy-D-glucose were used, and both were analyzed by nuclear magnetic resonance (NMR) for possible contamination by D-glucose. One source showed no detectable D-glucose, and the other contained <0.5% D-glucose. Both sources behaved identically in the trigger tests over the range of dilutions examined, indicating that the results obtained were not due to contaminating D-glucose (data not shown). The stabilizing "BOC" (butoxycarbonyl) group of BOC-L-phenylalaninal was removed by incubation with trifluoroacetic acid in dichloromethane (1:1 [vol/vol]) for 1 h at 20°C. The solution was then rotary evaporated, and the residue was washed in toluene and dried.

The molecular structures of all the substrates are provided in Table S1 in the supplemental material (CS ChemOffice Ultra 2002, version 7.0 [CambridgeSoft]).

***Aspergillus* strains and experimental conditions.** Two strains of *A. niger* were used: strain N402 (22) and a wild-type (WT) isolate (T1) from a food factory. In addition, two strains of *A. nidulans* were used for some studies: the WT, Glasgow strain G00 (FGSCA4), and a WT isolate (T31) from a food factory. The identities of the factory isolates were confirmed by sequencing the D1/D2 region of the 26S rRNA genes. T1 was *A. niger* (99.8% NRRL 326; GenBank accession no. U28812), and T31 was *A. nidulans* (100% NRRL 187; GenBank accession no. EF652427). All studies were conducted with *A. niger* strain N402, except where indicated as being repeated with the other *A. niger* strain and the two strains of *A. nidulans*. Six-day-old conidia were harvested from 1% (wt/vol) D-glucose AMM agar slopes, as previously described (21), and used immediately. To identify which compounds could be used by *A. niger* as nitrogenous sources to support conidial outgrowth, the following basal medium was used: 100 ml AMM containing 100 mM D-galactose (as the nontriggering, but growth-sustaining, carbon source), 100 μM 2-deoxy-D-glucose (as the trigger compound), and 10 mM potential nitrogen source (inorganic and organic forms; see Table S1 in the supplemental material for a list and structures) or 0.1 g/liter complex nitrogenous sources (peptone, yeast extract, tryptone, and Casamino Acids). AMM here refers to a solution of salts and trace elements containing (per liter of water) 0.52 g (each) potassium chloride and magnesium sulfate, 1.52 g potassium dihydrogen phosphate, 0.008 mg sodium tetraborate decahydrate, 1.6 mg zinc sulfate, and 0.16 mg (each) of copper sulfate pentahydrate, ferric orthophosphate monohydrate, manganese sulfate tetrahydrate, and sodium molybdate dihydrate. To identify which compounds could be used to trigger germination, AMM containing a nontriggering nitrogen source (10 mM sodium nitrate), a nontriggering carbon source (100 mM D-galactose), and 100 μM each nitrogen compound was added, including derivatives of L-amino acids (see Table S1 for a list and structures). Triggering was determined by hyphal outgrowth detected at 14 h. D-Trehalose breakdown was used as a measure of triggering at 1 h. In instances where complex nitrogen sources were investigated, 0.1 g/liter was used. All conidial suspensions (10^6 conidia/ml) and experimental cultures were prepared as described by Hayer et al. (21), with all cultures shaken at 150 rpm and incubated at 28°C.

Microscopy. The development of *A. niger* conidia was visualized using a GX microscope (Suffolk, United Kingdom) equipped with a GT Vision camera (Suffolk, United Kingdom). A 40× objective lens and a final magnification of ×400 were used. Microscope images were viewed and recorded using GXCapture version 7.3 software (Suffolk, United Kingdom), generally after either 14 h or 24 h of incubation at 28°C. Note that 14 h was sufficient time for all morphological markers to be established, but 24-h samples were also examined in case morphological changes were slow under some conditions. In all cases, the morphological states were identical. About 80% of the spores were in small clumps (4 to 25 spores), and 20% of the spores were not in clumps at these times. The clumped spores could be distinguished by microscopy and scored for morphology. A minimum of 100 spores for each sample were recorded, and their dimensions were measured to distinguish unswollen (dormant) from swollen spores and to detect the appearance of germ tubes. All samples were harvested from culture medium as previously described, although the pellets were resuspended in 1 ml AMM salt and trace element solution; 5 μl of each sample was then used for microscopy (21).

Flow cytometry. The swelling of 1×10^5 *A. niger* conidia at 1 h of incubation with either L-alanine or L-serine at different concentrations ranging from 0 to 10 mM was analyzed using a BD LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 488-nm laser for the detection of forward scatter (FSC), a measure of cell size. Samples were prepared and analyzed using BD FACSDiva software version 6.1.3 (BD Biosciences) as described previously (21).

Analysis of D-trehalose. The degradation of D-trehalose is an important process that occurs early during conidial germination (23, 24). Cytosolic extracts from dormant (0-h) and germinating (1-h) conidia were obtained as described by Novodvorska et al. (5), and the level of D-trehalose was assayed using a commercial kit (Megazyme International Ireland Ltd.). The protocol used was specified in the manual provided. The amounts of NADPH released in the assays were measured at 340 nm using an Eppendorf BioSpectrophotometer (Scientific Laboratory Supplies, Hamburg, Germany). A standard curve was carried out for the polyol using the standard provided with the kit (the optical density at 340 nm [OD_{340}] against the polyol concentration). The amount of D-trehalose in each sample was determined in duplicate samples.

L-[U-^{14}C]serine and L-[U-^{14}C]alanine uptake experiments. The uptake medium consisted of 15 ml of AMM containing 100 mM D-galactose, 10 mM sodium nitrate, 100 μM L-serine or L-alanine, and 10 μCi of L-[U-^{14}C]serine or L-[U-^{14}C]alanine (PerkinElmer, United Kingdom). Uptake studies were conducted as previously described by Hayer et al. (21), except that the filters were prewashed (5 ml) and postwashed (3 times with 5 ml each time) with the relevant uptake medium containing no ^{14}C. Hyamine hydroxide was also sampled to determine the amount of $^{14}CO_2$ produced, and the experiments were conducted in duplicate.

RESULTS

Defining the conditions for germination in *A. niger*. It was previously shown that the triggering of conidial germination in *A. niger* was induced by specific D-pyranose sugar structures. However, it was observed that germination could also be triggered by certain complex nitrogen components in a minimal medium lacking a pyranose trigger. Microscopy experiments were therefore carried out to confirm that AMM containing D-galactose and sodium nitrate alone or together could not trigger germination. The results showed that if present together, these compounds were able to support the germination of conidia into hyphae at 14 h and 24 h of incubation only if another compound, such as 2-deoxy-D-glucose, initially triggered the conidia to germinate (Table 1). Table 1 also confirms that 2-deoxy-D-glucose was a sufficient trigger for germination of *A. niger* conidia (but not a carbon source for

TABLE 1 Compositions of culture media used and conidial morphologies obtained by microscopy at 14 and 24 h[a]

Culture medium	Morphological outcome at 14 h and 24 h	
	A. niger	*A. nidulans*
AMM + sodium nitrate	Dormant	Dormant
AMM + D-galactose	Dormant	Germination
AMM + sodium nitrate + D-galactose	Dormant	Germination
AMM + D-galactose + 2-deoxy-D-glucose + sodium nitrate	Germination	Germination
AMM + sodium nitrate + 2-deoxy-D-glucose	Swelling	Swelling
AMM + D-galactose + 2-deoxy-D-glucose	Swelling	Germination
Sterilized distilled water + D-galactose + sodium nitrate + 2-deoxy-D-glucose	Germination	Germination

[a] The two strains of *A. niger* gave identical results as a pair, as did the two strains of *A. nidulans*. In all cases, dormant spores were recorded as 100% unswollen. Germination (into hyphae) and swelling were all >93%, except for swelling of *A. nidulans* conidia in AMM plus sodium nitrate plus 2-deoxy-D-glucose (70%).

growth), enabling conidial swelling at 14 h and 24 h of incubation only in the presence of sodium nitrate or D-galactose AMM. Furthermore, hyphal outgrowth after triggering did not occur in the presence of D-galactose but in the absence of sodium nitrate, presumably because there was no nitrogen source available, even though D-galactose was present as a carbon source. The AMM salt and trace element solution was also replaced by sterilized distilled water, and germination into hyphae occurred, suggesting that the salts/trace elements present in AMM were not essential for the germination of conidia (Table 1). The study summarized in Table 1 was repeated with a WT isolate of *A. niger*, and the results were identical to those with *A. niger* strain N402. Furthermore, for comparison, the study was repeated with two strains of *A. nidulans*. The two strains showed responses identical to each other, but there was divergence from the *A. niger* data, showing that fungal species do not respond identically to conditions for spore germination. In contrast to the lack of trigger response to galactose observed with *A. niger* conidia, galactose appeared to serve as both a trigger and a carbon source in *A. nidulans*. Also, *A. nidulans* conidia were able to trigger and germinate in the presence of galactose without nitrate as a nitrogen source. 2-Deoxy-D-glucose served as a swelling trigger in both *A. niger* and *A. nidulans*.

The results in Table 1, therefore, suggest that for full germination of *A. niger* conidia into hyphae to occur, three suitable compounds need to be present in the environment: a nitrogen source, a carbon source, and a germination trigger.

Based on the preliminary results (Table 1), nitrogen compounds that would enable the triggering of germination of *A. niger* conidia were tested in a medium containing AMM salt and trace element solution, D-galactose (a nontriggering carbon source), sodium nitrate (a nitrogen source), and a range of different compounds replacing 2-deoxy-D-glucose as a potential trigger. Initial studies using microscopy showed that L-serine at 1 mM was able to induce conidial outgrowth at 14 h but 1 mM L-alanine could not. This indicated that not all L-amino acids were able to trigger germination, possibly influenced by the concentration. The concentration required to trigger conidial germination was therefore tested at 1 h of incubation using flow cytometry, and L-serine and L-alanine were examined over a range of concentrations, 0 mM to 10 mM. The flow cytometry results showed that *A. niger* conidia in the presence of L-alanine did not swell at any concentration, as there was no increase in the FSC parameter compared to dormant conidia (control at 0 h). This response contrasted with the effect of L-serine, which led to a progressive increase in conidial swelling over a range of concentrations starting from 100 nM (Fig. 1). Visualizing conidia by microscopy at 14 h also confirmed these results showing that the swelling of conidia was initiated at 100 nM in the presence of L-serine and that outgrowth of hyphae from swollen spores occurred with concentrations as low as 10 μM.

Nitrogen compounds that can induce the triggering of germination. A summary of the results obtained from microscopy (Table 2) identifies some compounds that were able to trigger germination. This was detected by hyphal outgrowth from swollen spores (i.e., already triggered) (Fig. 2B) in media containing nontriggering nitrogen and carbon sources at 14 h and 24 h (Fig. 2C). They included 14 out of 20 proteinogenic L-amino acids (L-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glutamine, L-isoleucine, L-leucine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine) and L-ornithine, as well as complex nutrient mixtures that contained nitrogen compounds (yeast extract, peptone, tryptone, and Casamino Acids). Nontriggering nitrogen compounds included all the inorganic nitrogen compounds and urea, L-alanine, L-arginine, L-glycine, L-histidine, L-lysine, and L-methionine. *A. niger* conidia remained dormant when these compounds were added to the triggering medium (Table 2 and Fig. 2A; see Table S1 in the supplemental material for structures).

Assaying germination triggering by D-trehalose breakdown. D-Trehalose is present in dormant spores as a storage compound

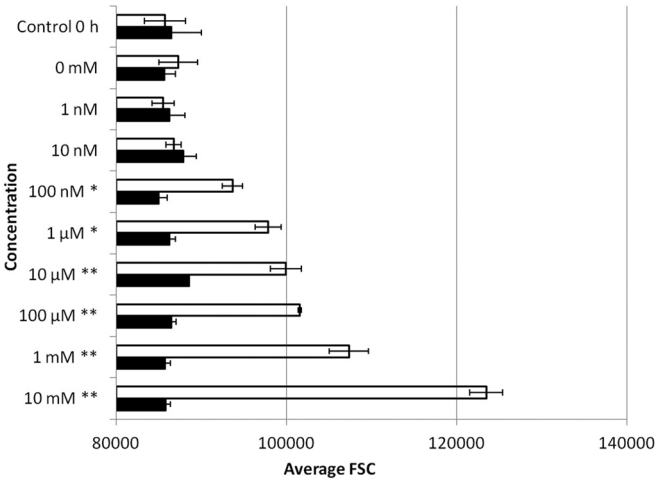

FIG 1 Conidial swelling tested using flow cytometry (average FSC, a measure of conidial size within the population). Control 0 h represents dormant conidia, and the bars represent *A. niger* conidia germinating in the presence of L-serine (triggering L-amino acid; white bars) or L-alanine (nontriggering L-amino acid; black bars) for 1 h at 28°C over a range of concentrations, 0 to 10 mM. Standard deviations for the duplicate samples are shown. *, L-serine concentrations that induced conidial swelling only; **, L-serine concentrations that induced hypha formation as revealed by microscopy at 14 h (images not shown). *t* tests comparing the sizes of dormant (0-h) conidia with those of conidia at 1 h at all the different concentrations of L-alanine showed no significant differences ($P = 0.56$ to 0.99). *t* tests comparing the sizes of dormant conidia with those of conidia at 1 h at L-serine concentrations of 0 mM to 10 mM also showed no significant differences ($P = 0.58$ to 0.91), while the sizes of 1-h swollen conidia were shown to be significantly different from those of 0-h conidia using L-serine concentrations of 100 nM to 10 mM ($P = 0.004$ to 0.04).

TABLE 2 Summary of results obtained by microscopy at 14 h and 24 h[a]

Nitrogen compound	Germination triggering[b]
Sodium nitrate	No
Sodium nitrite	No
Urea	No
Ammonium sulfate	No
L-Alanine	No
L-Arginine	No
L-Asparagine	Yes
L-Aspartic acid	Yes
L-Cysteine	Yes
L-Glutamic acid	Yes
L-Glutamine	Yes
L-Glycine	No
L-Histidine	No
L-Isoleucine	Yes
L-Leucine	Yes
L-Lysine	No
L-Methionine	No
L-Phenylalanine	Yes
L-Proline	Yes
L-Serine	Yes
L-Threonine	Yes
L-Tryptophan	Yes
L-Tyrosine	Yes
L-Valine	Yes
L-Ornithine	Yes
Peptone	Yes
Casamino Acids	Yes
Tryptone	Yes
Yeast extract	Yes
AMM (control)	No

[a] Both time points gave the same result. *A. niger* conidia were incubated with different nitrogen compounds potentially serving as triggers added to the medium containing nontriggering carbon and nitrogen sources (D-galactose and sodium nitrate respectively). Conidia developing in AMM (salt solution and trace elements) were used as a control.

[b] "Yes" indicates triggering of germination (90 to 96%), and "No" indicates that spores remained dormant (all 100% dormant) and outgrowth into hyphae from swollen spores did not occur.

and is known to be degraded upon spore germination (23, 24). The levels of D-trehalose in dormant spores (0 h) and germinating spores at 1 h were followed in the presence of the different potential triggering compounds, and D-trehalose levels were related to the microscopic appearance of the conidia (Fig. 3). Dormant conidia and 1-h conidia in the presence of nontriggering compounds had constantly maintained D-trehalose levels of ~100 to 123 pmol/100,000 conidia, while germinating 1-h conidia in the presence of triggering compounds had reduced D-trehalose contents of ~60 to 80 pmol/100,000 conidia. Figure 3 confirms that D-trehalose levels are a good marker for germination. It can also be observed from Fig. 3 that the compounds L-valine, L-isoleucine, and L-leucine, which triggered germination but were found not to be adequate nitrogen sources (see below), led to less degradation of D-trehalose and also had a smaller degree of conidial swelling in an hour than the other compounds that triggered conidial germination.

Generally, the compounds tested were described as having a purity of 98% to 99%, which raises the possibility that there may have been a small amount of contamination that was responsible for the responses observed. However, to minimize this possibility, the concentration of trigger was reduced to a low level, 100 μM, and as a control, ultrapure versions of L-serine, L-valine, and L-cysteine were also tested and found to support the same outcomes (data not shown).

Time course experiments were also set up in which the D-trehalose levels were checked over a longer time scale (up to 4 h) during conidial germination in the presence of L-serine and L-alanine (Fig. 4). The data confirm that L-alanine did not trigger even when the conidia were left to incubate longer, while L-serine triggered the early degradation of D-trehalose followed by its resynthesis between 3 and 4 h of germination.

Investigating the structure of the trigger. (i) Possible roles of amino and carboxyl groups. In order to determine the core structural features of the nitrogen compounds that can serve as triggers, a range of substrates were assessed to analyze the structures of those compounds recognized as triggers by the conidia. D-Trehalose degradation and microscopy assays at 1 h and 14 h, respectively, were used. The first question asked was whether either or both of the amine (NH_2) and carboxylic acid (COOH) groups were required for the triggering of germination. Therefore, selected L-amino acids—L-alanine, L-serine, L-threonine, L-leucine, and L-glycine—lacking either functional group were studied (Table 3; see Table S1 in the supplemental material for structures).

The microscopic and D-trehalose screens showed that in all cases (the compounds in Table 3) the conidia remained dormant at 14 h and did not have a reduction in D-trehalose content at 1 h (negative assay results), suggesting that none of the compounds were recognized as triggers for germination (data not shown). The fact that L-serine, L-leucine, and L-threonine each served as a trigger (Table 2 and Fig. 3) suggests that it is essential for a nitrogen compound to have both the NH_2 and COOH groups present in its structure in order to serve as a trigger. To test that supposition, studies were undertaken utilizing the two L-serine derivatives 2-amino-1-propanol and L-serine methyl ester, in which the COOH group was absent and replaced with a methyl group or the methyl group formed an ester in the structure (Table 3; see Table S1 in the supplemental material). In those examples, there were no visible signs of conidial germination or D-trehalose breakdown. However, replacing the carboxylic acid group with either an alcohol or an aldehyde (serinol or L-phenylalaninal) (see Table S1 in the supplemental material for structures) showed that both compounds functioned as germination triggers.

(ii) Proximity of the amino and carboxyl groups. Having established that the NH_2 and COOH groups are important (even if the carboxyl group was not essential in all cases [see above]), we then asked whether the two functional groups need to be on the first and second carbon, respectively, as is the case with naturally

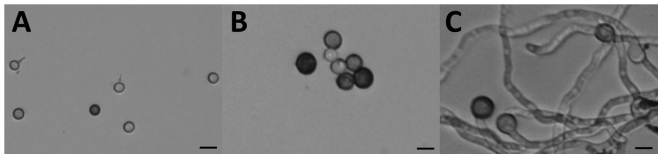

FIG 2 Representative microscope images (scale bars, 10 μm) of dormant conidia (nontriggering L-alanine in the presence of sodium nitrate) (A), swollen conidia (triggering L-serine but lacking other nitrogen sources) (B), and germinated cells (triggering L-serine in the presence of sodium nitrate) (C) at 14 h.

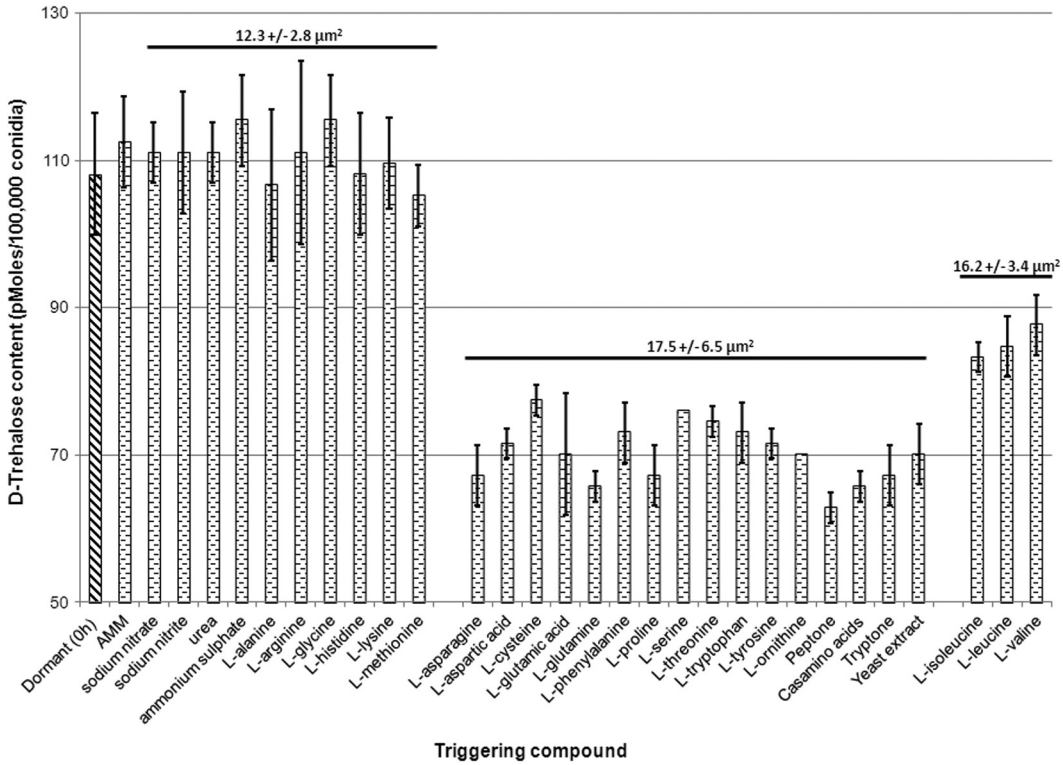

FIG 3 D-Trehalose levels in dormant (0-h) *A. niger* conidia (hatched bar) and at 1 h of conidial germination in the presence of AMM (salt solution and trace elements) and a range of potential trigger compounds at 100 μM. The results are grouped as either nontriggering compounds (e.g., L-alanine) (left), triggering compounds (e.g., L-serine) (middle), or triggering-only compounds (e.g., L-leucine) (right). *t* tests comparing 0-h and 1-h conidia showed no significant differences for nontriggering compounds ($P = 0.42$ to 1) but yielded significant differences for all the triggering compounds ($P = 0.01$ to 0.04). The average areas of 100 conidia determined at 1 h from representative microscope images of dormant spores (L-alanine) and swelling spores (L-serine and L-leucine) are also shown above the bars. The error bars indicate standard deviations.

occurring proteinogenic L-amino acids. Thus, in order to test this, the germination responses of *A. niger* conidia to DL-β-phenylalanine or DL-β-leucine were studied. These molecules are derivatives of two L-amino acids known to trigger germination (Table 2 and Fig. 3), but in the β conformation, which means that the amine group is shifted from the second carbon to the third carbon (see Table S1 in the supplemental material for structures). As with the previous compounds studied (Table 3), the results showed no triggering by β-amino acids (data not shown). The D-trehalose content was in the range of ~104 to 108 pmol/100,000 conidia,

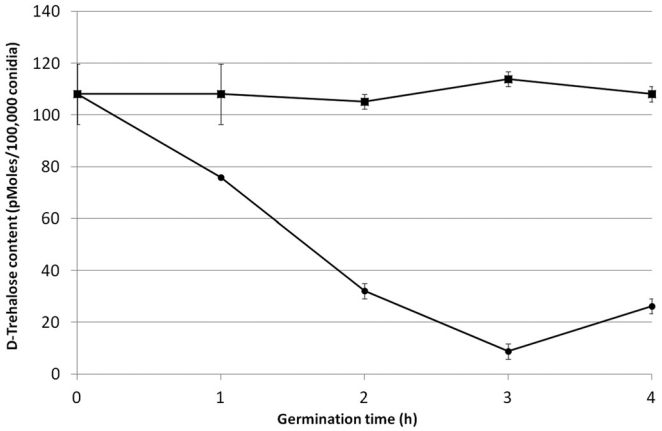

FIG 4 D-Trehalose levels in *A. niger* conidia over a longer course of germination (0 to 4 h). The level of conidial D-trehalose (found in dormant spores [0 h]) does not change in the presence of L-alanine (nontriggering L-amino acid) (squares), while the level of D-trehalose is initially reduced over the first 3 h and then resynthesized between 3 and 4 h in the presence of L-serine (triggering L-amino acid) (circles). The means and standard deviations for duplicate samples are shown.

TABLE 3 Compounds tested to identify whether the NH_2 and COOH groups are essential for triggering of conidial germination

Compound[a]	Variation from natural L-amino acid structure[b]
Methylamine	L-Glycine without COOH
Ethylamine	L-Alanine without COOH
Propionic acid	L-Alanine without NH_2 group
3-Hydroxypropionic acid	L-Serine without NH_2 group
Ethanolamine	L-Serine without COOH group
2-Amino-1-propanol	L-Serine with COOH group replaced by CH_3 (methyl) group
L-Serine methyl ester	L-Serine with CH_3 on COOH group forming ester
3-Hydroxybutryic acid	L-Threonine without NH_2 group
2-Hydroxypropyl-amine	L-Threonine without COOH group
4-Methyl-pentanoic acid	L-Leucine without NH_2 group
3-Methyl-butylamine	L-Leucine without COOH group

[a] None of the compounds served as germination triggers.
[b] How the compound differs from its natural L-amino acid (L-alanine, L-serine, L-threonine, L-leucine, and L-glycine) structure.

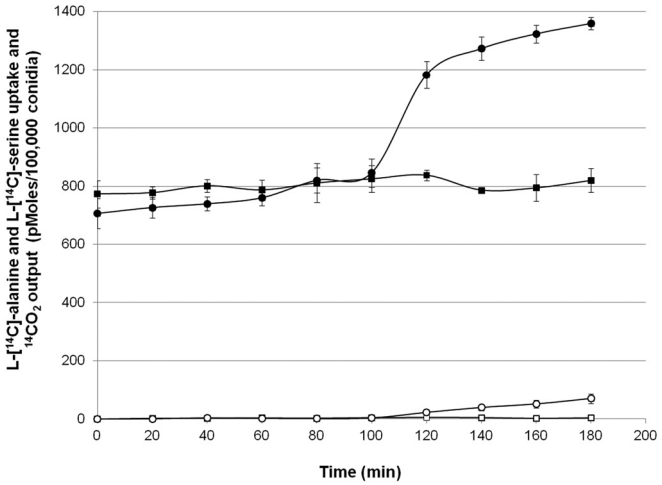

FIG 5 Uptake of L-[^{14}C]alanine (black squares) and L-[^{14}C]serine (black circles), together with the $^{14}CO_2$ ouput (white squares, L-alanine; white circles, L-serine) from 100,000 *A. niger* spores taken every 20 min over a 180-min course of germination. The means and standard deviations for duplicate samples are shown.

levels similar to those of the nontriggering L-amino acids identified in Fig. 3. Also, the conidia remained dormant, as represented in Fig. 2A, emphasizing the requirement for NH_2 and COOH to be positioned on adjacent carbons. Neither D-serine nor D-valine, amino acids in the D form, could trigger germination (data not shown), indicating that proximity in the correct orientation in space is important.

(iii) Length of the R group in amino acids. The final question asked was whether the length of the side chain was important to the structure of the trigger. Initial results suggested that size is a possible factor in determining whether a molecule could serve as a trigger for conidial germination. L-Glycine, L-alanine (both very short L-amino acids), and L-lysine (an L-amino acid possessing a long side chain) did not trigger germination, yet L-ornithine did (Table 2 and Fig. 3), and its structure is only one carbon shorter than that of L-lysine (see Table S1 in the supplemental material for structures). Therefore, a series of L-amino acid derivatives that differed in the lengths of their side chains, based on the number of carbon atoms, were studied. They included 2-aminooctanoic acid, 2-aminoheptanoic acid, 2-aminohexanoic acid, 2-aminopentanoic acid, and 2-aminobutyric acid compounds (see Table S1 in the supplemental material for structures). Germination of conidia and D-trehalose breakdown could be induced only by the last three compounds; those substrates with 4 to 6 carbons in their structures triggered germination, but not bigger (7 or 8 carbons) or smaller (2 or 3 carbons; L-glycine and L-alanine) substrates. Thus, it seems that there is a particular size range that compounds require in order to effectively induce germination.

Uptake of radiolabeled L-amino acids. The uptake of L-serine and L-alanine was followed over a course of 3 h, with samples taken every 20 min. The formation of $^{14}CO_2$ was also recorded (Fig. 5). The data in Fig. 5 show that the triggering compound, L-serine, was rapidly taken up after 100 min, while the nontriggering compound, L-alanine, was not taken up, even at 180 min (3 h). However, L-alanine could be taken up (again after 100 min) if a triggering compound, e.g., L-serine, was also present in the medium (see Fig. S1 in the supplemental material). The timing of the uptake was reinforced by the increased $^{14}CO_2$ output detected.

Nitrogen compounds that can act as nitrogen sources to support growth. As a result of the preliminary findings (Table 1) discussed above, nitrogen sources that would support the growth of *A. niger* were tested in a medium containing AMM salt and trace element solution, D-galactose (carbon source), and 2-deoxy-D-glucose (triggering compound), using a range of compounds as potential nitrogen sources. Microscopy was used to determine the morphology of conidia after 14 h and 24 h of incubation. It was found that the inorganic forms of nitrogen, sodium nitrate, sodium nitrite, and ammonium sulfate, as well as a range of organic nitrogen-containing compounds, including urea and most L-amino acids, were sufficient sources of nitrogen for growth. Thus, in the presence of urea, L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glutamine, L-glycine, L-histidine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, or L-ornithine and inorganic nitrogen compounds, the conidia were microscopically visualized to be germinating into hyphae at 14 h and 24 h. Complex nitrogenous nutrient sources, such as yeast extract, peptone, tryptone, and Casamino Acids, also supported mycelial growth. However, in the presence of L-valine, L-isoleucine, or L-leucine as the sole nitrogen source, conidia swelled but did not grow out into hyphae at 14 h or 24 h (data not shown).

DISCUSSION

Nitrogen metabolism is required for the synthesis of nucleic acids and proteins, processes that are known to occur early during the course of conidial germination (2, 8, 9). Germination responses to different L-amino acids may vary (19, 20), and that is confirmed here, but by using a minimal medium, it was possible to categorize the different nitrogen compounds into distinct groups: nontriggering compounds that acted as nitrogen sources and triggering compounds that did or did not act as nitrogen sources for germination. A summary of the data is presented in Table 4.

The use of different nitrogen compounds as the sole nitrogen source showed that inorganic and complex nitrogen compounds, as well as most L-amino acids, supported the outgrowth of *A. niger* conidia into hyphae. This observation was not unexpected, because it is well known that fungi utilize a broad range of nitrogen

TABLE 4 Division of nitrogen compounds into 3 different categories based on their effects on germination of *A. niger* conidia

Category	Nitrogen compounds
Nontriggering compounds that serve as nitrogen sources to support subsequent growth	Sodium nitrate, sodium nitrite, urea, ammonium sulfate, L-alanine, L-arginine, L-glycine, L-histidine, L-lysine, L-methionine
Triggering compounds that also support subsequent growth	L-Aspartic acid, L-asparagine, L-cysteine, L-glutamic acid, L-glutamine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-ornithine, peptone, Casamino Acids, tryptone, yeast extract
Triggering compounds that do not serve as nitrogen sources for subsequent growth	L-Isoleucine, L-leucine, L-valine

sources, among them ammonium, nitrate, and L-amino acids (25). Metabolism of nitrate, nitrite, and urea yields ammonium, which can be incorporated into the amino acid L-glutamic acid. Nitrogen from L-amino acids can also be converted into L-glutamic acid (26). This is especially true for the L-amino acids in the "glutamate family," L-arginine, L-ornithine, L-histidine, L-proline, and L-glutamine, all of which are typically converted to L-glutamic acid for their metabolism (12, 26). The cell wall component chitin, which contains both nitrogen and carbon, increases in amount during the isotropic expansion of conidia (6, 27). L-Valine, L-isoleucine and L-leucine did not act as nitrogen sources for growth. This unexpected result may relate only to this strain of *A. niger* or possibly to the species in general. Repeated experiments confirmed that these three L-amino acids did trigger germination but did not act as either carbon or nitrogen sources.

The initiation of germination of *A. niger* conidia requires a molecular trigger that breaks dormancy. A minimal medium containing water, salts, trace elements (AMM), D-galactose and sodium nitrate could not trigger the breaking of conidial dormancy. Some sugars having specifically shaped pyranose structures (21) can serve as triggers, and it was shown here that some L-amino acids can also be triggers. The minimum concentration of triggering sugars (10 nM) (21) is 10-fold lower than the concentration of triggering nitrogen compounds (100 nM) (Fig. 1) required to facilitate the initial swelling of spores at 1 h of germination. However, the concentrations of sugar and nitrogen compounds required to support the subsequent formation of germ tubes were similar (10 μM). Triggering of germination leads to the breakdown of D-trehalose and resynthesis later, and those events occur more quickly in the presence of triggering sugars than with L-amino acids. In the presence of L-serine, D-trehalose was gradually degraded over the course of 0 to 3 h and then resynthesized later (Fig. 4), whereas conidia in the presence of D-glucose degraded D-trehalose at a higher rate, depleting the store at 2 h of germination before replenishing the internal compound (data not shown). The mechanisms for sensing trigger compounds and signaling their presence are not wholly understood. G-protein-coupled receptors (GPCRs) can sense a range of nutritional compounds and can act to propagate extracellular signals into intracellular responses, e.g., germination (28). In *A. nidulans* the putative carbon sensor GPCRs are distinct from the nitrogen-sensing GPCRs (29). Lafon et al. (28) demonstrated a link between GPCR signaling and D-trehalose breakdown through the activation of neutral trehalase (the enzyme responsible for the catabolism of D-trehalose) (24). Deletions of individual G-protein subunits resulted in defective D-trehalose catabolism; the *ganB* deletion strain of *A. nidulans* showed a reduction in the rate of D-trehalose degradation, and Chang et al. (30) found that conidia from a *ganB* deletion strain of *A. nidulans* exhibited a reduced rate of swelling and germ tube formation. It is therefore likely that there is more than one triggering sensor on the surface of the conidium that recognizes carbon and nitrogen compounds separately to initiate germination by coupling the signal to D-trehalose breakdown. Both D-trehalose (substrate) and trehalase (enzyme) are found in dormant conidia. Horikoshi and Ikeda (31) showed that the activity of trehalase increases while the content of D-trehalose decreases during germination of *Aspergillus oryzae* conidia, while recent transcriptome data from *A. niger* (5) have determined that transcripts encoding enzymes involved in the metabolism of D-trehalose are also enriched in dormant conidia compared to germinants at 1 h. This suggests that the signal transduction from an external nutrient to D-trehalose breakdown could involve the rapid onset of translation of transcripts to synthesize trehalase or that the protein becomes activated by the GPCR-cyclic AMP (cAMP)-protein kinase A (PKA) pathway (28).

The structures of the nitrogen compounds that trigger germination of *A. niger* conidia were studied in detail using a range of L-amino acid derivatives, and the structures that a nitrogen compound can adopt in order to trigger the morphological and biochemical changes associated with germination were summarized. Small L-amino acids, such as L-glycine, and other small forms of nitrogen (urea, ammonium, nitrate, and nitrite) or large L-amino acids, such as L-arginine, did not trigger conidial germination, indicating that size is an important feature in determining whether a compound can serve as a trigger. The L-amino acids that were found to trigger all showed limited variation in size and shape, although non-size-based factors may also have roles. Note also that a nitrogen source, e.g., nitrate, was essential for the development of *A. niger* conidia beyond swelling to germination into hyphae. In contrast, *A. nidulans* conidia did not require nitrate for germination provided triggering had occurred, a finding previously published (9). The difference between *A. niger* and *A. nidulans* is not explained but probably reflects differences in nitrogen availability from stores within the conidia.

L-Proline was identified as a triggering compound, and it is commonly found in the environment. It is produced in large quantities in plants and reaches the soil following their decay, which suggests that the fungus has adapted mechanisms to utilize an abundant source of nitrogen (32). Two L-amino acids that did not trigger germination, L-histidine and L-methionine, both had sizes that conformed to the range of most trigger L-amino acids. This may indicate that the triggering process is more complex than has been described. It could be, for example, that the position of the —NH group (which is a difference between the ring structures of L-histidine and L-tryptophan) or the position and the interaction of the sulfur group of the L-amino acids L-methionine and L-cysteine (nontriggering and triggering compounds, respectively) with the receptor binding site also contribute to the ability of the triggering sensor to recognize certain structures.

The fact that uptake of L-serine occurred after 100 min suggests that the dormant conidia contain a pool of free L-amino acids that can be utilized for the very early stages of germination (7). Recent transcriptome data from *A. niger* conidia at the breaking of dormancy (0 to 1 h) highlighted the possibility that the proteasome may participate in the degradation of proteins that are possibly either damaged or no longer required, to yield a pool of L-amino acids that can serve as a free pool of building blocks for new proteins or as sources of carbon and nitrogen (5). Fungal vacuoles also have a store of L-amino acids, and such organelles have been found in dormant *A. oryzae* conidia as small, punctate vacuoles, and their morphology changes in parallel with conidial swelling, i.e., vacuoles enlarge as the conidium enlarges (33). Vacuolar proteinases can be induced by nitrogen starvation to make peptides accessible for primary metabolism (34). Thus, there are mechanisms available for the fungus to yield L-amino acids from internal stores before the onset of L-amino acid metabolism using external nitrogen sources.

The triggering sensor is probably external (as was speculated for the carbon source triggering sensor previously [21]), because detectable uptake of L-amino acids occurred after 1 h (Fig. 5; see

Fig. S1 in the supplemental material) while the swelling of conidia and D-trehalose breakdown occurred earlier, within the first hour (Fig. 3). The detection of $^{14}CO_2$ from the uptake studies with radiolabeled L-amino acids suggests that L-amino acids were also being metabolized as a carbon source.

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