Nonanoic Acid, a Fungal Self-Inhibitor, Prevents Germination of *Rhizopus oligosporus* Sporangiospores by Dissipation of the pH Gradient

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Germination of *Rhizopus oligosporus* sporangiospores is characterized by swelling of the spores and subsequent emergence of germ tubes. Changes in spore morphology and alterations in intracellular pH (pHi) of the sporangiospores were assessed during the germination process by flow cytometry. Sporangiospores were stained with carboxyfluorescein by incubation with carboxyfluorescein diacetate. The nonfluorescent carboxyfluorescein diacetate is passively transported into intact cells and subsequently cleaved by esterases, which results in intracellular accumulation of the fluorescent carboxyfluorescein. Given that the fluorescence of carboxyfluorescein is pH dependent, the pHi of the individual spores could be assessed simultaneously with spore size. For *R. oligosporus*, swelling of the sporangiospores was accompanied by an increase of pHi. In the presence of nonanoic acid, a self-inhibitor produced by various fungi, increase of the pHi was prevented and swelling was inhibited at concentrations of less than 1 mM. Octanoic acid and decanoic acid were equally effective. Acetic acid also inhibited germination but at much higher concentrations (>8 mM). The mechanism of action of these fatty acids is most likely dissipation of the pH gradient. A model is proposed in which the pHi plays a crucial role in the germination of *R. oligosporus* sporangiospores.

The development of fungal sporables can be arbitrarily divided into several stages: formation, maturation, dormancy, after-ripening, activation, and germination (11). Dormancy is a common strategy among fungi and bacteria to survive unfavorable external conditions. The morphology and physiology of dormant cells in nature are extremely diverse. To become germinable after dormancy, many (fungal) spores require an after-ripening period (e.g., a cold period) and/or an activation treatment. This treatment may include thermal activation, chemical activation (detergents, organic acids, and amino acids, etc.), or light activation (5, 11, 21). Generally, germination of fungal spores is characterized by swelling of the spores and the formation of germ tubes, accompanied by an increase in the respiratory rate and DNA, RNA, and protein synthesis (5, 11). Furthermore, the nutritional requirements for germination vary considerably; e.g., *Aspergillus* spores require only CO2, whereas *Rhizopus* spores need a carbon and nitrogen source (6, 11, 17).

A typical means of controlling germination in fungi, bacteria, and plants is self-inhibition. It has been previously observed that many fungal germinate poorly at high spore concentrations (crowding effect), and this suggested the presence of some inhibitory substance(s) produced by the organism (9, 16). Various self-inhibitors, such as nonanoic acid, 5-isobutyroxy-β-ionone, vanillic acid, and the cinnamic esters 3,4-dimethoxy-cinnamic acid methyl ester and methyl cis-ferulate have been identified after extraction from culture filtrates of fungi (9, 14, 16). Self-inhibition has the obvious advantage of preventing rapid germination of all spores at the same time and place, which ensures survival in fluctuating environmental conditions and stimulates dissemination in nature (16, 21). To date, the mechanism of action of self-inhibitors is not well understood. It is known that the cinnamic esters inhibit germination at the initiation of germ tube emergence, and it has been suggested that cinnamic esters may be preformed and remain bound to the cell surface (16). Nonanoic acid, on the contrary, is known to prevent swelling of the spores (14). Moreover, nonanoic acid appeared to be widely produced among fungi such as *Aspergillus niger*, *Syncephalastrum racemosus*, *Geotrichum candidum*, *Penicillium expansum*, *Rhizopus stolonifer*, and *Mucor plumbeus* (9, 14).

The criterion most frequently used to measure germination is emergence of the germ tubes, and traditionally, this has been determined microscopically. In this study, we have applied flow cytometry to monitor the germination process. In this system, particles pass one by one in a fluid stream through a measuring apparatus, which simultaneously analyzes scattered and fluorescence light from the particles. In combination with suitable fluorescent probes, this offers the possibility of determining various cellular parameters such as size, enzymatic activity, and intracellular pH.

In this work, the germination of *Rhizopus oligosporus* sporangiospores and the mechanism of action of nonanoic acid were investigated. Germination of *R. oligosporus* spores has previously been shown to be optimal at 37°C and at pH 4 and was stimulated by glucose and L-alanine (6, 17), but the physiological processes which take place during the germination process are not understood. We present evidence that intracellular pH (pHi) increase is associated with swelling and germination of *R. oligosporus* sporangiospores and that inhibition of germination by nonanoic acid is mediated by the capacity of this substance to dissipate the pH gradient.
MATERIALS AND METHODS

Organisms and growth conditions. *R. oligosporus* NRRL 5905 was grown for 5 to 7 days on malt extract agar slants at 30°C. Sporangiospore suspensions were obtained by adding 9 ml of dilution medium (0.5 g of NaCl per ml with 1 g of neutralized bacteriological peptone [Oxoid Ltd.] per ml) to the agar slant and vortexing the tube thoroughly. The spore suspensions were subsequently filtered through a 30-μm-pore-size nylon filter to remove mycelium and other debris and used immediately.

Fluorescence staining of spore suspensions. The *R. oligosporus* spore suspensions were washed twice by centrifugation in an Eppendorf centrifuge in Czapek and DOX synthetic medium for germination (CDM; Oxoid Ltd.), adjusted to pH 4.0 with lactic acid (final concentration, less than 0.05 mM). Subsequently, the suspension was incubated for 20 min in the presence of 5- and (6)-carboxyfluorescein diacetate (cFDA; 0.22 mM) at 40°C, washed twice with CDM, and resuspended in malt extract broth adjusted to pH 4.0 with lactic acid (unless indicated otherwise).

Determination of germination. Germination of *R. oligosporus* spores was determined microscopically and by flow cytometry at 1-h intervals. For microscopic analysis, at least 140 spores were counted at random, and germination was defined as the extension of a germ tube to one-half the diameter of the spore (17). The spore suspensions contained approximately 10^6 spores/ml. Samples for flow cytometric analysis were prepared by taking 100-μl aliquots, which were diluted into 2 ml of CDM (adjusted to pH 4.0 with lactic acid). The germination was evaluated at 37°C in malt extract broth in the absence and presence of various concentrations of nonanoic acid (the pH was readjusted to pH 4 with sodium hydroxide).

Flow cytometric analysis. Analysis of individual spores was performed with a PAS-II flow cytometer (Partec GMBH, Münster, Germany), equipped with an air-cooled argon ion laser (excitation wavelength, 488 nm), which was operated at 15 mW. The instrument was set up to collect four parameters, namely, scattered light (488 nm) at low and wide angles and fluorescent light at emission wavelengths of 515 and 560 nm. Low-angle light scatter (forward scatter) was used as an indicator of cell size, and wide-angle light scatter (90° or side scatter) was used as an indicator of cell shape. The results are represented in two-parameter dot plots in which the x axis and y axis are arbitrarily divided into 64 channels relative to the intensity of the incoming signal. A logarithmic amplification of the incoming signal was used to measure a wider range of signals (1.5- and 3-decade scales for scatter and fluorescence parameters, respectively). The sample analysis time was 2 min, during which approximately 10,000 particles were analyzed. The spores were separated from background by their side- and forward-scatter characteristics, and consequently, the total number of spores (fluorescent and nonfluorescent) was determined. The fluorescence intensity of the particles was calculated as the square root of the multiplication of the 515- and 560-nm fluorescence signals (after recalibration from log mode to linear mode).

Determination of intracellular pH. The intracellular pH of individual fluorescent cells was determined by flow cytometry from the ratio of the 515-nm signal (pH-sensitive wavelength) to the 560-nm signal (less-pH-sensitive wavelength) from cF in the cells. The incoming log mode signals were recalculated to the 560-nm signal (pH-sensitive wavelength) to the 560-nm signal. The calibration of the incoming signal was used to measure a wider range of signals (1.5- and 3-decade scales for scatter and fluorescence parameters, respectively). The results are represented in two-parameter dot plots in which the x axis and y axis are arbitrarily divided into 64 channels relative to the intensity of the incoming signal. A logarithmic amplification of the incoming signal was used to measure a wider range of signals (1.5- and 3-decade scales for scatter and fluorescence parameters, respectively). The sample analysis time was 2 min, during which approximately 10,000 particles were analyzed. The spores were separated from background by their side- and forward-scatter characteristics, and consequently, the total number of spores (fluorescent and nonfluorescent) was determined.

**RESULTS**

Germination of *R. oligosporus* sporangiospores. The germination of *R. oligosporus* sporangiospores in malt extract broth was determined microscopically and with flow cytometry. For flow cytometric analysis, the spores were stained with 5- (and 6)-carboxyfluorescein (cF) by incubation with cFDA. The nonfluorescent cFDA is passively transported into intact cells and subsequently cleaved by esterases, which results in intracellular accumulation of fluorescent cF (4). Fluorescence of cF at 515 nm is highly pH dependent, which allows measurement of the pH in individual cells. The ratio of the 515-nm signal to the much less-pH-dependent signal at 560 nm was used to determine intracellular pH. Following incubation in malt extract broth at pH 4.0 (Fig. 1), the forward scatter (indicating particle size), fluorescence intensity, and pH_in of the spores were determined simultaneously with flow cytometry. *R. oligosporus* sporangiospores are smooth walled, round to oval, and 5 to 10 μm in length. The spores were easily detected and enumerated in malt extract broth by flow cytometry (Fig. 2B), while initially only a small percentage (up to 35%) of these spores were fluorescently stained (Fig. 2A). This suggested that in at least a part of the dormant spores, intracellular esterases are active. The staining was not influenced by the absence or presence of glucose (data not shown).

In malt extract, the spores rapidly increased in size (swelling) as determined from the increase of the forward scatter in the side-scatter–forward-scatter dot plots (Fig. 2B). The fluorescent spores are depicted in the 515-nm fluorescence–forward-scatter dot plots (Fig. 2A). During incubation, the spores showed a synchronous increase in forward scatter and fluorescence intensity, and the dot plots after 3 h of incubation showed that almost all spores had become fluorescent, presumably because the esterase activity increases during incubation and cFDA is hydrolyzed to cF. All swollen (fluorescent) spores formed germ tubes approximately 2 h later. In spores which formed a germ tube, the fluorescence intensity and forward scatter were further increased (top right cells in dot plots measured after 5 h of incubation [Fig. 2]).

When the spores were incubated in CDM (Fig. 3), two distinct populations became increasingly evident in the side-scatter–forward-scatter dot plots, namely, small spores (events with low forward scatter) and swollen spores (events with increased forward scatter). The swollen spores represented about 30% of all spores. As illustrated in the 515-nm fluorescence–forward-scatter dot plots, which were measured simultaneously on the spores with increased forward scatter were fluorescent. The flow cytometry results and microscopic analysis showed that the swollen, fluorescent spores were smaller than those in malt extract broth. After 8 h, approximately 10% of the spores formed germ tubes, as determined by microscopic analysis.
Effect of nonanoic acid on germination of *R. oligosporus* sporangiospores. The germination of the *R. oligosporus* sporangiospores in malt extract broth (pH 4.0) at 37°C was determined in the absence and presence of nonanoic acid. In the absence of nonanoic acid, the spores started to form germ tubes after a lag time of about 3 h, and after 5 h, more than 50% of the spores had formed germ tubes, as determined microscopically (Fig. 4). In the presence of 0.25 mM nonanoic acid, the lag time was...
increased to approximately 6 h. In the presence of 1 mM nonanoic acid, emergence of germ tubes was inhibited for at least 7 h, but after 18 h, 47% of the spores germinated, whereas in the presence of 2 mM nonanoic acid, the germination after 18 h was less than 1% (data not shown). Moreover, transfer of the spores after 3 h to fresh malt extract broth without nonanoic acid did not show an increase in the number of germinated spores, which indicated that the effect of nonanoic acid is not reversible.

Simultaneously, the forward scatter (indicating size) of the spores was analyzed by flow cytometry (Fig. 5). In the absence of nonanoic acid (Fig. 5A), the forward scatter of the spores was increased after 3 h, which indicated swelling. After 5 h, a subpopulation of spores (representing approximately 40% of all spores) with a significant increased forward scatter could be distinguished, most likely indicating emergence of germ tubes. After 7.5 h, the forward scatter of the spores suggested that most spores were germinated. This is in agreement with observations made by microscopy. In the presence of 0.25 mM nonanoic acid (Fig. 5B), swelling was slightly suppressed and emergence of germ tubes was delayed. In the presence of 0.5 mM nonanoic acid (Fig. 5C), swelling was significantly inhibited and emergence of germ tubes within 7.5 h was prevented, whereas in the presence of nonanoic acid at 1 mM and higher concentrations (Fig. 5D and E), swelling was completely inhibited. In the presence of 2 mM nonanoic acid, the spores had even shrunk slightly, as determined from the decreased forward scatter (Fig. 5E).

In the same experiment, the pH$_{in}$ distribution of the spores in suspension was also determined at an extracellular pH of 4 (Fig. 6A). At the start of the incubation, the pH$_{in}$ values of the fluorescent spores ranged from 5 to 6.5. The mean pH$_{in}$ was approximately 5.7. Within 1 h, the pH$_{in}$ shifted approximately 0.5 pH unit to higher pH values. After 6 h of incubation, the pH$_{in}$ ranged approximately from 5.5 to 6.7, the mean pH$_{in}$ was 6.3, and the number of fluorescent spores was augmented from 40 to 90% of the total number of spores. In the presence of 1 mM nonanoic acid, the pH$_{in}$ shifted to lower pH values (Fig. 6B) and the number of fluorescent spores decreased to 20% of the total number. The mean pH$_{in}$ of the spores was calculated from the pH$_{in}$ of the individual cells (Fig. 7). In the presence of increasing concentrations of nonanoic acid, the pH$_{in}$ decreased gradually, and in the presence of 2 mM nonanoic acid, the pH gradient was almost completely dissipated. When nonanoic acid (1 mM) was added after 3 h to swollen spores, a decrease of the pH$_{in}$ could be observed (data not shown).

In addition, the effect of octanoic acid and decanoic acid on the germination of the spores was examined (Fig. 8). These medium-chain fatty acids were effective at low concentrations (1 mM). Transfer of spores incubated for 3 h in the presence of octanoic or decanoic acid to fresh malt extract broth did not augment the number of germinated spores (data not shown). This suggested that the negative effect of those acids is not reversible. The short-chain fatty acid acetic acid also inhibited germination but at much higher concentrations (>8 mM). Moreover, the inhibitory effect of acetic acid was completely reversible when the spores were resuspended in fresh malt extract broth after 3 h of incubation.

**DISCUSSION**

In this study, germination of *R. oligosporus* sporangiospores was evaluated by flow cytometry. A major advantage of flow cytometry is the possibility of analyzing simultaneously morphological and physiological parameters of a large number of cells. The low-angle light scatter (forward scatter) of the spores proved to be a very accurate indicator of size. Given that fluorescent spores hydrolyze CFDA and retain CF, the fluorescence intensity of the spores (due to accumulation of CF) could be used as an indication of metabolic (enzymatic) activity. Furthermore, the pH$_{in}$ could be determined effectively from the ratio of the 515-nm emission to the 560-nm emission. The use of the fluorescence ratio technique in combination with flow cytometry is the possibility of analyzing simultaneously multiple parameters of a large number of cells. The pH$_{in}$ of spores was calibrated by equilibration of the intracellular pH with the extracellular pH. This was achieved with acetic acid (2%), since the commonly used ionophores nigericin and valinomycin and the polyene antibiotics nystatin and amphotericin B were not effective (7, 15, 19). Given that the pH$_{in}$ of CF is about 6.3 (10), it is difficult to measure accurately pH values lower than approximately 5. However, because of the high sensitivity of the flow cytometer, the ratios could be determined accurately, and these in turn allowed an estimation of the corresponding pH$_{in}$ values. At the onset of filamentous growth in the germinated spores, formation of compartments was observed, most likely by emergence of vacuoles. Significantly, part of the CF was concentrated in vacuoles (data not shown). Under these conditions, the pH values determined represent an average pH$_{in}$, since the fluorescence of cytoplasmic and vacuolar CF contributes to the measured signal.

A schematic representation of the germination process of *R. oligosporus* sporangiospores is shown in Fig. 9. Germination is characterized by swelling (stage I) and subsequent emergence of germ tubes (stage II). Initially, the spores contain insufficient available endogenous carbon and/or energy sources, and an exogenously added carbon or energy source is needed for swelling (17). Furthermore, synthesis of fatty acids is required, as was demonstrated for *R. stolonifer* (18). This is most likely because in *Rhizopus* spp., a new inner wall layer, the germina-
FIG. 5. Size of *R. oligosporus* sporangiospores determined by flow cytometry. The results are plotted as two-parameter dot plots of forward scatter (indicating particle size) with side scatter. The spores were incubated in malt extract broth (pH 4, 37°C) in the absence (A) or presence of 0.25 (B), 0.5 (C), 1 (D), or 2 (E) mM nonanoic acid. The number of cells analyzed was approximately 10,000. Germinating spores with emerged germ tubes are depicted in the top right part of the two-parameter dot plots. The x and y axes are a 1.5-decade log scale divided arbitrarily into 64 channels.
tion wall (5), is formed during swelling (12, 28). During swelling, the oxygen consumption and RNA synthesis are significantly increased (5, 11). We have shown that swelling of *Rhizopus* spores is associated with an increase in fluorescence and pH. In the second stage, germ tubes are formed. The germ tube wall layer is continuous with the inner wall layer of the swollen spore, emerging through the original outer spore wall. *Rhizopus* spores require a nitrogen source for emergence of the germ tubes (17). During germ tube elongation, the intracellular ATP levels are increased significantly (unpublished data).

Until now, the mechanism by which germination of *Rhizopus* sporangiospores is triggered and the role of the pH in germination have been unclear. In *Rhizopus*, swelling of the spores and subsequent emergence of germ tubes are triggered by glucose (17), a phenomenon which has also been described in *Mucor racemosus* and *Pilobolus longipes* sporangiospores (1, 24). How glucose triggers germination in *Rhizopus* is not understood. Glucose is known to induce a (transient) increase in cyclic AMP (cAMP) (1, 3, 23), and a rise in cAMP has been reported to be responsible for breaking of

![FIG. 6. pH distribution of fluorescent *Rhizopus* sporangiospores in the absence (A) or presence (B) of 1 mM nonanoic acid after 0.15 (●), 0.75 (○), 3 (□), 4 (▲), 6 (●), and 7.5 (●) h of incubation in malt extract broth adjusted to pH 4 with lactic acid at 37°C. For comparison, the total number of spores (fluorescent and nonfluorescent) is standardized to 2,000. The pH was determined by flow cytometry from the ratio of the 515-nm signal to the 560-nm fluorescence signal. The excitation wavelength was 488 nm.](http://aem.asm.org/)

![FIG. 7. Mean pH of *Rhizopus* sporangiospores in malt extract broth (pH 4) in the absence (●) and presence of 0.25 (▲), 0.5 (■), 1 (▲), and 2 (●) mM nonanoic acid. The incubation was performed at 37°C. The pH was determined by flow cytometry from the ratio of the 515-nm signal to the 560-nm fluorescence signal. The excitation wavelength was 488 nm.](http://aem.asm.org/)

![FIG. 8. Effect of short- and medium-chain fatty acids on the germination of *Rhizopus* sporangiospores. The spores were incubated at 37°C in malt extract broth (pH 4) in the absence (●) and presence of 8 mM acetic acid (■), 1 mM octanoic acid (▲), and 1 mM decanoic acid (●). The spores were analyzed microscopically by counting at least 140 cells at random. Germination was defined as the extension of a germ tube to at least one-half the diameter of the spore.](http://aem.asm.org/)
dormancy in spores from Phycomyces blakesleeanus and Saccharomyces cerevisiae ascospores (2, 22, 25). It has also been reported that in S. cerevisiae and Fusarium oxysporum, uptake of glucose stimulates the plasma membrane-located H^+-ATPase (3, 8), resulting in extrusion of H^+. In S. cerevisiae, the glucose-induced H^+-ATPase activation was independent of the glucose-induced cAMP increase (20). Whether the initiation of germination in R. oligosporus sporangiospores is regulated by cAMP or activation of the H^+-ATPase remains to be elucidated. We suggest that an increase in the pH_{in} is crucial for germination and most likely stimulates various processes such as protein synthesis, enzyme activation, and cell wall synthesis. The critical role of the pH_{in} is supported by the inhibiting effect of nonanoic acid on the swelling and emergence of germ tubes. Our evidence suggests that the mechanism of action of nonanoic acid (pK_a 4.96, i.e., 89% undissociated acid at pH 4) is by dissipation of the pH gradient, thereby preventing the increase of the pH_{in} necessary for spore germination (Fig. 9). This nonspecific effect of nonanoic acid is supported by a similar effect on the germination of the spores by other medium-chain fatty acids, octanoic and decanoic acids. Moreover, Viegas et al. (27) demonstrated that dissipation of the pH gradient by these acids was also responsible for growth inhibition of S. cerevisiae. Other fatty acids such as acetic acid, citric acid, and palmitic acid inhibited germination less effectively (data not shown).

Self-inhibitors such as the cinnamic esters cis-3,4-dimethoxy-cinnamic acid methyl ester and cis-ferulic acid methyl ester did not inhibit swelling but prevented initiation of the germ tube elongation of urediniospores from rust fungi such as Uromyces phaseoli and Puccinia graminis (16). To date, the exact mode of action of these self-inhibitors has not been elucidated. The cinnamic esters were already effective at nanomolar concentrations, and it was suggested that enzymes associated with the newly formed germ tube which are necessary for germ tube extension were reversibly inhibited (13). Furthermore, several extracted phenolic compounds such as vanillic acid and p-hydroxybenzoic acid were reported to inhibit urediniospores of Puccinia graminis at millimolar concentrations (26), but detailed information about their mechanism of inhibition is lacking.

In conclusion, the viability of individual R. oligosporus sporangiospores could be efficiently assessed by flow cytometry. An increase in pH_{in} proved to be critical for swelling and subsequent emergence of the germ tubes. This was demonstrated by the effect of nonanoic acid and other weak acids, which inhibited germination of R. oligosporus by dissipation of the pH gradient.

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REFERENCES