



Chitosan permeabilizes the plasma membrane and kills cells of *Neurospora crassa* in an energy dependent manner

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ABSTRACT

Chitosan has been reported to inhibit spore germination and mycelial growth in plant pathogens, but its mode of antifungal action is poorly understood. Following chitosan treatment, we characterized plasma membrane permeabilization, and cell death and lysis in the experimental model, *Neurospora crassa*. Rhodamine-labeled chitosan was used to show that chitosan is internalized by fungal cells. Cell viability stains and the calcium reporter, aequorin, were used to monitor plasma membrane permeabilization and cell death. Chitosan permeabilization of the fungal plasma membrane and its uptake into fungal cells was found to be energy dependent but not to involve endocytosis. Different cell types (conidia, germ tubes and vegetative hyphae) exhibited differential sensitivity to chitosan with ungerminated conidia being the most sensitive.

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1. Introduction

Chitosan is a polymer of β -1,4-glucosamine subunits, and is a partly deacetylated form of chitin (Rabea et al., 2003). It is not toxic to mammals (Dodane and Vilivalam, 1998; Lee et al., 2004) and elicits plant defense mechanisms (Ait Barka et al., 2004; Benhamou et al., 1994; Lafontaine and Benhamou, 1996; Trotel-Aziz et al., 2006). Chitosan displays antibiotic activity against bacteria (Liu et al., 2001, 2004; Tikhonov et al., 2006) and fungi (Bautista-Banos et al., 2006; Bell et al., 1998; Laflamme et al., 1999; Palma-Guerrero et al., 2008; Park et al., 2002; Plascencia-Jatomea et al., 2003), and has been reported to damage the plasma membranes of both bacteria (Liu et al., 2004) and the yeast *Saccharomyces cerevisiae* (Zakrzewska et al., 2005). The deletion of genes encoding proteins that are involved in maintaining plasma membrane integrity was found to increase the sensitivity to chitosan (Zakrzewska et al., 2007). Plasma membrane damage has also been suggested to explain the fungicidal effects of chitosan on filamentous fungi (El

Ghaouth et al., 1992; Laflamme et al., 1999). The permeabilization of the plasma membrane by chitosan has been proposed to be caused by the interaction of the positive amino groups of chitosan with the negative charges on phospholipids (Liu et al., 2004). Recently, rhodamine-labeled chitosan was found to be taken up by conidia of plant pathogenic and nematophagous fungi in an energy dependent manner and not by passive diffusion (Palma-Guerrero et al., 2008). Previously the endocytic marker FM4-64 had been shown to be internalized by conidia of *Magnaporthe grisea* in an energy dependent manner (Atkinson et al., 2002), which suggests that chitosan may also be endocytically internalized.

The aim of the present study was first, to test the hypothesis that chitosan is endocytically internalized by fungal cells, and second, to obtain further insights into the mode of antifungal action by chitosan. For this purpose we used *Neurospora crassa*, a species in which endocytosis has been previously analyzed (Fischer-Parton et al., 2000; Read and Hickey, 2001; Read and Kalkman, 2003) and which is particularly amenable to live-cell analysis using confocal microscopy (e.g. Hickey et al., 2005). Our results show that chitosan enters and kills cells after plasma membrane permeabilization by an unknown energy dependent mechanism that does not involve endocytosis. We have also found that conidia, germ tubes, and vegetative hyphae exhibit differential sensitivity to the fungicidal effects of chitosan.

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2. Materials and methods

2.1. Strains and culture conditions

The *N. crassa* wild-type strain 74-OR23-IVA (FGSC #2489), and the *N. crassa* H1-GFP strain (N2283, Freitag et al., 2004) were grown and maintained on solid Vogel's agar medium (Davis, 2000). A transformed strain of *N. crassa* expressing codon-optimized aequorin (strain #272, Fungal Cell Biology Group, University of Edinburgh, UK; Nelson et al., 2004; Zelter, 2004) was stored as a stock culture on solid Vogel's agar medium containing 200 $\mu\text{g ml}^{-1}$ hygromycin B, but was grown on solid Vogel's medium lacking hygromycin B for the production of the conidia used in luminometry experiments.

2.2. Chemicals and dyes

Chitosan (T8s) with a molecular weight of 70 KDa and exhibiting 79.6% deacetylation was obtained from Marine BioProducts GmbH (Bremerhaven, Germany). Chitosan was dissolved in 0.25 mol l^{-1} HCl and the pH adjusted to 5.6 with 1 mol l^{-1} NaOH. The resulting solution was dialyzed for salt removal, and the dialyzed chitosan was autoclaved at 120 °C for 20 min (Palma-Guerrero et al., 2008).

Rhodamine-labeled chitosan (rhodamine-T8s chitosan) was kindly provided by Dr. V. Tikhonov (Laboratory of Physiologically Active Biopolymers, A.N. Nesmeyanov Institute of Organoelement Compounds, Russian Academy of Sciences, Moscow, Russia). After dialysis, the labeled chitosan was filtered through 3 KDa ultra-membranes in an Amicon Cell (Omega, Pall Corporation, Ann Arbor, MI, USA) and sterilized by filtration through a 0.2 μm pore-size syringe filter (Albet, Barcelona, Spain).

Sodium azide and the cell viability dyes, fluorescein diacetate (FDA) and propidium iodide (PI), were obtained from Sigma (St. Louis, MO, USA). The membrane selective fluorescent dye, FM4-64, was obtained from Invitrogen (Eugene, OR, USA). Stock solutions of FDA (1 mg ml^{-1} in acetone), PI (1 mg ml^{-1} in H_2O) and FM4-64 (10 mg ml^{-1} in DMSO) were all diluted in distilled H_2O and then added to the conidial inocula at final concentrations of 5 $\mu\text{g ml}^{-1}$, 5 $\mu\text{g ml}^{-1}$, and 5 μM , respectively.

2.3. Quantitation of conidial germination, conidial lysis and CAT fusion

Conidia were collected from 4 to 5 day old wild type cultures by adding 1 ml sterile distilled water and removing the conidial suspension with a pipette. The conidia were counted using a haemocytometer, diluted to the appropriate concentration, and immediately used in the bioassays. Conidial germination, conidial lysis and conidial anastomosis tube (CAT) fusion assays were carried out in 8-well slide culture chambers (Nalge Nunc International, Rochester, NY). Each well was filled with 200 μl of conidial suspension at a final concentration of 10^6 conidia ml^{-1} in potato dextrose broth (PDB) (Becton Dickinson and Company, Sparks, MD, USA) with chitosan at the appropriate final concentration. PDB (2.5 g l^{-1}) was used for the conidial germination and conidial lysis assays. PDB was replaced with liquid Vogel's medium (Davis, 2000) diluted 100 times for the CAT fusion assay. After incubation at 24 °C in continuous light, the number of non-lysed conidia were counted (for conidial lysis assays) or the percentage conidia that had not lysed and had germinated were quantified (for conidial germination assays). Percentage germination was defined as the percentage of conidia possessing one or more germ tubes and/or CATs. A 20 $\times$ dry or a 60 $\times$ water immersion plan apo objective with differential interference contrast (DIC) optics on an inverted TE2000E microscope (Nikon, Kingston-Upon-Thames, United Kingdom) was used in the germination and lysis assays. CAT fusion was only assessed with the 60 $\times$ objective on

the inverted microscope and CAT fusion was quantified as the percentage of conidia/conidial germlings involved in fusion. Three wells per treatment, with ten fields of view per well (each containing 100–300 conidia), were assessed for each of the three experimental assays, and each experiment was performed twice.

2.4. Monitoring rhodamine-chitosan internalization

Conidia were collected as described in Section 2.3 and were treated with 0.1 mg ml^{-1} rhodamine-chitosan for different times at room temperature (~ 22 °C). The conidia were washed three times with distilled water by centrifugation at 19,300g for 5 min to remove unbound rhodamine-chitosan. The resultant conidial suspension was then dispensed as 30–50 μl droplets onto glass coverslips, and conidia were immobilized by the inverted agar block method (Hickey et al., 2005) and imaged by confocal microscopy (Section 2.8) with a 100 $\times$ oil immersion objective.

2.5. Staining with fluorescent probes

Staining of conidia with FM4-64 or PI was carried out by adding FM4-64 (at a final concentration of 5 μM) or PI (at a final concentration of 5 $\mu\text{g ml}^{-1}$) to a conidial suspension prepared as described in Section 2.3. For the chitosan treatment, the conidia (to which FM4-64 or PI had been added) were treated with chitosan at a final concentration of 0.1 mg ml^{-1} . The conidial suspensions were inoculated, immobilized and imaged as described in Section 2.4.

2.6. Cell viability tests

A combination of FDA and PI was used to check cell viability after conidia and conidial germlings were treated with chitosan. Wells of the 8-welled culture chambers (Section 2.3) were filled with 100 μl of conidial suspension at a final concentration (after dye addition) of 10^6 conidia ml^{-1} . FDA and PI were added to the conidial suspension at a final concentration of 5 $\mu\text{g ml}^{-1}$. Once the culture chamber was mounted on the confocal microscope stage, 100 μl of chitosan was added at a final concentration of 0.1 mg ml^{-1} . Samples were immediately imaged using a 40 $\times$ dry or a 60 $\times$ water immersion objective, and images were captured every 30 s for 12 min. For the germling viability tests, 100 μl of conidial suspension in 2.5 g l^{-1} PDB was first incubated in each well of the 8-welled culture chamber for 6 h at 24 °C in continuous light. FDA, PI and chitosan were added as described in Section 2.5. Ungerminated conidia and 6 h old conidial germlings were imaged by confocal microscopy (Section 2.8) with a 20 $\times$ dry or a 60 $\times$ water immersion plan apo objective, and an image was captured every min for 1 h. The percentages of live and dead conidia or germlings were recorded at each time point and the percentage of live cells was determined. Three wells per treatment were recorded (100–300 conidia per field of view, 10 fields of view per well) and the experiment was performed twice.

FM4-64 was used to check the viability of vegetative hyphae after chitosan treatment. The wells containing 100 μl conidial suspension at a final concentration of 10^6 conidia ml^{-1} in 2.5 g l^{-1} PDB were incubated for 18 h at 24 °C in continuous light. FM4-64 was added to the vegetative hyphae at a final concentration of 5 μM . Chitosan was added at a final concentration of 0.1 mg ml^{-1} and samples were immediately imaged by confocal microscopy (Section 2.8) with a 20 $\times$ objective, and an image captured every min for 1 h.

2.7. Inhibition of energy dependent processes

Sodium azide and low temperature (4 °C) were used to inhibit ATP production and thus energy dependent processes (Atkinson et al., 2002; Hoffmann and Mendgen, 1998). Conidia or conidial germlings

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