

Energy-dependent uptake of benzo[a]pyrene and its cytoskeleton-dependent intracellular transport by the telluric fungus *Fusarium solani*

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Abstract In screening indigenous soil filamentous fungi for polycyclic aromatic hydrocarbons (PAHs) degradation, an isolate of the *Fusarium solani* was found to incorporate benzo[a]pyrene (BaP) into fungal hyphae before degradation and mineralization. The mechanisms involved in BaP uptake and intracellular transport remain unresolved. To address this, the incorporation of two PAHs, BaP, and phenanthrene (PHE) were studied in this fungus. The fungus incorporated more BaP into cells than PHE, despite the 400-fold higher aqueous solubility of PHE compared with BaP, indicating that PAH incorporation is not based on a simple diffusion mechanism. To identify the mechanism of BaP incorporation and transport, microscopic studies were undertaken with the fluorescence probes Congo Red, BODIPY[®]493/503, and FM[®]4-64, targeting different cell compartments respectively fungal cell walls, lipids, and endocytosis. The metabolic inhibitor sodium azide at 100 mM totally blocked BaP incorporation into fungal cells indicating an energy-requirement for PAH uptake into the mycelium. Cytochalasins also inhibited BaP uptake by the fungus and probably its intracellular transport into fungal hyphae. The perfect co-localization of BaP and BODIPY reveals that lipid bodies constitute the intracellular

storage sites of BaP in *F. solani*. Our results demonstrate an energy-dependent uptake of BaP and its cytoskeleton-dependent intracellular transport by *F. solani*.

Keywords Active transport · Benzo[a]pyrene · Confocal microscopy · Endocytosis · *Fusarium solani*

Introduction

In a search for indigenous soil filamentous fungi with the potential to degrade high-molecular-weight polycyclic aromatic hydrocarbons (PAHs), our laboratory has isolated a collection of telluric fungi from PAH-contaminated soil (Rafin et al. 2000, 2013; Potin et al. 2004a, b). We focused our attention on a fungus *Fusarium solani* (Mart.) Sacc. (1881) [teleomorph: *Haematonectria haematococca* (Berk. and Broome) Samuels and Rossman, Ascomycota, Hypocreales, Nectriaceae] which was able to metabolize and mineralize benzo[a]pyrene (BaP) (Rafin et al. 2000, 2006; Veignie et al. 2002). BaP degradation by this isolate led to the formation of distinct metabolites such as 6-hydroxybenzo[a]pyrene sulfate (Veignie et al. 2002) which is known to be produced during intracellular BaP detoxification processes mediated by cytochrome P450 monooxygenase and aryl sulfatases (Rafin et al. 2000, 2006). Moreover, quinones, reported in our previous studies as BaP metabolites, could also be obtained during PAH oxidation by P-450 cytochrome (Cavaliere and Rogan 1985, 1995).

These identified metabolites suggest that PAH degradation by *F. solani* occurs among others intracellularly which requires three successive steps: BaP uptake leading to its incorporation into fungal cells, intracellular transport of BaP to the storage sites, and BaP degradation. The mechanisms involved in uptake of hydrophobic organic compounds and their

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intracellular transport remain unresolved. While numerous works concern the uptake of alkanes by fungi (Cooney et al. 1980; Lindley and Heydeman 1983, 1986a, b) and by yeasts (Käppeli et al. 1984), these mechanisms were rarely considered in relation to PAH uptake by fungi (Wu et al. 2009; Deng et al. 2010). In the present study, we compared the incorporation of two PAHs, respectively BaP as a high-molecular-weight PAH and phenanthrene as a low-molecular-weight PAH, in this fungus as a model organism.

Concerning intracellular transport, recent research has introduced the novel concept of “hyphal pipelines” allowing intracellular fluorene and phenanthrene transport with vesicles in the oomycete *Pythium ultimum* (Furuno et al. 2012; Schamfuß et al. 2013). Uptake of contaminants by fungi has been also reported for various contaminants including radionuclides (Tobin et al. 1994), heavy metals (Clark and Zeto 2000), and organic pollutants (Käppeli et al. 1984). Cytological observations of similar vesicle-bound PAH were also noticed in our fungus model *F. solani* in previous studies (Rafin et al. 2000). To investigate the mechanisms involved in BaP intracellular transport and storage by this organism, microscopic studies based on wide field epifluorescence and laser scanning confocal microscopy were undertaken by comparing fluorescent cell marking triggered by BaP and fluorescence probes targeting different cell compartments such as Congo Red, BODIPY[®]493/503, and FM[®]4-64 (respectively cell walls, lipids, and endocytosis). Lastly, we focused our attention on the potential role of the cytoskeleton and energy requirement involved in the vesicles intracellular transport.

Materials and methods

Dyes and other chemicals

The dye Congo Red (disodium 4-amino-3-(4-(4-(1-amino-4-sulfonato-naphthalen-2-yl)diazenylphenyl)phenyl)diazenyl-naphthalene-1-sulfonate) was supplied by Sigma (Sigma-Aldrich, St Quentin Fallavier, France). BODIPY (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene BODIPY[®]493/503) was obtained from Life Technologies SAS (St Aubin, France). FM4-64 (N-(3-triethylammoniumpropyl-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide FM[®]4-64) was supplied by Interchim (Montluçon, France). BaP, PHE, and sodium azide (NaN₃) were purchased from Acros Organics (Noisy-Le-Grand, France). Cytochalasin B (7(S),20(R)-dihydroxy-16(R)-methyl-10-phenyl-24-oxa[14]cytochalasa-6(12),13(E),21(E)-triene-1,23-dione) was supplied by Santa-Cruz Biotechnology (USA). Hexane (HEX) was supplied by Sigma (St Quentin Fallavier, France); acetone (ACE), acetonitrile, and dimethyl sulfoxide (DMSO) by Panreac Química SA (Barcelona, Spain); and dichloromethane (DCM), methanol

(MeOH), and chloroform (TCM) by Merck (Darmstadt, Germany). Distilled deionized water was used throughout this work.

Fungal material, inoculum preparation, and cultures

F. solani, previously isolated from petroleum-contaminated soil, was supplied from our UCEIV mycology collection (Dunkerque, France). This isolate, numbered as isolate 14 (Rafin et al. 2013), was investigated in previous studies by Rafin et al. (2000, 2006, 2013) and Veignie et al. (2002, 2004, 2012). It was grown on Malt yeast extract agar at 22 °C under a photoperiod of 12 h/12 h. Cultures were grown in Erlenmeyer flasks in 50 mL liquid mineral salts medium (Veignie et al. 2002) with 1 g L⁻¹ yeast extract and 10 g L⁻¹ glucose. After sterilization (121 °C for 20 min), inoculation was performed by adding a spore suspension of *F. solani* (aged 7 days) prepared as described previously (Rafin et al. 2000), so as to obtain a final concentration of ~10⁴ spores mL⁻¹. After inoculation, Erlenmeyer flasks were incubated for 3 days at 22 °C on a reciprocating shaker (Infors, Massy, France) at 90 rpm before filtration. The filtered exponential phase growing mycelium was then used to inoculate cultures for PAH incorporation experiments and microscopy studies.

For PAH incorporation experiments, inoculation was performed with 150 mg of filtered exponential phase growing mycelium introduced in 25 mL MM containing 20 g L⁻¹ glucose. Cultures were grown for 48 h at 22 °C on a reciprocating shaker at 170 rpm. In treatments with PAHs, 250 µg of BaP or PHE initially dissolved in ACE (concentration of PAH stock solution was 0.8 mg mL⁻¹) were deposited into an Erlenmeyer flask. After total ACE evaporation, 25 mL MM containing 20 g L⁻¹ glucose was added before sterilization and inoculation. Each treatment was prepared in triplicate.

For microscopy studies, the inoculum was produced following the same protocol but in 5 mL MM for 24 h. In cultures exposed to pollutant, 10 µg BaP initially dissolved in ACE (concentration of PAH stock solution was 0.8 g L⁻¹) was deposited into each Erlenmeyer.

Extraction and HPLC analysis of PAH in incorporation experiments

At the end of the incubation, cultures were filtered onto a Büchner funnel. Mycelia were washed three times in 3 mL of DCM/HEX (1:1) to remove PAHs adsorbed onto hyphae cell walls. Cultures were then lyophilized for 24 h, and the dry weight biomass was obtained. The extraction consisted of five different steps each conducted in 5 mL of solvent for 4 min at 70 °C under rotation shaking (170 rpm) in the following order: TCM/MeOH (2:1), 2× TCM/MeOH (1:1), ACE/MeOH (1:1), and TCM/MeOH (1:1). Organic fractions were concentrated in 20 mL DCM/MeOH (1:1). PAH concentrations were

determined using an HPLC Waters 600 control system fitted with a Waters XTerra®, RP18 column (5 µm), and a Waters 996 Photo Diode Array Detector. Samples were separated using an isocratic solvent (acetonitrile/water, 90:10, v/v) at a solvent flow rate of 1.0 mL min⁻¹ for 10 min. Concentrations were determined by UV absorbance at 254 nm for PHE and 380 nm for BaP. PAH mass/dried biomass ratio (micrograms per milligram) was calculated. Results were expressed as mean value ± standard error for three replicates. Statistical analysis was performed by an unpaired Student's *t* test (at 99 % confidence).

Microscopy studies

The 5 µL Congo Red stock solution (10 µg mL⁻¹ in H₂O) was directly applied to 10 µL of the fungal culture sample onto a microscope slide prior to the observation. Stock solutions of BODIPY (5 mM in DMSO) and FM4-64 (1.65 mM in water/ethanol, 1:1) were added to the prepared fungal culture 4 h before the observations at final concentrations of 0.1 µM and 7.5 mM, respectively. Inhibitors were introduced into Erlenmeyer flasks containing MM without glucose for 4 h in the presence of the filtered exponential phase growing mycelium before introducing BaP for 24 h of incubation. Sodium azide was added directly as powder to MM at a final concentration of 100 mM before sterilization. Cytochalasin B was added to MM after sterilization at a final concentration of 0.1 g L⁻¹. All combinations with or without BaP or inhibitors were conducted.

Wide field epifluorescence microscopy was performed using an Axiostar plus HB050 microscope (exCitation 345 nm, emission 485 nm for BaP, 520 nm for Congo Red). Images were taken with a Canon Power Shot A640 digital camera. Confocal images were obtained using a LSM 780 laser scanning confocal microscope (Zeiss, Le Pecq, France; excitation 405 nm for BaP, 488 nm for BODIPY, 561 nm for FM4-64, and Congo Red) connected to a Zeiss AxioObserver Z1 with a Plan-Apochromat X40/1.3 oil Differential Interference Contrast (DIC) objective. Channels were separately excited and collected in line scan mode. The same instrumental conditions were used throughout the different experiments.

Results and discussion

Ratio of PAH incorporated per milligram of biomass versus PAH water solubility

The 48 h incubation of *F. solani* in MM containing PAH and glucose highlighted contrasting patterns between the two PAHs studied. In Fig. 1a, our results clearly indicated that the quantity of BaP incorporated into biomass was 1.2 µg mg⁻¹, 12.0-fold higher than the quantity of PHE

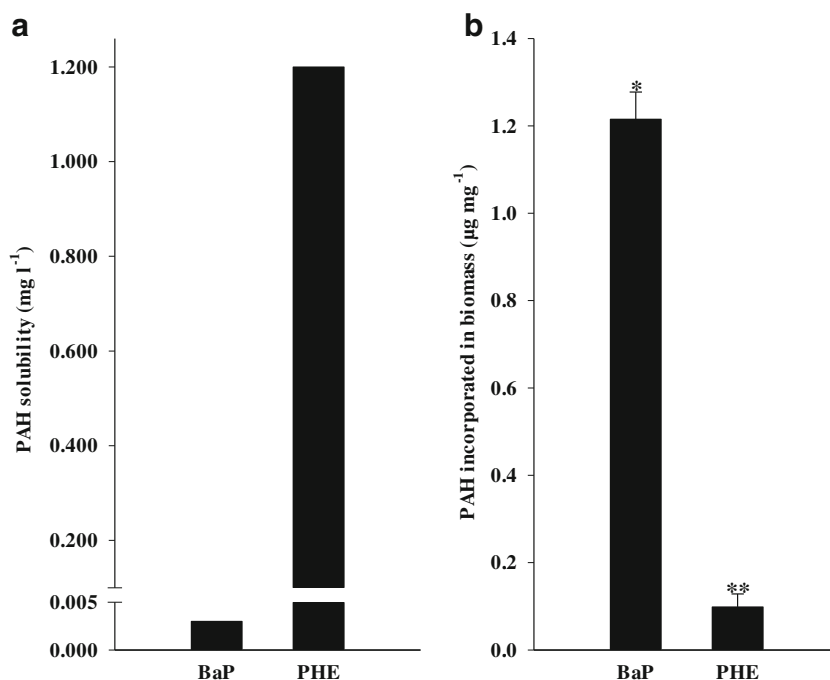
incorporated (0.1 µg mg⁻¹). This result was quite surprising considering that the solubility of BaP is 400-fold less than the solubility of PHE, at 0.003 and 1.2 mg L⁻¹, respectively (Fig. 1b). In a general manner, the uptake of low water solubility compounds in eukaryotic cells is far from clear and quite controversial. Three main hypotheses were put forward in the literature concerning hydrocarbons. First, Lindley and Heydeman (1983, 1986) suggested the existence of an active uptake mechanism of dodecane by the filamentous fungus *Cladosporium resinae*. Second, a partitioning phenomenon of PAH between two hydrophobic compartments (namely cell wall lipids and biological membranes) was suggested by Meissel et al. (1973) or between extracellular lipoproteins and the plasma membranes as described by Plant et al. (1985). Lastly, Verdin et al. (2005) proposed a passive diffusion process for BaP incorporation into *F. solani* hyphae. The results obtained in the present study strongly suggested the existence of an active mechanism involved in BaP transport by the *F. solani* cells. Indeed, the higher rate of incorporation of BaP compared with PHE into fungal cells, in spite of the 400-fold higher aqueous solubility of PHE compared with BaP, provides evidence against the principle of a simple diffusion mechanism. Moreover, 82.9 % of the initial quantity of BaP was incorporated into the fungal biomass after only 48 h. This result clearly indicated an intracellular accumulation of BaP against the concentration gradient which was not compatible with a simple diffusion process. To investigate the mechanism of BaP incorporation and transport by *F. solani*, we undertook further microscopic studies using fluorescent probes.

Localization and identification of BaP storage sites

Microscopic studies were conducted to compare observations of *F. solani* grown in the presence of BaP, used as an autofluorescent probe, by two different microscopic techniques: wide field epifluorescence and laser scanning confocal microscopy. Wide field epifluorescence microscopy clearly allowed the observation of blue-colored fluorescent vesicles associated with hyphae (Fig. 2a) after culturing the fungus for 24 h at 22 °C in contact with BaP. Such vesicles have already been reported to be associated with fungal hyphae (Rafin et al. 2000; Verdin et al. 2005; Wu et al. 2009; Thion et al. 2012). To confirm the intracellular localization of BaP, we used laser scanning confocal microscopy and Congo Red as a fluorescent marker of fungal cell walls (Roncero and Duran 1985; Slifkin and Cumbie 1988). Under these conditions, the images clearly showed the spatial location of BaP storage sites within the limits of cells (Fig. 2b) especially when those were highlighted by Congo Red (Fig. 2c).

The nature of the cellular compartment involved in BaP storage was investigated through the use of two different fluorescent probes with laser scanning confocal microscopy.

Fig. 1 Ratio of PAH incorporated per milligram of dried biomass compared with PAH water solubility. **a** Aqueous solubility (milligram per liter) of benzo[a]pyrene (BaP) and phenanthrene (PHE) as given in the literature. **b** Uptake of each PAH by exponentially growing *F. solani* cells after 48 h of incubation at 22 °C in PAH-containing liquid MM with glucose (mean value±standard error for triplicates). Statistical analysis performed by an unpaired Student's *t* test (at 99 % confidence)



As BaP possesses hydrophobic characteristics, it tends to concentrate in nonpolar cell compartments. Thus, we deliberately chose nonpolar fluorescent dyes for identifying BaP storage sites within the fungal hyphae. The water-soluble BODIPY fluorophore was first used offering an unusual combination of nonpolar structures that, upon binding to neutral lipid, emits a fluorescence signal with a narrow wavelength range, making it an ideal tool in multi-labeling experiments. The hydrophobic nature of this dye molecule and its low molecular weight promote a relatively fast diffusion rate through membranes into the nonpolar environment of cells (Johnson et al. 1996; Kamisaka et al. 1999; Saito et al. 2004). Staining with the BODIPY[®]493/503 has furthermore been described to be more specific for cellular lipid droplets than

staining with Nile red (Gocze and Freeman 1994). FM[®]4-64 has also been used in this study as this fluorescent membrane selective marker was reported to selectively stain yeast and fungi vacuolar membranes allowing the study of endocytosis in eukaryotic cells (Vida and Emr 1995; Fischer-Parton et al. 2000; Read and Kalkman 2003). The comparison of cell labeling triggered by BaP (Fig. 3a) and BODIPY (Fig. 3b) reveals a perfect co-localization of these compounds (Fig. 3d). This enables us to conclude that lipid bodies constitute the intracellular storage sites of BaP in *F. solani*. The BaP concentration in lipid bodies in *F. solani* was first hypothesized by Rafin et al. (2000) and further confirmed by other authors (Wu et al. 2009; Verdin et al. 2005; Thion et al. 2012). To the contrary, the blue fluorescence of BaP never localized with the

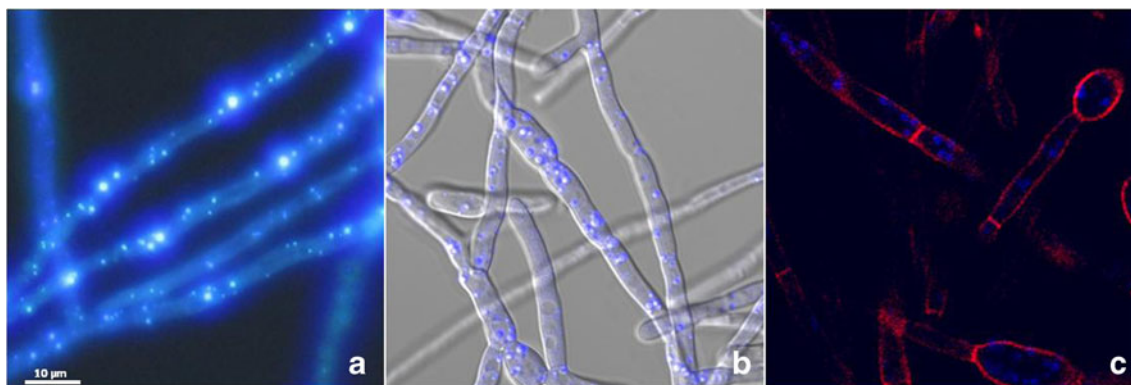
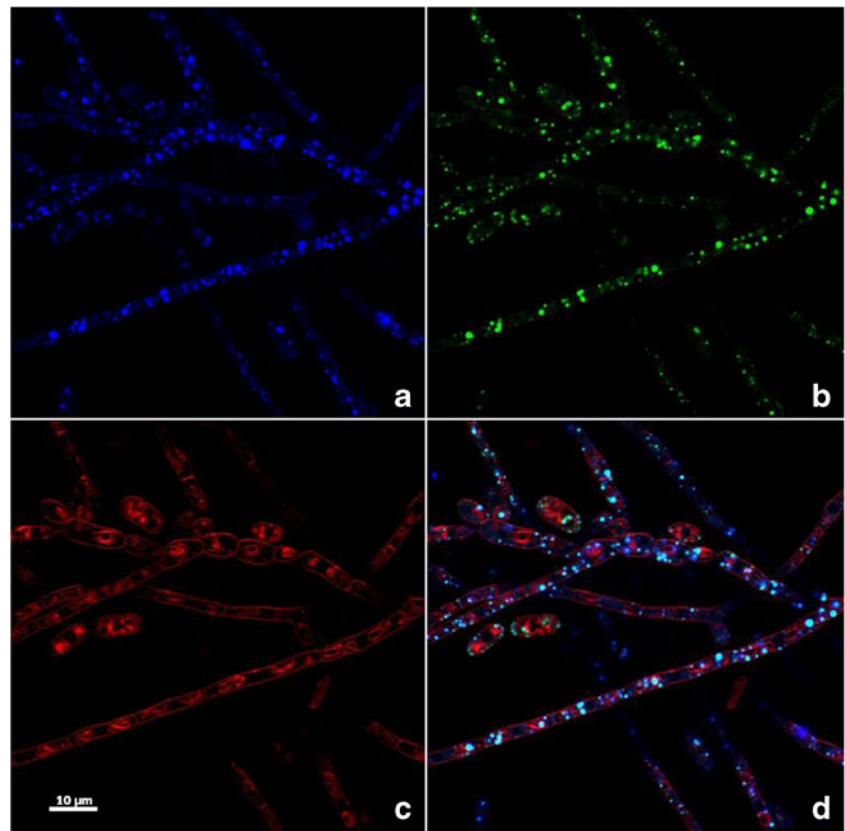


Fig. 2 Vesicles labeled by the blue fluorescence of BaP in *F. solani* viewed using three different microscopic techniques: **a** wide field epifluorescence microscopy. **b** confocal microscopy with DIC mode and 405 nm excitation. **c** Dual labeling of hyphae with Congo Red and

BaP, observed with laser scanning confocal microscope. BaP- and Congo Red-stained fungal vesicles in *blue* and fungal cell wall in *red*, respectively

Fig. 3 Confocal microscope images of the intracellular location of three fluorescent molecules in one *F. solani* hyphae optical section: **a** Benzo[a]pyrene (405 nm filter). **b** BODIPY (488 nm filter). **c** FM4-64 (561 nm filter). **d** Benzo[a]pyrene, BODIPY and FM4-64 merged images



red fluorescence of FM4-64 (Fig. 3c and d). This absence of co-localization indicated that a mechanism different from the associated fungal vacuole endocytic pathway used by FM4-64 was involved in BaP intracellular transport. Nevertheless, the presence of cell labeling by FM4-64 confirmed the existence of endocytosis by *F. solani*, as demonstrated in a large range of fungal species (Vida and Emr 1995; Yamashita and May 1998; Fischer-Parton et al. 2000; Atkinson et al. 2002; Read and Kalkman 2003).

Mechanisms involved in BaP uptake

To understand the mechanisms involved in BaP uptake by *F. solani* cells, the same fluorescent probes (BODIPY[®]493/503 and FM[®]4-64) were used with laser scanning confocal microscopy in the presence or absence of a metabolic inhibitor (NaN₃) utilized at a high concentration (100 mM). Three cases were observed (Fig. 4): (1) The incorporation of the fluorescent marker FM4-64 was totally blocked by this inhibitor (Fig. 4b and e). (2) No incorporation of BaP fluorescence into fungal cells was observed in the presence of NaN₃ (Fig. 4d) in comparison with the control (Fig. 4a). (3) As expected, the inhibitor had no effect on the BODIPY cell distribution confirming the occurrence of a simple diffusion process for this fluorescent probe (Fig. 4c and f).

The comparative kinetics of BaP and FM4-64 uptake was carried out over 24 h and observed with epifluorescence microscopy, allowing a temporal and spatial study of the accumulation of both probes into fungal cells (data not shown). Using FM4-64, cell labeling appeared quite rapidly in 5 min. For BaP, 25 min was necessary before the first intracellular staining was observed. The fluorescent signals appeared for both treatments first in hyphae apex and fungal branching areas. These observations reinforced the hypothesis of an active BaP uptake mechanism, as these particular zones (apical and branching segments) are well known to be involved in fungal growth and are among the most metabolically active areas. After 24 h, all the hyphae observed were fluorescently labeled. The two probes localized to separate intracellular storage sites, lipid bodies for BaP, and endocytosis vacuoles for FM4-64 (as observed in the section on “[Localization and identification of BaP storage sites](#)”). Finally, the relatively fast incorporation of BaP into fungal cells (allowing an intracellular staining observable at least after 25 min) might indicate that BaP used a constitutive pathway commonly used for natural hydrophobic molecules such as lipid fungal metabolism (Kamisaka and Noda 2001).

In a last experiment, to further characterize the intracellular mechanism involved in BaP transport to lipid bodies, the fluorescence of BaP and BODIPY[®]493/503 were followed with laser scanning confocal microscopy in the presence or

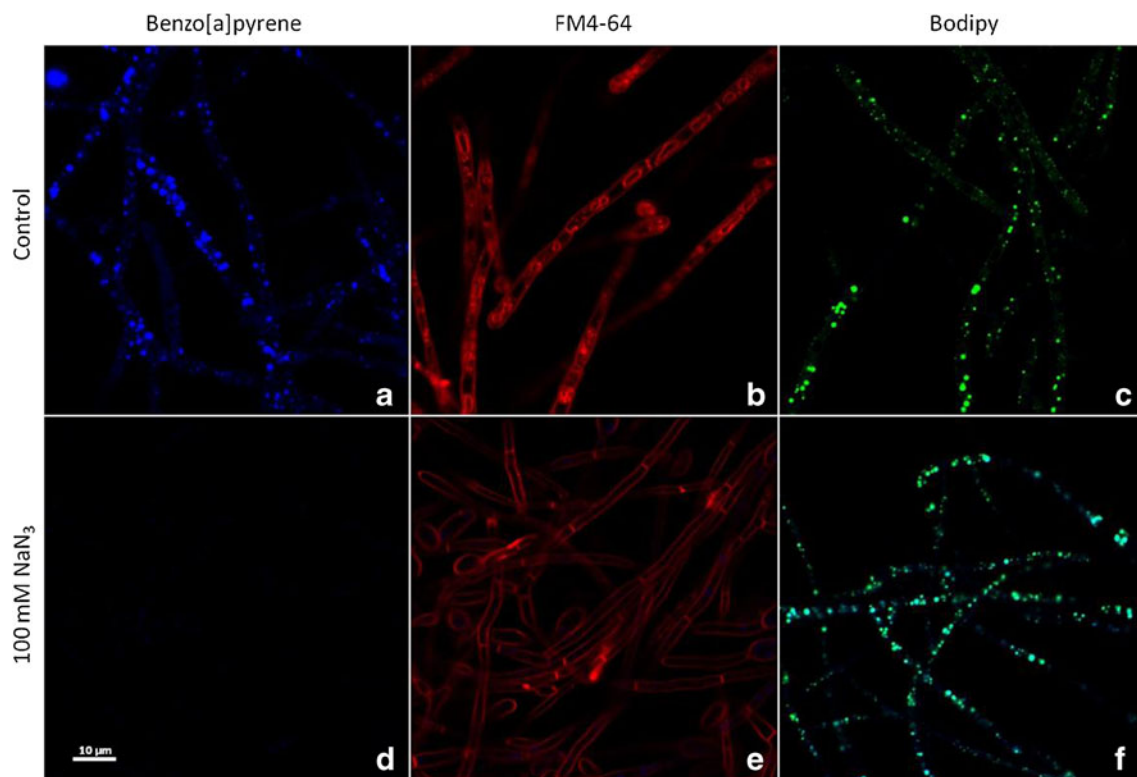


Fig. 4 Effect of 100 mM NaN_3 on the incorporation of BaP (405 nm filter), FM4-64 (561 nm filter), and BODIPY (488 nm filter) in *F. solani* hyphae (laser scanning confocal microscopy): **a, b, c** Control. **d, e, f** NaN_3 treatment

absence of a cytoskeleton inhibitor, cytochalasin B at a high concentration of 0.1 g L^{-1} . Cytochalasins are known as *in vivo* inhibitors of actin polymerization and can therefore be used to study the functions of the actin cytoskeleton in filamentous fungi (Torralba et al. 1998; Srinivasan et al. 1996). In the present study, the transport and concentration of BODIPY into lipid bodies were not altered by the cytochalasin treatment (Fig. 5g and h) in comparison with the control (Fig. 5e and f). As expected, the intracellular BODIPY localization was not perturbed by this treatment because of its known diffusion process. Moreover, the cell labeling in this treatment was observed instantaneously. Few lipid bodies were noticed corresponding in all probability to the pre-existent lipid bodies present before cytochalasin treatment. This result suggests that the actin cytoskeleton could be involved in the synthesis and motion of lipid bodies (Welte 2009). On the contrary, BaP incorporation into cells was totally inhibited in the presence of cytochalasin (Fig. 5c and d) in comparison with BaP control (Fig. 5a and b). This observation underlined the fundamental role of actin in BaP intracellular uptake in *F. solani* reinforcing the hypothesis of an energy-dependent transport mechanism for uptake and most probably BaP transport into fungal hyphae. A similar energy but not actin cytoskeleton-dependent uptake of other exogenous hydrophobic compounds such as phospholipids and storage into lipid bodies were described in an oleaginous fungus *Mortierella ramanniana* var.

angulispora (Kamisaka et al. 1999; Kamisaka and Noda 2001). Moreover, Lindley and Heydeman (1983) also demonstrated that the transport of hydrophobic *n*-alkanes across the cell membrane was an active uptake mechanism in *Cladosporium resinae*, the well-known jet fuel-degrading fungus. For the first time to our knowledge, the involvement of an energy-dependent endocytosis-like mechanism has been demonstrated for the uptake of a high-molecular-weight PAH (BaP) in *F. solani*. However, in the present research, the absence of colocalization of BaP and FM4-64 clearly demonstrated the occurrence of a fungal specific endocytic pathway for this pollutant, different from the classical endocytosis system usually described in yeast and fungal cells with vacuoles as end points of endocytic vesicles. Our results led to a hypothesis, in line with the vesicle flux model described by Zweytick et al. (2000), where BaP transport would occur through vesicle flux to pre-existing lipid particle structures functioning as a storage compartment. Such an intracellular PAH active transport mediated by lipid vesicles inside the hyphae was recently demonstrated in the oomycete *P. ultimum* (Schamfuß et al. 2013; Furuno et al. 2012). These studies promote the rule of mycelia network as a grid of “pipelines” allowing hydrophobic contaminant distribution in soil improving thereby pollutant bioavailability. Our present research confirmed the existence of such a “pipeline” network in a different fungal genus. Moreover, we clearly

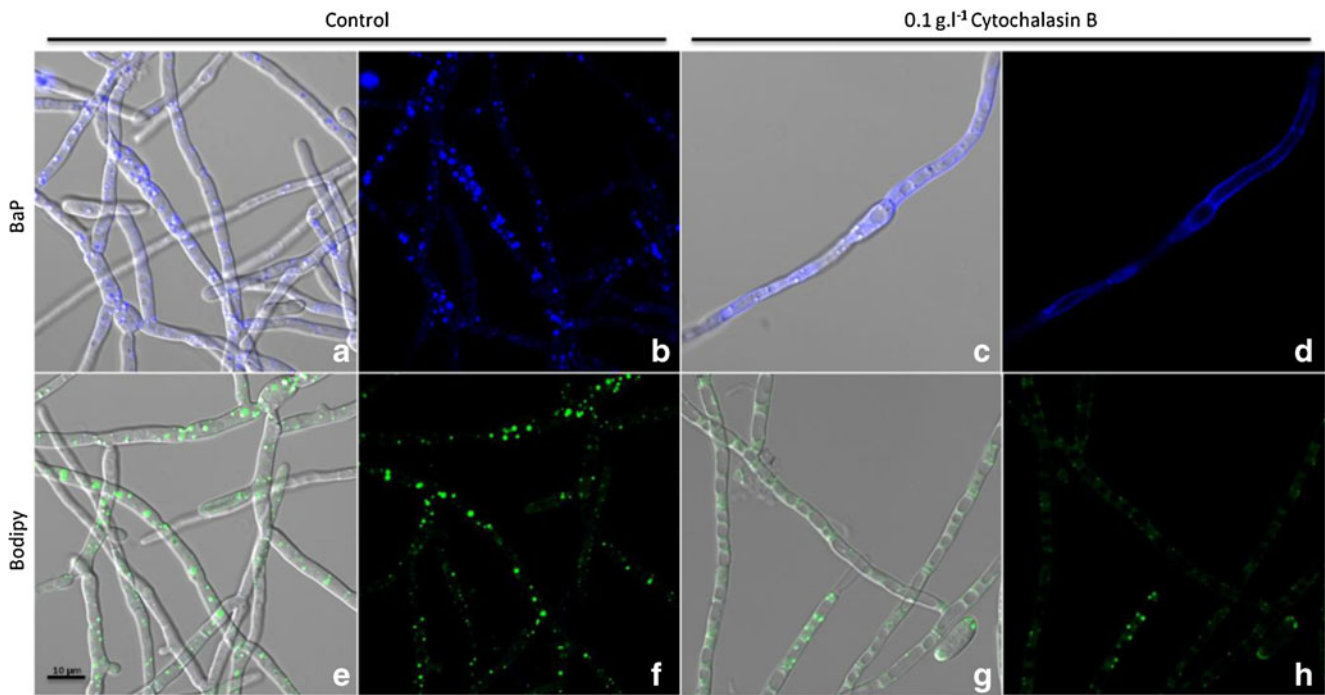


Fig. 5 Effect of the cytoskeleton inhibitor, cytochalasin B (0.1 g L^{-1}), on transport of BaP and BODIPY into *F. solani* lipid bodies. BaP control: DIC (a) and 405 nm laser line (b). BODIPY control: DIC (e) and 488 nm laser line (f). BaP with cytochalasin B treatment under DIC (c) and with

laser scanning confocal microscope (d). BODIPY with cytochalasin B treatment under confocal DIC (g) and with laser scanning confocal microscope (h)

demonstrated, contrary to the current consensus of fungal PAH uptake by diffusion, that *F. solani* cells used an energy-dependent process first for uptake of the pollutant and second for moving it into fungal hyphae. However, further studies could be undertaken in order to study this PAHs uptake in other environmental conditions such as oligotrophic and low energetic ones usually present in soils.

Finally, one limiting step of PAH incorporation into microorganisms is the pollutant bioaccessibility, especially for low water solubility pollutants. Once transfer across the membrane has occurred (defined as bioavailability), intracellular transport via vesicle flux, storage, transformation, degradation or further release can take place within the organism. In this study, in presence of the metabolic inhibitor NaN_3 , an intense red coloration was observed. It was localized in the fungal cell walls because of the accumulation of water diffusible FM4-64 at the biological membrane limits (Fig. 4e). In comparison, no BaP accumulation at the fungal cell walls was observed in NaN_3 treatment (Fig. 4d). This result indicated that the extracellular release of a pollutant with very low water solubility to a more accessible form and its transport to the fungal cells required energy contrary to FM4-64 which diffused passively. This microscopic observation reinforced the hypothesis that a biosynthesis of extracellular surfactant-like molecules could enhance BaP solubilization and its transfer to fungal cells before pollutant uptake across cellular wall and membrane (Veignie et al. 2012).

Conclusion

The use of filamentous fungi in bioremediation may be advantageous in particular in cases for which low substrate bioavailability limits access to competent microorganisms. In this work, we present evidence that an energy-dependent mechanism is involved in BaP, as a model of a high-molecular-weight PAH, entrance into the fungal hyphae of a telluric fungus *F. solani*. Further research is necessary to characterize the molecular mechanisms of this pollutant transmembrane transport and its eventual ubiquity throughout the fungal kingdom. In fungal bioremediation applied to PAH-polluted soil, a diffusion process occurring through the cell wall and plasma membrane has been currently proposed as a potential pollutant uptake mechanism (Schamfuß et al. 2013; Furuno et al. 2012). Such a passive process, being proportional only to the water soluble fraction of the compound, limits significantly the pollutant mass transfer into cells to degradation sites. On the contrary, an energy-dependent mechanism, as demonstrated in this work, may be a promising avenue for enhancing fungal biodegradation of organic pollutants for the following reasons. It would enhance the pollutant quantity incorporated into cells even for those compounds with low water solubility. Consequently, mycelia could then increase the pollutant intracellular transport through dense hyphal networks either to their own intracellular degradation sites if the species considered are themselves good pollutant-degrading

ones, or to other indigenous competent isolates (bacteria or fungi) living in the vicinity (Schamfuß et al. 2013; Furuno et al. 2012). This energetic entrance process could permit a faster PAH incorporation into fungal cells (i.e., 82.9 % of the initial quantity of BaP was incorporated into the fungal biomass after only 48 h). This advantage must be taken under consideration for developing promising monitored fungal-based technologies. The first steps (i.e., pollutant uptake and intracellular transport to storage sites) need to be completed by the study of pollutant degradation that could be occurred either by fungal cells themselves or by microorganisms living in their vicinity.

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