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Short Communication

Toxin production by bacterial endosymbionts of a *Rhizopus microsporus* strain used for tempe/sufu processing

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ABSTRACT

Mould fungi are not only well known for food spoilage through toxin formation but also for the production of fermented foods. In Asian countries, the fermentation of soy beans and tofu for tempe and sufu production with various *Rhizopus* strains is widespread. Here we report the finding of toxinogenic bacteria in a starter culture used for sufu production. By means of metabolic profiling of the fungus under standard conditions for tempe and sufu production, we found that toxins of the rhizoxin complex are produced in critical amounts. Considering that rhizoxins are severe toxins with strong antimitotic activity it is important to notice that our findings uncover a health-threatening symbiosis in food processing. A simple PCR method for detecting toxinogenic endofungal bacteria in starter cultures is proposed.

1. Introduction

Mould (filamentous) fungi are infamous for causing spoilage of food and production of toxic metabolites (Bohra and Purohit, 2003; Richard, 2007). Fungal toxins not only cause massive losses in the food industry, but – more importantly – threaten the health of consumers (Hussein and Brasel, 2001). Mass intoxifications of animals and humans such as the epidemic Turkey 'X' disease and the historical and modern cases of ergotism (St. Antony's Fire) are infamous examples of mycotoxicoses, underscoring the global impact of food spoilage by fungi (Franck, 1984; Möbius and Hertweck, 2009). Furthermore, even low concentrations of mycotoxins in food can elicit deleterious effects, such as chronic or acute toxic damage of liver and kidney (Bohra and Purohit, 2003; Fung and Clark, 2004). On the other hand, mould fungi also play a key role as sources of enzymes in the refinement of agricultural goods, such as cheese (e.g. *Penicillium* spp.) (Weidenbörner, 2008). In Asian countries, the use of Zygomycetes in the fermentation of soy beans to produce tempe and sufu ('soy bean cheese') is more common (Han et al., 2001). However, it is evident that microbial food fermentations may also be hazardous due to the usage of wrong media or unsuitable (i.e. toxigenic) strains, and/or possible microbial contaminations (Daniel and Fung, 2000). In the case of *Rhizopus* spp., two types of mycotoxins, the rhizoxins and the rhizonins, are known that were implicated as potential food contaminants (Jennessen et al., 2005).

Recently, we have discovered the first cases in which mycotoxins are actually not biosynthesized by the fungus, but by 'endofungal' bacteria residing within the cytosol (Partida-Martinez and Hertweck, 2005; Partida-Martinez et al., 2007a; Lackner et al., 2009). We found that various *Rhizopus microsporus* strains harbour bacterial symbionts such as *Burkholderia rhizoxinica* and *Burkholderia endofungorum* (Partida-Martinez et al., 2007c), which produce the antimitotic polyketide macrolide rhizoxin (Partida-Martinez and Hertweck, 2007; Scherlach et al., 2006), and the hepatotoxic cyclopeptide rhizonin (Partida-Martinez et al., 2007b), respectively. Rhizoxin has been shown to be toxic to a variety of human and murine tumor cell lines (Tsuruo et al., 1986). It effectively inhibits cell division by preventing the assembly of microtubules in eukaryotic cells (Takahashi et al., 1987). Rhizonin causes severe hepatic lesions and leads to 100% mortality in rats due to acute and chronic failure of the liver (Wilson et al., 1984).

Here we report the finding of a related toxinogenic symbiosis in a starter culture for sufu fermentation. We demonstrate that these antimitotic toxins are produced even under the standard conditions employed for soy bean fermentation.

2. Materials and methods

2.1. Detection and isolation of bacterial endosymbionts

R. microsporus var. *microsporus* Tieghem (CBS 111563) was cultivated at 30 °C and 80 rpm in VK medium composed of 1% corn

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starch, 0.5% glycerol, 1% gluten meal, 1% dried yeast, 1% corn steep liquor, and 1% $CaCO_3$, pH = 6.5. After 4 days, a small mycelial pellet (0.5 mL) was aseptically taken and submerged in 500 µL 0.85% NaCl. Using mechanical stress (pipetting), the mycelium was broken and then submitted to centrifugation (30 min, 13,200 rpm). A loop of the supernatant was then plated on nutrient agar (Becton, Dickinson and Company, USA) plates. The plates were incubated at 30 °C for several days, until the presence of mycelial or bacterial colonies could be confirmed. Once the first bacterial colonies appeared, they were picked up and cultivated in 1 mL of tryptic soy broth (TSB, Merck, Darmstadt, Germany) at 30 °C and 150–200 rpm until growth could be seen, usually after 2–3 days. Analysis of 16S rRNA genes and microscopy was performed to identify the isolates as *Burkholderia* sp. (Partida-Martinez et al., 2007a; Lackner et al., 2009). For generation of symbiont-free *R. microsporus*, the strain was constantly cultivated in the presence of ciprofloxacin (20–40 µg mL^{-1}, Bayer AG) (Partida-Martinez et al., 2007a).

2.2. Laser microscopy

A sterile cover slip was placed into a potato dextrose agar (PDA) plate, which was previously inoculated with *R. microsporus* var. *microsporus* Tieghem. The cover slip remained in the plate until several hyphae grew on it. The mycelium on the cover slip was incubated in the dark for 15 min with 0.025 µL of SYTO 9 green-fluorescent nucleic acid stain and an equal amount of propidium iodide stain dissolved in 50 µL trypton soy bouillon. Microscopic analysis was performed using a Zeiss LSM 510 Meta Laser Microscope at 480/500 nm (Partida-Martinez and Hertweck, 2005).

2.3. DNA isolation and PCR

Metagenomic DNA from fungal species was obtained using MasterPure™ Yeast DNA purification kit (Epicentre Biotechnologies) with several modifications for fungal strains (Schmitt et al., 2008; Partida-Martinez et al., 2008; Lackner et al., 2009). Mycelium obtained from a 3 d grown culture was separated from the media and after rinsing with water dried with a miracloth paper. The mycelium was ground with glass beads, mixed with 450 µL Yeast Cell Lysis solution and incubated for 60 min at 65 °C under heavy shaking. After 5 min on ice, 225 µL MPC protein precipitation solution (Epicentre Biotechnologies) were added and centrifuged at 10,000 rpm for 10 min. The DNA in the supernatant was precipitated with 750 µL isopropanol and pelleted by centrifugation. After washing with 70% ethanol, the pellet was dissolved in 50 µL TE buffer.

Bacterial DNA was obtained by chloroform/phenol extraction after 3 d incubation of the isolated bacteria in TSB at 30 °C. Bacterial cells were lysed using a sucrose containing buffer, lysozyme and proteinase K. After incubation with RNase A two rounds of phenol/chloroform extraction followed. The DNA was precipitated with isopropanol, washed with 70% ethanol and dissolved in water.

Amplification of polyketide synthase (PKS) genes in extracts of fungal and bacterial DNA was performed using degenerated KS (ketosynthase) primers as described previously (Brendel et al., 2007; Partida-Martinez and Hertweck, 2007).

2.4. Fermentation and metabolic profiling

A. Sufu. 26 g of 2 cm sized commercially supplied tofu chunks were filled into a 300 mL Erlenmeyer flask. To each flask 2 mL 0.8% citric acid solution and 2 mL 2% NaCl solution were added. The flasks were capped with cotton wool and sterilized at 100 °C for 15 min. The tofu chunks were inoculated with a small amount of mycelium of *R. microsporus* var. *microsporus* Tieghem obtained from a 3 d culture grown on a potato-dextrose agar plate. Fermentation of the tofu was accomplished at 23 °C for 24 h to 7 d (Weidenbörner, 2008).

B. Tempe. 500 g of dried soy beans were soaked in water overnight and afterwards cooked by boiling for 1 h. After peeling of the hull, 80 g soy beans were filled in a polypropylene bag and inoculated with *R. microsporus* var. *microsporus* Tieghem as described above. The polypropylene bag was perforated to assure oxygen supply and incubated at 30 °C for 24 h to 96 h (Nout and Kiers, 2005).

The fermented sufu/tempe was chopped into small pieces and extracted with 150 mL ethyl acetate for 24 h. The extract was dried with sodium sulfate and concentrated under reduced pressure. 200 µL of the fatty residue were dissolved in 500 µL methanol. Toxin production was monitored by HPLC analysis. Analytical HPLC was performed on a Shimadzu HPLC system consisting of an autosampler, high-pressure pumps, column oven and a diode array detector (DAD). HPLC conditions (Scherlach et al., 2006): C18 column (Grom Sil 100 ODS 0AB, 3 µm, 250 × 4.6 mm) and gradient elution (MeCN/0.1% TFA (H_2O) 25/75 5 min, in 35 min to MeCN/0.1% TFA (H_2O) 80/20, in 5 min to 100% MeCN), 25 °C, flow rate 0.9 mL min^{-1}, injection volume 20 µL. LC-MS measurements were performed using a Surveyor HPLC system (Thermo Electron, Bremen) coupled to a Finnigan LCQ benchtop mass spectrometer with an electrospray ion source. HPLC conditions: gradient elution (MeCN/0.1% HCOOH 25/75 5 min, in 35 min to MeCN/0.1% HCOOH 80/20, in 5 min to 98% MeCN); flow rate 0.6 mL min^{-1}). All solvents used were of analytical or HPLC grade.

Toxin concentration was measured by integration of peak areas of rhizoxin derivatives at 311 nm and calculated in relation to the peak area of rhizoxin S2 authentic standard (Scherlach et al., 2006).

3. Results and discussion

Zygomycetes, in particular *Rhizopus* spp., are commonly used for the production of fermented soy foods such as tempe and sufu pehtze (Han et al., 2001, 2004). Prompted by our discovery of a toxinogenic *Rhizopus–Burkholderia* symbiosis in the context of rice seedling blight, we have screened a collection of *Rhizopus* spp. representing highly diverse ecological niches and geographic origins for the occurrences of associated bacteria (Lackner et al., 2009). The fungal collection also included a *R. microsporus* var. *microsporus* Tieghem (CBS 111563) from a “rice wine tablet” used as a sufu fermentation starter culture. PCR using universal primers (16S rRNA genes) and total DNA obtained from this strain indicated the presence of bacteria. Sequencing and taxonomic classification revealed that these microorganisms were related to the previously identified toxin-producing bacteria *B. rhizoxinica* and *B. endofungorum* (Partida-Martinez et al., 2007c). The rod-shaped endofungal bacteria were visualized inside the mycelium by confocal laser scanning microscopy using a live/dead stain (Fig. 1). Toxins produced under standardized fermentation conditions for both the isolated symbionts and the fungus harbouring endobacteria were monitored by HPLC-DAD/MS. In both cases, we noted the production of significant amounts of rhizoxin derivatives 1–5. The structures of the metabolites were deduced from retention time, UV and MS in comparison with reference compounds (Scherlach et al., 2006). These results unequivocally prove that the fungal strain used as sufu starter culture harbours toxinogenic endobacteria and thus has, in principle, the potential to secrete toxins into the soy bean fermentation.

However, it is well known that the production of microbial secondary metabolites strongly depends on the fermentation conditions applied and the media used (Scherlach and Hertweck, 2009). In the context of food processing, a famous example is the terpenoid PR-toxin from various strains of *Penicillium roqueforti*, which is used in cheese refinement. However, the toxin is not produced and/or rapidly inactivated under the conditions employed for cheese production (Bullerman and Bianchini, 2007; Sieber, 1978). Likewise, roquefortine from *P. roqueforti* and cyclopiazonic acid from *Penicillium camemberti* are not detected in harmful amounts in blue mould cheese and camembert cheese (Kokkonen et al., 2005; Burdock and Flamm,