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Inhibition of mycotoxin-producing fungi by *Bacillus* strains isolated from fish intestines

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ABSTRACT

Bacillus strains isolated from the aquatic environment of the Brazilian Amazon region were tested for their activity against mycotoxigenic fungi. All tested bacteria showed antifungal activity, inhibiting at least 7 indicator fungi. Four *Bacillus* strains showing promising antifungal results were subsequently evaluated for their activity in reducing mycelial growth rate, sporulation, spore germination percentage, and mycotoxin production. *Bacillus* sp. P1 and *Bacillus* sp. P11 had a remarkable antifungal effect on toxigenic fungi. Washed bacterial cell suspension of strains P1 and P11 (10^7 CFU/ml) reduced by >70% the fungal colony diameters, including a complete inhibition of ochratoxin A (OTA) producing *Aspergillus* spp. Significant reduction of growth rate, sporulation and spore germination were also observed. The bacteria influenced the production of mycotoxins, causing a reduction around 99 and 97% in AFB1 and OTA concentration, respectively. Chromatographic analysis revealed the presence of lipopeptides (iturin A and surfactin isomers) in butanol extracts of cell-free supernatants and cell pellets of strains P1 and P11. Furthermore, antifungal activity of these extracts was confirmed against *A. flavus* A12 and *A. carbonarius* ITAL293, producers of AFB1 and OTA, respectively. These bacterial strains could be promising biocontrol agents against toxigenic fungi.

1. Introduction

Mycotoxins are harmful substances whose occurrence in food, even at low concentrations but consumed for long periods, can result serious injury to human and animal health (Proietti et al., 2014). They represent a group of toxic substances that display varied chemical, physical and toxicological properties, but share the fact that they are secondary metabolites from biosynthetic routes of certain fungi (Pitt and Hocking, 2009; Marin et al., 2013; Ashiq et al., 2014).

Mycotoxin contamination of foodstuffs and agricultural commodities has been reported worldwide. Climatic conditions, associated with inadequate agricultural practices, may determine the occurrence of fungal species and the type of mycotoxins produced (Kumar et al., 2008; Pitt and Hocking, 2009). Species of genera *Aspergillus*, *Penicillium* and *Fusarium* are among the most important toxigenic fungi and are frequently reported in the literature due to the occurrence in foods and potential for mycotoxin production (Barrett, 2000; Simas et al., 2007).

Among all the relevant mycotoxins in food, special mention is made for aflatoxin *B*1 (AFB1) and ochratoxin A (OTA) because of their high toxicity as well as for their carcinogenic, mutagenic and teratogenic properties (Marin et al., 2013; Ashiq et al., 2014; De Ruyck et al., 2015). The International Agency for Research on Cancer (IARC) assessed the carcinogenic potential of these compounds and classified aflatoxins, including AFB1, as carcinogens to humans (Group 1), whereas OTA is categorized into group 2B, a possible carcinogenic agent to humans (IARC, 1993 and 2012). Furthermore, some mycotoxins should receive more attention because of their co-occurrence, as is the case of citrinin, frequently associated with OTA (CAST, 2003; Marin et al., 2013). These toxins may act synergistically increasing the probability of toxic effects on an organism (Gayathri et al., 2015).

In addition to health problems, significant economic losses are associated with mycotoxin contamination at all levels of the food chain. Many strategies have been used to prevent mycotoxin contamination. Chemical, physical and biological methods have been investigated to reduce or eliminate mycotoxins from different foods. However, such methods are frequently limited by economic reasons, partial efficiency, or alteration of food quality parameters (Jouany, 2007; Klich, 2007). Therefore, the prevention of mycotoxin synthesis remains best way to avoid food contamination. The use of chemical fungicides may be a useful strategy to control fungal growth. However, these agents must be completely lethal, otherwise they may stimulate the production of mycotoxins (D'Mello et al., 1998; Matthies et al., 1999), or constant use can result in the development of fungal resistance (Krid et al., 2012). Moreover, there is a need to find safe means of controlling these pathogens due to the impact of fungicides on human health and the environment.

The inhibition of toxigenic fungi by antagonistic microorganisms is an interesting alternative to control mycotoxin contamination in food. Among the most promising candidates to be used as biological control

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agents, the genus *Bacillus* has been intensively investigated due to certain characteristics such as high growth rate, recognition of GRAS status (Generally Recognized as Safe) of some species, ability to form endospores, and production of a large number of antimicrobial substances (Schallmey et al., 2004). The potential of *Bacillus* species to produce lipopeptides with ability to inhibit fungal growth is already known (Chen et al., 2009; Zhao et al., 2010). These compounds are non-ribosomal peptides that have low toxicity, high biodegradability and are classified into three major families: iturins, fengycins and surfactins (Ongena and Jacques, 2008; Caldeira et al., 2011). Iturins and fengycins have strong antifungal activity against a large number of phytopathogenic fungi (Romero et al., 2007a; Arrebola et al., 2010; Yánez-Mendizábal et al., 2011). Although surfactins are not antifungal substances, they have a synergistic action with iturins and fengycins, increasing their antifungal activity (Coutte et al., 2010).

Bacillus-based products are commercially available as biocontrol agents against phytopathogenic fungi (Choudhary and Johri, 2009; Wang et al., 2015). Living cells and natural products derived from *Bacillus* are employed in field crops and greenhouses, at either preharvest or postharvest. They can be applied to the soil or sprayed on the surface of plants, including seeds (Pérez-García et al., 2011; Mnif and Ghribi, 2015). However, these products are not always completely effective in fungal control (Zhang et al., 2016) and therefore the search for new *Bacillus* antagonistic strains is needed.

In recent years, some *Bacillus* strains from the aquatic environment of the Amazon region were investigated for their antimicrobial activity against bacteria (Cladera-Olivera et al., 2004; Motta et al., 2004), viruses (Silva et al., 2014) and non-toxigenic fungi (Velho et al., 2011). However, the knowledge about the antifungal potential of these isolates is still limited, since no studies were conducted on the control of toxigenic fungi and mycotoxin production. The aim of this study was to evaluate the ability of *Bacillus* strains isolated from fish intestines to affect the growth parameters (mycelial growth rate, sporulation and spore germination percentage) of toxigenic fungi *in vitro* and the production of mycotoxins. The ability of these bacterial strains to produce lipopeptides iturin A and surfactins was also investigated.

2. Materials and methods

2.1. Microorganisms and maintenance

Bacillus strains, isolated from intestines of typical fishes of the Brazilian Amazon region (Table 1), were kindly provided by Prof. Spartaco Astolfi-Filho (Universidade Federal do Amazonas, Manaus, Brazil). Some isolates were selected based on their inhibitory activity against phytopathogenic fungi (Velho et al., 2011). *B. subtilis* ATCC 19659 (American Type Culture Collection, Rockville, USA) was also included in the study as a reference strain (Velho et al., 2011).

The following toxigenic isolates of fungi were used in this investigation: *Aspergillus flavus* A12, *Aspergillus* sp. A65 and *A. parasiticus* 30BL (aflatoxin *B*1 producers); *A. carbonarius* ITAL293, *Aspergillus* sp. UMO1B, *Aspergillus* sp. UCO1A, *Aspergillus* sp. UCO2A and *Aspergillus* sp. UCS2B (ochratoxin A producers); *Penicillium citrinum* ITAL197 and *Monascus purpureus* NRRL1992 (citrinin producers); *P. chrysogenum* IFL1 and *P. chrysogenum* IFL2 (roquefortine C producers) and *Fusarium graminearum* (fusarenon X producer). All fungi are kept in the culture collection of the Laboratório de Toxicologia de Alimentos (Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil). The strains *A. carbonarius* ITAL293 and *P. citrinum* ITAL197 were kindly provided by Instituto de Tecnologia de Alimentos (ITAL, Campinas, Brazil).

Table 1
Bacterial strains tested for inhibition of toxigenic fungi.

Bacterial isolates	Origin
Bacillus subtilis ATCC 19659	American Type Culture Collection
Bacillus sp. P1	*Potamorhina latior*
Bacillus sp. P7[a]	*Piaractus mesopotamicus*
Bacillus sp. P11[a]	*Leporinus* sp.
Bacillus sp. P34[a]	*Leporinus* sp.
Bacillus sp. P39A	*Leporinus* sp.
Bacillus licheniformis P40[a]	*Leporinus* sp.
Bacillus subtilis P45B[a]	*Piaractus mesopotamicus*
Bacillus sp. P51	*Semaprochilodus* sp.
Bacillus sp. B312	*Myletus edulis*

[a] Bacteria tested by Velho et al. (2011) against phytopathogenic fungi.

The bacterial cultures were stored on Brain Heart Infusion (BHI; Oxoid, Basingstoke, UK) agar plates at 4 °C or for long term storage at −20 °C in BHI broth with 20% (*v*/v) glycerol and routinely propagated twice from frozen stocks before use. The fungi were maintained on potato dextrose agar (PDA; Acumedia, Lansing, MI, USA) slants covered with mineral oil at 4 °C and subcultured periodically. Working isolates were cultivated onto PDA agar plates.

2.2. Inocula production

Inoculations of bacterial cells were made from the working cultures into BHI broth and incubated at 37 °C for 24 h with constant shaking (125 rpm). The concentration of cell suspension was determined by spectrophotometry (optical density at 600 nm) and viability confirmed by standard plate count method using BHI agar. The concentration of each bacterial suspension was adjusted to obtain approximately 10^7 CFU/ml.

To prepare fungal inoculum, each isolate was grown on PDA plates at 25 °C for 7 days and 12 h photoperiod. Spores were harvested from plates by adding 5 ml of sterile distilled water containing 0.05% (v/v) Tween 80 and with the aid of a Drigalski loop. The suspension was collected and transferred to a sterile tube. Then, the material was filtered through sterile gauze to ensure spores free of any debris. Finally, spores were counted with a Neubauer chamber and the suspensions were used in the experiments.

2.3. Initial selection of potentially antagonistic bacteria

Bacterial isolates were screened for antagonism *in vitro* against the toxigenic fungi. The method described by Benitez et al. (2010) was followed with minor modification. Fifteen milliliters of sterile PDA at 45 °C containing fungal spore suspension (1×10^6 spores/ml) were transferred to Petri dishes and allowed to solidify. The PDA plates were surface inoculated with 20 μl of *Bacillus* liquid culture (10^7 CFU/ml), cultured previously on BHI broth at 37 °C and 125 rpm for 48 h.

The Petri dishes were incubated at 25 °C for 5 days, then the areas of inhibition were measured with a digital caliper. The antagonistic activity was evaluated according to the scale proposed by Bacon and Hinton (2002): −, no antagonism; +, weak antagonism (clear zones of inhibition <3 mm); ++, moderate antagonism (clear zones of inhibition ≥3–9 mm); +++, strong antagonism (clear zones of inhibition >9 mm). The bacteria with more representative results were subjected to further studies.

2.4. Bacterial effect on fungal growth

The most promising four bacterial strains were further tested to determine the effects on fungal growth, colony characteristics and sporulation of two representative producers of AFB1, OTA or citrinin. This evaluation was adapted from Nesci et al. (2005) and Gandomi et al. (2009). Bacteria cells were obtained by centrifugation at 10,000 g for 15 min after growing as previously described. Each bacterial suspension (10^7 CFU/ml) was mixed with 15 ml of molten PDA medium and poured into Petri plates (80 mm diameter). After the plates were cooled, a sterile paper disc (5 mm diameter) was placed at the center of agar surface and 10 μl of fungal spore suspension (10^6 spores/ml) were spotted onto the discs. The control plates were inoculated with test fungi but without bacteria. *B. subtilis* ATCC 19659 was also tested following the same