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Inducing secondary metabolite production by the soil-dwelling fungus *Aspergillus terreus* through bacterial co-culture

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ABSTRACT

The soil-dwelling fungus *Aspergillus terreus* was isolated from sediment collected from the lake of Wadi El Natrun in Egypt. Co-cultivation of *A. terreus* with the bacteria *Bacillus subtilis* and *Bacillus cereus* on solid rice medium resulted in an up to 34-fold increase in the accumulation of constitutively present fungal natural products (**4**–**15**) compared to axenic cultures of *A. terreus*. The fungal products included two new butyrolactone derivatives, isobutyrolactone II (**1**) and 4-O-demethylisobutyrolactone II (**2**), together with the known N-(carboxymethyl)anthranilic acid (**3**) that were not present in axenic fungal controls and were only detected during co-cultivation with *B. subtilis* or with *B. cereus*. The structures of all compounds were unambiguously elucidated by 1D and 2D NMR spectroscopy, and by HRESIMS measurements, as well as by comparison with the literature. In a second set of experiments, *A. terreus* was co-cultured with *Streptomyces lividans* and with *Streptomyces coelicolor*. These co-cultivation experiments failed to induce fungal natural product accumulation in contrast to co-cultures with *Bacillus* sp. Compounds **5** and **14** showed weak inhibition of *B. cereus* with minimal inhibitory concentrations (MICs) of 64 μg/mL, whereas only **8** showed moderate cytotoxicity against the murine lymphoma (L5178Y) cell line with inhibition of 80% at a dose of 10 μg/mL.

1. Introduction

Fungi and bacteria co-exist in complex ecosystems such as soil, water or in the living tissues of plants, where they compete and communicate with each other as well as with other organisms such as algae, protozoans and even their metazoan hosts (in the case of endophytes) (Strobel et al., 2004; Aly et al., 2011; Brakhage and Schroeckh, 2011). It is generally accepted that one of the roles of secondary metabolites is to provide biological advantage for the producer in response to its environment, which implies the presence of sensing mechanisms to control production of metabolites (Chiang et al., 2011). In mimicking the natural microbial environment, co-cultivation of different microbes in one culture vessel (also called mixed cultivation) may lead to an enhancement of the accumulation of constitutively present natural products (Oh et al., 2007; Schroeckh et al., 2009; Nuetzmann et al., 2011) or may trigger the expression of silent biosynthetic pathways yielding new compounds (Oh et al., 2005; Cueto et al., 2001) due to microbial crosstalk and chemical defense (Pettit, 2011).

During our previous studies on inducing new secondary metabolites by co-cultivation, Ola et al. reported that co-culture of the endophytic fungus *Fusarium tricinctum* with *Bacillus subtilis* resulted in an up to 78-fold increase in the accumulation of constitutively present secondary metabolites, in addition to four compounds, including three new compounds, which were not present in axenic fungal or bacterial controls (Ola et al., 2013). These latter compounds were suspected to arise from induction of cryptic biogenetic gene clusters.

In this study, we report on the metabolic response of the soil fungus *Aspergillus terreus* during co-cultivation with *B. subtilis*,

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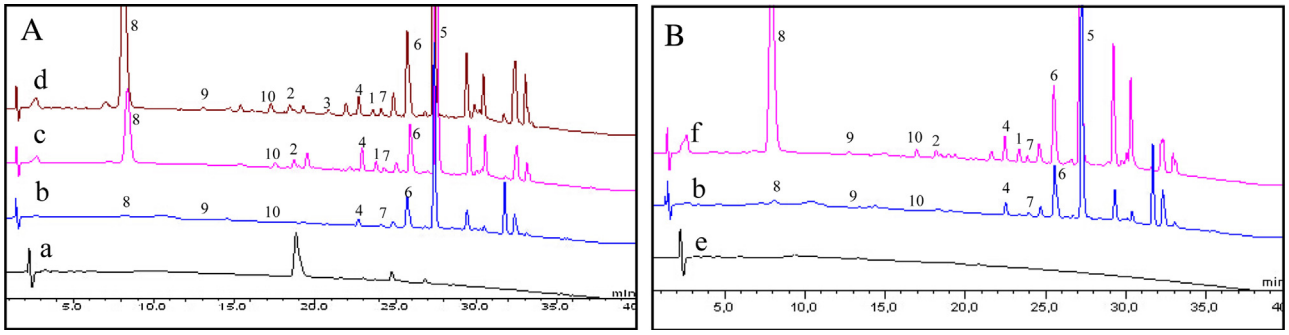

Fig. 1. HPLC chromatograms of EtOAc extracts from co-culture experiments (detection at UV 235 nm): (A) (a) *B. subtilis* control, (b) *A. terreus* control, (c) co-culture of *A. terreus* with autoclaved *B. subtilis*, (d) co-culture of *A. terreus* with viable *B. subtilis*; (B) (b) *A. terreus* control, (e) *B. cereus* control, (f) co-culture of *A. terreus* with *B. cereus*.

Bacillus cereus, *Streptomyces coelicolor* or with *Streptomyces lividans*. The strain of *A. terreus*, which was used for our study, had been isolated from sediment collected from the lake of Wadi El Natrun in Egypt. We found an up to 34-fold increase in the accumulation of constitutively present fungal natural products (**4**–**15**) compared to axenic cultures of *A. terreus* when the fungus was co-cultured either with *B. subtilis* or with *B. cereus*. In addition, three metabolites (**1**–**3**) including the two new compounds isobutyrolactone II (**1**) and 4-O-demethylisobutyrolactone II (**2**), were not present in axenic fungal controls and were only detected during co-cultivation (Fig. 1). Interestingly, when *A. terreus* was co-cultured either with *S. coelicolor* or with *S. lividans*, no induction of fungal natural product accumulation was observed hinting at a specificity of the fungal response toward different bacteria.

2. Results and discussion

A. terreus is well known for the production of butyrolactones (Nuclear et al., 2010). When *A. terreus* was cultured axenically on solid rice medium, average yields of the main butyrolactone derivatives per culture flask were 0.65 mg for butyrolactone II (**4**) (Nitta et al., 1983), 117.1 mg for butyrolactone I (**5**) (Kiriyama et al., 1977; Rao et al., 2000), 19.6 mg for butyrolactone III (**6**) (Rao et al., 2000), and 0.86 mg for butyrolactone VI (**7**) (Nuclear et al., 2010). During co-cultivation of *A. terreus* with *B. subtilis*, with autoclaved *B. subtilis* or with *B. cereus* a strong enhancement of butyrolactone accumulation was observed (Table 1). During co-cultivation with *B. subtilis*, the average production of butyrolactones per flask reached 1.94 mg for butyrolactone II (**4**), 290.3 mg for butyrolactone I (**5**), 56.87 mg for butyrolactone III (**6**), and 2.04 mg for butyrolactone VI (**7**), which accounted for a 2.4–3.0 fold increase of the latter metabolites compared to axenic fungal controls (Table 1). Interestingly, when *A. terreus* was co-cultivated with autoclaved *B. subtilis*, a similar induction of butyrolactone accumulation was observed which resulted in an average production per flask of 2.16 mg for butyrolactone II (**4**), 390.6 mg for butyrolactone I (**5**), 30.6 mg for butyrolactone III (**6**), and 1.39 mg for butyrolactone VI (**7**) (1.5–3.3 fold increase). These data indicate that even heat sterilized bacterial biomass and culture media cause an induction of fungal natural products accumulation similar to adding live bacterial cultures. Co-culturing of *A. terreus* with *B. cereus* resulted in a similar induction of butyrolactone accumulation as observed before, accounting for a 1.8–3.3 fold increase compared to controls (Table 1), which is in accordance with the aforementioned data (Table 1).

A similar trend was observed with regard to the induction of orsellinic acid (**10**) (Xu et al., 2014) and terrein (**8**) (Dunn et al., 1975) during co-cultivation of *A. terreus* with *B. subtilis* or with *B. cereus*. The latter compound, which is a typical constituent of *A. terreus*, was strongly enhanced during co-cultivation leading to an up to 34-fold increase during co-cultivation of *A. terreus* with *B. subtilis*, with autoclaved *B. subtilis* or with *B. cereus*, respectively. However, no clear induction was detected for dihydroterrein (**9**) (Hosoe et al., 2009) compared to the fungal control, indicating that the effects of co-culturing are not uniform for all fungal compounds.

In addition to the increase of constitutively present metabolites, two new compounds (**1** and **2**) that were only observed in co-cultures of *A. terreus* with *B. subtilis*, with autoclaved *B. subtilis* or with *B. cereus*, as well as the known N-(carboxymethyl)anthranilic acid (**3**) that was only detected when co-culturing *A. terreus* with *B. subtilis*, were isolated (Fig. 2).

Compound **1** was obtained as a yellow gel. The molecular formula was determined as $C_{18}H_{16}O_6$ on the basis of the prominent ion peak at *m/z* 329.1018 $[M+H]^+$ observed in the HRESIMS spectrum, requiring 11 degrees of unsaturation. Compound **1** displayed UV absorbances at λ_{max} 201, 224, and 313 nm, typical for butyrolactone derivatives. Inspection of the ^{1}H NMR of **1** indicated the presence of four symmetrical doublets at δ_H 7.72 (2H, d,

Table 1
Yield of induced metabolites per flask during co-culture of *A. terreus* and *Bacillus.* sp. ($n = 4$) vs axenic controls of *A. terreus* ($n = 4$).

Compound	Control[a] (mg)	*A. terreus* vs. *B. subtilis* (mg)	Increase (fold)	*A. terreus* vs. autoclaved *B. subtilis* (mg)	Increase (fold)	*A. terreus* vs. *B. cereus* (mg)	Increase (fold)
1	n.d.	2.33 ± 0.35		2.89 ± 0.47		3.01± 0.42	
2	n.d.	1.57 ± 1.79		2.15 ± 1.18		1.45± 0.98	
3	n.d.	1.61 ± 0.00011		n.d.		n.d.	
4	0.65 ± 0.05	1.94 ± 0.001	3.0	2.16 ± 0.009	3.3	2.13 ± 0.07	3.3
5	117.1 ± 0.27	290.3 ± 6.68	2.5	390.6 ± 6.68	3.3	439.4 ± 37.1	3.7
6	19.59 ± 2.81	56.87 ± 4.35	2.9	30.64 ± 2.27	1.5	44.13 ± 2.36	2.3
7	0.86 ± 0.24	2.04 ± 0.31	2.4	1.39 ± 0.20	1.6	1.57± 0.38	1.8
8	11.7 ± 6.66	380.4 ± 72.2	32.5	184.5 ± 31.1	15.7	397.5 ± 185.9	33.9
9	0.28 ± 0.31	0.33 ± 0.35	1.2	n.d.		0.27 ± 0.29	1.0
10	0.36 ± 0.23	1.68 ± 0.29	4.8	0.97 ± 0.09	2.7	0.99 ± 0.18	2.8

n.d.: not detected.
[a] *A. terreus* axenic control.