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Taxonomy of mycelial actinobacteria isolated from Saharan soils and their efficiency to reduce aflatoxin B1 content in a solid-based medium

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Abstract Aflatoxin B1 (AFB1) is a carcinogenic compound produced by filamentous fungi. In order to reduce AFB1 occurrence in foodstuffs, 13 strains of mycelial actinobacteria were tested *in vitro* for the efficacy to reduce AFB1 content, all were isolated from the Saharan soils of Algeria. Firstly, morphological study and molecular analysis, based on the 16S rRNA gene, indicated that these strains belong to *Actinomadura*, *Nocardiopsis*, *Nonomuraea*, *Saccharothrix* and *Streptomyces* genera. Secondly, strains efficacy to reduce pure AFB1 content was studied in ISP2-medium. After a 4-day incubation at 30 °C on AFB1-supplemented medium (5 ppm of AFB1), AFB1 was extracted and quantified. AFB1 content was reduced by all the strains (42.9 to 97.6 %). The three most efficient reducers (94.9 to 97.6 %) were two strains belonging to the genus *Streptomyces* and one to the genus *Saccharothrix*. Among those latter, ACD6 and ABH19 strains showed no adsorption mechanism involved, suggesting a potential degradation mechanism. These findings led us to suggest that these actinobacterial strains could be used as decontamination treatments for the reduction of AFB1 content.

Keywords Mycelial actinobacteria · Taxonomy · Molecular identification · Aflatoxin B1 decontamination

Introduction **Revoir ce qui est jaune (ligne 51) « Recently, was detected ???)**

Aflatoxin B1 (AFB1) is a secondary metabolite produced mainly by two closely related fungi, *Aspergillus flavus* and *A. parasiticus* (Ellis et al. 1991) This mycotoxin is carcinogenic, mutagenic, hepatotoxic and immunosuppressive (Guengerich et al. 1996; Hussein and Brasel 2001) and its presence is reported worldwide in several food and feed. Recently, was detected in wheat grains in India (Toteja et al. 2006), maize in Italy and Iran (Giorni et al. 2007; Karami-Osboo et al. 2012) and dried fruit in Pakistan (Ghosia and Arshad 2011). Thus, numerous physical and chemical methods for AFB1 decontamination were investigated, including thermal inactivation, gamma irradiation, UV irradiation (Ghanem et al. 2008; Herzallah et al. 2008; Ashik 2015, Jalili 2016) and ammoniation, acid treatment, ozonation and chlorine (Bozoğlu 2009; Ashik 2015; Jalili 2016), respectively. Unfortunately, these methods have their limits including: high stability of AFB1, potential toxic residues and treatment cost.

Biological treatments are promising approaches against AFB1 accumulation with a low risk of residual toxicity or modification of food properties. Several bacteria reviewed in Verheecke et al. 2016, non-mycelial bacteria [e.g.: *Bacillus subtilis*, *Pseudomonas solanacearum* (Nesci et al. 2005)], yeast [e.g.: *Candida albicans*, *Pichia anomala* (Hua 1999)], non-toxigenic *A. flavus* (Ehrlich 2014) and *Streptomyces* strains (Verheecke et al. 2014) revealed

promising results as biological treatment against AFB1 accumulation. However, non-*Streptomyces* actinobacteria have never been tested for their biological treatment against AFB1 contamination.

To selectively isolate rare mycelial actinobacteria several methods have been used including dry heating of soil samples (Zakharova et al. 2003), freezing of marine sediment samples (Bredholdt et al. 2007), phenolic treatment (Istianto et al. 2012) and selection media such as chitin-vitamin medium (Hayakawa and Nonomura 1987), which contains chitin as the sole source of carbon and nitrogen. Actinobacteria better degrade this polymer compared to other microorganisms. Moreover, the addition of water soluble B vitamins in this medium promote growth of rare actinobacterial strains, which are generally auxotrophic. This medium is successfully used for the isolation of new taxa of rare mycelial actinobacteria from Algerian Saharan soils (Saker et al. 2014; Bouras et al. 2015; Meklat et al. 2015).

Algerian Saharan soils in arid climate are attractive sources of several non-*Streptomyces* genera, such as *Actinomadura*, *Actinopolyspora*, *Amycolatopsis*, *Nocardiopsis*, *Saccharopolyspora* and *Saccharothrix* (Sabaou et al. 1998). In these extreme habitats, these microorganisms have already been identified as source of bioactive compounds (Merrouche et al. 2010; Aouiche et al. 2012; Boubetra et al. 2013). These non-*Streptomyces* genera could also be promising biological treatment for AFB1 decontamination.

The present study aimed to isolate and identify mycelial actinobacteria, and to detect their ability to reduce AFB1 content in a solid-based medium.

Materials and Methods

Isolation of actinobacterial strains ligne 86 (en jaune: est-ce qu'on met « of prevalent » ? On ajoute « of » ou non ?

Mycelial actinobacteria strains were isolated from five soils samples collected from three Algerian Saharan regions, Adrar (latitude, 27°53'N; longitude, 0°17'), Béni-Abbès (latitude, 30°7'N, longitude, 2°10'W) and Tamanrasset (latitude, 22°47'N, longitude, 5°31'E). To promote the growth prevalent and rare mycelial actinobacteria, the isolation was made on chitin-vitamin agar medium by using the standard dilution plate method, (Hayakawa and Nonomura 1987). The medium was supplemented with 80 mg/L of cycloheximide to suppress the growth of fungi. After 21 days of incubation at 30 °C, the actinobacterial strains were picked, purified and preserved on International *Streptomyces* Project (ISP) 2 medium (Shirling and Gottlieb 1966) at 4 °C.

Taxonomic study of actinobacterial strains

Morphological study

The morphological and cultural characteristics of actinobacterial strains were determined on the ISP media: yeast extract-malt extract agar (ISP2), oatmeal agar (ISP3) and inorganic salts-starch agar (ISP4) (Shirling and Gottlieb 1966), and also on the Bennett medium (Waksman 1961). After incubation at 30 °C for 14 days, colors of aerial mycelia, substrate mycelia and diffusible pigments were determined using the ISCC-NBS color name chart (Kelly and Judd 1976). The micromorphology and sporulation of strains were examined by naked eye and by light microscope (Motic, B1 Series, Hong Kong).

Molecular study for actinobacteria identification

The actinobacterial colonies were removed aseptically from ISP2 medium and transferred to 1.5 mL sterile Eppendorf tubes. Genomic DNA was extracted according to the method of Liu et al. (2006). PCR amplification of the 16S rRNA gene sequence was performed by using two universal primers: 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'). The 16S rRNA gene was PCR-amplified in 50 µl of reaction mixture, using 25 to 50 ng of genomic DNA, 0.5 µM of each primer, 1X PCR buffer, 10 µM deoxynucleoside triphosphate mixture and 0.4 U *Taq* DNA polymerase. The amplification was performed as follows, initial DNA denaturation at 98 °C for 4 min, 30 cycles of: denaturation at 94 °C for 1 min, primers annealing at 52 °C for 1 min and extension at 72 °C for 2 min and a final elongation at 72 °C for 10 min before cooling at 4 °C. The PCR products were analyzed by agarose gel electrophoresis, and shipped to Beckman Coulter Genomics (United Kingdom) for purification and sequencing.

The sequences obtained were compared with sequences in EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al. 2012). Phylogenetic analyses were conducted using MEGA version 5 (Tamura et al. 2011). 16S rRNA gene sequences of the 13 strains were aligned against neighboring nucleotide sequences using CLUSTAL W (with default parameters) (Thompson et al. 1994). A phylogenetic tree was constructed by using the neighbor-joining (Saitou and Nei 1987) with Jukes and Cantor (1969) model. To evaluate the reliability of the tree topology, a bootstrap analysis (Felsenstein 1985) was performed.

Effect of actinobacterial strains on pure aflatoxin B1

Culture media

Five milligrammes of pure AFB1 (Sigma-Aldrich, Saint-Quentin-Fallavier, France) were solubilized in 5 mL of methanol. The solution was added to one litre of ISP2 medium to obtain a final concentration of 5 ppm (Verheecke et al. 2014). The following two controls were included: (a) sterile ISP2 solid medium, (b) sterile ISP2 solid medium supplemented with 5 ml/L of methanol. Spores of actinobacterial strains were inoculated on the media by completely covering the Petri dish surface and were incubated at 30 °C for 96 h. The experiment was performed twice in triplicates.

Extraction and quantification of residual AFB1

AFB1 was extracted as described by Verheecke et al. (2014). A volume of 10 µL of each sample was injected into the HPLC system (Ultimate 3000, Dionex, FR) coupled with a coring cell (Diagnostix Gmbh, GE). A reverse phase C18 column (250 × 4.6 mm; 3 µm, Kinetex, Phenomenex) was used. The mobile phase consisted of acetonitrile: methanol: water (20: 20: 60, v/v/v) with 119 mg/L of potassium bromide and 100 µL/L of 65 % nitric acid. The total run time was 35 min at a flow rate of 0.8 mL/min at 25°C. The AFB1 absorbance was measured by a fluorescence detector (Ultimate 3000, RS Fluorescence Detector, Dionex) at an excitation wavelength λ = 362 nm and an emission wavelength λ = 440 nm. Areas under AFB1 absorbance peak were used to estimate the AFB1 residual content in medium (rcm). The analyses were carried out using Chromeleon software thanks to standards of AFB1. The recovery ratio was 50 %.

AFB1 adsorption tests

The adsorption tests were performed as described by Verheecke et al. (2015). Briefly, strains ACD6 and ABH19 were placed in a glass vial at a concentration of 10^6 spores/mL containing an AFB1 concentration of 1 µg/mL. After 1 or 60 min incubation at 30 °C, the supernatant is filtered and transferred into vial. The filter is rinsed twice: once with sterile water (1 mL) and once with methanol, and the rinse liquids were also quantified by HPLC.

Statistical analysis

All the data are graphically presented as mean ± standard deviation (n = 6). Non parametric statistical analysis was performed using R (version 3.2.2); the package 'nparcomp' was used and the contrast method was Tukey with a confidence level of 95 % and a logit transformation.

Results

Strains isolation

The mycelial actinobacteria strains from Saharan soils were isolated using chitin-vitamin agar medium. After 3 weeks of incubation at 30 °C, 13 colonies presenting micromorphological characteristics of actinobacteria were picked out and purified.

Taxonomic characterization of actinobacterial strains

Morphological and cultural characteristics

According the morphological tests (especially micromorphological characteristics) the 13 actinobacteria strains were classified into three groups.

The first group includes 7 strains: ABH1, ABH2, ABH5, ABH9, ABH11, ABH16 and ABH25. Their aerial mycelium is white, yellowish to pale brown, with the exception of ABH1 (yellowish pink) and ABH5 (yellowish orange). Their substrate mycelium is yellowish to pale brown except for ABH1 (pink to orange-pink). Each strain formed a well-branched substrate mycelia with little fragmentation on agar media. Aerial mycelium is well developed, fragmented anarchically and is often with zigzag into long chains of non-motile and elongated spores of different sizes. These characteristics belong to *Nocardiopsis* and/or *Saccharothrix* genera (Hozzein and Trujillo 2012; Labeda 2012).

The second group contains 4 strains: ABH21, ACD2, ACD7 and ACD12. The aerial mycelium is white for ABH21, ACD2 and ACD12, and is yellowish/pink for ACD7. The substrate mycelium is orange to brownish orange for ABH21, and white to pale beige for ACD2, ACD12 and ACD7. These four strains were found to form a sterile substrate mycelium. However, the aerial mycelium was found to bore short chains of spores on short sporophores; these chains are straight, flexuous to hooked (ABH21, ACD2 and ACD7) or irregular spirals (ACD12). All these characteristic belong to *Actinomadura* and/or *Nonomuraea* genera (Hozzein and Trujillo 2012; Labeda 2012).

The third group contains 2 strains, ABH19 and ACD6. Their aerial mycelium is pinkish white to yellowish pink and the substrate mycelium is light beige. These strains have both a non-fragmented substrate mycelium and a well-developed aerial mycelium. The latter has long chains of spores that are irregular spirals and carried by long sporophores. These are characteristic of *Streptomyces* genus (Kämpfer 2012a).

16S rRNA gene sequencing and phylogenetic analyses

The morphology of the strains was confirmed by the phylogenetic study (Fig. 1). Indeed, the strains of group 1 belong to *Nocardiopsis* and *Saccharothrix* genera, those of group 2 to *Actinomadura* and *Nonomuraea* genera, and those of group 3 to *Streptomyces* genus.

The strains ABH1, ABH2 and ABH11 were related to *Nocardiopsis sinuspersici*, with a similarity of 99.4, 98.8 and 99.0 %, respectively. Strain ABH5 was similar by 100 % to *Nocardiopsis halotolerans*. Strain ABH9 is closely related to *Nocardiopsis arvandica* (99.4 %), while strain ABH16 likely belongs to *Nocardiopsis dassonvillei* subsp. *dassonvillei* (99.8 %). Moreover, strain ABH25 was related to *Saccharothrix carnea* with a similarity of 99.7 %.

Strains from the second morphological group were related to the *Actinomadura* and *Nonomuraea* genera. Strain ABH21 exhibited 100 % sequence similarity with *Nonomuraea dietziae*. Strains ACD2, ACD7 and ACD12 were most closely related to *Actinomadura meyerae* (99.1 %), *Actinomadura apis* (99.5 %) and *Actinomadura sputi* (98.3 %).

Strains from the third morphological group (ABH19 and ACD6) showed both 99.6 and 99.7 % sequence similarities to *Streptomyces smyrnaeus*.

For each of the 16S rRNA gene sequences from actinobacterial strains, a GenBank accession numbers was assigned (Fig. 1). Dois t-on mettre: "…… strains, GenBank accession numbers were assigned" (a Genbank ou GenBank? Et was ou were?).

Reduction of of AFB1 content by actinobacteria

Thirteen actinobacterial strains were inoculated in an AFB1-supplemented medium (5 ppm) and incubated for 4 days at 30 °C. All strains showed no macroscopic difference between the two control media (sterile ISP2 medium and sterile ISP2 medium supplemented with methanol). Supplementation of AFB1 led to an inhibition of aerial mycelium development in two strains, ABH16 and ABH21.

The final AFB1 content in the medium was analysed for each strains. HPLC results are shown in Table 1. Strains ACD12, ABH5 and ACD2 moderately reduced the AFB1 content (rcm between 57.03 and 53.60 %). Strains ABH11, ABH9, ABH2, ABH21, ABH1, ACD7 and ABH16 showed a more significant reduction in the AFB1 content (rcm between 44.93 and 27.37 %). Finally, strains ABH25, ACD6 and ABH19 were extremely efficient in the reduction of AFB1 content (rcm between 5.10 and 2.40 %) and revealed a peak emergence in their HPLC profile (Fig. S1). Moreover, ABH19 and ACD6 strains were further tested for adsorption. For both strains, the results showed no significant AFB1-adsorption in comparison to the control.

Discussion

La phrase en bas signifie: Treize souches d'actinobactéries mycéliennes ont fait l'objet tout d'abord d'une étude morphologique (on devrait mettre en principe : Thirteen strains of mycelial actinobacteria were first subjected to a morphological study). Est-ce ça ?

In this study, 13 strains of mycelial actinobacteria were obtained and sustained in the first step of the morphological study.

According to the micromorphological characteristics, the 13 actinobacterial strains belong to *Nocardiopsis* (strains ABH1, ABH2, ABH5, ABH9, ABH11 and ABH16) (Meyer 1976), *Saccharothrix* (strain ABH25) (Labeda et al. 1984), *Actinomadura* (strains ACD2, ACD7 and ACD12) (Lechevalier and Lechevalier 1970), *Nonomuraea* (strain ABH21) (Zhang et al. 1998) and *Streptomyces* (strains ABH19 and ACD6) genera (Holt et al. 1994).

The most closely related species were determined by molecular study based on the 16S rRNA gene. The obtained results showed that some strains may be new species. Indeed, ACD12 showed a similarity value under 98.65 %, the threshold to new species proposed by Kim et al. (2014). While some strains, such as ABH5, ABH16, ABH25 and ABH21, have very high percentages of similarity with some species of *Nocardiopsis*, *Nonomuraea* and *Saccharothrix* (99.7 to 100 %), other strains (in addition to ACD12), such as ABH2, ABH11, ABH9, ABH1 and ACD2, have relatively low percentages of similarity with species of *Nocardiopsis* and *Actinomadura* (98.8 to 99.4 %). High 16S rRNA gene similarity values were found between representatives of validly described *Nocardiopsis* and *Actinomadura* species, such as the type strains of *Nocardiopsis valliformis* and *N. exhalans* (99.9 %) (Yang et al. 2008), *N. sinuspersici* and *N. arvandica* (99.9 %) (Hamedi et al. 2011), *N. halophila* and *N. baichengensis* (99.9 %) (Li et al. 2006), *N. litoralis* and *N. kunsanensis* (99.6 %) (Chun et al. 2000), *N. metallicus* and *N. exhalans* (99.4 %) (Schippers et al. 2002), *Actinomadura kijaniata* and *A. namibiensis* (99.2 %) (Wink et al. 2003) and *A. coerulea* and *A. verrucosospora* (99.2 %) (Preobrazhenskaya et al. 1975). This strongly suggests the presence of several new

246 species of *Nocardiopsis* and *Actinomadura*, especially for the strains ACD12, ABH2 and ABH11 (similarity below
247 or equal to 99.0 %), but also ABH1, ABH9 and ACD7 (similarity between 99.1 and 99.4 %).
248 In our study, AFB1 inhibited aerial mycelium in strains ABH16 and ABH21. The same observation was
249 reported by Reiss (1971) on fungal strains exposed to AFB1 and other aflatoxins. Verheecke et al. (2014) observed
250 in the presence of AFB1 a lack of pigmentation in *Streptomyces* strains.
251 The 13 tested actinobacterial strains strongly reduce the AFB1 rcm in the medium (rcm between 57.03 and 2.4
252 %). Two strains of *Streptomyces* (ABH19 and ACD6) and a strain of *Saccharothrix* (ABH25) showed efficient
253 reduction in AFB1content (rcm between 5.10 and 2.40 %), with emergence of a new peak in their chromatograms.
254 This new peak was not detected in presence of the other ten actinobacterial strains (related to *Actinomadura*,
255 *Nocardiopsis* and *Nonomuraea* genera) and in controls (methanol extraction of strains grown in the absence of
256 AFB1). The ability of *Streptomyces* strains to reduce AFB1 contamination was previously reported (Zucchi et al.
257 2008; Verheecke et al. 2014; Harkai et al. 2016). Tests have shown that AFB1 reduction by *Streptomyces* is not
258 linked to adsorption mechanisms (Verheecke et al. 2015). In the present work, we also report no adsorption by the
259 two *Streptomyces* strains, ACD6 and ABH19. This is the first time that a strain in the genus *Saccharothrix* has been
260 shown to reduce AFB1. The new peaks could represent side-products generated from potential degradation of AFB1
261 or a metabolite produced by the actinobacterial strains in the presence of AFB1 (Wang et al. 2011; Eshelli et al.
262 2015).
263
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268
269 **Conflict of Interests**
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271 The authors declare that there is no conflict of interests regarding the publication of this paper.
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Table 1 Impact of actinobacterial strains on AFB1 content in a solid medium

Strain	AFB1 (rcm* in %)
Control	100.00 ± 5.59[a]
ACD12	57.03 ± 7.68[b]
ABH5	53.98 ± 7.07[b,c]
ACD2	53.60 ± 8.44[b,c]
ABH11	44.93 ± 7.96[c]
ABH9	41.41 ± 3.6[c,d]
ABH2	36.60 ± 5.18[c,d]
ABH21	35.60 ± 6.74[c,d]
ABH1	34.93 ± 3.85[d]
ACD7	33.73 ± 5.96[c,d]
ABH16	27.37 ± 7.02[d]
ABH25	5.10 ± 0.67[e]
ACD6	4.53 ± 0.92[e,f]
ABH19	2.40 ± 1.00[f]

Data with the same letter are not significantly different ($P < 0.05$)

* rcm: residual content in the medium

Legends of figures

Fig. 1 Neighbor-joining phylogenetic tree based on almost complete 16S rRNA gene sequences showing taxonomic position of actinobacterial isolates of Saharan soils. Bootstrap values (>50%) based on 1,000 resamplings are shown at branch nodes. The numbers between brackets are the accession numbers. Bar, 0.01 substitutions per site

Electronic Supplementary Material

Legends of figures

Fig. S1 HPLC chromatograms showing AFB1 peaks. (A): AFB1 peak in uninoculated AFB1-supplemented medium, (B): AFB1 peak after inoculation by the strain *Saccharothrix* sp. ABH25. The arrow indicates the emerged peak

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