RESEARCH LETTER

An endophytic taxol-producing fungus from *Taxus x media*, *Aspergillus candidus* MD3

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Abstract

An endophytic taxol-producing fungus (strain MD3) isolated from the inner bark of *Taxus x media* was identified as *Aspergillus candidus* according to its morphological characteristics, physiological and biochemical characteristics, and 18S rRNA gene sequence analysis. Taxol produced by *A. candidus* MD3 was shown to be identical to authentic taxol analyzed by UV, HPLC, MS and nuclear magnetic resonance spectroscopy. The gene encoding the 10-deacetylbaccatin III-10-*O*-acetyl transferase, which catalyzes formation of the last diterpene intermediate in the taxol biosynthetic pathway, has been cloned from *A. candidus* MD3 for the first time and possesses high homology to the same gene found in *Taxus* spp.

Introduction

Taxol was originally isolated and characterized from the bark of *Taxus brevifolia* (Wani *et al.*, 1971). It is an important anticancer agent used widely for clinical applications and found in extremely small quantities in all *Taxus* species (Wang *et al.*, 2000). Since 1993, observations of taxol-producing fungi, *Taxomyces andreanae* and *Pestalotiopsis microspora*, have been reported, demonstrating that microorganisms can produce taxol (Stierle *et al.*, 1993; Strobel *et al.*, 1996; Zhou *et al.*, 2007). So far, more than 30 taxol-producing fungi have been reported globally, most of them endophytes of *Taxus* spp. belonging to ascomycetes and imperfect fungi (Ji *et al.*, 2006). Thus, fermentation processes using taxol-producing microorganisms may be an alternative way to produce taxol (Wang *et al.*, 2000).

Recently, an endophytic taxol-producing fungus has been successfully isolated from the inner bark of *Taxus x media* in our laboratory. The purpose of this work was to identify this taxol-producing endophytic fungus (strain MD3), provide evidence of taxol production by strain MD3 and clone the 10-deacetylbaccatin III-10-*O*-acetyl transferase (DBAT) gene from strain MD3.

Materials and methods

Isolation of strain MD3

Strain MD3 was one of 30 endophytic fungi isolated from the inner bark of *T. media* obtained on the campus of Huazhong University of Science and Technology (114°31′E, 30°36′N), Hubei province, central China. The yew bark was cut into pieces (*c.* $0.5 \times 0.5 \times 0.5$ cm). The bark pieces were treated with 70% (v/v) ethanol, washed with sterilized water and the outer bark was removed with a sterilized sharp blade. Small pieces of the inner bark were placed on the surface of potato dextrose agar (PDA) medium supplemented with 50 μg mL^{-1} ampicillin in Petri dishes. After several days, fungi were observed growing from the bark fragments. Individual hyphal tips of the various fungi were removed from the agar plates,

placed on new PDA medium, and incubated at 25 °C for at least 10 days. Each fungal culture was checked for purity and transferred to another PDA plate by the hyphal tip method (Strobel *et al.*, 1996). The fungal isolates were numbered and stored on the surface of PDA plate at 4 °C or as spores and mycelia in 15% (v/v) glycerol at − 70 °C.

Morphological observation

Strain MD3 was grown on the surface of Czapek yeast autolysate agar (CYA) medium at 25 °C for 2 weeks for identification based on the morphology of the fungal colony, the mechanism of spore production and the characteristics of the spores.

For scanning electron microscopy, the spores of strain MD3 were suspended in 0.9% sodium chloride solution and coated on a coverslip. The samples were then critically point-dried, gold-coated with a sputter coater and observed and photographed with an FEI Sirion 200 field emission scanning electron microscope.

18S rRNA gene sequence analysis

The genomic DNA of strain MD3 was extracted using the sodium dodecyl sulfate–cetyl trimethyl ammonium bromide method (Kim & Mauthe, 1990). A reaction mix of PCR contained 30–100 ng of the genomic DNA of strain MD3, 100 μM each of 18S-F1 (5′-GATCCTGCCAGTAGTCA TATGC-3′) and ITS2 (5′-GCTGCGTTCATCG ATGC-3′), 150 μM dNTPs in 1 × PCR reaction buffer, 2.5 U *Taq* DNA polymerase (New England Biolabs), and the total volume was brought to 50 μL with deionized water. The thermal cycling condition was hot started for 6 min at 95 °C followed, after adding 2.5 U *Taq* polymerase, by 30 cycles of 50 s at 94 °C, 50 s at 53 °C and 2 min at 68 °C, and additionally 10 min at 68 °C. The PCR products were purified using the Gel Extraction Kit (Watson, China) ligated to the sequencing vector pGEM-T easy vector, and sequenced by Beijing Aoke Biological Engineering Technology and Service Co. Ltd. The 18S rRNA gene sequence of strain MD3 was analyzed online with BLAST in the GenBank database (http:/www.ncbi.nlm.nih.gov/BLAST).

Taxol isolation and quantification

Strain MD3 was inoculated into a 1-L Erlenmeyer flask containing 300 mL potato dextrose liquid medium and cultured with 120 r.p.m. shaking at 25 °C for 10 days. The mycelia of strain MD3 were then harvested by centrifugation at 12 000 ***g*** for 10 min and dried at 45 °C overnight. Dried mycelia were crushed and extracted with 6 mL methanol : chloroform (1 : 1, v/v) three times. The extracts were concentrated under reduced pressure at 50 °C and dissolved in 1 mL 100% methanol (v/v), and individual components were separated by thin layer chromatography (Strobel *et al.*, 1996; Wang *et al.*, 2000). The band corresponding to authentic taxol (Sigma, Cat. No. T-7402) was dissolved in 0.5 mL 100% methanol. After purification, the fungal taxol isolated from strain MD3 was estimated by its UV absorption at 273 nm with a Beckman DU7500 spectrophotometer. Its millimolar absorption coefficient, ε, at this wavelength is 1.7 (Strobel *et al.*, 1996).

HPLC

A C_{18} column (3.9 × 300 mm, 10 μm, Waters, uBondpak) was used to determine the behavior of the fungal taxol with HPLC. Samples in 10 μL of methanol were injected and elution was done with methanol : H_2O (65 : 35, v/v). A variable wavelength recorder set at 228 nm was used to detect compounds eluting from the column.

Spectroscopic analyses

Mass spectroscopy was done on taxol samples using the electrospray technique with an Agilent 1100 LC/MSD trap. The sample was dissolved in 100% methanol and was injected with a spray flow of 2 μL min^{-1} and a spray voltage of 2.2 kV using the loop injection method.

Nuclear magnetic resonance (NMR) spectroscopy was done on taxol samples dissolved in 100% deuterated methanol with a Bruker 400 MHz instrument. The sample was subjected to 2048 scans with a sweep width of 6024 and 8 K real points.

The cloning of *dbat* gene

The *dbat* gene of strain MD3 was amplified with degenerate primers designed according to the *dbat* gene (GenBank accession no. EF028093) from *T. media*. The reaction mix contained 30–100 ng the genomic DNA of strain MD3, 100 μM each of *dbat*-F (ATGGCDGGVTCHACDGAN) and *dbat*-R (RRAWGGYTTWGTKACATATT), 150 μM dNTPs in 1 × PCR reaction buffer, and 2.5 U *Taqplus* DNA polymerase, and the total volume was brought to 50 μL with deionized water. The thermal cycling condition was predenaturing at 95 °C for 6 min, repeating for 30 cycles of denaturing at 93 °C for 50 s, annealing at 55 °C for 40 s, extension at 68 °C for 1.5 min and additional extension at 68 °C for 10 min. The amplified DNA fragments were sequenced and analyzed online with BLAST in the GenBank database.

Results and discussion

Identification of strain MD3

Strain MD3 was identified as *Aspergillus candidus*. It grew slowly on CYA at 25 °C and was unable to grow at 37 °C. The

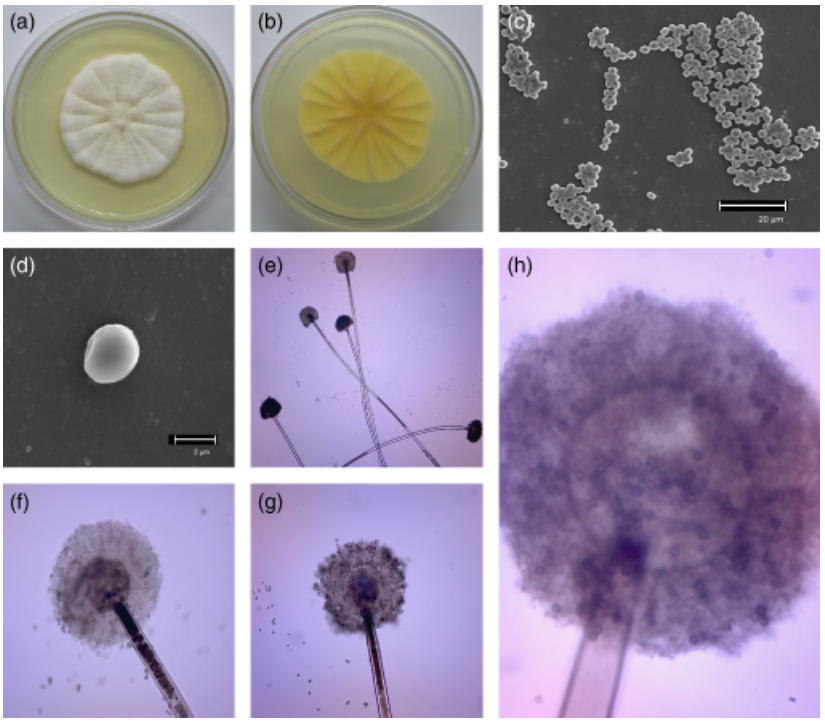

Fig. 1. Morphological observation of *Aspergillus candidus* MD3. (a) The colonies after 14 days at 25 °C on CYA; (b) reverse side of the colony; (c, d) scanning electron micrographs of the conidia; (e–h) the light micrographs of the conidial heads and conidiophores. Magnification: e, × 100; f, g, × 400; h, × 1000.

colonies are white and 4–5 cm in diameter after 2 weeks, and the colony reverse is cream–yellow (Fig. 1a and b). Conidia are single, colorless, smooth, globulose and 2.5–3.5 μm in diameter (Fig. 1c and d). Strain MD3 can produce typical *Aspergillus* conidial heads (Fig. 1e–h). Conidial heads are 20–50 μm in diameter, biseriate, white, at first globose, with spore chains later adherent in loose divergent columns, and produce diminutive heads. Conidiophores are 600–900-μm long, thick-walled and smooth. Vesicles are 40–50 μm in diameter, globose, colorless, and phialides cluster on one side of the vesicle. The morphological characteristics of strain MD3 are similar to those of *A. candidus* described by Varga *et al.* (2007). The detailed morphological and physiological properties of strain MD3 are shown in Table 1. The analysis results of the 18S rRNA gene sequence (GenBank accession no. EU883597) from train MD3 showed that it shares 99.6% identity with that from *A. candidus* (GenBank accession no. AB002065.1). The identity of the fungus strain MD3 was determined by Dr Zhang Luo-zheng and Xin Xiao-hong of the China Center for Type Culture Collection (CCTCC). *Aspergillus candidus* MD3 was deposited at CCTCC No. 208002.

Taxol from *A. candidus* MD3

The fungal compound isolated from *A. candidus* MD3 yielded a UV absorption spectrum that was similar to authentic taxol, and gave a single peak when eluting from the C_{18} HPLC column, with about the same retention time of 15.4 min as authentic taxol. Further convincing spectroscopic evidence for the identity of taxol was obtained by electrospray mass spectroscopy. The fungal compound produced an identical electrospray mass spectrum as authentic taxol, with the major molecular ion being $(M+Na)^+ = 876$ (Stierle *et al.*, 1993; Edward *et al.*, 1994) (Fig. 2). Both HPLC and MS analyses demonstrated that the fungal compound is taxol. To show the chemical structure of the fungal taxol isolated from *A. candidus* MD3, a further analysis by NMR was done and showed that the fungal taxol produced an ^1H NMR spectrum identical to that of authentic taxol (Fig. 3). These results demonstrate that *A. candidus* MD3 can produce taxol.

By measuring the absorption coefficient of the fungal taxol, the mean yield of taxol produced by strain *A. candidus* MD3 was about 112 μg g^{-1} (taxol per dry wt of mycelium) when it was cultured in 300 mL potato dextrose liquid medium at 25 °C with 120 r.p.m. shaking for 10 days.

The *dbat* gene from *A. candidus* MD3

The *dbat* gene (GenBank accession no. EU883596) from strain *A. candidus* MD3 was cloned with the degenerate primers. It consists of 1545 bp and shares 99% identity with the *dbat* gene (GenBank accession no. EF028093) found in *T. media* and 97% identity with that found in *Taxus wallichiana* var. *mairei* (GenBank accession no. AY931014.1).

The *dbat* gene encodes the DBAT transferase that catalyzes formation of baccatin III in the taxol biosynthetic

Table 1. Morphological and physiological properties of *Aspergillus candidus* MD3

Characteristics	Properties	Characteristics	Properties	Characteristics	Properties
Morphological		Carbon source utilizing		Carbon source utilizing	
Colony color	White	D-Arabinose	+	D-Galacturonic acid	−
Colony reverse	Yellowish	L-Arabinose	+	Gentiobiose	+
Aerial hypha	Fertile	D-Arabitol	+/−	D-Gluconic acid	−
Branch	+	Arbutin	+	D-Glucosamine	−
Secondary branching	+	D-Cellobioside	+	α-D-Glucose	−
Mycelia septa	+	β-Cyclic dextrin	−	D-Glucuronic acid	−
Conidial shape	Smooth,	i-Erythritol	−	Glycerol	+
Conidial shape	(sub)Globule	D-Fructose	+	Glycogen	+
Conidial color	White	L-Trehalose	+	m-Inositol	+/−
Size of conidia	2.5 − 4.0 μm	Sucrose	+	α-D-Lactose	−
Sclerotia	Black	Turanose	+	Lactulose	+/−
		Xylitol	−	Maltitol	−
		D-Galactose	+	Maltose	+

+, positive; −, negative.

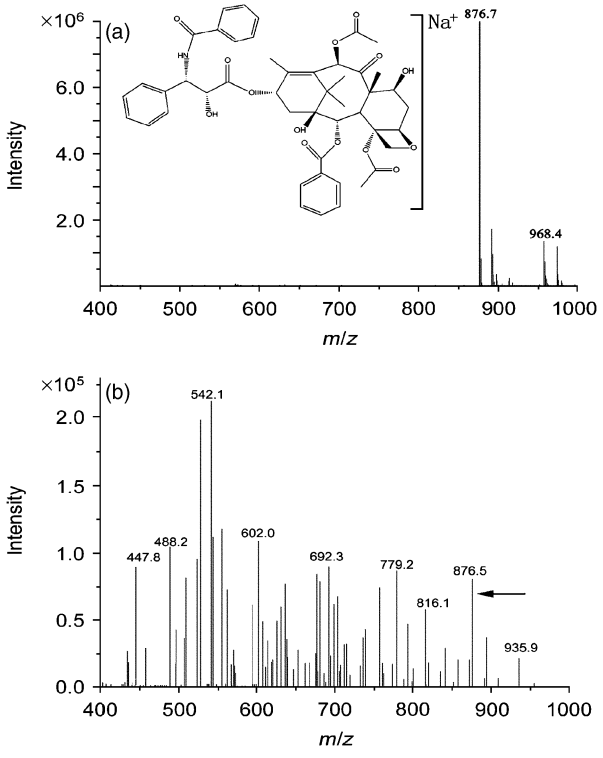

Fig. 2. Electrospray mass spectra of taxol. (a) Authentic taxol; (b) fungal taxol isolated from *Aspergillus candidus* MD3. An arrow shows the electrospray mass peak with $(M+Na)^+ = 876$ of the fungal taxol.

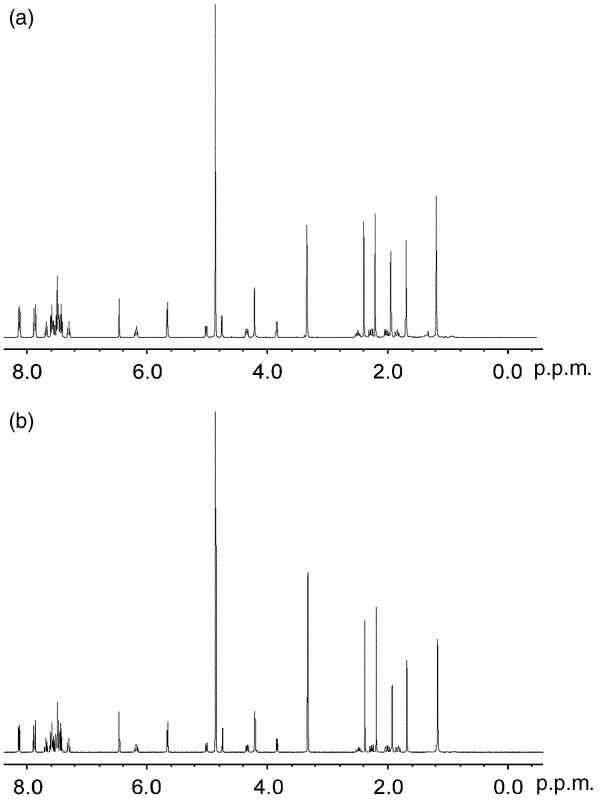

Fig. 3. ^{1}H NMR spectra of taxol. (a) Authentic taxol; (b) fungal taxol isolated from *Aspergillus candidus* MD3.

pathway in *Taxus* spp. (Wakler & Croteau, 2000). Croteau and his colleagues proposed that the pathway of taxol consisted of 19 enzymatic steps by cloning and characterization of a dozen genes involved in taxol biosynthesis from *Taxus cuspidate* (Jennewein *et al.*, 2004). However, the molecular basis of taxol synthesis and the taxol biosynthetic pathways are still unknown in any fungi. In this study, the *dbat* gene from *A. candidus* MD3 is shown to possess high homology to the same gene found in *Taxus* spp. Other genes involved in taxol biosynthesis from *A. candidus* MD3 will be cloned and characterized in future research, and we believe that those results will provide insights into the taxol biosynthetic pathway in *A. candidus* MD3.

Aspergillus candidus is a fungal species found throughout the world (Perrone *et al.*, 2007; Varga *et al.*, 2007), and it has been reported to produce several secondary metabolites including candidusins, terpreniss and chlorflavonin (Varga *et al.*, 2007). As other strains of *A. candidus* except *A. candidus* MD3 have not been reported to produce taxol, how does *A. candidus* MD3 have the capability to produce taxol? One idea was that certain endophytic microorganisms of *Taxus* species may produce and tolerate taxol to better compete and survive in association with these trees, and the acquisition of the metabolic machinery to produce taxol is either by coevolution or by genetic transfer (Young *et al.*, 1992). Comparative results of the genomes of *A. candidus* MD3 and other strains of *A. candidus* may shed light on the mechanism of acquiring the capability of taxol production for *A. candidus* MD3. This mechanism may be related to its ability to respond to environmental conditions, as such responses may represent a ready exchange of genetic information, which allows this organism to mimic the chemical environment of its host and, ultimately, to gain a number of evolutionary advantages (Li *et al.*, 1996). Further study of *A. candidus* MD3 should provide insights into the mechanism of molecular communication between *Taxus* spp. and *A. candidus* MD3.

Aspergillus candidus MD3 is valuable for both basic research and industrial interest. Strain *A. candidus* MD3 can produce about 112 $\mu g\, g^{-1}$ (taxol per dry wt of mycelium) when it is cultured under the conditions described in this study. However, it is only a wild strain, and we believe that the taxol yield of *A. candidus* MD3 will be increased by strain improvement and optimization of the fermentation media.

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Authors' contribution

P.Z. and P.-P.Z. contributed equally to this work.

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