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ORIGINAL ARTICLE

Aspergillus niger var. *taxi*, a new species variant of taxol-producing fungus isolated from *Taxus cuspidata* in China

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Keywords
Aspergillus niger var. *taxi,* classification, endophytic fungi, taxol, *Taxus cuspidata*.

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Abstract

Aims: To characterize and identify a new taxol-producing fungal strain HD86-9 isolated from *Taxus cuspidata* in China.
Methods and Results: Taxol extracted from strain HD86-9 was identified by HPLC and MS analyses. Strain HD86-9 was cultured and its morphology and phenotypes were described. HD86-9 displayed morphology most similar to that of *Aspergillus niger* but presented differences in the shape and size of the conidia. The growth evaluation showed that the maximal tolerable temperature of the new strain was 43°C, higher than that of the model *Aspergillus niger*. The 18S rDNA and the internal transcribed spacer region including the 5·8S rDNA of HD86-9 were amplified by PCR; molecular analysis of these sequences revealed their high similarity of 98% to those of *Aspergillus niger*.
Conclusions: The morphology and molecular analysis identified HD86-9 as a new variant of taxol-producing endophytic fungi, and it was named *Aspergillus niger* var. *taxi* D.P. Zhou, K. Zhao and W.X. Ping, var. *nov.*
Significance and Impact of the Study: As the first report of a taxol-producing variant of *Aspergillus niger* species, this study offers important information and a new resource for the production of an important anticancer drug by endofungus fermentation.

Introduction

Taxol is a diterpenoid with anticancer activities. It was first isolated from the bark of *Taxus brevifolia* Nutt by Wani *et al.* (1971), and is still mainly extracted from the bark of yews at the present time. However, this method cannot meet the increasing demand for taxol on the market because yews grow very slowly and are a rare and endangered species belonging to first-level conservation plants in China. Recently, increasing efforts have been made to develop alternative means of taxol production, such as using complete chemical synthesis, semi-synthesis and the *Taxus* spp. plant cell culture.

Several endophytic fungi that produce taxol have been isolated (Stierle *et al.* 1993; Strobel *et al.* 1996a,b). Since 1993, the authors have isolated five new endophytic fungal species that produce taxol by screening samples from the inner bark (phloem-cambium) and xylem of *Taxus cuspidata* Sieb. et Zucc. These fungi are *Nodulisporium sylviforme* D.P. Zhou *et al.* (Zhou *et al.* 2001), *Pleurocytospora taxi* J.Q. Sun, D.P. Zhou and W.X. Ping (Sun *et al.* 2003), *Alternaria taxi* J.P. Ge *et al.* (Ge *et al.* 2004), *Botrytis* (Zhao *et al.* 2008a), and strain HD86-9 (CCTCC M 206137). Since strain HD86-9 is a new species of unknown identity, in this study, we characterized its culture phenotypes and molecular properties, and determined its classification according to these characteristics.

Materials and methods

Culture of strain HD86-9

The taxol producing strain HD86-9 was grown on potato dextrose agar medium composed of peeled and cut potato 200 g l^{-1}, glucose 10 g l^{-1} and agar 15 g l^{-1} (Shen *et al.* 2000) at 28°C for 3–5 days.

Characterization of taxol produced by strain HD86-9

The taxol product that was previously extracted from fermented fungal culture of stain HD86-9 by methanol and ethyl acetate method (Christen *et al.* 1991) was further analysed by high performance liquid chromatography (HPLC) and mass spectrometry (MS) in this study. A Waters Millennium32 HPLC work station equipped with a photodiode array detector was used for quantitative analysis. An aliquot of taxol extract (10 μl) was lyophilized and dissolved in 1% methanol and was injected onto a 250 mm × 4·6 mm Taxsil-3 C_{18} reverse phase column. The mobile phase was a mixture of methanol-water (60 : 40) and the flow-rate was set at 1·0 ml min^{-1}. Taxol in the eluent was detected by measurement of the absorbance at 227 nm where taxol has the maximum absorbance. Qualification was achieved by using the standard curve generated from the taxol standard over a concentration range of 0·05–1·00 mg ml^{-1} at which both the peak area and height showed linear relationships with the absorbance (r = 0·9988). The structure of the newly extracted taxol was confirmed with a Waters triple quadrupole tandem LC-MS system (Waters, MA, USA). The HPLC portion was run isocratically with acetonitrile : water (49 : 51) as mobile phase. The sample was loaded onto a 250 × 4·6 mm Taxsil-3 C_{18} reverse phase column (Metachem, Co. Ltd, CA) and separated at a flow rate of 0·8 ml min^{-1} with the column temperature at 35°C. The MS scanning ranged from 100 to 1000 m/z, and the shell gas (N_2) and assistant gas (N_2) were 65 international units (IU) and 20 IU, respectively. The discharge current was 5 μA. The evaporator and capillary temperatures were 465°C and 180°C, respectively.

Morphological examination of strain HD86-9

Strain HD86-9 was activated at 28°C on a plate of potato dextrose agar (PDA) medium (Shen *et al.* 2000). The mycoflora were inoculated at three different spots in a 9 cm plate with Czapek Yeast Agar (CYA) medium (Shen *et al.* 2000) and incubated at 28°C in the dark for 1 week. The spore-producing filamentous fungi were detected and identified to the genus and species levels based on morphological characteristics as previously described (Raper and Fennell 1965; Qi and Kong 1997; Klich 2002). Colony diameters were measured using a ruler. Morphology of the endophytic fungus HD86-9 was examined with both a light microscope and a scanning electron microscope. Digital micrographs of colonies were taken with a Nikon Coolpix 995 camera.

Growth response of strain HD86-9 after 7-day incubation at 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 40°C on malt extract agar (MEA) media in plastic Petri dishes has also been analysed (Samson *et al.* 2007).

Molecular analysis of strain HD86-9

Culture and collection of mycelium were carried out as previously described (Zhao *et al.* 2004). For sequence analysis, DNA was extracted from taxol-producing fungus HD86-9 and identified using methods previously described (Guillemant and Drouard 1992; He 2000). The 18S rDNA sequences and the an internal transcribed spacer (ITS) region including the 5·8S rDNA were amplified by PCR with primer pairs 5′-GGATCAGAATT CTATTCTGGTTGATCCTGCCAG-3′ and 5′-CTCAGTA AGCTTGATCCTTCCGCAGGTTCACC-3′, and 5′-TCCG TAGGTGAACCT GCGG-3′ and 5′-TCCTCCGCTTATT GATATGC-3′, respectively. PCR reactions were carried out as one cycle of heat treatment at 94°C for 10 min, a total of 30 cycles of denaturation at 94°C for 10 s, annealing at 55°C for 30 s, extension at 72°C for 1·5 min, followed by a final extension at 72°C for 7 min. The PCR products were stored at 4°C, later analysed by 0·8% agarose gel electrophoresis, and then sequenced. Sequencing of the PCR products was performed by the service of Bo-ya Company Ltd (Shanghai, China). The two fragments of the 18S rDNA and ITS region were sequenced in both directions using an ABI PRISM 377-18 DNA Sequencer (Applied Biosysterns) according to the manufacturer's instructions. The sequences obtained were submitted to GenBank for homology search with BLAST (http://rdp.cme.msu.edu and http//ncbi.nim.nih.gov). The sequences of the 18S rDNA and the ITS region were aligned with those of related fungal strains retrieved from the GenBank databases using CLUSTALX. A phylogenetic tree was constructed from the evolutionary distance data by PHYLIP software, ver. 3.57c (distributed by Felsenstein, J., Department of Genetics, University of Washington, Seattle, WA, USA).

Results

Taxol production from strain HD86-9

Strain HD86-9 was previously isolated from this laboratory; it could produce taxol at a high amount of 273·46 $\mu g\ l^{-1}$. This ability remained stable after the fungus being cultured for five generations. Extracted from fermented fungal culture, taxol product showed the characteristic peaks for taxol in the HPLC chromatograph (Fig. 1a,b), and its structure was also confirmed with the mass spectrum (Fig. 2).

Morphologic characterization

Strain HD86-9 grew rapidly on the CYA medium at 25°C, reaching a size of 43–60 mm in 5 days. The

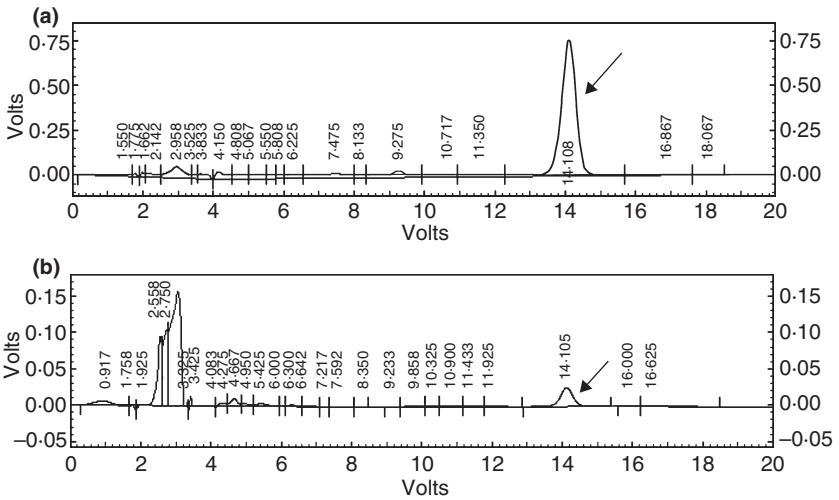

Figure 1 HPLC chromatograms of taxol extracted from strain HD86-9. Arrows indicate the taxol-specific peaks. (a) Taxol molecule standard; (b) Taxol sample extracted from strain HD86-9.

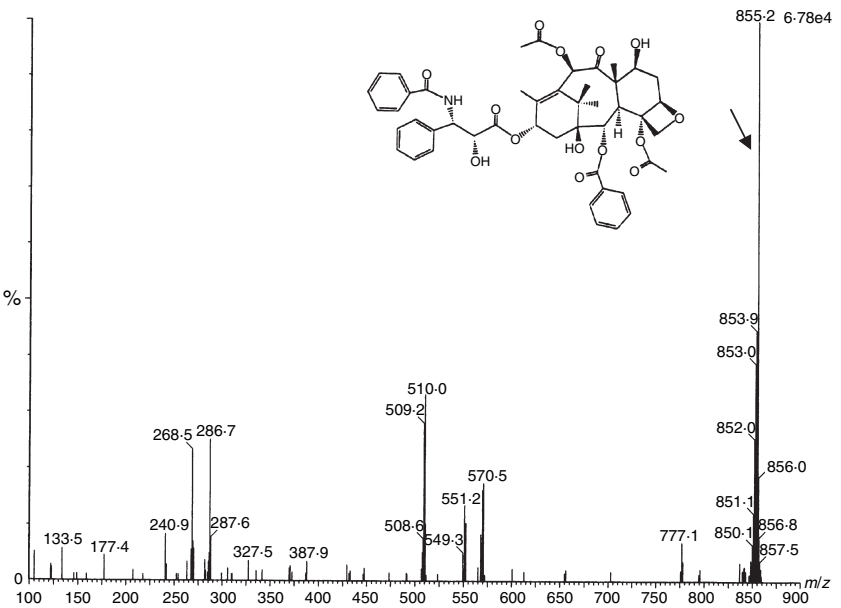

Figure 2 Mass spectrum of taxol extracted from strain HD86-9. Arrow indicates the molecular ion of taxol at *m/z* 855 $[M + H]^+$.

mycelium surface was velvety and smooth with regular or irregular radial furrow ornamentation. There were massive conidiospores. Occasionally only a few were present in or near the margin. The spore had a head of carbon black colour and without any transudate. The back of the fungus appeared yellowish or yellow brownish. The middle of the colony was yellow brownish to pitchy. The conidial head was globular at an early stage with a diameter of 150–500 μm; it broke into several cylindrical textures with a diameter of 800 μm or larger at the later stage. The top vesicle was globular or oval, blastic on the surface, with a diameter of 30–80 μm. The conidial fructification was found to be double layered. The conidiophores grew larger when the fungus grew; the sizes of conidiophores were usually in the range of (12–35) × (5–10) μm, and the biggest one was up to 80 μm. They turned brown in colour at the late stage of growth. The sizes of phialide were in the range of (8–12) × (2–5) μm. The conidiophores were globular or ovular with a diameter of 20–33 μm, and the parietes appeared very rough. The conidiospores showed obvious ridges and pitches on the surface.

As shown in Fig. 3, the morphological characteristics of this strain were most close to those of *Aspergillus niger*, but also with some distinctions. For example, the conidiophores of the new strain appeared substantially larger, with a diameter of 20–33 μm, and the walls were rougher. In addition, HD86-9 did not grow at 6°C or 9°C on malt

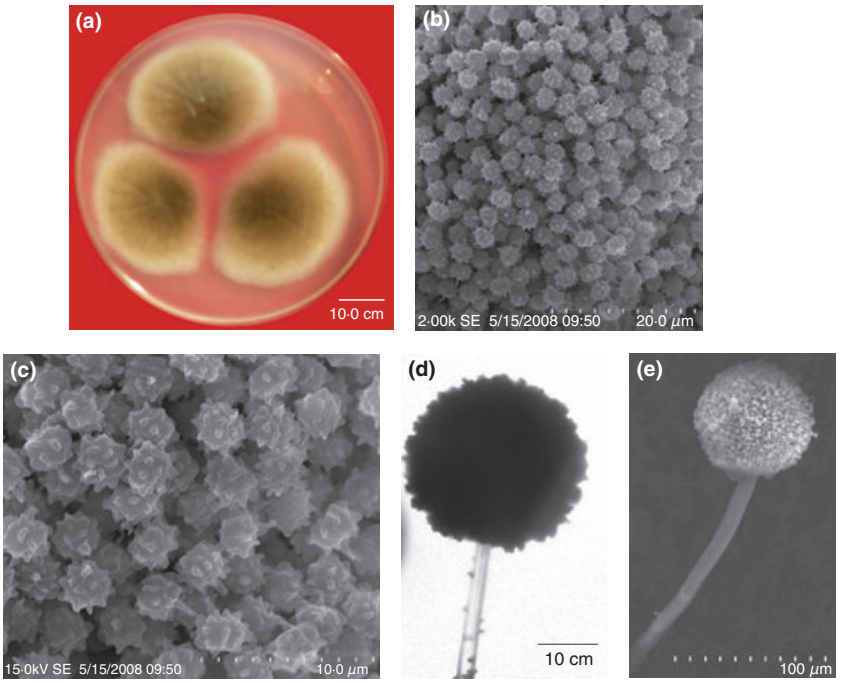

Figure 3 Morphology of taxol-producing endophytic fungus HD86-9. (a) Colony growing on PDA medium at 28°C for 3 days; (b, c) conidia viewed under a scanning electron microscope at the magnitudes of 2000 (b) and 5000 (c); (d, e) conidiophores viewed under a light microscope at the magnitudes of 1000 (d) and 370 (e).

extract agar media, but grew well at 40°C. Its maximum tolerable growth temperature was determined to be 43°C.

Molecular analysis

The 18S rDNA and ITS region including the 5·8S rDNA of HD86-9 were successfully amplified by PCR with expected sizes of about 1755 and 583 bp, respectively (Fig. 4). After sequencing, these newly identified sequences were submitted to and deposited into GenBank under accession number EU853156 for the 18S rDNA and number EU853157 for ITS region including the 5·8S rDNA. After homology searching against the GenBank or the proprietary fungal DNA databases, the new sequences of HD86-9 were found to share 98% similarity with those of *Aspergillus niger*, in both in the 1755 bp 18S rDNA and in the 583 bp ITS region. A phylogenetic relationship was established through the alignment and cladistic analysis of homologous nucleotide sequences among these fungal species (Fig. 5a,b), HD86-9 was shown to be closest to the genus *Aspergillus*. According to this phylogenetic analysis, strain HD86-9 was classified to the genus *Aspergillus* as a species variant of *Aspergillus niger*.

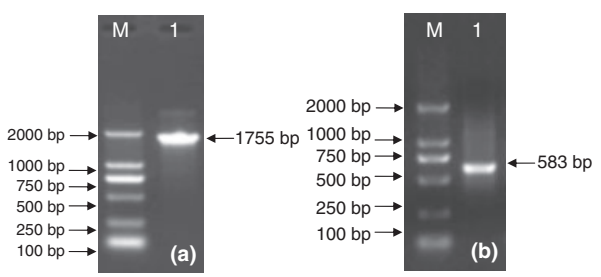

Figure 4 Agarose gel electrophesis for PCR products of the 18S rDNA and ITS region including the 5·8S rDNA amplified from strain HD86-9. (a) The 18S rDNA; (b) The ITS region. Lane M: DNA molecular weight marker DL2000.

Discussion

Taxol, or by a generic name 'paclitaxel', is a mitotic inhibitor that has been used in chemotherapy for many types of cancers since 1970s. It is known to be produced by a number of endophytic fungi, including the following species or genera from the current literature (Zhao *et al.* 2008b): *Taxomyces andreanae*, *Taxomyces* sp., *Trichoderma*, *Tubercularia* sp., *Monochaetia* sp., *Fusarium lateritium*, *Pestalotiopsis microspora*, *Pestalotiopsis guepinii*, *Pithomyces* sp., *Pestalotia bicilia*, *Papulaspora* sp.1, *Pseudomonas aureofaciens*, *Pleurocytospora taxi*, *Cephalosporium* spp., *Chaetomium*, *Martensiomyces* spp., *Mycelia sterilia*, *Nodulisporium sylviforme*, *Rhizoctonia* sp., *Penicillium*, *Alternaria* sp., *Alternaria taxi*, *Ectostroma* sp.1, *Botrytis* sp.1, *Alternaria alternate*, and *Botrytis taxi* (Zhao *et al.* 2008a).

The endophytic fungus *Aspergillus niger* from *Taxus* spp. has never been reported to be able to produce taxol. Our present study is the first report for the isolation, characterization and identification of a new variant of the *Aspergillus niger* from *Taxus cuspidata* in China that is able to produce taxol at a high amount of 273·46 $\mu g\ l^{-1}$.

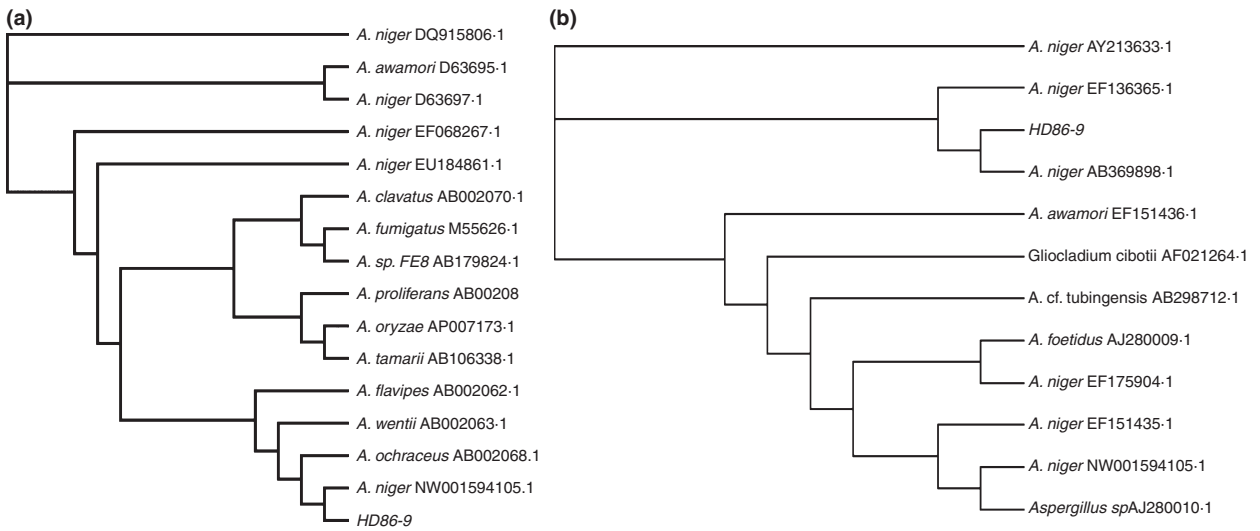

Figure 5 Phylogenetic trees showing relationship of strain HD86-9 with other related fungal species retrieved from GenBank based on their sequence homologies of 18S rDNA (a) and ITS region including the 5·8S rDNA (b).

This new strain differs from the model species of *Aspergillus niger* in the shape and size of the conidia. Its phenotypes are also different from those of *Aspergillus niger* published in literature (Qi and Kong 1997). In addition, Samson *et al.* (2007) reported that all black aspergilli (*Aspergillus* section *Nigri*) strains are not able to grow at 6°C and 9°C, with the exception of *Aspergillus carbonarius*, which can grow at 9°C for up to 96 h. The maximum temperature that *Aspergillus niger* could tolerate was 40°C. In this study, the new strain HD86-9 was not able to grow at 6°C or 9°C on the malt extract agar media, but it survived at the maximum tolerable temperature of 43°C.

In addition to the morphological observation, 18S rDNA sequencing and analysis of the ITS region including the 5·8S rDNA also showed a close relationship of HD86-9 to the genus *Aspergillus*. Molecular identification has been increasingly used as a supplementary tool for the traditional systematic classification. The agreement of the classical morphological identification and the molecular biological analysis in this study determined that HD86-9 represented a new strain of taxol-producing endophytic fungi and was named *Aspergillus niger* var. *taxi* D.P. Zhou, K. Zhao and W.X. Ping, var. nov. The isolation of the strain provides an excellent opportunity for large scale production of taxol.

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