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ORIGINAL ARTICLE

Identification and characterization of a special species of *Paecilomyces*

Qinglin Dong · Huarui Wang · Xiangying Xing · Shaodong Ji

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Abstract A special purple filamentous fungus, TD16, was isolated from the contaminated culture broth of cyanobacteria (chroococcaceae sp). The sequence of the ITS1-5.8S-ITS2 region of its rDNA suggests that it belongs to the genus *Paecilomyces* with the highest homology of 99%. Further phylogenetic analysis (GenBank accession no: JN243772) indicates that the fungus is related more closely to *Paecilomyces lilacinus* than to other species of *Paecilomyces*. However, its phialides consist of a very thin neck (diameter less than 0.5 μm), approximately half that of the *Paecilomyces lilacinus* reported previously. Accordingly, the species is perhaps a variety or subspecies of *Paecilomyces lilacinus*. Furthermore, this special *Paecilomyces* secreted polysaccharides composing of galactose only and a lilac water-soluble pigment into the broth.

Keywords Sequence · Phylogenetic · Paecilomyces · Polysaccharide

Introduction

The genus *Paecilomyces* was established by Bainier (1907), revised by Brown and Smith (1957) and modified by Samson (1974). *Paecilomyces variotii* was the first species accepted in *Paecilomyces*, until now more than 100 species have been recognized in the genus (He et al. 2011). *Paecilomyces* is a common filamentous fungus usually found in soil, decaying plants, and food products (Chen et al. 2010; Gumus et al. 2010; Samson et al. 2009). Some species of *Paecilomyces* are isolated from insects, an it is even a cause of infection for man (Kalkar et al. 2006; Luangsa-ard et al. 2011; Schooneveld et al. 2008). The common and well-studied species of the genus are *Paecilomyces variotii and Paecilomyces fumosoroseus*, of which *P. variotii* has been found to be an important source of thermotolerant enzymes (Michelin et al. 2008, 2010), while *P. fumosoroseus* is the fungus most widely tested for the control of whitefly (Wraight et al. 2000). *Paecilomyces lilacinus* is an emerging pathogen that causes severe human infections, and also being used as an important biological control agent against root-knot nematodes (Pastor and Guarro 2006; Anastasiadis et al. 2008). However, little is known about its metabolic characteristics.

We have previously isolated from the contaminated culture broth of cyanobacteria a special fungus, TD16, with some similar characteristics to *Paecilomyces* (De Hoog et al. 2000). Thus, in the present study, we first identify the fungus according to morphology observation, internal transcribed spacer (ITS) sequences as well as phylogenetic analysis, and then study its extracellular metabolites.

Materials and methods

Morphological observation

Strain TD16 was transplanted onto potato dextrose agar (PDA). After incubating at 4, 25 or 37°C for 14 days, colony diameters were measured and cultures were investigated with a light microscope. Morphological analyses of the strain were carried out based on colony characters, hypha, conidiophore structure, sporogenous structure and conidium.

Q. Dong (✉) · H. Wang · X. Xing · S. Ji
Department of Biochemical Engineering, School of Chemical Engineering, Hebei University of Technology, Tianjin 300130, China
e-mail: qldong@hebut.edu.cn

DNA extraction and sequencing

Genomic DNA was extracted from mycelium cultured in potato dextrose liquid medium according to Graham et al. (1994) with some modifications. PCR primers ITS1 5′-TCCGTAGGT GAACCTGCGG-3′ and ITS4 5′-TCCTCCGCTTATT GATGC-3′ were designed to amplify regions of the genes for the ITS regions of the rDNA gene. Primers were synthesized by Beijing Sanbiotech (Beijing, China). Template DNA (2–4 μl; 100 ng) was amplified in a 50 μl PCR reaction consisting of 20 mM Tris-HCl (pH 8.4), 50 mM KCl; 0.2 mM each dATP, dGTP, dCTP, and dTTP; 1.2–1.6 mM $MgSO_4$; 0.2 pmol of (each) primer and 1 U *Taq* DNA polymerase. The reaction was set up as follows: initial denaturation at 94°C for 3 min. Each of the 30 cycles is composed of a denaturation step of 94°C for 15 s, an annealing step of 55°C for 30 s, and an extension step of 72°C for 30 s, and the last cycle was followed by a final extension at 72°C for 3 min. Products were visualized on a 1.5% agarose gel. The amplicons were purified with a PCR purification kit. The purified PCR products were sequenced bidirectionally with ITS1 and ITS4 by Beijing Sanbiotech.

Phylogenetic analysis

The sequences of the ITS regions were submitted to GenBank for homology analysis. The highest homology and representative strains of *Paecilomyces* were selected for phylogenetic analysis. Alignment of the sequences obtained was performed with Clustal 1.81. The phylogenetic tree was constructed with the MEGA 4.1 by the neighbor-joining method (N-J) with 1,000 bootstrap replicates.

Polysaccharide analysis

Strain TD16 was grown in Czapek liquid medium cultured on a rotary shaker incubator at 25°C, 130 rpm. After fermentation for 7 days, the culture broth was centrifuged at 8,000 rpm for 10 min. The supernatant was mixed with 4 volume of 95% ethanol. After standing overnight, the cotton-like precipitate was collected by centrifugation. The precipitation was dissolved in distilled water with ultrasonic treatment and deproteined using the Sevage method (chloroform: *n*-butanol at 4:1, v/v). The solution was dialysed against running water for 48 h and distilled water for 24 h, after that freeze-dried completely.

The lyophilized extracellular polysaccharide (5 mg) was hydrolyzed with 2 mL 2 mol/L H_2SO_4 for 6 h at 100°C, in sealed tubes. The excess acid was neutralized completely by $BaCO_3$, and then centrifuged to eliminate insoluble material. The evaporated supernatant was cooled to room temperature and then used for paper chromatography by capillary tube. The developing solvent employed for the separation was butanol: acetone: water 4: 3: 1 (v/v). After 4 h, aniline-phthalic acid was sprinkled on the paper as a color development reagent and the paper was then dried at 105°C for 5 min. D-Glucose, D-fructose, D-galactose and D-xylose were prepared as standards and subjected to paper chromatography in the same way.

A major structural analysis of the purified extracellular polysaccharide was carried out using a Fourier transform infrared (FTIR) spectrophotometer. The lyophilized sample was ground with KBr and then pressed into pellets for FTIR measurement at a ratio of 1:20. The FTIR spectra were recorded in the range of 4,000–400 cm^{-1} and processed by Origin 8.0.

Pigment analysis

Strain TD16 was grown in Czapek liquid medium cultured for 7 days; culture broth was centrifuged at 8,000 rpm for 10 min. The supernatant was mixed with 20% (w/w) polyethylene glycol 1,000 and 8% potassium dihydrogen phosphate (w/w). The mixture was vibrated gently until the solid material dissolved. The solution was centrifuged at 8,000 rpm for 10 min at 4°C; an aqueous two-phase system consisting of polyethylene glycol and potassium dihydrogen phosphate was obtained. The pigmented phase was collected. A preliminary analysis of the separated pigment was conducted using an ultraviolet–visible spectrophotometer.

Results

Growth characteristics and morphology

Colonies on PDA were fast growing, attaining a diameter of 25 mm after 7 days at 25°C, with no or restricted growth at 4°C and 37°C. Colonies were rounded, central bulged, dense floccose, thick, with orderly margins and radiating ring. Early stage colonies were white, later changing to wine red; reverse mostly dark brown, and then with the appearance of a lavender pigment (Fig. 1a, b).

Vegetative hyphae smooth-walled, hyaline, septate, 1.5–2.8 μm wide (Fig. 1c). Conidiophores arising from submerged hyphae, 15–25 μm in length, occasionally forming tufts up to 50 μm high, verticillate branches with whorls of three to six phialides (Fig. 1d). Phialides 2.0–2.5×6.5–9.0 μm, consisting of a swollen columnar basal portion tapering into a slightly crooked distinct neck; the neck thin and short, 1.0–3.0 μm long and diameter less than 0.5 μm (Fig. 1e). Conidia were produced from the phialide neck and formed divergent chains, which sometimes become scat-

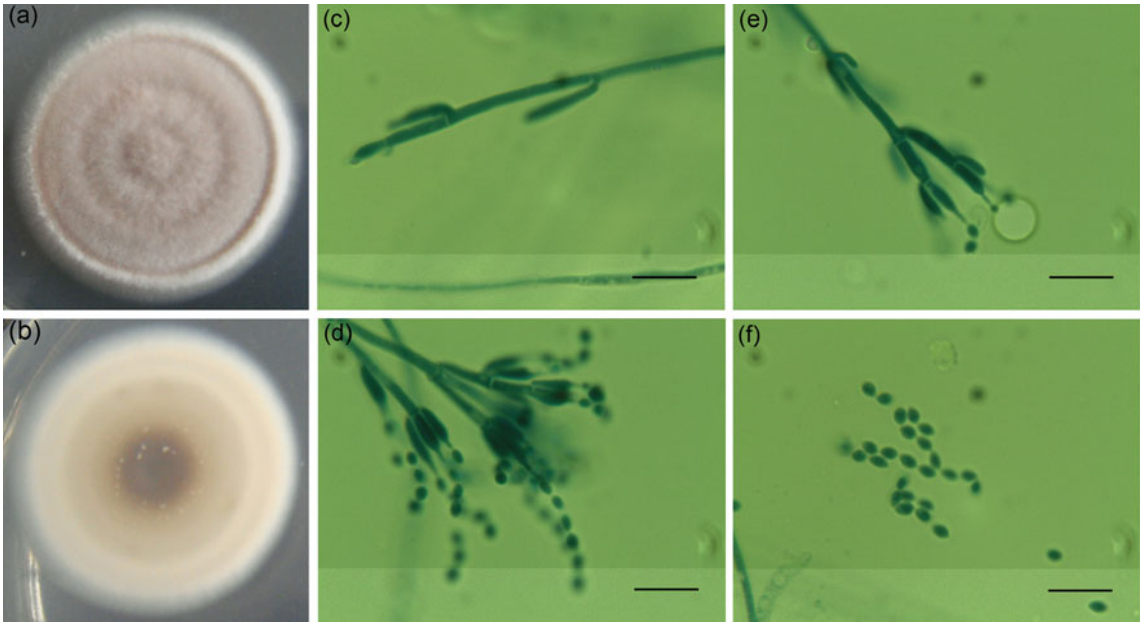

Fig. 1a–f Morphological characters of fungal strain TD16. Species TD16 was cultured on potato dextrose agar (PDA) at 25°C for 7 days. **a**, **b** Colony and the reverse. **c** Hyphae: ramose and septate. **d** Conidiophores: verticillate branches with whorls of three to six phialides. **e** Phialides consist of a cylindrical inflated base, tapering into a long, very thin, and sometimes incurved neck. **f** Conidia formed divergent chains. *Bars* **c**–**f** 10 μm

tered, ellipsoidal to fusiform, smooth-walled, hyaline, 1.8–2.1×2.3–2.8 μm (Fig. 1f).

DNA sequence analysis

The ITS1-5.8S-ITS2 region was successfully amplified and was sequenced as 534 bp. The sequences obtained were submitted to GenBank (accession no: JN243772). BLAST analysis demonstrated that the strain belonged to *Paecilomyces*. The highest homology is 99%. The phylogenetic tree as shown in Fig. 2 was constructed with the MEGA 4.1 by the N-J method with Kimura 2-parameters.

The phylogenetic tree based on ITS rDNA shows that the strain TD16 with *Paecilomyces lilacinus* and *Paecilomyces nostocoides* on the same branch, and supported by bootstrap analysis as 100%. TD16 is related most closely to *Paecilomy-*

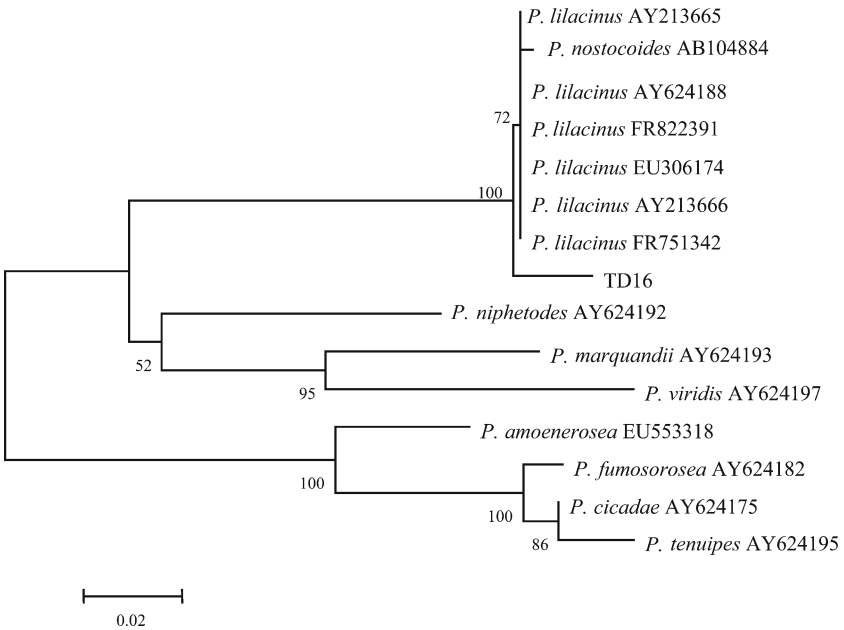

Fig. 2 Phylogenetic relationships between TD16 and other related species inferred from ITS sequences. The accession number in GenBank of each fungus is indicated after the species name. Numbers at the branch nodes are the confidence values above 50% obtained from 1,000-replicate bootstraps. *Bar* Number of base substitutions per site

Fig. 3 Paper chromatogram of hydrolyzed exopolysaccharide. Lanes: *A* hydrolysate, *B* fructose, *C* xylose, *D* galactose, *E* glucose

ces lilacinus and *Paecilomyces nostocoides*, with the distance to other *Paecilomyces* being farther.

Extracellular product analysis

Paper chromatography of hydrolyzed exopolysaccharide and four kinds of standard monosaccharide (Fig. 3) illustrates that the exopolysaccharide was composed of galactose only.

Figure 4 illustrates the FTIR spectrum of the exopolysaccharide. The large absorption peak at 3,447 cm^{-1} was the OH stretching peak. The absorption bands at 1,775 cm^{-1} and 1,735 cm^{-1} were absent, indicating a product without acetyl ester. The absorption peak at 1,636 cm^{-1} caused by vibration of CO illustrates that the monosaccharide has a carbonyl. The weak absorption peak at 1,384 cm^{-1} resulted from CH angle vibration. Absorption in the range of 1,200–1,000 cm^{-1} suggested that the monosaccharide has a pyran structure. The IR spectrum lacked an obvious characteristic absorption at 810 cm^{-1} and 870 cm^{-1}, which was attributed to the lack of mannose. The absorption peak at 890 cm^{-1} indicated that the exopolysaccharide did not contain any β-configuration of sugar units.

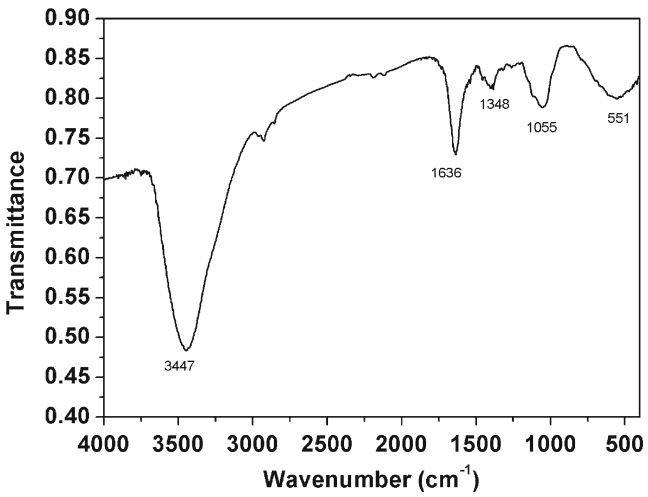

Fig. 4 Fourier transform infrared (FTIR) spectrum of the purified exopolysaccharide obtained from fermented TD16 in the range 4,000–400 cm^{-1}

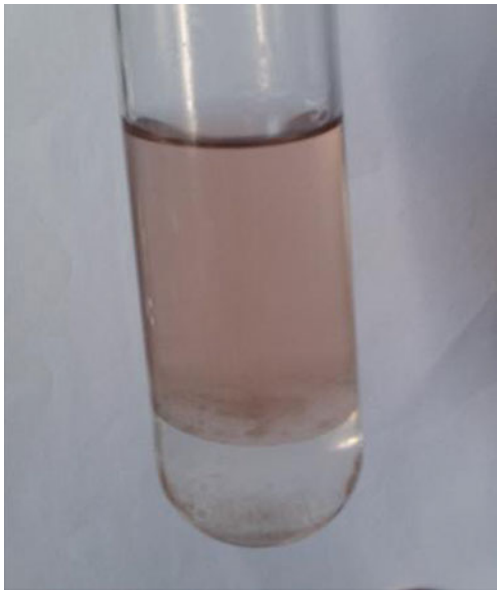

Fig. 5 Lilac pigment of TD16 extraction in aqueous two-phase system consisting of 20% PEG 1000/8% KH_2PO_3

On the basis of the analysis mentioned above, the monosaccharide lacks any special modification and the sole component of the extracellular polysaccharide is galactose.

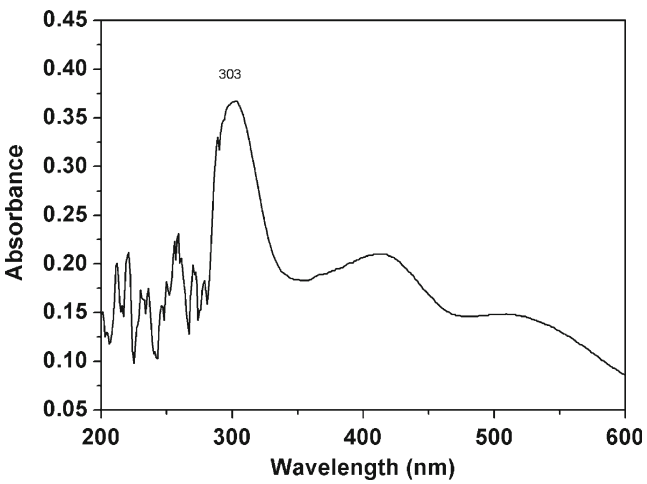

Fig. 6 UV–vis absorption spectra of the lilac pigment

The pigment secreted by the strain, which was lilac in color, was extracted by two-aqueous phase extraction according to its water-solubility. The pigment was dissolved in the top phase, at the same time, proteins and other impurities entered the lower phase (Fig. 5). The extracted pigment was evaluated by its ultraviolet–visible spectrum and displayed a distinct absorption at 303 nm (Fig. 6). Moreover, the lavender pigment is sensitive to heat and light, and varies with pH (date not shown).

Discussion

The conidiophores of TD16 have divergent whorls of phialides, and a cylindrical or inflated base tapering into a distinct neck. These features reveal that TD16 belongs to the genus *Paecilomyces*. Further genetic analysis and construction of a phylogenetic tree showed that *P. lilacinus*, *P. nostocoides* and TD16 isolates formed a distinct and closely related subgroup, with TD16 in a branch of its own, parallel to the clade containing *P. lilacinus* and *P. nostocoides*. However, the conidia of *P. nostocoides* come in two sizes, and while one forms chains (Dunn 1983), conidia of TD16 form a uniform divergent chain, which distinguishes it from *P. nostocoides*. Obviously TD16 differs from *P. nostocoides*. A BLAST search with the sequence of the ITS region of the rDNA gene of TD16 showed a 99% homology with *P. lilacinus*, indicating that strain TD16 is related more closely with *P. lilacinus*. Furthermore, from our morphological observations, TD16 is more similar to *P. lilacinus* due to the presence of fusiform conidia in divergent chains. Nevertheless, *P. lilacinus* can grow at 37°C and is usually considered as a biological control agent for root-knot nematodes, and is even a pathogen for human beings (Takayasu et al. 1977; Kiewnick and Sikora 2006; Ciecko and Scher 2010), whereas strain TD16 was isolated from a quite different source, and exhibited no or restricted growth at 37°C. Moreover, phialides of *P. lilacinus* consisted of an inflated basal portion, tapering into a short neck about 1 μm wide (Samson 1974). The neck diameter of TD16 is less than 0.5 μm, approximately half of that previously reported. These results strongly suggest that TD16 is perhaps a subspecies or, at least, a variety of *P. lilacinus*.

With regard to extracellular metabolites, the polysaccharide excreted by this special *Paecilomyces* TD16 was composed of galactose only, whereas most polysaccharides secreted by *Paecilomyces* contain glucose (Lu et al. 2007). These results suggest that strain TD16 differs from other species of *Paecilomyces* in its intracellular carbohydrate metabolic networks. Furthermore, although *Paecilomyces* exhibits a particular lilac color, it has attracted no attention, and no information has been available concerning this pigment previously. In the present study, the lilac water-soluble pigment has been separated for the first time using an aqueous two-phase extraction system. The lilac pigment is unique compared to known fungal pigments (Mapari et al. 2005), including the ultraviolet absorption at 303 nm, sensitivity to heat and light, varying with pH; the pigment may be of potential use in bio-industry.

In conclusion, based on morphology observation and genetic analysis, the special fungus TD16 was identified as a variety or subspecies of *P. lilacinus* that produces an extracellular polysaccharide comprised only of galactose, and a lilac water-soluble pigment.

Further detailed research on the polysaccharide and pigment is ongoing.

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