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Chemosystematics and Molecular Phylogeny of a New Bioflocculant-Producing *Aspergillus* Strain Isolated from Korean Soil

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Abstract The ubiquinone and G+C contents of the bioflocculant-producing fungus, a new *Aspergillus* strain, were determined using high-performance liquid chromatography. The internal transcribed spacers 1 and 2 (ITS1 and ITS2), and the 5.8S ribosomal DNA (rDNA) of the strain were amplified and sequenced. The strain contained ubiquinone-10(H_2) as a major quinone and the G+C content was 49 mol%. A phylogenetic analysis of the ITS regions indicated that the strain belonged to the genus *Aspergillus* according to its previously classified morphological characteristics. Based on a sequence homology search, the strain was most closely related to *Petromyces muricatus* (anamorph, *A. muricatus*; accession number, AJ005674). The sequence of a new *Aspergillus* strain in ITS1 and ITS2, and 5.8S rDNA showed 97% homology to *P. muricatus*. Therefore, the strain is believed to be a new bioflocculant-producing *Aspergillus* strain.

Key words: Chemosystematics, phylogeny, bioflocculant, *Aspergillus*

Recently, because of industrial development and the increased concern about environmental pollution, the development of biodegradable and safe flocculants has become a priority. In this respect, bioflocculants from microorganisms could be very useful candidates. In a previous paper, the authors reported on some microbial flocculants from fungi [5, 6] isolated from soil samples collected throughout Korea. One of the isolates, the strain which exhibited high ability to flocculate kaolin clay, was identified based on the following phenotypic information; conidial head loosely columnar and yellowish-brown, conidiophores smooth-walled, 4-5 mm long and colourless, and mostly present as metulae. The aim of the present study was to examine detailed chemotaxonomic characterizations and to determine the phylogenetic relationships among strains of *Aspergillus* and related taxa by comparing nucleotide sequences of the two internal transcribed spacers (ITS1 and ITS2) and the 5.8S ribosomal RNA coding gene (rDNA).

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Microorganism and Experimental Methods

The strain was grown aerobically at 25°C in Potato Dextrose Broth (Difco). The cells were harvested by filtration on a Buchner funnel, washed twice with distilled water, and freeze-dried. The strain has been deposited in both the Korean Collection for Type Cultures (KCTC 16780) and the American Type Culture Collection (ATCC 201291).

The ubiquinones were extracted and isolated according to the method of Yamada and Kondo [14]. The purified ubiquinones were identified by their retention times in high performance liquid chromatograms [11] previously described [8, 9] with a slight modification: instrument, Hitachi L-5000 LC Controller; pump, Hitachi L-6000; column, YMC-Pack ODS-AM (4.6ϕ × 250 mm, YMC Co., Ltd., Japan); column temperature, 30°C; eluent, methanol/isopropyl ether (3:1); flow rate, 1 ml/min. The ubiquinones were detected by monitoring at 275 nm with a Tosoh UV-8011 detector. The data were analyzed using a Hitachi D-2000 chromato-Integrator.

The DNA was isolated by the method of Zu *et al.* [15]. The G+C content in the DNA was determined by high-performance liquid chromatography (HPLC) [3] under slightly different conditions from that for the ubiquinone. The conditions were as follows; mobile phase, [$(NH_3)H_2PO_4$:acetonitrile = 40:1]; UV absorbance, 270 nm.

The ITS1 and ITS2, and 5.8S rDNA were amplified using ITS 5F and ITS 4R primers derived from the conserved regions of the 18S and 28S rDNA, respectively [13]. The PCR product was purified using a QIAEX gel extraction kit (Qiagen, Chatsworth, U.S.A.). The sequences of the ITS1 and ITS2, and 5.8S rDNA were determined with an Applied Biosystems (Foster City, U.S.A.) model 377A automatic DNA Sequencer. The DNA sequence determined was aligned with representatives of the genus *Aspergillus* and related taxa using the CLUSTAL W software [8]. For distance analysis, DNADIST in PHYLIP

version 3.5 [2] was used to obtain a matrix of Kimura's two-parameter distance [4]. The distance matrix was then analyzed by NEIGHBOR with algorithms based on Saitou and Nei's neighbor-joining method [7]. The strength of the internal branches of the resulting trees was statistically tested by bootstrap analysis [1] from 1,000 bootstrap replications. The ITS1 and ITS2, and 5.8S rDNA sequences determined in the present study have been deposited in the NCBI data library under accession number AF 134810. The EMBL/GenBank/DDBJ accession numbers for the ITS sequences (including ITS1, ITS2, and 5.8S rDNA) are as follows: *A. flavus*, AB008414; *A. niger*, L76748; *A. fumigatus*, AF078889; *A. sojae*, AB008419; *A. oryzae*, AB008417; *A. wentii*, L76806: *P. muricatus*, AJ005637; *Trichoderma harzianum*, AF030395. These sequences have the highest similarity in the genus *Aspergillus* compared with a new *Aspergillus* strain.

Chemosystematics and Phylogenetic Position

The strain contained a dihydrogenated ubiquinone with 10 isoprene units [Q-10(H_2)] as the major quinone (Fig. 1). The complexity of *Aspergillus* is emphasized by the diversity of teleomorphs and the heterogeneity of the ubiquinone systems. Three major ubiquinones, Q-9, Q-10, and Q-10(H_2), occurred in *Aspergillus* [10]. Accordingly, the genus *Aspergillus* has been divided into three subgroups based on a ubiquinone system. The heterogeneity of the ubiquinone system suggests that the taxa of *Aspergillus* are in need of revision. The strain had a 49 mol% G+C content (Fig. 2). The G+C content in strains belonging to the genus *Aspergillus* ranges from 49 to 57 mol%. The ITS1 and ITS2, and 5.8S rDNA were amplified successfully by a PCR and fully sequenced by the PCR sequencing method, followed by automated fluorescence detection. The determined sequence had 501 residues, consisting of ITS1 (168 residues), 5.8S rDNA (158 residues), and ITS2 (175 residues) (Fig. 3). The strain sequence was compared with a data set consisting of 7 (Q-9, 10, 10(H_2) groups of *Aspergillus*) reference sequences derived from various databases. Since the morphology, ubiquinone system, and DNA G+C content all suggested that the strain was a member of the genus *Aspergillus*, as described above, the reference sequences were selected from those species belonging to this phylogenetic group. The phylogenetic analysis showed that the strain is a member of the genus *Aspergillus* and most closely related to *P. muricatus* (accession number: AJ005674) (Fig. 4). About 97% similarity of ITS1 and ITS2, and the 5.8S rDNA between a new *Aspergillus* strain and *P. muricatus* sequences were higher than any of the other *Aspergillus* species used in this study. However, it was clear that the new *Aspergillus* strain was different from *P. muricatus*. Based on a sequence homology search from the GenBank and other databases, no other species was yet identified as having the same sequences as the new *Aspergillus* strain. In addition, the

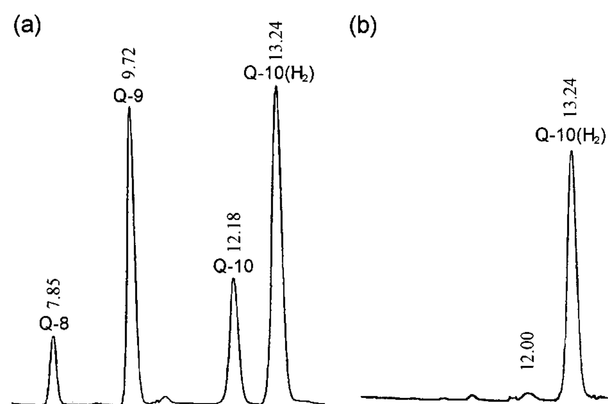

Fig. 1. HPLC chromatogram of (a) ubiquinone standards and (b) ubiquinone-10(H_2) from a new *Aspergillus* strain.

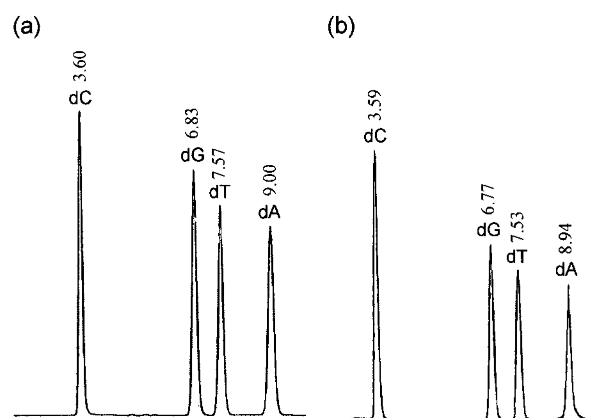

Fig. 2. HPLC chromatogram of (a) G+C content standards (51%) and (b) G+C content from a new *Aspergillus* strain.

|⇒ITS1
ACTGAGTGAGGGTCCCTCGGGGCCCAACCTCCACCCGTGTATACCGTACC

TTGTTGCTTCGGCGAGCCCGCCCCCTTTTTCTTTTAGGGGGCACAGCGCTC

GCCGGAGACACCAACGTGAACACTGTCTGAAGTTTTGTCGTCTGAGTCGAT

|⇒5.8S rDNA
TGTATCGCAATCAGTTAA|AACTTTCAACAATGGATCTCTTGGTTCCGGCAT

CGATGAAGAACGCAGCGAAATGCGATAATTAATGTGAATTGCAGAATTCAG

TGAATCATCGAGTCTTTGAACGCACATTGCACCCCCTGGTATTCCGGGGGG

|⇒ITS2
TATGCCTGTCCGAGCGTCAT|TGCTGCCCTCAAGCACGGCTTGTGTGTTGGG

TCGTCGTCCCCCCCCAGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCG

CGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTTGTAGGCCCG

GCCGGCTGCTGGCCGACGCTGAAAAGCAACCAACTATTTTTCCAGG

Fig. 3. The sequences of ITS1 and ITS2, and 5.8S rDNA of a new *Aspergillus* strain.

ITS1 spans from 1 to 168 bp; the 5.8S coding region is from 169 to 326 bp; and ITS2 is from 326 to 501 bp.

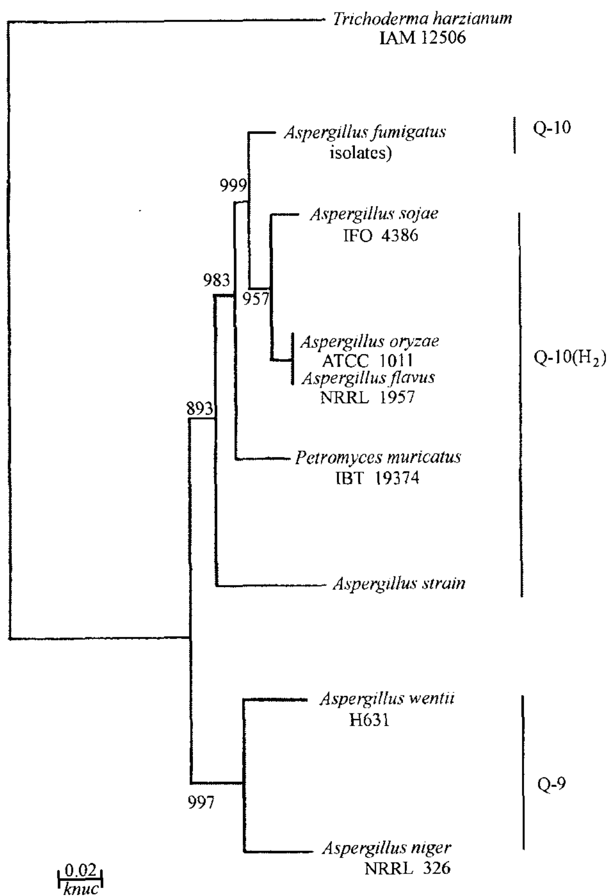

Fig. 4. Phylogenetic tree showing the relationship between a new *Aspergillus* strain and species related to the fungi.
The bar represents 2 nucleotide substitutions per 100 nucleotides in the ITS1 and ITS2, and 5.8S rDNA sequences. The bootstrap probabilities were indicated at the branch points.

strain differed from *A. sojae* which had previouly been known to produce bioflocculants in the morphological and molecular features. Accordingly, the new *Aspergillus* strain is believed to be a new biofluocculant-producing *Aspergillus* strain. In particular, this strain is expected to be useful in various industrial applications for flocculating organic materials such as charcoal.

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