

Screening of Myxobacteria Carrying Tubulysin Biosynthetic Genes

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Tubulysins are a group of secondary metabolites produced by myxobacteria that inhibit the function of the eukaryotic cytoskeleton. We developed a pair of PCR primers that specifically amplified tubulysin biosynthetic genes. Using these primers, eight out of the eighty-one strains of myxobacteria belonging to the *Cystobacteraceae* family that harbored putative tubulysin biosynthetic genes were screened through PCR analysis. The selected strains included two *Archangium gephyra*, two *Stigmatella* sp., two *Vitiosangium cumulatum*, and two unidentified myxobacteria. LC-MS analysis of the culture extracts from the selected strains revealed that *A. gephyra* KYC4066 produced putative tubulysin A and B.

Keywords: *Archangium gephyra*, myxobacteria, secondary metabolite, tubulysin

Introduction

Myxobacteria are delta-proteobacteria that produce diverse bioactive secondary metabolites. More than 600 different metabolites have been isolated from myxobacteria [1–3]. Among these metabolites isolated from myxobacteria, seven groups of metabolites exhibit inhibitory activity against the function of the cytoskeleton in eukaryotic cells. Substances inhibiting the function of the cytoskeleton possess the potential to be developed as anticancer drugs. For example, epothilones are a group of the substances produced by myxobacteria that inhibit cytoskeleton function [4]. The United States Food and Drug Administration approved Ixabepilone, a semisynthetic derivative of epothilone B, as an anticancer treatment in 2007, as it exhibits excellent effects on cancer cells that possess multi-pharmaceutical resistance [5].

Tubulysins are another group of substances that inhibit the function of the cytoskeleton and have been

studied for its superior anticancer activity [6–9]. Tubulysins promote the depolymerization of tubulin polymers [10], while epothilones promote the polymerization of tubulin [4]. Tubulysins are attractive lead structures for development as antineoplastic agents; however, tubulysin-producing strains have not been isolated and reported in Korea.

Tubulysins were previously reported to be produced by the myxobacteria *Angiococcus disciformis*, *Archangium gephyra*, and *Cystobacter* sp. [6, 11]. Tubulysin biosynthetic gene clusters have been identified and reported from *A. disciformis* An d48 and *Cystobacter* sp. SBCb004 [11, 12] (Fig. 1). DNA sequences homologous to those of the tubulysin biosynthetic genes are also found in the genome of *Vitiosangium* sp. GDMCC 1.1324 (GenBank accession number: NZ_PZOX01000000). *Angiococcus*, *Archangium*, *Cystobacter*, and *Vitiosangium* genera belong to the *Cystobacteraceae* family of myxobacteria [13]. Myxobacteria belonging to *Cystobacteraceae* are distinguished from other myxobacterial families in the shape of vegetative cells, myxospores, and swarm colonies. Vegetative cells of *Cystobacteraceae* are slender,

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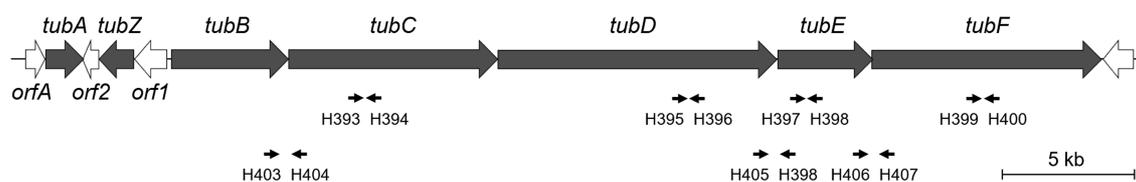


Fig. 1. PCR primers used to detect tubulysin biosynthetic genes. Locations of the primers are marked on the tubulysin biosynthetic gene cluster from *Cystobacter* sp. SBCb004 [11].

flexible long rods with tapering ends. Myxospores are short, optically refractile rods or spheroids. Swarm colonies quickly adsorb Congo red and turn purple red [13].

The Myxobacteria Bank at Hoseo University possesses approximately 2,600 myxobacterial strains that were isolated in Korea. In this study, we attempted to identify tubulysin-producing strains among the myxobacterial strains in the Myxobacteria Bank by screening strains carrying tubulysin biosynthetic genes.

Materials and Methods

Strains, culture conditions, and chemicals

All myxobacterial strains used in this study were isolated from Korean soil by the Myxobacteria Bank at Hoseo University [14, 15]. Among the strains used for this study, another strain number of KYC2615 was MEHO 001, and the accession number for this strain provided by the International Depository Authority was KCTC 13929BP. Another strain number of KYC4066 was MEHO 003, and the accession number was KCTC 14258BP. VY/3 medium [16] was used for the vegetative growth of the strains, and CYS medium [16] was used for the preparation of culture extracts. All strains were cultured at 32°C. Synthetic tubulysin (TAM1446) was purchased from Tub Pharmaceuticals GmbH, Austria.

Polymerase chain reaction (PCR)

Two oligonucleotides, 5'-GTGCAGAAAYCTGCGGCTCTT-3' (H403) and 5'-AGCTGCTCCTGGAACCACA-3' (H404) were used to detect tubulysin biosynthetic genes using PCR. Two oligonucleotides, 5'-GAGTTTGATCCTGGCTCAG-3' (27f) and 5'-AGAAAGGAGGTGATCCAGCC-3' (1525r) that were reported previously [17] were used for PCR amplification of 16S rRNA genes. PCR was performed using 2X *Taq* PCR Pre-Mix (Solgent, Korea). For the PCR analysis, 35 cycles that included denaturation at 94°C for 40 sec, annealing at 60°C for 20 sec, and

extension at 72°C for 90 sec were performed. The PCR products were analyzed by agarose gel electrophoresis.

DNA sequences of the PCR products were determined by Macrogen (Korea) and were analyzed using the BLAST program [18]. The phylogenetic analysis of the strains based on 16S rRNA sequences was performed using the MEGA-X program [19] and the 16S rRNA database from EZBioCloud [20].

Preparation of culture extracts

Myxobacterial strains were cultured in CYS liquid media with 1% Amberlite XAD-16 resin (Sigma, USA) for 7 days. The resin was harvested and extracted using methanol. Methanol was evaporated from the extract, and the residue was dissolved in a mixture of ethyl acetate and water in a 1:1 ratio (v/v). After the ethyl acetate and water layers were separated, the ethyl acetate layer was recovered and dried. The dried residue was dissolved in methanol.

High-performance liquid chromatography (HPLC)

HPLC was performed using an HPLC system (1260 VL Infinity Series; Agilent, USA) equipped with a Zorbax SB-C18 column (4.6 × 150 mm, 5 μm) (Agilent). Mobile phase A was water and mobile phase B was acetonitrile, and both mobile phases contained 0.1% formic acid. The gradient elution at a flow rate of 0.5 ml/min was performed as follows: 0–5 min 30% B (isocratic), 5–25 min 30–60% B (linear gradient), 25–30 min 60–100% B (linear gradient), and 30–35 min 100% B (isocratic).

LC-MS analysis

Liquid chromatography mass spectrometry (LC-MS) was performed using an Accelar UHPLC (Thermo Scientific, USA) equipped with an Acquity UPLC® BEH C18 column (2.1 × 150 mm, 1.7 μm) and an LTQ-Orbitrap XL high-resolution mass analyzer located at Gyeonggi

Bio-Center. Mobile phase A was water, mobile phase B was acetonitrile, and both mobile phases contained 0.1% formic acid. The gradient elution at a flow rate of 0.4 ml/min was performed as follows: 0–1 min 20% B (isocratic), 1–20 min 20–80% B (linear gradient), 20–24 min 80–100% B (linear gradient), and 24–27 min 100% B (isocratic).

Results

Development of PCR primers for the detection of tubulysin biosynthesis genes

In this study, we used PCR analyses in an attempt to select a tubulysin-producing strain by screening strains with tubulysin biosynthetic genes from among the 81 strains of *Cystobacteraceae* owned by the Myxobacteria Bank, and following selection, we confirmed the production of tubulysins. To achieve this, PCR primers that specifically detect only tubulysin biosynthesis genes are required. However, as such primers have not been previously characterized, we first endeavored to develop primers that specifically detect the tubulysin biosynthetic genes.

To develop PCR primers specific for the tubulysin biosynthesis genes, the nucleotide sequences of the tubulysin biosynthesis genes from *A. disciformis* An d48 and *Cystobacter sp.* SBCb004 (GenBank accession numbers: AJ620477, GU002154) were aligned. Conserved sequence regions in both strains were selected and used to design 7 pairs of PCR primers (Fig. 1, Table S1). These primer pairs were first used for PCR amplification on 10 strains that were randomly selected to test if they specifically amplified the tubulysin biosynthetic genes. The analysis of PCR products by agarose gel electrophoresis revealed that excessive amounts of nonspecific bands were generated from the six pairs of primers, and thus, strains with and without tubulysin biosynthetic genes could not be distinguished. However, the H403/H404 primer pair specifically amplified the end of the *tubB* gene and the beginning of the *tubC* gene. Therefore, the H403 and H404 primer pairs were selected as primers for detecting the tubulysin biosynthetic genes.

Screening of strains carrying tubulysin biosynthesis genes by PCR

When PCR was performed on 81 strains using the

H403 and H404 primer pair, it was found that a PCR product of approximately 1.4 kb in size was generated from 8 strains (KYC1032, KYC2615, KYC2714, KYC2827, KYC2836, KYC2837, KYC2840, and KYC4066). Analysis of the nucleotide sequences of the resulting PCR products indicated that all of the PCR products possessed the end of the *tubB* gene and the beginning portion of the *tubC* gene, and these products exhibited greater than 83% similarity to those from *Cystobacter sp.* SBCb004. For example, the sequence of the PCR product (1,399 bp) obtained from the KYC4066 strain was 85% identical to that of the *tubB-tubC* gene site of *Cystobacter sp.* SBCb004 (Fig. S1) This confirmed that the H403 and H404 primer pair specifically amplified the tubulysin biosynthesis genes.

Phylogenetic analysis of the strains

Six of the 8 selected strains (KYC1032, KYC2615 (= MEHO 001), KYC2714, KYC2836, KYC2837, and KYC4066 (= MEHO 003)) were pure isolated strains, while KYC2827 and KYC2840 were not. The 27f and 1525r primers were used to amplify the 16S rRNA gene of 6 pure isolated strains by PCR. The nucleotide sequences of the resulting PCR products were determined and then used to identify the strains according to phylogenetic analysis. The nucleotide sequences of the 16S rRNA genes from the KYC2615 and KYC4066 strains were 99.11% and 99.79% identical, respectively, to that of DSM 2261^T, which is the type strain of *Archangium gephyra* (Fig. 2). The KYC1032 and KYC2714 strains possessed 16S rRNA gene sequences that were 99.52% and 99.93% identical, respectively, to those of ATCC 25190^T, ATCC 25191^T, and DSM 14722^T, the type strains of *Stigmatella* (Fig. 2). The nucleotide sequences of the 16S rRNA genes of the KYC2836 and KYC2837 strains were 99.31% and 99.83% identical, respectively, to that of MCy10943^T, the type strain of *Vitosangium cumulatum* (Fig. 2). Therefore, among the six strains, two strains appeared to be *A. gephyra*, another two strains were *Stigmatella sp.*, and the remaining two strains were *V. cumulatum*.

Analysis of culture extracts

To test if the 8 strains selected through PCR detection actually produce tubulysins, culture extracts were prepared and analyzed by HPLC. When the culture extract

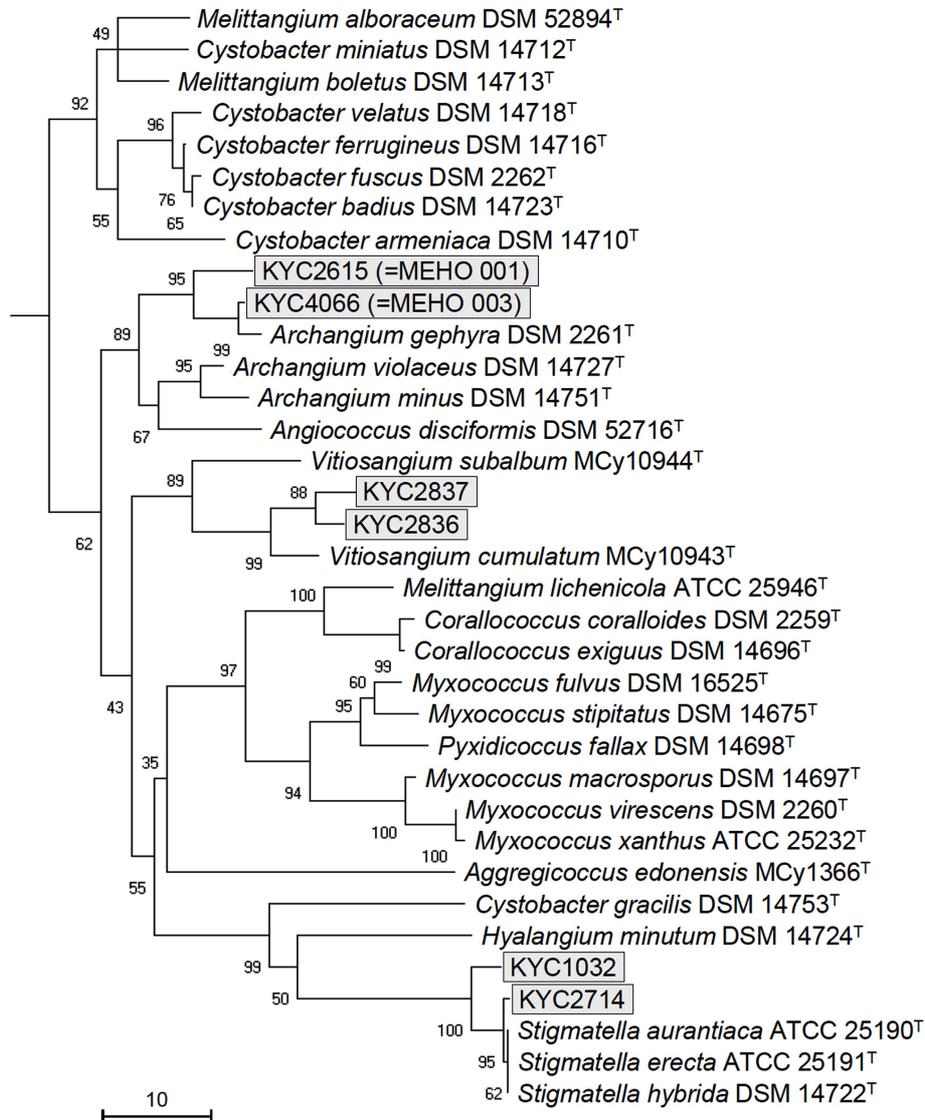


Fig. 2. Phylogenetic dendrogram of the strains based on 16S rRNA sequences. The phylogenetic dendrogram was generated using the Neighbor-Joining method [23]. The bootstrap values are expressed as percentages of 1,000 replications. *Anaeromyxobacter dehalogenans* 2CP-1^T (AF382396) was used as an out-group (not shown). Bar, 10 substitutions in 1,452 nucleotide positions.

of the KYC4066 strain was analyzed, the peak substances detected at 22.58 and 25.21 min exhibited a PDA spectrum that was highly similar to that of TAM1446, a synthetic tubulysin (Fig. 3). Subsequently, these peak substances were separated by HPLC and then analyzed by mass spectrometry. The 22.58 min peak substance exhibited an m/z value of 830.4334 ($[M+H]^+$) that was highly similar to the theoretical molecular weight of tubulysin B (830.4368 ($[M+H]^+$)), and the 25.21 min

peak substance possessed a m/z value of 844.4487 ($[M+H]^+$) that was highly similar to the theoretical molecular weight of tubulysin A (844.4525 ($[M+H]^+$)) (Fig. 4). When all the possible chemical formulas possessing a molecular weight within Delta (ppm) ± 5 were analyzed, no other substances produced by myxobacteria other than tubulysin A and B were found. Therefore, the 22.58 min and 25.21 min peak substances detected in the culture extract from the KYC4066 strain were highly

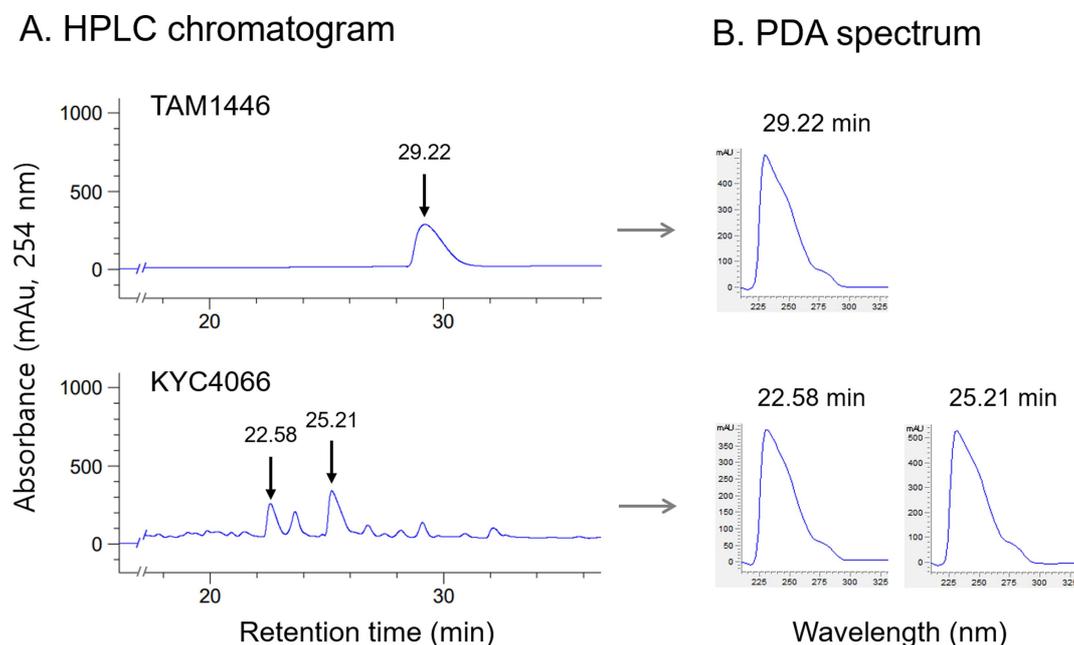


Fig. 3. HPLC chromatogram of the culture extract from *Archangium gephyra* KYC4066 and PDA spectrums of HPLC peaks. The culture extract from *A. gephyra* KYC4066 was separated using an HPLC method, and the PDA spectrum of the peaks was compared to that of TAM1446, a synthetic tubulysin.

likely to be tubulysin B and tubulysin A, respectively.

Discussion

Searching for microorganisms that produce specific substances requires a great deal of time and effort. Even more efforts are required when the microorganisms that produce a specific target substance are not common or when the substance is very difficult to analyze due to small production amounts. Tubulysins inhibit the cytoskeleton and exhibit excellent anticancer activity. Therefore, many studies examining this substance have been conducted. However, only a few of the strains producing tubulysins are known worldwide. Additionally, the tubulysin producing strains produce trace amounts of tubulysins. In this study, we developed a primer pair that specifically detects the tubulysin biosynthesis genes, and used it in PCR analyses to select 8 strains containing the tubulysin biosynthesis gene. By analyzing the culture extracts of these strains, we confirmed that *A. gephyra* KYC4066 produced substances that were presumed to be tubulysin A and B. Selection of microbial strains by this method is expected to be useful in the future for the selection of strains that produce var-

ious other substances.

Of the 81 strains used in this study, 24 were presumed to be *A. gephyra*. Of these, only two strains, KYC2615 and KYC4066, were detected to possess the tubulysin biosynthesis gene, while most of the other *A. gephyra* strains were detected not to have the tubulysin biosynthesis gene. We also could not locate the tubulysin biosynthesis genes in the genome of DSM 2261^T, the type strain of *A. gephyra* (GenBank accession number: CP011509). Some secondary metabolic biosynthesis genes of myxobacteria, including the carotenoid biosynthesis genes, are commonly found in almost all myxobacterial strains [3]. However, some genes were found only in specific strains. For example, chivosazol and etnangien biosynthetic genes are found in the So ce56 strain of *Sorangium cellulosum*; however, they were not found in the So0157-2 strain belonging to the same species [21, 22]. It appears that tubulysin is another example in which only a few strains possess a certain biosynthetic gene, while most of the other strains do not despite belonging to the same species.

Tubulysins were first reported to be produced by the myxobacteria *A. disciformis* and *A. gephyra* [6], and it was later reported to be produced by *Cystobacter* sp. [11].

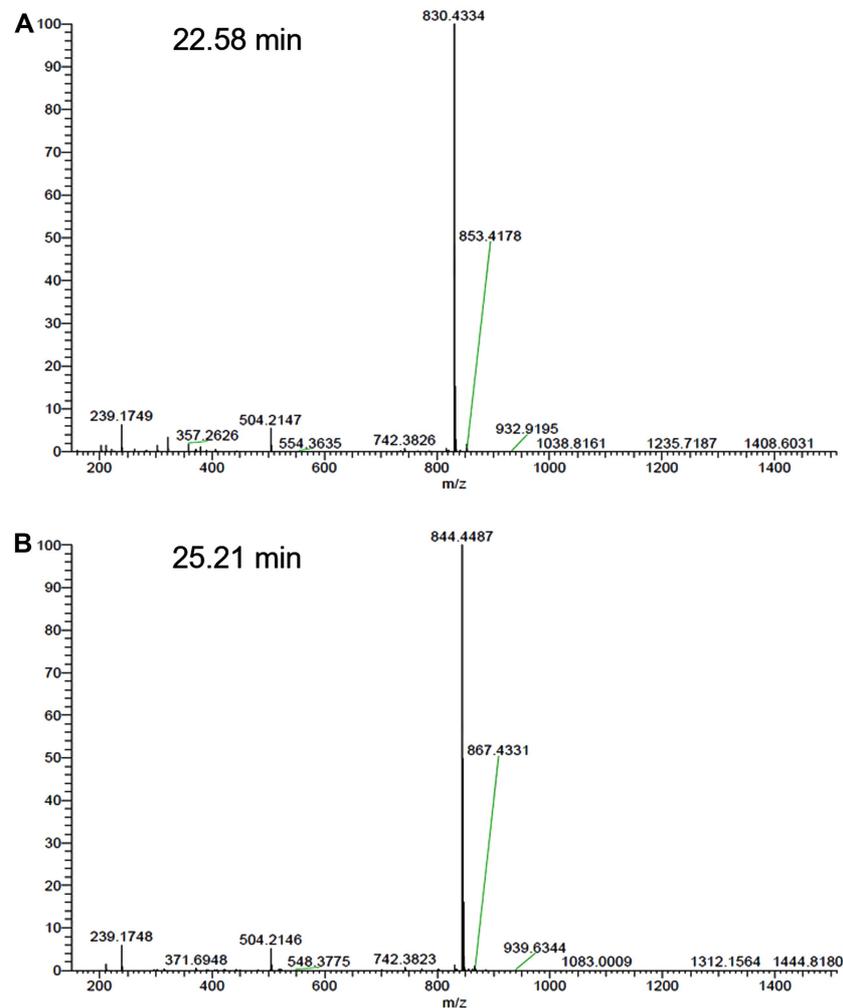


Fig. 4. Mass spectroms of the HPLC peak substances produced by *A. gephyra* KYC4066.

Sequences similar to those of the tubulysin biosynthetic genes were also found in the genome of a strain belonging to the genus *Vitiosangium* (GenBank accession number: NZ_PZOX01000000), and some gene fragments were found in the genome of *S. aurantiaca* [11]. In this study, it was determined that two strains of *A. gephyra*, two strains of *Stigmatella*, two strains of *V. cumulatum*, and two strains of unidentified myxobacteria possessed tubulysin biosynthesis genes. As tubulysins are produced in a very small amount while the culture extracts contain many other substances, it was difficult to determine if the strains produced tubulysins by the simple HPLC analysis of the culture extract. Therefore, it remains unknown if all these strains produce tubulysins. It is also unknown if all these strains possess

a complete set of tubulysin biosynthetic genes. However, we found that KYC4066, one of the selected strains, produced putative tubulysin A and B. Thus, we expected that it is highly likely that these strains produce tubulysins.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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