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Isolation and Identification of a Marine Bacterium, *Pseudomonas* sp. BK1 Producing Extracellular Enzymes Capable of Decomposing Multiple Complex Polysaccharides

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Abstract

A marine bacterium (strain BK1) that produces extracellular enzymes capable of decomposing complex polysaccharides, such as agar, chitin, carboxymethylcellulose, xylan and mannan, was isolated from the marine red alga *Porphyra dentata*. Strain BK1 was gram-negative, aerobic, catalase- and oxidase-positive, polarly flagellated bacilli that produce gelatinase and urease, but not decarboxylases. The G+C content of the DNA was 51.6 mol%. The major isoprenoid quinone component was identified as an ubiquinone-8, and the major cellular fatty acids were C16:0, C16:1 w6c and C18:1 w7c. Comparative 16S rRNA sequence analysis placed strain BK1 with members of the genus *Pseudomonas*. On the basis of phenotypic and genotypic data, the strain BK1 was shown to be a member of the subgroup of *Pseudomonas*, and named as *Pseudomonas* sp. BK1.

Key words – Polysaccharides, *Porphyra*, *Pseudomonas*

Introduction

During the past decades, marine microorganisms such as bacteria and microalgae have been increasingly recognized as an important and untapped resource for novel bioactive materials[2]. The oceans cover greater than 70% of the earth's surface and, taking this into account by volume, represent better than 95% of the biosphere. Given this fact, the oceans present themselves as an unexplored area of opportunity for the discovery of pharmacologically active compounds. However, it is important to pursue basic research on the marine environment in order to permit the continued isolation of unique microorganisms. There is still limited knowledge of the physiological requirements of most marine microorganisms, and a greater understanding of their conditions for growth will offer new insights into the complex world of marine microbiology.

The marine red alga, *Porphyra*, is abundantly cultivated in eastern Asia including Japan, Korea and China for food use. Porphyran originates from the cell wall and intercellular region of *Porphyra*, and is known to be closely related to agarose in its basic structure, whereas

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it is very different in terms of having L-galactose-6-sulfate. The primary structure of porphyran shows alternating 1,4-linked 3,6-anhydro-L-galactose units and 1,3-linked β-D-galactose units, which sometimes occur as the L-galactose-6-sulfate and 6-*O*-methyl derivative, respectively[11,13]. It has been reported that porphyran has some physiological functions such as antitumor activity[14], antihypertensive and antihyperlipidemic effects[15], and macrophage stimulation activity[19]. Despite of its biomedical applications, however, *Porphyra* farming has frequently suffered great economic damage due to several bacterial and fungal diseases. Therefore, breeding improvements using biotechnological approaches, such as protoplast fusion and gene manipulation, are expected to help resolve these problems. The establishment of effective techniques for protoplast production might offer useful tools to improve the breeding of *Porphyra* species. Since the cell wall of *Porphyra* is mainly composed of complex polysaccharides such as xylan, mannan, porphyran and cellulose[3], the development of enzymes capable of decomposing components of *Porphyra* cell wall will facilitate more efficient production of *Porphyra* protoplast.

In the present study, we isolated and characterized a marine bacterium from *Porphyra dentata* which produces extracellular enzymes capable of decomposing multiple complex polysaccharides.

Materials and Methods

Isolation, media and culture conditions

To isolate the algal polysaccharide-decomposing bacteria, *Porphyra dentata* with disease symptom of green spot rot-like deterioration on their tissues were collected from a farm of laver in Haenam, Korea. *Porphyra dentata* tissue showing the severe disease symptom was excised and then suspended in filtrated seawater with shaking for 2 h. After centrifugation at 5000×g for 10 min, the supernatant was diluted and spread on A medium containing 1.2% agar. A medium consists of the following components per seawater liter (pH 7.5); 10 g polypeptone, 1 g yeast extract, 0.1 g K_2HPO_4, 0.6 mg $FeCl_3$ and 1 g Tris. Firstly, agar-decomposing clones were selected by visual inspection from the agar plate and finally detected by staining the plate with I_2/KI solution[5]. These clones were incubated at 23℃ for 3 days in A medium (pH 7.5) and the supernatants obtained by centrifugation were used for enzyme assays related to decomposition of complex polysaccharides.

Biochemical and physiological characterization

The general characteristics of the isolated strain were determined according to *Bergey's Manual of Systematic Bacteriology*[9] and other physiological tests including carbon-source utilization were performed using API 20E system (BioMerieux, France), which were prepared according to the manufacturer's specifications.

Morphological characteristics

Morphology of the isolated strain was examined by phase-contrast microscopy and transmission electron microscopy. For transmission microscopy, the cells were fixed with 5% glutaraldehyde and 1% osmium tetroxide. Ultrathin sections of the sample embedded in epoxy resin were prepared with an ultramicrotome, stained with uranyl acetate and lead citrate. Electron micrograph was taken using a model JEM-1200 EX transmission electron microscope (JEOL Ltd, Tokyo, Japan).

Cellular fatty acid analysis

Bacterial cells were harvested, saponified, and methylated. The fatty acid methyl esters (FAMEs) were analyzed by gas chromatography (HP 6890, Hewlett-Packard Co., CA, U.S.A.), and identified using a Microbial Identification System (MIDI, Inc., NewYork, U.S.A.) to determine the fatty acid composition.

Quinone analysis

The quinones were extracted from the cell pellet washed with 50 mM phosphate buffer (pH 7.0) using

chloroform/methanol (2:1, v/v), and analyzed by thin-layer chromatography (TLC) on Kieselgel $60F_{254}$ plate (Merck, Germany) using a mixture of hexane:diethyl ether (85:15, v/v) as the developing solvent. The quinone bands were detected under UV wavelength, scraped from the TLC plate, and recovered in acetone. The isoprenoid quinine composition was determined by high-performance liquid chromatography (HPLC) under the following conditions: column, Spherisorb 5 μm ODS2 (4.6×250 mm); eluent, methanol/isopropyl ether (3:1, v/v) for ubiquinones, methanol/isopropyl ether (4:1, v/v) for menaquinones; flow rate, 1 ml/min. The ubiquinones and menaquinones were detected by monitoring at 275 nm and 270 nm, respectively, using a UV detector.

G+C content of DNA

The G+C content of the DNA was analyzed by HPLC under the following conditions: C18 symmetry column (4.6×250 mm); 0.5 M triethylamine phosphate (pH 5.1):water:methanol (1:18:5, v/v/v); mobile phase flow rate of 1 ml min^{-1} and UV detector at 254 nm.

Analysis of 16S rDNA gene sequence

For the sequence analysis, bacterial genomic DNA was extracted and purified using a Wizard Genomic DNA Prep. Kit (Promega Corp., Madison, U.S.A.). Two primers annealing at the 5′ and 3′ end of the 16S rDNA genes were 5′-GAGTT TGATC CTGGC TCAG-3′ (positions 9 to 27 [*E. coli* 16S rDNA numbering]) and 5′-AGAAA GGAGG TGATC CAGCC-3′ (positions 1542 to 1525 [*E. coli* 16S rDNA numbering]), respectively. PCR amplification was performed in a final reaction volume of 100 μl, and the reaction mixture contained each primer at a concentration of 0.5 μM, each deoxynucleoside triphosphate at a concentration of 200 μM, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, and 2.5 U of *Taq* DNA polymerase. The PCR reaction was run for 35 cycles on a DNA thermal cycler (Model No. 9700, Perkin-Elmer Co., Norwalk, USA). The following thermal profile was used for the PCR: denaturation at 94℃ for 1 min, primer annealing at 60℃ for 1 min and extension at 72℃ for 2 min. The final cycle included extension for 10 min at 72℃ to ensure full extension of the products. The amplified PCR products were then analyzed on a 1.0% (w/v) agarose gel, excised from gel, and purified. The purified products were cloned into pGEM-T Easy vector (Promega Corp., Madison, U.S.A.) and subsequently sequenced using ALF Red automated DNA sequencer (Pharmacia, Sweden). The 16S rDNA sequence of the strain BK1 was aligned with those in the GenBank database. Multiple alignment of sequences and calculations of levels of sequence similarity was performed by using CLUSTAL W[18]. The neighbor-joining phylogenetic analysis was carried out with MEGA program[10].

Enzyme assays

Agarase activity was measured by the method of Ha *et al.*[5] with some modifications. Standard assay conditions were as follows. The enzyme sample (10 μl) was added to 90 μl of 0.2% (w/v) melted low-melting-point-agarose solution in 20 mM Tris-HCl buffer (pH 8.0). After incubation at 30℃ for 30 min, the reaction was stopped by immersion in boiling water for 10 min. Amounts of the released reducing sugars were determined by the Somogyi-Nelson method[16] using D-galactose as a standard. One unit of agarase activity was defined as the amount of enzyme which produces 1 μmol of galactose per minute.

Mannanase activity was assayed by the method of Kataoka and Tokiwa[7] using 0.5% (w/v) locust bean gum as substrate. The substrate was suspended in 20 mmol l^{-1} phosphate buffer (pH 7.0) by heating at 121℃ for 20 min and the insolubles were removed by centrifugation. The enzyme sample (0.1 ml) was incubated with 0.9 ml of the substrate solution at 30℃ for 20 min. Amounts of the released reducing sugars were determined by the Somogyi-Nelson method[16] using D-mannose as a standard. One unit of mannanase activity was defined as the amount of enzyme which produces

1 μmol of mannose per minute.

Xylanase activity was determined by measuring the released reducing sugars as xylose by the dinitrosalicylic acid[12]. One milliliter of enzyme sample was mixed with 1 ml citrate/phosphate buffer (pH 7.0) and 1 ml 1% xylan. The reaction mixture was incubated at 40℃ for 5 min. The reaction was stopped by addition of 3 ml dinitrosalicylic acid reagent. One unit of xylanase activity was defined as the amount of enzyme which produces 1 μmol of xylose per minute.

Chitinase activity was assayed in a reaction mixture containing 1.4 ml of 0.2% glycol chitin in 10 mM Tris-HCl (pH 6.8) and 0.1 ml of enzyme sample. After incubation at 25℃ for 20 min, 2 ml of 0.5 M sodium carbonate containing 0.05% potassium ferricyanide was added to the reaction solution, which was then boiled for 15 min. Amounts of the released reducing sugars were determined by the method of Imoto and Yagishita [6]. One unit of chitinase activity was defined as the amount of enzyme which produces 1 μmol of *N*-acetyl-glucosamine per minute.

Cellulase activity was assayed according to the method of Thomas and Bhat[17] using 1% (w/v) carboxymethylcellulose (CMC) as substrate. One unit of cellulase activity was defined as the amount of enzyme which produces 1 μmol of glucose per minute.

Results and discussion

Isolation of strain BK1

Five strains exhibiting agarase activity were selectively isolated from *Porphyra dentata* tissue showing the severe disease symptom. After incubation at 23℃ for 3 days, extracellular enzyme activities for mannanase, chitinase, xylanase and CMCase were measured using the supernatants obtained from each strain as enzyme samples. Among these strains, as shown in Table 1, strain BK1 revealed highest activities for five enzymes tested, and was finally selected for further study.

Morphological, biochemical and physiological properties

As shown in Fig. 1, the strain BK1 was found to be a Gram-negative, rod-shaped with several polar flagella, motile, non-spore forming bacteria. The results obtained for the biochemical and physiological characteristics of strain BK1 are summarized in Table 2. The strain BK1

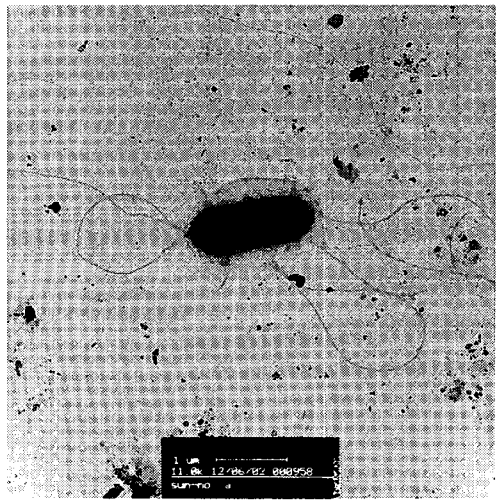

Fig. 1. Transmission electron micrograph of strain BK1. Scale bar = 1 μm.

Table 1. Comparison of enzyme activities of the five isolates.

Strain	Enzyme activities (U/ml of supernatant)				
	Agarase	Chitinase	CMCase	Xylanase	Mannanase
BK1	3.82	12.72	10.13	11.84	2.46
BK2	2.61	8.53	8.50	9.22	1.97
BK3	2.24	10.23	7.67	10.23	2.13
BK4	3.27	9.34	9.14	7.75	2.06
BK5	3.15	9.67	8.16	9.45	1.93

Table 2. Biochemical and physiological characteristics of strain BK1.

Characteristics	Strain BK1
Catalase	+
Oxidase	+
Arginine dihydrolase	-
Lysine decarboxylase	-
Ornithine decarboxylase	-
Gelatinase	+
Urease	-
Assimilation of	
Glucose	+
Arabinose	+
Mannitol	-
Sorbitiol	-
Rhamnose	+
Sucrose	-
Melibiose	+
Trehalose	+
Gluconate	+

+, positive; -, negative.

exhibited positive results with catalase, oxidase and gelatinase tests, while showing negative results with arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and urease. In addition, strain BK1 was able to utilize glucose, arabinose, rhamnose, melibiose, trehalose and gluconate as carbon sources. Accordingly, these morphological, biochemical and physiological properties suggested that strain BK1 was closely related to *Pseudomonas* subgroup[9].

Quinone component

The major isoprenoid quinone component of strain BK1 was found to be ubiquinone-8 (Fig. 2). Members of the genus *Pseudomonas* generally contain ubiquinone Q-9 as their major isoprenoid quinine components, while *Pseudomonas denitrificans* and some unnamed *Pseudomonas* spp. possess major amount of Q-10[1]. Some species of the *Pseudomonas* subgroup like *Pseudomonas fluorescens* and *Pseudomonas maltophilia* also contain Q-8 as the major isoprenoid quinine component[1].

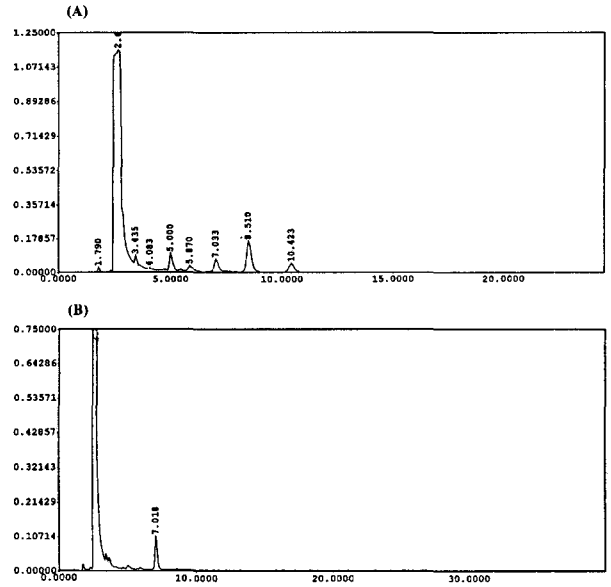

Fig. 2. HPLC chromatogram of the isoprenoid quinone composition of standard ubiquinones (A) and quinones extracted from strain BK1 (B).

Fatty acid composition

The cellular fatty acid analysis of strain BK1 is shown in Table 3 and Fig. 3. The major fatty acids of strain BK1 were C16:0 (26.09%), C16:1 w6c (24.48%), C18:1 w7c (11.18%) and C15:0 2OH, whereas low levels of fatty acids were C10:0 3OH, C12:0 2OH, C18:1 w6c and C18:0. This result was similar to that of *Pseudomonas* sp. which is a gram-negative strain[8].

16S rDNA sequence and G+C content

The 1,492 bp sequence obtained from strain BK1 was

Table 3. Major cellular fatty acids in strain BK1.

Retention time	Fatty acids	%
4.048	C 10:0 3OH	4.81
5.930	C 12:0 2OH	5.57
6.032	C 12:0 3OH	3.54
9.904	C 15:0 2OH	9.20
9.965	C 16:1 w6c	24.48
10.205	C 16:0	26.09
13.358	C 18:1 w7c	11.18
13.418	C 18:1 w6c	6.00
13.666	C 18:0	4.75

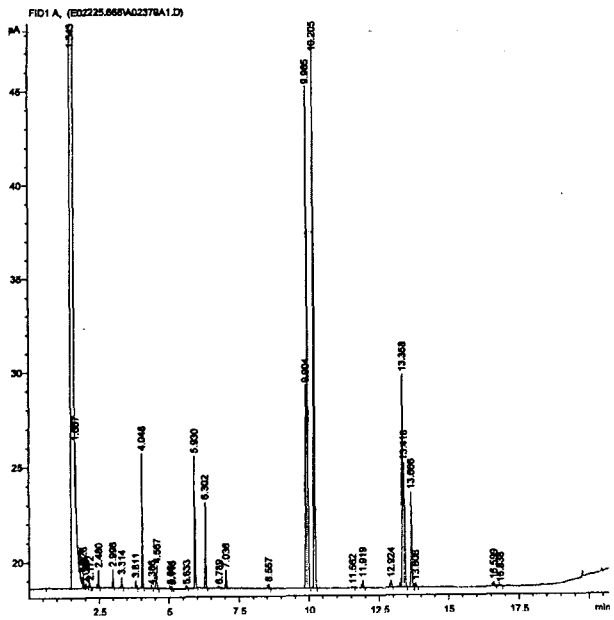

Fig. 3. Gas chromatogram of fatty acid methyl esters (FAMEs) from strain BK1.

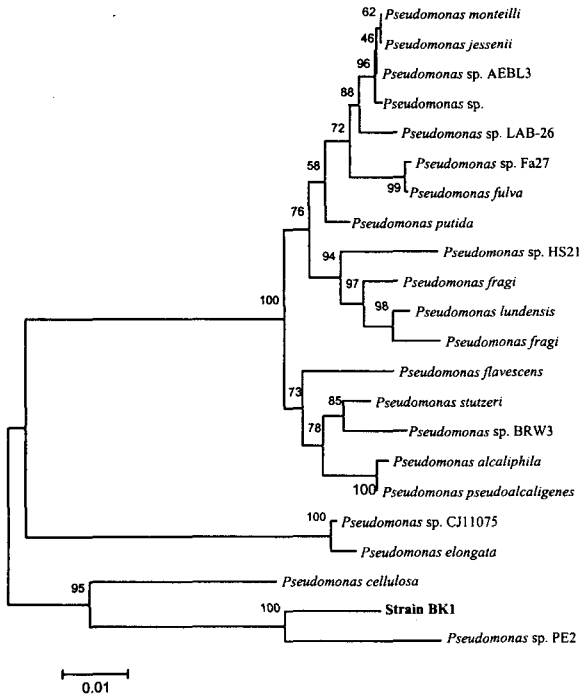

Fig. 4. Phylogenetic comparison between strain BK1 and most closely related bacteria. The values at branch-points indicate the percentage support for a particular node after 100 bootstrap replications. The phylogenetic tree was constructed based on the alignment of 16S rDNA sequences.

aligned with all the available 16S rDNA gene sequences in GenBank databases. As a result, a phylogenetic tree was constructed (Fig. 4). The phylogenetic analysis of strain BK1 using its 16S rDNA sequence data showed that this strain possesses 16S rDNA sequences similar to *Pseudomonas*. The levels of similarity of the strain BK1 with *Pseudomonas* ranged from 85.2% to 96.0%. The strain BK1 sequences were most identical to the sequences of *Pseudomonas* sp. PE2 (similarity, 96.0%). The G+C content of strain BK1 DNA was 51.6 mol%. This value was different from that of the genus *Pseudomonas* showing 58 ~71 mol%[9].

In conclusion, the strain BK1 was found to be closely related to the *Pseudomonas* subgroup based on its morphology, physiological properties, fatty acid composition, and quinine component. The phylogenetic analysis of strain BK1 using its 16S rDNA sequence data also supported the isolate closely related to the *Pseudomonas* subgroup. Accordingly, the isolated bacterium BK1 was named as "*Pseudomonas* sp. BK1". This organism was deposited with Korean Federation of Culture Collections under accession no. KFCC-11308.

Extracellular hydrolytic enzymes such as xylanase, mannanase and cellulase are useful for structural analysis of the cell walls of marine algaes and for protoplast isolation, which is an important technique for cell fusion and gene manipulation of algae. Extracellular hydrolytic enzymes such as cellulase, chitinase and mannanase consist of discrete domains, which can be arranged in different orders in different proteins[20].

Purification of these enzymes is currently in progress in order to clarify their enzymatic properties. Further research is planned to clone the genes for these enzymes and deduce the complete primary structures from their nucleotide sequences. Manipulation of the genes will provide detailed information on the multiplicity and activity domains of these enzymes.

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초록 : 복합 다당류 분해 효소들을 생산하는 해양미생물 *Pseudomonas* sp. BK1의 분리 및 특성

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한천, 키틴, 셀룰로오스, 자일란, 만난과 같은 복합다당류들에 대한 분해능을 나타내는 효소들을 생산하는 해양미생물을 홍조류인 *Porphyra* dentata로부터 분리하였다. 분리균 BK1은 그람음성, 호기성 간균으로 DNA의 G+C함량은 51.6 mol%를 나타내었다. 주요 isoprenoid quinine 구성성분은 ubiquinone-8로 확인되었고, 주요 세포 지방산은 C16:0, C16:1 w6c, C18:1 w7c로 밝혀졌다. 16S rRNA서열의 비교분석 결과는 분리균 BK1이 *Pseudomonas* 속의 일원인 것으로 확인되었다. 이러한 결과를 바탕으로 분리균 BK1은 *Pseudomonas* sp. BK1으로 명명하였다.