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Characterization of Two Algal Lytic Bacteria Associated with Management of the Cyanobacterium *Anabaena flos-aquae*

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Abstract Various microorganisms were isolated from the surface waters and sediments of eutrophic lakes and reservoirs in Korea to enable an investigation of bacteria having algal lytic activities against *Anabaena flos-aquae* when water blooming occurs and to study enzyme profiles of algal lytic bacteria. Two bacterial strains, AFK-07 and AFK-13, were cultured, characterized and identified as *Acinetobacter johnsonii* and *Sinorhizobium* sp., respectively. The *A. johnsonii* AFK-07 exhibited a high level of degradatory activities against *A. flos-aquae*, and produced alginase, caseinase, lipase, fucodian hydrolase, and laminarinase. Moreover, many kinds of glycosidase, such as β-galactosidase, β-glucosidase, β-glucosaminidase, and β-xylosidase, which hydrolyzed β-*O*-glycosidic bonds, were found in cell-free extracts of *A. johnsonii* AFK-07. Other glycosidases such as α-galactosidase, α-*N*-Ac-galactosidase, α-mannosidase, and α-L-fucosidase, which cleave α-*O*-glycosidic bonds, were not identified in AFK-07. In the *Sinorhizobium* sp. AFK-13, the enzymes alginase, amylase, proteinase (caseinase and gelatinase), carboxymethyl-cellulase (CMCase), laminarinase, and lipase were notable. No glycosidase was produced in the AFK-13 strain. Therefore, the enzyme system of *A. johnsonii* AFK-07 had a more complex mechanism in place to degrade the cyanobacteria cell walls than did the enzyme system of *Sinorhizobium* sp. AFK-13. The polysaccharides or the peptidoglycans of *A. flos-aquae* may be hydrolyzed and metabolized to a range of easily utilized monosaccharides or other low molecular weight organic substances by strain AFK-07 of *A. johnsonii*, while the products of polysaccharide degradation or peptidoglycans were more likely to be utilized by *Sinorhizobium* sp. AFK-13. These bacterial interactions may offer an alternative effective approach to controlling the water choking effects of summer blooms affecting our lakes and reservoirs.

Keywords: hydrolytic-enzymes, algal lytic bacteria, *Acinetobacter johnsonii*, *Sinorhizobium* sp., *Anabaena flos-aquae*

INTRODUCTION

Many cyanobacteria are ecologically important microorganisms because of their nitrogen-fixing properties [1-3]. However, water blooms (mass development) of the cyanobacteria create severe problems in many freshwater lochs and reservoirs throughout the world [4-6]. The cyanobacteria water blooms, particularly those due to the genera *Microcystis* and *Anabaena*, are widely distributed in natural freshwater ecosystems during the summer season. Their adverse effects on water quality include deterioration because of the generation of toxic substances [3,4,7] and offensive odors [3,8,9] which affect the water supply to humans and livestock as well as affecting recreational use. Several approaches have been taken in an attempt to control harmful cyanobacteria blooms. Most frequently used are the algicidal agents, copper sulfide [10] and simazine [6], which prevent photosynthesis but these agents are also expensive and potentially damage the freshwater environment. An alternative approach is to reduce the nutrient required by algae for growth, but this is difficult to control because of the variable amount of eutrophic nutrient that reaches the lakes and reservoirs. Neither of these methods have been successful so far [11,12]. As a result, various biological controls have been introduced as a more suitable approach to regulating the algal blooms. These newer controls include bacterial pathogens that inhibit the growth of the cyanobacteria [6,13,14]. The nature of the antagonistic mechanisms involved, however, have not been elucidated [11,12]. Moreover, the role of physiological adaptation and selection by an algal lytic bacterial species with respect to algal degradation cannot easily be investigated in a natural ecosystem due to the complex interactions involving other resident microbial communities [15]. In order to obtain new microorganisms for controlling cyanobacterial blooms, several bacterial strains with known algal lytic activities against *Anabaena flos-aquae* have been isolated

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and identified. The enzyme profiles and the activities of these algal lytic bacteria have been investigated, are the subject of this report, and include protease (in particular gelatinase and caseinase), lipase, amylase, carboxymethyl-cellulase (CMCase), chitinase, agarose, fucodian hydrolase, laminarinase, alginase, β-glucosidase, α- and β-galactosidase, β-*N*-acetyl-glucosaminidase, β-xylosidase, and α-mannosidase.

MATERIALS AND METHODS

Algal Culture Conditions

A. flos-aquae NIES-75 was used as a host for algal lytic bacteria and was kindly supplied by the National Institute for Environmental Studies (NIES) of Japan. A clonal axenic culture was developed, cultivated and maintained in BG-11 medium which included $NaNO_3$ 1.5 g, K_2HPO_4 0.04 g, $MgSO_4 \cdot 7H_2O$ 0.075 g, $CaCl_2 \cdot 2H_2O$ 0.036 g, EDTA-disodium 0.001 g, Na_2CO_3 0.02 g, citric acid 0.006 g, ferric ammonium citrate 0.006 g, and micronutrient 1 mL per liter [16] with continuous illumination (cool white fluorescent lamps, incident light intensity of 35 μE/m^2/s) and at 25 ± 0.2°C with agitation (150 rpm in a rotary shake incubator). The composition of micronutrient was H_3BO_3 2.86 g, $MnCl_2 \cdot 4H_2O$ 1.81 g, $ZnSO_4 \cdot 7H_2O$ 0.222 g, $Na_2MoO_4 \cdot 2H_2O$ 0.39 g, $CuSO_4 \cdot 5H_2O$ 0.079 g, $Co(NO_3)_2 \cdot 6H_2O$ 0.0494 g per liter [17].

Isolation of Algal Lytic Bacteria

Various surface waters and sediment cores were collected from the Pal'tang Reservoir and Lake of Daecheong, Korea. The dominant phytoplankton of these eutrophic lakes during the summer season is the cyanobacteria. Algal lytic bacteria were isolated by the soft agar overlayer technique [18]. Axgenic cultures of *A. flos-aquae* NIES-75 were grown in BG-11 medium for one week. One mL of the *A. flos-aquae* cultures was mixed with 1 mL of filtered (200 μm filter) suspensions of surface water or the sediment samples, molten BG-11 soft agar and maintained at 50°C. The mixture was immediately poured onto a BG-11 soft agar plate. After the agar had solidified, the plates were incubated at 25 ± 0.2°C with continuous illumination of cool white fluorescent lamps giving an incident light intensity of 35 μE μE/m2/s.

Bacterial colonies that produced clear zones on lawns of *A. flos-aquae* NIES-75 were picked, purified, and maintained on additional BG-11 agar plates. Pure cultures of selected bacteria were stored at −80°C in BG-11 medium containing 0.1% (v/v) yeast extract supplemented with 20% (v/v) of glycerol.

Determination of Biochemical and Physiological Characteristics of Bacterial Isolates

Purified bacterial isolates were precultured on nutrient agar (peptone 5.0 g, NaCl 5.0 g, yeast extract 2.0 g, beef extract 1.0 g, and agar 15.0 g per liter) for the following tests. The biochemical and physiological characteristics were determined using the method of Gerhardt *et al.* [19] and further investigated using carbon utilization, the optimum pH and temperature for growth, a hemolytic test, and gram straining. Oxidase and catalase activities, gelatin liquefaction, arginine dehydrolase, ornithine decarboxylase, *etc.* were further examined. Average guanine-plus-cytosine (G+C) values were determined by using the thermal denaturation method to establish DNA base composition [20] and calculations were made using the equation of Marmur and Doty [21], as modified by De Lay [22].

Analysis of Fatty Acid Composition

The amounts of fatty acids in whole-cell hydrolysates were determined as methyl esters by temperature programmed gas chromatographic separation (model 5890 Series II Hewlett Packard, Avondale, PA, USA) with a flame ionization detector and hydrogen as the carrier gas. The ethers were prepared according to procedures described by MIDI Inc. (www.midi-inc.com) in technical notes supplied with the microbial identification system. An approximate 10 mg dry mass of cells were harvested and washed, saponified with sodium hydroxide methylated with acidic methanol extracted in a mixture of *n*-hexane/methyl *tert*-butyl ether, and then washed with base. The fatty acids were identified using standards, and derivatisation procedures and mass spectrometry as described by Haertig *et al.* [23]. The degree of saturation was defined as the ration of saturated fatty acids to the total amount of fatty acids. For fatty acid analysis, cells were grown for 40 h on Columbia agar (Oxoid Ltd., Basingstoke, UK) that contained 5% (v/v) defibrinated horse blood. The bacterial isolates were grown at 25 ± 0.2°C.

Tests for Antibiotic Sensitivity

As chemical compounds, antibiotics are commonly released into freshwater ecosystems, so it is important to choose an antibiotic resistant bacterium for use in a wastewater treatment system. The suspensions of bacterial isolates precultured for 24 h were spread onto the surface of Antibiotic Medium II (Difco, Becton, Dickinson and Company, MD, USA.) agar plates using sterile swabs to create the bacterial lawns. Thirteen different types of antibiotic discs (Difco, Becton, Dickinson and Company, MD, USA) were then carefully placed on the bacterial lawns using sterile forceps. The plates with a bacterial lawn and antibiotic disc were then incubated at 25 ± 0.2°C for 24 to 48 h to verify antibiotic resistance. The antibiotics used for this study were bacitracin, carbenicillin, cephalothin, chloramphenicol, clindamycin, erythromycin, gentamycin, kanamycin, lincomycin, penicillin G, rifampin, streptomycin, and tetracycline.

Identification of Bacterial Isolates with Phylogenetic Analysis

The chromosomal DNA was isolated using a method

described by Yoon *et al.* [24]. Cells grown in nutrient broth at 30°C for 8 h were used for genomic DNA extraction with the Wizard Genomic DNA purification kit (Promega Co., San Luis Obispo, CA, USA) according to the manufacture's protocol, using 10 mg/mL of a final lysozyme concentration. The amplification of the 16S rDNA was conducted using two primers according to Stackebrandt and Liesack [25] 5′-GAGTTTGATCCTGGCTCAG-3′ and 5′-AGAAAGGAGGTGATCCAGCC-3′. A PCR was run for 35 cycles in a DNA thermal cycler (model Genetic analyzer 377, Perkin-Elmer, Boston, MA, USA) employing the thermal profile according to Yoon *et al.* [24]. The 16S rDNA sequence of the bacterial isolates AFK-07 and AFK-13 was aligned using CLUSTAL W software [26]. The evolutionary distance matrices were calculated with the DNADIST program in the PHYLIP package [27]. The sequence of representative species of the genus *Acinetobacter* or *Sinorhizobium* and related taxa were cited using the DDMJ/EMBL/GenBank Database. The values of 16S rDNA similarity were calculated from the alignment, while the evolutionary distances were calculated using a Kimura two-parameter correction. A phylogenetic tree was constructed using the neighbor-joining method [28] based on the calculated distance matrix.

Algal Lytic Activity Test of AFK-07 and AFK-13 on *A. flos-aquae*

The algal lytic bacteria AFK-07 and AKF-13 grown under the above conditions were inoculated into test tubes (50 mL capacity) which included 25 mL of *A. flos-aquae* NIES-75, and cultured for 5 days. To measure the algal lytic activity of the isolates, the concentration of chlorophyll *a* was measured by *in vivo* Xuorometry (Tuner Designs, Sunnyvale, CA, USA). All tests were repeated three times.

Preparation of Cell-Free Extracts

Cells of the bacterial isolates AFK-07 and AFK-13 were harvested by centrifugation at 10,000 × g and 4°C for 20 min, washed twice with 50 mM phosphate buffer (pH 7.2), and resuspended in the same buffer. The cells were homogenized by sonication (MSE 100 watt Ultrasonic Disintegrator, MSE, London, UK) and centrifuged at 15,000 × g for 20 min at 4°C to remove cell debris. The supernatant was used as the crude enzyme solution. The cell-free extracts were kept at −20°C until used.

Enzyme Assays

To determine the activities of chitinase, laminarinase (β-1,3-D-glucanase), alginase, agarase, and carboxymethyl-cellulase (CMCase), their respective substrates chitin, laminarin, alginate, agar, and CM-cellulose were purchased from Sigma-Aldrich Ltd., USA. Most enzyme activities were measured by colorimetric analysis of the reducing sugar content determined by the procedure of Somogyi [29] and Nelson [30]. Chitinase and alginase were determined by development of clear zones around the colonies. Congo red was used to detect *in vivo* CMCase activity as follows: colonies of AFK-07 and AFK-13 on agar plates were lysed by exposure to chloroform vapor (15 min). The plates were overlaid with 5 mL of 50 mM phosphate citric acid buffer, pH 5.2, at 50°C supplemented with agar (0.5%, w/v) and CM-cellulose (1%, w/v) at 50°C. After 12 h incubation at 30°C, the plates were flooded with Congo red (1%, w/v) for 20 min and washed with 1 M NaCl. Active clones were surrounded by yellow halos on a red background. One unit of cellulase activity corresponded to one μmol D-glucose equivalent released per min. Laminarinase activity was determined by measuring the amount of reducing sugar released from laminarin. A standard assay mixture (1 mL) contained enzyme solution properly diluted, 4 mg of laminarin, and 50 mM potassium acetate buffer at pH 5.5. The reactions were run for 30 min at 30°C and stopped by boiling for 5 min; reducing-sugar content was determined as described previously. A unit of laminarinase activity was defined as the amount of enzyme catalyzing the release of one μmol of glucose equivalent per minute. Glycosidase activities were determined in 100 mM phosphate buffer, pH 7.0 at 28°C and 0.05 mL of *p*-nitrophenyl glycoside in 50 mM phosphate buffer with a pH of 7.0 and 0.1 mL of cell-free extract mixed thoroughly and 0.1 mL of 1 M Na_2CO_3 then added to stop the reaction. Specific activities of glycosidase were expressed as the amount of enzyme that converted 1 μmol of *p*-nitrophenol per hour. Enzyme and substrate blanks were also included for all respective assays. A unit of enzyme activity was defined as the amount of enzyme catalyzing the release of one μmol of correspondent substrate per 1 mg of protein. Protein concentrations were measured by the method of Bradford [31] with bovine serum albumin as the standard.

RESULTS

Isolation of Algal Lytic Bacteria against *A. flos-aquae* NIES-75

A total of 178 bacterial strains from the surface waters and sediments of eutrophic lakes and reservoirs in Korea were screened and isolated according to their different colony morphologies on agar plates. Among these bacteria, only nine isolates exhibited inhibitory activities towards the cyanobacterium *A. flos-aquae* NIES-75 on agar. Of these nine, the isolates AFK-07 and AFK-13 were selected to show strong algal lytic activity when 5 mL (1×10^5 cfu/mL) of the isolates were inoculated into 100 mL of *A. flos-aquae* cultures (1×10^8 cells/mL) a second time for a 10-day period. The characteristics of the AFK-07 and AFK-13 isolates are shown in Table 1. These two bacteria were characterized as Gram-negative, rod type, non-pigmented with a slow gliding feature in nutrient broth (NB) medium and 1.5% agar. The optimal temperature and pH for their growth were determined as 28~30°C and 6~8, respectively.

Table 1. Morphological, physiological, and biochemical characteristics of the bacterial isolates AFK-07 and AFK-13

Characteristics	AFK-07	AFK-13
Cell shape	Short rod	Rod
Cell diameter	0.8~1.4 μm	0.5~1.0 μm
Gram staining	–	–
Optimum temperature	28°C	30°C
Growth on 42°C	–	+
Optimum pH	6~8	6~8
Motility	+	+
Catalase	+	+
Oxidase	–	+
Arginine	+	–
Ornithine	+	–
Lysine	+	–
Citrate utilization	+	+
Hemolysis	–	–
Production of		
Amylase	–	+
Caseinase	+	+
Gelatinase	–	+
Agarase	–	–
Carboxymethyl-cellulase (CMCase)	–	+
Lipase	+	+
Chitinase	–	+
Alginase	+	–
Laminarinase	+	+
Fucodian hydrolase	+	–
Carbohydrate utilization of		
D-Lactate	+	–
D-Mannose	–	+
Lactose	–	+
D-Arabinose	–	+
D-Galactose	–	+
Sorbitol	+	–
D-Fucose	–	–
D-Ribose	+	+
D-Xylose	+	+
Glycerol	–	–
Mannitol	–	+
Cellobiose	–	+
Trehalose	–	+
Sucrose	–	–
Coenzyme Q	Q-7	Q-10
G+C contents (mol %)	44.3%	60.3%

Characterization of Algal Lytic Bacteria

Enrichment cultures were obtained from freshwater sediments with the algal lytic bacteria added to favor the growth of hydrolytic enzyme-producing microorganisms.

Table 2. Fatty acid composition of the bacterial isolates AFK-07 and AFK-13 grown on Columbia agar that contained 5% (v/v) defibrinated horse blood at 25 ± 0.2°C

Fatty acid (FA)	FA (%) of AFK-07	FA (%) AFK-13
12:0	7.90	5.30
Unsaturated	1.14	1.29
12:0 2OH	2.90	0
12:0 3OH	5.67	0
13:0	0	1.32
14:0	0.57	5.89
14:0 3OH	3.42	0
15:0	0	4.91
16:1 alcohol	0.85	0
16:1 w9c[a]	1.21	0
16:1 w7c[a]	28.85	16.01
16:0	17.28	23.18
17:0	0	2.30
17:1	0	0.30
18:1 alcohol	0.20	0
18:1 w9c	33.18	0
18:1 w9t	0.92	0
18:0	1.07	0
19:0 CYCLO w8c	0	0.40
Summed feature 7[b]	3.51	15.32

[a]There is currently a trend away from use of "systematic" names and toward the use of the omega positions of the double bonds.
[b]18:1 w7c/w9t/w12t, 18:1 w12t/w9t/w7c, 18:1 w9c/w12t/w7c.

Only two bacterial stains were clearly picked on the plates during monitoring of the algal degradation. The isolate AFK-07 was gram-negative, rod-shaped, catalase positive and oxidase negative; arginine dehydrolase, lysine and ornithine decarboxylase, lipase, caseinase, alginase, laminarinase (β-1,3-glucanase), and fucodian hydrolase were positive. The Coenzyme Q was ubiquinone Q-7, and the G+C contents of the DNA similar to the genus *Acinetobacter* (38~47 mol%) were 44.3 mol%.

In the AFK-13 isolate, amylase, protease (caseinase and gelatinase), CMCase, lipase, and laminarinase (β-1,3-glucanase) were detected while ubiquinone Q-10 was listed as coenzyme, and the G+C contents of the DNA were 60.3 mol%. Additional phenotypic features for AKF-13 are listed in Table 1. Thus, both AFK-07 and AFK-13 were found to be very similar to *Acinetobacter* sp. and *Rhizobium* sp. based on their biochemical and physiological characteristics, respectively. The antibiotic sensitivity tests of the two isolates are provided in Table 3. AFK-07 was sensitive only to lincomycin and tetracycline.

Analysis of the Fatty Acid Methyl Ester (FAME) Profiles of AKF-07 and AKF-13

The fatty acids composition of AFK-07 is given in Table 2. The major components are even-numbered saturated, *cis*-monounsaturated, and hydroxyl straight-chain

Table 3. Antibiotic sensitivities of the bacterial strains AFK-07 and AFK-13 to 13 antibiotics

Antibiotic	Bacterial strain	
	AFK-07	AFK-13
Bacitracin	R	S
Carbenicillin	R	S
Cephalothin	R	S
Chloramphenicol	R	S
Clindamycin	R	S
Erythromycin	I	S
Gentamycin	R	R
Kanamycin	R	R
Lincomycin	S	S
Penicillin G	R	S
Rifampin	R	R
Streptomycin	R	R
Tetracycline	S	S

S: Sensitivity; R: Resistance; I: Intermediate reaction.

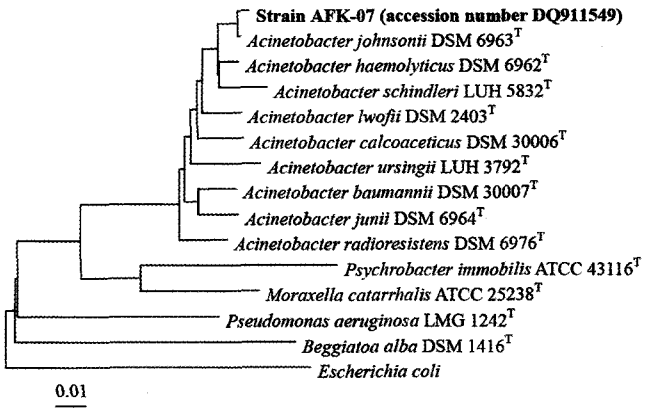

Fig. 1. Phylogenetic tree based on the 16S rDNA sequences showing the positions of the isolate AFK-07, the type strains of *Acinetobacter* species and representatives of some other related taxa. The scale bar represents 0.01 substitutions per nucleotide position.

fatty acids. Oleic acid (18:1 w9c, 33.18%), palmitoleic acid (16:1 w7c, 28.85%), and palmitic acid (16:0, 17.28%) were the major components, which generally accounted for greater than 90% of the total fatty acids composition. The FAME of AFK-13 contained 18 fatty acids (Table 2). Only fatty acid 16:0 and the unresolved fatty acid mixture in Summed Features 7 were present in over 15% of the total fatty acids. The main components were 23.18% for 16:0, 16.01% for 16:1 w7c, and 15.32% for Summed Features 7. In addition, 19:0 CYCLO w8c was 0.40%. According to their FAME profile characteristics, AFK-07 was tentatively identified as an *Acinetobacter* sp. and AFK-13 as a *Sinorhizobium* sp.

Phylogenetic Analysis of Two Algal Lytic Bacterial Strains

The 16S rDNA sequence was analyzed to determine which known species matched the AFK-07 and AFK-13 isolates with the highest homology among the *Acinetobacter* (99.9%) and *Sinorhizobium* species (98.1%) that are contained in the DDMJ/EMBL/GenBank, respectively. The 16S rDNA sequences of strain AFK-07 and AFK-13 have been deposited in the GenBank database under accession numbers DQ911549 and DQ911548, and in that order. The phylogenetic tree constructed using the neighbor-joining method is shown in Figs. 1 and 2. The sequencing data was aligned to construct a phylogenetic tree. The phylogenetic position of AFK-07 was then compared with *Acinetobacter* species and related taxa in a dendrogram. In the phylogenetic tree, AFK-07 was closest to *Acinetobacter johnsonii* DSM 6963^T and part of a robust monophyletic cluster with *A. haemolyticus* DSM 6962^T, *A. schindler* LUH 5832^T, *A. lwofii* DSM 2403^T, and *A. calcoaceticus* DSM 30006^T. The level of sequence similarity of AFK-07 in the monophyletic cluster was greater than 97% (Fig. 1). The sequence of AFK-07 was almost identical to that of *A. johnsonii* DSM 6963^T with a 99.8% similarity. Hence, this appears to be the first report that *A. johnsonii* AFK-07 (accession number DQ911549) has a powerful algal lytic ability against *A. flos-aquae* NIES-75.

The 16S rDNA sequences of AFK-13 were compared with the 16S rDNA sequences of the type strains belonging to the genus *Sinorhizobium* and representatives of other related genera. The highest homology with the sequences of *Sinorhizobium medicae* A321^T exhibited 98.1% with AFK-13. This phylogenetic relationship is consistent with phenotypic and chemotaxonomic evidence and allowed us to suggest that AFK-13 be designated as *Sinorhizobium* sp. AFK-13 (accession number DQ911548). As the value of DNA-DNA homology is a conclusive factor in identifying bacterial strains, values higher than 70% homology as the basis for including in the same species [32]. According to reports on the correlation between the DNA-DNA homology and 16S rDNA sequence homologies [33,34], strains that reveal a DNA-DNA homology higher than 70% when strains show sequence homology higher than 99.5%. Consequently, the strain AFK-13 should not be identified as *S. medicae*, but rather recognized as a *Sinorhizobium* species.

Algal Lytic Effects of *A. johnsonii* AFK-07 and *Sinorhizobium* sp. AFK-13 on *A. flos-aquae*

The cyanobacterium *A. flos-aquae* was clearly suppressed by treatment with both algal lytic bacteria *A. johnsonii* AFK-07 (Fig. 3A) and *Sinorhizobium* sp. AFK-13 (Fig. 3B). After 2 days of bacterial strains AFK-07 and AFK-13 exposure, the cyanobacteria biomass was suppressed by 58.2 and 44.1% and after 7 days, and by 81.5 and 85.8% respectively by each strain. Cell concentrations of the algal lytic bacterial strains was determined by direct measurement of the divergence between the total cell concentrations of the cyanobacterium co-cultured with the algal lytic bacterium minus the cell concentration

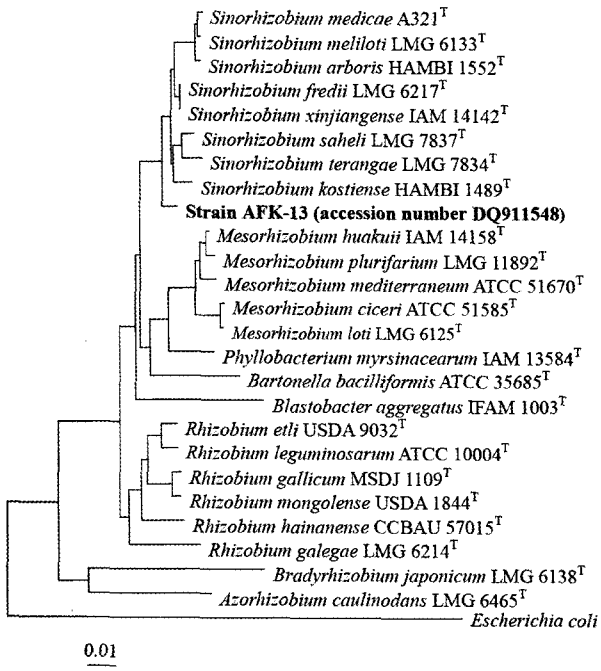

Fig. 2. Phylogenetic tree based on the 16S rDNA sequences showing the positions of the isolate AFK-13, the type strains of *Sinorhizobium* species and representatives of some other related taxa. The scale bar represents 0.01 substitutions per nucleotide position.

of cyanobacterium at the same density. In contrast, the cell concentrations of the algal lytic bacteria showed a dramatic increase, reaching a maximum concentration of 8.9×10^9 cfu/mL (data not shown).

Enzyme Profiles of *A. johnsonii* AFK-07 and *Sinorhizobium* sp. AFK-13

We analyzed the hydrolytic enzyme profiles of *A. johnsonii* AFK-07 and *Sinorhizobium* sp. AFK-13 in this study. The two species, ordinarily free-living bacteria, expressed proteases (caseinase or gelatinase or both), lipase, and the prominent laminarinases (β-1,3-glucanase), and the majority of *A. johnsonii* AFK-07 also exhibited caseinase, alginase, fucodian hydrolase, β-galactosidase, β-glucosidase, β-glucosaminidase, and β-xylosidase that mainly hydrolyzed β-glycoside bonds (Fig. 4). Other glycosidase enzymes such as α-galactosidase, α-fucosidase, and α-mannosidase, which degrade α-glycoside bonds, were not detected. In contrast, *A. johnsonii* AFK-07 exhibited complex enzyme systems for the hydrolysis of cyanobacteria or algal polysaccharides, while *Sinorhizobium* sp. AFK-13 produced a protease, amylase, laminarinases (β-1,3-glucanase) and CMCase that likely act to degrade cell wells of *A. flos-aquae* NIES-75 (Fig. 4).

DISCUSSION

To monitor the effectiveness of algal lytic bacteria to

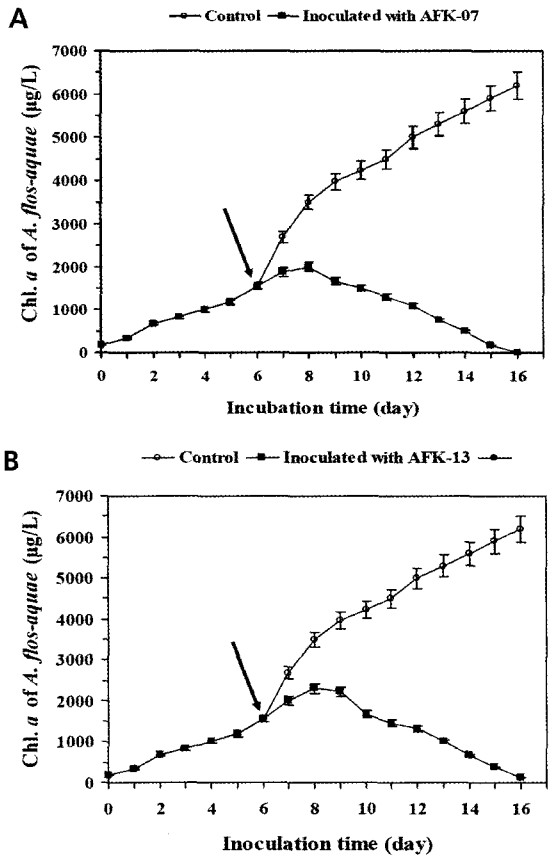

Fig. 3. Density of *A. flos-aquae* in cultures incubated with (■) algal lytic bacterium AFK-07 (A) and AFK-13 (B) and (○) in the absence of algal lytic bacteria. The arrow indicates the time of algal lytic bacterium inoculation. Data are the mean ± S.D. from at least three independent assays.

control cyanobacteria that form water blooms, numerous aquatic samples were collected from lakes and reservoirs characterized by algal blooming due to *Anabaena* spp. and *Microcystis* spp. Our identification of only two algal lytic bacteria among nine isolates selected from 178-screened bacterial isolates suggests that such activities may be common among freshwater bacteria. Indeed, Daft and Stewart [35] isolated only four strains of Myxobacteriales which caused the lyses of 40 strains of cyanobacteria. A recent report by Yamamoto *et al.* [13] in a study of 83 isolated actinomycetes lethal to the toxin-producing cyanobacteria *Microcystis aeruginosa*, provides additional evidence for the effectiveness of algal lytic microorganisms in bacterial lyses. Low densities of algal lytic bacteria in the environment may be due to insufficient inorganic fertility that affect DNA replication and repair in bacteria and causes mutations in microorganism [36]. In this study, the two algal lytic bacteria AFK-07 and AFK-13 isolated from the surface waters. The point made early on was to examine water and sediment) of eutrophic lakes and reservoirs are associated with a bloom population of the target algal species and exhibited strong algal lytic activity against the test *A. flos-aquae* (Fig. 3). The fatty acid compositions of the two algal lytic bacteria were

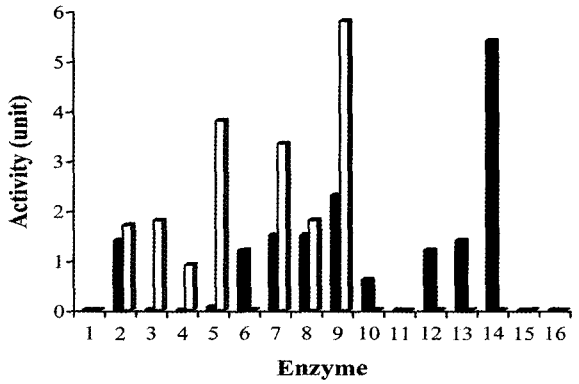

Fig. 4. Comparative enzyme activities and enzyme profiles of *A. johnsonii* AFK-07 (■) and *Sinorhizobium* sp. AFK-13 (□). Values are indicated as enzyme activities. One unit of glycanase or glycosidase activity of enzyme is described as that which liberates 1 μmol from reducing ends of corresponding monosaccharides or 1 μmol from *p*-nitrophenol of corresponding substrates per h, respectively. 1, Agarase; 2, Alginase; 3, Amylase; 4, Chitinase; 5, CMCase; 6, Fucodian hydrolase; 7, Laminarinase; 8, Lipase; 9, Protease; 10, β-Glucosaminidase; 11, α-Galatosidase; 12, β-Galactosidase; 13, β-Glucosidase; 14, β-Xylosidase; 15, α-Fucosidase; 16, α-Mannosidase.

characteristic for Rhizobiaceae [37-39]. Three major components identified for AFK-07 were oleic acid (18:1 w9c, 33.18%), palmitoleic acid (16:1 w7c, 28.85%), and palmitic acid (16:0, 17.28%). These fatty acids are common to the genus *Acinetobacter*. Eight other common fatty acids, namely 14:0, 15:0, 16:1 w7c, 16:0, 17:0, 17:1, C19:0, CYCLO w8c, and Summed Feature 7 are found in most species of *Rhizobium* [17] and are noted in AFK-13. However, 18:1 alcohol, 18:1 w9c, 18:1 w9t, and 18:0 were not detected in AFK-13, which suggests that AFK-13 is, clarified the genus *Rhizobium*. The AFK-07 bacterium was isolated from surface waters of the Lake of Daecheong, and found to degrade the cyanobacterium *A. flos-aquae* AKF-07 was subsequently identified as *A. johnsonii* based on its 16S rDNA sequencing analysis (Fig. 1). The AKF-07 produces several enzymes catalyzing hydrolysis of complex polysaccharides or the peptidoglycans of cyanobacteria. Some of the corresponding glycanase were detected in cell free extracts of *A. johnsonii* AFK-07 and included alginase, fucodian hydrolase, and glycanase including β-galactosidase, β-glucosidase, β-glucosaminidase, and β-xylosidase that primarily hydrolyze β-glycoside bonds (Table 1 and Fig. 4). The AFK-13 bacterium was isolated from the Pal'tang Reservoir and was subsequently designated as *Sinorhizobium* sp. (Fig. 2) owing to a 98.1% similarity with *S. medicae* A321^T. The enzyme profiles of *Sinorhizobium* sp. AFK-13 are showed high activities of protease [40], laminarinase and CMCase in Fig. 4. There was no evidence of glycanase production. Recently, two species of *Flexibacter* that lysed the cyanobacteria *Oscillatoria williamsii* were shown to produce a lysozyme as one of their cell inhibitory compounds [41]. Also, Burnham *et al.* [11] has reported on *Myxococcus xanthus*, which degraded the cyanobacterium *Phormidium luridum* var. *olivacea*, to lyses cells through the release of a lysozyme-like enzyme. Kim *et al.* [42] reported on *Moraxalla* sp. CK-1, which has been known to inhibit the growth of *Anabaena cylindrica*, and produces aminodase, or an endopeptidase. Carotenoids, peptidoglycans-associated proteins, and lipopolysaccharide (LPS), laminarinase and cellulose are one of the major constituents of cyanobacteria including the genera *Microcystis* and *Anabaena* [43]. The peptidoglycans are to be covalently linked to a wall polysaccharide. In our results, the amylase and fucodian hydrolase were not active and the highest activity of both β-glucosidase and laminarinase were present in the two AFK strains. Laminarins, alginic acids, cellulose, and the fucodians are the major constituents of algal cell walls, up to 50~80% of defatted algal mass [43,44]. However, it is mainly laminarinase in microorganisms. It has been speculated that this occurrence is likely due to an important defense mechanism role whereby the digestion of cell walls (*e.g.* fungi) releases an oligosaccharide that switches the production of antifungal compounds [45] and their ability to hydrolyze the reserved β-1,3-glucan [46]. In Fig. 4, the glycanase in *A. johnsonii* AFK-07, the CMCase, laminarinase, and protease in *Sinorhizobium* sp. AFK-13 and the lipase in both strains show high algal lytic activities. The enzymic proteins such as lysozyme, protease, and lipase that are extracted from microorganisms cause bacterial cell lysis [12,14,47,48]. Consequently, the results obtained from this study suggest that *A. johnsonii* AFK-07 most likely acts to degrade polysaccharides or the peptidoglycans of the algal cell walls in the initial stages, and that *Sinorhizobium* sp. AFK-13 is able to metabolize the peptidoglycans and utilize the products of polysaccharide degradation. The present study results enlarge and enhance an understanding of the functional interactions between algal cell structure and the enzymes of algal lytic bacteria during the degradation of the cyanobacterium *A. flos-aquae*. The further identification and purification of the most active enzymes are now in progress in our laboratory. The elucidation of the mechanism of selective alga lytic enzyme action against the harmful accumulation of the blue-green algae in our waters is necessary if we are to control the blooms. Thus, the algal lytic bacteria AFK-07 and AFK-13 offer promise as potential biocontrol agents for harmful algal blooms.

CONCULSION

Two heterotrophic bacterial strains have been shown to have strong algal lytic activities against the blue green algae *A. flos-aquae*. A two-species bacterial community of cultural proteobacteria consisted of *Acinetobacter* and *Sinorhizobium*. The first member of the community, *A. johnsonii* AFK-07, was highly active in metabolic, algal lytic and hemolytic activities, and produced alginase, caseinase, lipase, fucodian hydrolase, laminarinase, β-galactosidase, β-glucosidase, β-glucosaminidase, and β-xylosidase. The second member of the community, *Sinorhizobium* sp. AFK-13, produced alginase, amylase,

protease, carboxymethyl-cellulase, laminarinase, and lipase. Both member of the algal lytic bacterial community utilized a range of easily assimilated monosaccharide and other low molecular weight organic substances. Our study results provide evidence for the complex metabolic interrelationship between two members of this community. *A. johnsonii* AFK-07, likely plays an important role in the initial stage of algal degradation while *Sinorhizobium* sp. AFK-13, is resistant to the bacteriolytic activities of *A. johnsonii* AFK-07 and is able to utilize the peptidoglycans of harmful alga or the products of polysaccharide degradation in general. Therefore, our study helps to understand the complex structural and functional relationships amongst freshwater algal lytic bacterial communities during algal degradation.

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