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Characteristics and Effects of Algal-Lytic Bacterium for Control of Fresh Water Microalgae

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Abstract: The aim of this study was to isolate and identify algal-lytic bacterium that tends to kill the harmful Microcystis aeruginosa and to determine the algal-lytic activity of the bacterial culture filtrate. In the screening of algal-lytic bacteria, we isolated a bacterium which had potent algal-lytic effects on fresh water algal blooms. Among of algal-lytic bacteria isolated in this study, isolate JS-9 was the strongest algal-lytic activity against *M. aeruginosa*. Isolate JS-9 was identified on the basis of morphological and biochemical characteristics. Thus, this isolate was designated as *Pseudomonas* sp. JS-9. The optimal culture conditions were 25°C, initial pH 7.0, and 1.0% (w/v) NaCl concentration. *Pseudomonas* sp. JS-9 showed algal-lytic activity through indirect attack, which excreted active substance into the culture filtrate. When 10% culture filtrate of JS-9 was applied to *M. aeruginosa*, 90% of algal cells were destroyed within 3 days. In addition, the algal-lytic activities were increased in dose and time dependent manners. Taken together, our results imply that *Pseudomonas* sp. JS-9 and its secreted compounds could play an important role in regulating the onset and development of algal blooms in the natural fresh water environments.

Key words: Algal blooms, algal-lytic activity, fresh water microalgae, *Microcystis aeruginosa*, *Pseudomonas* sp. JS-9, substance

INTRODUCTION

Most Harmful Algal Blooms (HABs) in freshwater ecosystems are dominated by *Microcystis aeruginosa*, a cyanobacterium that produces potent microcystins that have been implicated in the deaths of microalgae (Li *et al*., 2015). In the past decades increasing eutrophication has led to frequent outbreaks of cyanobacterial blooms (mainly *M. aeruginosa*) in many lakes around the world. Growth and decay of these blooms caused off-flavor episode and these are becoming a worldwide problem in aquatic environments, especially in eutrophic lakes and reservoirs (Yu *et al*., 2009). It also has triggered a serious drinking water pollution incident when a dense cyanobacterial bloom occurred in the lakes.

In order to solve these problems of HABs, many methods have been implemented such as chemicals (Elder and Horne, 1978; Jeong *et al*., 2003) and microwaves (Li *et al*., 2011). Although, these methods can take effect on HABs, their high cost and the secondary damage make them impractical and infeasible for HABs control (Singh, 1974). Therefore, biological agents including viruses (Cai *et al*., 2011) and bacteria (Li *et al*., 2014; Zhang *et al*., 2011) are considered as effective and biosecure ways to mitigate HABs and the close relationship between bacteria and microalga has received particular interest in recent years (Zheng *et al*., 2013). And bacteria living with microalgae have been suggested to affect algal population dynamics and toxicity (Wang *et al*., 2010; Croft *et al*., 2005). Due to the fact that bacteria associated with algae typically show species specificity, many studies have been conducted on algicidal bacteria related to HABs (Mayali and Doucette, 2002; Amaro *et al*., 2005). These bacteria may be valuable as biotic agents for controlling HABs without causing negative effects on other organisms. Some bacteria even produce secondary metabolites with a diverse range of algicidal activities to inhibit or kill algal cells (Luo *et al*., 2013). So, far, many algicidal bacteria have been isolated from the environment and identified (Li *et al*., 2013).

In this study, the bacterium with algal-lytic effects against *M. aeruginosa* was isolated and identified from an eutrophic Daechung Lake in Korea. And also, the algal-lytic effect of the culture filtrate of *Pseudomonas* sp. JS-9 against *M. aeruginosa* was investigated.

MATERIALS AND METHODS

Sampling and isolation of algal-lytic bacteria: Seawater samples were collected at a depth of 1 m using a Van Dorn sampler in Daechung Lake (in Korean Peninsula). To isolate bacteria with algal-lytic effects

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against *M. aeruginosa*, samples were serially diluted and 0.1 mL aliquots of each dilution were spread onto Zobell 2216E agar plates which were then incubated for 7 days at 25°C. Individual colonies of distinct morphology were streaked onto Zobell 2216E agar plates for purification and subsequently frozen at -70°C in 20% (v/v) glycerol.

In order to isolate the strains with algal-lytic effects against *M. aeruginosa*, 10 mL aliquot of each isolate was inoculated in triplicate into 20 mL of logarithmic-phase *M. aeruginosa* cultures and 10 mL aliquot of Zobell 2216E medium was added to algal cultures as a control. The survival of *M. aeruginosa* cells in each well was assessed daily using a microscope (Axiovert 40 CFL; Zeiss) at a magnification of 600×. Meanwhile, the Chlorophyll-a (Chl-a) concentration of the samples was periodically measured and the color of *M. aeruginosa* was also periodically observed. Bacteria from wells exhibiting algal-lytic activity against *M. aeruginosa* were isolated for further study. As a result, ten algal-lytic strains were isolated in this investigation. Finally, one strain was isolated which showed the high algicidal activity and this strain was designated JS-9.

Algal and bacterial cultures: *M. aeruginosa* (NIES-298) was obtained from the National Institute of Environmental Studies (NIES), Japan. Before used as an inoculant, it was cultured for 7 days to reach the log phase under the following conditions: sterilized BG11 medium 3000 lux white light, light: dark = 12 h:12 h; 25°C. For analysis of algal-lytic characteristic, the isolate JS-9 was grown in sterilized Zobell 2216E medium at 25°C and reached logarithmic growth phase. Then, strain JS-9 at logarithmic growth phase was used unless specially mentioned.

Chlorophyll-a measurement and algal-lytic activity of culture filtrates of isolate JS-9: After staining with Lugol's solution, Microcystis cells were enumerated in a Sedgewick-Rafter chamber using a Zeiss Axiovert 40 CFL microscope at 600×magnification. Chl-a concentrations in *M. aeruginosa* cells were measured after extraction overnight in 80% acetone at 4°C in the dark. The supernatant was collected by centrifugation and then analyzed at 660 nm using a spectrophotometer (UV-1601, Shimadzu, Japan) with an 80% acetone blank. The concentrations of Chl-a were calculated according to the method of MacGregor *et al.* (2001).

The algicidal effect of the culture filtrates of isolate JS-9 against *M. aeruginosa* was investigated at various concentrations of filtrates. The bacterial and *M. aeruginosa* cultures were prepared as above. The culture filtrates of isolate JS-9 were added to *M. aeruginosa* cultures (ca. 8.0×10^5 cells/mL) at concentrations of 1.0, 3.0, 5.0 and 10.0%, respectively. Zobell 2216E medium was added as the control and the bioassay plates were incubated as above. After incubation for 3, 6, 9, 12, 15 and 18 days, the viable cells in each well were counted with a Sedgewick-Rafter chamber using a microscope.

Identification of algal-lytic bacterium: Isolate JS-9, algal-lytic bacterium was grown at 25°C for 3 days and morphological characteristics were observed on Zobell 2216E agar. Standard physiological and biochemical characteristics were examined according to the methods of MacFaddin (1980). Additional biochemical tests were performed using API kits (API 20E, API 50CHB/E and API ZYM; BioMerieux, Marcy L'Etoile, France) and carbon utilization test based on Biolog GN2 MicroPlates (MicroLog System) were utilized according to the manufacturer's instructions. API 20E, API 20NE and Biolog GN2 plates were examined after 48 h and API ZYM after 4 h of incubation. Miniaturized test results were recorded in triplicate.

RESULTS AND DISCUSSION

Isolation of bacteria demonstrating algal-lytic effects against *M. aeruginosa*: To isolate bacterial strains exhibiting algal-lytic activity, we identified test tubes in which 50% of *M. aeruginosa* cells were dead. In total, 208 colonies showing distinct color and morphology were selected. Each isolate was cultured and inoculated once again into *M. aeruginosa* cultures to confirm its algal-lytic activities. A total of 60 algal-lytic strains were isolated of which isolate JS-9 exhibited the strongest effect against *M. aeruginosa* cells.

Identification of isolate JS-9: The morphological and biochemical characteristics of isolate JS-9 are summarized in Table 1. This algicidal bacterium was gram-negative and rod-shaped (0.5-0.9 μm wide×1.0-2.2 μm long). Cells were non-spore-forming and motile by a single polar flagellum, as seen after flagella staining (Heimbrook *et al.*, 1989). After 48 h incubation at 25°C on Zobell 2216E agar plates, colonies showed a topaz color and were circular (approximately 1.2 mm in diameter), convex with regular edges. Isolate JS-9 produced fluorescein but not pyocyanin, on King B and King A media, respectively (King *et al.*, 1954). The optimal conditions for growth were 25°C, at pH 7.0 in the presence of 1.0% (w/v) NaCl. Isolate JS-9 did not grow at temperatures below 5°C or above 45°C, pH values below 5.0 or above 9.0 and NaCl concentrations above 6.0%. Oxidase and catalase test were positive. Gelatin was hydrolyzed and D-Glucose was utilized. Nitrate was not reduced to nitrite. Morphological and biochemical characteristics supported

Table 1: The morphological and biochemical characteristics of *Pseudomonas* sp. JS-9. All data were obtained under the same conditions. Miniaturized test results were recorded in triplicate+, positive; – negative

Characteristics	JS-9	Characteristics	JS-9
Colony color	Topaz	Growth at 45°C	–
surface	Smooth	6% NaCl	–
elevation	Convex	Fluorescent pigments	+
Cell form	Rod	King B medium (fluorescein)	+
Motility	+	King A medium (phycocyanin)	–
Flagelation	A Single polar	Assimilation of (API 20NE)	
Gram stain	–	L-arabinose	+
Catalase, Oxidase	+	D-mannose	–
Activity of enzymes (API 20NE and API 20E)		D-mannitol	–
Hydrolysis of gelatin	+	N-acetyl-glucosamine	–
Reduction of nitrates	–	Enzymatic activities (API ZYM)	
Acid produced from (API 20NE)		Esterase C8	+
D-glucose	–	Leucine arylamidase	–

the identification of isolate JS-9 as a member of the genus *Pseudomonas*. Thus, this isolate was designated as *Pseudomonas* sp. JS-9.

The genus *Pseudomonas* is one of the most commonly reported bacteria and has had an important role in the biotechnology and pharmaceutical industries due to its metabolic versatility (Gross and Loper, 2009). To date, there are more than 150 species described in this genus. Pseudomonas is one of the most diverse and ubiquitous bacterial genera and representatives have been isolated worldwide given their extraordinarily versatile metabolism. The antibiotic and cytotoxic effects of Pseudomonas strains have frequently been reported. The vast metabolic diversity of *Pseudomonas* sp. is reflected on the production of a wide variety of bioactive molecules such as antimicrobial compound xantholysins described recently (Li *et al*., 2013).

Algal-lytic activity of *Pseudomonas* sp. JS-9 culture filtrates against *M. aeruginosa*: The algal-lytic effect of *Pseudomonas* sp. JS-9 filtrates against *M. aeruginosa* cultures (ca. 8.0×10^5 cells/mL) was measured at concentrations of 1.0-10.0% (Fig. 1). After 3 days, almost all *M. aeruginosa* cells remained at a concentration of 1.0%. However, at concentrations >3.0%, significant differences between the control and culture filtrates were observed after incubation for 3 days. Using 3% filtrates, over 50% of cells had died after 3 days while in the presence of 10% filtrates, over 90% of cells were killed within 3 days. T hese results suggest that culture filtrates of *Pseudomonas* sp. JS-9 inhibit the growth of *M. aeruginosa* in a concentration and time-dependent manner. The algal-lytic process induced by the filtrate was observed under light microscopy.

In this study, an algal-lytic bacterium against *M. aeruginosa* was isolated from fresh water in Daechung Lake, South Korea. The identification and characterization of both the bacterium and the mode of algal-lytic action strongest algal-lytic activity against *M. aeruginosa*. This

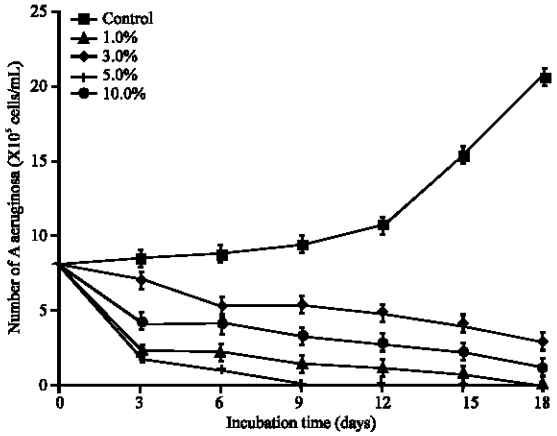

Fig. 1: Algal-lytic activity of the culture filtrate of *Pseudomonas* sp. JS-9 against *M. aeruginosa* at various concentrations. *Pseudomonas* sp. JS-9 was cultured at 25°C for 3 days in Zobell 2216E medium. Each culture filtrate was added to *M. aeruginosa* cultures (ca. 8.0×105 cells/mL) at the logarithmic growth phase. The filtrate of Zobell 2216E medium was used as a control. Data are expressed as the mean±standard deviation from triplicate assays

JS-9 was tentatively identified to be *Pseudomonas* sp. and designated as *Pseudomonas* sp. JS-9. Bacteria play an important role in the interactions of microalgae in the sense of decreasing and developing algal blooming (Yoshinaga *et al*., 1995) and various free-living bacteria also coexist in algal cultures maintained in the laboratory (Hold *et al*., 2001). And also, bacteria are considered one of the key biological agents in the dramatic termination of algal blooms (Schoemann *et al*., 2005).

At the present, algicidal bacteria is classified into four groups including members of the genus Cytophaga (renamed Cellulophag) and Saprospira (phylum Bacterioidetes) and the genera Pseudoalteromonas and Alteromonas (phylum γ-Proteobacteria) (Mayali and

Azam, 2004). Other researchers Su *et al.* (2007) have observed that the phylogenetic diversity of algicidal bacteria was limited to Proteobacteria (α and γ) and the Cytophaga-Flavobacter-Bacteroides (CFB) group. Three bacterial strains, with one belonging to each of the groups Cytophaga (*Cytophaga* sp. AMA-01), γ-Proteobacteria (*Pseudoalteromonas* sp. AMA-02) and α-Proteobacteria (*Ruegeria atlantica* AMA-03) were found to be potentially lytic to algae in high-nutrient content media (Amaro *et al*., 2005).

Some putative algicides are resistant to autoclaving and are thus unlikely to be enzymes (Skerratt *et al*., 2002), but their chemical structures remain uncharacterized. Algicidal bacteria kill their prey by two main mechanisms: direct contact or algicide release (Mayali and Azam, 2004). Saprospira prey upon bacteria as well as algae by attaching to their prey (Lewin, 1997). It was noted that Cytophagales are often particle-associated (Kirchman, 2002) in contrast, algicidal bacteria such as Alteromonas and Pseudoalteromonas are killed by releasing dissolved substances (Mayali and Azam, 2004). Many Pseudoalteromonas produce extracellular bioactive molecules (Holmstrom and Kjelleberg, 1999) and release dissolved algicide we used culture filtrate in experiments testing algicidal activity. This result revealed that *Pseudomonas* sp. JS-9 killed *M. aeruginosa* through algicide release with motion of indirect attacks. The heat-stable algicidal activity suggests that a thermal stable algicide(s) produced by *Pseudomonas* sp. JS-9 could not be enzymatic, since, the filtrate, after treatment at 100°C for 30 min, exhibited similar algicidal ability. Ahn *et al.* (2003) reported that a culture broth of *Bacillus subtilis* completely inhibited the growth of *M. aeruginosa*, a bloom-forming cyanobacterium found in highly eutrophic lakes. Recently, Mu *et al.* (2007) also reported that the secreted metabolites of Bacillus fusiformis showed algicidal activity against *M. aeruginosa*.

The isolation, purification and characterization of algicidal compounds are difficult due to their various characteristics across different species of algicidal bacteria (Skerratt *et al*., 2002). These compounds could be proteases (Lee *et al*., 2002), peptides (Imamura *et al*., 2000), bacillamide (Jeong *et al*., 2003), biosurfactants (Wang *et al*., 2005) or antibiotic-like substances (Dakhama *et al*., 1993). Algicidal compounds could be heat-labile (Baker and Herson, 1978) or heat-stable (Skerratt *et al*., 2002). These results support that some algicidal bacteria kill their algal prey using proteases: we had expected *Pseudomonas* sp. JS-9 to break down *M. aeruginosa* cells using algicidal compounds similar to proteases, peptides, antibiotic-like substances or biosurfactants excreted by the bacteria.

The identification and characterization of the responsible molecules should help understand the decreasing of algal bloom phenomenon. In future study, we will carry out the structure elucidation of released algicides to understand how algal-lytic bacteria kill *M. aeruginosa* cells.

CONCLUSION

Frequent outbreaks of harmful *M. aeruginosa* blooms have become a major environmental problem worldwide. Among of algal-lytic bacteria isolated in this study, isolate JS-9 was the strongest algal-lytic activity against *M. aeruginosa*. *Pseudomonas* sp. JS-9 showed algal-lytic activity through indirect attack which excreted active substance into the culture filtrate. When 10% culture filtrate of JS-9 was applied to *M. aeruginosa*, 90% of algal cells were destroyed within 3 days. The ideal method for the microbial control of harmful *M. aeruginosa* should have the characteristics of high efficiency and a low risk of secondary pollution. Based on our findings, *Pseudomonas* sp. JS-9 may have applications in the eutrophication of fresh water.

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