Influence of Glucose Concentrations on Biofilm Formation, Motility, Exoprotease Production, and Quorum Sensing in Aeromonas hydrophila

IQBAL KABIR JAHID,1,2 NA-YOUNG LEE,1 ANNA KIM,1 AND SANG-DO HA1,8

1School of Food Science and Technology, Chung-Ang University, 72–1 Nae-Ri, Daedeok-Myun, Ansung, Kyunggido 456–756, South Korea; and 2Department of Microbiology, Jessore Science and Technology University, Jessore 7408, Bangladesh

ABSTRACT

Aeromonas hydrophila recently has received increased attention because it is opportunistic and a primary human pathogen. A. hydrophila biofilm formation and its control are a major concern for food safety because biofilms are related to virulence. Therefore, we investigated biofilm formation, motility inhibition, quorum sensing, and exoprotease production of this opportunistic pathogen in response to various glucose concentrations from 0.05 to 2.5% (wt/vol). More than 0.05% glucose significantly impaired (P < 0.05) quorum sensing, biofilm formation, protease production, and swarming and swimming motility, whereas bacteria treated with 0.05% glucose had activity similar to that of the control (0% glucose). A stage shift biofilm assay revealed that the addition of glucose (2.5%) inhibited initial biofilm formation but not later stages. However, addition of quorum sensing molecules, C4-HSL and N-3-hexanoyl-L-homoserine lactone, significantly restored protease production, indicating that quorum sensing is controlled by glucose concentrations. Thus, glucose present in food or added as a preservative could regulate acyl-homoserine lactone quorum sensing molecules, which mediate biofilm formation and virulence in A. hydrophila.

Aeromonas hydrophila is opportunistic and a primary pathogen of amphibians, reptiles, fish, and humans (1). A. hydrophila causes various human infections such as gastrointestinal tract syndromes, wound and soft tissue infections, and bloodstream dyscrasias (29). A. hydrophila recently has been categorized as an emerging foodborne pathogen (17), with food and/or water as possible sources of human infection (14).

Biofilms are architecturally complex assemblies of microorganisms that form on biotic or abiotic surfaces or at interfaces. Biofilms are characterized by interactions between microorganisms embedded in a matrix of extracellular polymeric substances created by the microbial populations and exhibit altered phenotypes with respect to growth rate and gene transcription (7). Like many other microorganisms, A. hydrophila can form biofilms in laboratory settings on stainless steel (23), glass (40), and vegetables (8). A. hydrophila possesses two distinct types of flagella, polar and lateral, for swimming and swarming, respectively. Both flagellar systems play vital roles in pathogenesis (34) and optimal biofilm formation (18). A. hydrophila secretes metalloprotease, zinc protease, and serine protease, which play a vital role in invasiveness and generation of infection (5).

Bacterial genes and their respective phenotypes are regulated in a population-dependent manner with the aid of small diffusible signal molecules through a phenomenon referred to as quorum sensing (QS) (36). Gram-negative bacteria produce acyl-homoserine lactone (AHL) QS systems to regulate a variety of physiological processes, including virulence. A. hydrophila produces N-3-butanoyl-L-homoserine lactone (C4-HSL) synthase, encoded by ahyl, and N-3-hexanoyl homoserine lactone (C6-HSL) synthase, encoded by ahylR, at a ratio of 70:1 (35). Modulation of virulence, exoprotease production, T3SS and T6SS protein expression, and biofilm development by QS has been previously reported (15, 23, 36). Khajanchi et al. (14) also reported that AHLs and cytotoxic activity levels were higher in clinical strains than in strains isolated from water, establishing the importance of QS in human infection.

Because biofilms are directly related to virulence (31) and sessile cells are resistant to antibiotics and various disinfectants (24), a better approach is to inhibit biofilm formation rather than attempt to kill bacteria already in established biofilms (32). Blockage of QS is a new phenomenon, and many antibacterial agents have been identified recently to combat disease in this manner (2). Furukawa et al. (10) reported that sugar fatty acid esters inhibited biofilm formation by foodborne pathogens. Similarly, EDTA (3), bacteriocins, and competition for nutrients (31) inhibited biofilm formation by Listeria monocytogenes.
Glucose also has been reported as having antibiofilm effects on *Escherichia coli* (13) and *Bacillus cereus* (33).

Chestnut honey (38) and vanillin (30) also have been reported as having antibiofilm and anti-QS effects on *A. hydrophila*. The present study was conducted to examine the effect of various glucose concentrations on biofilm formation, motility, exoprotease production, and QS in *A. hydrophila*.

**MATERIALS AND METHODS**

**Bacterial strains, culture media, and growth conditions.** The strains used for the study were *A. hydrophila* strains KCTC 2358 (isolated from a can of milk with a fishy odor), KCTC 11533 (isolated from surface water), and KCCM 32586 (a clinical isolate). The bioreporter strain *Chromobacterium violaceum* CV026 (Animal, Plant and Fisheries Quarantine and Inspection Agency, Seoul, Korea) also was included. A 20% (wt/vol) glucose (Merck, Darmstadt, Germany) solution was prepared by filter sterilization with 0.22-μm-pore-size filters (Millipore Corporation, Billerica, MA). Nutrient broth (NB; Difco, BD, Sparks, MD) was used for all the experiments except the violacein assay, for which we used Luria-Bertani (LB) medium (Difco, BD). Before each experiment, the cultures were activated by transferring from the −80°C freezer on nutrient agar plates to 30°C for overnight incubation. A single colony from the plate was inoculated in 5 ml of NB and incubated overnight at 30°C for 48 h without shaking and then diluted 1:50 in NB or LB containing 0, 0.05, 0.25, 0.5, 1.0, and 2.5% (wt/vol) glucose. Bacteria were grown at 30°C unless otherwise indicated.

**Quantitative biofilm formation assay.** The quantitative biofilm assay was performed according to the procedure described by O’Toole (28) with minor modifications. The cultures were grown in NB for 48 h without shaking and then diluted (1:50) in NB containing various concentrations of glucose, and 100-μl aliquots were plated in each well of a 96-well polystyrene microtiter plate (BD, Franklin Lakes, NJ). The microtiter plate was incubated at 30°C for 72 h without shaking. After incubation, the absorbance was determined as an indicator of cell growth at 600 nm unless otherwise indicated.

**Motility assay (swimming and swarming).** Swimming is defined as flagellum-directed movement in an aqueous environment, and swarming is defined as multiple, lateral flagellum-directed rapid movement on solid surfaces. Both forms of motility were examined in the present study using protocols described elsewhere (6, 12) with slight modifications. For swimming motility, 1.5 μl of the same OD cultures were spotted at the center of a plate containing 25 ml of NB and 0.3% Bacto-agar (Difco, BD) and incubated face up for 24 h at 25°C. The assay was performed after the plates were allowed to dry for 12 h. To measure swarming, the same culture volume was inoculated on the surface of an agar plate containing LB and 0.5% Bacto-agar (Difco, BD) and incubated at 25°C for 48 h. After incubation, the diameter of the area of motility of the strains was measured, and the plates were photographed.

**Exoprotease assay.** Exoprotease activity was assessed using a Fluoro protease assay kit (G-bioscience, St. Louis, MO). Overnight cultures were diluted (1:50) with cultures with the same OD in different concentrations of glucose containing fresh NB and incubated for 48 h with shaking at 200 rpm. After incubation, supernatants were collected by centrifugation at 16,060 × g. The supernatant (50 μl) from each glucose condition was added to 150 μl of fluorescein isothiocyanate–conjugated substrate and incubated at room temperature for 1 h. Fluorescence was measured at 485 nm excitation and 530 nm emission with a fluorescence microplate reader (Spectra Max Gemini EM, Molecular Devices). The data were interpreted using the trypsin standard supplied with the kit. The medium with fluorescent substrate was used as the negative control.

**Production of AHLs.** Violacein production was quantified using procedures similar to those previously described (20) with some modifications. The *A. hydrophila* strains were grown for 48 h, and *C. violaceum* CV026 was grown in LB for 24 h at 28°C with shaking at 200 rpm. The *A. hydrophila* strains were streaked perpendicularly to the *C. violaceum* CV026 on LB agar plates with different concentrations of glucose and incubated for 24 h at 28°C. All of the culture grown on the *C. violaceum* CV026 plate was collected by rubbing it off plate, and this culture was mixed with 500 μl of dimethyl sulfoxide (DMSO; Sigma Aldrich, St. Louis, MO), which resulted in release of the violacein pigment. After centrifugation at 16,060 × g for 15 min, 200 μl of colored DMSO from each condition was evaluated at 585 nm with a microplate reader.

**SEM of biofilm experiments.** Biofilm formation of *A. hydrophila* strain KCTC 11533 was observed using scanning electron microscopy (SEM) after incubation for 72 h on polystyrene coupons (2 by 2 cm) prepared in house in 5 ml of NB containing different concentrations of glucose. The selected coupons were rinsed three times with phosphate-buffered saline (PBS; pH 7.2). The adhered cells were fixed in 4% glutaraldehyde in PBS for 2 h and then washed three times for 15 min with PBS. The fixed cells were serially treated with ethanol (50% for 15 min, 60% for 15 min, 70% for 15 min, 80% for 15 min, 90% for 15 min, and 100% two times for 15 min each) and successively dehydrated by soaking in 33, 50, 66, and 100% hexamethyldisilazane in ethanol for 15 min each. The dehydrated samples were examined under a scanning electron microscope (S-3400, Hitachi High Technologies, Tokyo, Japan) according to the procedure described previously (20).

**Biofilm inhibition determined with the stage shift assay.** For the stage shift biofilm assay, we followed the procedure described by Chang et al. (3). *A. hydrophila* KCTC 11533 was inoculated without glucose into NB, 48-h cultures were diluted to...
1:50 in NB, and 100-μl aliquots were inoculated into a microtiter plate and incubated at 30°C for 6 days. At various time intervals, 2.5% (wt/vol) glucose was added to the microtiter wells, and biofilm inhibition was calculated using equation 2:

\[
\text{biofilm reduction} (\%) = \left( \frac{A_{2.5} - B_{2.5}}{A_0 - B_0} \right) \times 100
\]

where \( A_{2.5} \) denotes the average BFI at specific times with the addition of 2.5% glucose and bacteria, \( B_{2.5} \) denotes the BFI of the specific time with the addition of 2.5% glucose in blank wells, \( A_0 \) denotes the BFI after 6 days in the control (0% glucose), and \( B_0 \) denotes the BFI of blank wells.

**Induction of exoprotease inhibition using exogenous AHLs.** N-Acyl homoserine lactones C4-HSL and C6-HSL were purchased commercially (Cayman Chemical Company, Arbor, MI), and 100 mmol stock solutions were prepared by dissolving the AHLs in DMSO according to the manufacturer’s instructions and stored at −20°C until further use. *A. hydrophila* KCTC 11533 was inoculated into NB containing different concentrations of glucose, and final concentrations of 20 μmol of C4-HSL and C6-HSL, both singly and in combination, were used to examine protease activity using the Fluoro protease assay kit according to the procedure described for exoprotease activity. The percent induction by exogenous AHLs with and without the addition of HSL and blank values with or without HSL were calculated using equation 3:

\[
\text{induction protease} (\%) = \left( \frac{P(HC - HB)}{P(C - B)} \right) \times 100
\]

where \( HC \) denotes the average absorbance in the supernatant with the addition of cells, HSL, and glucose; \( HB \) denotes the absorbance value in a blank with glucose and HSL without the addition of cells; \( C \) denotes the average absorbance concentration in control (0%) glucose with cells without HSL; and \( B \) denotes the average of 0% glucose blank absorbance values without bacterial cells and HSL. The values were converted to protease concentrations at specific glucose concentrations and HSL, which are denoted as \( P(HC - HB) \) and control glucose as \( P(C - B) \).

**Statistical analysis.** To determine the inhibitory effect of glucose on motility, biofilm formation, exoprotease activity, and QS, all experiments were repeated three times. The data were analyzed with an analysis of variance using SAS software (version 9.1, SAS Institute Inc., Cary, NC) for a completely randomized design. When the effect was significant \((P < 0.05)\), means were separated with Duncan’s multiple range test.

**RESULTS**

**Biofilm formation.** The results of biofilm formation after exposure to different glucose concentrations, based on CV staining on the polystyrene solid surface of 96-well microtiter plates, are shown in Figure 1. The representative results from *A. hydrophila* KCTC 2358 showed a gradual reduction of biofilm formation with the addition of glucose in NB (Fig. 1a). Addition of 0.05% glucose did not significantly reduce biofilm formation compared with the control, whereas 0.25% glucose significantly \((P < 0.05)\) inhibited biofilm formation of all three strains (Fig. 1b). Glucose at 2.5% completely inhibited biofilm formation (Fig. 1b) without any significant effect \((P > 0.05)\) on planktonic growth; therefore, biofilm reduction by glucose was not due to growth inhibition. The mean planktonic growth values, as indicated by OD_600 values, were 0.22, 0.75, and 0.34 for strains KCTC 2358, KCTC 11533, and KCCM 32586, respectively, in control broth (0% glucose). The mean growth (OD_600) values were 0.21, 0.72, and 0.33, respectively, with 0.25% glucose and 0.19, 0.71, and 0.29, respectively, with 2.5% glucose. The average BFI scores for biofilm formation were 1.3, 1.04, and 0.31 for strains KCTC 2358, KCTC 11533, and KCCM 32586, respectively, in control broth (0% glucose) and were reduced to 0.33, 0.39, and 0.074, respectively, in 0.25% glucose. The results also showed significant \((P < 0.05)\) strain-specific BFI values; strain KCCM 32586 had low biofilm formation in control broth. In general, the results indicate that regardless of strain concentrations of glucose higher than 0.05% had an inhibitory effect on biofilm formation.

**Swarming and swimming motility.** Because motility is one of the contributing factors for virulence and biofilm formation and because *A. hydrophila* cells have both lateral...
and polar flagella, motility was tested using semisolid agar with incubation for 24 h for swarming motility and 48 h for swimming motility. Figure 2 shows both types of motility of all three *A. hydrophila* strains of different origins. A slow reduction in bacterial motility was found in medium supplemented with increasing concentrations of glucose (Fig. 2a). No movement from the site of inoculation was observed with 2.5% glucose. Motility of all strains was significantly impaired (*P* < 0.05) by the addition of glucose (Fig. 2b and 2c), following the same trend as observed for the biofilm inhibition assay. Mean diameters of swarming motility (Fig. 2b) were 39.7, 18.3, and 38.3 mm for strains KCTC 2358, KCTC 11533, and KCCM 32586, respectively, on control plates, which was significantly reduced to 10.7, 7.0, and 11.3 mm, respectively, with the addition of 0.25% glucose. Mean diameters of swimming motility (Fig. 2c) were 61.6, 20.7, and 46.7 mm, respectively, on control plates, with significant reductions to 17.7, 14.3, and 5.7 mm, respectively, with the addition of 0.25% (wt/vol) glucose, indicating the same inhibition trend as seen for swarming. Motility also was strain specific; strain KCTC 11533 was significantly less motile (*P* < 0.05).

**Exoprotease production.** The pathogenic and virulence characteristics of *A. hydrophila* are related to exoprotease production. In control and 0.05% added glucose treatments, exoprotease production was induced, but higher glucose concentrations (≥0.25%) significantly inhibited (*P* < 0.05) the exoprotease activity in the *A. hydrophila* supernatant (Fig. 3). Strain KCTC 2358 had significantly lower protease production in the control broth than did the other two strains. Protease concentrations were 10.9, 23.2, and 38.9 ng/ml for strains KCTC 2358, KCTC 11533, and KCCM 32586, respectively, in control supernatants and were reduced to 0.2, 7.5, and 1.4 ng/ml, respectively, with 0.25% glucose. Glucose concentrations of 1.0 and 2.5% inhibited the secretion of exoprotease to below the detection limit of the assay (Fig. 3).
Violacein production. The QS regulation effect was determined using the biosensor C. vilaceum CV026 C6-HSL transposon mutant strain. This mutant strain is negative for violet color in the absence of AHLs but produces color when AHLs are externally supplied. When A. hydrophila strains are grown with CV026, the mutant produces color because violacein is produced in response to AHLs secreted by the test strains, and the intensity of the color is proportional to the AHL concentration. Figure 4a shows violacein production by CV026 cultures grown with strain KCTC 11533 at 1.0 and 2.5% glucose in one experiment, which did not result in color production. Figure 4b shows violacein production for multiple experiments. Mean violacein production was 0.922 and 0.720 with strains KCTC 11533 and KCCM 32586, respectively, under control conditions, and addition of 0.25% glucose significantly reduced (P < 0.05) the values to 0.679 and 0.223, respectively. Violacein values were 0.075 and 0.046, respectively, at 0.5% glucose, but addition of 1.0% glucose did not result in color production. Culture with strain KCTC 2358 did not result in production of color by the biosensor strain at any glucose concentration. These findings indicate that in general, AHL production by A. hydrophila strains was repressed by glucose.

Biofilm observation by SEM. Figure 5 shows images of biofilm formation by strain KCTC 11533 with different concentrations of glucose; this strain produced significantly more biofilm and induced more violacein production than did the other A. hydrophila strains. Three-dimensional, thick, and firmly attached mushroom-like structures were noted for the control culture and that with 0.05% glucose (Fig. 5a and 5b), whereas addition of 0.25 and 0.5% glucose resulted in only cell monolayers (Fig. 5c and 5d). Very few bacteria were attached to the polystyrene surface in cultures with 1.0 and 2.5% glucose (Fig. 5e and 5f). The bacterial cell shapes also differed at noninhibiting concentrations (≥0.05%) and inhibiting concentrations (≥0.25%) of glucose (Fig. 5g through 5i). The cells in the control biofilms (Fig. 5g) and the 0.05% glucose biofilms (Fig. 5h) were oval, short rods, and those grown with 1.0% glucose were elongated rods (Fig. 5i). These SEM results further support biofilm inhibition by glucose at concentrations ≥0.25%.

Stage shift biofilm assay. Figure 6 shows that the addition of glucose (2.5%) at different stages of growth of A. hydrophila KCTC 11533 significantly inhibited (P < 0.001) biofilm formation within 48 h. The mean decreases in the BFI compared with that at 6 days were 89.9, 83.3, 67.4, 50.1, and 18.4% at 0 h, 6 h, 12 h, 1 day, and 2 days, respectively. The addition of glucose did not significantly affect biofilm inhibition after 2 days.

Induction of protease inhibition with exogenous AHLs. The exogenous addition of 20 μmol C4-HSL and 20 μmol C6-HSL, both singly and in combination, resulted in significantly higher (P < 0.05) protease production. Figure 7 shows that in A. hydrophila KCTC 11533 the combination of C4-HSL and C6-HSL resulted in higher protease activity than obtained when each AHL was added alone. The combination of both molecules restored protease production to 83.4, 39.8, and 33.5% for media containing 0.25, 0.5, and 1.0% glucose, respectively. The external supply of QS molecules did not restore this activity in medium with 2.5% glucose.

DISCUSSION

Kirov (17) reported on the potential of A. hydrophila as a human foodborne pathogen. Because biofilms may play a vital role in virulence, we assessed the potential role of QS and inhibition of QS in biofilm formation, motility, and exoprotease activity in A. hydrophila. Other researchers have noted inhibition of QS and biofilm formation by compounds such as brominated furanones (11), α-amino acids (19), cis-2-decenolic acid (4), and 7-hydroxyindole (21). Although vanillin and honey have been reported to inhibit biofilm formation in A. hydrophila (30, 38), we
FIGURE 5. Scanning electron micrographs of biofilms formed on polystyrene coupons by A. hydrophila KCTC 11533 with different concentrations (wt/vol) of glucose: (a) 0%; (b) 0.05%; (c) 0.25%; (d) 0.5%; (e) 1.0%; (f) 2.5%; (g) 0%, higher magnification (10 µm); (h) 0.05%, higher magnification (10 µm); (i) 1.0%, higher magnification (10 µm).

FIGURE 6. Stage shift biofilm assay of A. hydrophila KCTC 11533 grown with different concentrations of glucose. Values are the mean ± standard error of the mean of three independent experiments. Within each variable, values with the same letter are not significantly different according to Duncan’s multiple range test (P > 0.05).

FIGURE 7. Induction of protease inhibition in cultures of A. hydrophila KCTC 11533 plus different concentrations of glucose by addition of exogenous C4-HSL and C6-HSL. Values are the mean ± standard error of the mean of three independent experiments. Within each variable, values with the same letter are not significantly different according to Duncan’s multiple range test (P > 0.05).
found that glucose at low concentrations (0.25%) also inhibited biofilm formation (Fig. 1) without affecting planktonic growth. In a recent study, 0.5% (wt/vol) honey reduced *E. coli* O157:H7 biofilm formation by 98% through inhibition of the expression of biofilm-related curli genes, QS genes, and virulence genes (22). Kierek and Watnick (16) found that biofilm formation was induced in *Vibrio cholerae* by monosaccharides (glucose, galactose, and mannose).

O’Reilly and Day (27) observed reduced production of protease by *A. hydrophila* grown in 0.5% (wt/vol) glucose, whereas fructose and sucrose did not influence protease production. However, 0.25% (wt/vol) glucose significantly reduced protease production by *A. hydrophila* in our study (Fig. 3).

The findings in our study suggest that glucose may inhibit production of lateral and polar flagella under the control of QS (Figs. 2 and 4). QS may moderate both swarming and swimming motility, which were completely inhibited by addition of 1.0 and 2.5% glucose; the strains did not diffuse from the origin of inoculation (Fig. 2), and no violacein was produced (Fig. 4) under these conditions by *C. violaceum*. Polar and lateral flagellar systems for swimming and swarming, respectively, in *A. hydrophila* are associated with virulence (39, 41). Yu et al. (41) found an interconnection between T3SS and T6SS proteins under the control of temperature, RpoN, AxsA/ExsA, and QS. The QS regulator (AhyR) is responsible for the upregulation of T3SS and T6SS (15, 39).

Although *C. violaceum* CV026 is a C6-HSL mutant, it produces the violacein color when exposed to C8-AHL, C8-3-oxo-AHL, and C4-AHL (9, 26). *A. hydrophila* secretes C4-HSL and C6-HSL (35). The QS experiments (violacein production) revealed that 0.5% glucose inhibited approximately 95% of QS activity, based on the response of the CV026 biosensor (Fig. 4b). Culture of CV026 with *A. hydrophila* KCTC 2358 did not result in color production.

The activity of both serine protease and metalloprotease in *A. hydrophila* is under the control of QS mechanisms (36), which is also the case for protease production in *Aeromonas salmonicida* (35). However, we found a correlation between violacein production and exoprotease production inhibition by glucose (Figs. 3 and 4). Scanning electron micrographs of control cultures and those grown with 0.05% glucose revealed three-dimensional mushroom-like structures of sessile cells previously noted by other researchers for wild types but not QS mutants (23). The difference in cell shape as revealed by SEM (Fig. 5) also is correlated with previous findings of the inhibitory effect of honey on *E. coli* O157:H7 cells (22). The stage shift biofilm assay results also revealed that glucose inhibited early stage biofilm formation because QS is active at the initial stage of biofilm formation (Fig. 6). *A. hydrophila* ahyR mutant strain (this gene encodes a protein responsible for the synthesis of C4-HSL) had substantially reduced serine protease and metalloprotease activity, which could be restored by the addition of exogenous C4-HSL (36). We found a significant increase in protease production with the addition of exogenous C4-HSL and C6-HSL (Fig. 7). These findings support our hypothesis that glucose regulates QS, which in turn influences motility, biofilm formation, and exoprotease activity.

In conclusion, glucose concentrations starting at 0.25% (wt/vol) inhibited biofilm formation, motility, and protease production by *A. hydrophila* via the signal cascade QS-inhibiting pathway (Fig. 8). Glucose might bind to the master PhoB/AxsA regulators of polar and lateral flagella, which are also controlled by QS mechanisms. The regulation of AHLs further modulates protease production, motility, and biofilm formation. The four characteristics (biofilm formation, motility, protease production, and QS) of *A. hydrophila* strains that were originally isolated from food, surface water, and a clinical setting had different phenotypic patterns, and regulation was strain specific. Strain KCTC 2358 had significantly higher biofilm production and high motility.
with less evidence of QS and protease production. Strain KCCM 32586 had less biofilm formation but higher protease production and QS activity, and strain KCTC 11533 had higher biofilm formation, QS, and exoprotease activity but less motility. Thus, none of these strains had the same phenotypic profile for these four characteristics. Inhibitory activities were noted when glucose was added at concentrations ≥0.25% (wt/vol), whereas no inhibitory effect was observed with ≤0.05% (wt/vol) added glucose (Fig. 8).

The present findings indicate that glucose regulates biofilm formation and virulence properties. Because food is a complex matrix with many components, further studies are required to elucidate the effects of glucose in various foods on biofilm formation and virulence properties of *A. hydrophila*.

**ACKNOWLEDGMENT**

This research was supported by the Chung-Ang University Excellent Freshman Scholarship grants.

**REFERENCES**


