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ARTICLE

Insecticidal Activity of Rhamnolipid Isolated from *Pseudomonas* sp. EP-3 against Green Peach Aphid (*Myzus persicae*)

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Microorganisms capable of growth on oils are potential sources of biopesticides, as they produce complex molecules such as biosurfactants and lipopeptides. These molecules have antimicrobial activity against plant pathogens, but few data are available on their insecticidal activity. The present study describes the insecticidal activity of a rhamnolipid isolated from diesel oil-degrading *Pseudomonas* sp. EP-3 (EP-3). The treatment of cell-free supernatants of EP-3 grown on glucose−mineral medium for 96 h led to >80% mortality of aphids (*Myzus persicae*) within 24 h. Bioassay-guided chromatography coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MADLDI-TOF MS) and (^{1}H, ^{13}C) nuclear magnetic resonance (NMR) analyses was employed to isolate and identify the EP-3 insecticidal metabolites. Dirhamnolipid, with molecular formulas of $C_{32}H_{58}O_{13}$ and $C_{34}H_{62}O_{13}$, was identified as a main metabolite exhibiting insecticidal activity against aphids. Dirhamnolipid showed a dose-dependent mortality against aphids, producing about 50% mortality at 40 μg/mL and 100% mortality at 100 μg/mL. Microscopy analyses of aphids treated with dirhamnolipid revealed that dirhamnolipid caused insect death by affecting cuticle membranes. This is the first report of rhamnolipid as an insecticidal metabolite against *M. persicae*. Rhamnolipid shows potential for use as a pesticide to control agricultural pests.

KEYWORDS: Aphid; biosurfactant; biopesticide; *Pseudomonas* sp. EP-3; rhamnolipid

INTRODUCTION

Aphids, one of the most important insect pests of many crops worldwide, are a major cause of crop damage related to reduced crop production and quality. Direct and indirect effects of aphids on crops induce deformed leaves and spread plant pathogens and viral diseases (*1*, *2*). Aphids are mostly controlled by chemical insecticides, but increased insect resistance and undesirable environmental effects have prompted research on naturally occurring and more environmentally friendly means of aphid control. The environmentally friendly organic farming program of the Korean government strongly recommends that farmers decrease their use of synthetic pesticides. Thus, finding environmentally safe chemicals is of great interest to agricultural scientists. In Korea, there is growing interest in the use of microbial metabolites for insect control as they are generally more biodegradable and more environmentally friendly than synthetic chemicals.

Oil-degrading microorganisms have received much attention in agriculture, as they produce complex molecules such as biosurfactants that exhibit antimicrobial activities against plant pathogens. Sophorolipid, a biosurfactant produced by *Candida bombicola* and *Pseudomonas aeruginosa*, exhibits antimicrobial activity against Gram-positive pathogens causing gray and softened spots in apple and tomato (*3*, *4*). Sophorolipid has been found to significantly inhibit the growth of algal cells by inducing membrane perturbations and cell lysis (*5*). Rhamnolipid, a biosurfactant from *Pseudomonas* strains, has antagonistic effects on zoosporic plant pathogens (*6*, *7*) and is also a potential source of algicidal chemicals that completely inhibit the growth of harmful algal blooms (*8*). The culture broth of *Bacillus subtilis* C1 containing the biosurfactant surfactin completely inhibits the growth of the bloom-forming cyanobacterium *Microcystis aeruginosa* (*9*). Nevertheless, few studies have examined the insecticidal activity of biosurfactants produced by microorganisms. Rhamnolipid is expected to penetrate the cuticle membrane of insects because its molecular structure consists of lipids and sugars. In the present study, the aphicidal activity of dirhamnolipid produced by diesel oil-degrading *Pseudomonas* sp. EP-3 was investigated. The effects of dirhamnolipid on aphids were demonstrated by microscopy analyses of aphid membrane.

MATERIALS AND METHODS

Chemicals. The solvents used in this study were of HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals

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were of analytical grade and commercially available from Sigma-Aldrich Co. (St. Louis, MO), unless otherwise stated.

Bacterial Isolation and Identification. Diesel oil-degrading microorganisms were isolated by enrichment culture techniques using an agricultural soil that had been contaminated with agricultural machine oils. A 2 g soil sample was suspended in 100 mL of mineral salt medium (MSM) containing 1% (v/v) diesel oil as a sole source of carbon and the following constituents (in grams per liter, pH 7.2): K_2HPO_4, 7.5; KH_2PO_4, 2.0; NH_4NO_3 1.0; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.02; and 10 mL of trace elements containing 20 mg of $Na_2MoO_4 \cdot 2H_2O$, 50 mg of H_2BO_3, 30 mg of $ZnCl_2$, 10 mg of $CuCl_2$, and 20 mg of $FeCl_3$ per liter. The suspension was incubated at 25 °C for 7 days on a shaking incubator at 150 rpm and then was transferred to a fresh MSM. After four weekly transfers, the cultures were diluted serially with the MSM and plated onto MSM agar plates that had been uniformly plated with 100 μL of diesel oil. Following microbial growth on the plates at 25 °C for 7 days, visible colonies were obtained and purified by respectively plating on Luria−Bertani (LB, Difco) agar. Individual colonies were further tested for oil degradation by incubation in 100 mL of the MSM containing 1% diesel oil as the sole carbon source. Bacterial identifications were carried out by 16S rRNA sequence analyses by comparing their sequences to other 16S rRNA sequences available from a BLAST search of the DDBJ database as described previously (*10*).

Screening of Microorganisms with Aphicidal Activity. The isolates were grown in LB medium overnight and inoculated at 1.0% (v/v) to MSM containing 0.5% (w/v) glucose as the sole carbon source. The cultures were then incubated for 7 days and centrifuged at 12000*g* for 20 min. The resulting supernatants were used for bioassays against green peach aphid, *Myzus persicae*. In vitro mortality bioassays against *M. persicae* were performed in 2-week-old Chinese cabbages (*Brassica rapa* subspecies *pekinensis* and *chinensis*). Chinese cabbage seeds were disinfected with 70% ethanol for 1 min followed by rinsing with sterile distilled water. The disinfected seeds were planted in commercial compost in square, plastic insect-breeding dishes (80 × 80 × 100 mm) and grown in a greenhouse at 25 ± 2 °C and 60 ± 5% relative humidity for 2 weeks. Twenty second-instar nymphs were reared on the Chinese cabbage leaves and allowed to settle onto the leaves for 18 h prior to spray application. Subsequently, the plants were sprayed uniformly to runoff using small hand-held sprayers. After drying, the plants were then held in the growth chamber at 25 ± 2 °C and 65 ± 5% relative humidity with a photoperiod of 16:8 h light/dark. The plants received only the MSM for negative control samples. The plants received imidacloprid at a recommended level for positive control samples. Aphid mortalities were investigated 24 h after spray applications by Abbott's formula (*11*).

Isolation of Insecticidal Metabolites. An isolate that showed the strongest insecticidal activity among the isolates was grown in the MSM containing 0.5% (w/v) glucose as the sole carbon source as described above. The cultures were centrifuged at 12000*g* for 20 min, and the supernatants were extracted twice with 2 times the volume of ethyl acetate. The organic extracts were evaporated to dryness in an EYELA model N-1000 rotary evaporator (Tokyo Rikakikai Co., Ltd., Japan) at 40 °C. The dried extracts were dissolved in ethyl acetate. A portion of the dried extracts was diluted at 100 μg/mL in 10% (v/v) aqueous solvent mixture of dimethyl sulfoxide and methanol (4:1, v/v) for aphid mortality assays as described above. The extracts dissolved in ethyl acetate were subjected to silica gel (Kiesel gel 60, 230−400 mesh, Merck) column chromatography. An open glass column (3.5 mm i.d. × 500 mm in length) was slurry-packed with silica gel in ethyl acetate. The extracts were dissolved in ethyl acetate and loaded onto the column. The column was eluted with a solvent mixture of ethyl acetate and methanol, in which the methanol concentration was increased at 5% (v/v) in each elution step. Each eluate was evaporated in the evaporator at 40 °C and examined for aphid mortality as described above. The fractions with aphicidal activity were collected together and further purified by Sephadex LH-20 column chromatography (3.3 mm i.d. × 500 mm in length). The column was eluted with 4 times the bed volume of methanol, and the eluate was examined for aphid mortality as described above. The eulates with aphicidal activity were injected onto a Waters PrepLC 2000 model preparative HPLC equipped with a Dionex Corona CAD universal detector. The HPLC column was a Capcell Pak C18 stainless column (20 mm i.d. × 250 mm in length, particle size = 5 μm). The mobile phase consisted of methanol and water, and the methanol concentration was increased from 20 to 100% (v/v) over 40 min at a flow rate of 8 mL/min. Peaks were collected and subjected to aphid mortality bioassays as described above. The active fraction was then subjected to instrumental analyses for identification of an insecticidal metabolite.

Microscopy Analyses. Microscopy analyses were employed to investigate the effects of the isolated metabolite on aphids (*12*). Briefly, for scanning electron microscopy analysis, aphid samples were fixed with 2% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 50 mM cacodylate buffer at pH 7.4 for 48 h at room temperature. After fixing, the samples were washed three times in the cacodylate buffer and dehydrated through 50, 70, 90, and 100% ethanol for 5 min in each stage. The samples were dried and examined with a Hitachi S-2400 model scanning electron microscope. For light and transmission electron microscopy analyses, aphid samples were immersed in 2.5% (v/v) glutaraldehyde in 0.1 M PBS buffer at pH 7.4 for 48 h at 4 °C. Following washing with the PBS buffer, the samples were immersed in a mixture of 2% osmium tetroxide and 3% (v/v) ferrocyanide (1:1, v/v) in the same buffer for 1 h at 4 °C. After a washing with the PBS buffer, the samples were dehydrated in a series of ethanol solutions and embedded in Epon 812. Semithin sections were mounted on glass slides and examined with an Olympus TH4-200 model light microscope (Olympus U.K. Ltd., London, U.K.) after staining with 1% (w/v) aqueous toluidine blue for 2 min. Ultrathin sections were mounted on Formvar-coated nickel grids (200 mesh) and stained with aqueous uranyl acetate and alkaline lead citrate for 5 min. The samples were then examined with an FEI Techai sprit G2 model transmission electron microscope with an accelerating voltage of 120 kV.

Greenhouse Assays of Aphid Mortality. Aphid mortality assays *in vivo* were performed on green peach aphids, occurring naturally on greenhouse pepper plants, at Naju, Republic of Korea, in May 2009. Ten pepper plants infested with aphids were sprayed to runoff with 2 L of the cell-free supernatant using small hand-held sprayers. The cell-free supernatant was used within 48 h after cultivation. Aphid mortalities were investigated 24 h after spray applications as follows: mortality (%) = 100[1− (number of living aphids on plant leaves after application/number of living aphids on plant leaves before application)]. Control aphids received only the MSM.

Statistical Analysis. Data were analyzed by ANOVA using IBM SPSS Statistics 19 software (Somers, NY). The significance of the observed data was determined by Duncan's multiple-range test ($P = 0.01$).

Biosurfactant Activity. To investigate if the isolate produce biosurfactants, surface interfacial tension values were measured using a Du Nouy model 3010 tensiometer (Du Nouy, Japan). Biosurfactant measurements were performed in triplicate on cell-free supernatant obtained by centrifugation at 12000*g* for 20 min. The instrument was calibrated with air and water to a reading of 72.75 mN/m.

Instruments. Mass spectra were obtained using a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems) operating in positive ion mode (*13*). A Shimazu model QP2010 GC-MS system equipped with a DB-5 capillary column (0.25 i.d. × 30 m in length, 1.0 μm film thickness) was also used. The carrier gas was helium, and the flow rate was 1.0 mL/min. Injection was performed in a split mode of 20:1. The initial column temperature was set for 2 min at 100 °C, followed by ramping at a rate of 10 °C/min to 280 °C. The injector temperature was 280 °C. Mass spectrometry was performed in the chemical ionization mode with isobutane as a chemical gas and electron impact mode with an ionizing voltage of 70 eV. GC-MS chemical library databases (Wiley7, NIST27, and NIST147) were used to characterize the target compound. A Varian model Unity INFINITY plus 200 MNR (200 mHZ) and a Varian model Unity INOVA NMR (500 mHz) were used for ^{13}C and ^{1}H analyses, respectively.

RESULTS

Characterization of a Microbial Isolate Exhibiting Insecticidal Activity. Forty microorganisms capable of growth on diesel oil as the sole carbon source were isolated. Among them, an isolate that showed the best growth on diesel oil was designated *Pseudomonas* sp. EP-3 and selected for further study. The 16S rRNA sequence analysis of EP-3 showed 95% similarity with *P. aeruginosa* LMG 1242^T (accession no. Z76651). EP-3 is now deposited in the

GenBank database under accession no. HM031115. EP-3 is capable of growing in MSM containing diesel oil up to 20% (v/v) as the sole carbon source on the basis of colony-forming unity. When EP-3 was incubated in glucose MSM, the growth of EP-3 was accompanied by a decrease in surface tension values (Supporting Information, Supplemental 1), giving a value 35.2 mN/m at 96 h and suggesting that EP-3 produced biosurfactant during growth on glucose MSM. Aphid mortalities were accompanied by EP-3 during growth on glucose−MSM (**Figure 1**), exhibiting the highest mortality after a 96 h incubation. In preliminary experiments, EP-3 cultural supernatant exhibited consistent mortality after autoclaving at 121 °C for 20 min, suggesting that the involvement of cultural proteins in aphid mortality would be negligible. These results suggested that insecticidal metabolites produced by EP-3 are related to biosurfactant production during growth. Greenhouse assays of aphid mortality showed mortalities ranging from 70 to 100% (Supporting Information, Supplemental 2). No phytotoxicity on pepper plant leaves was observed by visual observations after treatment of EP-3 supernatant.

Isolation and Identification of Insecticidal Metabolite. TOF-MS and NMR analyses were employed to identify the insecticidal metabolite produced by EP-3. MADLDI-TOF MS analysis of the isolated metabolite detected $(M + Na)^+$ peaks at m/z 673.2864 and 701.2791 and $(M - H + Na_2)^+$ peaks at m/z 695.2392 and 723.2468 (**Figure 2**), indicating molecular masses of 650.7951 and 678.8433, respectively. The mass spectrometry data resembled those of dirhamnolipid reported previously (*13*). When the metabolite was hydrolyzed under acidic conditions, 3-hydroxydecanoic acid (3OH-C_{10} fatty acid) and 3-hydroxydodecanoic acid (3OH-C_{12} fatty acid) were detected by GC-MS analyses in the electron impact and chemical ionization modes (Supporting Information, Supplemental 3). The molecular ion peaks of the fatty acids were not detected in EI mode, exhibiting the fragment ion peaks $C_5H_{11}^+$ and $(C_5H_{11} + H_2O)^+$ at m/z 71 and 89, respectively (Supporting Information, Supplemental 3A and 3C). The fragment ion peak at m/z 117 was suggested to be $(C_7H_{15} + H_2O)^+$ (Supporting Information, Supplemental 3C). GC-MS analysis of 3-hydroxydecanoic acid in CI mode detected an $(M + H)^+$ peak at m/z 189, exhibiting two main fragment ion peak at m/z 171 and 153 resulting from subsequent loss of water molecules (Supporting Information, Supplemental 3B), whereas the analysis of 3-hydroxydodecanoic acid detected an $(M + H)^+$ peak at m/z 217, exhibiting two main fragment ion peaks at m/z 171 and 153 resulting from the loss of water and CO molecules (Supporting Information, Supplemental 3D). The ^1H and ^{13}C NMR chemical shifts and coupling constant data of the isolated metabolite are presented in **Table 1**. Two anomeric protons (H-1, H-8) at 3.79 and 4.94 ppm were correlated to anomeric carbons at 72.00 and 104.4 ppm, respectively. Two methyl protons (H-6 and H-12) were assigned to the same chemical shifts (1.29 ppm). Two hydroxymethine protons (H-13, H-17) were detected at 4.12 and 5.32 ppm. The α protons of carbonyl groups were coupled at 2.60 ppm (H-14) and 2.51 ppm (H-18). The ester (C-15) and acid (C-19) carbons were detected at 172.72 and 175.32 ppm, respectively. From heteronuclear multiple bond coherence coupling (HMBC), the C-15 was coupled adjacent to H-14 (2.60 ppm) and H-13 (4.12 ppm), and C-19 was coupled adjacent to H-18 (2.51 ppm) and H-17 (5.32 ppm). C-13 was coupled to H-7 (4.97 ppm). The 3, 4, 9, and 10 carbons and protons were not identified due to complicated peaks. Alcohol and carboxylic acid hydroxy protons were not detected probably due to deuterium exchange with CD_3OD.

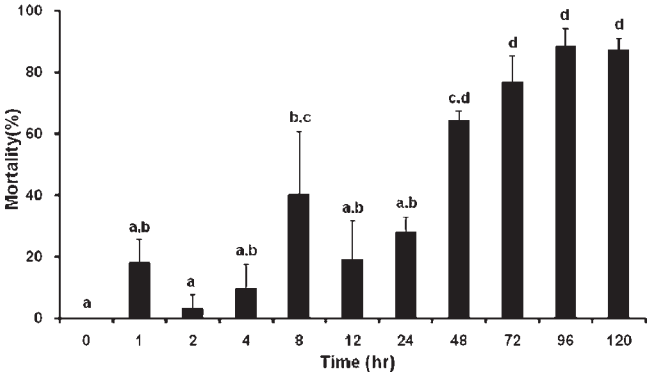

Figure 1. Aphid mortalities of *Pseudomonas* sp. EP-3 cultural supernatants during growth on glucose−mineral medium. Data are means ± SD of triplicates. Data showing the same letter are not significantly different on the basis of Duncan's multiple-range test at $P = 0.01$.

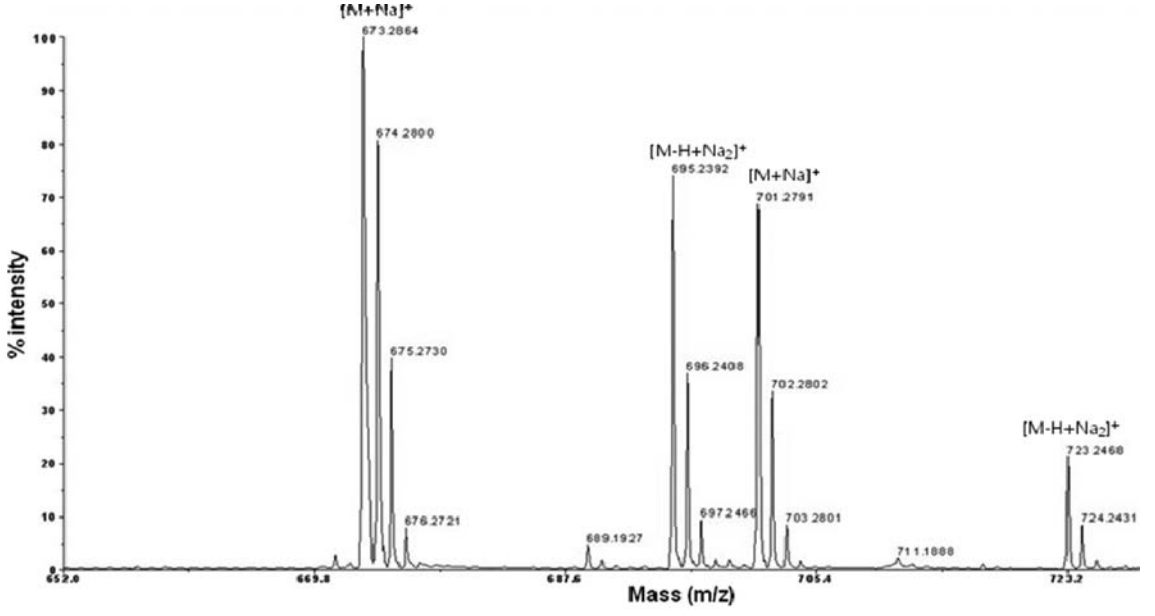

Figure 2. MALDI-TOF mass spectra of the insecticidal metabolite isolated from cell-free supernatant of *Pseudomonas* sp. EP-3 grown on glucose−mineral medium.

Table 1. NMR Data of the Insecticidal Metabolite Isolated from Cell-Free Supernatant of *Pseudomonas* sp. EP-3 Grown on Glucose−Mineral Medium

proton	δ_H	carbon	δ_C
H-1	4.94	C-1	104.40
H-2	3.79	C-2	80.71
H-3	*a*	C-3	*a*
H-4	*a*	C-4	*a*
H-5	3.74	C-5	70.42
H-6	1.29	C-6	18.23
H-7	4.97	C-7	99.13
H-8	3.79	C-8	72.00
H-9	*a*	C-9	*a*
H-10	*a*	C-10	*a*
H-11	3.70	C-11	70.30
H-12	1.29	C-12	18.19
H-13	4.12	C-13	75.30
H-14	2.60	C-14	41.40
		C-15	172.72
H-16	1.60	C-16	25.97
H-16′ (CH_2)	1.36	C-16′ (CH_2)	34.24
H-16″ (CH_3)	0.95	C-16″ (CH_3)	14.60
H-17	5.32	C-17	72.80
H-18	2.51	C-18	40.71
	a	C-19	175.32
H-20	1.67	C-20	26.39
H-20′ (CH_2)	1.36	C-20′ (CH_2)	34.24
H-20″ (CH_3)	0.95	C-20″ (CH_3)	14.60

[a] Not identified due to the complicated peaks.

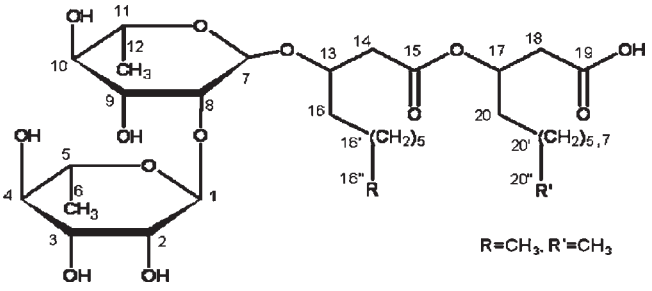

Figure 3. Chemical structure of dirhamnolipid isolated from *Pseudomonas* sp. EP-3.

The NMR data patterns resembled closely those of dirhamnolipid reported in a previous study (*13*). On the basis of MS and NMR data, the metabolite was identified as dirhamnolipid with formulas $C_{32}H_{58}O_{13}$ and $C_{34}H_{62}O_{13}$ consisting of two rhamnose molecules and two fatty acids (**Figure 3**).

Bioassays of Dirhamnolipid against *M. persicae*. Dirhamnolipid led to about 50% aphid mortality at 40 μg/mL and about 100% aphid mortality at 100 μg/mL (**Figure 4**), which was comparable to 100% mortality of imidacloprid, as a typical commercial insecticide, at a recommended level of 40 μg/mL (data not shown). The dose-dependent mortality of dirhamnolipid demonstrated that it clearly affected aphids. The microscopy data of aphids treated with MSM or the isolated rhamnolipid are presented in **Figure 5**. Scanning electron microscopy (SEM) analyses of aphids treated only with MSM showed a mosaic-like cuticular structure (**Figure 5A**), whereas clear evidence of damage to the cuticle was observed in aphids treated with the isolated dirhamnolipid (**Figure 5B**). A clearly apparent view of cuticular aphid membranes treated only with MSM was observed by light microscopy (**Figure 5C**), demonstrating that the cuticular membranes are well-organized for cellular components. However, the microscopy analyses showed that the cuticular membranes of aphids became much thinner after dirhamnolipid treatment (**Figure 5D**), and the membranes were

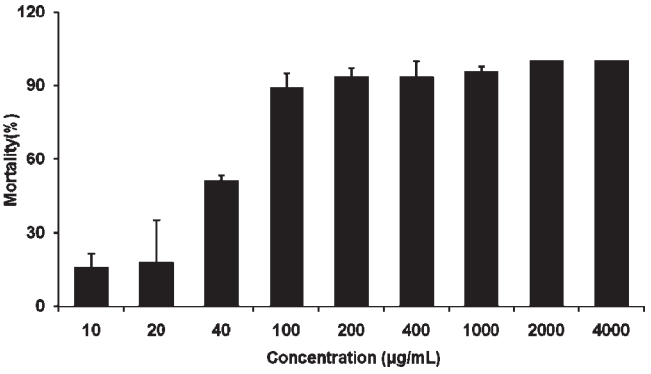

Figure 4. Dose-dependent aphid mortalities of dirhamnolipid isolated from cell-free supernatant of *Pseudomonas* sp. EP-3 grown on glucose−mineral medium. Data are the mean ± SD of triplicates.

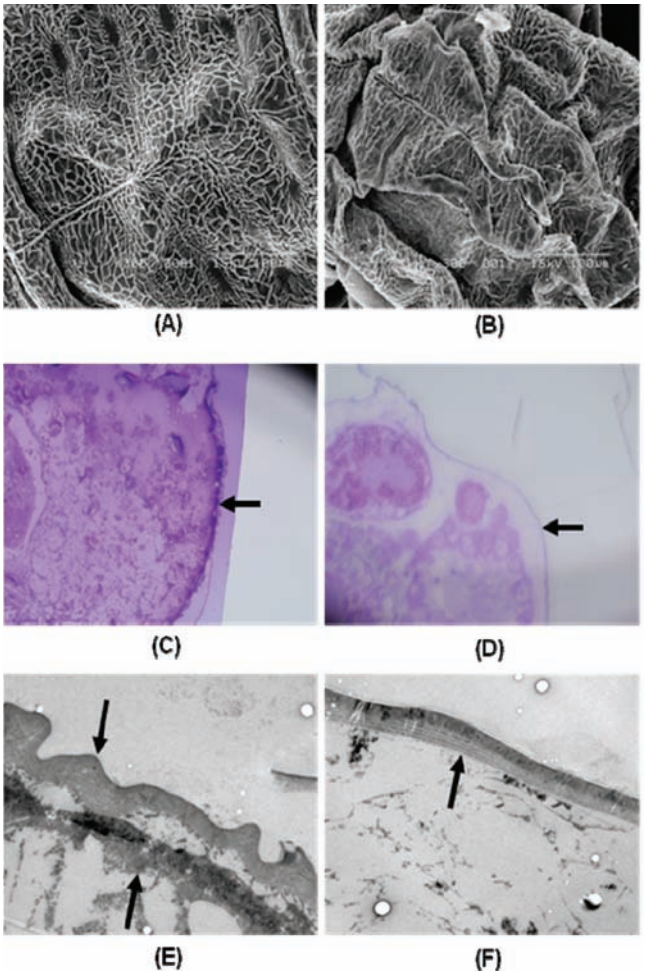

Figure 5. Micrographs of scanning electron microscopy (**A**, **B**), light microscopy (**C**, **D**), and transmission electron microscopy (**E**, **F**) of the aphids treated only with glucose−mineral medium (**A**, **C**, **E**) and the isolated dirhamnolipid (**B**, **D**, **F**). Arrows indicate aphid membranes.

dehydrated and detached from cellular components. Transmission electron microscopy confirmed two crinkly layers of cuticular membranes in aphids treated with MSM (**Figure 5E**). The outer was probably old cuticle, whereas the inner was newly synthesized cuticle. The cuticles were lost and not clear after dirhamnolipid treatment (**Figure 5F**). These observations suggest that dirhamnolipid affects the cuticular membrane to cause aphid death.

DISCUSSION

Rhamnolipids are surface-active glycolipidic biosurfactants produced by a variety of microorganisms by renewable feedstock (*14*, *15*). Most attractively, they are biodegradable and can be used as environmentally friendly chemicals for agricultural purposes. Thus, they have received much attention for the control of plant pathogens (*16*−*18*), but few studies have examined their insecticidal activity. In the present study rhamnolipid was examined for insecticidal activity against *M. persicae*. The bioassay-guided isolation and the instrumental analyses of the extracts of EP-3 cell-free supernatant identified dirhamnolipid as a main insecticidal metabolite against *M. persicae*. Most synthetic insecticides usually target nerve functions and the biochemical enzymes of insects (*19*, *20*). In our study, dirhamnolipid was supposed to target the cuticle membrane of aphids, which suggests the possibility that dirhamnolipid may be used for the control of insects that are resistant to those synthetic pesticides.

Significant dehydration was observed in the cuticle membrane of aphids treated with dirhamnolipid. Rhamnolipids are known to titrate as a function of their partition into the cell membranes of plant pathogens (*7*, *21*), which would not be exceptional in our study because the aphid membranes were significantly changed by dirhamnolipid treatment. The aphid membranes consist of a complex mixture of alkanes, wax esters, fatty acids, and phospholipids (*22*). These molecules are lipophilic chemicals that interact with surface active rhamnolipids including both a lipophilic and hydrophilic moiety (*23*, *24*). Thus, we hypothesize that the biosurfactant property of the rhamnolipids affected the membrane lipids of the aphids, as evidenced by the microscopy observations results, and led to leakage or dehydration of the aphid cellular components. Further study is required to characterize the exact mode of action of dirhamnolipid against aphids. It would be also interesting to examine the mortality of dirhamnolipid against another type of aphid. In this study, we report for the first time dirhamnolipid as an insecticidal metabolite against *M. persicae*. Dirhamnolipid may have potential for use as an environmentally friendly agricultural pesticide.

Supporting Information Available: Supplemental 1, surface tension values of cell-free supernatants of *Pseudomonas* sp. EP-3 during growth on glucose−mineral medium (data are means ± SD of triplicates); Supplemental 2, aphid mortalities of cell-free supernatant of *Pseudomonas* sp. EP-3 grown on glucose−mineral medium under greenhouse conditions; Supplemental 3, GC-MS spectra of 3-hydroxydecanoic acid (A, B) and 3-hydroxydodecanoic acid (C, D) of the insecticidal metabolite in EI (A, C) and CI (B, D) modes. This material is available free of charge via the Internet at http://pubs.acs.org.

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