Entomological Research

Entomological Research **47** (2017) 337–343

SHORT COMMUNICATION

Identification of lipopeptide xantholysins from *Pseudomonas* sp. DJ15 and their insecticidal activity against *Myzus persicae*

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Received 27 April 2017;
accepted 29 April 2017.

doi: 10.1111/1748-5967.12241

Abstract

Myzus persicae is an important insect pest that reduces crop production worldwide. The use of pesticides for aphid control has generated much concern related to insect resistance and undesirable environmental effects. In an effort to discover new alternatives to counter *M. persicae*, we found that *Pseudomonas* isolate DJ15 produced insecticidal metabolites. To isolate the insecticidal metabolites, a cell-free supernatant of DJ15 was extracted and subjected to bioassay-guided chromatography. Based on the structures elucidated in instrumental analyses, the metabolites were identified as xantholysins A and B. The metabolites showed strong insecticidal activity against *M. persicae* with 50% mortality at levels of 13.4 and 24.6 μg/mL for xantholysins A and B, respectively. This is the first study to identify xantholysins as insecticidal metabolites against *M. persicae*.

Key words: aphid, biopesticide, biosurfactant, lipopeptide, xantholysin.

Introduction

Myzus persicae is a typical aphid that reduces crop production worldwide. Aphids damage crops directly through phloem feeding and indirectly through transmission of plant viruses (Sylvester 1989; James & Perry 2004). The damage to greenhouse crops, including fruits and vegetables, is often serious. Several conventional chemicals have been used for aphid control, but their extensive use causes concern related to insect resistance and undesirable environmental effects. Consequently, aphid control research seeking new alternatives to conventional chemicals has received much attention.

Aphid numbers can increase rapidly *via* asexual reproduction, and aphids can produce multiple generations in a short time. To maximize control efficiency while minimizing the risk of insect resistance, a structurally diverse group of chemicals is required to reduce aphid populations quickly. Biosurfactants should have diverse effects on aphids because they consist of a wide range of molecules, such as glycolipids, lipopeptides, polysaccharide–protein complexes, and lipids (Lang & Wagner 1987). Recently, biosurfactants were introduced as alternative agents for aphid control (Kim *et al.* 2010; Yang *et al.* 2017). Because biosurfactants are generally safer environmentally than other chemicals are, they are a potentially sustainable tool in pest management programs (Crombie 1999). Biosurfactants are amphiphilic compounds that contain hydrophobic head and hydrophilic tail moieties. Due to their amphiphilic nature, biosurfactants may cause aphid mortality by interacting with cell membranes.

In our search for new alternatives to conventional chemicals, we screened microorganisms for insecticidal activity against *M. persicae* and found that DJ15, an isolate of *Pseudomonas* species, produced metabolites with insecticidal activity. Using bioassay-guided fractioning and instrumental analyses, the metabolites were isolated and identified as xantholysins A and B. The xantholysins showed strong insecticidal activity against *M. persicae*, causing 50 % mortality (LC_{50}) at levels of 13. 4 and 24.6 μg/mL, respectively. Here, we report for the first time xantholysin metabolites that are insecticidal against *M. persicae*.

Materials and methods

Microbial isolation

Microorganisms were isolated by an enrichment culture method using a coast soil contaminated with crab shell waste. A 1.0 g sample of the soil was suspended in 100 mL of 0.05 % (*w*/*v*) swollen chitin containing 0.2 % (*w*/*v*) yeast extract (CY). The swollen chitin was prepared as described previously (Pranav & Deshpande 1989). The suspension was incubated at 30°C for 4 days on a shaking incubator at 150 rpm and plated onto CY agar plates. Following microbial growth on the plates at 30°C for 4 days, visible colonies were individually obtained and purified by plating repetitively on the agar plates. For further experiments, individual colonies were incubated in 100 mL of mineral salt medium (MSM) containing 2.0 % (*w*/*v*) glucose as a sole source of carbon and the following constituents (in grams per liter, pH 7.0): Na_2HPO_4, 2.0; KH_2PO_4, 1.0; $(NH_4)_2SO_4$ 0.4; $MgSO_4{\cdot}7H_2O$, 0.4; 2 mL of trace elements containing 0.1 g of $Al(OH)_3$, 50 mg of $SnCl_2{\cdot}2H_2O$, 50 mg of KI, 50 mg of LiCl, 80 mg of $MnSO_4{\cdot}4H_2O$, 50 mg of H_3BO_3, 0.1 g of $ZnSO_4{\cdot}7H_2O$, 0.1 g of $CoCl_2{\cdot}6H_2O$, 0.1 g of $NiSO_4{\cdot}6H_2O$, 0.1 g of $BaCl_2$, 50 mg of $(NH_4)_6Mo_7O{\cdot}4H_2O$, 80 mg of $FeSO_4$ per liter. Microbial identification was performed 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 (Kim *et al.* 2004).

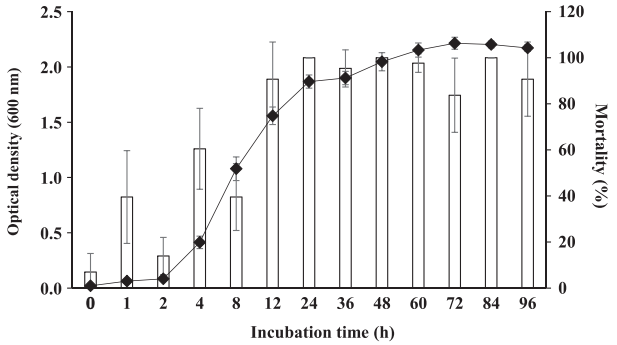

Figure 1 Aphid mortalities (bar graph) of the cell-free supernatant of *Pseudomonas* sp. DJ15 during its growth (solid line) on glucose-mineral medium. The data are means ± SD of triplicate.

Extraction and identification of insecticidal metabolites

Microorganisms with insecticidal activity were screened by mortality bioassays against *M. persicae*. Microbial cultures obtained as above were centrifuged at 12,000 x g for 20 min, and the resulting supernatant was used for bioassays as described earlier (Yang *et al.* 2017). An isolate that exhibited the strongest insecticidal activity among the isolates was tested for the isolation of insecticidal metabolites. The isolate was grown in the MSM as described above, and the cultural supernatant was extracted twice with the same volume of ethyl acetate. The organic extracts were evaporated to dryness in a rotary evaporator (EYELA N-1000, Tokyo Rikakikai Co., Tokyo, Japan) at 40°C and dissolved again in a solvent mixture of chloroform and methanol (55:45, *v*/v) for silica gel column chromatography. The extracts were loaded onto a glass column (10 mm i.d. × 50 cm in length) packed with silica gel (Kiesel gel 60, 230–400 mesh, Merck). The column was then eluted with the solvent mixture, in which every 7 mL-fraction was collected. Each fraction was evaporated to dryness by a gentle stream of nitrogen gas at 40°C and dissolved at a concentration of 1,000 μg/mL in

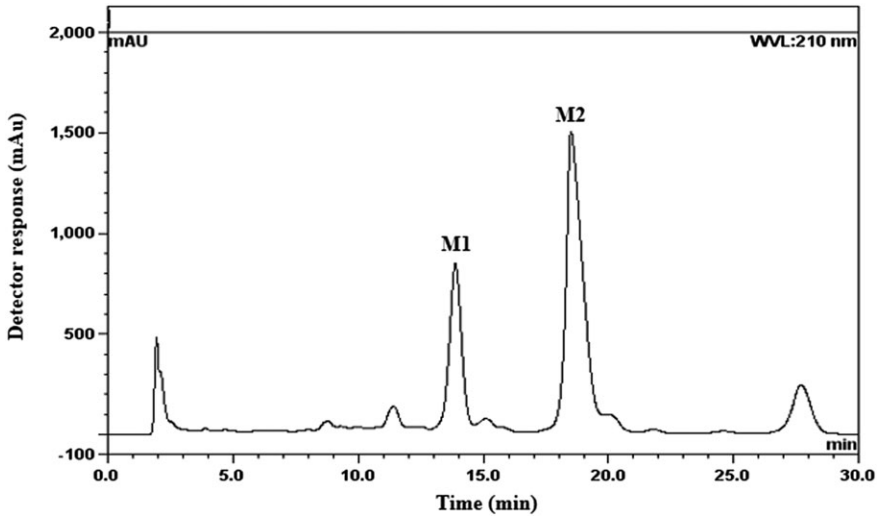

Figure 2 HPLC chromatograms of the insecticidal metabolites M1 and M2 isolated from extracts of the cell-free supernatant of *Pseudomonas* sp. DJ15.

10 % (*v*/v) aqueous solvent mixture of dimethyl sulfoxide and methanol (4:1, *v*/v). The fractions were then examined for aphid mortality assays as described above. The fractions with insecticidal activity more than 90 % at 1,000 μg/mL were combined and purified by solid phase extraction (SPE) cartridge chromatography (Superclean LC-18, Supelco). The cartridge columns were pre-washed with two times the bed volume of methanol and water before loading the fractions. The cartridge column was then eluted with two times the bed volume of aqueous methanol, in which the methanol concentration was increased at 15 % (*v*/v) in each elution step. Each fraction was evaporated as above, and a part of each fraction was subjected to mortality bioassays. The fractions with insecticidal activity were combined in methanol and purified by preparative high-performance liquid chromatography (prep-HPLC). Peaks were repetitively collected and subjected to insecticidal bioassays as described above. The active fraction was analyzed by mass spectrometer (MS) and nuclear magnetic resonance (NMR) analyses for chemical structure elucidation.

Mortality bioassays

Mortality bioassays against *M. persicae* were performed as previously (Kim *et al.* 2010). Briefly, aphids at 2nd instar were reared on the cabbage leaves (30 × 30 mm) that had been placed on the microbial plates with wet filter papers (No 6). The aphids were allowed to settle onto the leaves overnight prior to application and topically treated with 1.0 μL of the cultural supernatant or the metabolite samples obtained as above. The plates were placed in the growth chamber at 25 ± 2°C, 65 ± 5 % relative humidity and a photoperiod of 16:8 h L:D. The MSM or 10 % (*v*/v) aqueous solvent mixture of dimethyl sulfoxide and methanol (4:1, *v*/v) was used as negative control samples. Aphid mortality was investigated 24 h after application by Abbott's formula (Abbott 1925) as: mortality (%) = [(A-B) / A] × 100, where A and B represent the number of live aphids in the control samples and the number of live aphids in the treated samples, respectively. All experiments were repeated in triplicate, unless otherwise stated.

Table 1 Summary of proton chemical shifts of the metabolites M1 and M2

Metabolite	Sequence	HN	Hα	Hβ	Others
M1	LEU1	8.42	4.23	1.65, 1.61	Hγ: 1.61; Hδ: 0.85
	GLU2	8.69	4.20	1.94, 2.06	Hγ: 2.4
	GLN3	8.30	4.22	2.04	Hγ: 2.30; Hε: 7.47, 6.86
	VAL4	7.66	4.00	2.18	Hγ: 0.89
	LEU5	7.91	4.13	1.62	Hγ: 1.62; Hδ: 0.84
	GLN6	8.22	4.08	1.95, 2.13	Hγ: 2.21; Hε: 7.27, 6.72
	SER7	7.90	4.66	4.27	
	VAL8	8.27	4.14	2.36	Hγ: 0.88, 0.99
	LEU9	8.12	4.16	1.73, 1.45	Hγ: 1.45; Hδ: 0.83
	GLN10	7.91	4.23	2.00	Hγ: 2.30; Hε: 7.36, 6.73
	LEU11	7.99	4.18	1.54, 1.72	Hγ: 1.54; Hδ: 0.88, 0.80
	LEU12	7.87	4.15	1.61	Hδ: 0.84
	GLN13	7.35	4.12	1.87	Hγ: 2.15, 2.27; Hε:7.38, 6.85
	VAL14	7.63	4.24	2.04	Hγ: 0.79
M2	LEU1	8.44	4.22	1.65, 1.61	Hγ: 1.61; Hδ: 0.85
	GLU2	8.72	4.20	1.94, 2.06	Hγ: 2.40
	GLN3	8.31	4.21	2.04	Hγ: 2.28, 2.33; Hε: 7.48, 6.85
	VAL4	7.67	3.99	2.18	Hγ: 0.87, 0.91
	LEU5	7.92	4.12	1.63	Hγ: 1.63; Hδ: 0.84
	GLN6	8.21	4.07	1.95	Hγ: 2.21; Hε: 7.28, 6.72
	SER7	7.89	4.64	4.26	
	VAL8	8.27	4.15	2.38	Hγ: 0.88, 1.00
	LEU9	8.17	4.13	1.74	Hγ: 1.46; Hδ: 0.84, 0.77
	GLN10	7.91	4.23	1.99	Hγ: 2.31; Hε: 7.37, 6.74
	LEU11	7.94	4.20	1.54, 1.73,	Hγ: 1.54; Hδ: 0.88, 0.81
	LEU12	7.88	4.14	1.61	Hδ: 0.84
	GLN13	7.24	4.10	1.87	Hγ: 2.14, 2.26; Hε:7.37, 6.96
	ILE14	7.56	4.30	1.81	Hγ: 1.10, 1.29; Hδ: 0.78

Instruments

A Dionex model P680 preparative high-performance liquid chromatography (prep-HPLC) equipped with a Dionex model PDA-100 photodiode array detector at 210 nm was used for the isolation of insecticidal metabolites. The column was a μ-Bondapak C18 stainless steel column (7.8 mm i.d. × 30 cm in length). The mobile phase consisted of 84 % (*v*/v) aqueous methanol at a flow rate of 2.0 mL/min. High-performance liquid chromatography mass spectrometer (HPLC/MS) was an AB Sciex model API 2000 equipped with electron spray ionization (ESI) source mode in positive mode. The mobile phase consisted of 85 % aqueous acetonitrile containing trifluoroacetic acid at a flow rate of 1.0 mL/min. An UltraflexExtrem model time of flight mass spectrometer (TOF-MS, Bruker Daltonics) with the FlexControl software package (version 3.4, Bruker Daltonics) was used for accurate mass analysis. Spectra were recorded in the positive mode calibrated as previously (Sassi *et al.* 2015). A Varian VNMRS 600 mHz spectrometer was used for ^{1}H–NMR analysis. The samples used for 2D ^{1}H–NMR experiments were dissolved in 99.5 % DMF-d7. The spectra of two-dimensional (2D) DQF-COSY, TOCSY and NOESY were acquired at 298 K. NOESY spectra were recorded with mixing time of 200 ms.

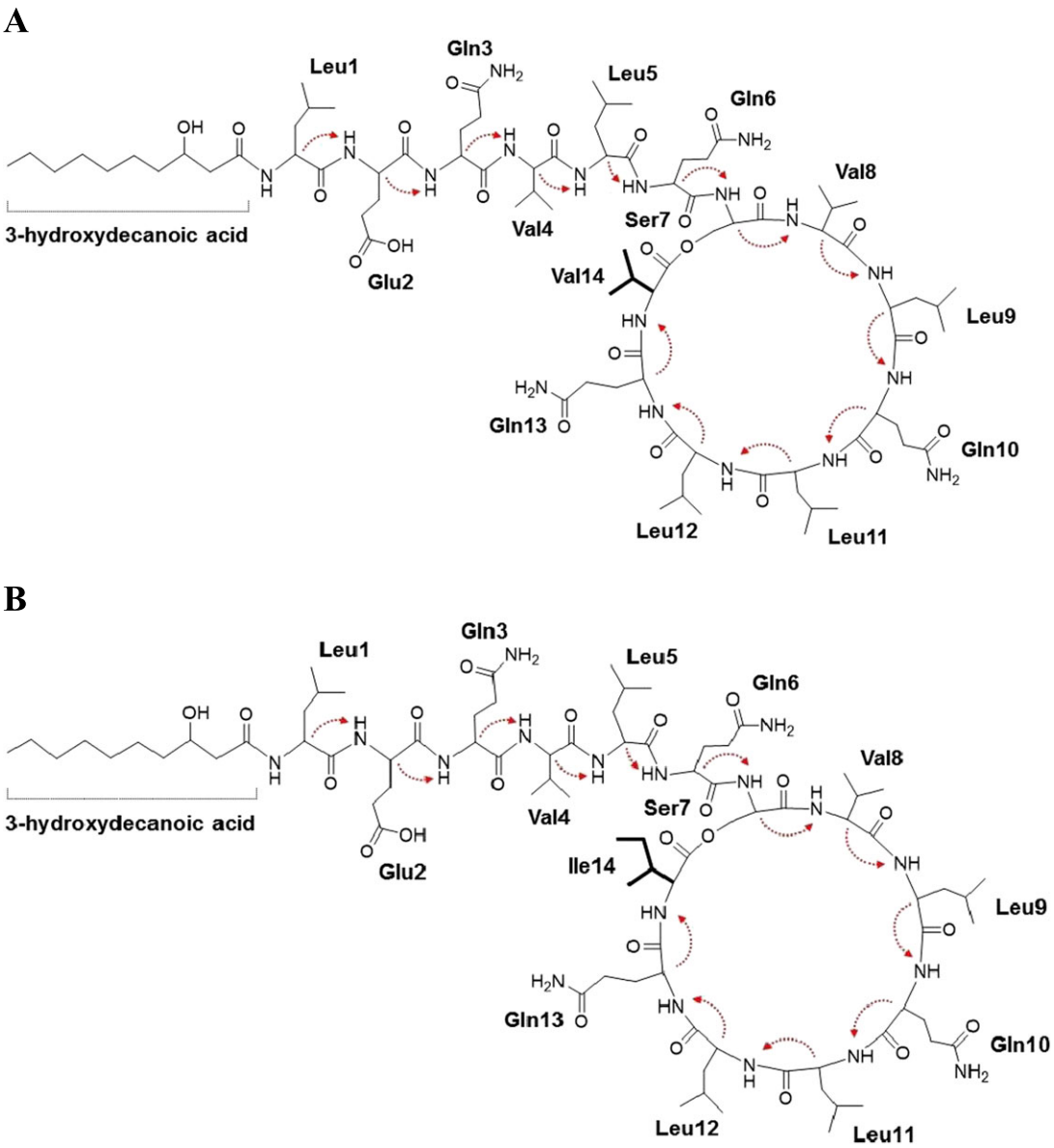

Figure 3 Chemical structures of the metabolites M1 (A) and M2 (B) with sequential NOEs observed from 2D ^{1}H-^{1}H NOESY spectra with 200 ms mixing time. Amino acids were labeled with residue name and number, and Hα-HN NOEs were indicated by the red dotted arrow.

NMR data were processed and analyzed using NMRPipe (Delaglio *et al.* 1995) and NMR View (Johnson & Blevins 1994) software.

Statistical analysis

Data were analyzed by Analysis of Variance (ANOVA) using IBM SPSS Statistics 19 software (Somers, NY, USA). The concentration-dependent mortality data were obtained by probit analysis (95 % confidence limits) using the SAS program.

Results and discussion

The microorganism that showed the strongest insecticidal activity among the isolates, designated *Pseudomonas* sp. DJ15, was used for further study. Isolate DJ15 has a 16S rRNA sequence with 96 % similarity to that of *Pseudomonas mosselii* CIP 105259^T (Supporting information, Figure S1), and its 16S rRNA sequence is now available in GenBank under accession number KY817593. The growth of DJ15 was accompanied by an increase in aphid mortality, with the highest mortality after a 48-h incubation (Fig. 1). These results demonstrated that DJ15 produced insecticidal metabolites during its growth.

HPLC analyses after a series of chromatography steps led to the isolation of two major metabolites from extracts of the cell-free supernatant of DJ15 (Fig. 2). The structure of the insecticidal metabolites was elucidated using MS and NMR analyses. HPLC/MS analyses detected ion peaks with charge $z = 2$ at m/z 881.6 and 888.2 for metabolites M1 and M2, respectively (Supporting information, Figure S2). TOF-MS analyses of M1 and M2 detected $(M + Na)^+$ peaks at m/z 1784.0956 and 1798.0403, respectively (Supporting information, Figure S3). Based on high-resolution mass spectroscopy data, metabolite M1 was determined to have the molecular formula $C_{83}H_{144}N_{18}O_{23}Na$, and metabolite M2 to have the molecular formula $C_{84}H_{146}N_{18}O_{23}Na$.

The ^{1}H NMR analyses of the metabolites confirmed the presence of peptides, with α-proton resonances between 4.4 and 3.9 ppm, side-chain protons at 2.5–0.8 ppm (Table 1). The chemical structures of the metabolites were elucidated by 2D NMR spectroscopy. The amino acid spin system was determined based on scalar coupling patterns using double-quantum filtered correlation spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY), and complementary nuclear Overhauser effect spectroscopy (NOESY) measurements. Sequence-specific assignments were obtained from the NOESY spectra. The proton 2D TOCSY spectrum of M1 and M2 in DMF-d7 allowed the identification of 14 residues (Supporting information, Figure S4). The presence of a 3-hydroxydecanoic acid (HAD) moiety was determined from the analysis of proton TOCSY spectra. Figure 3 shows the chemical structures of the metabolites determined from the NMR data, with sequential nuclear Overhauser effects. The MS and NMR data of the metabolites were identical to those of reported xantholysins (Li *et al.* 2013). Therefore, metabolites M1 and M2 were identified as xantholysins B and A, respectively.

Metabolites M1 and M2 caused dose-dependent mortality in aphids, with LC_{50} values of 24.6 and 13.4 μg/mL for M1 (xantholysin B) and M2 (xantholysin A), respectively (Fig. 4). Metabolite M1 contains valine at position 14 in its chemical structure, and metabolite M2 has isoleucine at position 14. Studies have demonstrated that the hydrophobicity of a chemical structure affects insect mortality (Magee 1983; Yamamoto & Powell 1993; Mullin *et al.* 2015). Consequently, the more hydrophobic isoleucine likely

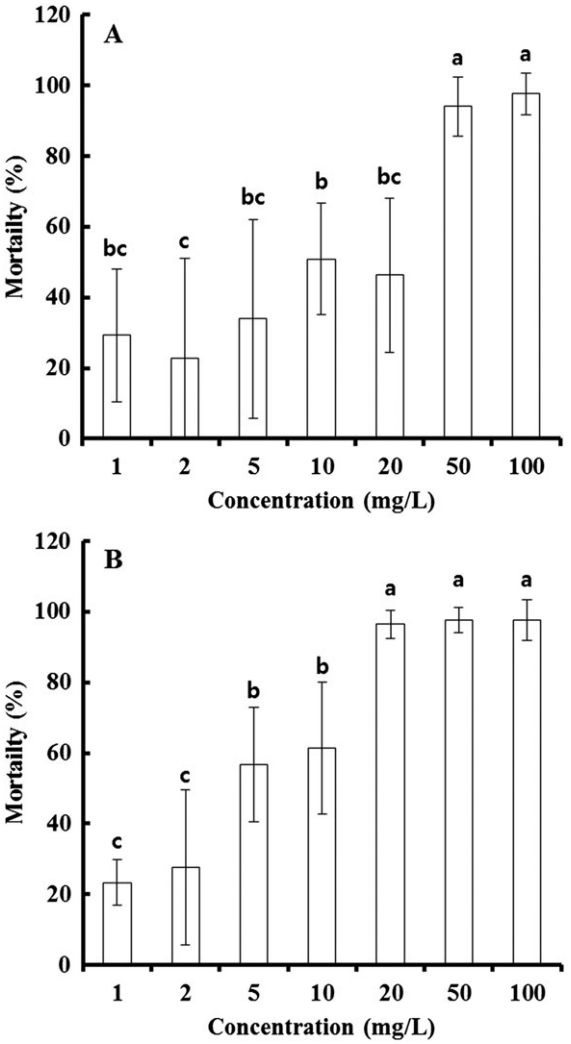

Figure 4 Dose-dependent aphid mortalities of the metabolites M1 (A) and M2 (B) isolated from extracts of the cell-free supernatant of *Pseudomonas* sp. DJ15. The data are means ± SD of triplicate.

contributes to the lower LC_{50} value of M2 compared with that of M1.

Xantholysins are antimicrobial cyclic lipopeptides produced by the rhizosphere bacterium *Pseudomonas putida* BW11M1 (Li *et al.* 2013). Xantholysins show broad activity against Gram-negative and Gram-positive bacteria (Molina *et al.* 2015), but few studies have examined their insecticidal activity. This study is the first to demonstrate insecticidal activity of xantholysins. Bioassay-guided isolation coupled with instrumental analyses successfully identified xantholysins in the cell-free supernatant of DJ15. Xantholysins consist of a hydrophilic peptide and a hydrophobic fatty acid, and are biosurfactants with surface tension activity. Studies have demonstrated that biosurfactants lead to cellular component leakage or dehydration by affecting biological membranes (Wang *et al.* 2005; Kim *et al.* 2010; Jang *et al.* 2013; Mnif & Ghribi 2015). Xantholysins with hydrophobic and hydrophilic moieties would interact with cell membrane molecules, disrupting the aphid cuticle membrane. Further work should examine this hypothesis to increase our understanding of the mode of action of xantholysins against *M. persicae*. Xantholysins and other lipopeptide biosurfactants are potential insecticidal agents in pest management programs.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2015R1D1A3A01019576).

References

Abbott WS (1925) A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology* **18**: 265–267.

Crombie L (1999) Natural product chemistry and its part in the defence against insects and fungi in agriculture. *Pesticide Science* **55**: 761–774.

Delaglio F, Grzesiek S, Vuister W, Zhu G, Pfeifer J, Bax A (1995) NMRPipe: A multidimensional spectral processing system based on UNIX pipes. *Journal of Biomolecular NMR* **6**: 277–293.

James CKNG, Perry KL (2004) Transmission of plant viruses by aphid vectors. *Molecular Plant Pathology* **5**: 505–511.

Jang JY, Yang SY, Kim YC *et al.* (2013) Identification of orfamide A as an insecticidal metabolite produced by *Pseudomonas protegens* F6. *Journal of Agricultural and Food Chemistry* **61**: 6786–6791.

Johnson B, Blevins RA (1994) NMR View: A computer program for the visualization and analysis of NMR data. *Journal of Biomolecular NMR* **5**: 603–614.

Kim IS, Ryu JY, Hur HG, Gu MB, Kim SD, Shim JH (2004) *Sphingomonas* sp. strain SB5 degrades carbofuran to a new metabolite by hydrolysis at the furanyl ring. *Journal of Agricultural and Food Chemistry* **52**: 2309–2314.

Kim SK, Kim YC, Lee S, Kim JC, Yun MY, Kim IS (2010) Insecticidal activity of rhamnolipid isolated from *Pseudomonas* sp. EP-3 against green peach aphid (*Myzus persicae*). *Journal of Agricultural and Food Chemistry* **59**: 934–938.

Lang S, Wagner F (1987) Structures and properties of biosurfactants. In: Kosaric N, Cairns WL, Gray NCC (eds) *Biosurfactants and Biotechnology*, pp 21–45. Marcel Dekker Inc., New York.

Li W, Rokni ZH, Vleeschouwer MD *et al.* (2013) The antimicrobial compound xantholysin defines a new group of *Pseudomonas* cyclic lipopeptides. *PloS One* **8**: 1–16.

Magee PS (1983) Chemicals affecting insects and mites. In: Topliss JG (ed.) *Quantitative structure-activity relationships of drugs*, pp 393–433. Academic Press, New York.

Mnif I, Ghribi D (2015) Lipopeptides biosurfactants: Mean classes and new insights for industrial, biomedical, and environmental applications. *Peptide Science* **104**: 129–147.

Molina SC, Udaondo Z, Daddaoua A *et al.* (2015) Efflux pump-deficient mutants as a platform to search for microbes that produce antibiotics. *Microbial Biotechnology* **8**: 715–725.

Mullin CA, Chen J, Fine JD, Frazier MT, Frazier JL (2015) The formulation makes the honey bee poison. *Pesticide Biochemistry and Physiology* **120**: 27–35.

Pranav V, Deshpande MV (1989) Chitinase production by *Myrothecium verrucaria* and its significance for fungal mycelia degradation. *Journal of General Applied Microbiology* **35**: 343–350.

Sassi M, Arena S, Scaloni A (2015) MALDI-TOF-MS platform for integrated proteomic and peptidomic profiling of milk samples allows rapid detection of food adulterations. *Journal of Agricultural and Food Chemistry* **63**: 6157–6171.

Sylvester ES (1989) Viruses transmitted by aphids. In: Minks AK, Harrewijn P (eds) *Aphids, Their Biology, Natural Enemies and Control (2C)*, pp 65–87. Elsevier, Amsterdam.

Wang X, Gong L, Liang S, Han X, Zhu C, Li Y (2005) Algicidal activity of rhamnolipid biosurfactants produced by *Pseudomonas aeruginosa*. *Harmful Algae* **5**: 433–443.

Yamamoto T, Powell GK (1993) *Bacillus thruringiensis* crystal proteins: recent advances in understanding its insecticidal activity. In: Kim L (ed.) *Advanced engineered pesticides*, pp 1–42. Marcel Dekker Inc., New York.

Yang SY, Lim DJ, Noh MY, Kim JC, Kim YC, Kim IS (2017) Characterization of biosurfactants as insecticidal metabolites produced by *Bacillus subtilis* Y9. *Entomological Research* **47**: 55–59.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1 Phylogenetic tree of *Pseudomonas* sp. DJ15 based on 16S rRNA sequence.

Figure S2 LC/MS spectra of the insecticidal metabolites M1 (A) and M2 (B) isolated from extracts of the cell-free supernatant of *Pseudomonas* sp. DJ15.

Figure S3 TOF-MS spectra of the insecticidal metabolites M1 (A) and M2 (B) isolated from extracts of the cell-free supernatant of *Pseudomonas* sp. DJ15.

Figure S4 ^{1}H-^{1}H TOCSY 2D NMR spectrum of the metabolites M1 (A) and M2 (B) in DMF-d7 at 298 K, revealing the characteristic amino acid spin system patterns.