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Characterization of an Antibiotic Produced by *Bacillus subtilis* JW-1 that Suppresses *Ralstonia solanacearum*

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Bacillus subtilis JW-1 was isolated from rhizosphere soil as a potential biocontrol agent of bacterial wilt caused by *Ralstonia solanacearum*. Seed treatment followed by a soil drench application with this strain resulted in >80% reduction in bacterial wilt disease compared with that in the untreated control under greenhouse conditions. The antibacterial compound produced by strain JW-1 was purified by bioactivity-guided fractionation. Based on mass spectroscopy and nuclear magnetic resonance spectral data (^{1}H, ^{13}C, ^{1}H-^{1}H correlation spectroscopies, rotating frame nuclear Overhauser effect spectroscopy, and heteronuclear multiple-bond correlation spectroscopy), the structure of this compound was elucidated as a cyclic lipopeptide composed of a heptapeptide (Gln-Leu-Leu-Val-Asp-Leu-Leu) bonded to a β-hydroxy-*iso*-hexadecanoic acid arranged in a lactone ring system.

Keywords: Bacterial wilt, *Ralstonia solanacearum*, biocontrol agent, antibacterial compound, cyclic lipopeptide, surfactin isoform

Introduction

Bacterial wilt, caused by *Ralstonia solanacearum* (*R. solanacearum*), is a serious soilborne disease infecting economically important crops such as tomato and pepper and causes severe yield and quality reductions in the southern part of South Korea. The bacterium enters plant roots *via* wounds or where secondary roots emerge. After infection, the bacterium colonizes vascular tissue in the stem, causing wilt and decay of infected tissues [3, 5]. Because *R. solanacearum* has a wide host range and can survive in soil and water for considerable periods of time in the absence of the main susceptible crop [18], it is difficult to control with current integrated strategies, including the use of resistant cultivars, soil fumigation, and crop rotation. Thus, alternative and more efficient disease management strategies are urgently needed.

Biological control through the use of natural antagonistic microorganisms has drawn attention as a promising strategy for effective disease management and for environmental protection from chemical pesticides [4, 17]. However, the development of effective biocontrol measures against *R. solanacearum* has not yet been achieved.

In our continued screening for a potential antagonistic strain against *R. solanacearum*, we isolated the JW-1 strain from rhizosphere soil, which showed strong antibiosis *in vitro* and induced a significant bacterial wilt disease suppression effect *in vivo*. In this study, we report on the isolation and the structural elucidation of an antibacterial compound, responsible for the inhibition of *R. solanacearum*, from JW-1 strain culture broth.

Materials and Methods

Isolation of Strain JW-1 and Culture Conditions

The JW-1 strain was isolated from rhizosphere soil of pepper plants collected from bacterial wilt suppressed fields in Kyunggido, South Korea and identified using biochemical, physiological, and 16S rDNA sequence analyses. The 16S rDNA gene was amplified using the universal primers fD1 and rP2. The polymerase chain reaction product was purified using the QIA Quick Gel Extract Kit (Qiagen, USA) and sequenced with an ABI PRISM 310 Genetic Analyzer (Perkin Elmer, USA).

The JW-1 strain was cultured at 28°C for 24 h in medium containing 1% soluble starch, 2% glucose, 0.1% meat extract, 0.4% yeast extract, 0.2% NaCl, 0.005% K_2HPO_4, and 2.5% soybean meal at pH 7.3. One milliliter of the seed culture was inoculated into the

same medium (100 ml) and incubated at 28°C for 5 days on a rotary shaker (200 rpm). Aliquots were taken at various time intervals to measure cell growth and antibiotic activity.

In Vitro Antibiosis Test

An isolate of *R. solanacearum* was obtained from the Korea National Academy of Agricultural Science and maintained at −80°C in tryptic soy broth (Difco Laboratories, USA) containing 20% glycerol. An antibiotic activity test of the strain against the pathogen was conducted using the agar diffusion method. A paper disc (8 mm) was impregnated with 50 µl of culture broth from the strain and placed on a pathogen-seeded tryptic soy agar plate. The plate was incubated for 48 h, and the diameter of the inhibition zone was measured.

Seed Bacterization and Rhizosphere Colonization

The seeds were coated with the bacteria by mixing 2.5 ml of bacterial suspension (10^9 CFU/ml) with 5 g of seeds. The coated seeds were dried overnight under a stream of sterile air at room temperature. Seed treatment with a strain resulted in the population densities of 10^7 to 10^8 CFU per seed, which was verified by the dilution plating method before planting. Seeds bacterized with the strain were allowed to germinate in the water agar plates. The root colonization ability is detected by the presence of a turbid zone in the agar plate around the roots, and the root length was measured 5 days after germination at 28°C and compared with those of the non bacterized seeds.

Greenhouse Pot Assays

The suppressive effects of the JW-1 strain on bacterial wilt of tomato and pepper plants were evaluated under greenhouse conditions by seed treatment and/or the soil drench application method. Bacteria were added to seeds (10^7 to 10^8 CFU per seed) according to the method of Kim and Kim [9]. Bacterized and nonbacterized seeds of pepper and tomato were planted in plastic pots containing soilless pro-mix that was not autoclaved. When the plants were 30 days old, the JW-1 strain was introduced at a density of 10^7 CFU/g potting soil. Two days after adding the JW-1 strain, the plants were challenged with 5 ml of *R. solanacearum* suspension (10^8 CFU/ml). Water drench was used on the untreated control. Two to 3 weeks after pathogen challenge, wilt disease was visually rated by assessing the percentage of wilted leaves. Biocontrol efficacy was calculated using the following formula: biocontrol efficacy = ([disease incidence of control − disease incidence of treatment group]/disease incidence of control) × 100. Analysis of variance was performed and differences between treatment means were determined by Tukey's test using SAS software (SAS Institute, USA).

Isolation of the Antibacterial Compound

Bioactivity-guided fractionation of the JW-1 strain culture broth led to isolation of the antibacterial compound (compound J). The culture broth (5 liters) was centrifuged at 8,000 rpm for 25 min, and the cell-free supernatant was extracted with ethyl acetate. The organic fraction was dried over Na_2SO_4 and concentrated to dryness. The oily residue (3.42 g) was dissolved in a small volume of methanol, applied to silica gel column chromatography, and eluted with $CHCl_3$-CH_3OH (80:20). The active fractions were combined and submitted to a Sephadex LH-20 column using methanol for elution. Evaporation of the solvent from the active fractions gave a crude active compound as a colorless powder (0.97 g). The final purification was performed by reverse-phase semipreparative high-performance liquid chromatography (HPLC) (C_{18} µBondapak column; Waters, USA) and eluted with a gradient of 10–65% CH_3CN in 0.1% trifluoroacetic acid at a flow rate of 4 ml/min. The elution was monitored by absorbance at 215 nm. The active compound was eluted as a single peak (retention time, 34.5 min), and the eluates yielded active compound J (86 mg) as a white powder.

Amino Acid Analysis

The purified compound J (1 mg) was hydrolyzed with 6 N HCl at 110°C for 24 h, and the reaction mixture was extracted with $CHCl_3$. The water layer of the hydrolysate was analyzed according to Nimura and Kinoshita [13]. Amino acid composition and chirality were determined by HPLC of *o*-phthalaldehyde-*N* acetyl-L-cysteine derivatives of amino acids using a Kromasil C_{18} column (EKa Chemicals, Sundsvall, Sweden) and by comparison with standard amino acid enantiomers. The elution was performed with a gradient solvent system of 50 mM sodium acetate-methanol (100:0 – 40:60) and detected at 254 nm. The chirality of a specific amino acid in the sequence was determined by comparing the chiral composition of the partial hydrolysis product with that of the intact peptide.

Analytical Methods

The ultraviolet (UV) spectrum was recorded on a Shimadzu model UV-160 spectrophotometer. High-resolution fast atom bombardment (FAB) mass spectra were obtained with a JEOL model JMS-AX505 HA spectrometer. ^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR spectra were recorded on a Brucker AV 500 spectrometer operating at 500 and 125 MHz, respectively. Two-dimensional correlation spectroscopy (COSY), rotating frame nuclear Overhauser effect spectroscopy (ROESY), and heteronuclear multiple-bond correlation spectroscopy (HMBC) spectra were recorded in DMSO-d_6 at 27°C.

Results and Discussion

Characteristics of the Strain JW-1

The JW-1 strain was selected from 250 microbial candidates based on performance as a biocontrol agent of *R. solanacearum*. The JW-1 strain exhibited a strong *in vitro* antibiosis against *R. solanacearum* (inhibition zone diameter >26 mm). The JW-1 strain also produced a turbid zone around the root in

Table 1. Characteristics of strain JW-1.

Morphological characteristics	
Gram staining	Positive
Spore formation	Formed
Motility	Present
Biological characteristics	
Range of growth temperature	5–55°C
Optimum growth temperature	30–38°C
Range of growth pH	5.0–8.0
Indole production	Positive
Catalase	Positive
Voges-Proskauer response	Positive
Starch catabolic ability	Positive
Gelatin catabolic ability	Positive

the agar plate and resulted in more than 10% enhancement of root growth, indicating effective colonizing on the rhizosphere. The strain JW-1 was identified as *Bacillus subtilis* JW-1 based on morphological, biochemical, and 16S rDNA sequence analyses (data not shown). The strain was gram-positive and motile. The oxygen requirement, spore formation, and catalase reaction were also examined (Table 1). The JW-1 strain could grow at temperatures ranging from 5°C to 55°C, but had the optimal temperature of 30–38°C for antagonistic activity to the pathogen.

Fermentation was carried out at 30°C for 5 days and the culture broth was taken at various time intervals for measurement of cell growth and antibiotic activity. Production of the antibacterial compound by the JW-1 strain was monitored using the paper disc method. Dry cell weight measurements were made by membrane filtration (0.45 µm in pore size, 47 mm in diameter). Five milliliter of culture broth was filtered through the membrane under vacuum. The membrane was then dried in an oven and weighed. The antibiotic was produced by strain JW-1 near the end of the bacterium's early exponential growth phase (Fig. 1). At the death phase (5 days), the production of the antibacterial compound was at its highest with no significant change in the levels before/after that time.

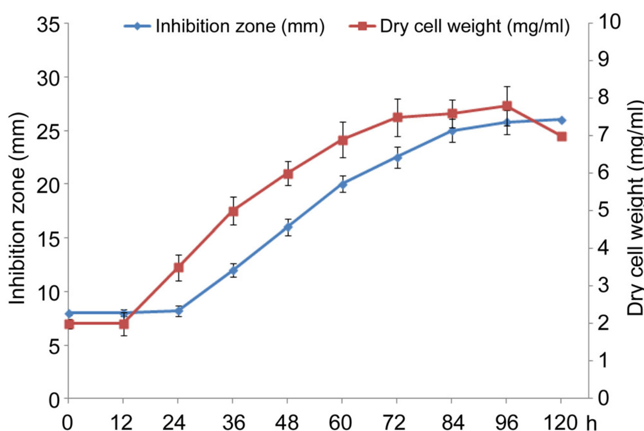

Fig. 1. Typical time course of the growth of and antibiotic production by JW-1 strain.

Suppression of Bacterial Wilt by the JW-1 Strain

The biocontrol efficacy of the JW-1 strain on the suppression of bacterial wilt was assessed through the greenhouse pot assay. Treatment with the JW-1 strain induced significant suppression of bacterial wilt, compared with that in the untreated control (Table 2). In the control treatment, initial symptoms of wilting during the daytime and recovering at night occurred 3 days after the challenge, and the wilt developed throughout the whole plant thereafter. The wilted leaves maintain their green color and do not fall off as disease progresses. Three modes of strain application were tested: seed treatment, soil drench, and seed treatment and soil drench. The soil drench method resulted in improved suppression compared with that in the seed treatment. However, seed treatment followed by a soil drench application appeared to be more effective than that of a single treatment, resulting in disease suppression of up to 80%. This indicated that the mechanism of disease suppression by the JW-1 strain was antibiotic secretion. To ascertain that the suppressive effect of JW-1 was related to antibiotic secretion, three types of inoculation (culture broth, washed cell suspension, and cell-free culture filtrate) were introduced into soil and the suppressive effects were compared (Table 3). The highest disease suppression was achieved in the culture broth treatment. The centrifuged

Table 2. Suppression of bacterial wilt in tomato and pepper plants by treatment with the JW-1 strain.

Treatment	Biocontrol efficiency (%)[a]	
	Pepper	Tomato
Seed treatment[b]	31.7 ± 2.4	23.8 ± 3.2
Soil application[c]	75.3 ± 5.2	70.1 ± 3.8
Seed+ soil treatment	80.4 ± 5.8	78.5 ± 4.9

[a]Data represent means of 10 replications ± standard deviation of each treatment. Treatments were arranged in a randomized complete block design. The experiment was conducted in triplicates. Biocontrol efficacy was based on comparisons to nonbacterized but pathogen-challenged controls.

[b]Pepper and tomato seeds were coated with the bacterial cell suspension and planted.

[c]Plants were grown in potting soil and were drenched with the bacterial cell suspension.

Table 3. Suppression of bacterial wilt of tomato and pepper plants by treatment of the JW-1 strain.

Treatment	Biocontrol efficacy (%)[a]	
	Pepper	Tomato
Culture broth	79.7 ± 3.7	79.3 ± 4.1
Centrifuged culture filtrate	78.4 ± 2.1	79.1 ± 4.3
Washed cell suspension	29.3 ± 1.9	25.8 ± 3.1
Washed cell + compound J	77.1 ± 3.8	76.8 ± 2.1

[a]Data represent means of 10 replications ± standard deviation of each treatment. The experiment was repeated three times. Disease severity was rated 20 days after inoculation with *R. solanacearum*. Biocontrol efficacy is based on comparisons with the nonbacterized but pathogen-challenged controls.

culture filtrate showed the same level of suppression as nonfractionated culture broth, whereas disease suppression by inoculating the washed cell suspension was reduced to 25%. Furthermore, when the washed cell suspension was co-inoculated with compound J (25 μg/ml), the same level of disease suppression as culture broth treatment was obtained. The results herein suggested that the major mechanism of disease suppression by the JW-1 strain appeared to be secretion of an antibacterial compound in culture broth, even though other mechanisms may have acted in concert to achieve control.

Isolation and Structural Elucidation of the Active Compound

The purified active compound J showed a single band (rf value, 0.83) on thin layer chromatography (silica gel 60 F_{254}) using butanol-acetone-water (32:48:8, by vol.) under UV light. Compound J was negative to ninhydrin but positive to 4,4′-*bis*(dimethylamino) diphenylmethane reagent and bromocresol green, indicating that it contained a cyclic peptide moiety and a free carboxyl group. A white spot formed with the same rf value when the plate was sprayed with water; thus, indicating that the compound was lipophilic. The molecular weight of 1,048 for compound J was deduced from the FAB mass spectrum, which displayed a $[M+H]^+$ peak at *m/z* 1,049 and a $[M+Na]^+$ peak at *m/z* 1,071, respectively. An amino acid analysis of the water layer of the hydrolysate showed the presence of 2 moles each of L-Leu and D-Leu, and 1 mole each of L-Glx, L-Val, and L-Asp. These data suggested that compound J was a cyclic lipopeptide composed of seven amino acid residues and a lipophilic moiety.

The nine amide protons (δ_H 6.7–9.8) and seven $C_\alpha H$ protons (δ_H 4.09–4.74) were clearly identified in the ^{1}H-NMR spectrum of compound J. The ^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY, ROESY, and HMBC experiments revealed that the nine NH signals among seven doublet signals represented seven NH peptide backbone and two singlet NH signals that were identified as a glutamine (Gln) ε-NH and ε-NH′, confirming that the Glx residue in the hydrolysate was Gln. Ten carbonyl carbons in the ^{13}C-NMR spectrum were easily attributed to their intra residue connectivities to amino acids and one acyl residue. Thus, a heptapeptide structure including Gln was confirmed. The sequence of seven amino acids was determined by the connections between the α proton of residue *i* with the amide proton of residue *i*+1 from the ROESY experiment. ROESY correlations were observed between the α proton of Gln (1) (δ 4.42) and NH of Leu (2) (δ 7.99), between the αH of Leu (2) (δ 4.19) and NH of Leu (3) (δ 7.72), between the αH of Leu (3) (δ 4.09) and NH of Val (δ 8.24), between αH of Val (δ 4.09) and NH of Asp (δ 8.15), between αH of Asp (δ 4.74) and NH of Leu (6) (δ 7.53), and between αH of Leu (6) (δ 4.51) and NH of Leu (7) (δ 8.52). Therefore, the amino acid sequence was determined to be Gln-Leu-Leu-Val-Asp-Leu-Leu.

The $CHCl_3$ layer of the hydrolysate containing a lipophilic compound showed a negative ninhydrin reaction and gave a $[M+H]^+$ ion peak at *m/z* 255 on FAB-MS. As $C_{38}H_{66}N_8O_{10}$ was assigned to the seven amino acid residues, $C_{16}H_{30}O_2$ was allotted to the fatty acid moiety by considering the molecular formula $C_{54}H_{96}N_8O_{12}$. After the proton and carbon signals of the seven amino acid residues were assigned, one carbonyl carbon (δ_C 169.9), one oxygenated methine (δ_H 4.88), one methine, 11 methylenes, and two methyl groups remained. The β-hydroxy fatty acid was confirmed based on an oxygenated methine signal at δ_C 71.1 (corresponding to δ_H 4.88), whereas an oxygenated methine signal for α-hydroxy acid was close to δ_C 80 [7]. The ^{13}C-NMR chemical shifts and the corresponding distortionless enhancement by polarization transfer (DEPT) spectra confirmed the presence of an *iso* alkyl chain by the branching CH signal at δc 28.4 ppm. Although the *iso* CH_3 peak could not be unambiguously assigned, the *iso* branching CH signal at δ_C 28.4 was easily identified as an *iso* chain type [10]. Based on the above FAB-MS and NMR data, the fatty acid component was elucidated as β-hydroxy-*iso*-hexadecanoic acid. The molecular ion peak of *m/z* 255 $(M+H)^+$ of the fatty acid moiety in the hydrolysate, which was smaller than that of β-hydroxy-*iso*-hexadecanoic acid by 18 mass units, can be explained by dehydration during acid hydrolysis. Furthermore, the HMBC correlation between the carbonyl carbon (δ_C 171.8) of Leu (7) and the β-methine proton (δ_H 4.88) of the lipophilic moiety indicated the presence of an ester linkage between the hydroxyl group of the fatty acid moiety and the carbonyl group of

Table 4. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectral assignments of the active compound J in DMSO-d_6.

Residue	Position	δ_C	δ_H (*J* in Hz)
L-Gln (1)	C=O	170.9	
	NH		8.04(d, 8.0)
	αCH	52.5	4.42 (m)
	βCH_2	27.0	1.84 (m)/ 1.95 (m)
	γCH_2	29.8	1.82 (2H, m)
	δCO	173.8	
	εNH_2		7.20 (s)/6.70(s)
L-Leu (2)	C=O	172.6	
	NH		7.99 (d, 5.7)
	αCH	51.7	4.19 (m)
	βCH_2	39.5	1.47 (2H, m)
	γCH	24.2	1.51 (m)
	δCH_3/ CH_3	23.0/22.9	0.85 / 0.82 (m)
D-Leu (3)	C=O	172.9	
	NH		7.72 (d, 5.7)
	αCH	51.8	4.09
	βCH_2	39.0	1.43/1.31 (m)
	γCH	24.2	1.3 (m)
	$\delta CH_3/CH_3$	22.8/22.6	0.71 /0.70 (m)
L-Val (4)	C=O	170.5	
	NH		8.24 (d, 8.3)
	αCH	58.6	4.09 (m)
	βCH	30.0	2.19 (m)
	$\gamma CH_3/CH_3$	17.8/19.0	0.81/0.85 (m)
L-Asp (5)	C=O	171.6	
	NH		8.15 (d, 7.4)
	αCH	49.6	4.74 (m)
	βCH_2	35.7	2.96/2.81
	γCO	170.0	
D-Leu (6)	C=O	171.8	
	NH		7.53 (d, 7.8)
	αCH	50.9	4.51 (m)
	βCH_2	41.5	1.43/1.25 (m)
	γCH_2	24.0	1.43
	$\delta CH_3/CH_3$	21.6/21.6	0.84/0.84
L-Leu (7)	C=O	171.8	
	NH		8.52 (d, 8.0)
	αCH	50.7	4.21 (m)
	βCH_2	38.9	1.53 (m)
	γCH_2	24.0	1.37 (m)
	$\delta CH_3/CH_3$	21.2/21.1	0.85/0.85 (m)

Table 4. Continued.

Residue	Position	δ_C	δ_H (*J* in Hz)
Alkyl chain	1CO	169.9	
	$2CH_2$	40.9	2.83/2.29 (m)
	3CH	71.1	4.88
	$4CH_2$	32.9	1.62/1.54
	$5CH_2$	23.8	1.32
	6-10 CH_2	27,9-29.3	1.21
	$11CH_2$	35.6	1.05/1.25 (m)
	12 CH_2	33.2	1.28 (m)
	13 CH_2	26.3	1.21
	14CH	28.4	1.20
	15-16 CH_3	22.7	0.79

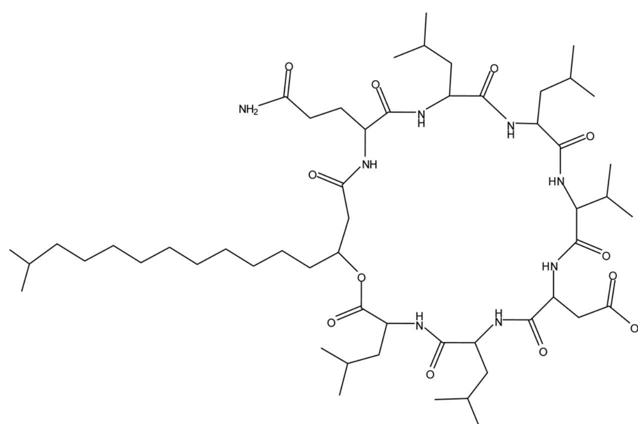

Fig. 2. Chemical structure of compound J.

Leu (7). A ^1H-^{13}C correlation was observed between NH of Gln (1) and CO of the fatty acid residue in the HMBC spectrum, and the NH of Gln (1) was correlated with α-CH_2 and β-methine protons in the fatty acid residue in the ROESY spectrum. Full assignments of all protons and carbons in compound J by DEPT, ^1H-^1H COSY, ^1H-^{13}C COSY, ROESY, and HMBC analyses are described in Table 4. Based on these results, the structure of the active compound J was elucidated as shown in Fig. 2

Compound J belongs to the surfactin family, which is a heptapeptide interlinked with a β-hydroxy fatty acid to form a cyclic lactone ring structure. The differences in the amino acid composition and lipid portion from standard surfactin were as follows: compound J was composed of Gln instead of Glu as the N-terminal amino acid and contained an *iso*-type β-hydroxy hexadecanoic acid moiety, instead of *n*- or *anteiso*-type mixtures of C_{13}–C_{16} fatty acids

[2]. *Bacillus subtilis* produces surfactin isoforms depending on the specific strain and fermenting conditions. The isoforms differ slightly in chain length and branching of the hydroxy fatty acid component as well as amino acid substitutions in the peptide ring [2, 15]. Surfactin isoforms are powerful biosurfactants owing to their amphiphilic nature, and display hemolytic, antiviral, antimycoplasma, and antibacterial activities [8, 11, 14].

We have shown that the antagonistic JW-1 strain produced compound J, a novel surfactin isoform, under our fermentation conditions, and that secretion of compound J was a principle mechanism for the suppressive effect against *R. solanacearum*.

Lipopeptides such as surfactin act as spreaders by facilitating the formation of a stable biofilm; thus, favoring root colonization by plant-associating bacilli. Deleting surfactin expression in the *Bacillus* strain led to the inability to form biofilms and reduced the biological control of plant disease [1, 12]. Surfactin has recently been identified as a bacterial determinant for eliciting induced systemic resistance in the host plant, leading to suppression of plant diseases [6, 16]. In this context, compound J may have additional roles in biological control besides antibiosis, such as induction of a resistance response and contributions to plant root colonization.

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