JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 4287–4292 4287
DOI:10.1021/jf900164h

# Isolation and Characterization of Leu$^7$-Surfactin from the Endophytic Bacterium *Bacillus mojavensis* RRC 101, a Biocontrol Agent for *Fusarium verticillioides*

Maurice E. Snook, Travor Mitchell, Dorothy M. Hinton, and Charles W. Bacon*

Toxicology and Mycotoxin Research Unit, Russell Research Center, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 5677, Athens, Georgia 30604

*Bacillus mojavensis* is an endophytic bacterium patented for control of fungal diseases in maize and other plants. Culture extracts and filtrates from this bacterium were antagonistic to the pathogenic and mycotoxic fungus *Fusarium verticillioides*. However, the identity of the inhibitory substance from extracts of this bacterium has not been determined. An HPLC-MS analysis of the culture filtrate showed a major ion peak that was identified as a cyclic lipopeptide. Furthermore, collisional ion dissociation (CID) analysis indicated that this lipopeptide was surfactin, a cyclic heptapeptide linked to a $\beta$-hydroxy fatty acid. A CID analysis of the peptide moiety was established by deduction and indicated that the peptide sequence consisted of two acidic amino acids and five hydrophobic amino acids with a sequence of Leu-Leu-Asp-Val-Leu-Leu-Glu. These spectra indicated that this bacterium produced Leu$^7$-surfactin, which was toxic to *F. verticillioides*. Production of this cyclic lipopeptide is a characteristic of several species of *Bacillus*, but this is the first report of this very powerful biosurfactant from this endophytic species.

**KEYWORDS: *Bacillus mojavensis*; bacterial endophyte; biocontrol; biosurfactants; *Fusarium verticillioides*; fumonisins; lipopeptides; surfactin**

## INTRODUCTION

*Fusarium verticillioides* (Saccardo) Nirenberg (=*F. moniliforme* Sheldon), Holomorph: *Gibberella moniliformis* Wineland, is an important plant pathogen. This fungus also produces the fumonisin mycotoxins, especially fumonisin $B_1$, which is toxic to livestock and poultry. Furthermore, *F. verticillioides*-contaminated maize is associated with human esophageal cancer and has been recently implicated in human neural tube defects (*1*, *2*). This class of mycotoxin is produced in maize and maize products, worldwide, where biotic and abiotic factors influence the amount of toxin accumulated. Whereas *F. verticillioides* is reported as a pathogen of maize, it is better described as a biotrophic parasite because in most instances it is associated with maize as a symptomless endophyte (*3*). This endophytic state makes it particularly difficult to control.

Most strains of *Bacillus mojavensis* Roberts, Nakamura, and Cohan (*4*) are endophytic, intercellular, and nonpathogenic spore formers (*5*). Strain RRC 101 (ATCC 55732) was further characterized by its ability to enhance plant growth and to colonize plants endophytically following the topical application to seed, and this strain was subsequently patented as a control of maize diseases (*5*). The endophytic infection remains during the entire cereal growing season following the one-time application to seed. Maize seedlings infected with this bacterium have reduced fumonisins concentrations (*5*−*7*). We examined all available strains of *B. mojavensis* and have found that all of these strains are endophytic and antagonistic to *F. verticillioides* and other fungi on culture media (*7*), indicating a natural grouping of bacterial endophytes that offer immense biocontrol potential. These studies suggest that this bacterium has the potential as a useful antagonist to assist in the control of the endophytic infection and resulting diseases produced by *F. verticillioides*. This bacterium can inhibit growth and fumonisin production by the fungus (*6*), but the substance responsible for this inhibition is unknown. Preliminary results (*8*) from culture extracts prepared from *B. mojavensis* suggested that this substance was similar to the lipopeptide biosurfactants and other related antimicrobial substances that have been reported from strains of *Bacillus subtilis* (*9*); the species complex *B. mojavensis* was removed (*4*). The objectives of this research are to isolate and characterize the nature of the inhibitor produced by this species of bacterium and to test the identified substance for its effectiveness against the target species *F. verticillioides*.

## MATERIALS AND METHODS

**Microorganisms.** *B. mojavensis* RRC101, the patented endophytic strain (ATCC 55732) (*5*) shown to confer disease protection and to reduce the level of fumonisin mycotoxin in maize seedlings (*6*), was used in this study to evaluate lipopeptide production. Stock cultures were stored on silica gel at −30 °C. The working stock culture was maintained on nutrient agar (Difco Inc., Detroit, MI), and stored at 4 °C until used. The three strains of *F. verticillioides* used in this work included RRC 826 and

*Corresponding author [telephone (706) 546-3142; fax (706) 546-3116; e-mail charles.bacon@ars.usda.gov].



Published on Web 4/17/2009

RRC 408, both of which produced symptomless infections on maize, and RRC Patgus, which is highly pathogenic to maize and wheat seedlings. All fungi were stored on potato dextrose agar plates (PDA, Difco Inc.) at 4 °C. Macroconidia were produced from these fungi by culturing on a carboxyl methyl cellulose (CMC) medium containing CMC, 15.0 g; $NH_4NO_3$, 1.0 g; $KH_2PO_4$, 1.0 g; $MgSO_4$, 0.5 g; yeast extract, 1.0 g; and distilled water, 1000 mL, for 5 days in shake culture.

**Surfactin Isolation from Liquid Cultures.** Bacterial cells were removed from cultures by centrifugation (2000*g*). The supernatant was filter-sterilized with a sterile 0.2 $\mu$m filtering unit and assayed for biological activity against *F. verticillioides*, RRC PAT Gus, 408, and 826. Surfactin in this cell-free supernatant was concentrated by preparative reverse-phase C-18 chromatography. Approximately 100 g of Waters BondaPak C18 bulk packing materials (Millipore Corp., Milford, MA) was packed into a glass chromatography column (54 × 2.54 cm, Chrome column, Andrews Glass Co., Vineland, NJ; 15 psi nitrogen pressure used to aid flow), washed with MeOH and recycled to distilled water. The supernatant was eluted with solutions of methanol (0, 25, 50, 75, and 100%), and 125 mL fractions were collected for each percent methanol. The methanol was removed under vacuum; the residue was taken up in sterile nutrient broth and assayed for inhibitory activity against the three strains of *F. verticillioides*. Lipopeptides were precipitated from cell-free supernatants of 48 h bacterial nutrient broth cultures by adjusting the pH to 2.0 with concentrated HCl. The acid precipitate was collected by centrifugation (2000*g*); the pellets were dried, placed in methanol or methylene chloride overnight, and centrifuged (2000*g*). The solvents were removed, and portions of the crude extract were placed in nutrient broth solutions and assayed for inhibition to *F. verticillioides* as described above.

**Large-Scale Preparation of Surfactin.** Because conventional liquid media known for surfactin production (*9*, *16*−*18*, *22*, *24*) did not allow production by *B. mojavensis*, the large-scale production of surfactin was accomplished as solid state cultures in aluminum pans (28 cm × 18 cm × 3.5 cm), each containing 300 mL of sterilized nutrient agar (Difco Inc.). Nutrient agar was prepared by adding 0.5% agar to Difco Nutrient Broth. This high-water-content, modified nutrient agar preparation allowed for the production of the inhibitory substance and for the complete dissolution and removal of the agar upon freezing after the bacterium had been cultured. The bacterium was inoculated over the surface of the medium, which was then incubated at 25 °C in the dark for 5 days. The trays were frozen overnight at −20 °C, thawed to room temperature, and centrifuged at 2000*g*; the supernatant was filter-sterilized through a 0.2 $\mu$m filtering unit, and the filtrate was stored at 4 °C until analyzed as described above for surfactin isolation.

**Crude Biosurfactant Analyses.** Crude biosurfactant analyses in cell-free RRC 101 cultural supernatants and RRC 101 acid-precipitated and methanol-extracted samples were performed on a HP1090 diode array high performance liquid chromatograph (Agilent Technologies, Santa Clara, CA). A Beckman Ultrasphere ODS C-18 column, 250 × 4.6 mm i.d., was used to separate crude biosurfactants. Bound surfactants were eluted with a gradient program that consisted of a mobile phase of 50% methanol/water to 100% methanol performed during a linear gradient over 35 min by volume with 0.1% $H_3PO_4$ in the solvent. The flow rate was 1 mL/min, and the injection volume was 20 $\mu$L. Crude biosurfactants were monitored at 210 nm, and individual peaks were collected manually. Peaks with the highest biosurfactant activity were pooled, the solvent was removed, and then bioassays for toxicity to *F. verticillioides* were performed.

**Surfactin Chemical Analysis.** Standards of surfactin (from *B. subtilis*, approximate purity of 98%) and iturin A were obtained from Sigma-Aldrich (St. Louis, MO). The UV-MS data of these standards were consistent with those previously reported (*10*). A Finnegan LCQ Duo with electrospray ion source was used to obtain the mass spectra for the analyses. The system was equipped with a SpectraSystem P2000 HPLC pump, a SpectraSystem AS3000 autoinjector, and a SpectraSystem UV6000LP UV−vis detector for tandem UV−MS analyses. With the instrument in positive ion mode, the mass spectrometer parameters were tuned on the 1036.7 (M + H) ion obtained by direct injection of the surfactin standard. The instrument was similarly tuned in negative ion mode using the 1034.6 (M − H) ion, again obtained by direct injection of the standard.

A Beckman Coulter Ultrasphere ODS column was used to provide separations. The column was 250 × 4.6 mm i.d. and contained a 5 $\mu$m packing. The solvent system used was methanol/water, with 1% formic acid added. A linear gradient was used, beginning at 50% methanol and increasing to 100% over 45 min. The solvent was then held at 100% methanol for another 45 min. For HPLC analyses, Xcalibur, the system control software, was run in data-dependent mode, isolating and fragmenting the most intense ion (by collisional ion dissociation, CID, 35%) found in each scan.

**Microbial Inhibition and Antagonism Assays.** Antagonism was assessed by two methods. The first method consisted of a radial agar diffusion assay, which was used to measure the in vitro toxin production and inhibition to the strains of *F. verticillioides* (*7*). The target fungi were applied as a 0.5 cm plug from a specific strain taken from a 14-day-old culture grown on PDA, and the antagonism was measured on a nutrient agar plate after incubation as described above (*7*). These experiments were repeated at least twice for each fungal strain, each replicate was a single Petri dish, and each strain was replicated five times per experiment.

The second method of measuring antagonism consisted of measuring growth rates with and without known concentrations of surfactins. These assays were performed with an automated turbidometer, the Microbiology Bioscreen C Reader (Labsystems, Helsinki, Finland), which was performed in 100-well sterile microplates. Each well contained 190 $\mu$L of nutrient broth, to which was added 10 $\mu$L of bacterial or fungal inoculum or both ($10^3$ CFU/mL, each) in nutrient broth, and 100 $\mu$L of culture extract or solvent control. The concentrations of surfactin and iturin A used to measure the in vitro toxicity ranged from 10 to 200 $\mu$g/mL. The cultures were incubated at 30 °C with constant shaking, and the $OD_{600}$ was measured at 30 min intervals over the incubation period of 48 h. Data were analyzed with the Bioscreen C Reader software package (Research Express, version 1.00). Percentage inhibition was determined by comparing the $OD_{600}$ obtained in the treatment groups with the control groups. All experiments were performed at least in duplicate and were repeated independently.

**Statistical Analysis.** Data are reported as averages. Statistical analysis was carried out with the SigmaStat statistical software package version 2, and significant treatment differences were separated by a *t* test.

## RESULTS

*B. mojavensis* produced an antagonistic zone (*7*) that inhibited the mycelial growth of all three strains of *F. verticillioides*. The zones of inhibition were wider when assays were conducted on nutrient agar, suggesting a greater concentration of inhibitor production (data not shown). Therefore, this medium was selected for the analysis of surfactin production. Fungi grown in the absence of the bacterium produced hyphae and conidia that had uniform cell wall thickness without bulging of the outer walls and had organized cytoplasmic structures. The bacterial inhibitor caused enlargement and bulging of the hyphae and degradation of the cellular matrix and cell membranes of hyphae and conidia of the fungi whenever there was contact with the antifungal compounds or direct contact with bacterial cultures on agar medium (data not shown).

The bacteria-free supernatant was applied to a C18 column and eluted with methanol/water (0−100%). The fractions eluted in the 100% methanol fraction appeared to contain most of the antifungal inhibitory activity during a 16 h observation period (**Table 1**). Cell-free supernatants from 5-day-old liquid cultures and the surfactant standards in nutrient broth had inhibitory effects on the growth of *F. verticillioides*, which is typified by RRC 408 during bioassays. However, turbidometer measurements of the growth of *F. verticillioides* indicated that iturin A was not inhibitory (**Table 2**). Identical spectra of activity were observed from the two symptomless strains and the pathogenic strain when tested against the crude inhibitor extract (**Table 1**) and the commercial standard (**Table 2**). Only surfactin and culture filtrates from cells grown on nutrient agar were effective in

**Table 1.** Growth Inhibition Produced by *Fusarium verticillioides* RRC 408 from Methanol Fractions from a $C_{18}$ Column of 5-Day-Old Cultures of *Bacillus mojavensis* Grown on Nutrient Agar

| fraction | $OD_{600}$[a] |
|---|---|
| nonfractionated culture broth[b] | 0.772 a |
| methanol 0% | 0.624 a |
| methanol 25% | 0.636 a |
| methanol 50% | 0.686 a |
| methanol 75% | 0.692 a |
| methanol 100% | 0.125 b |

[a] Determined after 48 h of incubation. Values are the means of 10 determinations; different letters indicate significant differences ($P < 0.05$). [b] Growth of *B. mojavensis* before addition to the column (initial control).

**Table 2.** Inhibition of Growth of *Fusarium verticillioides* Produced by Surfactin, Iturin A, and Extracts from *Bacillus mojavensis*

| treatment[a] | average $OD_{600}$[b] |
|---|---|
| *F. verticillioides* 408 (control) | 0.415 b |
| iturin A | 0.310 b |
| surfactin | 0.135 a |
| *B. mojavensis* 101 extract | 0.184 a |

[a] 20 $\mu$g/mL was the concentration used for iturin A and surfactin. [b] Average of 10 determinations; different letters indicate significant differences ($P < 0.05$)

reducing the growth of the fungi. Fractions produced in 5−12-day-old nutrient broth shake cultures and extracted with methanol or methylene chloride at pH 2 did not show any inhibitory activity against the fungi (data not shown). Thus, solid-state culture, that is, cultured on nutrient agar, favored the production of surfactin by this strain. The HPLC-MS chromatography of the bacterial supernatant from the nutrient agar culture of *B. mojavensis* prepared as described above revealed a peak with a molecular ion of 1036.7 $[M + H]^+$ that corresponded in both retention time and mass to an identical molecular ion in the surfactin standard (**Figure 1**).

The CID fragmentation of the 1036.7 $[M + H]^+$ ion produces ions of $m/z$ 923.4, 810.4, 695.3, 596.2, 483.1, and 370.0 (**Figure 2**). These correspond to the sequential loss of amino acid residues Leu/Ile-Leu/Ile-Val-Asp-Leu/Ile-Leu/Ile, agreeing with the CID fragmentation of the surfactin standard, and suggested the following sequence: Glu-Leu-D-Leu-Val-Asp-D-Leu-Leu. However, the above fragmentation would be indistinguishable by mass spectrometry from $Ile^7$-surfactin, which has Ile substituted for Leu at the terminal residue (*10*). Summarizing these results, the structure of the inhibitory lipopeptide produced by *B. mojavensis* is a seven-residue peptide of Glu-Leu-Leu-Val-Asp-Leu-Leu with a lactone bond linking the terminal $Leu^7$ to the $\beta$-hydroxyl fatty acid moiety, creating a cyclic peptide. The structure of the major lipopeptide is identical to that for $Leu^7$-surfactin reported by Kakinuma et al. (*11*). Thus, the major lipopeptide structure supported by these data is indicated in **Figure 3**.

Also of note, the relative percentages of the various surfactin $\beta$-hydroxy fatty acid chain lengths detected in the extract differ significantly from those detected in the standard. Using the relative intensities of the ions from the spectra obtained by direct infusion of the standard and of the extract, the relative percentages for major species were calculated (**Table 3**). These spectra reveal the C-15 isomer to be the dominant isomer in the extract, whereas the standard is a mix of the C-12−C-16 isomers. Several other analogues with greater or lesser fatty acid side chain lengths also were identified in the extract and in the surfactin standard but were of minor abundance.

## DISCUSSION

The genus *Bacillus* is known for the production of over 24 antibiotics, several of which are fungicidal (*9*, *10*) with potential control of plant diseases (*12*, *13*). Direct analysis of culture filtrate by mass spectrometry established that *B. mojavensis*, which has strong inhibitory properties toward *F. verticillioides*, produced $Leu^7$-surfactin as well as mixtures of several other isomers. CID analyses of culture filtrates correspond to surfactin spectral analyses reported for culture filtrates of other surfactin-producing bacteria (*14*−*16*). In vitro toxicity tests with the standard mix of the surfactins (**Table 3**) confirmed toxicity of the surfactins to *F. verticillioides*. The configuration of the major $C_{15}$ $\beta$-O fatty acid is uncertain but is expected to be iso and anteiso in variable relative proportions (*9*). Surfactin was first isolated several decades ago as a bacterial cyclic lipopeptide with exceptional surfactant power (*11*, *17*). To our knowledge this is the first report establishing that *B. mojavensis* produces surfactin and that surfactin suppresses the growth of *F. verticillioides*. This result is consistent with earlier work that this bacterium produces substances in culture that reduce the growth of this fungus, as well as reduce the in planta production of the mycotoxin fumonisin in maize seedlings infected with *F. verticillioides* (*6*). However, we cannot exclude the possibility of other interactive mechanisms, for example, competitive exclusion, which might also be involved with the in planta inhibitory response. Nevertheless, the data indicated that *B. mojavensis* is one of several *Bacillus* species reported as producing this biosurfactant (*9*, *10*, *15*, *18*, *19*) and is the second endophytic species (*15*).

In addition to surfactin, iturin is another lipopeptide produced by *Bacillus* species, although it is coproduced with surfactin by only a few species. Of these two, surfactin was toxic to *F. verticillioides*, whereas iturin was not toxic at the concentrations tested. However, there is a report that iturin is very effective against several other phytopathogenic fungi (*10*).

Surfactin belongs to a powerful group of biosurfactants, and it has a wide spectrum of activity. These activities include the ability to serve as a cation carrier across organic barriers, to produce ion channels in uncharged membranes, and to cause membrane dissolution (*20*, *21*). Surfactin has the ability to be effective at very low concentrations, and it is effective against some Gram-positive and most Gram-negative bacteria; it is antiviral, antifungal, antitumor, and antimycoplasma (*9*, *15*, *22*). In addition to its antibiotic effects, surfactin can be used in textile manufacturing, environmental bioremediation, and fossil fuel recovery. Therefore, this compound has enormous potential for biotechnological and biopharmaceutical applications. Surfactin is one of a few viral antibiotics that are produced by bacteria. Its mode of action is based on its detergent-like activity in dissolving several types of lipid membranes. It is effective at concentrations from 12 to 50 $\mu$g/mL (*20*). The concentration selected for use in this work was 20 $\mu$g/mL, and the $LD_{50}$ was not determined. The biological activity of a surfactant depends on both the chain length and the branching type. Although we have no direct proof for the precise branching types of the $\beta$-O fatty acid chain for this isomer produced by *B. mojavensis*, Yakimov et al. (*9*, *23*) established that the order of activity was highest for normal, followed by iso and finally anteiso. Furthermore, the $C_{14}$ chain length was more active than the $C_{15}$. The order of amino acids influences the activity of surfactin, with the maximum hydrophobic activity associated with the α-amino acid in the fourth position. A surfactant is more active when hydrophobic amino acids are at this position, such as leucine or isoleucine (*24*). The minor surfactins, observed in the culture fluids (**Table 3**), might represent isomers with this greater biological activity,

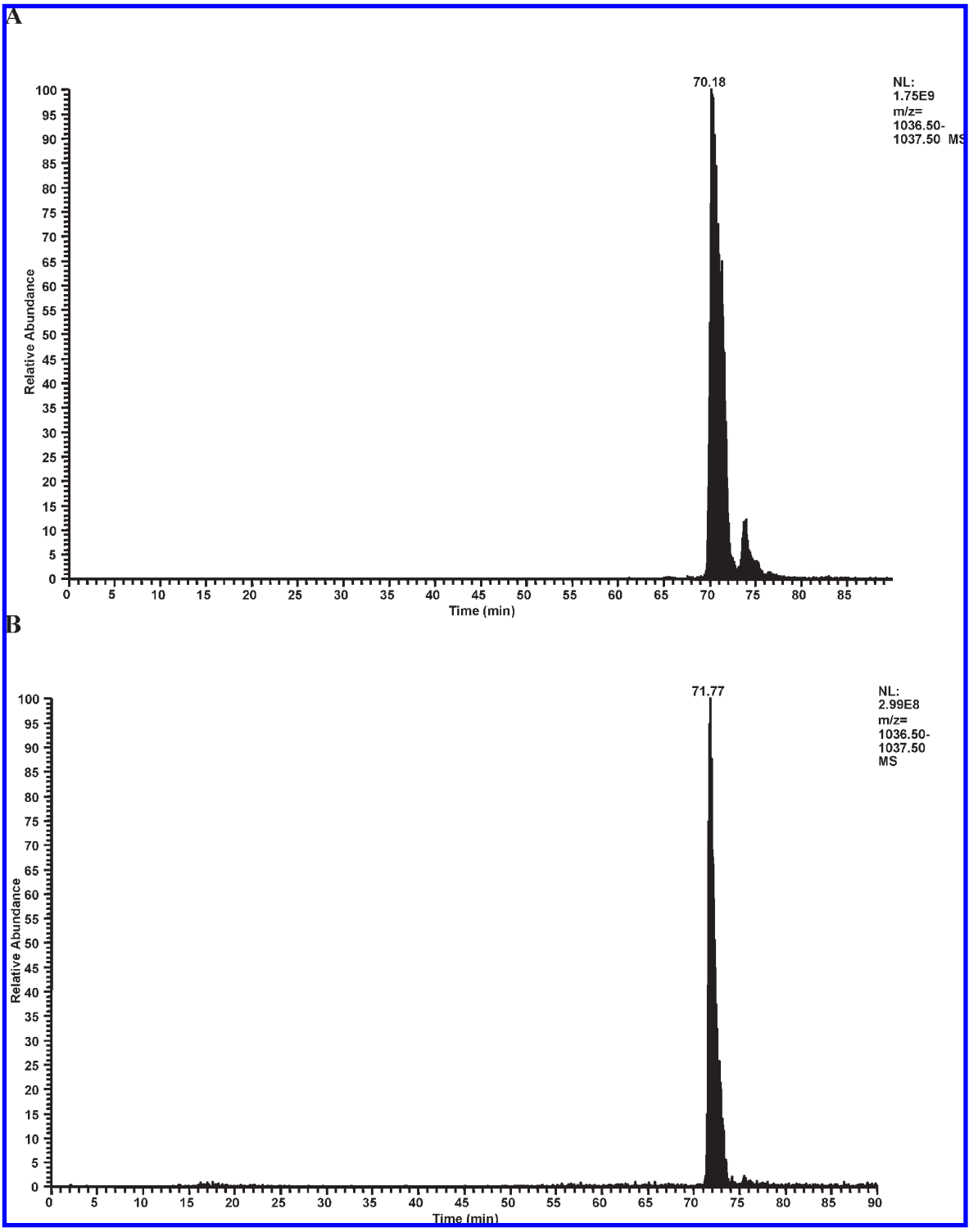


**Figure 1.** HPLC-MS chromatogram of (**A**) the surfactin standard (mass to charge range of 1036.5−1037.5) and of (**B**) the bacterial extract (mass to charge range of 1036.5−1037.5).

which would be highly desirable for biocontrol purposes. Their interactions are expected to be synergistic.

*F. verticillioides* infects maize as a symptomless biotrophic endophyte during most of its association with maize, but under less than ideal culture conditions, the fungus becomes virulent and rapidly colonizes senescing or injured tissue as a saprophyte, producing higher concentrations of the fumonisins, particularly in the ear and kernels (*3*). Two approaches are used to control *F. verticillioides* and its mycotoxins in maize, plant resistance and biological controls. Attempts to control the endophytic infection of *F. verticillioides* involves the use of several biocontrol agents, especially endophytic bacteria (*6*, *12*, *13*), although there are compounding biotic and abiotic environmental factors that might reduce the efficiency of such organisms. A key to understanding the biocontrol potential of such organisms would be to understand the inhibitory system responsible for the control observed exerted by this bacterial endophyte.

Whereas surfactin exerts its toxic effects by altering membrane integrity, the degree to which this activity is accomplished is concentration dependent (*9*). At very low concentrations, it

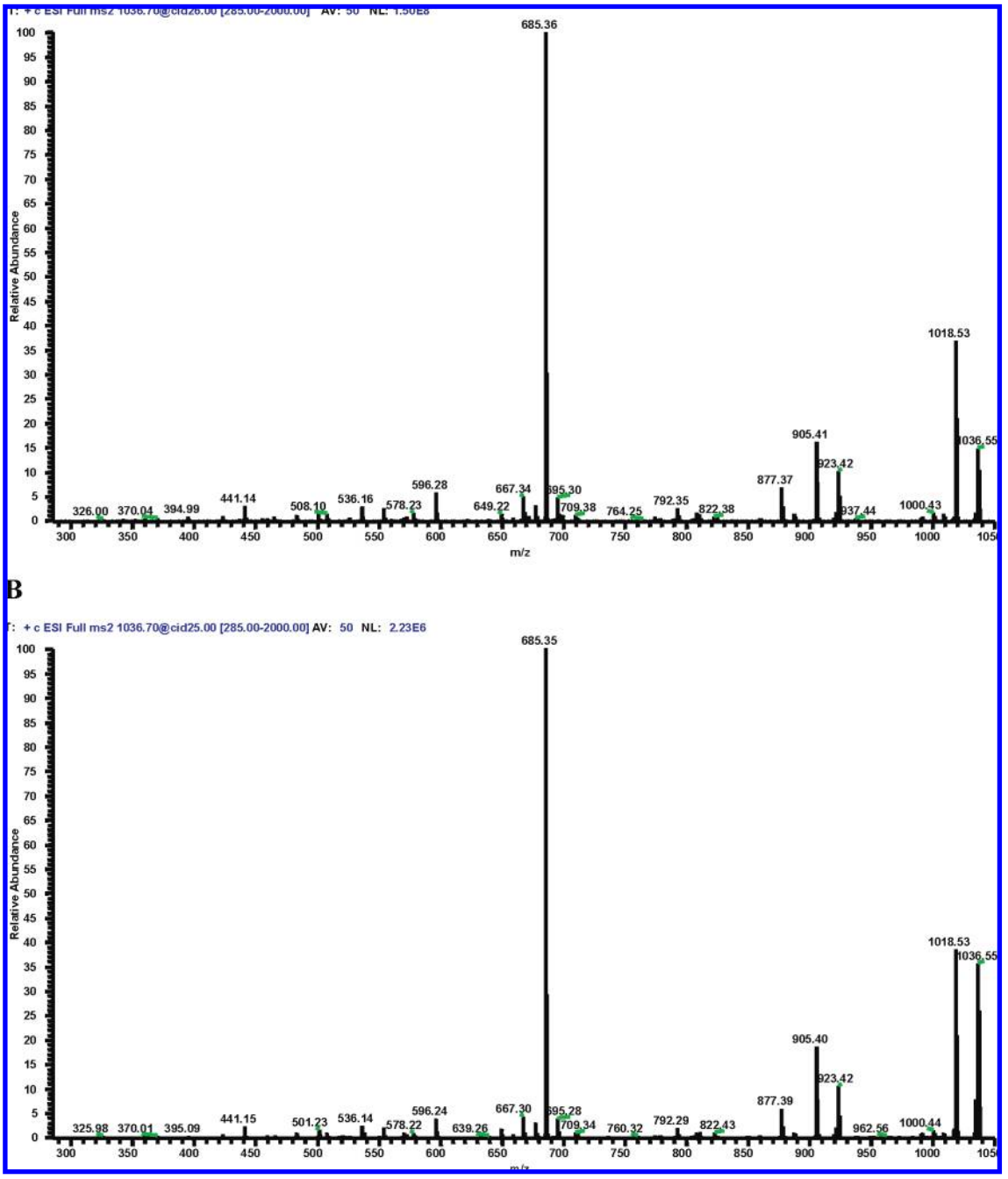


**Figure 2.** Fragmentation ($MS^2$) of the 1036.7 ion from the surfactin standard (**A**) and the culture filtrate (**B**).

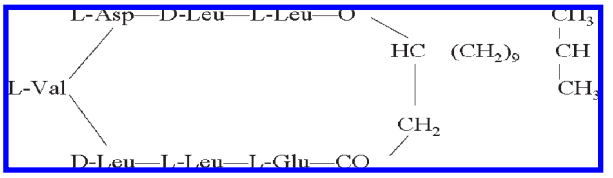


**Figure 3.** Structure of surfactin represented as the iso form for the $\beta$-hydroxy fatty acid moiety, although the normal and anteiso are equal possibilities.

produces conformational changes within the phospholipids. At intermediate concentrations, it causes segregation within the bilayers, resulting in channels that are permeable to cations. At high concentrations, it produces a detergent effect resulting in complete membrane disruption. Regardless of concentration effects, the final toxicity expression is limited in environments in which protein and lipid concentrations are high (*9*, *10*), indicating a possible upper limit to its activity intracellularly based on nutrient concentration. However, because *B. mojavensis* and *F. verticillioides* are both intercellular biotrophs and the apoplastic fluid that they live in does not contain high concentrations of these nutrients (*25*−*27*), the action of surfactin in planta should not be reduced. Compared with chemical pesticides,

**Table 3.** Results of CID Analysis of the Relative Percentages of the Major Surfactin $\beta$-Hydroxy Fatty Acid Chain Lengths in the Surfactin Standard and *B. mojavensis* Extract (±15% Relative Error)

| $\beta$-hydroxy fatty acid chain length | standard (%) | extract (%) |
|---|---|---|
| C-12 | 2.63 | |
| C-13 | 10.02 | 7.18 |
| C-14 | 47.48 | 17.52 |
| C-15 | 36.75 | 73.52 |
| C-16 | 3.12 | 1.78 |

surfactins have low toxicity, are highly biodegradable, and have other environmentally friendly characteristics, which should enhance *B. mojavensis* as an effective biocontrol agent for maize and other plant diseases and for mycotoxin reduction.

## LITERATURE CITED


(1) Riley, R. T.; Norred, W. P.; Bacon, C. W. Fungal toxins in foods: recent concerns. *Annu. Rev. Nutr*. **1993**, *13*, 167–189.

(2) Voss, K. A.; Gelineau-van Waes, J.; Riley, R. T. Fumonisins: current research trends in developmental toxicology. *Mycotox. Res*. **2006**, *22*, 61–69.

(3) Bacon, C. W.; Hinton, D. M. Symptomless endophytic colonization of maize by *Fusarium moniliforme*. *Can. J. Bot*. **1996**, *74*, 1195–1202.

(4) Roberts, M. S.; Nakamura, L. K.; Cohan, F. M. *Bacillus mojavensis* sp. nov., distinguishable from *Bacillus subtilis* by sexual isolation, divergence in DNA sequence, and differences in fatty acid composition. *Int. J. Syst. Bact*. **1994**, *44*, 256–264.

(5) Bacon, C. W.; Hinton, D. M. Use of Bacillus subtilis as an endophyte for the control of diseases caused by fungi. Patent 5,994,117, US Patent & Trademark Office, Nov 30, 1999.

(6) Bacon, C. W.; Yates, I. E.; Hinton, D. M.; Meredith, F. Biological control of *Fusarium moniliforme* in maize. *Environ. Health Perspect*. **2001**, *109*, 325–332.

(7) Bacon, C. W.; Hinton, D. M. Endophytic and biological control potential of *Bacillus mojavensis* and related species. *Biol. Control* **2002**, *23*, 274–284.

(8) Bacon, C. W.; Hinton, D. M.; Snook, M. E. Tentative identification of *Bacillus mojavensis* antifungal inhibitor. *Phytopathology* **2005**, *95* (Suppl.), s5.

(9) Peypoux, E.; Monmatin, J. M.; Wallach, J. Recent trends in the biochemistry of surfactin. *Appl. Microbiol. Biotechnol*. **1999**, *51*, 553–563.

(10) Bonmatin, J.; Laprevote, O.; Peypoux, F. Diversity among microbial cyclic lipopeptides: Iturins and surfactins. Activity−structure relationships to design new bioactive agents. *Comb. Chem. High Throughput Screen*. **2003**, *6*, 541–556.

(11) Kakinuma, A.; Hori, M.; Isono, M.; Tamura, G.; Arima, K. Determination of amino acid sequence in surfactin, a crystalline peptidolipid surfactant produced by *Bacillus subtilis*. *Agric. Biol. Chem*. **1969**, *33*, 971–997.

(12) Lodewyckx, C.; Vangronsveld, J.; Porteous, F.; Moore, E. R. B.; Taghavi, S.; Mezgeay, M.; van der Lelie, D. Endophytic bacteria and their potential applications. *Crit. Rev. Plant Sci*. **2002**, *21*, 583–606.

(13) Cavaglieri, L.; Orlando, J.; Rodríguez, M. I.; Chulze, S.; Etcheverry, M. Biocontrol of *Bacillus subtilis* against *Fusarium verticillioides* in vitro and at the maize root level. *Res. Microbiol*. **2005**, *156*, 748–754.

(14) Vater, J.; Kablitz, B.; Wilde, C.; Franke, P.; Mehta, N.; Cameotra, S. S. Matrix-assisted laser desorption ionization-time of flight mass spectrometry of lipopeptide biosurfactants in whole cells and culture filtrates of *Bacillus subtilis* C-1 isolated from petroleum sledge. *Appl. Environ. Microbiol*. **2002**, *68*, 6210–6219.

(15) Sun, L.; Zhaoxin, L.; Bie, X.; Lu, F.; Yang, S. Isolation and characterization of a co-producer of fengycins and surfactins, endophytic *Bacillus amyloliquefaciens* ES-2, from *Scutellaria baicalensis* Georgi. *World J. Microbiol. Biotechnol*. **2006**, *22*, 1259–1266.

(16) Kowall, M.; Vater, J.; Klluge, B.; Stein, T.; Franke, P.; Ziessow, D. Separation and characterization of surfactin isoforms produced by *Bacillus subtilis OKB 105*. *J. Colloid Interface Sci*. **1998**, *204*, 1–8.

(17) Arima, K.; Kakinu; Tamura, G. Surfactin, a crystalline peptide lipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. *Biochem. Biophys. Res. Commun*. **1968**, *31*, 488–494.

(18) Georgiou, G.; Lin, S. G.; Sharma, M. M. Surface-active compounds from microorganisms. *Bio/Technology* **1992**, *10*, 60–65.

(19) Perez, C.; Suarez, C.; Castro, G. R. Antimicrobial activity determined in strains of *Bacillus pumilus* A-1, and cloning and nucleotide sequence of the regulatory gene, psf-1. *Folia Microbiol*. **1993**, *38*, 25–28.

(20) Heerklotz, H.; Seelig, J. Detergent-like action of the antibiotic peptide surfactin on lipid membranes. *Biophys. J*. **2001**, *81*, 1547–1554.

(21) Deleu, M.; Bouffioux, O.; Tazafindralambo, H.; Paquot, M. Interaction of surfactin with membranes: a computational approach. *Langmuir* **2003**, *19*, 3377–3385.

(22) Desai, J. D.; Banat, I. M. Microbial production of surfactants and their commercial potential. *Microbiol. Mol. Biol. Rev*. **1997**, *61*, 47–64.

(23) Yakimov, M. M.; Fredrickson, H. L.; Timmis, K. N. Effect of heterogeneity of hydrophobic moieties on surface activity of lichenysin A, a lipopeptide biosurfactant from *Bacillus licheniformis*. *Biotechnol. Appl. Biochem*. **1996**, *23*, 13–18.

(24) Peypoux, F.; Michel, G. Controlled biosynthesis of Val 7- and Leu7-surfactins. *Appl. Microbiol. Biotechnol*. **1992**, *36*, 515–517.

(25) Canny, M. J. Apoplastic water and solute movement: new rules for an old space. *Annu. Rev. Plant Physiol. Mol. Biol*. **1995**, *46*, 215–236.

(26) Tejera, N.; Ortega, E.; Rodes, R.; Lluch, C. Nitrogen compounds in the apoplastic sap of sugarcare stem: some implications in the association with endophytes. *J. Plant Physiol*. **2006**, *163*, 80–85.

(27) Satoh, S.; Lizuka, C.; Kikuchi, A.; Fujii, I. Proteins and carbohydrates in xylem sap from squash root. *Plant Cell Physiol*. **1992**, *33*, 841–847.


**Received for Review January 15, 2009. Revised manuscript received March 18, 2009. Accepted March 31, 2009.**