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Characterization of endophytic strains of *Bacillus mojavensis* and their production of surfactin isomers

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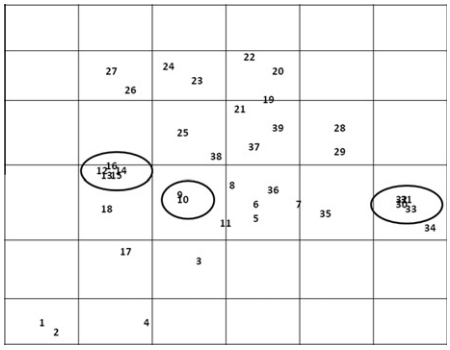
HIGHLIGHTS

- We identified endophytic strains of *Bacillus mojavensis* by rep-PCR.
- The strains apparently exist both as clones and as distinct ecotypes.
- We demonstrated the ability of strains to produce several surfactins.
- The antagonistic effect of surfactin on *Fusarium verticillioides* was demonstrated.

GRAPHICAL ABSTRACT



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ABSTRACT

Bacillus subtilis consists of a large collection of strains from which several cryptic species have been delineated, and most of these along with strains within the species are important biocontrol agents. *Bacillus mojavensis*, a species recently distinguished from this broad *B. subtilis* group of bacteria, was determined to be endophytic and to have biocontrol potential due to its inhibition of the maize mycotoxic fungus *Fusarium verticillioides*. Recently, we reported that a patented strain *B. mojavensis* produced the biosurfactant Leu^7-surfactin that was inhibitory to fungi. In this study, the first objective was to validate the identity of strains, and analyze genotypically a collection of *B. mojavensis* strains, which involved analysis of repetitive-PCR amplified *Bacillus* DNA sequences with a PCR genotyping bar system. In an effort to understand further surfactin production, a second objective was to screen this collection of *B. mojavensis* strains for surfactin analogs. The results indicated that all strains are valid *B. mojavensis*, and that there was genotypic diversity among strains from the great deserts. Further, the study established that most strains can produce a mixture of surfactins that was comprised of acyl chain lengths ranging from C-11 to C-17. These experiments indentified high producers of C-15 surfactin, the most biologically active isoform. However, the *in vitro* inhibition observed did not necessarily relate to total surfactin concentrations, suggesting a complex mechanism for inhibition and/or the presence of other unknown factors.

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1. Introduction

Bacillus mojavensis Roberts, Nakamura, Cohan was distinguished from *Bacillus subtilis* by fatty acid composition, DNA divergence, and sexual isolation (Roberts et al., 1994) following its initial designation as a *B. subtilis* strain discovered from soils and plant debris from the Mojave Desert (Roberts et al., 1994). The

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identification of *B. mojavensis* as a new *Bacillus* species (Cohan et al., 1991; Roberts et al., 1994) led to the re-analysis of *Bacillus* strains in research laboratories, which resulted in the subsequent identification of the strains used in this study as *B. mojavensis* (Bacon and Hinton, 2002). However, confusion remains to be a problem over the identity of this apparent cryptic strain within the *B. subtilis* complex (Logan et al., 2009; Ruiz-Garcia et al., 2005; Wang et al., 2007). Nevertheless, one strain was identified as *B. mojavensis* and as a maize endophyte that possesses biocontrol potential by its actions in conferring growth enhancing and anti-fungal capabilities to maize plants (Bacon and Hinton, 1999). This endophytic bacterium–plant relationship has a potential for the control of the important maize endophytic and oftentimes pathogenic fungus *Fusarium verticillioides* (Saccardo) Nirenberg (= *Fusarium moniliforme* Sheldon), Holomorph: *Gibberella moniliformis* Wineland.

Studies have shown an inhibitory effect of *B. mojavensis* on the growth of this fungus and a reduction of its mycotoxins, the fumonisins (Bacon and Hinton, 1999; Bacon et al., 2001). Recently, we isolated and identified from a patented strain of *B. mojavensis*, RRC101, Leu^7-surfactin (Snook et al., 2009; Fig. 1), which is surfactin A, one of three surfactins. The others are surfactin B and C, differing in the amino acid at the 7-carbon as valine and isoleucine, respectively. These cyclic lipopeptides are powerful biosurfactants, and are known to be toxic to bacteria, viruses, mites, and fungi (Arima et al., 1968; Desai and Banat, 1997; Georgiou et al., 1992; Peypoux et al., 1999; Seydlova and Svobodova, 2008). Further, surfactins are being modified to design new and more specific biosurfactants (Bonmatin et al., 2003).

The overall aim of the current work was to understand further any biocontrol interactions between the endophytic strains of *B. mojavensis* and *F. verticillioides*. Our first objective was to validate the identity of strains in our collection of *B. mojavensis* strains along with an attempt to detail the existence of any ecotype diversity. Our second objective was to determine the production of surfactin potential by these strains, along with the diversity of isomers of surfactin produced by strains with the aim of identifying strains capable as biocontrol agents based on surfactin structure activity relationships. These experiments, when coupled with phenotypic analysis of the plant–bacteria interaction, should assist in procedures for their identification, and in the selection of *B. mojavensis* strains with the best biocontrol potential along with providing further insight into the endophytic and anti-fungal characteristics of *B. mojavensis*.

2. Materials and methods

2.1. Sources of microorganisms

Twenty-nine strains of *B. mojavensis* from different geographical origins were used in this study (Table 1) and see Roberts et al. (1994) for specific locations within a desert as well as the procedure used for their isolation from plant, plant parts or soil. The Russell Research Center (RRC) strains included RRC 111 (an endophytic antagonistic and auxotrophic transformed hybrid (*trp*C2, t*hr*5) produced by transforming the non-endophytic and non-antagonistic but auxotrophic (*trp*C2, t*hr*5) *B. subtilis* BD170 (ATCC 33608) with DNA from the endophytic patented strain RRC 101; RRC 112 (a rifampicin mutant of RRC 101); RRC 112fa (a rifampicin–fusaric acid tolerant mutant); and RRC 113 and RRC 114 (two strains derived from *B. mojavensis* RRC 111 as UV-induced mutants randomly selected on the basis of being non-antagonistic and antagonistic, respectively, to *F. verticillioides*). The other species of bacteria used included the type *B. subtilis* ATCC 6051, *B. subtilis* BD 170, a strain developed for transformation studies; and ATCC 6633, a randomly selected strain used for media testing. Stock cultures of bacteria were stored on silica gel at −30 °C, and working stock cultures were maintained on nutrient agar (Difco Inc., Detroit, MI), and stored at 4 °C until used. *F. verticillioides* Patgus, which is highly pathogenic to maize and wheat seedlings was the fungal strain used in this study. This fungus was stored on potato dextrose agar plates (PDA, Difco, Inc.) at 4 °C. Macroconidia were produced from the fungus by culturing it on 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.

2.2. Fungal and bacterial growth inhibition assay

Bacterial inocula (10^3 CFU/ml) were prepared from 16-h nutrient broth cultures of *B. mojavensis* strains that were obtained from

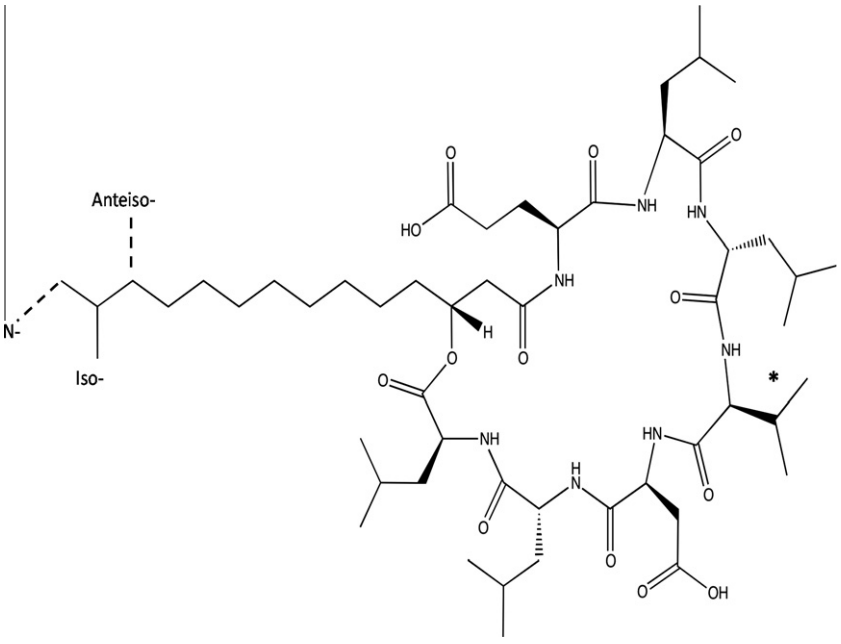

Fig. 1. Structure of iso-leu^7-surfactin A, also indicated in broken lines are points of variation for the anteiso- and normal-configurations; *, indicates location of leucine at the 7 position.

Table 1
Total surfactin A production by strains of *Bacillus mojavensis* cultured in nutrient broth medium or Pharmamedia, compared with the assay for antagonism to *F. verticillioides* Patgus measured on nutrient agar after 14 days.

Strains[a]	Source	Surfactin[b] (μg/ml)		Antagonism[c] (mm)
		Pharmamedia	Nutrient broth	
RRC 112	Mutant	0.73 ± 0.02	0.00	10.86 ± 0.23a
RRC 112fa	Mutant	0.63 ± 0.02	0.00	10.12 ± 0.33a
RRC 111	Mutant	2.31 ± 0.43	0.00	9.31 ± 0.34a
RRC 113	Mutant	2.63 ± 0.52	0.00	0.00
RRC 114	Mutant	2.18 ± 0.31	0.00	10.02 ± 0.54a
ATCC 51516	Mojave	22.55 ± 2.91	3.39 ± 1.12a	2.69 ± 0.42b
NRRL 14699	Mojave	0.21 ± 0.01	0.00	0.00
NRRL 14700	Mojave	15.22 ± 1.77	2.41 ± 1.22a	0.00
NRRL 14701	Mojave	1.18 ± 0.06	0.00	8.61 ± 0.65a
NRRL 14702	Mojave	22.94 ± 2.31	5.03 ± 1.34b	9.15 ± 0.45a
NRRL 14703	Gobi	2.42 ± 0.65	0.00	4.40 ± 0.67c
NRRL 14704	Gobi	2.06 ± 0.55	0.00	2.39 ± 0.24d
NRRL 14705	Gobi	37.21 ± 3.54	0.00	8.75 ± 0.23a
NRRL 14706	Gobi	10.11 ± 1.22	0.00	7.35 ± 0.24d
NRRL 14707	Gobi	0.29 ± 0.02	0.72 ± 0.03c	9.41 ± 1.09a
NRRL 14708	Gobi	89.30 ± 3.7	6.28 ± 0.67b	0.00
NRRL 14709	Gobi	30.27 ± 2.92	9.85 ± 0.89d	7.69 ± 1.20d
NRRL 14710	Gobi	0.10 ± 0.01	0.00	1.92 ± 0.02b
NRRL 14711	Gobi	27.16 ± 2.41	1.71 ± 1.9a	6.90 ± 0.2d
NRRL 14712	Gobi	17.55 ± 1.19	10.51 ± 2.1d	9.89 ± 0.2a
NRRL 14713	Gobi	36.89 ± 2.84	1.60 ± 1.7a	0.00
ATCC 51517	Gobi	11.38 ± 1.43	3.14 ± 1.2d	11.07 ± 0.2a
NRRL 14714	Sahara	37.29 ± 2.13	10.25 ± 1.6d	12.36 ± 0.6a
NRRL 14715	Sahara	27.64 ± 1.7	35.94 ± 1.3e	0.00
NRRL 14716	Sahara	28.69 ± 1.2	7.00 ± 1.8b	8.53 ± 0.3a
NRRL 14718	Sahara	20.07 ± 1.1	7.19 ± 1.6b	10.98 ± 0.5a
NRRL 14719	Sahara	0.00	0.00	10.45 ± 0.4a
NRRL 14824	Sahara	25.59 ± 1.1	29.63 ± 1.4f	9.80 ± 0.3a
NRRL 14817	Arizona	0.00	0.00	7.99 ± 0.2da

[a] RRC, Russell Research Center culture collection, Athens, Ga: ATCC, American Type Culture Collection; NRRL, Northern Regional Research Center, Peoria, IL. Origin of RRC isolates and mutants are described in the text.

[b] Surfactin determined on either nutrient broth or Pharmamedia, but antagonisms were determined on nutrient agar and measured in mm. The data on surfactin analyses are from three independent experiments for each strain, and are presented as means ± standard error, and different letters within a column indicate significant differences, $P < 0.05$, based on the standardized condition of our chemical protocol.

[c] Zones of antagonism were measured after 14 days at 25 °C and values are combined means of two separate experiments, each replicated five times for each experiment. Values represent the means and standard deviations of six replicates per strain, and different letters within the column indicate statistically significant differences between strains according to the LSD test at $P < 0$ .05.

50 ml of nutrient broth contained in 125-ml shake flasks and incubated on an Innova 4300 incubator shaker (New Brunswick Scientific). The inoculum consisted of cells that were washed in sterile distilled water by centrifugation at 2000*g*. The assay was performed measuring growth curve rates in sterile 100-well microplates. Each well contained 185 μl of the designated surfactant media to which was added 15 μl of the bacterial inoculum. The cultures were incubated at 30 °C with constant shaking and the OD_{600} was measured at 30 min intervals over the incubation period in an automated turbidometer, the Microbiology Bioscreen C Reader (Labsystems, Helsinki, Finland). The data were analyzed with the Bioscreen C Reader using the Transgalactic software package, Research Express version 1.00. Each experiment had 10 replications and all bacterial growth experiments were repeated.

To determine the antagonism of surfactins produced in culture medium, a comparison was made on the growth of *F. verticillioides* cultured in media containing authentic surfactin, and iturin, another chemically related lipopeptide surfactant. Standards of surfactin A (from *B. subtilis*, approximate purity of 98%) and iturin A were obtained from Sigma–Aldrich (St. Louis, MO). These experiments were performed on an automated turbidometer, the Microbiology Bioscreen C Reader, following previous methods (Snook et al., 2009). Briefly, each well contained 190 μl of nutrient broth, to which was added 10 μl of bacterial or fungal inoculum or both (10^3 CFU/ml, each) in nutrient broth, and 100 μl of culture extract or solvent control. The concentrations of surfactin and iturin A ranged from 10 μg/l to 200 μg/ml; however, the data are reported on 20 μg/ml of each over a 21 h observation period. Each data point represents the means of five replicates and each experiment was repeated twice.

2.3. Antagonism assay

A radial agar diffusion assay was used to measure the *in vitro* toxin production and inhibition of the bacterial strains to *F. verticillioides*, RRC Patgus at 25 °C during a 14 day incubation period following prior procedures (Bacon and Hinton, 2002). J-broth medium (tryptone, 5.0 g; yeast extract, 15.0 g; K_2HPO_4, 3.0 g; glucose, 2.0 g; distilled water, 1000 ml) was inoculated with the designated *B. mojavensis* strains, placed in a shaker, 30 °C at 160 rpm for 16 h. The cultures were placed in 50-ml centrifuged tube, centrifuged at 2000*g*, and the cells were washed by resuspending in sterile water and re-centrifuged. The concentrations of cells were adjusted to 10^3 with sterile distilled water, which were used as inocula. The center of nutrient agar plates were streaked with bacteria, incubated 5 days at 25 °C, and *F. verticillioides* were inoculated along the outer edges of a plate by applying a 0.5 cm square plug of the fungus obtained from a 14-day-old culture grown on PDA. The plates were incubated in the dark at 25 °C and antagonism was determined by measuring the zone in mm between the bacteria and the fungus at day 14 with electronic digital calipers. Control plates were only inoculated with the fungus or the designated bacterial strain. Each data point represents five replicates and the experiments were repeated twice.

2.4. Molecular characterization of strains

2.4.1. DNA extraction and rep-PCR

The strains were grown in 50 ml Erlenmeyer flasks, overnight in 10 ml of nutrient broth at 30 °C inoculated with a single colony. Genomic DNA was isolated from the bacterial strains using the Mo Bio UltraClean purification kit (Mo Bio, Carlsbad, CA, USA) according to the manufacturer's protocol. DNA purity and concentration was assessed spectrophotometrically. DNA fingerprint analysis using repetitive polymerase chain reaction (rep-PCR) was performed using the repetitive sequence-based PCR DNA fingerprinting kit according to the manufacturer's recommendations (Bacterial Barcodes, Inc., Athens, GA, a division of BioMeriuex). This system has been successfully used to distinguish bacterial species and strains (Cleland et al., 2007; Healy et al., 2005). Briefly, genomic DNA from the *Bacillus* species were subjected to repetitive element PCR amplification using a Peltier Thermal Cycler (PTC-200, MJ Research/Bio-Rad, Hercules, CA, USA). Fingerprint DNA fragments were obtained by adding 2 μl of genomic DNA to a PCR reaction mix that included rep-PCR min MM1 (DiversiLab Kit) and a *Bacillus* primer mix (DiversiLab kit). The PCR reaction was denatured at 94 °C for 120 s, followed by 35 cycles run under the following conditions: denaturation, 94 °C for 30 s; annealing, 55 °C for 30 s; extension, 70 °C for 90 s. The 35 cycles were followed by a final extension step at 70 °C for 180 s.

2.4.2. DNA fragment separation and phylogenetic analysis

Separation of the amplicons was accomplished by using a microfluidics DNA LabChip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The process involved pipetting 1 μl of rep-PCR product and a gel-dye/marker mix onto the microfluidics chip according to manufacturer's protocol and loading the

chip onto the Bioanalyzer for DNA fragment analysis. The resulting fingerprint patterns were generated as a fluorescent peak graphical displayed, along with virtual DNA gel patterns. The data were collected as separate data files on the Bioanalyzer, which was analyzed, and uploaded to the Bacterial Barcodes website (www.bacbarcodes.com) where the web-based software performed a statistical analysis of the data with the addition of web-based profiles of sufficient quality from an internal database. Comparison of samples was done using the DiversiLab software, which uses the Kullback–Leibler distance approximation to compare samples with one another. Results from the similarity analysis were plotted as dendrograms.

2.5. Surfactin production

The bacterial strains were cultured in either liquid or agar media. The media listed below and used to screen strains for surfactin production were dissolved in a liter of distilled water. Nutrient broth medium consisting of 16.0 g of nutrient broth (Difco, Detroit Mi); and Pharmamedia 3.0 g amended with nutrient broth, 8.0 g. Pharmamedia is a cottonseed derived protein rich nutrient medium (Traders Protein, Southern Cotton Oil Company, P.O. Box 80367, Memphis, TN 38108). When an agar medium was desired, 15.0 g of agar per liter was added to each of the medium indicated above.

Nutrient broth cultures, 16 h-old, of the designated bacterial strains were centrifuged and the cell pellets used to prepare the bacterial inocula. The bacterial suspensions were adjusted to 10^3 CFU and 1 ml of each strain was added to 125-ml flasks containing 50 ml of nutrient broth or the Pharmamedia. The flasks were placed in the incubator shaker at 160 rpm for a total of 5 days in the dark at 30 °C. At the end of the fifth day, the cultures were centrifuged to remove bacterial cells, and each supernatant filter sterilized (Nalgene filter unit, 50 mm diameter membrane, 0.2 μm pore size) and stored at 4 °C until surfactin analyses.

2.5.1. Surfactin isolation and identification

A standard of surfactin (from *B. subtilis,* approximate purity of 98%) was obtained from Sigma–Aldrich (St. Louis, MO) and the UV-MS data of these standards were consistent with those reported by Bonmatin et al. (2003). The procedures for isolating and purifying the surfactins were according to Snook et al. (2009). This included a Finnegan LCQ Duo with electrospray ion source that was used to obtain the mass spectra for the analyses. The system was equipped with a SpectraSystem P2000 HPLC pump, a SpectraSystem AS3000 auto injector and a SpectraSystem UV6000LP UV–vis detector for tandem UV-MS analyses. The mass spectral data were made with the instrument in the positive mode, and 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-μm 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, XCaliber, the system control software, was run in the data-dependent mode, isolating, and fragmenting the most intense ion (35%) by collisional ion dissociation found in each scan.

2.5.2. Quantification of surfactin

For quantification of each isoform, a chromatogram was produced by filtering from the total ion current, the sum of the ion intensity for the m/z corresponding to M+H, which is due to a protonated ion, and the ion intensity for the m/z corresponding to each of the first through fourth singly charged sodium adducts. Though the mass of each isomer and its sodium adducts was unique, the M+1 or M+2 isotopes of some isoforms would correspond to the mass of another. Since isoforms overlapped chromatographically, this isotopic overlap had to be accounted for. In order to accommodate this, the following procedure was used. From the chromatographic summation, the ion intensity of the m/z for the M+H of any other isoform was subtracted if the m/z of the +1 or +2 isotope corresponded to the m/z of the M+H or any of the sodium adducts for the isoform being quantitated. The isoform was then quantitated using the response of the surfactin standard relative to the internal standard 5,7-dimethoxycoumarin. The data on surfactin analyses are from three independent experiments for each strain, and quantification of surfactin was performed by combining these experiments and presented as means based on a standardized condition of our chemical protocol.

2.6. Data analysis

Surfactin analyses were performed on duplicate samples per experiment and each experiment was repeated three times. All analyses were performed using software of the GraphPad PRISM, Inc., version 4.03. Data from repeated experiments in each test were pooled and analyzed statistically. Analysis of variance (ANOVA) was used to compare the biocontrol efficacy of endophytic strains and surfactant treatments, and multiple comparisons of treatment means were separated by Fisher's protected least significant difference at $P < 0.05$. Results from the rep-PCR analysis for selected individual strains were repeated three or more times and the DNA fingerprint of each replicate was indistinguishable for a strain and in terms of similarity coefficients of different batches (>95%), and we found consistent strain-level identification for all randomized strains selected. These results indicate that the rep-PCR amplified products provided by the DiversiLab system were reproducible at both the species and strain levels.

3. Results

3.1. Molecular characterization of species

The 29 *B. mojavensis* strains analyzed in this study were collected from different sources indicated in Table 1 and analyzed by The DiversiLab for identification and characterization. All strains subjected to this procedure were identified as *B. mojavensis*. The amplified sequences using the *Bacillus* rep-PCR kit followed by statistical analysis using the summarized Kullback–Leibler distance approximation of the *Bacillus* strains provided data that distinguished between the *B. mojavensis* strains and the four *B. subtilis* strains (Fig. 2). This analysis produced two broad clades of strains, which were identified as *B. mojavensis* with a 95% similarity or higher. A separate clade was observed accommodating the four strains of *B. subtilis*, which included the type species ATCC 6051, the Marburg strain (Fig. 2). Fig. 2 also shows that the laboratory hybrid strain RRC 111 grouped with *B. mojavensis*, although this strain was transformed under congressional conditions with DNA from the patented endophytic strain RRC 101 and *B. subtilis* 170, producing an auxotrophic, endophytic and antagonistically 'hybrid' strain (Ganova-Raeva et al., 1998). Two of these four *B. subtilis* strains had 95% similarity, whereas the *B. subtilis* strain, 6633, was only 85% similar to the other *B. subtilis* strains, while strain *B. subtilis* 170 although was 95% similar was distinctly grouped separately when this data was scrutinized and presented along with the scatter plot analysis (Fig. 3).

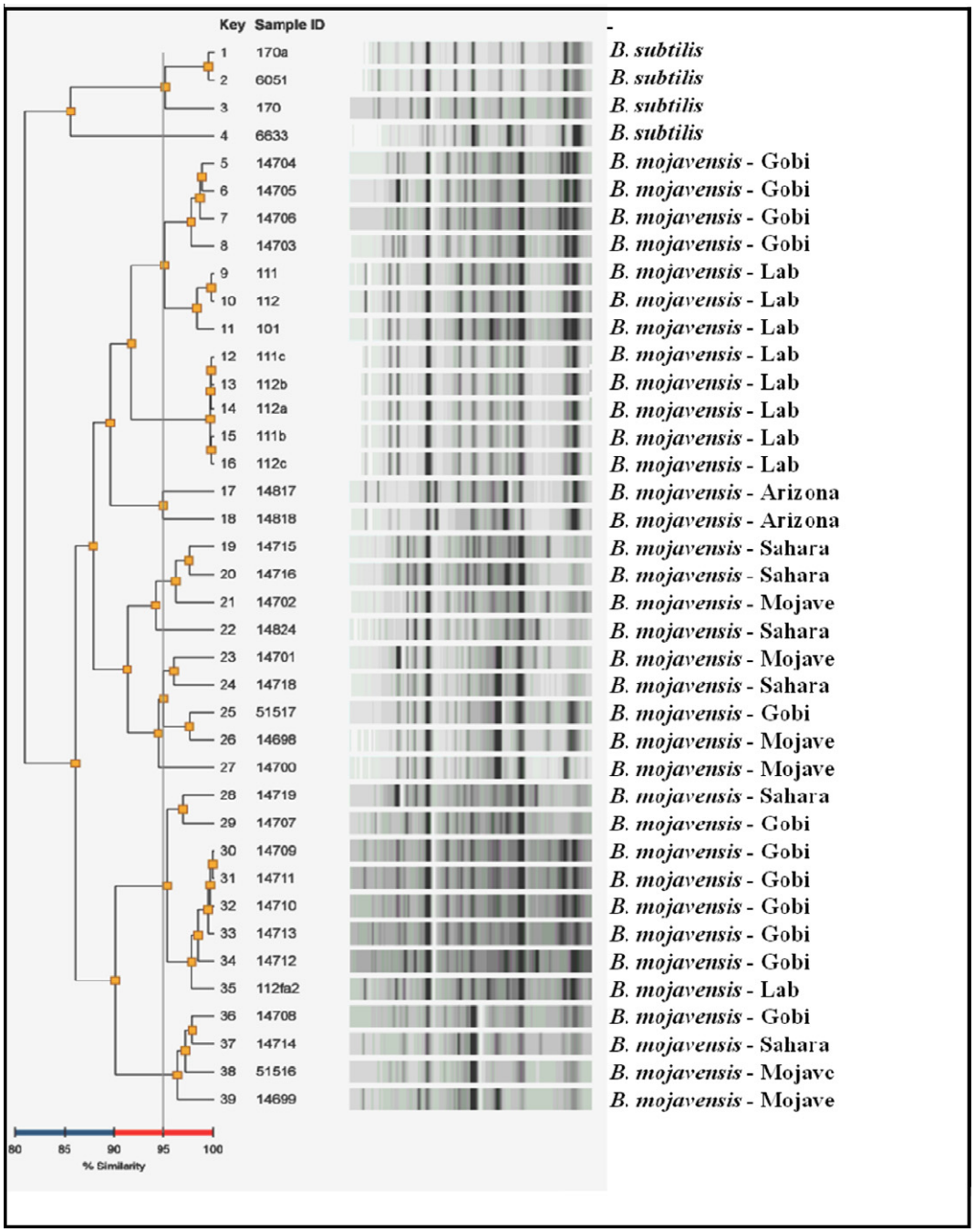

Fig. 2. Dendrogram and gel-like images resulting rep-PCR amplicons of *B. mojavensis* and *B. subtilis* strains. A pair-wise similarity matrix was generated using the Kullback–Leibler distance approximation to compare samples. The vertical line on the dendrogram indicates 95% similarity cutoff.

The mutant strains of *B. mojavensis* developed in our laboratory were placed into two clades with greater than 95% similarity within each group (Fig. 2). The two strains originating from the Arizona desert fell into their own group of almost 95% similar, while the strains from the Mojave and Sahara deserts could not be distinguished into a particularly identifiable group other than two Sahara strains (NRRL B-14715, NRRL B-14716), which fell into a group of greater than 95% similarity.

The scatter plot data of this rep-PCR procedure show that several species group within clusters, and also to show a two-dimensional representation of heterogeneity among isolates in which samples that are close to each other have a higher level of similarity than those that are farther apart therefore discriminating below the species level (Fig. 3). The scatter plot analysis of the strains of *B. mojavensis* indicates that while there are four major clusters. However, most of the strains are outliers (5-8, 17-29, 36-39) and distinct from each other representing higher degrees of differentiation at the strain level than the clusters strains (Fig. 3). The data indicate that the Arizona strains formed a loose cluster, while some of the strains from the Gobi, the patented strain, and its laboratory mutants form very tight similarity clusters. The only strains showing no close similarity were those isolated from the Sahara and Mojave Deserts.

3.2. Surfactin analysis

A comparison of the effects on growth of *F. verticillioides* by the surfactin standard and a sterile culture filtrate of *B. mojavensis*,

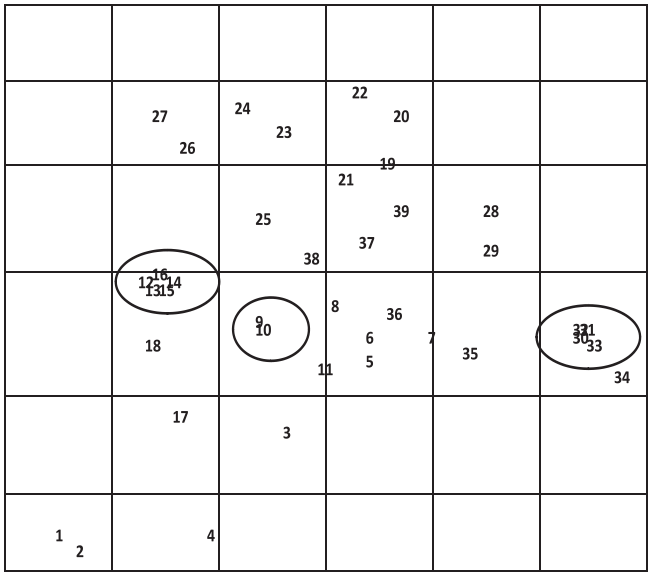

Fig. 3. Scatter plot summary based on rep-PCR analysis of *Bacillus mojavensis* and *B. subtilis* strains grouped according to their relative similarity or dissimilarity patterns that is not related to surfactin accumulation patterns. Strains 1–4 represent *B. subtilis*. Three clusters of *B. mojavensis* are indicated. The oval far left consists of strains 12–16, representing a group of related mutants with strong similarities but are distinctly separated from the strains from which they were derived. Two clonal origins of stains are suggested in the far right oval consisting of strains 30–33, and the oval 9, and 10. The other strains (3, 5–8, 17–29, and 34–39) represent higher degrees of differentiation and dissimilarities at the strain level than those in the clusters suggesting that they are distinct ecotypes. All numbers refer to strains identified in Fig. 2.

along with another cyclic peptide similar in properties to surfactin, iturin, are presented in Fig. 4. Both purified surfactin and 5-day-old culture filtrate of *B. mojavensis* inhibited the growth of *F. verticillioides* in liquid culture. Both surfactin and the culture filtrate caused an approximate 10-h lag period. While iturin was inhibitory, its effects were significantly less than that of surfactin ($P = 0.05$). However, iturin was not found in any of the bacterial extracts (data not shown). While the concentration of the surfactin in the culture extract was not determined, the data in Fig. 4 suggest that the extract of 5-day-old *B. mojavensis* contains at least 20 μg of surfactin, which is inhibitory to the growth of *F. verticillioides*.

There was no correlation between total surfactin production on the two media and *in vitro* inhibition of *F. verticillioides* observed on nutrient broth agar (Table 1). Some strains produced strong antagonisms but no detectible surfactin produced, while other showed the reverse. The marked variation in the production of isoforms of surfactin on the two media indicates the influence on cultural conditions. The antagonisms were measured on nutrient agar, i.e., solid-state fermentation as opposed to submerge fermentations achieved on nutrient broth or Pharmamedia amended with nutrient broth and reported in Tables 2 and 3. Surfactin concentration was not measured in the nutrient agar during the antagonism studies (Table 1).

The strains of *B. mojavensis* produced different types and total amounts of surfactin on the Pharmamedia and the nutrient broth medium (Tables 2 and 3). Some strains were either completely negative for surfactin (NRRL 14719, NRRL 14817, NRRL14710, NRRL 14699) or very low producers (NRRL 14711, NRRL 14713, and NRRL 14707) on the nutrient broth medium (Table 1). However, some of these strains were positive for the surfactins on the Pharmamedia amended with nutrient broth (Table 3). Strains also varied with the total amounts produced on each medium. For example, strain *B. mojavensis* NRRL 14715 was the highest producer of total surfactins (combined isoforms) on nutrient broth, while strain NRRL 14708 produced the most surfactin on the Pharmamedia (Table 1). Strain NRRL B-14708 was the highest producer of acyl chain lengths of C-15 surfactin, the most biologically active isoforms, which occurred on the Pharmamedia (Table 3), whereas NRRL B-14715 produced the most of this biologically active C-15 isoform on nutrient broth (Table 2). Of all the desert strains, NRRL 14699 was either a non-producer or the weakest producer of surfactin, producing only the C-15 isomer and only on Pharmamedia (Table 2). However, strain NRRL 14817, an Arizona isolate, was negative for surfactin production on both media (Tables 2 and 3).

The surfactin isoforms are present in most complexes in variation in acyl chain length ranging from C-13 to C-15. However, our studies revealed only the synthesis of acyl chain length of C-11 and above on the nutrient broth. On the Pharmamedia no isolates were identified that produced isomers with an acyl chain length of C-11 (Table 3). The ratio of specific isoforms within a surfactin mixture varied according to strain, although most strains produced higher amounts of C-13 isoforms than of the C-14 and C-15 acyl chain lengths, especially on the Pharmamedia where almost all strains produced more C-13 than the C-14 isoforms (Table 2 and 3).

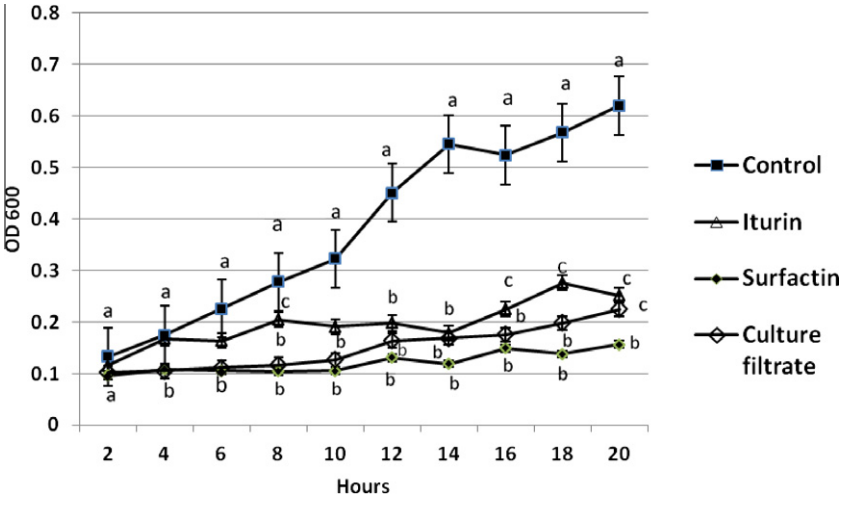

Fig. 4. Effects of commercially purchased surfactin A and iturin (20 μg/ml of each), and 5-day-old culture filtrate prepared from a nutrient broth culture medium of *Bacillus mojavensis* on the growth of *F. verticillioides* RRC Patgus cultured in nutrient broth. Each bar represents the standard error of the means of five replications. Different lowercase letters at a given time indicate significant differences between treatments at $P < 0.05$.

Table 2
Production of surfactin A isoforms by *B. mojavensis* strains cultured in nutrient broth medium with most biological isoform, C-15, for fungicidal activity in bold.

Strains	Surfactin production by acyl chain length (μg/ml[a])						
	C-11	C-12	C-13	C-14	C-15	C-16	C-17
RRC 112	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00
RRC 112fa	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00
RRC 111	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00
RRC 113	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00
RRC 114	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00
ATCC 51516	0.00	0.00	0.42 ± 0.02	0.24 ± 0.01	**1.55 ±** 0.03	0.00	0.00
NRRL 14699	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00
NRRL 14700	0.00	0.00	0.79 ± 0.03	0.38 ±	**0.17 ±** 0.02	0.00	0.00
NRRL 14701	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00
NRRL 14702	0.52 ± 0.02	0.00	0.74 ± 0.03	0.39 ± 0.03	**1.49 ±** 0.02	0.00	0.00
NRRL 14703	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00
NRRL 14704	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00
NRRL 14705	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00
NRRL 14706	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00
NRRL 14707	0.00	0.00	0.54 ± 0.02	0.00	**0.18 ±** 0.02	0.00	0.00
NRRL 14708	0.00	0.69 ± 0.03	0.83 ± 0.03 0.02	0.39 ± 0.04	**3.37 ±** 0.54	0.00	0.00
NRRL 14709	0.00	0.00	0.85 ± 0.02	0.54 ± 0.02	**5.84 ±** 0.87	0.00	0.63 ± 0.02
NRRL 14710	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00
NRRL 14711	0.00	0.00	0.26 ±	0.00	**0.58 ±** 0.04	0.00	0.00
NRRL 14712	0.72 ± 0.04	0.18 ± 0.02	1.13 ± 0.05	0.63 ± 0.03	**5.46 ±** 0.92	0.00	0.00
NRRL 14713	0.00	0.00	0.12 ± 0.02	0.18 ± 0.02	**0.32 ±** 0.03	0.00	0.00
NRRL 51517	0.00	0.00	0.28 ± 0.05	0.17 ± 0.03	**1.62 ±** 0.04	0.00	0.00
NRRL 14714	0.53 ± 0.03	0.86 ± 0.02	1.72 ± 0.05	0.63 ± 0.05	**5.30 ±** 0.33	0.00	0.00
NRRL 14715	0.20 ± 0.5	0.33 ± 0.04	4.17 ± 0.32	2.66 ± 0.45	**22.48 ±** 1.32	0.37 ± 0.03	0.18 ± 0.04
NRRL 14716	0.80 ± 0.21	0.99 ± 0.01	0.99 ± 0.04	0.33 ± 0.05	**2.31 ±** 0.22	0.00	0.00
NRRL 14718	0.75 ± 0.04	0.16 ± 0.04	1.47 ± 0.08	0.44 ± 0.05	**3.68 ±** 0.43	0.00	0.00
NRRL 14719	0.00 ±	0.00	0.00	0.00	**0.00**	0.00	0.00
NRRL 14824	0.31 ± 0.04	0.35 ± 0.02	3.76 ± 0.07	2.53 ± 0.50	**18.47 ±** 1.02	0.35 ± 0.22	0.17 ± 0.03
NRRL 14817	0.00	0.00	0.00	0.00	**0.00**	0.00	0.00

[a] Isoforms of surfactin analyses are from three independent experiments for each strain, and quantification of surfactin was performed by combining these experiments and presenting the mean ± standard errors based on a standardized condition of our chemical protocol.

Table 3
Production of surfactin A isoforms by *B. mojavensis* strains cultured on Pharmamedia amended with nutrient broth with most biological isoform, C-15, for fungicidal activity in bold.

Strains	Surfactin production by acyl chain length (μg/ml[b])					
	C-12[a]	C-13	C-14	C-15	C-16	C-17
RRC 112	0.00	0.00	0.00	**0.73 ±** 0.09	0.00	0.00
RRC 112fa	0.00	0.11 ± 0.02	0.00	**0.52 ±** 0.03	0.00	0.00
RRC 111	0.00	0.38 ± 0.03	0.00	**1.93 ±** 0.09	0.00	0.00
RRC 113	0.00	0.33 ± 0.04	0.17 ± 0.02	**2.13 ±** 0.54	0.00	0.00
RRC 114	0.00	0.34 ± 0.03	0.21 ± 0.01	**1.63 ±** 0.42	0.00	0.00
ATCC 51516	0.29 ± 0.03	5.42 ± 0.03	3.09 ± 0.21	**13.20 ±** 0.99	0.38 ± 0.09	0.00
NRRL 14699	0.00	0.00	0.00	**0.21 ±** 0.03	0.00	0.00
NRRL 14700	0.51 ± 0.08	5.02 ± 0.05	2.07 ± 0.30	**7.45 ±** 0.98	0.00	0.00
NRRL 14701	0.00	0.44 ± 0.05	0.00	**0.74 ±** 0.05	0.00	0.00
NRRL 14702	0.33 ± 0.02	6.16 ± 0.40	3.24 ± 0.10	**13.09 ±** 0.78	0.00	0.00
NRRL 14703	0.00	0.56 ± 0.03	0.21 ±	**1.65 ±** 0.04	0.00	0.00
NRRL 14704	0.00	0.52 ± 0.04	0.21 ±	**1.33 ±** 0.03	0.00	0.00
NRRL 14705	0.42 ± 0.05	8.14 ± 0.98	7.24 ± 0.89	**19.70 ±** 1.02	1.02 ± 0.02	0.37 ± 0.06
NRRL 14706	0.00	2.20 ± 0.29	1.08 ± 0.12	**6.75 ±** 0.98	0.00	0.00
NRRL 14707	0.00	0.07 ± 0.02	0.00	**0.22 ±** 0.02	0.00	0.00
NRRL 14708	4.82 ± 0.94	19.36 ± 1.0	27.54 ± 1.23	**32.28 ±** 1.32	2.37 ± 0.04	0.75 ± 0.14
NRRL 14709	0.45 ± 0.06	6.79 ± 0.99	6.12 ± 0.78	**15.88 ±** 1.22	0.66 ± 0.10	0.21 ± 0.02
NRRL 14710	0.00	0.00	0.00	**0.10 ±** 0.05	0.00	0.00
NRRL 14711	0.73 ± 0.02	5.50 ± 0.12	5.96 ± 0.42	**14.48 ±** 1.22	0.34 ± 0.12	0.15 ± 0.06
NRRL 14712	0.12 ± 0.01	5.06 ± 0.32	2.13 ± 0.90	**9.73 ±** 1.45	0.29 ± 0.02	0.03 ± 0.01
NRRL 14713	0.58 ± 0.03	8.92 ± 0.53	7.74 ± 1.09	**17.97 ±** 1.34	0.86 ± 0.03	0.35 ± 0.01
NRRL 51517	0.06 ± 0.02	3.10 ± 0.73	1.53 ± 0.04	**6.67 ±** 1.33	0.00	0.02 ± 0.01
NRRL 14714	2.32 ± 0.82	9.27 ± 0.69	9.43 ± 1.09	**14.09 ±** 1.2	0.46 ± 0.03	0.17 ± 0.03
NRRL 14715	0.39 ± 0.03	7.76 ± 0.93	4.78 ± 0.76	**13.82 ±** 0.92	0.39 ± 0.03	0.16 ± 0.02
NRRL 14716	0.65 ± 0.03	9.18 ± 0.98	4.88 ± 0.85	**12.72 ±** 0.98	0.49 ± 0.03	0.15 ± 0.02
NRRL 14718	1.93 ± 0.09	11.57 ± 0.88	3.71 ± 0.82	**10.55 ± 0.77**	0.18 ± 0.5	0.00
NRRL 14719	0.00	0.00	0.00	**0.00**	0.00	0.00
NRRL 14824	0.71 ± 0.04	7.38 ± 0.87	3.83 ± 0.45	**13.05 ±** 1.23	0.35 ± 0.13	0.00
NRRL 14817	0.00	0.00	0.00	**0.00**	0.00	0.00

[a] No isolates produced the C-11 acyl chain length on this medium.
[b] Isoforms of surfactin analyses are from three independent experiments for each strain, and quantification of surfactin was performed by combining these experiments and presenting the means ± standard errors based on the standardized condition of our chemical protocol.

4. Discussion

Recognition of strains or subspecies within *B. subtilis* is difficult since very little phenotypic characters can be used to separate strains/subspecies within the species, resulting in most identification within this species actually referring to strains of *B. subtilis sensu lato*. Using primarily DNA sequence analysis, Roberts et al. (1994) described *B. mojavensis* as a cryptic species of *B. subtilis*, which along with three other species resulted in the designation of the type species as *B. subtilis* subsp. *subtilis* to clearly identify *B. subtilis* isolates and not the broad definition of this species (Nakamura et al., 1999). Their definition of the new species indicated that *B. mojavensis* was identical in typical phenotypic analyses to *B. subtilis* (morphological, metabolic and physiological data), but was readily distinguished on the basis of DNA relatedness data, sequence divergence, and transformation experiments (Roberts et al., 1994). Such sole distinctions, even in the presence of DNA-based technology, particularly the lack of support by phenotypic differences, have been criticized as being inappropriate for speciation by Logan et al. (2009). A case in point was the use of DNA relatedness data that led to the creation of two additional species within *B. subtilis* (Ruiz-Garcia et al., 2005). These two species were later shown under a rigorous treatment of DNA-relatedness threshold values to be invalid and to represent heterotypic synonyms of *B. mojavensis* (Wang et al., 2007).

The strains in our work are all phenotypically endophytic (Bacon and Hinton, 2002), antagonistic to fungi, most producers of surfactin media, and clearly exhibit 95% and higher relatedness to the type *B. mojavensis*, 14698 (Fig. 2). Thus, these strains represent heterotypic synonyms of *B. mojavensis*, and following the conclusions of Logan et al. (2009), the minimal requirements for describing new taxa of aerobic, endospore-forming bacteria are met. These molecular analyses do validate maintaining *B. mojavensis* as a distinct species, agreeing with the work of Roberts et al. (1994) and Wang et al. (2007). This work substantiates the ability of this rep-PCR procedure to identify genotypic differentiation of a species and at the strain level, which was established earlier for several Eubacteria and Archaea (Cleland et al., 2007; Healy et al., 2005; Versalovic et al., 1991).

Multiple clades of *B. mojavensis* were observed, which did not reflect their deserts origins, suggesting that these strains are not clonal in origin. Strains from the Gobi desert represented the greatest diversity of surfactin among all. One clade from the Gobi desert on Pharmamedia media amended with nutrient broth was totally devoid of surfactin production on nutrient broth, which is suggestive of degrees of biochemical specializations at the strain level, and would have to be habitat delineated. If this speculation is correct, these clades represent ecotypes within any desert location and not clonal lines within a specific desert. The scatter plot of the strains suggests some indications of ecotypes with three major groups of ecotypes but also the presence of 25 distinct and separate ecotypes. However, these ecotypes are not defined on the basis of surfactin. With the exception of one isolate, NRRL B-14719, the strains from the Sahara desert represented the most consistent number of surfactin producing strains of all the desert strains (Table 1), although one strain, NRRL 14708, from the Gobi Desert produced the most surfactin of the 26 desert isolates on Pharmamedia amended with nutrient broth.

The surfactin fraction consists of a mixture of surfactins with varying acyl chain lengths. However, an actual limit of the chain length apparently is a function of a strain. Further, the C-15 acyl chain length was more active on membrane disruption, or biocontrol potential than the C-14, which is more responsible for physical activity such as reduction of surface activity as in bioremediation properties, e.g., oil spill cleanups (Bonmatin et al., 2003). In general, the Pharmamedia amended medium favored the production of larger amounts of these two isoforms over the nutrient medium. However, on this medium no strain produced the C-11 isoforms possibly due to the higher nutrient content of the Pharmamedia. Cultural ingredients and their amounts affect both the quantitative and qualitative components of the surfactin complex (Desai and Banat, 1997; Landy et al., 1948; Peypoux and Michel, 1992). When these two media were amended with several concentrations of nitrogen and carbohydrate sources, surfactin production was greatly reduced or not produced at all (results not reported).

It is not established if the terminal carbon atoms of the isomers reported in this work are in the normal (Fig. 1), the iso- or the anteiso configuration. Both the surfactant and biological activity are dependent on the chain length and its branching configuration. Yakimov et al. (1996) established that the order of activity was highest for normal, followed by iso- and finally the lowest was the anteiso-configuration. The order of amino acids also influences the activity of surfactin, with the maximum hydrophobic activity associated with the α-amino acid in the fourth position (Fig. 1). Further, surfactin is more active when hydrophobic amino acids are at the fourth position, such as leucine or isoleucine (Yakimov et al., 1996). However, since the components of the surfactin complex consist of several isoforms, the resulting *in planta* interactions are expected to be synergistic and to vary.

The inhibition of *F. verticillioides* growth by bacterial strains indicates no strict relationship to *in vitro* surfactin production. On the surfactin production media (a submerged fermentation), substrate utilization, and abundance are not the same as substrates abundance and availability on nutrient agar (a stationary fermentation). Nutrient limited environments influence not only the quantity of surfactins but also their qualitative expression (Peypoux and Michel, 1992). Further, the lack of inhibition might reflect the kinetics of surfactin production which is strain specific and differing from the majority of strains. That is, most strains produced surfactin primarily during the early logarithmic stage of fermentation and only under nitrogen and carbon starvation. However, some strains rapidly remove or absorb the surfactin immediately during the early logarithmic stage, and presumably decompose it (Lin, 1996). Thus, such cultures might not be inhibitory as bioassayed in these experiments on solid agar, but nevertheless will be scored positive for surfactin production during the early stage of fermentation but negative during the late stage when we performed our analysis.

The combinatorial chemistry of the surfactin molecule extends the activity spectrum to include both physical and biological applications. The physical activities include the ability to lower the surface tension of water, which extends its uses into a variety of environmental remediating uses such as fossil fuel recovery. Others include the ability to serve as a cation carrier across organic barriers, to produce ion channels in uncharged membranes, and to cause membrane dissolution (Desai and Banat, 1997; Georgiou et al., 1992). The biological uses are primarily those related to biocontrol potential, which is related to the ability of surfactins to cause membrane dissolution of pathogenic microbes and this ability is effective at very low concentrations. Thus, as a biocontrol agent, surfactin is effective against some Gram-positive and most Gram-negative bacteria, further it is anti-viral, anti-fungal, antitumor and anti-mycoplasma (Desai and Banat, 1997; Heerklotz and Seelig, 2001; Peypoux et al., 1999). Surfactin is one of a few viral antibiotics that are produced by bacteria. The revelations that the surfactins at very low concentrations can serve as elicitors of induced systemic resistance in plants (Jourdan et al., 2009; Ongena et al., 2007) adds complexity to mechanisms of action for the surfactins as biocontrol substances. A role in induced systemic resistance increases the value of the surfactins as a plant protector,

and further suggests that precise stoichiometric amounts of surfactin might not relate to *in vivo* biocontrol activity.

In summary, the results reported here characterized for the first time that strains of *B. mojavensis* produce a considerable number of isoforms of surfactin A. Thus, we identified another species of *Bacillus* as a producer of the surfactins, supporting the contention, *a priori*, that biosynthesis of this group of lipopeptide is characteristic of members of the genus *Bacillus*. These data indicate that 93% of isolates was capable of producing surfactin A on one or both media, although the isoforms varied, particularly those associated with strong biocontrol characteristics such as those with acyl chain lengths of C-15. There was a medium-induced production of surfactin as 42% of the strains were non-producers on nutrient broth as opposed to 6% were non-producers on the Pharmamedia. The molecular analysis of strains used in this study identified all as being strains of *B. mojavensis*, and that there are possible ecotypes. Further, these strains varied in their ability to produce isoforms of surfactins regardless of the desert from which they were isolated. The results suggest that the biocontrol potential of surfactins produced by this endophytic species may be as simple as involving events resulting from transient channeling of host plasma membrane to events at a higher level of complexity leading to defensive host reactions such as systemic resistance (Jourdan et al., 2009; Ongena et al., 2007). Data are needed documenting the endophytic production of surfactin and its relationship to plant protection, fungus infection and plant colonization. Finally, the data suggest that other substances might be involved indicating the need for additional chemistry of the inhibitory fractions.

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