The Journal of Microbiology (2011) Vol. 49, No. 4, pp. 603-609
DOI 10.1007/s12275-011-1044-y

Cyclic Lipopeptide Profile of Three *Bacillus subtilis* Strains; Antagonists of *Fusarium* Head Blight

Christopher A. Dunlap[1*], David A. Schisler[1], Neil P. Price[2], and Steven F. Vaughn[3]

[1]Crop Bioprotection, [2]Bioproduct and Biocatalysis, [3]New Crops and Processing, Research Units, National Center for Agricultural Utilization Research, Agricultural Research Service, USA
[‡]United States Department of Agriculture, 1815 North University Street, Peoria, Illinois 61604, USA

(Received January 27, 2011 / Accepted April 13, 2011)

The objective of the study was to identify the lipopetides associated with three *Bacillus subtilis* strains. The strains are antagonists of *Gibberella zeae*, and have been shown to be effective in reducing Fusarium head blight in wheat. The lipopeptide profile of three *B. subtilis* strains (AS43.3, AS43.4, and OH131.1) was determined using mass spectroscopy. Strains AS43.3 and AS43.4 produced the anti-fungal lipopeptides from the iturin and fengycin family during the stationary growth phase. All three strains produced the lipopeptide surfactin at different growth times. Strain OH131.1 only produced surfactin under these conditions. The antifungal activity of the culture supernatant and individual lipopeptides was determined by the inhibition of *G. zeae*. Cell-free supernatant from strains AS43.3 and AS43.4 demonstrated strong antibiosis of *G. zeae*, while strain OH131.1 had no antibiosis activity. These results suggest a different mechanism of antagonism for strain OH131.1, relative to AS43.3 and AS43.4.

***Keywords*:** *Bacillus*, iturin, fengycin, wheat, biocontrol, lipopeptides

Bacillus subtilis strains produce a broad spectrum of antimicrobial compounds (Mannanov and Sattarova, 2001). This activity has made them candidates for use as biological control agents against many plant pathogens (McSpadden Gardener and Driks, 2004). The ability of diverse set of *Bacillus* species to control plant diseases has led to registration and commercial development of several disease control products based in these strains. The integration of these products into integrated pest management systems has been shown to be an effective disease control strategy (Jacobsen *et al.*, 2004). The biological activity of these strains has often been associated with the production secondary metabolites, particularly antimicrobial cyclic lipopeptides.

Cyclic lipopeptides are a large class of biosurfactants, which are surface-active and have antimicrobial activity. This class consists of surfactins, fengycins (Vanittanakom *et al.*, 1986), iturins (Besson *et al.*, 1978) and similar compounds (Walker and Abraham, 1970; Peypoux *et al.*, 1986) and their structures are presented in Fig. 1. These compounds consist of a cyclic peptide or a peptide macrolactone of L and D amino acids and a variable hydrophobic alkyl chain. This lipophillic tail and hydrophilic head group are responsible for its surface-active and membrane binding activities. Compounds of the iturin family are lipoheptapeptides, which have strong antifungal and hemolytic activities (Bonmatin *et al.*, 2003). Fengycins are cyclic lipodecapeptides with antifungal activity (Vanittanakom *et al.*, 1986). In general, each lipopeptide family consists of several different isoforms arising from variable lengths and branching of their alkyl chains and conservative changes in their amino acids (e.g. Ala to Val) or stereochemistry. A more thorough review of their structural traits, physicochemical properties and application to biocontrol applications can be found in a recent review (Ongena and Jacques, 2008).

Recent advances in mass spectroscopy have provided a reliable method of identifying secondary metabolites in microbial systems (Erhard *et al.*, 1997). The application of mass spectroscopy research with *Bacillus* species is rapidly emerging due to its numerous secondary metabolites (Vater *et al.*, 2003) and its use to rapidly identify different *Bacillus* strains (Elhanany *et al.*, 2001). Cyclic lipopeptides are often identified using

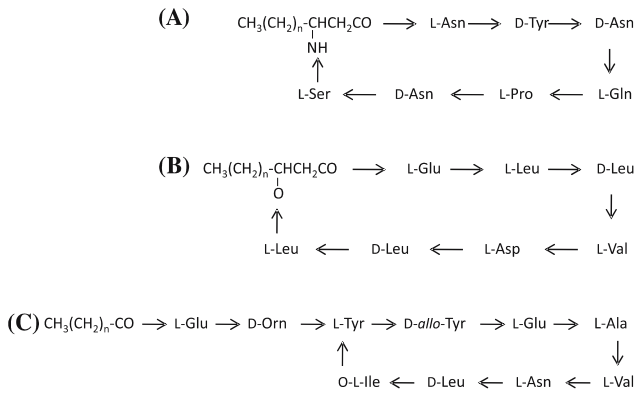

Fig. 1. Structures of common lipopeptides from *B. subtilis.* (A) Iturin, n=10-13; (B) Surfactin, n=10-12; (C) Fengycin, n=13-17.

* For correspondence. E-mail: christopher.dunlap@ars.usda.gov; Tel.: +1-309-681-6339; Fax: +1-309-681-6693
‡ Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer

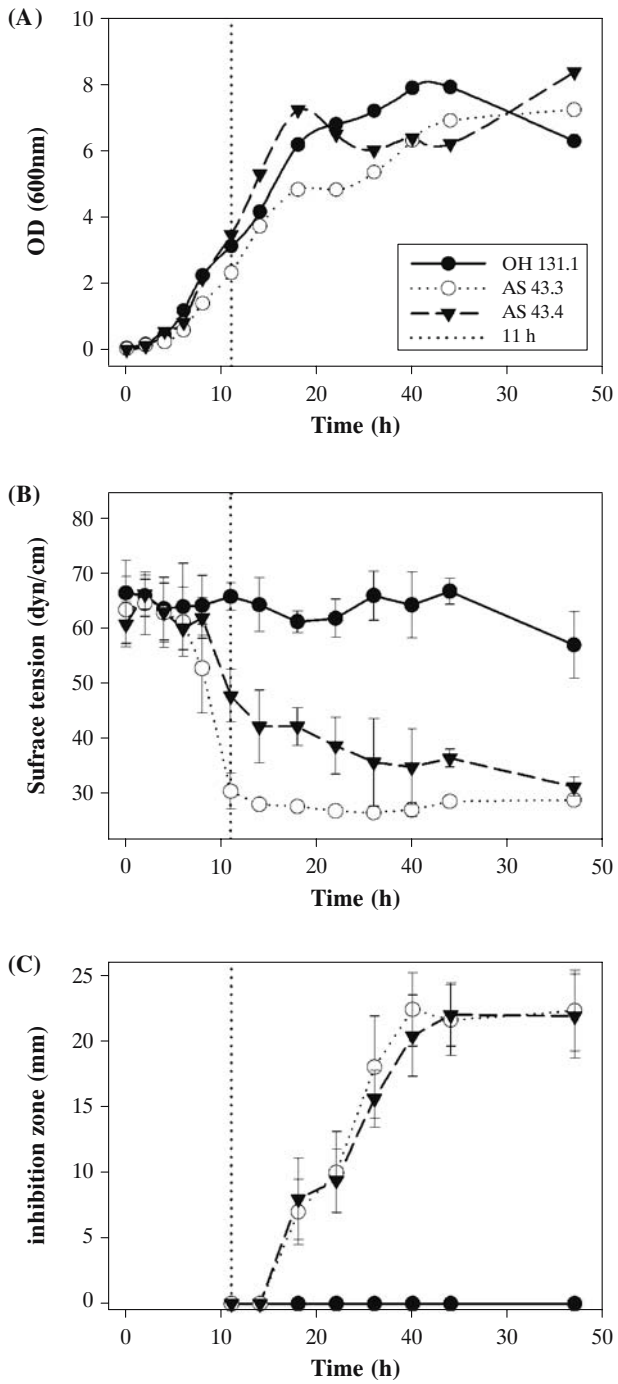

Fig. 2. Forty-eight hour growth curves, surface tension and anti-fungal activity of three *B. subtillis* strains: OH 131.1 (●), AS 43.3 (○), and AS 43.4 (▼). (A) Optical density measured at 600 nm (B) Surface tension of cell-free supernatants diluted 1:10 with water. (C) Zone of inhibition produced by cell-free supernatants against *G. zeae*. The results are an average of at least 6 replicate cultures. Error bars are represented at one standard deviation.

mass spectroscopy and are useful biomarkers for studies of *Bacillus* strains. In addition, mass spectroscopy has identified active metabolites in several *Bacillus* anti-fungal biocontrol systems, for recent examples: *Fusarium graminearum* (Wang *et al.*, 2007), *Gloeosprium gloeosporioides* (Cho *et al.*, 2003), *Rhizoctonia solani* (Yu *et al.*, 2002), *Collectotrichum demantium* (Hiradate *et al.*, 2002), and *Pythium ultimum* (Ongena *et al.*, 2005).

Our laboratory has previously identified three *B. subtilis* strains which are antagonists of *Gibberella zeae* (anamorph: *Fusarium graminearum*), the primary causal agent of *Fusarium* head blight in the United States (Khan *et al.*, 2001). These strains were isolated from the anthers of wheat, which are the site of infection onset. These strains have been shown to be effective in reducing disease incidence and severity (Khan *et al.*, 2001, 2004; Schisler *et al.*, 2002a, 2002b). The objective of the current study was to determine the identity of any cyclic lipopeptides associated with the antagonist activity of *G. zeae*. If present, our second objective was to assess their level of production during the time course of culturing.

Materials and Methods

Preparation of Bacillus cultures

B. subtilis strains AS 43.3 (NRRL B-30210), AS 43.4 (NRRL B-30211) and OH 131.1 (NRRL B-30212) were produced in a semi-defined liquid culture medium (SDLC) (Slininger *et al.*, 2007) for all assays. Samples of bacterial cultures frozen at -80°C in 10% glycerol were streaked for purity onto one-fifth strength tryptic soy broth agar, pH 6.8 (Difco Laboratories, USA). After 24 h incubation at 28°C, cells were removed from the surface of colonized plates using sterile cotton swabs and utilized to initiate precultures of each strain. Ten milliliter of SDLC in 50 ml Erlenmeyer flasks were inoculated to an optical density of approximately 0.2 at 620 nm wavelength light (A_{620}) for precultures of each antagonist. Precultures then were incubated in a shaker incubator (Inova 4230, New Brunswick Scientific, USA) at 25°C with a throw of 2.5 cm and 250 rpm for 24 h. Precultures of each strain were used to inoculate test cultures composed of 50 ml of SDLC in 250 ml Erlenmeyer flasks to an optical density of 0.1 (A_{620}). Test cultures were incubated as described for precultures and harvested at various stages of culture growth as needed for conducting analyses and bioassays.

Radial diffusion assay

Cell-free supernatants and isolated lipopeptides were analyzed for antifungal activity by placing one 25 µl drop on a V8 agar (CV8 agar) plate. The drops were allowed to absorb completely. Macroconidia of *G. zeae* isolate Z-3639, originally isolated from wheat in Kansas, were produced on CV8 agar under a regime of 12 h/day fluorescent light for 7 days at 24°C (Schisler *et al.*, 2002a). A conidial suspension of *G. zeae* isolate Z-3639 was prepared by overwhelming the surface of a colonized CV8 agar with 10 ml of 25 mM phosphate buffer (pH 7.0) and then drawing the suspension into a sterile pipet for transfer to a sterile centrifuge tube. The suspension was lightly sprayed onto the plates using a Sprā tool (Aervoe Industry, USA) so that a light mist coated the plate. The plates were then placed into the fluorescent light box for 4 days. The zone of inhibition was then measured at the widest radius using a ruler to the nearest mm.

Minimum inhibitory concentration of individual lipopeptides

The minimum inhibitory concentration of the lipopeptides was determined by the dilution method on CV8 agar plates, similar to the method described above. One 25 µl drop of each lipopeptide solution

(pH 7.0) was placed on the plate and allowed to absorb. Solutions started at 1,000 nM and were serially diluted by a factor of two until a concentration of 1.95 nM was reached (9 solutions). The plates were sprayed with a suspension of *G. zeae* and allowed to develop as described above. The minimum concentration that inhibited growth was recorded. The experiment was repeated five times and the median value reported.

Purification of antifungal cyclic lipopeptides

Lipopeptides were precipitated from cell-free supernatants with the addition concentrated HCl until pH 2.0 was reached. The precipitate was extracted with dichloromethane and the solvent was removed with a rotary vacuum evaporator. The recovered material was redissolved in water by the addition of NaOH until pH 7.0 reached. Individual lipopeptides were isolated from this solution using HPLC (Thermo Separations P2000 system) methods similar to those previously described (Gueldner *et al.*, 1988). Breifly, the aqueous solution was loaded onto Luna C18 10 μm, 100 Å column, 25 cm×4.6 mm (Phenomenex, USA) and eluted with a 30-100% gradient with acetonitrile (containing 0.1% trifluoroacetic acid) at 1 ml/min. Absorbance was monitored at 215 nm and fractions manually collected. Analytical determination of the cell-free supernatant lipopeptides was determined using the same chromatography methods. The supernatant was injected directly on to the column without any pretreatment. The previously isolated compounds were used as standards.

Matrix-assisted laser desorption ionization mass spectroscopy

MALDI-TOF mass spectra were recorded on a Bruker Daltonics Omniflex instrument (USA). Cell-free supernatants and isolated lipopeptides were mixed with an equal volume of matrix medium [10 mg/ml α-cyan-4-hydroxycinnamic acid in 70% aqueous acetonitrile containing 0.1% trifluoroacetic acid (v/v)]. The solution was spotted (1-2 μl) on the sample target and allowed to air dry. Ions were produced with a nitrogen laser (337 nm) and accelerated at 20 kV. Each mass spectra was produced by averaging more than 100 individual laser shots. External and internal calibration was performed with dextran oligomers (DP 4-10).

Surface tension measurements

The surface tension of cell-free supernatants was determined using the pendant drop method. Cell-free supernatants were diluted 1:10 with deionized distilled water and analyzed with FTA 4000 surface tension instrument (First Ten Angstroms Inc., USA). Measurements were made with a 22 gauge blunt needle with a 7 μl drop. The values reported represent an equilibrium surface tension determined 60 sec after drop formation. The reported values are the average of a minimum of 6 separate cultures.

Results

Growth curves, surface tension, and antagonism of *G. zeae* with the culture supernatant

Growth curves are reported in Fig. 2A for three *B. subtilis* strains: OH 131.1, AS 43.3, and AS 43.4. The strains exhibited similar growth profiles with each reaching the stationary phase in about 24 h. All three strains also reached similar final optical densities, around 7 OD (A_{620}) under these conditions. For latter comparisons, 11 h is considered mid-exponential growth phase, while 24 h and 48 h are considered early and late stationary phase, respectively.

The surface tension of the culture supernatant for each strain was monitored over the growth time course. Surface tension data can provide an estimate of lipopeptide production during the course of culturing (Gerson and Zajic, 1979). The results are reported in Fig. 2B. The results show OH 131.1 produced very little lipopeptides under these conditions, while both AS 43.3 and AS 43.4 show production of lipopeptides beginning in the early to mid log growth phase.

The ability of the cell-free supernatants of each strain to inhibit *G. zeae* was tested using a radial diffusion assay. The zone of inhibition produced from cell-free supernatants of each strain is reported in Fig. 2C. The results suggest OH 131.1 supernatant did not inhibit *G. zeae* under these conditions. Supernatants from strains AS 43.3 and AS 43.4 inhibited *G. zeae* in a similar manner. In all cases, there was little to no antifungal activity during the mid log growth phase (11-14 h).

Accumulation of individual lipopeptides

The accumulated lipopeptides in the cell-free supernatants for each strain were quantified using HPLC. The assignment of lipopeptides was on the basis of molecular weight using MALDI-TOF mass spectrscopy. Samples were analyzed at the

Table 1. Supernatant lipopeptides produced by three Bacillus strains during mid-exponential (11 h), early stationary (24 h) and late stationary (48 h) growth phase

Lipopeptide[a]	OH 131.1			AS 43.3			AS 43.4		
	11 h (mM)	24 h (mM)	48 h (mM)	11 h (mM)	24 h (mM)	48 h (mM)	11 h (mM)	24 h (mM)	48 h (mM)
Surfactins									
Leu/Ile-7, C_{13}	n/d	0.006±0.003	0.011±0.003	0.037±0.021	0.012±0.004	n/d	n/d	0.008±0.002	0.012±0.003
Leu/Ile-7, C_{14}	n/d	0.015±0.005	0.022±0.008	0.053±0.019	0.045±0.017	0.009±0.005	n/d	0.012±0.003	0.025±0.012
Leu/Ile-7, C_{15}	n/d	0.019±0.007	0.040±0.013	0.074±0.025	0.041±0.15	n/d	n/d	0.012±0.003	0.037±0.017
Iturins									
Asn-1, C_{14}	n/d	n/d	n/d	n/d	0.38±0.15	0.57±0.19	n/d	0.42±0.18	0.59±0.24
Asn-1, C_{15}	n/d	n/d	n/d	n/d	0.27±0.12	0.36±0.16	n/d	0.32±0.14	0.43±0.23
Fengycins									
Ala-6, C_{16}	n/d	n/d	n/d	n/d	1.77±0.42	1.92±0.37	n/d	1.63±0.32	2.24±0.35
Ala-6, C_{17}	n/d	n/d	n/d	n/d	1.63±0.26	1.88±0.42	n/d	1.57±0.38	2.28±0.56
Ala-6, C_{18}	n/d	n/d	n/d	n/d	0.46±0.17	0.57±0.14	n/d	0.55±0.15	0.70±0.25

[a] under these HPLC conditions, the limit of detection was 0.003 mM with detection at 205 nm (n/d<0.003 mM)

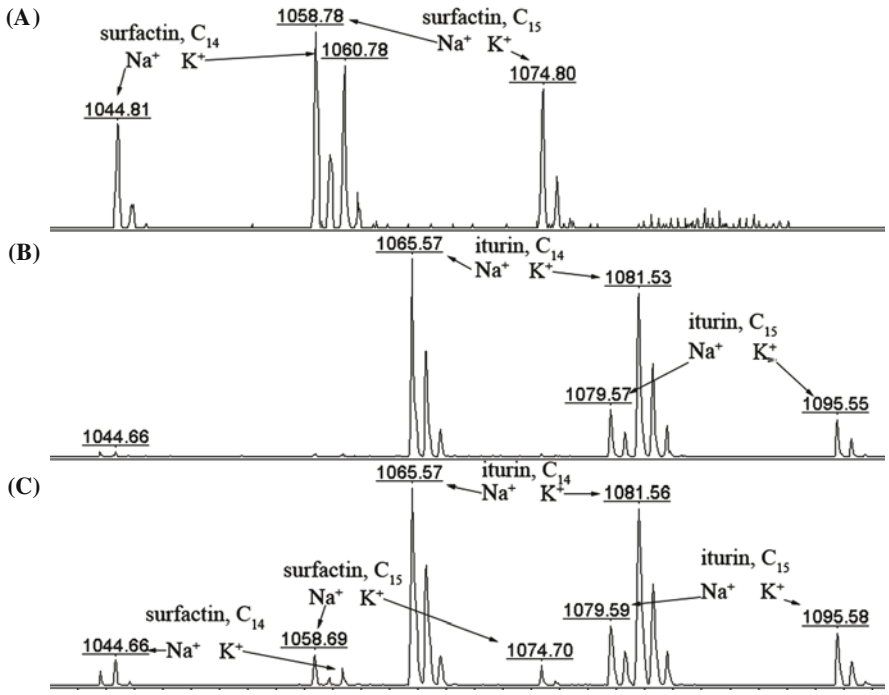

Fig. 3. MALDI-TOF mass spectra of the stationary phase culture media (48 h) of three *B. subtillis* strains: (A) OH 131.1, (B) AS 43.3, and (C) AS 43.4.

following time points: mid-log growth phase 11 h, early stationary phase 24 h, and late stationary phase 48 h. A summary of the accumulated lipopeptides at each timepoint for each strain is reported in Table 1. The results show strain OH 131.1 only produced one class of lipopeptides, surfactins. Strains AS 43.3 and AS 43.4, both produced surfactins, iturins, and fengycins. Figure 3 shows the partial mass spectra of the culture supernatant for the three strains after 48 h of culturing. In this experiment, MALDI mass spectroscopy detects the positive ions generated from the sample. The positive ions typically observed in these experiments are the parent molecule plus a cation, such as, $[M+H]^+$, $[M+Na]^+$, or $[M+K]^+$. The sodium and potassium adducts of surfactins and iturins can be seen for the three strains (Fig. 3). Figure 4 shows the fengycins, only found in strains AS 43.3 and AS 43.4. The iturins and fengycin family of lipopeptides increased in concentration for strains AS 43.3 and AS 43.4 with increasing culture time (Table 1). The surfactins produced by AS 43.4 and OH 131.1 also slowly accumulated, while surfactin production peaked for strain AS 43.3 during the exponential growth phase. The HPLC data of the exponential growth phase shows surfactins present in AS 43.3 and not detected in AS 43.4 or OH 131.1, this is consistent with surface tension data for the samples. AS 43.3 had a sharp drop in surface tension during the exponential growth phase, while AS 43.4 and OH 131.1 had modest changes in surface tension during this period. A notable observation is the fengycin is much less prominent than iturin in the mass spectra (~5 fold), even though HPLC shows fengycins in higher concentrations than iturins in the supernatant. Differences in ionization efficiencies between analytes have long been seen with all forms of mass spectroscopy. MALDI has technical limitations related to ionization efficiency, ion suppression for complex samples and co-crystallization behavior (Yates III, 2004), so the mass spectra do not always represent the relative concentration of analytes.

Minimum inhibitory concentration of individual lipopeptides

The anti-fungal activity against *G. zeae* was determined for each isolated lipopeptide, the results are summarized in Table 2. The results show the iturins were slightly more inhibitory than the fengycins, while the surfactins demonstrated no activity at the highest concentration (1 μM) tested. The values obtained are similar to those previously reported for fengycin

Table 2. Minimum inhibitory concentration of lipopeptides against *G. zeae*

	(nM)[a]
Surfactins	
Leu/Ile-7, C_{13}	not inhibitory at 1,000 nM
Leu/Ile-7, C_{14}	not inhibitory at 1,000 nM
Leu/Ile-7, C_{15}	not inhibitory at 1,000 nM
Iturins	
Asn-1, C_{14}	32.0
Asn-1, C_{15}	16.0
Fengycins	
Ala-6, C_{16}	64.0
Ala-6, C_{17}	64.0
Ala-6, C_{18}	32.0

[a] Values reported are the median of 5 observations

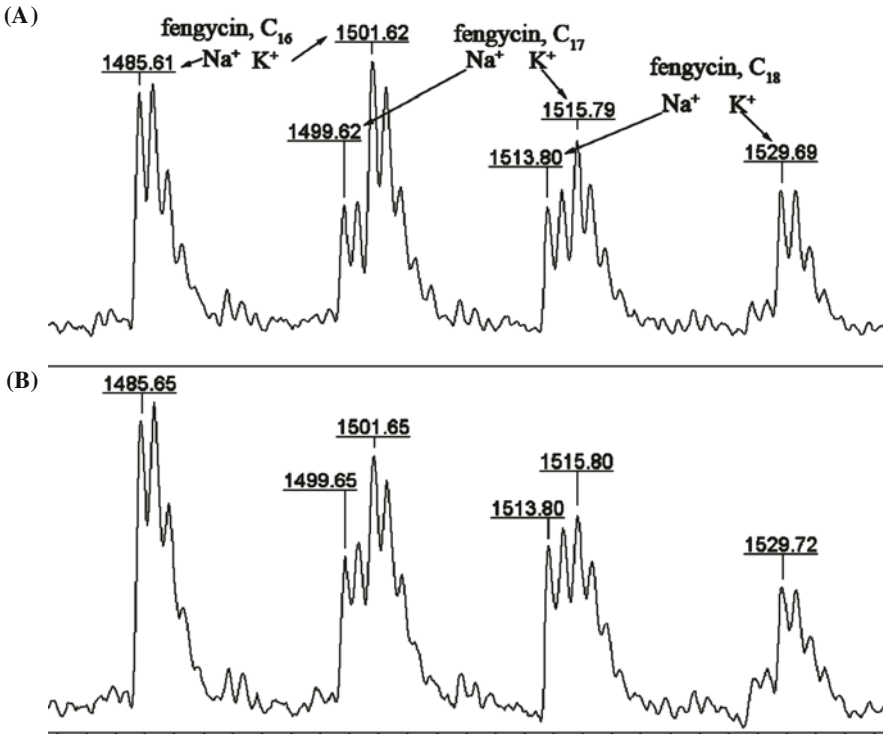

Fig. 4. MALDI-TOF mass spectra of the fengycins compounds produced by two *B. subtillis* strains in the staionary phase (48 h): (A) AS 43.3 and (B) AS 43.4.

(Vanittanakom *et al.*, 1986; Hu *et al.*, 2007) and iturin (Besson *et al.*, 1978) compounds against *Fusarium* species.

Discussion

Understanding the mode of action is important in developing a successful biological control agent. *B. subtilis* strains are known to produce more than two dozen antibiotics with peptide antibiotics the most prominent class (Stein, 2005). The importance of these antibiotics in biological control systems is well established and studied (Jacobsen *et al.*, 2004). Two recent studies have identified fengycin as the prominent lipopeptide in *B. subtilis* strains which antagonize *G. zeae* (Ramarathnam *et al.*, 2007; Wang *et al.*, 2007). The overall importance of antibiotic production to the survival of *B. subtilis* can be seen in its genomic contributions, with an estimated 5% of the genome dedicated to these tasks (Stein, 2005).

The three *B. subtilis* strains currently tested exhibited different lipopeptide profiles during their growth courses. The OH 131.1 strain was probably the most interesting strain in that it produced very little lipopeptides based on the surface tension and HPLC data. The mass spectroscopy data showed the only notable lipopeptide produced was surfactin. Surfactin has limited anti-fungal properties and the OH 131.1 culture supernatant did not inhibit *G. zeae* in our assays. This suggests OH 131.1 does not utilize lipopeptides in its antagonism of *G. zeae* or relies upon a signaling event to induce antibiotic production. OH 131.1 may antagonize *G. zeae* through direct competition of nutrients or through other mechanisms. *B. subtilis* strains have been previously shown to suppress plant disease through inducing host plant defenses (Kloepper *et al.*, 2004; Ryu *et al.*, 2007).

Both *B. subtilis* strains AS 43.3 and 43.4 produced surfactin, iturin and fengycin lipopeptides. The AS 43.3 culture rapidly reduced the surface tension of the culture media starting during the early exponential growth phase. While the AS 43.4 strain took until the late stationary phase to reach comparable levels of surface activity. This difference in surface tension can be attributed to higher surfactin production by AS 43.3 during the exponential growth phase. However, by the late stationary phase (48 h), AS 43.3 had reduced levels of surfactin in the supernatant, based on HPLC analysis. Determining the potential role or importance of surfactin in a biological control system is complicated by several factors. Surfactin production is widespread across most *B. subtilis* strains (Hofemeister *et al.*, 2004) and is closely connected to the competence development pathway (Hamoen *et al.*, 2003). The competence development pathway is an adaptive process, which allows DNA transfer between microorganisms. In addition, surfactin has been shown to be required for biofilm development, another adaptive strategy (Hofemeister *et al.*, 2004). Surfactin may also synergistically impact the anti-fungal activity of other lipopeptide, such as iturin (Hiraoka *et al.*, 1992). Iturin and fengycin production have been reported to be induced by starvation and regulated by the gene products associated with sporulation and the competence development pathway (Stein, 2005). Both AS 43.3 or 43.4 exhibited similar biocontrol efficacies in greenhouse and field assays (Schisler *et al.*, 2002b; Khan *et al.*, 2004). These strains were typically more successful in greenhouse assays which lacked overhead irrigation. This dif-

ference in activity has been attributed to the rain fastness of the antifungal lipopeptides. Rain events or overhead irrigation in field trials can easily wash away the water soluble lipopeptides. Ironically, rain and high humidity conditions during wheat anthesis are well correlated with disease outbreak (McMullen *et al.*, 1997). Strain OH 131.1 typically demonstrates the opposite behavior, where it performs less efficaciously in a greenhouse environments and better in a field environment (Khan *et al.*, 2001, 2004; Schisler *et al.*, 2002b). These differences in field and greenhouse efficacies, in addition to differences *in vitro* lipopeptide production, suggests strain OH 131.1 may utilize a different mode of action than strains AS 43.3 and AS 43.4.

This work identifies the lipopeptides profile associated with three *B. subtilis* strains during different growth stages. To our knowledge this represents the first quantitative lipopeptide profile (including each individual alkyl chain length isomers) of *B. subtilis* strains originally isolated from wheat heads. This work also reports a comprehensive list of minimum inhibitory concentrations of lipopeptides against *G. zeae*. This work identifies the iturin and fengycin lipopeptides as the antifungal compounds associated with the antibiosis activity of strains AS 43.3 and AS 43.4. The mechanism of antagonism for strain OH 131.1 remains unresolved and will be addressed in future research.

References

Besson, F., F. Peypoux, G. Michel, and L. Delcambe. 1978. Identification of antibiotics of iturin group in various strains of *Bacillus subtilis*. *J. Antibiot.* 31, 284-288.

Bonmatin, J.M., O. Laprevote, and F. Peypoux. 2003. Diversity among microbial cyclic lipopeptides: Iturins and surfactins. Activity-structure relationships to design new bioactive agents. *Comb. Chem. High Throughput Screening* 6, 541-556.

Cho, S.J., S.K. Lee, B.J. Cha, Y.H. Kim, and K.S. Shin. 2003. Detection and characterization of the *Gloeosporium gloeosporioides* growth inhibitory compound iturin A from *Bacillus subtilis* strain KS03. *FEMS Microbiol. Lett.* 223, 47-51.

Elhanany, E., R. Barak, M. Fisher, D. Kobiler, and Z. Altboum. 2001. Detection of specific *Bacillus anthracis* spore biomarkers by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* 15, 2110-2116.

Erhard, M., H. Von Dohren, and P. Jungblut. 1997. Rapid typing and elucidation of new secondary metabolites of intact cyanobacteria using MALDI-TOF mass spectrometry. *Nat. Biotechnol.* 15, 906-909.

Gerson, D.F. and J.E. Zajic. 1979. Microbial surfactants. *Process Biochem.* 14, 20-29.

Gueldner, R.C., C.C. Reilly, P.L. Pusey, C.E. Costello, R.F. Arrendale, R.H. Cox, D.S. Himmelsbach, F.G. Crumley, and H.G. Cutler. 1988. Isolation and identification of iturins as antifungal peptides in biological control of peach brown rot with *Bacillus subtilis*. *J. Agric. Food Chem.* 36, 366-370.

Hamoen, L.W., G. Venema, and O.P. Kuipers. 2003. Controlling competence in *Bacillus subtilis*: shared use of regulators. *Microbiology* 149, 9-17.

Hiradate, S., S. Yoshida, H. Sugie, H. Yada, and Y. Fujii. 2002. Mulberry anthracnose antagonists (iturins) produced by *Bacillus amyloliquefaciens* RC-2. *Phytochemistry* 61, 693-698.

Hiraoka, H., O. Asaka, T. Ano, and M. Shoda. 1992. Characterization of *Bacillus subtilis* RB14, coproducer of peptide antibiotics iturin a and surfactin. *J. Gen. Appl. Microbiol.* 38, 635-640.

Hofemeister, J., B. Conrad, B. Adler, B. Hofemeister, J. Feesche, N. Kucheryava, G. Steinborn, and *et al*. 2004. Genetic analysis of the biosynthesis of non-ribosomal peptide- and polyketide-like antibiotics, iron uptake and biofilm formation by *Bacillus subtilis* A1/3. *Mol. Genet. Genomics* 272, 363-378.

Hu, L.B., Z.Q. Shi, T. Zhang, and Z.M. Yang. 2007. Fengycin antibiotics isolated from B-FS01 culture inhibit the growth of *Fusarium moniliforme* Sheldon ATCC 38932. *FEMS Microbiol. Lett.* 272, 91-98.

Jacobsen, B.J., N.K. Zidack, and B.J. Larson. 2004. The role of *Bacillus*-based biological control agents in integrated pest management systems: Plant diseases. *Phytopathology* 94, 1272-1275.

Khan, N.I., D.A. Schisler, M.J. Boehm, P.E. Lipps, and P.J. Slininger. 2004. Field testing of antagonists of Fusarium head blight incited by *Gibberella zeae*. *Biol. Control.* 29, 245-255.

Khan, N.I., D.A. Schisler, M.J. Boehm, P.J. Slininger, and R.J. Bothast. 2001. Selection and evaluation of microorganisms for biocontrol of Fusarium head blight of wheat incited by *Gibberella zeae*. *Plant Dis.* 85, 1253-1258.

Kloepper, J.W., C.M. Ryu, and S. Zhang. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* 94, 1259-1266.

Mannanov, R.N. and R.K. Sattarova. 2001. Antibiotics produced by *Bacillus* bacteria. *Chem. Nat. Compd.* 37, 117-123.

McMullen, M., R. Jones, and D. Gallenberg. 1997. Scab of wheat and barley: A re-emerging disease of devastating impact. *Plant Dis.* 81, 1340-1348.

McSpadden Gardener, B.B. and A. Driks. 2004. Overview of the nature and application of biocontrol microbes: *Bacillus* spp. *Phytopathology* 94, 1244.

Ongena, M. and P. Jacques. 2008. Bacillus lipopeptides: versatile weapons for plant disease biocontrol. *Trends Microbiol.* 16, 115-125.

Ongena, M., P. Jacques, Y. Toure, J. Destain, A. Jabrane, and P. Thonart. 2005. Involvement of fengycin-type lipopeptides in the multifaceted biocontrol potential of *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.* 69, 29-38.

Peypoux, F., M.T. Pommier, and D. Marion. 1986. Revised structure of mycosubtilin, a peptidolipid antibiotic from *Bacillus subtilis*. *J. Antibiot.* 39, 636-641.

Ramarathnam, R., S. Bo, Y. Chen, W.G.D. Fernando, G. Xuewen, and T. De Kievit. 2007. Molecular and biochemical detection of fengycin- and bacillomycin D-producing *Bacillus* spp., antagonistic to fungal pathogens of canola and wheat. *Can. J. Microbiol.* 53, 901-911.

Ryu, C.M., J.F. Murphy, M.S. Reddy, and J.W. Kloepper. 2007. A two-strain mixture of rhizobacteria elicits induction of systemic resistance against *Pseudomonas syringae* and cucumber mosaic virus coupled to promotion of plant growth on *Arabidopsis thaliana*. *J. Microbiol. Biotechnol.* 17, 280-286.

Schisler, D.A., N.I. Khan, and M.J. Boehm. 2002a. Biological control of Fusarium head blight of wheat and deoxynivalenol levels in grain via use of microbial antagonists. *Adv. Exp. Med. Biol.* 504, 53-69.

Schisler, D.A., N.I. Khan, M.J. Boehm, and P.J. Slininger. 2002b. Greenhouse and field evaluation of biological control of Fusarium head blight on durum wheat. *Plant Dis.* 86, 1350-1356.

Slininger, P.J., D.A. Schisler, L.D. Ericsson, T.L. Brandt, M.J. Frazier, L.K. Woodell, N.L. Olsen, and G.E. Kleinkopf. 2007. Biological control of post-harvest late blight of potatoes. *Biocontrol Sci. Technol.* 17, 647-663.

Stein, T. 2005. *Bacillus subtilis* antibiotics: Structures, syntheses and specific functions. *Mol. Microbiol.* 56, 845-857.

Vanittanakom, N., W. Loeffler, U. Koch, and G. Jung. 1986. Fengycin - A novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3. *J. Antibiot.* 39, 888-901.

Vater, J., X. Gao, G. Hitzeroth, C. Wilde, and P. Franke. 2003. "Whole cell" - Matrix-assisted laser desorption ionization-time of flight-mass spectrometry, an emerging technique for efficient screening

of biocombinatorial libraries of natural compounds - Present state of research. *Comb. Chem. High Throughput Screening* 6, 557-567.

Walker, J.E. and E.P. Abraham. 1970. The structure of bacilysin and other products of *Bacillus subtilis*. *Biochem. J.* 118, 563-570.

Wang, J., J. Liu, H. Chen, and J. Yao. 2007. Characterization of *Fusarium graminearum* inhibitory lipopeptide from *Bacillus subtilis* IB. *Appl. Microbiol. Biotechnol.* 76, 889-894.

Yates III, J.R. 2004. Mass spectral analysis in proteomics. *Annu. Rev. Biophys. Biomol. Struct.* 33, 297-316.

Yu, G.Y., J.B. Sinclair, G.L. Hartman, and B.L. Bertagnolli. 2002. Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. *Soil Biol. Biochem.* 34, 955-963.