Identification of antifungal antibiotics of *Bacillus* species isolated from different microhabitats using polymerase chain reaction and MALDI-TOF mass spectrometry

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Abstract: Although many *Bacillus* species are known to be good antibiotic producers capable of acting as biocontrol agents, the underlying antimicrobial mechanisms are often poorly understood. In this study, 21 *Bacillus* strains, demonstrating over 50% mycelial inhibition against *Sclerotinia sclerotiorum* as well as significant control in plant assays, were examined for the presence of antibiotic biosynthetic genes. Primers specific for bacillomycin D, iturin A, surfactin, mycosubtilin, fengycin, and zwittermicin A were used to amplify biosynthetic genes from these bacteria using PCR. The majority of strains harbored surfactin (21/21) and iturin A (20/21) biosynthetic genes. Three strains (*Bacillus subtilis* 3057, *Bacillus amyloliquefaciens* BS6, and *Bacillus mycoides* 4079) were positive for bacillomycin D, whereas 4 strains (*B. subtilis* H-08-02, *B. subtilis* 3057, *B. amyloliquefaciens* BS6, and *B. mycoides* 4079) showed the presence of the fengycin biosynthetic gene. The zwittermicin A gene was detected in *B. mycoides* S, *Bacillus thuringiensis* BS8, and *B. amyloliquefaciens* BS6. Sequence analysis of purified PCR products revealed homology with corresponding genes from other *Bacillus* sp. in the GenBank database. Production of particular antibiotics in strains BS6, H-08-02, 3057, and 4079 was confirmed through matrix-assisted laser desorption ionization – time of flight – mass spectroscopy (MALDI-TOF-MS). This study revealed the equivalent capability of different *Bacillus* strains from various microhabitats to produce the above-mentioned antibiotics and highlights the possibility of using some strains as potential biocontrol agents under different microhabitats distant from their original habitat. Furthermore, it will enable researchers to develop rational strategies for the application of the antagonists and their metabolites within an agroecosystem. To the best of our knowledge, this is the first report of a *B. mycoides* strain that carries biosynthetic genes and produces fengycin and surfactin.

Key words: *Bacillus* spp., surfactin, iturin A, bacillomycin D, fengycin, zwittermicin A, PCR-detection, MALDI-TOF-MS.

Résumé : Même si plusieurs espèces de *Bacillus* sont reconnues comme étant de bonnes productrices d'antibiotiques capables d'agir comme agents de contrôle biologique, les mécanismes antimicrobiens sous-jacents sont souvent peu compris. Dans cette étude, 21 souches de *Bacillus* montrant un pouvoir d'inhibition de la croissance mycéliale de *Sclerotinia sclerotiorum* de plus de 50 % ainsi qu'un pouvoir de contrôle significatif lors d'analyses sur végétaux, ont été examinées relativement à la présence de gènes de biosynthèse d'antibiotiques. Des amorces spécifiques à la bacillomycine D, à l'iturine A, à la surfactine, à la mycosubtiline, à la fengycine et à la zwittermicine A ont été utilisées pour amplifier par réaction en chaîne par polymérase (PCR) les gènes de biosynthèse présents chez ces bactéries. La majorité des souches possédaient les gènes de biosynthèse de la surfactine (21/21) et de l'iturine A (20/21). Trois souches (*Bacillus subtilis* 3057, *Bacillus amyloliquefaciens* BS6 et *Bacillus mycoides* 4079) étaient positives à la bacillomycine D alors que 4 souches (*B. subtilis* H-08-02, *B. subtilis* 3057, *B. amyloliquefaciens* BS6 et *B. mycoides* 4079) révélaient la présence du gène de biosynthèse de la fengycine. Le gène de la zwittermicine A a été détecté chez *B. mycoides* S, *Bacillus thuringiensis* BS8 et *B. amyloliquefaciens* BS6. L'analyse de la séquence des produits de PCR purifiés a révélé une homologie avec les gènes correspondants chez d'autres espèces de *Bacillus* dans la base de données GenBank. La production d'antibiotiques particuliers dans les souches BS6, H-08-02, 3057 et 4079 a été confirmée par spectroscopie MALDI-TOF-MS. Cette étude a révélé que différentes souches de *Bacillus* provenant de microhabitats variés possèdent des capacités équivalentes de production des antibiotiques mentionnés plus haut et souligne la possibilité d'utiliser quelques unes de ces souches comme agents de contrôle biologique potentiels dans différents microhabitats éloignés de leur habitat original. De plus, elle permettra d'entreprendre de la recherche pour développer des stratégies rationnelles visant l'application d'antagonistes et de leurs métabolites dans un écosystème agricole. À notre connaissance, il s'agit de la première description d'une souche de *B. mycoides* portant des gènes de biosynthèse et produisant de la fengycine et de la surfactine.

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Mots-clés : *Bacillus* spp., surfactine, iturine A, bacillomycine D, fengycine, zwittermicine A, détection par PCR, MALDI-TOF-MS.

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Introduction

Many *Bacillus* species are capable of producing a wide variety of secondary metabolites that are diverse in structure and function. The production of metabolites with antimicrobial activity is one determinant of their ability to control plant diseases (Silo-suh et al. 1994). These metabolites can be ribosomal compounds such as subtilin (Zuber et al. 1993), subtilosin A (Babasaki et al. 1985), TasA (Stöver and Driks 1999), and sublancin (Paik et al. 1998). A variety of non-ribosomally produced small lipopeptides belonging to the surfactin family: surfactin and lichenysins (Kluge et al. 1988); the iturin family: iturin A, C, D, and E, bacillomycin D, F, and L, and mycosubtilin (Maget-Dana and Peypoux 1994); and the fengycin family: fengicins and plipastatins (Vanittanakom et al. 1986); as well as aminopolyols such as zwittermycin A (Milner et al. 1996), are also common. Antibiotics from the iturin family show strong antifungal and hemolytic activities with limited antibacterial activity (Maget-Dana and Peypoux 1994), whereas the activity shown by fengycin is specific against filamentous fungi and inhibits phospholipase A_2 (Nishikiori et al. 1986). Surfactin shows antiviral and antimycoplasma activities (Vollenbroich et al. 1997*a*, 1997*b*). Zwittermicin A is a linear aminopolyol (He et al. 1994) having a broad spectrum of activity against certain gram-positive, gram-negative, and eukaryotic microorganisms (Silo-Suh et al. 1998), and it also has an insecticidal activity similar to the protein toxin produced by *Bacillus thuringiensis* (Broderick et al. 2000, 2003).

There are reports that *Bacillus* spp. especially *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* have been effective against plant and fruit diseases caused by soil-borne, aerial, or post-harvest fungal diseases (Broggini et al. 2005; Szczech and Shoda 2004; Yoshida et al. 2002; Havenga et al. 1999; Korsten and De Jager 1995; Siddiqui and Mahmood 1992). In most situations, the involvement of antibiotics has been established (Stabb et al. 1996; Asaka and Shoda 1996; Moyne et al. 2001; Leclère et al. 2005). Detection of antibiotic production by a particular bacterium is important in determining its capability to be a good biocontrol agent for plant diseases. Screening candidate strains for particular antibiotic-encoding sequences followed by direct detection of the antibiotic profile of a particular bacterium provides a rapid approach in comparison with the traditional method of selection (de Souza and Raaijmakers 2003). PCR-based detection of antibiotic biosynthetic genes and MALDI-TOF-MS analysis are very appropriate approaches in this regard. MALDI-TOF-MS is an easy and less time consuming method for confirmation of antibiotic production when compared with the biochemical extraction, purification, and characterization of lipopeptides.

Previous studies carried out in our lab showed that *Bacillus* species isolated from different microhabitats (Table 1) had strong antifungal properties against *Sclerotinia sclerotiorum* and *Fusarium graminearum*. Duncan et al. (2006) isolated *Bacillus* species from sclerotia collected from sunflower fields in Manitoba. From them, 29 species showed in vitro antagonism to *S. sclerotiorum*. In a separate study, *B. subtilis* H-08-02, *B. cereus* L-07-01, and *B. mycoides* S-07-01 showed strong antifungal activity against *F. graminearum* (Table 1, Fernando et al. 2002; Ramarathnam et al. 2007). In a greenhouse study, Zhang (2004) found that *B. amyloliquefaciens* BS6 and *B. thuringiensis* BS8 almost completely reduced the leaf and stem infection caused by *S. sclerotiorum* in canola (*Brassica napus* L.). These interesting findings led us to hypothesize that one of the biocontrol mechanisms could be antibiotic production. Therefore, the current study was designed to detect the presence of antibiotic biosynthetic genes for the antibiotics surfactin, iturin A, bacillomycin D, fengycin, mycosubtilin, and zwittermicin A by specific PCR using 21 *Bacillus* species, which have shown >50% mycelial inhibition or leaf/stem infection reduction. Furthermore, the production of these antibiotics was confirmed through MALDI-TOF-MS analysis (Vater et al. 2003). Since the *Bacillus* strains used in this study were isolated from different microhabitats, this study would be important for the understanding of the niche specificity of the antibiotics produced by *Bacillus* species.

Materials and methods

Bacterial isolates and culture conditions

Twenty-one strains of *Bacillus* species with different origins, together with reference strains for each antibiotic tested (Table 1), were used in this study. Pure cultures of each bacterium were maintained in Luria–Bertani broth (LBB), amended with 20% glycerol (Fisher Scientific, Fair Lawn, New Jersey), and stored at –80 °C. New cultures of each bacterium were started by streaking them onto Luria–Bertani agar (LBA; Difco Laboratories, Detroit, Michigan) and incubated at 28 °C for 24 h.

DNA extraction, PCR analysis, and BLAST search

Each strain was inoculated into 5 mL aliquots of LBB and incubated at 32 °C on a rotary shaker at 180 r/min for 16–18 h. DNA extraction was done using a cetyltrimethylammonium bromide (CTAB)-based miniprep protocol (Ausubel et al. 1995) with slight modifications. PCR reactions were carried out using the methodology established by Ramarathnam et al. (2007) with the positive controls (Table 1) and the antibiotic specific primers (Table 2). Products were sequenced at the MacrogenUSA (Rockville, Maryland). Specific homologies for the sequences were searched for in the GenBank database through the NCBI n-BLAST search.

MALDI-TOF-MS analysis of the antibiotics present in the cell-surface extracts of bacterial strains *B. amyloliquefaciens* BS6, *B. subtilis* 3057, and *B. mycoides* 4079

Among the strains tested, BS6, H-08-02, S-07-01, L-07-

Table 1. A list of the bacterial isolates (*a*) and a list of the positive strains (*b*) used in the current study.

(*a*) Bacterial isolates.

Bacterium	%Inhibition	Origin	Reference
Bacillus subtilis 240	78.1	Sunflower sclerotia, Winnipeg, Manitoba	Duncan et al. 2006
B. subtilis 2031	66.5	Sunflower sclerotia, Winnipeg, Manitoba	Duncan et al. 2006
B. subtilis 2090	70.6	Sunflower sclerotia, Winnipeg, Manitoba	Duncan et al. 2006
B. subtilis 3057	78.2	Sunflower sclerotia, Winnipeg, Manitoba	Duncan et al. 2006
B. subtilis H-08-02	60.0	Wheat head	Fernando et al. 2002; Ramarathnam et al. 2007
Bacillus amyloliquefaciens 226	67.6	Sunflower sclerotia, Winnipeg, Manitoba	Duncan et al. 2006
B. amyloliquefaciens 248	79.5	Sunflower sclerotia, Winnipeg, Manitoba	Duncan et al. 2006
B. amyloliquefaciens 265	82.6	Sunflower sclerotia, Winnipeg, Manitoba	Duncan et al. 2006
B. amyloliquefaciens 268	76.4	Sunflower sclerotia, Winnipeg, Manitoba	Duncan et al. 2006
B. amyloliquefaciens 2033	78.5	Sunflower sclerotia, Winnipeg, Manitoba	Duncan et al. 2006
B. amyloliquefaciens BS6	100.0*	Canola leaves, Saskatchewan	Zhang 2004
Bacillus licheniformis 223	88.5	Sunflower sclerotia, Winnipeg, Manitoba	Duncan et al. 2006
B. licheniformis 266	74.1	Sunflower sclerotia, Winnipeg, Manitoba	Duncan et al. 2006
B. licheniformis 3039	56.0	Sunflower sclerotia, Winnipeg, Manitoba	Duncan et al. 2006
Bacillus mycoides 4079	55.5	Sunflower sclerotia, Winnipeg, Manitoba	Duncan et al. 2006
B. mycoides S-07-01	55.0	Wheat rhizosphere	Fernando et al. 2002; Ramarathnam et al. 2007
Bacillus thuringiensis BS8	87.0*	Canola leaves, Saskatchewan	Zhang, 2004
Bacillus cereus L-07-01	52.0	Wheat phyllosphere	Fernando et al. 2002; Ramarathnam et al. 2007
Strain G20	Unpublished	Alfalfa field soil, Carman, Manitoba	R. Li, unpublished data
Strain LRC	Unpublished	Wheat field soil, Carman, Manitoba	R. Li, unpublished data
Strain BLA	Unpublished	Wheat field soil, Carman, Manitoba	R. Li, unpublished data

(*b*) Positive strains used in the current study.

Reference strain	Antibiotic tested
Bacillus subtilis ATCC 13952	Iturin A and fengycin
B. subtilis ATCC 6633	Surfactin
B. subtilis strain Bs49	Bacillomycin D
Bacillus cereus strain UW85	Zwittermicin A

*Indicates the % inhibition of leaf infection; other values indicate the % inhibition of mycelial growth on PDA.

01, 3057, and 4079 showed the presence of multiple biosynthetic genes of antifungal lipopeptides surfactin, iturin A, bacillomycin D, and fengycin. Therefore, the surface extracts of these strains were subjected to MALDI-TOF-MS analysis for the detection of the production of the antifungal lipopeptides surfactin, iturin A, bacillomycin D, and fengycin. *Bacillus subtilis* strain ATCC13952 was used as the reference control for iturin A and fengycin. *Bacillus subtilis* strain ATCC6633 was used for surfactin and *B. subtilis* strain Bs49 was used for bacillomycin D. Preparation of bacterial surface extracts was done according to the methodology described by Vater et al. (2003). Strains were grown on Landy agar (LA; 20 g glucose, 5 g L-glutamic acid, 0.5 g $MgSO_4$, 0.5 g KCl, 1 g KH_2PO_4, 0.15 g $Fe_2(SO_4)_3 \cdot 6H_2O$, 5 mg $MnSO_4 \cdot H_2O$, 0.16 mg $CuSO_4 \cdot 5H_2O$, 15 g agar, 1000 mL distilled water, pH 6) at 28 °C for 2 days. One to two loops of bacterial cells from the LA plates were suspended in 500 µL of 70% acetonitrile with 0.1% trifluoroacetic acid for 1–2 min. The suspensions were vortexed gently to provide homogenous suspensions. The bacterial

Table 2. Specific primer sequences used for iturin A, bacillomycin D, surfactin, zwittermicin A (Ramarathnam 2007), and fengycin (Ramarathnam et al. 2007) in this study.

Antibiotic	Primer name	Primer sequence
Zwittermicin A	ZWITF2	TTGGGAGAATATACAGCTCT
	ZWITR1	GACCTTTTGAAATGGGCGTA
Iturin A	ITUD1F	GATGCGATCTCCTTGGATGT
	ITUD1R	ATCGTCATGTGCTGCTTGAG
Bacillomycin D	BACC1F	GAAGGACACGGCAGAGAGTC
	BACC1R	CGCTGATGACTGTTCATGCT
Surfactin	SUR3F	ACAGTATGGAGGCATGGTC
	SUR3R	TTCCGCCACTTTTTCAGTTT
Fengycin	FEND1F	TTTGGCAGCAGGAGAAGTTT
	FEND1R	GCTGTCCGTTCTGCTTTTTC
	FENA1F	GACAGTGCTGCCTGATGAAA
	FENA1R	GTCGGTGCATGAAATGTACG
	FENB2F	CAAGATATGCTGGACGCTGA
	FENB2R	ACACGACATTGCGATTGGTA

cells were pelleted by centrifugation and the cell-free supernatant was transferred to a new mocrocentrifuge tube and stored at 4 °C for further analysis. One microlitre of the surface extract was spotted onto the target of the mass spectrometer with an equal volume of matrix solution (dihydroxy benzoic acid (DHB); 50 mg in 1 mL of 70% acetonitrile / 0.1% trifluoroacetic acid. Samples were air-dried and subjected to MALDI-TOF-MS analysis. The MALDI-TOF-MS analysis was performed at the Department of Physics and Astronomy, University of Manitoba, Winnipeg, Manitoba. Analysis by MALDI-TOF-MS was performed on the QqTOF prototype mass spectrometer.

Results

PCR analysis and BLAST search

PCR products amplified from all strains using primer pair SUR3F/3R showed a 441 bp band corresponding to the surfactin antibiotic biosynthetic gene (Fig. 1A). Sequenced products of BS6, S-07-01, H-08-02, 4079, 3057, 223, 266, 2031, and 2033 showed a high similarity to GenBank accession No. AF534916.1 (surfactin synthetase gene cluster of *Bacillus* sp. CY22), also confirming the presence of that gene in other strains positive for surfactin. All strains, except 3039, showed a 647 bp band corresponding to the iturin A antibiotic biosynthetic gene, and the sequenced PCR products of strains BS6, BS8, H-08-02, L-07-01, 223, 248, 266, 3057, and 4079 (Fig. 1B) using primer pair ITUD1F/1R were highly homologous to the iturin A synthetase gene cluster of *Bacillus* sp. (GenBank acc. No. AF534617.1). Only strains BS6, 3057, and 4079 were positive for the presence of bacillomycin D biosynthetic genes showing the band at 875 bp (Fig. 1C) and their sequences were homologous to that of the *B. subtilis* bacillomycin D operon (GenBank acc. Nos. AY137375.1 and AJ576102.1). For zwittermicin A, 2 primer pairs were used, 677/678 for the zwittermicin A self-resistant protein (*ZmaR*) and ZWITF2/R1 for the zwittermicin A biosynthetic gene cluster. Strains BS6, BS8, and S-0701 showed the 1 kb band responsible for the *ZmaR* gene (Fig. 1D), whereas strains BS6 and BS8 only showed the presence of the zwittermicin A biosynthetic gene cluster (Fig. 1E). The sequences of the PCR products from strains BS6, BS8, and S-07-01 that were amplified using primers 677/678 showed high similarity to the zwittermicin A resistant protein gene of *B. thuringiensis* (GenBank acc. No. AY083683). Only 3 strains (H-08-02, 3057, and 4079) showed the 964 bp band corresponding to the fengycin biosynthetic gene cluster (Fig. 1D). This particular band from H-08-02 was amplified using primers FEND1F/1R and the bands for strains 3057 and 4079 were amplified using the primers FENB2F/2R and FENA1F/1R, respectively. The sequence of the corresponding band amplified from H-08-02 was homologous to the *fenD* gene of *B. subtilis* in the GenBank database (acc. No. AJ011849.1). None of the 21 strains was positive for the presence of the mycosubtlin biosynthetic gene.

MALDI-TOF-MS analysis

Strains showing the presence of multiple genes (BS6, BS8, H-08-02, S-07-01, 3057, and 4079) together with the positive strains were subjected to MALDI-TOF-MS analysis. Mass spectra obtained from strains BS6, H-08-02, 3057, and 4079 showed very clear peak clusters (Figs. 2 and 3). The particular mass peaks and the corresponding antibiotics are listed in Table 3. However, an interesting phenomenon could be observed in the mass spectra of 4 different strains, BS6, H-08-02, 4079, and 3057. In the spectrum of BS6, peaks corresponding to surfactin, iturin A, and bacillomycin D could be detected in more or less similar intensities, whereas, in the spectrum of H-08-02, only peaks representing surfactin were prominent. In the spectra of 3057 and 4079, peaks representing bacillomycin D and iturin A were prominent, respectively (Figs. 2 and 3). The group of peaks at m/z = 1449.7, 1463.8, 1477.8, and 1491.8 detected in the mass spectra of strains BS6, H-08-02, 4079, and 3057 could be attributed to the protonated forms of C15–C17 fengycins, and the peaks at m/z = 1501.7, 1515.7, 1529.7, 1539.7, and 1543.8 in the mass spectra of the these strains corresponded to sodium and potassium adducts of C16–C17 fengycin (Fig. 4). The result of this study suggests that the major antibiotic produced by a particular strain might be induced by the environmental signals and affected by the genetic makeup of the bacterium.

Discussion

All the strains used in the current study have shown >50% mycelia inhibition of *S. sclerotiorum* and (or) *F. graminearum* under laboratory conditions and, in addition, some have shown significant greenhouse and field control of stem rot disease in canola and fusarium head blight in wheat (Fernando et al. 2002; Zhang 2004; Duncan et al. 2006). Furthermore, the buried sclerotia treated with strains 2031, 2033, 4079, 266, and 223 showed significant reduction in their germination ability when they were recovered from soil after a period of time and cultured on PDA (W.G.D. Fernando and P. Parks, unpublished data). Therefore, one of the mechanisms of control was hypothesized to be antibiosis. This study was carried out to test this hypothesis where the presence of biosynthetic genes encoding the antimicrobial antibiotics surfactin, iturin A, bacillomycin D, fengycin, and zwittermicin A was investigated by PCR, and their pro-

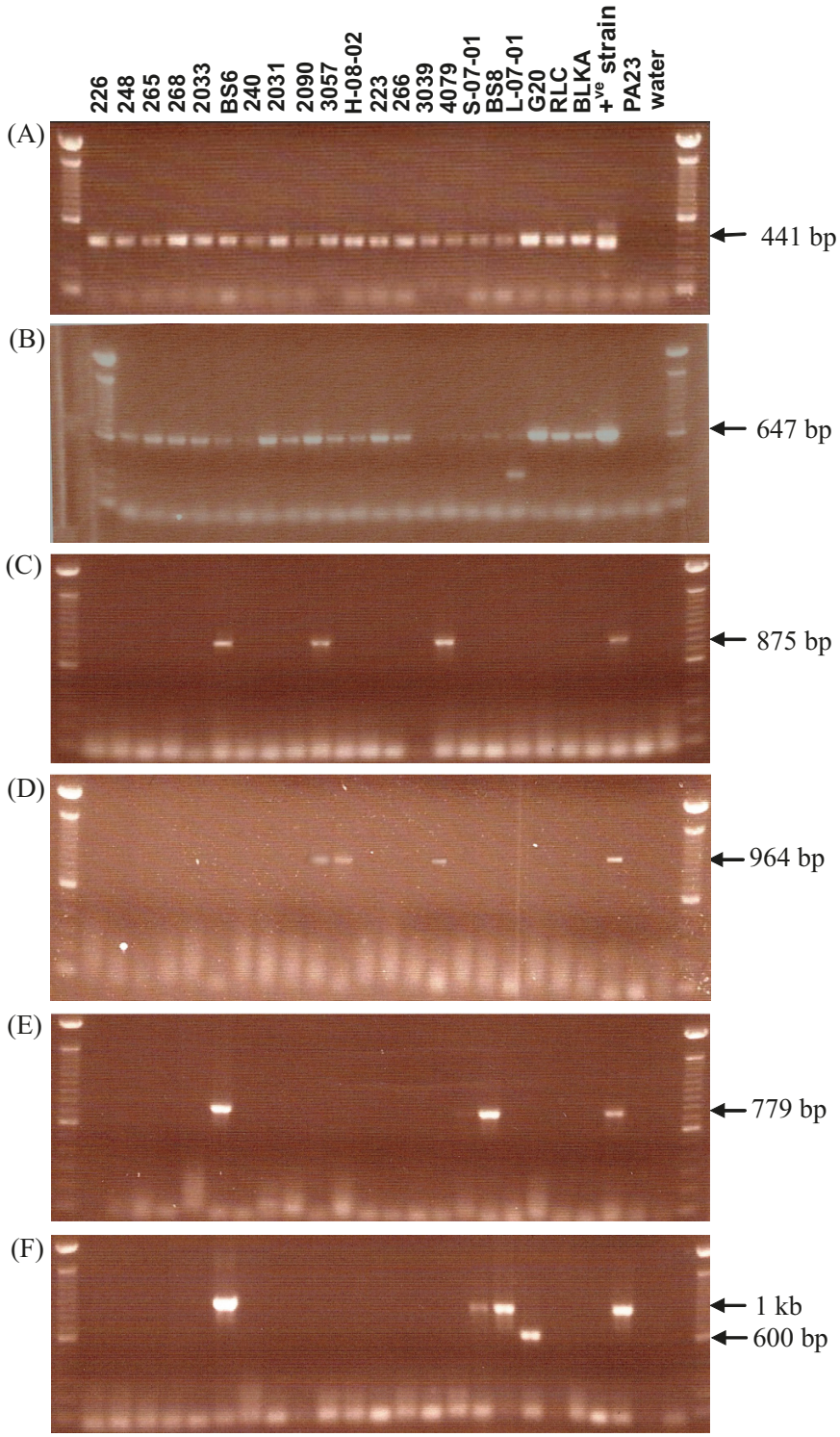
Table 3. Peak masses corresponding to particular antibiotics produced by the bacterial strains *Bacillus amyloliquefaciens* BS6, *Bacillus mycoides* 4079, *Bacillus subtilis* 3057, and *B. subtilis* H-08-02 detected by MALDI-TOF-MS analysis.

Strain	Peak mass (*m*/*z*)	Antibiotics
BS6	1008.6, 1022.6, 1036.6; 1046.6, 1060.6, 1074.6; 1053.6, 1067.6, 1081.6, 1069.5, 1083.5, 1097.5, 1095.5, 1111.6; 1449.7, 1463.8, 1477.8, 1491.8; 1501.7, 1515.7, 1529.7, 1539.7, 1543.8	Surfactins; surfactins; bacillomycin D; fengycins; fengycins
4079	1008.6, 1022.6, 1036.6; 1046.6, 1060.6, 1074.6; 1053.6, 1067.6, 1081.6, 1069.5, 1083.5, 1097.5, 1095.5, 1111.6; 1071.6, 1085.6; 1093.6, 1096.6, 1098.6, 1107.6, 1109.6, 1123.6; 1449.7, 1463.8, 1477.8, 1491.8; 1501.7, 1515.7, 1529.7, 1539.7, 1543.8	Surfactins; surfactins; bacillomycin D; iturin A; iturin A; fengycin; fengycin
3057	1008.6, 1022.6, 1036.6; 1046.6, 1060.6, 1074.6; 1031.5, 1045.6, 1059.6; 1053.6, 1067.6, 1081.6, 1069.5, 1083.5, 1097.5, 1095.5, 1111.6; 1449.7, 1463.8, 1477.8, 1491.8; 1501.7, 1515.7, 1529.7, 1539.7, 1543.8	Surfactins; surfactins; bacillomycin D; bacillomycin D; fengycin; fengycin
H-08-02	1008.6, 1022.6, 1036.6; 1046.6, 1060.6, 1074.6; 1071.6, 1085.6; 1449.7, 1463.8, 1477.8, 1491.8	Surfactins; surfactins; iturin A; fengycin

duction was confirmed by MALDI-TOF-MS. PCR-based detection of specific antibiotic-producing bacteria is favored over the time-consuming and laborious method of random isolation and screening procedures (de Souza and Raaijmakers 2003). The detection of a particular antibiotic biosynthetic operon in a bacterial strain would signify the function of the operon and the production of the antibiotics. Any loss of function of the operon would be an exception to the rule (McSpadden Gardener et al. 2001). MALDI-TOF-MS confirms the function of the operon detected by PCR. In the current study, production of antibiotics by some of the strains was not observed through MALDI-TOF-MS even though the specific genes were detected by PCR. A similar observation was made by Leenders et al. (1999) where they detected conserved genes specifying surfactin and fengycin synthetase (Kunst et al. 1997) in *B. subtilis* 168, but they did not produce the corresponding antibiotics because of a mutation in the gene encoding 4′-phosphopantetheinyl transferase (Mootz et al. 2001). The same reason might be involved in the observation made in the current study where there could have been mutations occurring in any gene of the gene cluster or this simply explains the failure in the method. In the current PCR screening, almost all isolates, including *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. mycoides*, and *B. cereus/thuringiensis*, were positive for the antibiotics surfactin and iturin A. This result supports earlier findings in which these 2 antibiotics were detected from a wide variety of *Bacillus* sp. (Vater et al. 2002; Mikkola et al. 2004; Thaniyavarn et al. 2003; Goursaud 1989; McInerney et al. 1990; Ramarathnam 2007), including all the above-mentioned species except from *B. mycoides*. This implies that surfactin and iturin A are among the most common lipopeptide antibiotics produced by *Bacillus* sp. The specific surface- and membrane-active properties of the surfactins help bacteria to form biofilms; therefore, surfactin is thought to perform developmental functions rather than defense functions in the environment (Hofemeister et al. 2004). This might be the reason that most *Bacillus* strains produce surfactins. To the best of our knowledge, this study reports the production of surfactin and iturin A by a *B. mycoides* strain for the first time. Detection of *zmaR* genes in *B. thuringiensis* BS8, *B. cereus* L-07-01, and *B. mycoides* S-07-01 is in accordance with the findings of a previous study (Zhang et al. 2006). In addition, the present study detected the presence of *zmaR* and antibiotic biosynthetic genes for zwittermicin A and surfactin in *B. amyloliquefaciens*, which had not been detected by Ramarathnam (2007). The presence of the fengycin biosynthetic gene and the production of the antibiotic in the cell-surface extract were observed only in a few strains. According to previous studies, fengycin was reported to be produced by *B. subtilis* (Vater et al. 2002; Ramarathnam et al. 2007) and *B. amyloliquefaciens* (Koumoutsi et al. 2004). In the current study a *B. mycoides* strain (4079) was observed to contain the biosynthetic gene and produce fengycin, which has not been recorded yet in the literature. Although the antibiotic biosynthetic gene for fengycin in strain BS6 was not amplified using any of the 7 primer pairs used, MALDI-TOF-MS analysis showed a group of mass peaks corresponding to isoforms of fengycin. One possible explanation for this observation might be the failure of the PCR method. As reported in other previously published research, bacillomycin D was detected in *B. subtilis* (Moyne et al. 2001; Ramarathnam et al. 2007) and *B. amyloliquefaciens* (Ramarathnam et al. 2007) strains in the current study. However, its production by a *B. mycoides* strain (4079), as observed in the current study, is not recorded in the literature. The detection of very low percentages of bacillomycin D, fengycin, and zwittermicin A producing bacteria among the studied isolates indicates that a comparatively low percentage of bacteria in the environment has the ability of producing these 3 antibiotics and, within the same species, the capability is rather variable. Not detecting the mycosubtilin biosynthetic gene cluster in any of the strains tested might be an indication of the extreme rareness of mycosubtilin production among *Bacillus* antagonists.

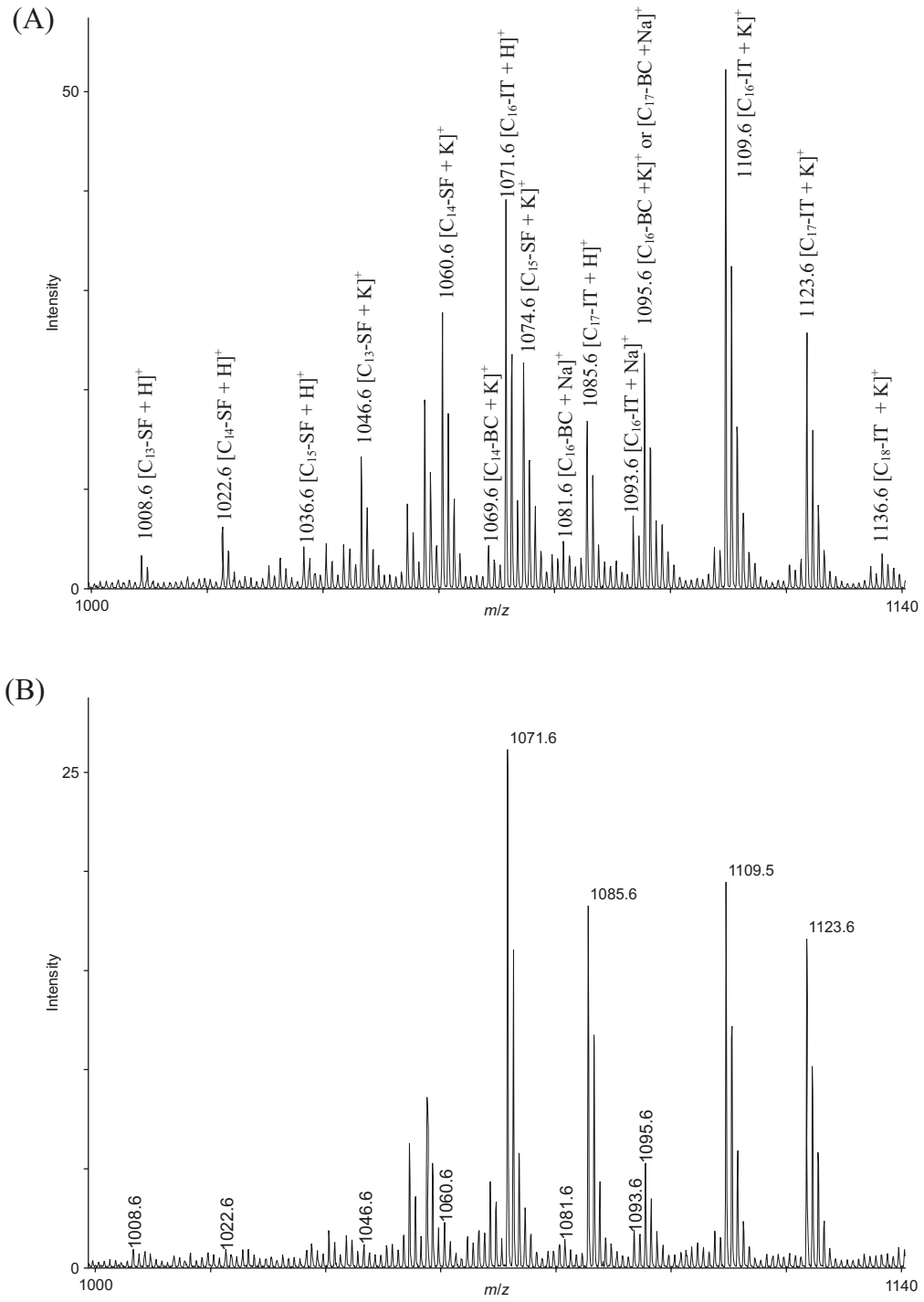
The detection of antibiotic biosynthetic genes and their production by the *Bacillus* strains in the current study revealed that one of the mechanisms responsible for biocontrol exhibited by these strains could be antibiosis. Among the strains tested, the contribution of antibiotics in biocontrol could be very high for strains BS6, H-08-02, 3057, and 4079, in which several antibiotics were detected. However, there are strains that have shown >75% mycelia inhibition or control of *S. sclerotiorum* and (or) *F. graminearum* (Fernando et al. 2002; Duncan et al. 2006) but were detected as

Fig. 1. PCR amplification of biosynthetic genes corresponding to surfactin (A), iturin A (B), bacillomycin D (C), fengycin (D), zwittermicin A (E), and zwittermicin A self-resistant protein, ZmaR (F). Positive controls were *Bacillus subtilis* (for iturin A and fengycin ATCC 13952), *B. subtilis* ATCC 6633 (for surfactin), *B. subtilis* strain Bs49 (for bacillomycin D), and *Bacillus cereus* strain UW85 (for zwittermicin A). *Pseudomonas chlororaphis* strain PA23 was an additional negative control. PCR products were separated on 1% agarose gel for 30 min and visualized by a transilluminator.



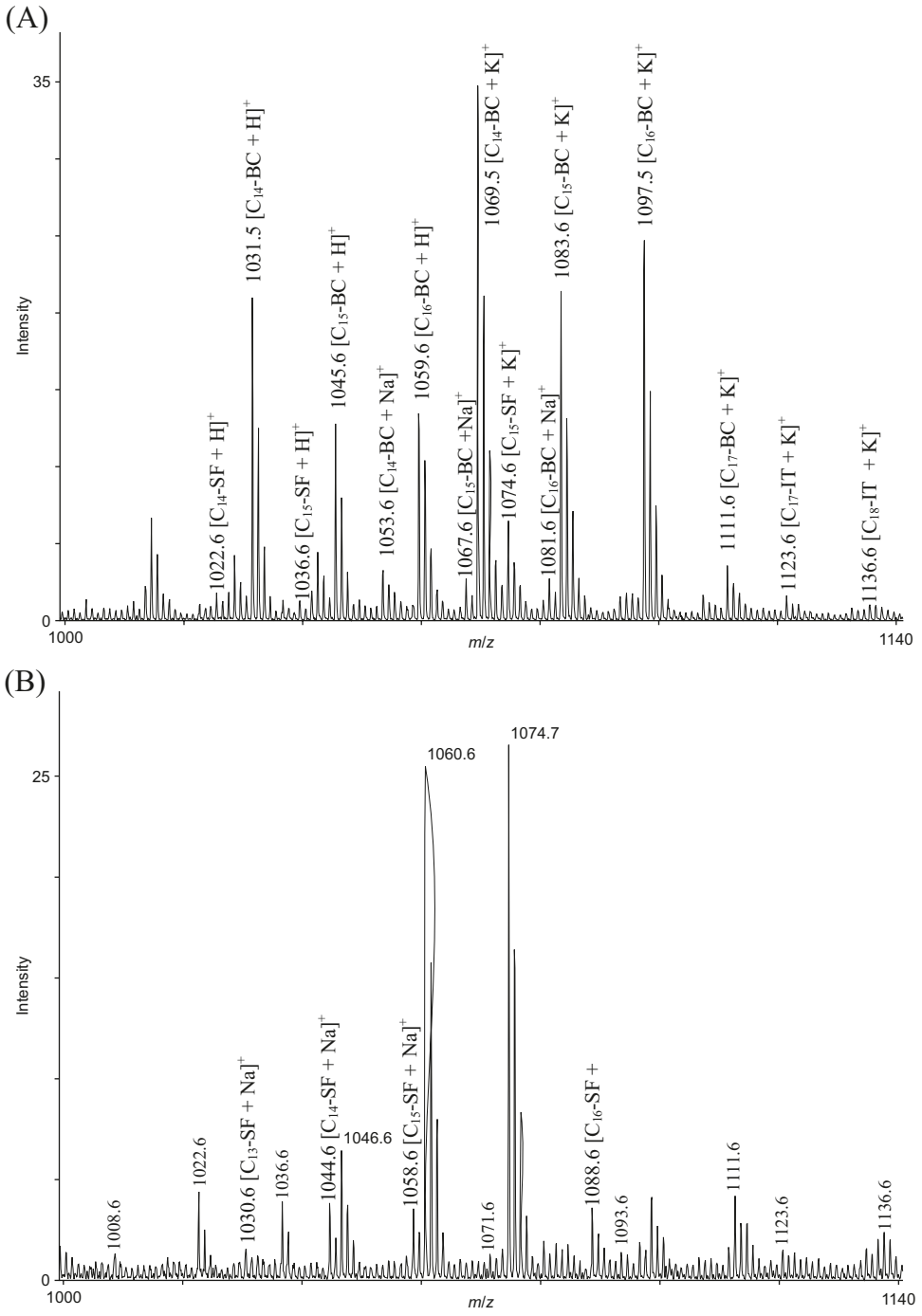
producing only surfactin and iturin A. Therefore other types of antibiotics, which may be novel (not reported in literature), could be involved in the biocontrol ability of those strains or different mechanisms such as competition for an ecological niche or a substrate, and induced systemic resistance (ISR) might be involved. For example, bacterial volatiles induced systemic resistance in *Arabidopsis* (Ryu et al. 2004). Mari et al. (1996) reported that the reduction of gray

Fig. 2. MALDI-TOF mass spectra of bacterial cell-surface extracts obtained from strains *Bacillus amyloliquefaciens* BS6 (A) and *Bacillus mycoides* 4079 (B) analyzed for the presence of the lipopeptide antibiotics surfactin (SF), iturin A (IT), and bacillomycin D (BC). Mass ranges were (*m*/*z*) 1008–1074, 1070–1150, and 1030–1111 for SF, IT, and BC, respectively.



mold caused by *Botrytis cinerea* in pears by *B. amyloliquefaciens* 2TOE is due to competition for nutrients. In the phyllosphere, competition for nutrients mainly affects the spore germination of the pathogen (Yoshida et al. 2002). Furthermore, the level of production of surfactin and iturin A by these strains could be higher than other strains. On the other hand, surfactin and iturin (iturin A, bacillomycin D, etc.) are found to exhibit strong antifungal activity (Maget-Dana and Peypoux 1994; Peypoux et al. 1999), and surfactin is considered the most powerful biosurfactant known, which causes detergentlike action on biological membranes (Carrillo et al. 2003). Surfactin and members of the iturin family, therefore, might have the ability to exert significant pressure on pathogens. In several other studies, the biocontrol ability of surfactin and iturin A were documented, which supports this suggestion. For example, Phae et al. (1990) reported that more than 23 types of plant pathogens were suppressed in vitro by an iturin A and surfactin pro-

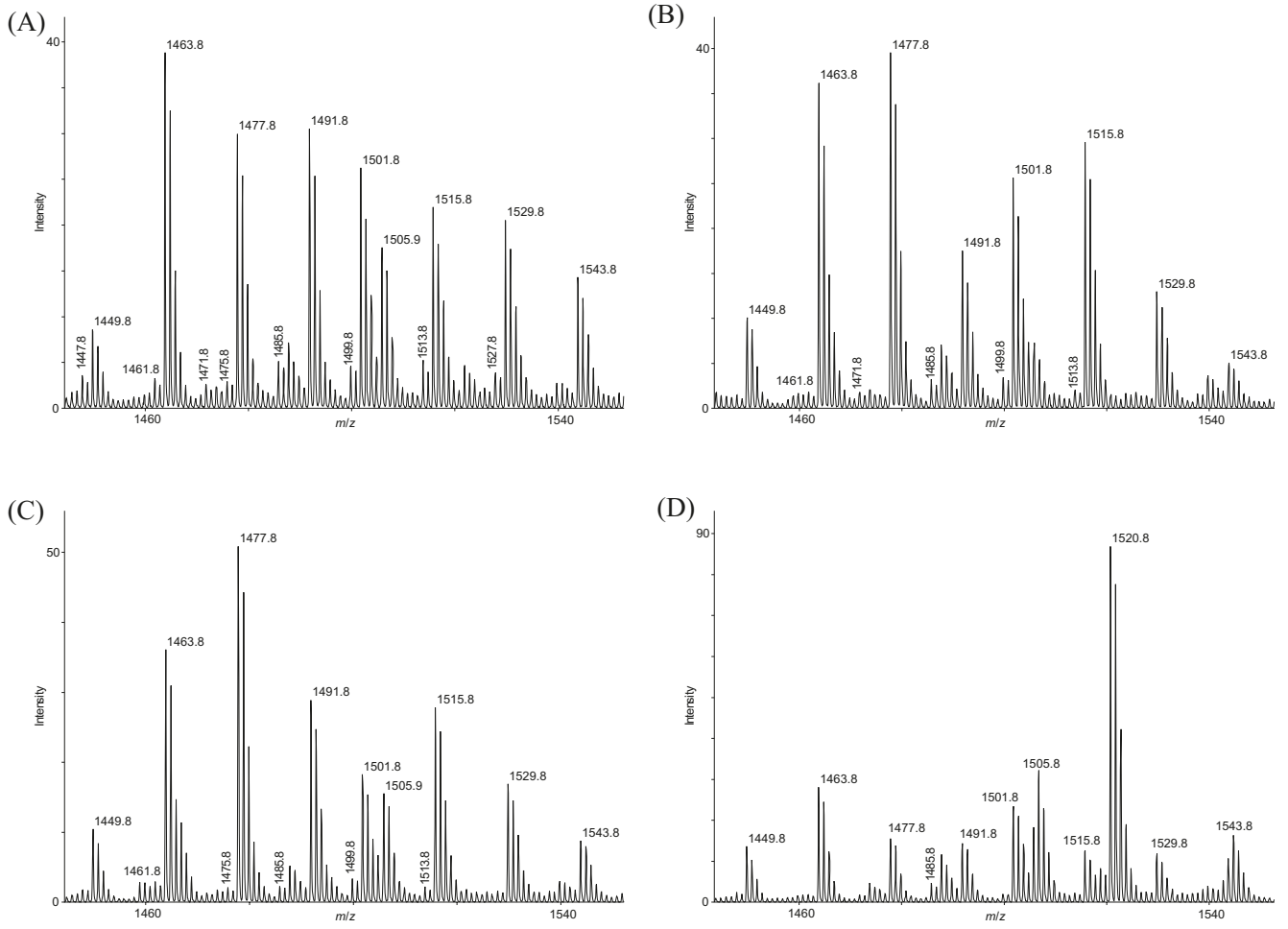
Fig. 3. MALDI-TOF mass spectra of bacterial cell-surface extracts obtained from strains *Bacillus subtilis* 3057 (A) and *Bacillus subtilis* H-08-02 (B) analyzed for the presence of the lipopeptide antibiotics surfactin (SF), iturin A (IT), and bacillomycin D (BC). Mass ranges were (*m*/*z*) 1008–1074, 1070–1150, and 1030–1111 for SF, IT, and BC, respectively.



ducing (Hiraoka et al. 1992; Asaka and Shoda 1996) *B. subtilis* isolate. There are also instances in which some antibiotics showed stronger activity than others. Asaka and Shoda (1996) observed a significant stronger suppressive activity of iturin A against plant pathogens than for surfactin. Kim et al. (2004) reported a stronger antifungal activity for fengycin-producing strains in comparison with those producing surfactin and iturin A. Koumoutsi (2006) reported a comparatively higher effect of bacillomycin D than of fengycin against various phytopathogenic fungi. The inconsistent performances exhibited by the same antibiotics in different studies suggest that their performance might be altered by various undetermined factors. Although the reasons have yet to be explored, differences in the media used could be one possible explanation.

Another interesting phenomenon observed in this study

Fig. 4. MALDI-TOF mass spectra of bacterial cell-surface extracts obtained from strains *Bacillus subtilis* H-08-02 (A), *Bacillus amyloliquefaciens* BS5(B), *Bacillus mycoides* 4079 (C), and *Bacillus subtilis* 3057 (D) analyzed for the presence of the lipopeptide antibiotic fengycin. The mass range was (*m*/*z*) 1047–1543. Mass peaks 1449.8, 1461.8, 1463.8, 1475.8, 1477.8, 1491.8, and 1505.8 correspond to protonated forms of C15–C17 fengycin, whereas the other mass peaks correspond to the sodium and potassium adducts of C15–C17 fengycin.



was the difference in the types of antibiotics dominating in the mass spectra of different strains. For example, in the spectrum of BS6, peaks corresponding to surfactin, iturin A, and bacillomycin D were prominent, whereas in the spectrum of H-08-02, peaks representing surfactin are prominent. In the spectra of 3057 and 4079, peaks representing bacillomycin D and iturin A dominated, reflecting the higher intensity values of these antibiotics in the particular surface extracts. This indicates that, although these strains have biosynthetic genes for surfactin, iturin A, and bacillomycin D, at a certain time only 1 or 2 antibiotics are produced at high concentrations, and the major antibiotic in action under a given set of conditions may vary from strain to strain. Since all the strains were subjected to similar conditions in the current study, it can be inferred that the extent to which a particular strain captures the environmental signals that induce the expression of different antibiotic genes differ among strains. Alternatively, the production of some antibiotics can be delayed relative to others (Hofemeister et al. 2004), which might have caused the differences in peak intensities. Although a particular strain can produce more than 1 antibiotic, a synergistic performance of the antibiotics is occasional (Koumoutsi 2006). This may be due to the lower production of some antibiotics over others under a given set of conditions. Strain 4079 is a good example of this where it was found to produce 4 of the antibiotics tested, but the amount of *S. sclerotiorum* mycelia inhibition shown was only 55%. The MALDI-TOF mass spectrum of strain 4079 showed high intensities for only iturin A and fengycin.

The results of this study together with those of other relevant studies emphasize the synergistic effect of several mechanisms in biocontrol along with antibiotics. However, at a certain time and under certain environmental conditions, antibiotic production may play the main role in its biocontrol of plant pathogens. This study revealed the equal capability of different *Bacillus* strains from various microhabitats to produce a variety of antibiotics and highlights the possibility of using some strains as potential biocontrol agents under diverse microhabitats distant from their origin. Using the *Bacillus* sp. as a biocontrol agent has several advantages

over other organisms because of its resistance to heat and desiccation (Hou et al. 2006). Understanding the mechanisms of biocontrol at the molecular and biochemical level will enable us to develop rational strategies for the application of the antagonists and their metabolites within an agroecosystem.

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