Mass spectrometric analysis of lipopeptides from *Bacillus* strains isolated from diverse geographical locations

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Received 31 October 2006; revised 16 January 2007; accepted 21 February 2007.
First published online 10 April 2007.

DOI:10.1111/j.1574-6968.2007.00702.x

Editor: Simon Silver

Keywords
Bacillus; MALDI; MS; lipopeptides; biomarkers; surfactins; iturins.

Abstract

Matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF MS) has been applied to characterize lipopeptide biomarkers from 54 different strains of *Bacillis* from most taxa within the *Bacillis subtilis–Bacillis licheniformis* clade, isolated from seven geographic locations on five continents. Even the most narrowly defined taxa are diverse in terms of the lipopeptide profiles. Many strains produce previously identified compounds with known antimicrobial properties (e.g. polymyxins and bacitracins), whereas other compounds represent novel classes that were hitherto unknown. Of particular interest is the novel 942/958 Da biomarkers produced by *B. s.* spizizeni desert strains and several type strains.

Introduction

Several reports have shown the effectiveness of matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF MS) to identify small molecule biomarkers that distinguish and characterize different *Bacillus* strains and species (Hathout *et al.*, 1999; Gebhardt *et al.*, 2002; Bonmatin *et al.*, 2003; Madonna *et al.*, 2003; Vater *et al.*, 2003; Pittenauer *et al.*, 2006). Most identified *Bacillus* biomarkers are cyclic peptides and lipopeptides, typically in the mass range of 800–5000 Da, and several have distinct antimicrobial properties. Prominent among these are surfactins, iturins (bacillomycins), polymyxins, fengycins (plipastatins), kurstakins, and bacitracins (Stein, 2005). MALDI-TOF MS profiles of lipopeptides have been used to identify rapidly various strains of *Bacillus* sp. (Ryzhov *et al.*, 2000; Williams *et al.*, 2002; Madonna *et al.*, 2003; Pabel *et al.*, 2003). The approach of identifying species- and strain-specific biomarkers is particularly convenient when combined with MALDI-TOF MS fingerprinting of whole bacterial cells (Leenders *et al.*, 1999; Vater *et al.*, 2002).

The chemical structures, biosynthesis, and properties of *Bacillus* nonribosomal peptide antibiotics have been ably reviewed (Stein, 2005). Iturins and surfactins are composed of a seven-member cyclic peptide backbone containing an integral 3-amino or 3-hydroxy acyl group. The specific residues in the peptide part are significantly different between iturin and surfactin families. The major iturins A and B differ at residue 1 (L-Asn or L-Asp, respectively), and the related bacillomycins have considerably more structural diversity. Residues D-Tyr-2, D-Asn-3, and L-Thr/L-Ser-7 are conserved for both families, but bacillomycins D, F, and L also differ in residues 1, 4, 5, and 6. The surfactins are comprised of an L-Glu-1, L-Leu-2, D-Leu-3, L-Val-4, L-Asp-5, D-Leu-6 backbone, with the only diversity arising in residue 7 (L-Leu, L-Val, or L-Ile for surfactins A, B, and C, respectively). A fourth cyclic lipopeptide, called mycosubtilin, is structurally similar to bacillomycins D and F. These structural differences underlie the respective antifungal and antibacterial properties of the iturins (bacillomycins) and surfactins (Stein, 2005). The acyl chains for these compounds are typically 10–15 carbons long, and include several branched-chain fatty acids (Bland, 1996). Recently, a new class of lower mass (about 900 Da) lipopeptides called kurstakins were identified from *Bacillus thuringiensis* (Hathout *et al.*, 2000). These are characterized by an internal lactone linkage between the C-terminus and an internal Ser residue and, like iturins and fengycins, have pronounced antifungal activity. Higher mass cyclic lactone lipopeptides called maltacines (C1a, 1504.8 Da; C1b 1518.9 Da) have also been isolated from *Bacillus subtilis* species (Hagelin, 2005).

Polymyxins, fengycins, and bacitracins are cyclic nonapeptides, decapeptides, and dodecapeptides, respectively. At least eleven different polymyxins are known, many of which are obtained commercially from *Bacillus polymyxa*. The cyclic peptide backbone of the polymyxins is rich in γ-diaminobutyric acid, with the N-terminal diaminobutyric acid amide-linked to 6-methylheptanoyl or 6-methyloctanoyl groups. Bacitracins are also produced commercially as topical antibacterial agents, and are synthesized by both *B. subtilis* and *Bacillus licheniformis* species. The commercial preparation is a mixture of at least nine bacitracins, with bacitracin A ($C_{66}H_{103}N_{17}O_{16}S$) as the major constituent. A characteristic feature of the bacitracins is an N-terminal amide linkage to thiazoline-4-carboxylic acid. The fengycins are distinguished from the polymyxins and bacitracins by their biological activity against pathogenic fungi. Fengycins are characterized by an ester-linkage between the C-terminal Ile residue and the phenolic group of D-Tyr, the third residue in the peptide chain. Like most other lipopeptides, the fengycins are terminally *N*-acylated with a β-hydroxy fatty acid, typically containing 15, 16, or 17 carbon atoms. The molecular formulae and calculated accurate masses for these compounds are shown in Table 1. Typically, each compound gives rise to several MS peaks, due to sodium and potassium adduct ($[M+Na]^+$ and $[M+K]^+$) ions and protonated $[M+H]^+$ ions.

Here, the typing of 54 different *Bacillus* strains is described by MALDI-TOF MS analysis of their lipopeptide biomarkers. The strains included in the study were isolated from a diversity of habitats from five continents. The strains represent most of the recognized species and subspecies of the *B. subtilis–B. licheniformis* clade (Palmisano *et al.*, 2001).

Table 1. Molecular Formulae and Calculated Masses* for Lipopeptide Biomarkers from *Bacillus* species

Lipopeptide	Mr (Da)	Lipopeptide	Mr (Da)
Kurstakins		Fengycins	
1. $C_{39}H_{64}N_{11}O_{12}$	878.47	Val-7, C_{16}	1462.80
2, 3. $C_{40}H_{66}N_{11}O_{12}$	892.41	Val-7, C_{17}	1476.82
4. $C_{41}H_{68}N_{11}O_{12}$	906.50	Val-7, C_{18}	1490.84
Surfactins		Iturins	
Leu/Ile-7, C_{13}	1007.65	Asn-1, C_{10}	986.48
Leu/Ile-7, C_{14}	1021.66	Asn-1, C_{12}	1014.51
Leu/Ile-7, C_{15}	1035.68	Asn-1, C_{14}	1042.54
		Asn-1, C_{15}	1056.56
Polymyxins			
B_1, $C_{56}H_{98}N_{16}O_{13}$	1202.75	Bacitracins	
B_2, $C_{55}H_{96}N_{16}O_{13}$	1188.73	A, $C_{66}H_{103}N_{17}O_{16}S$	1421.75
D_1, $C_{50}H_{93}N_{15}O_{15}$	1143.70	B, $C_{65}H_{101}N_{17}O_{16}S$	1407.73
D_2, $C_{49}H_{91}N_{15}O_{15}$	1129.68	C, $C_{64}H_{99}N_{17}O_{16}S$	1393.72
E_1, $C_{53}H_{100}N_{16}O_{13}$	1168.77	D, E, $C_{63}H_{97}N_{17}O_{16}S$	1379.70
E_2, $C_{52}H_{98}N_{16}O_{13}$	1154.75		

*Typically, $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$ ions are observed at 1.008, 22.9898, and 38.9637 mU above the molecular masses. The monoisotopic masses were calculated using IsoPro 3.0.

Moreover, the strains include closely related ecotypes that were first demarcated by analysis of DNA sequences and then confirmed by differences in their microhabitats (Cohan, 2006). Several biomarkers of pharmaceutical interest have also been identified in multiple strains.

Experimental

Bacillus strains and growth conditions

All strains were obtained from the US Department of Agriculture's Agricultural Research Service (USDA–ARS) Culture Collection in Peoria, Illinois, where they are permanently archived (http://nrrl.ncaur.usda.gov). The accession numbers for these strains are listed in Table 2. Strains were grown on tryptone-glucose-yeast extract (TGY) medium (5 g tryptone, 5 g yeast extract, 1 g glucose, 1 g K_2HPO_4, sterile H_2O to 1000 mL, final pH 7.0) at 28 °C in both liquid culture and on agar plates. The strains originate from seven geographical regions: Paulett Island, Antarctica; the Athabasca Glacier, Canada; Tierra del Fuego, Argentina; Evolution Canyon, Israel; Mojave Desert, USA; Death Valley, USA; and the Sahara Desert, Tunisia. The geographic source of each strain is listed in Table 2. This study includes 49 isolates, as well as the types strain of five taxa: *Bacillus atrophaeus*, *B. licheniformis*, *Bacillus vallismortis*, *B. subtilis* ssp. *subtilis*, and *B. subtilis* ssp. *spizizenii*.

MALDI-TOF MS

MALDI-TOF mass spectra were recorded on a Bruker-Daltonic Omniflex instrument operating in reflectron mode. Ion source 1 was set to 19.0 kV, and source 2 to 14.0 kV, with lens and reflector voltages of 9.20 and 20.00 kV, respectively. Samples were typically dried under a lamp onto a conventional 49-place stainless-steel target. The matrix used was 2,5-dihydrobenzoic acid (2,5-DHB). A 200-ns pulsed ion extraction was used with matrix suppression up to 200 Da. The instrument was calibrated externally on a series of malto-oligosaccharides (degree of polymerization 3–13). Laser excitation was at 337.1 nm, typically at 60% of 150 μJ maximum output, and 80 shots were accumulated.

Results and discussion

Whole bacterial cells of 54 *Bacillus* strains and species from seven different geographical locations, and their corresponding liquid culture-grown supernatants, were analyzed by MALDI-TOF MS. The compounds found fell into three mass ranges: 850–950 m/z, which includes kurstakins; 1000–1100 m/z, which includes surfactins and iturins; and 1450–1550 m/z, which includes fengycins, polymyxins, and bacitracins. Examples of the mass spectra of compounds are shown in Figs 1 and 2.

Table 2. Lipopeptide biomarkers identified from various *Bacillus* species* by MALDI-TOF MS

Strain	Kurstakins	942, 958	Iturins	Surfactins	1093, 1107	1121, 1137	Polymyxins	1304, 1320	Bacitracin	Fengycins	Other
B. sp.; Athabasca Glacier											
B-41086	–	–	–	c, m	c	c	–	–	–	–	–
B-41087	–	–	–	c, m	c	c	–	–	–	–	–
B-41088	–	–	–	c, m	c	c	–	–	–	c	–
B-41089	–	–	–	c, m	–	c	c, m	–	–	–	–
B-41090	–	–	–	c	–	c, m	m	m	–	c, m	–
B. sp.; Tierra del Fuego											
B-41091	–	–	–	c, m	–	–	m	–	–	c	–
B-41092	–	c	c	c	c	m	m	m	–	–	–
B-41093	–	–	–	c, m	–	c, m	c, m	–	–	–	–
B. sp.; Antarctica											
B-41094	c	–	m	–	–	–	–	m	–	–	–
B-41095	c	–	–	–	–	–	m	m	–	–	–
B-41096	–	–	–	c, m	–	m	m	–	–	–	–
B-41097	–	–	–	c	–	m	m	–	–	–	–
B-41098	c	–	–	c	–	m	m	–	m	–	–
B-41099	–	–	–	–	–	–	–	–	m	–	–
B. s. spizizenii; Mojave, Death Valley, and Sahara											
B-23055	–	c	–	c, m	c	m	m	–	–	–	–
B-23056	c	c	c	c, m	–	–	m	m	c, m	c, m	–
B-23057	c	c	–	c	–	–	–	–	–	–	–
Type strains†											
B-23049	–	c	–	c	c	–	m	–	–	–	–
NRS-213	–	c	c	–	c	m	m	–	–	c,m	–
NRS-1264	c	c	–	c	–	m	m	–	–	–	c
B-4219	c	c	–	c	–	m	m	m	–	–	–
B-14893	c	c	–	c, m	–	–	m	–	–	c	–
Evolution Canyon, Israel											
B-41277	–	–	c	c, m	–	m	m	m	–	m	–
B-41279	–	–	c	c, m	c	c, m	c, m	c, m	–	m	–
B-41282	–	–	c	c, m	c	c	–	–	c	c	c
B-41284	–	–	–	c, m	m	m	–	–	–	m	c
B-41288	c	–	c, m	c, m	c	m	m	–	c, m	m	–
B-41294	–	–	c, m	c, m	c	c, m	m	m	m	m	m
B-41295	–	–	c	–	c	c, m	m	m	–	–	–
B-41299	c	–	c, m	c, m	c, m	–	m	–	c	–	c
B-41300	c	–	c	c	c	c	m	–	c	–	c
B-41304	c	–	c	c	–	m	m	–	–	m	m
B-41305	–	–	–	c, m	c	c	–	–	–	c	–
B-41307	c	–	–	c, m	–	–	m	–	c	c	c
B-41311	c	–	c	c, m	–	m	m	–	c, m	m	–
B-41315	c	–	–	c, m	–	–	m	–	c	c	–
B-41317	–	–	–	c, m	–	m	m	m	–	m	–
B-41318	c	–	c	c	–	m	–	–	–	–	–
B-41320	–	–	c	c, m	c	c, m	m	m	–	–	m
B-41321	–	–	–	c, m	c	c, m	m	m	–	c, m	m
B-41323	c	–	–	m	–	m	m	m	–	m	–
B-41326	–	–	c	c, m	–	m	–	–	–	–	–
B-41327	c	–	c	c, m	–	m	–	m	c	c, m	m
B-41330	c, m	–	c	c, m	–	–	–	–	–	c, m	c
B-41331	–	–	c	c	c, m	c, m	m	m	–	–	m
B-41335	–	–	–	m	–	m	m	–	–	m	m
B-41336	–	–	–	m	–	m	m	m	m	m	m
B-41337	c, m	–	c, m	c, m	–	–	m	–	–	–	c
B-41339	–	–	c	c, m	–	m	m	m	m	m	m
B-41342	–	–	c	c, m	c	m	m	m	–	c, m	m
B-41344	–	–	c	c, m	–	c, m	m	m	–	m	m

Table 2. Continued.

Strain	Kurstakins	942, 958	Iturins	Surfactins	1093, 1107	1121, 1137	Polymyxins	1304, 1320	Bacitracin	Fengycins	Other
B-41346	–	–	c	c, m	–	c, m	c, m	m	–	m	m
B-41349	–	–	–	c, m	–	–	m	–	m	–	m
B-41351	–	–	c	c, m	–	m	–	m	–	c, m	m

*Note that all strain numbers are prefixed by 'NRRL' in the USDA-ARS culture collection (http://nrrl.ncaur.usda.gov).
†B-23049, *B. s. spizizenii*; NRS-213, *B. atrophaeus*; NRS-1264, *B. licheniformis*; B-4219, *B. s. subtilis*; B-14893, *B. vallismortis*.

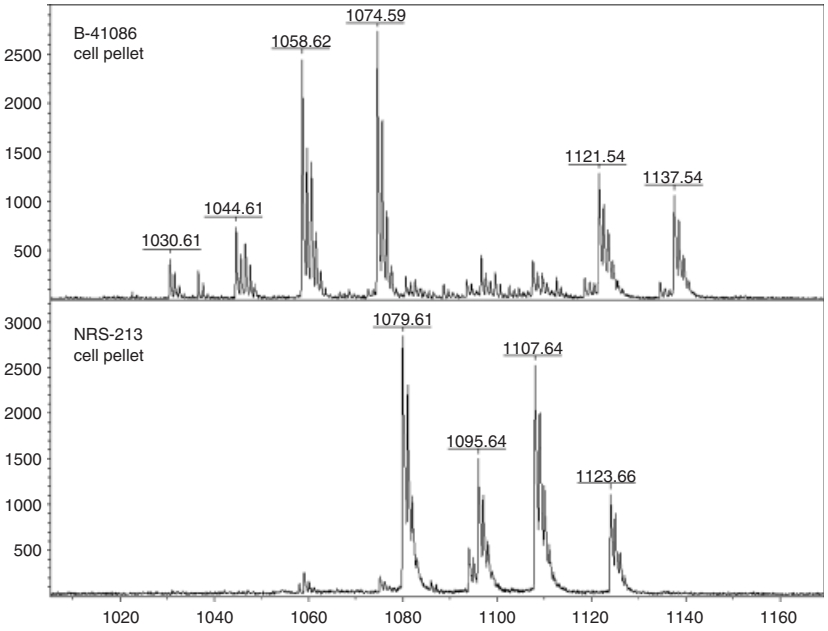

Fig. 1. MALDI-TOF mass spectra of a representative surfactin-producing strain (B-41086) and an iturin-producing strain (NRS-213). (Top panel) For strain B-41086, molecular ions are apparent for surfactins (Leu/Ile-7, C_{15} ($[M+Na]^+$ at 1058.62, $[M+K]^+$ at 1074.59); Leu/Ile-7, C_{14} ($[M+Na]^+$ at 1044.61); and Leu/Ile-7, C_{13} ($[M+Na]^+$ at 1030.61). Small peaks are assigned as 'Other' biomarkers in Table 2. (Lower panel) For strain NRS-213, iturin Asn-1, C_{15} is assigned from $[M+Na]^+$ at 1079.61 and $[M+K]^+$ at 1095.64, and a second iturin is apparent 28 mass units higher ($[M+Na]^+$ at 1107.64 and $[M+K]^+$ at 1123.66).

Ten major groups of lipopeptide biomarkers were identified, representatives of which were produced by all 54 strains tested (Table 2, and Additional Information on line). In addition to kurstakins, iturins, surfactins, polymyxins, bacitracins, and fengycins, four new groups were identified that, in lieu of further characterization, have been designated 942/958, 1093/1107, 1121/1137, and 1304/1320 Da according to the observed mass of their molecular ions. The following discussion pertaining to the *Bacillus* strains is arranged in order of increasing mass of their lipopeptide biomarkers.

Biomarkers in the 880–1100 Da mass range

Of the 54 strains tested, 20 were found to produce kurstakins, the lowest molecular weight group of lipopeptides. These were typically identified by the molecular ions m/z 889, 905, 917, and 933. Except for strains B-41330 and B-41337, the kurstakins were predominantly found in the bacterial colonies on agar plates as opposed to having been secreted in liquid culture, suggesting that they are generally retained by the cells and not secreted. All except four kurstakin-producing strains (B-23057, B-41318, B-41323, and B-41327) also produced polymyxins. A previously undescribed biomarker product, 942/958 Da, was identified in nine strains, and its production was closely correlated with the type strains and the *B. subtilis*. spizizeni desert strains, B-23049, B-23055, B-23056, and B-23057 (Fig. 2, Table 2). Indeed, the B-41091 strain isolated from the Tierra del Fuego was unusual in being the only other 942/958 producer.

Iturin biomarkers were produced by 25 strains, and except for four cases (B-14288, B-14294, B-14299, and B-41337) it was also primarily retained in the cell colonies. Most of the iturin producers also made polymyxins and the 1121/1137 Da product, but both of these were generally secreted. The surfactins were present in the colonies and culture supernatants for 49 of the 54 strains tested. Only five strains did not produce surfactins (NRS-213, B-41295, B-41094, B-41095, and B-41099). The latter three were the only strains found that produced neither iturins nor surfactins, and B-41099 was unusual in that it produced only one biomarker, identified as bacitracin.

Biomarkers in the 1090–1240 Da mass range

Biomarker compounds in the mass range 1090–1240 Da included 1093/1107 and 1121/1137 Da products, and the polymyxins. The 1093/1107 Da biomarkers were produced

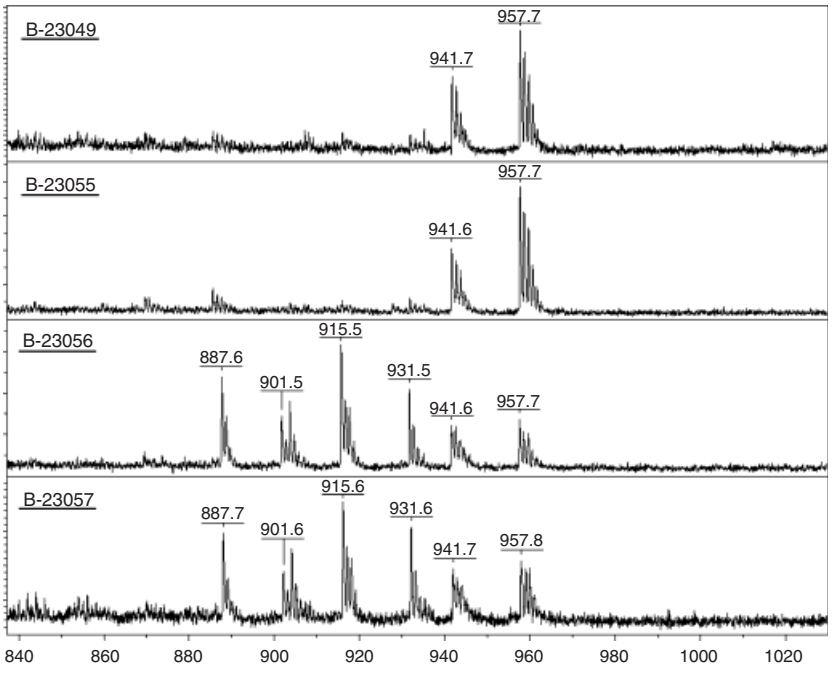

Fig. 2. Production of novel 942/958 Da biomarkers by *Bacillus subtilis* spizizeni desert strains. Other MALDI-TOF MS ions from B-23056 and B-23057 strains are assigned as kurstakin biomarkers (see Table 1).

by 20 *Bacillus* strains and, except for B-41284, were generally restricted to the cell colonies. These unassigned biomarkers are in a mass range similar to several mycosubtilins (Bonmatin *et al*., 2003; Vater *et al*., 2003). Another previously unidentified biomarker, the 1121/1137 Da compound, was produced by 41 of the strains tested. The polymyxins were also produced by 41 strains, but were found almost exclusively in the culture supernatants. Only four strains were identified that produced polymyxin in colonies and supernatant medium, and none in colonies alone.

Biomarkers in the 1300–1550 Da mass range

Compounds identified in the 1300–1550 Da mass range included another novel biomarker, 1304/1320 Da, plus the commercially important bacitracins and fengycins. Twenty strains made the 1304/1320 Da product, and there is a strong correlation for coproduction with polymyxins and the 1121/1137 Da material. All but two (B-41327 and B-41351) of the 1304/1320 Da-producing strains also made polymyxins, and all except one (B-23056) also made the 1121/1137 Da product. Furthermore, it is noticeable that almost always these products were found in the culture supernatants and are presumed to be predominantly secreted. Fifteen strains were identified that make the commercially important antibiotic bacitracin, including one strain (B-41099) for which it is apparently the only lipopeptide produced. Fengycins are also of commercial interest, and 30 strains were identified that are fengycin producers. All but one of these strains (NRS-213) also made surfactins, and all but seven of the fengycin-producers also secreted polymyxins.

Other biomarkers

Apart from the 10 major biomarker groups identified above (Table 2), several minor ion peaks were also characterized by MALDI-TOF MS. In total, 22 *Bacillus* strains were identified that produce these additional minor products, all of which coproduce surfactins. Twelve *Bacillus* strains produced a minor product that gave rise to a molecular ion at m/z 1620.5, most with a coproduct ion at m/z 1642.5, 22 mass units larger. These probably correspond to $[M+H]^+$ and $[M+Na]^+$ ions for a biomarker compound with a mass close to 1619.5 Da. For some strains, these ions are accompanied by several others at m/z 1598, 1620, 1636, 1658, and 1664 that may represent structural variants within this series of compounds. Ion clusters around m/z 1940–2004 and 2072–2223 indicate that other higher mass biomarkers may be present, and similarly for lower mass compounds in the ranges m/z 1100–1160 and m/z 1510–1570.

Conclusions

Several studies have indicated the potential for using MALDI-TOF MS for the identification of low-mass biomarkers in single bacterial colonies (Leenders *et al*., 1999; Vater *et al*., 2002). This technique has been particularly valuable for typing bacterial strains, especially those of the *Bacillus* species. The present report represents the largest study of this kind, a survey of the compounds produced by 54 *Bacillus* species and strains deposited at the USDA–ARS Culture Collection. Ten groups of lipopeptide biomarkers were identified from these strains, including previously described kurstakins, iturins, surfactins, polymyxins,

bacitracin, and fengycins. Four new groups of biomarker were also identified, in the molecular ion mass ranges 942/958, 1093/1107, 1121/1137, and 1304/1320 Da.

The 942/958 Da biomarker group is particularly interesting. The characteristic m/z 941.7 and 957.7 ions may arise from two separate compounds differing by 16 mass units, or may be $[M+Na]^+$ and $[M+K]^+$ adduct ions from a single compound with a mass of 918.7 Da. It may therefore represent a novel component(s) of the kurstakin group of compounds. It was found in the *Bacillus* species type strains that were analyzed as well as in several strains of *B. subtilis spizizenii*, but it was not present in the strains that belong to unidentified species (denoted *B.* sp. in Table 2). These strains are closely related to *B. subtilis* but are ecologically and genetically distinct enough so as to warrant unique species status and can be considered members of the '*B. subtilis* species group' (Rooney *et al.*, 2005). From this viewpoint, it is worthwhile to note that the 942/958 Da biomarker is not produced by any of the *B.* sp. strains except one, B-41092. Perhaps, this is because B-41092 is genetically closer *B. subtilis* than the other unidentified *B. sp*. strains. It is speculated that the lipopeptide differences may themselves be partly responsible for ecological differentiation, by allowing populations to compete against microorganisms in different microhabitats, a hypothesis that can be tested by testing for associations between microhabitats and lipopeptide biomarkers from future collections.

Acknowledgements

We thank Chris Dunlap (ARS, Peoria) for discussion and Trina Hartman (ARS, Peoria) for technical assistance.

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Supplementary material

The following supplementary material is available for this article online:

Table S1. Lipopeptide biomarkers produced by various strains of *Bacillus* species in the USDA-ARS culture collection.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6968.2007.00702.x (This link will take you to the article abstract).

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