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Rapid profiling of antimicrobial compounds characterising *B. subtilis* TR50 cell-free filtrate by high-performance liquid chromatography coupled to high-resolution Orbitrap™ mass spectrometry

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RATIONALE: Several *Bacillus* strains, typically isolated from different food sources, represent renowned producers of a multitude of low and high molecular weight compounds, including lipopeptides and macrolactones, with an importance for their antimicrobial activity. The high homology shared by many of these compounds also occurring as closely related isoforms poses a challenge in their prompt detection.
METHODS: Identification and structural elucidation is generally achieved by matrix-assisted laser desorption/ionization (MALDI) or liquid chromatography (LC) coupled to mass spectrometry (MS) after a pre-fractionation and/or purification step of the extract. In this paper we report the application of a method based on LC separation and high-resolution Orbitrap™-based MS for the rapid screening of raw filtrate of the strain *Bacillus subtilis* TR50 endowed with antimicrobial activity, without requiring any sample pre-treatment.
RESULTS: Upon direct analysis of the cell-free filtrate of *Bacillus subtilis* TR50 by high-resolution mass spectrometry (HRMS), different compounds families, that proved to exert a remarked antimicrobial activity against several foodborne pathogens, can be readily displayed along the chromatographic run. Among them, three different classes were identified and characterized belonging to the iturin, fengycin and surfactin groups. The high resolving power and accurate mass accuracy provided by the HRMS system in use ensured an enhanced selectivity compared to other mass spectrometers. In addition, after activation of the HCD cell, the HR-MS/MS spectra can provide insights in the structural elucidation of several compounds.
CONCLUSIONS: The acquisition of HRMS spectra of raw filtrates of *subtilis* strains allows untargeted analysis of the major classes of compounds produced to be performed, thus facilitating identification of other unknown bioactive molecules after retrospective analysis. These features make this approach a fast tool applicable to the rapid screening and further identification of antimicrobial compounds released by *Bacillus* strains in raw filtrates. Copyright © 2015 John Wiley & Sons, Ltd.

Bacillus subtilis are gram-positive endospore-forming bacteria. Different *Bacillus subtilis* strains are renown producers of a broad spectrum of bioactive metabolites and for this reason are largely used for biotechnological and biopharmaceutical applications.[1,2]

Subtilis strains are usually present in several foods, such as milk and cheeses,[3,4] meat and sausages[3,5,6] and seafood,[7] acting as fermenting or spoiling microflora.

With the exception of some pathogenic species (*B. cereus* and *B. anthracis*), the genus *Bacillus* encompasses species with **GRAS** (Generally Recognised As Safe)/**QPS** (Qualified Presumption of Safety) properties.[8] It is also well known that many *Bacillus* strains (in particular *B. subtilis* strains) display resistance to the physical-chemical conditions occurring in the human upper gastrointestinal tract and are also sources of antimicrobial compounds typically employed as probiotics in animal feeds, in registered medicines and as human dietary supplements.[9]

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The classifications of these compounds include ribosomally synthesised bacteriocins and post-translationally modified lantibiotic-like peptides[10] that exhibit antimicrobial activity against several Gram-positive and -negative bacteria, yeast, and fungi; moreover, non-ribosomal compounds could be synthesised through a multistep mechanism including the selection and condensation of amino acid residues, originating cyclic lipopeptides (iturin group) and macrolactones (surfactins, fengycins and plipastatins). These latter molecules occur as families of closely related isoforms that differ in the length and branching of the fatty acid side chains and in the amino acid substitutions in the peptide ring.[11] Other antimicrobial peptides with low molecular weight (bacilysin) and non-peptide-based antibiotics with different chemical structures (amicoumacins, macrolactin) were also identified.[12–14]

The wide chemical variability shown by these metabolites and their related antimicrobial efficacy depend on both the specific strain producer and the nutritional/environmental growth conditions.[10,15,16] The difficulty in reliably identifying the antimicrobial compounds released by a single *Bacillus* strain hampers their application in many fields such as controlling food microbial spoilage or fighting the emergence of antibiotic resistance to human pathogens.

Analytical methodologies typically employed for microorganism fingerprinting or metabolite identification are mainly based on the coupling between matrix-assisted laser desorption/ionisation (MALDI) and time-of-flight (TOF) MS systems. In general, this has proven to be the most suitable MS platform for rapid microorganism identification and compound elucidation. Several authors reported the use of MALDI-TOF systems for the identification and rapid characterisation of bacteriocins and lipopeptides (typically in the mass range of 800–5000 Da) in culture filtrates of several *Bacillus* strains.[12,17–19] Electrospray ionisation coupled with tandem mass spectrometry (ESI-MS/MS) has also been applied for this purpose and, in particular, tailored to the identification of lipopeptide homologues[20,21] and amino acid sequences contained in the cyclic lactone ring.[22] Likewise, fast-atom bombardment mass spectrometry (FAB-MS) was successfully used to identify a group of plipastatins and surfactins.[23]

In general, most of the above-mentioned mass spectrometric techniques were usually preceded by purification steps based on HCl or ammonium sulphate precipitation, ultrafiltration, solid-phase extraction or thin-layer chromatography (TLC).[24–28]

A few investigations have been carried out on whole bacterial cells for the rapid typing of different microorganisms, including *subtilis* strains isolated from different sources mainly based on MALDI-TOF analysis.[29,30] Pecci *et al.*[31] reported for the first time the characterisation of lipopeptide biosurfactants produced by a strain of *Bacillus licheniformis* by ESI-MS and ESI-MS/MS direct infusion experiments into an ion trap mass spectrometer.

In particular, *B. subtilis* strain TR50, previously isolated in our laboratory,[5] has been proven to exert a strong antimicrobial activity against several foodborne pathogens, regardless of the identity of the active metabolites produced. Interestingly, the treatment of its cell-free supernatant (CFS) with proteinases or lipases caused a differential loss in its inhibitory activity against target microbial pathogens, thus suggesting the production of multiple antimicrobial compounds.[15]

In this paper, we describe a high-throughput untargeted method for the rapid screening of antimicrobial compounds from *Bacillus* strains, using high-performance liquid chromatography (HPLC) separation coupled to an Orbitrap™-based mass spectrometer equipped with a collision chamber, enabling fragmentation experiments to be performed.

This approach is herein described and proposed as a high-throughput tool for the rapid identification of antimicrobial and antifungal compounds occurring in the CFS of *B. subtilis* strains.

EXPERIMENTAL

Chemicals and microbiological media

Formic acid, solvents and reagents used for HPLC/MS analysis were obtained from Sigma-Aldrich (Milan, Italy).

All media and supplements were purchased from Oxoid Ltd (Basingstoke, Hampshire, UK), except PDB (DIFCO, Lawrence, KS, USA).

Culture conditions for the production of antibacterial compounds and antimicrobial assay

Bacillus subtilis TR50, stored at −80°C in the bacterial collection of the Institute of Sciences of Food Production, was inoculated in tryptone soy broth (TSB) containing 0.5% glucose and incubated overnight at 30°C in an orbital shaker at 130 rpm; 2% of this overnight culture was used to inoculate 200 mL of potato dextrose broth (PDB) contained in 1-L flasks and incubated at 30°C in an orbital shaker at 60 rpm. The 9-day-old broth culture was centrifuged (9000 *g* for 20 min at 4°C), and, after discarding the bacterial pellet, the supernatant was filtered through a 0.22-µm syringe cellulose acetate filter (Whatman International Ltd, Maidstone, Kent, UK). The CFS, freeze-dried and concentrated 20-fold (CFS-20X) with ultrapure MilliQ water (Millipore, Marlborough, MA, USA), was assayed for antimicrobial activity[32] against *Salmonella enterica* ATCC13311 and *Staphylococcus aureus* NCTC8325 cells grown in brain-heart infusion (BHI) broth at 37°C and 150 rpm for 16 h. Tetracycline-embedded disks (10 µg/disk; Kairosafe Srl, Duino Aurisina, Italy) and un-inoculated 20-fold concentrated PDB (PDB 20×) were used as positive and negative controls, respectively. Halos without growth around the disks were digitally acquired as areas (mm^2) by the ChemiDoc system (Bio-Rad Laboratories S.r.l., Milan, Italy) and processed using Quantity One 4.3.1 software (Bio-Rad).

HPLC/MS apparatus and operating conditions

An HPLC/UHPLC pump equipped with an autosampler (Accela, Thermo Fisher Scientific, Waltham, MA, USA) was used for the chromatographic analysis by injecting 10 µL of 10× diluted (CFS-20X concentrated) *Bacillus subtilis* TR50 filtrate onto a Kinetex™ column (C18, 100 mm length, 2.1 mm i.d., 2.7 µm packing, 100 Å; Phenomenex, CA, USA). The following binary elution gradient was used for the separation: 95% A for 2 min; a linear ramp to 50% solvent A in 35 min; and then isocratic for 2 min and down to 20% A in 1 min. This composition was maintained for 5 min before subsequent reconditioning at 95% A for 10 min prior to the next injection. The solvents used were (A) H_2O and (B) CH_3CN/H_2O (95:5 v/v), each containing 0.1% HCOOH (v/v), and the flow rate was set at 200 µL/min. MS analyses were performed on an Exactive™ high-resolution mass spectrometer (Thermo Fisher Scientific) integrated with a high-energy collision-dissociation chamber (HCD) and equipped with an ESI interface. The spectrometer was operated at a resolution as high as 50,000. All data were processed by using XCalibur software version 1.1.

A 5 µg/mL standard solution of GFP (Glu-fibrinopeptide) in acetonitrile/H_2O (50:50 v/v) containing 0.1% HCOOH was infused into the instrument to optimise the electrospray parameters for peptide detection. Infusion experiments into the source were carried out by means of an automatic pump delivering the standard solution at a flow rate of 5 µL/min. The mass accuracy of the instrument was assessed by comparing the experimental *m/z* ratio with that expected for the peptide ion and was found to be always accurate to 5 ppm.

Each acquisition run was divided in two full scan MS events, one in the mass range of 150–2000 *m/z* and the other in the mass range of 150–1500 *m/z*, with the HCD collision energy set at 40 eV.

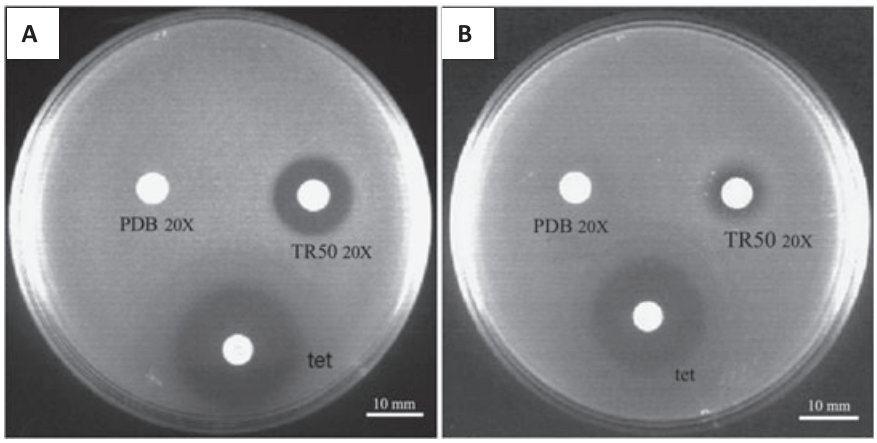

Figure 1. Antimicrobial activity of the TR50 cell-free 20-fold enriched supernatant (TR5020X) from 9 days of growth in PDB against *Staphylococcus aureus* NCTC8325 (A) and *Salmonella enterica* ATCC13311 (B). Positive control: commercial disk with 10 µg of tetracycline (tet); negative control: un-inoculated PDB 20× concentrated (PDB 20×).

The optimised MS instrumental parameters were as follows: resolution, high; microscans, 1 Hz; AGC, balanced 1×10^6; maximum IT, 100 ms; sheath gas, 15; auxiliary gas, 5; spray voltage, 4 V; capillary temperature, 250°C; capillary voltage, 32.50 V; tube lens, 130 V; skimmer voltage, 30 V; and heater temperature, 30°C.

RESULTS AND DISCUSSION

Antimicrobial activity of *Bacillus subtilis* TR50

As the first step, CFS-20X filtrate was checked for its antimicrobial activity prior to the HRMS analysis.

Different inhibition halos were displayed when filtrates were tested against two pathogens, as shown in Fig. 1, giving rise to considering the CFS 20-fold concentrated TR50 filtrate as that containing the highest amount of antimicrobial compounds, as demonstrated in a previous preliminary investigation.[15]

HRMS and HCD-MS/MS analysis of the cell-free supernatant

To quickly identify and characterise the most abundant lipopeptides present in the *Bacillus subtilis* filtrate, a 1:10 dilution with a mobile phase ($ACN/H_2O + 0.1\%$ FA; 50:50) prepared from the CFS-20× was directly analysed by HPLC and HRMS using an Orbitrap™-based system equipped with an integrated collision chamber to perform fragmentation experiments. The sample after chromatographic separation (final concentration 2× of CFS) was injected into the MS system without any further sample pre-treatment. Figure 2 (a) shows a typical full scan HRMS chromatogram following the injection of 10 µL of the CFS of *B. subtilis* TR50 strain. As highlighted in the figure, three different clusters representing bacillomycin analogues, fengycin and surfactin families could be displayed along the trace whose simplified molecular structures are reported in Fig. 2(b). In order to reduce the complexity of the chromatographic run and for a faster peak attribution, the total ion current trace could be easily split up into three time slots, as schematised in Table 1: section

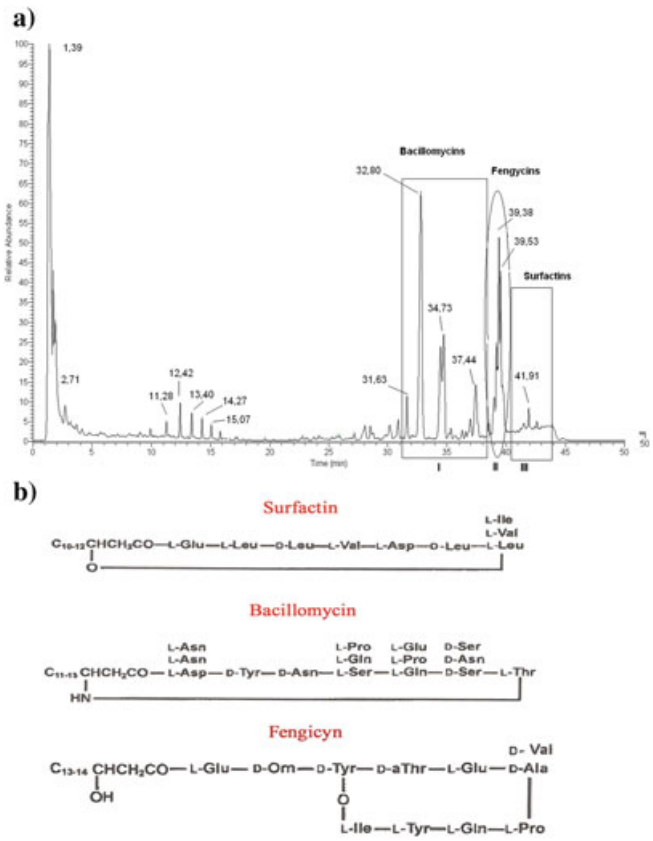

Figure 2. (a) Chromatographic profile of a cell-free supernatant of *Bacillus subtilis* TR50 in full scan HRMS mode. (b) Scheme of the molecular structures referred to the three main families detected in *subtilis* cell-free broth media.

I = 31.5–38.3 min, section II = 38.3–39.6 min, and section III = 41–44 min. Also reported in the same table are both the experimental and calculated mass and the mass measurement error on the base of a single analysis ($m_{experim} - m_{exact}/m_{exact} \times 10^6$ expressed in ppm).

Table 1. Major lipopeptides families identified upon LC/HRMS analysis of a cell-free filtrate of *B. subtilis* TR50 strain along with the most prevalent adducts detected

LIPOPEPTIDE FAMILIES	Elemental composition	Experimental mass $[M+H]^+$	Exact mass $[M+H]^+$	Mass error (ppm)	Adduct $[M+Na]^+$	Time windows (min)
SURFACTINS	$C_{47}H_{72}N_{10}O_{15}$	1017.5228	1017.5251	0.002	1039.5122	I (31.5–38.3)
BACILLOMYCIN D analogues						
C13 fatty acid						
C14 fatty acid	$C_{48}H_{74}N_{10}O_{15}$	1031.5370	1031.5408	3.7	1053.5196	
C15 fatty acid	$C_{49}H_{76}N_{10}O_{15}$	1045.5531	1045.5564	3.1	1067.5358	
C16 fatty acid	$C_{50}H_{78}N_{10}O_{15}$	1059.5687	1059.5721	3.2	1081.5514	
C17 fatty acid	$C_{51}H_{80}N_{10}O_{15}$	1073.5841	1073.5877	3.3	1095.5668	
FENGYCINS	$C_{72}H_{110}N_{12}O_{20}$	1463.7989	1463.8032	2.9		II (38.3–39.7)
Fengycin B C14 fatty acid						
Fengycin A C15 fatty acid	$C_{71}H_{108}N_{12}O_{20}$	1449.7817	1449.7876	4.0		
Fengycin A C16 fatty acid	$C_{72}H_{110}N_{12}O_{20}$	1463.7956	1463.8032	5.2		
Fengycin A C17 fatty acid	$C_{73}H_{112}N_{12}O_{20}$	1477.8107	1477.8189	5.5		
Fengycin B C16 fatty acid	$C_{74}H_{114}N_{12}O_{20}$	1491.8260	1491.8345	5.7		
Fengycin B C17 fatty acid	$C_{75}H_{116}N_{12}O_{20}$	1505.8432	1505.8502	4.6		
Fengycin B C18 fatty acid	$C_{76}H_{118}N_{12}O_{20}$	1519.8580	1519.8658	5.1		
SURFACTINS (*m/z*)		$+[H]^+$			$+[Na]^+$	III (39.9–44)
C12 fatty chain	$C_{50}H_{87}N_7O_{13}$	994.6424	994.6435	1.1	1016.6251	
C13 fatty chain	$C_{51}H_{89}N_7O_{13}$	1008.6580	1008.6591	1.1	1030.6407	
C14 fatty chain	$C_{52}H_{91}N_7O_{13}$	1022.6730	1022.6748	1.8	1044.6557	
C15 fatty chain	$C_{53}H_{93}N_7O_{13}$	1036.6884	1036.6904	1.9	1058.6711	

In the first section, a cluster characterised by five ions, corresponding to *m/z* 1017, 1031, 1045, 1059 and 1073 spaced by 14 Da, prevailed in the recalled MS spectra. By exploiting some options provided by the XCalibur software it was possible to retrieve information on the elemental composition of unknown compounds based on the accurate mass of selected ions detected in the HRMS spectrum. This class of compounds was assigned to the putative bacillomycins of the iturin family that represent antifungal agents contained in the culture medium of some *Bacillus subtilis* strains.

As shown in Table 1, several molecules were detected along the chromatographic run, differing by CH_2 residues and thus reflecting the difference in the fatty acid length. The most abundant ions dominating the MS spectra retrieved at each retention time are represented by two principal ions attributed to bacillomycin D-1 analogues[33] with mass units of 1031 $[M+H]^+$ and 1053 for the adduct $[M+Na]^+$ (equivalent to the C_{15}-β-amino fatty chain), and 1045 $[M+H]^+$ and its adduct 1067 $[M+Na]^+$ (equivalent to the C_{14}-β-amino fatty chain). By recalling the elemental composition of the *m/z* ions detected along the MS spectrum, a list of different possible candidate compounds was generated by the software along with the associated mass error. In order to achieve a more trustful assignment for each compound, only those ranking at the highest positions in the list were taken into consideration (meaning showing the lowest mass error). In particular, ions with *m/z* 1045 and 1059 were assigned to bacillomycin D bearing C_{15}- and C_{16}-β-amino fatty acids, respectively, without determining their iso-/anteiso-configuration as also reported by Tanaka *et al.* in another work.[26] Figure 3 shows a typical molecular assignment referred to bacillomycin D, C15 fatty acid accomplished on the basis of the accurate mass of the detected ion; in the same panel is also reported a comparison between the theoretical and the experimental isotopic patterns for that putative compound (elemental composition $C_{49}H_{77}O_{15}N_{10}$).

The close similarity shown by molecules belonging to lipopeptide families make purification and isolation of single classes quite a challenging task, as was highlighted by several authors.[23–25,28] In particular, Elkahoui *et al.*[24] described the analysis of three methanolic fractions (respectively 20%, 60% and 80% of methanol) carried out on a triple-quadrupole mass spectrometer for the identification of bacillomycin D, C16 (1059. Da) and C15 (1045 Da) and of two iturins D (C_{13} and C_{14}) identified in the 80% fraction.

In this paper, we demonstrate that the identification of lipopeptide families could be easily obtained by a single method based on the coupling between HPLC and a HRMS system.

The relative abundance of each bacillomycin homologue was also estimated based on the sums of the peak areas obtained from the extracted ion chromatograms of all bacillomycin molecular ions detected. The results provided the relative contents in the ratio of 1:56:32:10:1, as detailed in Table 2. Identification was accomplished by overlaying the isotopic pattern experimentally found with that theoretically

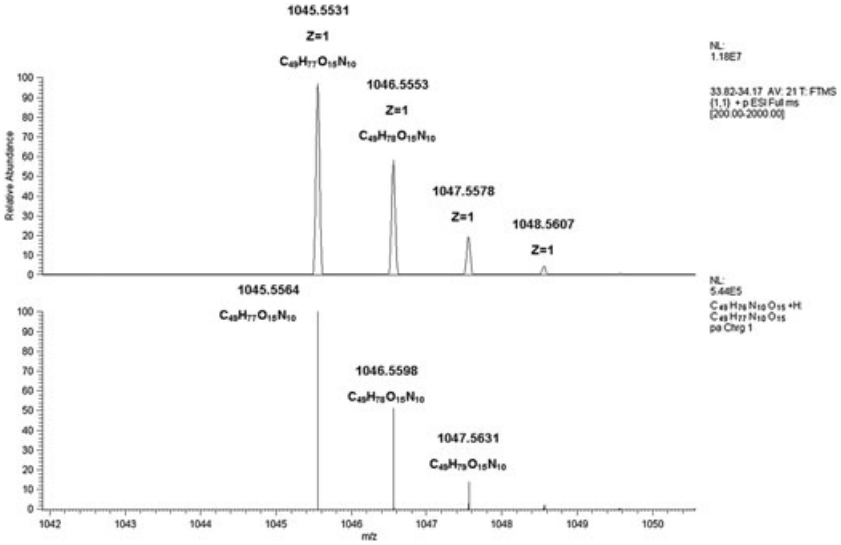

Figure 3. Elemental composition and isotopic distribution calculated for Bacillomycin (C15) detected at retention time 34 min. Experimental (top) and theoretical isotopic (bottom) distribution for the assigned compound $C_{49}H_{77}O_{15}N_{10}$.

Table 2. Relative abundances of the different lipopeptides belonging to the same class along the chromatographic run

Lipopeptide classes	$[M+H]^+$	Relative abundance (%)
SURFACTINS		
BACILLOMYCIN		
D analogues (*m/z*)		
C13 fatty acid	1017.5251	1
C14 fatty acid	1031.5369	56
C15 fatty acid	1045.5531	32
C16 fatty acid	1059.5687	10
C16 fatty acid	1073.5841	1
FENGYCINS		
C15 fatty acid (A)	1449.7817	2
C16 fatty acid (A)	1463.7989	16
C17 fatty acid (A)	1477.8107	22
C16 fatty acid (B)	1491.8260	36
C17 fatty acid (B)	1505.8399	22
C18 fatty acid (B)	1519.8580	2
SURFACTINS (*m/z*)		
C12 fatty chain	994.6424	-
C13 fatty chain	1008.6580	13
C14 fatty chain	1022.6730	61
C15 fatty chain	1036.6884	26

calculated by the software. When the bacillomycin class was analysed in full ion fragmentation mode, the resulting HCD-MS spectra did not provide any peculiar fragment that can be considered as a valid ion marker able to identify this class of compounds in the cell-free supernatant.

Identification of fengycin classes

Time window II (38.3–39.6 min) was characterised by the elution of the putative fengycin class, with the most abundant ions detected being *m/z* 1463, 1477, 1491 and 1505. Apart from these dominant ions, minor levels of other forms, including *m/z* 1519 and 1449, were also detected (see Table 2 for details of relative abundances).

The electronic mass filtration on the full scan MS acquisition based on the accurate mass of each class of compounds allows retrieval of an extracted ion chromatogram filtered on the specific mass of interest, reducing the complexity of the multiscreening analysis. Figure 4 reports typical chromatographic traces referring to the analysis of the fengycin family, where the application of mass current filtration on the total ion current (TIC) enables selective traces to be obtained referring to different homologues belonging to the same fengycin class and characterised by a progressive elution time dependent on the alkyl chain length (C_{15}, C_{16}, C_{17} of fengycin A and C_{16}, C_{17}, C_{18} of fengycin B). Furthermore, upon activation of the fragmentation chamber in the so-called 'all ion fragmentation mode', each eluted analyte could undergo fragmentation with N_2 in the collision cell, thus providing a fragmentation pattern of the original molecule.

To unequivocally identify the class of compounds belonging to the fengycin family, HCD-MS experiments were performed on the peaks eluted from the analytical column upon activation of the high-energy collision dissociation chamber (energy set at 40 eV). In particular, some typical fragments characterising the fengycin classes A and B were noted. Figure 5 reports an overlay of a full scan HRMS chromatogram and the respective HCD fragmentation spectrum recalled at retention time 39.4 min, corresponding to the elution of the precursor ion *m/z* 1505. As shown in the lower panel of Fig. 5, fengycin lipopeptide 1505 was preferentially detected as a doubly charged ion at *m/z* 753, thus bearing $2H^+$. However, the fragmentation pattern obtained for this category of compounds produced some relevant fragment ions that were common to the whole fengycin family and could be potentially used to trace the presence of this family for a quick analysis of *Bacillus* raw extracts. The dominant markers emerging from the HCD-MS spectra, 1108, 994 and 883, were confirmed for all

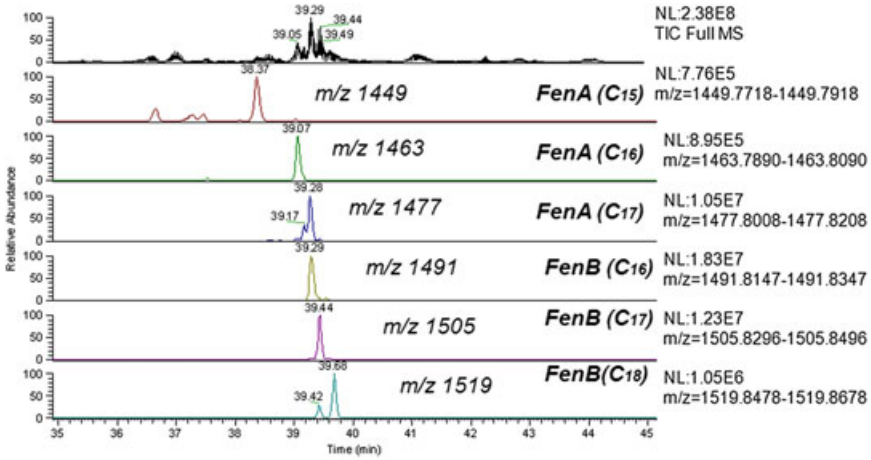

Figure 4. Elution profile of the major lipopeptides belonging to the fengycin family (extraction mass window 0.02 Th).

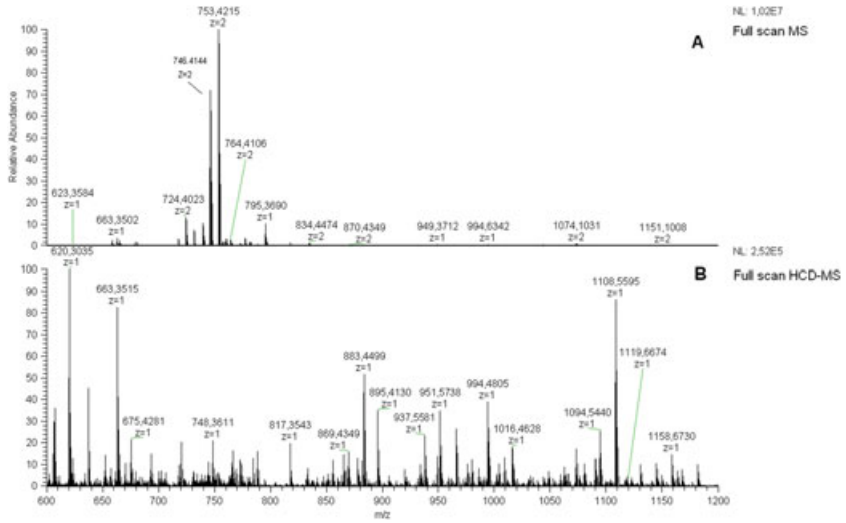

Figure 5. Overlay of a full scan HRMS chromatogram (A) and the respective HCD fragmentation spectrum (B) in the selected mass range 600–1200 Da recalled at retention time 39.4 min corresponding to fragmentation of fengycin *m/z* 1505.

the precursor ions 1491, 1505 and 1519 and they belonged to the fengycin B class, as reported in Table 3. However, a minor component was also observed by a careful analysis of the MS spectra and was represented by the ion masses 1449, 1463 and 1477, putatively attributed to lipopeptides belonging to the fengycin A class. Fragments 1080 and 663 appeared to be the most prevailing ions in the HCD-MS spectrum, thus designing these fragments as potential markers of the fengycin A class.

These results are in agreement with those recently reported by Pecci *et al.*,[31] although a different ratio of the fengycin mixture was observed in this specific *Bacillus* CSF-20X filtrate. Moreover, two additional compounds were for the first time detected at retention times of 38.3 and 38.6 min, with *m/z* 1449 and 1477, respectively. Both were characterised by the most abundant common marker at *m/z*=1095, in the HCD-MS spectrum.

In conclusion, some markers common to all different fengycin classes can be noted in the respective HCD spectra, thus suggesting their potential use for the prompt and reliable detection of this family of lipopeptides in *subtilis* raw extracts.

Identification of surfactin classes

The last part of the chromatographic run, section III (41–44 min), was dominated by four molecular ions with *m/z* 994, 1008, 1022 and 1036. Molecular mass *m/z* 1036 at retention time 44 min is attributed to surfactin bearing an heptapeptide ring and a C_{15}-β-hydroxy fatty acid esterified with a glutamic acid residue,[34] and the remaining ions represent C_{12}–C_{14} surfactins, spaced by 14 Da. Surfactins produced by *B. subtilis* are surface-active agents endowed with antibiotic properties.[35] As they can be released by *B. subtilis* strains under different isoforms, their

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Table 3. Most dominant MS/MS ions obtained upon fragmentation in the HCD cell of the major surfactin and fengycin precursors

Molecular ions (*m/z*)	MS/MS fragments	Candidate marker
SURFACTINS		
1008.6580	*y-ions* 990.6471 [1008-H_2O]	*m/z*
$[M+H]^+$	895.5736 [1008-L]	685.4473
	782.4898 [1008-LL]	
	667.4608 [1008-LLD]	
	568.3947 [1008-LLDV]	
	b-ions 685.4473 [1036-fatty chain]	
1022.6724	*y-ions* 1004.6615 [1022-H_2O]	
$[M+H]^+$	909.5879 [1022-L]	
	796.5042 [1022-LL]	
	681.4777 [1022-LLD]	
	582.4095 [1022-LLDV]	
	b-ions 685.4473 [1036-fatty chain]	
1036.6880	*y-ions* 1018.6777 [1036-H_2O]	
$[M+H]^+$	923.6043 [1036-L]	
	810.5205 [1036-LL]	
	695.4936 [1036-LLD]	
	596.4257 [1036-LLDV]	
	b-ions 685.4473 [1036-fatty chain]	
FENGYCINS		
FENGYCIN A		
1449.7813	1080.5322; 663.3541; 937.5615	*m/z*
1463.7956	1080.5322; 663.3541, 937.5615	663.3541
1477.8107	1080.5322; 663.3541, 937.5615	
FENGYCIN B		
1491.8399	1108.5634; 994.4840; 883.4528	*m/z*
1505.8399	1108.5634; 994.4840; 883.4528	994.4840
1519.8658	1108.5634; 994.4840; 883.4528	

identification in the CFS filtrate is a fundamental step before their use for different applications. By comparing the relative abundances, the highest peak was found to be the ion carrying the C_{14} fatty chain (C_{13}:C_{14}:C_{15} in a ratio 13:61:26). As reported in Table 1, the main molecular ion along with the respective Na^+ and K^+ adducts were identified for the three classes of surfactins.

Interestingly, an ion with mass 1036 *m/z* was also detected at retention time 39.9 min; this molecule resembled a surfactin C_{14} with a seven-residue peptide containing a methylated glutamic acid produced by a *B. subtilis* strain from petroleum-contaminated soil.[36] When surfactins underwent fragmentation, the structural elucidation of each compound was achieved by the detection of *y*- and *b*-ions in the HCD-MS spectra retrieved. As shown in Table 3, the *b*-ion at *m/z* 685 corresponded to the C_{15} fatty chain and proved to be the ion marker common to the three surfactins detected. When surfactins were analysed by HRMS by other authors, the ion detected at *m/z* 1058 was attributed to the sodium adduct of a surfactin homologue bearing a C_{15}-β-hydroxy fatty acid side chain. Its protonated form was *m/z* 1036. After fragmentation, the production of a set of ions with *m/z* 1058 → 945 → 832→ 717→ 618 was reported, suggesting the loss of the amino acid sequence Leu–Leu–Asp–Val from the C-terminus, whereas the product ions with *m/z* 707 → 594 → 481 → 382 indicated the loss of the amino acid sequence Leu–Leu–Val from the middle.

In conclusion, in this work, we propose a new rapid and high-throughput HRMS method using a benchtop Orbitrap™-based system for the fast multi-screening of multiple antimicrobial compounds in *Bacillus subtilis* untreated broth media. Such an approach skipped any purification or fractionation step and enabled the prompt identification of different classes of antimicrobial lipopeptides with a mass accuracy better than 5 ppm.

CONCLUSIONS

The simultaneous production and excretion of different lipopeptide classes, such as iturins, surfactins and fengycins, in broth media culture of *Bacillus* species has been widely reported in the literature.[10,21,24] In particular, the increase in antifungal activity shown by *B. subtilis* RB14 and *B. amyloliquefaciens* BNM 122 was attributed to the production of surfactins and iturins, respectively.[37] Although several studies have been accomplished on this topic, particularly to characterise the metabolic or peptidic profile of *Bacillus subtilis* strains by MS detection, the majority of the methods developed typically employed a pre-fractionation step followed by purification before the MS analysis.

In this paper, we demonstrate the applicability of a benchtop HR Orbitrap™-based mass spectrometer for rapid *Bacillus subtilis* TR50 lipopeptidic profiling. The implementation of such a forward and high-throughput strategy only employing one dilution step of the raw filtrate enabled the rapid identification of three different classes of antimicrobial lipopeptides, bacillomycin D analogues, surfactins and fengycins, in one run. Thanks to the high resolving power offered by the system in use and the high mass accuracy provided (≤5 ppm), enhanced selectivity was ensured in the cell-free extract analysed. The presence of the HCD chamber integrated within the system capable of providing HR-MS/MS spectra also allowed further information on fragment markers to be obtained, thus facilitating the identification of other unknown compounds in the cell-free supernatant. These features make this approach a reliable and fast tool applicable to the broad screening of a multitude of antimicrobial compounds produced by *Bacillus* strains.

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