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RESEARCH ARTICLE

Characterization by Tandem Mass Spectrometry of Biologically Active Compounds Produced by Bacillus Strains

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A methodology relying on LC-ESI-MS/MS has been applied to the characterization of biologically active compounds produced by Bacillus strains grown in liquid culture media. This approach enabled off-line evaluation of biological activities and complementary MALDI-MS/MS analysis. In this way, major limitations of direct MALDI analysis such as spectral discrimination and lack of information on low molecular weight species were circumvented. Fragmentation patterns combined with accurate mass determination led to reliable structure assignments for amicoumacins and major lipopeptide series: surfactins and fengycins. In this latter series, LC-ESI-MS/MS correlated with activity tests confirmed the biological activities of fengycins, whereas linear forms of fengycins were inactive.

Keywords: lipopeptides, MS, MS/MS, liquid chromatography, bacillus strains.

Introduction

Among Gram-positive bacteria, Bacillus strains are known to produce numerous compounds that display a large spectrum of biological activities [1]. Owing to their production of antimicrobial substances, Bacillus strains can act as biological control agents for plant diseases [2] and some of these substances have an effect on plant pathogens [3]. Furthermore, these bacteria have beneficial effects on human health that are directly connected to their production of anti-microbial substances [4] and probiotics based on Bacillus bacteria contribute to the prevention and treatment of infectious diseases [5] and gastrointestinal microbial disorders [6]. Many compounds produced by Bacillus strains are characterized by an alkyl chain of variable length linked to a cyclic peptide, and thus can be classified as lipopeptides.

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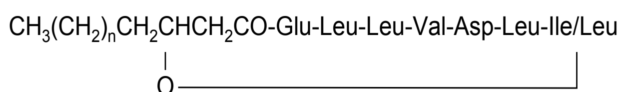
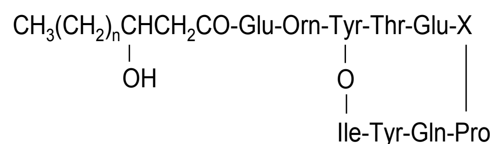
Their amino acid composition and fatty acid chain length are variable, as well as the type of ring closure, thus leading to the occurrence of numerous isoforms and isobaric species **(Figure 1)**. Surfactins are powerful biosurfactants with exceptional emulsifying and foaming properties [7]. This family encompasses structural variants, but all members are heptapeptides interlinked with a β-hydroxy fatty acid to form a cyclic lactone ring structure. Fengycins, which are also called plipastatins, are lipodecapeptides with an internal lactone ring in the peptidic moiety linked to a β-hydroxy fatty acid chain that can be saturated or unsaturated. Fengycins are less hemolytic than surfactins but retain a strong fungitoxic activity [7].

Other compounds synthesized by Bacillus strains were listed as antibiotic compounds. In 1974, Berdy reported 167 antibiotics produced by members of the Bacillus genus [8]. Since then, many new antibiotics have been isolated from strains of the Bacillus genus and have found applications in the pharmacology, veterinary and food industries [9]. These compounds include low molecular weight species (below 500 Da) such as amicoumacins

SURFACTINS A/C

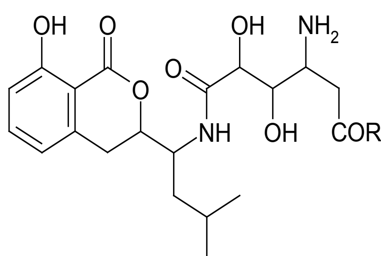


FENGYCINS



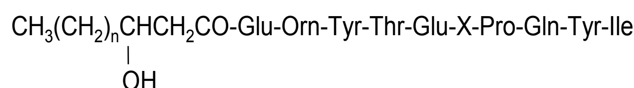
X=Ala, Fengycins A
X=Val, Fengycins B

AMICOUMACINS



$R=NH_2$, Amicoumacins A
R=OH, Amicoumacins B

LINEAR FENGYCINS



X=Ala, Linear Fengycins A
X=Val, Linear Fengycins B

Figure 1. Structures of amicoumacins and some major lipopeptide series. In this study, $9<n<14$

(Figure 1) that have antibacterial, anti-inflammatory and anti-ulcer activity [10].

Several analytical methods have been developed for the detection of lipopeptides, such as capillary electrophoresis (CE), thin layer chromatography (TLC), liquid chromatography coupled to an UV detector (LC-UV) or also immunological detection by competitive ELISA assays for the quantification and localization of lipopeptides on the surface of plant tissues. However, none of these methods provides enough information for a safe identification of individual lipopeptides, whereas mass spectrometry turned out to be a more informative methodology for the characterization of lipopeptide structures.

Matrix Assisted Laser Desorption Ionization - Mass Spectrometry (MALDI-MS) has been widely used for the screening of bacterial strains [11-13], whereas electrospray ionization (ESI) has been less used [14-16]. MALDI-MS is simple, fast and sensitive, but three major drawbacks limit its application to the characterization of biologically active substances produced by bacteria, namely spectral discrimination, loss of information for small molecules with molecular weights below 700 Da and dominance of cationic adducts among molecular species. The first two points largely explain the fact that the full panel of biological activities displayed by a given strain is rarely reflected by the compounds revealed by MALDI-MS fingerprinting. For the third point, the occurrence of multiple ionic species for a single molecule complicates full scan spectra and is directly related to the composition of culture media; furthermore, MS/MS of $[M+Na]^+$ and $[M+K]^+$ precursor ions most often leads to a low content of structure information than hinders structure characterization.

An analytical method based on LC-ESI-MS/MS (liquid chromatography coupled to tandem mass spectrometry) could have the double advantage of the separation of the different families of lipopeptides and of mass measurement combined to fragment ion spectra for structure identification.

Therefore, we designed a more reliable methodology for rapid characterization of biologically active compounds produced by Bacillus strains that associates measurement of biological activities and structure determination.

Materials and methods

Reference compounds

Surfactin A (mixture of compounds with different alkyl chain lengths), and Glu-fibrinopeptide were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Culture media and strains

Bacillus subtilis strains were grown in Difco sporulation medium (Fisher Scientific, Illkirch, France) and Landy medium (laboratory-made), MgSO4, MnSO4 and FeSO4 (VWR International, Fontenay-sous-Bois, France), Glutamic acid, KCl, CuSO4 and KH_2PO4 (Sigma-Aldrich, Lyon, France) and D-glucose (Biosolve Chimie, Dieuze, France) for 18 and 70 h at 30°C, at 200 rpm, with an estimation of 109 bacteria/mL for the Difco and 108 bacteria/mL for the Landy medium. Culture broths (10 mL) were centrifuged at 9,500 rpm for 15 min at 4°C. Bacterial cells left in the supernatant were removed by filtration (0.45μm membrane, Millipore, Molsheim, France). Supernatants were stored at -20°C.

Extraction

Bacteria grown in liquid medium (10 mL cultures) were harvested by centrifugation and resuspended in 200 μL of sterile physiological water. A 100 μL aliquot of the suspension was extracted with 400 μL of a 1/1 solution (v/v) of acetonitrile/isopropanol containing 0.1% formic acid. Solvent was removed under vacuum (SpeedVac, Savant, Holbrook, NY, USA) prior to re-dissolution and LC/MS/MS analysis.

Solubilisation of extracts

Lipopeptides tend to remain adsorbed on the wall of Eppendorf tubes. Optimal re-dissolution of bacterial extracts was obtained with 50 μL of 1/1/2 (v/v/v) acetonitrile/isopropanol/H_2O solution containing 0.1% formic acid, followed by treatment in an ultrasonic bath (two 30 s period, with 30 s pause time).

LC-ESI-MS and MS/MS analysis

A C4 column (3.9 x 150 mm, 5μm particles, 100 Å pore size; Waters, St Quentin en Yvelines, France) was used for reverse phase liquid chromatography (RPLC) at 1 mL/min flow rate with a linear gradient from 0% to 100% B in 40 min (eluent A: 0.1% formic acid; eluent B: 80% acetonitrile, 20% isopropanol with 0.1% formic acid). The injection volume was 100 μL of bacterial extract diluted 1/4 in 0.1% aqueous formic acid. Using a post-column split, 90 % of the effluent was collected (1 min fractions) for further characterization and 10% was analyzed by ESI-MS/MS (LCQ Advantage, ThermoFisher, San Jose, CA, USA) in the positive ion mode. MS/MS spectra were acquired in data dependent scan mode with dynamic exclusion for 1 min after 3 scans. High flow rate settings were used for the ion source: 270°C capillary temperature; voltages were 10 V for the capillary and 4.5 kV for the spray. The analyses were achieved in triplicate.

MALDI-MS/MS analysis

A MALDI Q-ToF Premier instrument (Waters, Manchester, UK) fitted with a nitrogen laser (337 nm) was used for accurate mass and MS/MS analysis of fractions collected from the C4 column. The matrix was α-cyano-4-hydroxycinnamic acid (3.6 mg/mL in 50% acetonitrile containing 0.1% TFA). Glu-Fibrinopeptide was used for single point mass correction (Lockmass). Mass accuracy for pseudo-molecular ions was better than 5 ppm.

Measurement of biological activities

Solvent extracts were separated by reversed phase liquid chromatography on a micro-preparative scale. This step was followed by the measurement of biological activities on collected fractions. This methodology is suited to the characterization of active compounds produced by Bacillus strains in liquid culture media. Antifungal activities were tested against *Botrytis cinerea* and *Fusarium oxysporum* by the agar diffusion method [17]. Fungi were deposited in the center of Petri dishes containing MALT-Agar solidified medium. Once fungi had started development, a 20 μL aliquot of the solutions to be tested was deposited on the surface of the agar medium. Petri dishes were incubated at 25°C in an aerobic atmosphere for 48-72 h. Growth inhibition was determined by measurement of the halo diameters around the deposit.

Results and discussion

Extraction of lipopeptides

A *Bacillus subtilis* strain producing a large panel of biologically active compounds including low molecular weight amicoumacins was used for the selection of the best extraction solvent. C14 surfactin was added as a standard for quantitative evaluation of the extraction process, while using a constant volume of bacterial suspension and extraction solvent. The best extraction solvent composition was 1/1 acetonitrile/isopropanol (v/v) containing 0.1% formic acid.

Limitations of fast screening by MALDI

Direct analysis by MALDI mass spectrometry is widely used for bacterial typing because it gives a rapid overview of the different species present in a sample. However, selective desorption-ionisation effects prevent its application to the exhaustive identification of active substances produced by a bacterial strain [11,18]. Isobaric species cannot be differentiated by MALDI-MS and the presence of different adducts (Na^+ and K^+) in lipopeptides [11-12] complicates considerably the identification of these lipopeptides.

Furthermore, low molecular weight compounds such as

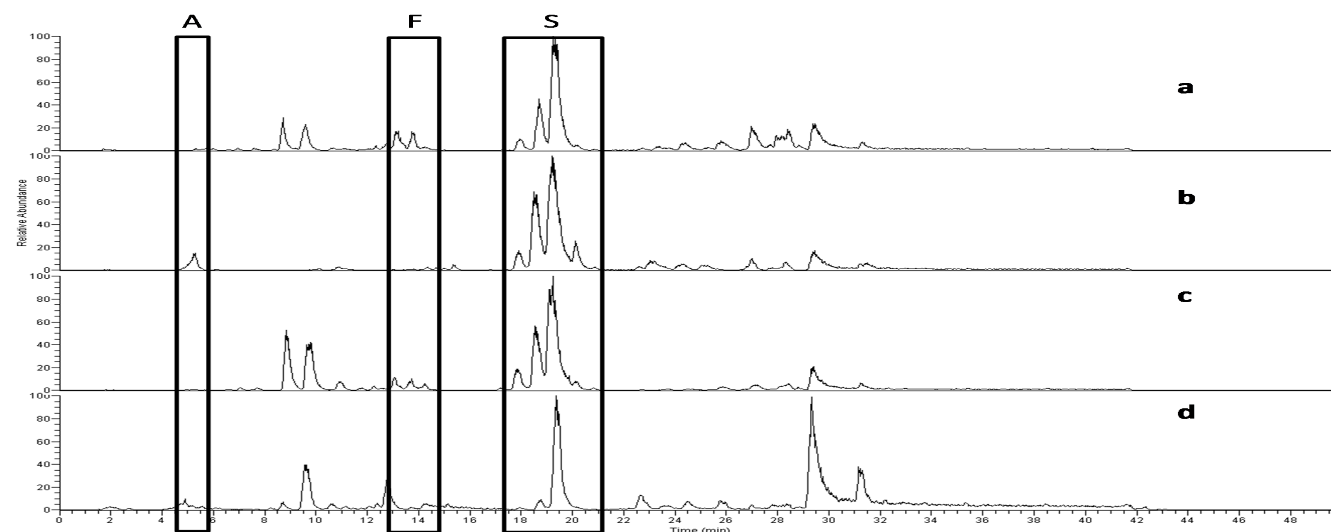


Figure 2. LC/MS base peak traces for lipopeptides extracted from *Bacillus subtilis* strains 1, 2 and 3 (panels a, b and c, respectively), grown in Landy medium for 70h. On panel d, bacteria from strain 1 were grown in Difco sporulation medium for 70h. A, F and S represent the retention time ranges of Amicoumacins, Fengycins and Surfactins, respectively.

amicoumacins are hardly detectable by MALDI-MS in a mass range where clusters of matrix ions are dominant. Considering these limitations, we have designed an analytical strategy relying on micro-preparative liquid chromatography combined to ESI-MS/MS analysis. Collected fractions were assayed for antifungal activity, and used for accurate mass determination and MALDI-MS/MS with a Q-ToF instrument when structure assignments were ambiguous.

Chromatographic conditions

As lipopeptides of the surfactin series are strongly retained by most C18 grafted supports, a C4 support with 0.1 nm porosity was preferred. A clear separation of surfactins and fengycins was observed, with enough selectivity for separating surfactins and fengycins having various fatty acid chain lengths **(Figure 2)**. LC/MS analysis of various Bacillus strains provided evidence for the variability of compositions in the amicoumacin, fengycin and surfactin series **(Figure 2a-c, frames A, F and S)**. LC/MS analysis also highlighted the influence of culture conditions on the relative abundance of compounds produced by Bacillus strains. For the same strain, a higher concentration of fengycins and surfactins was obtained for the Landy medium **(Figure 2a)**, compared to Difco sporulation medium **(Figure 2d)**. Similarly, a higher concentration of fengycins and surfactins was obtained for longer culture times (data not shown). As shown for instance for surfactins, compositions according to the alkyl chain length also vary from one strain to the other, and from one culture condition to the other **(Figure 2, frame S)**.

Handling of collected fractions

Fractions collected (900 μL) from the C4 column were dried under vacuum and redissolved in 50 μL of solvent (concentration factor was 18). Then, 20 μL of this solution was used to measure biological activities. The use of C14 surfactin as a standard for a quantitative evaluation revealed that a simple addition of solvent such as 1/1 acetonitrile/water was insufficient to bring lipopeptides back into solution, most of the material being lost on the wall of Eppendorf tubes used for fraction collection. At best, a 70 ± 5 % re-dissolution yield was obtained with 1/1/2 (v/v/v) acetonitrile/isopropanol/water containing 0.1% formic acid. This solvent composition was also suited for biological activity assays without further sample handling.

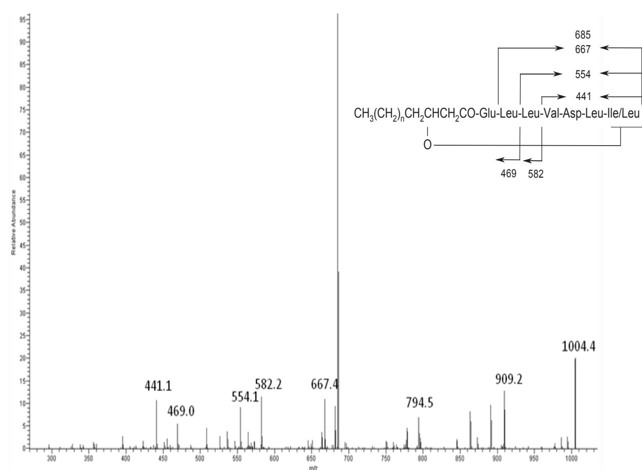


Figure 3. ESI-MS/MS spectrum (fragmentation of $[M+H]^+$ ion at m/z 1022.7 at a collision energy of 38 (arbitrary unit, ion trap mass spectrometer) of a C14 surfactin A/C (where n=9) and its fragmentation scheme.

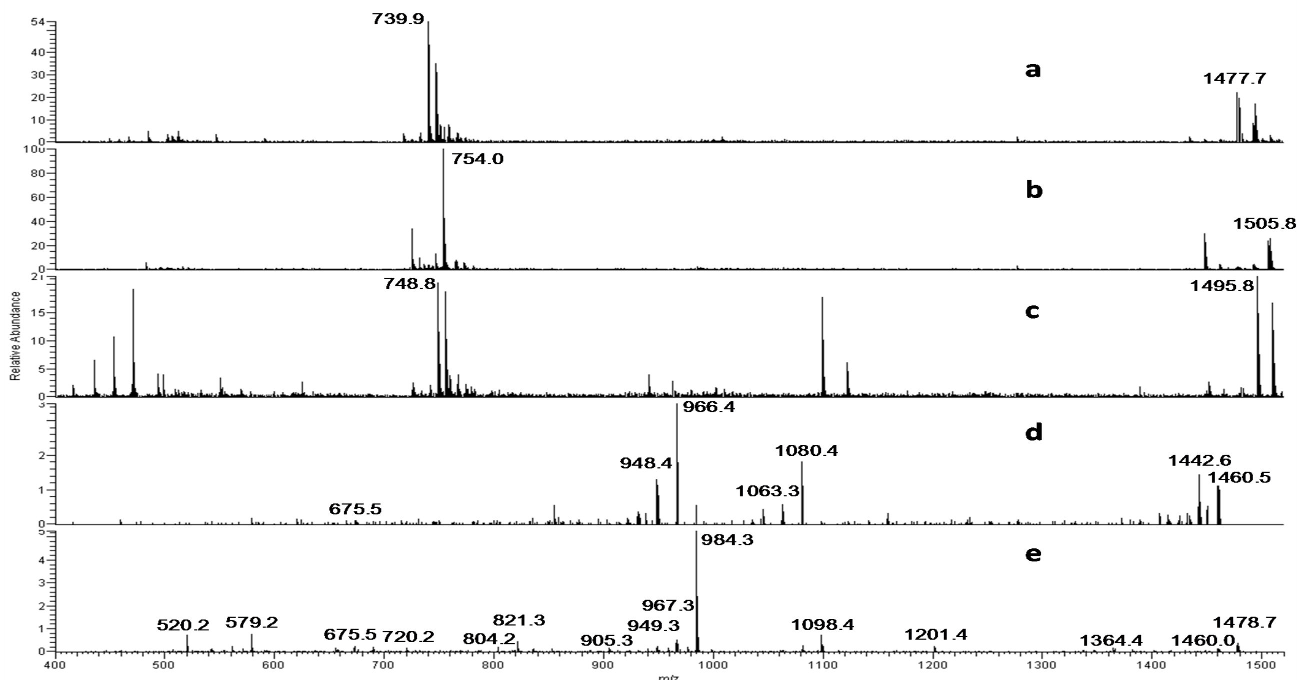
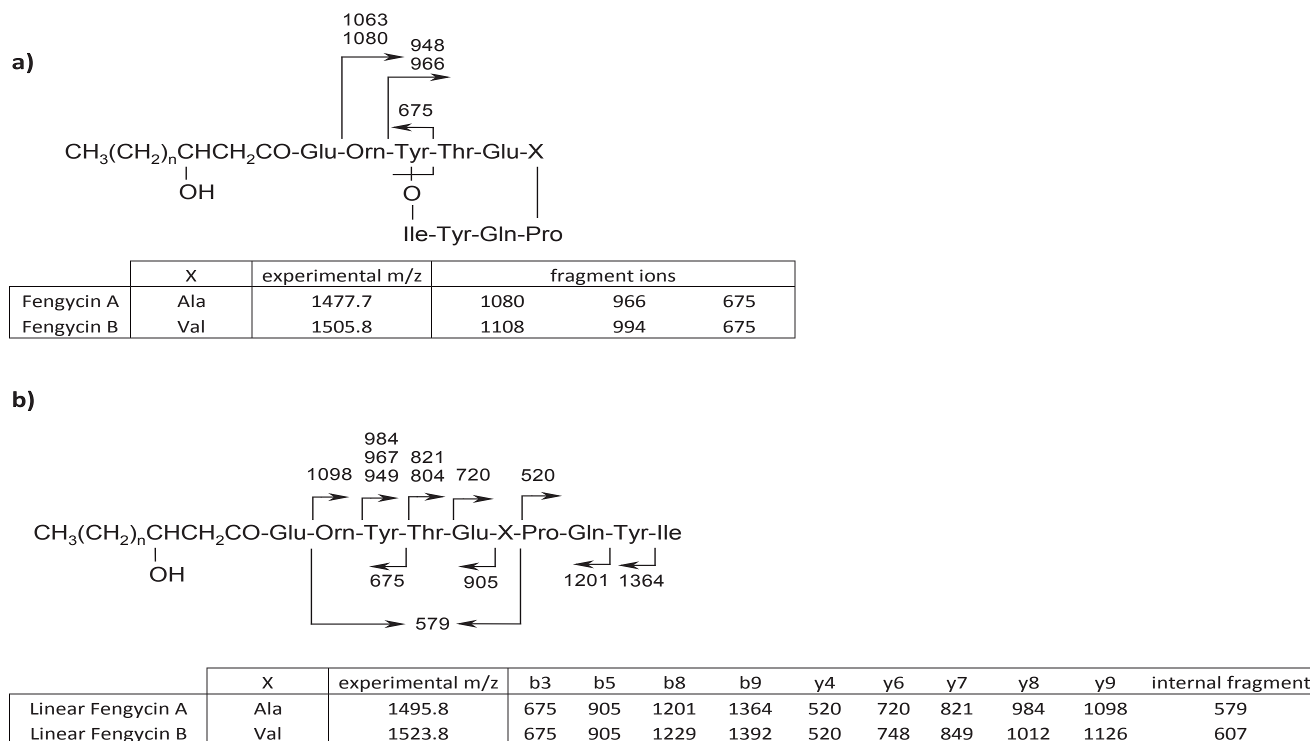


Figure 4. ESI-MS spectra of C17 fengycin A, C17 fengycin B and linear C17 fengycin A (panels a, b and c, respectively), with $[M+H]^+$ ion at *m/z* 1477.7, 1505.8 and 1495.8 and $[M+2H]^{2+}$ ion at *m/z* 739.9, 754.0 and 748.8, respectively. ESI-MS/MS spectra (fragmentation of $[M+H]^+$ ions at *m/z* 1477.7 and 1495.8) of a C17 fengycin A and a linear C17 fengycin A (where n=13), respectively



	X	experimental m/z	fragment ions		
Fengycin A	Ala	1477.7	1080	966	675
Fengycin B	Val	1505.8	1108	994	675

	X	experimental m/z	b3	b5	b8	b9	y4	y6	y7	y8	y9	internal fragment
Linear Fengycin A	Ala	1495.8	675	905	1201	1364	520	720	821	984	1098	579
Linear Fengycin B	Val	1523.8	675	905	1229	1392	520	748	849	1012	1126	607

Figure 5. Fragmentation schemes of a C17 fengycin A and linear C17 fengycin A (n=13, X=Ala) (a and b, respectively), corresponding to the ESI-MS/MS spectra of **Figure 4**. Tables represent the equivalent fragment ions for fengycins A and B, and linear fengycins A and B (a and b, respectively).

Identification of Amicoumacins

Amicoumacin A is characterized by an amidated terminal group whereas amicoumacin B bears a carboxylate end group **(Figure 1)**. These compounds were never detected by direct MALDI-MS analysis, but were easily characterized by LC-ESI-MS/MS. Amicoumacins A and B had the same retention time on the C4 column (5.3 min, **Figure 2**) and the same fragment ions in MS/MS. They were thus differentiated by the sole *m/z* value of the pseudomolecular ion (*m/z* 424.2 and *m/z* 425.2 for Amicoumacin A and B, respectively). In this study, only Amicoumacin B was found in strain 2.

Identification of surfactins

LC-ESI-MS/MS analysis resolved most spectral discrimination problems encountered with direct MALDI-MS analysis and particularly provided a clear separation between surfactins and other lipopeptides **(Figure 2)**. Identification within the surfactin series by means of LC-ESI-MS/MS relies on fragment ions that characterize the residues of the peptidic cycle (*m/z* 441, 554, 667 and 685) as well as the length of the β-hydroxy fatty acid chain bound to it (*m/z* 469 and 582) **(Figure 3)**. Surfactins can be separated by RPLC on the C4 column according to their fatty acid chain length: C12, C13, C14, C15, C16 and C17 surfactins were eluted at 17.2, 17.8, 18.5, 19.2, 20.1 and 20.8 min, respectively **(Figure 2)**. However, surfactins A and C (Leu/Ile substitution) cannot be distinguished by this low energy MS/MS approach. Only surfactins A and/or C were found in the investigated Bacillus strains.

Identification of cyclic and linear fengycins

The presence of ornithine in the structure of fengycins leads to abundant doubly charged ions in ESI mode **(Figure 4a-b)**. Ala/Val isoforms, corresponding to fengycins A and B, were easily distinguished by ESI and MALDI-MS/MS by their fragment ions **(Figure 5a)** [14], and by their LC retention times (for instance C16 fengycin A eluted at 13.1 min, whereas C16 fengycin B eluted at 13.7 min) **(Figure 2)**.

In Bacillus strain 2, we detected a series of compounds eluted from the C4 column close to fengycins, but with molecular masses in excess of 18 amu **(Figure 4c)**. Accurate mass measurements allowed attributing this mass difference to a water molecule, suggesting that these compounds were linear forms of fengycins. Sequence ions obtained by MS/MS confirmed the absence of the Tyr-Ile bond, and located Ile at the C-terminus of the linear peptide chain **(Figures 4e and 5b)**. The C16-C18 linear fengycins A and a C17 linear fengycin B were eluted before their cyclic counterparts (from 11.7 to 12.9 min, **Figure 2**).

Activity tests run on a fraction containing only the linear forms of fengycins did not reveal any antifungal activity (data not shown). Furthermore, we noticed that lesser amounts of linear fengycin forms were found with longer culture times. These observations are in agreement with the fact that cyclisation is the last step of fengycin synthesis, and is an absolute requirement for the biological activity of these compounds [19].

Conclusions

The analytical methodology that has been set up is adapted to the characterization of the structure and evaluation of the activity of compounds produced by Bacillus. It allowed separating the surfactin series from the cyclic and linear fengycin series. Notably, the fact that linear forms of fengycins are devoid of antifungal activity confirmed that cyclisation is essential for the activity of fengycins.

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