**Title**: characterization of new fengycin cyclic lipopeptides produced by *Bacillus amyloliquefaciens* (ET) originating from a salt lake of Eastern Algeria

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**Abstract**

Fengycin cyclic lipopeptides are widely known for their important role in plant diseases biocontrol. This is due to their potent antimicrobial activity and involvement in plant systemic resistance elicitation. The main objective of this study is to characterize fengycin variants produced by the *B. amyloliquefaciens* strain (ET). LC-MS analysis of fengycin extract shows several group of molecular ion peaks (MH+ /MH2+), corresponding to conventional and some new types. Further characterization of these fengycin molecules was carried out by MS.MS (CID) analysis. This technique allows fragmenting the fengycin molecules at different positions, under collision induced dissociation (CID) conditions. The obtained product ions corresponding to a cleavage of Glu1-Orn2 (B) and Orn2-Tyr3 (A) were used as diagnostic ions for identifying fengycin variants. The diagnostic product ions at (A= 921.5 / B=1080.5) and (A= 949.4/ B=1108.5) correspond to fengycin A and B, respectively. The diagnostic ion (A=980/B=1094), may correspond to fengycin C, D, S or B2. Interestingly, unknown diagnostic product ions at (A= 951/B=1065) and (A= 979/B= 1093) were detected for the first time in this study, which correspond to new fengycin variants fengycin X and Y, respectively. The diversity of the fengycin types produced by ET strain will help the elucidation of its biocontrol mechanisms.

**1- Introduction**

*Bacillus* *subtilis* strains are a rich source of antimicrobial compounds having a high potential for biofertilizer and biocontrol applications. A great deal of interest has centered on the antibiotics produced by non-ribosomal peptides synthetases (NRPSs) (Stein, 2005; Caboche et al., 2010). Cyclic lipopeptides (CLPs) are among the most NRPSs antibiotics produced by *Bacillus* *subtilis* and other related *Bacillus* species (Aric *et al.,* 2001; Pueyo *et al.,* 2005). Based on their structure, the CLPs are generally classed into three families, i.e., surfactin, iturin and fengycin (Ongena and Jacques, 2008).

Fengycins show strong fungitoxic activity, specifically against filamentous fungi [Tao *et al.,* 2011]. They are involved in plant systemic resistance elicitation [Ongena *et al.,* 2007]. The fengycin family is also called the plipastatin family. Their structure consists of a- hydroxy fatty acid connected to the N-terminus of a decapeptide [Ongena and Jacques, 2008]. The C-terminal residue of the peptide moiety is linked to the tyrosine residue at position 3, forming the branching point of the acylpeptide and the eight-membered cyclic lactone [Ongena and Jacques, 2008]. Two classes of fengycins, A and B, which differ by an Ala to a Val change at position 6, have been reported thus far [Vanittanakom *et al.,* 1986]. Nowadays, the peptide sequences of fengycin variants from various *Bacillus* strains have been determined by partially hydrolyzed fengycins and tandem mass spectrometry. Two mass spectrometry approaches have been reported, i.e., matrix assisted laser desorption ionization (MALDI) mass spectrometry and liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC-ESI-MS) (Vateret *al.,* 2002; Pueyo *et al.,* 2005). Fragment ions (m/z 1080, m/z 966, m/z 1108 and m/z 994) are used as fingerprints to quickly detect fengycin A and fengycin B, respectively (Bie *et al.,* 2009). Further heterogeneity in these tow fengycins types is observed in the length of the β-hydroxy fatty acids (C14-C17). The presence of unsaturated β-hydroxy fatty acids in the fengycins has also been reported previously (Kim *et al.,* 2004). In addition, diverse fragment ions corresponding to several fengycine types were described recently. they are fengycin C, D and S (Yu Li *et al.,* 2012) and fengycin A2, B2 and C (Pathak *et al.*, 2012) (scheme 1). The results of the present study establish the sequences of some new fengycin variants produced by *B. amyloliquefaciens* strain (ET).

**2- Material and methods**

**2.1- Microorganism strain and production of fengycin**

The *Bacillus amyloliquefaciens* (ET) was isolated from the salt lake of Ain Mlila (Algeria). It was used for producing fengycin lipopeptide. This strain was inoculated in a 250 mL shake flask containing 50 mL of opt medium as described by Jaques *et al.* (1999), incubated at 30°C and 180 rpm during 72 h. At the end of cultivation, the culture broth was centrifuged at 15,000×g for 20 min to remove bacterial cells. The pH of the supernatant was adjusted to 2.0 by adding 6 N HCl until a white precipitate appears. Then the supernatant was centrifuged at 11,000×g for 10 min and the precipitate was collected. 80% acetonitrile was added to the precipitate to suspend fengycin. The fengycin suspension was centrifuged several times to obtain a pure fengycin extract (Khyati *et al.,* 2012).

**2.2- Characterization of Fengycin**

LC-MS analysis were carried out using a nanoHPLC Dionex Ultimate 3000 system, coupled to an Amazon speed ETD mass spectrometer (Bruker Daltonics). The sample injection volume was 20 µl. Before injection, fengycin extracts were diluted 30 x in acetonitrile 30%, formic acid 0.1%.The LC separation was carried out using aqueous 0.1% (v/v) formic acid and acetonitrile 2% and 80% as solvent A and B, respectively, with the following gradient: 00-05 min:37.5% B ; 05-27 min : 37.5-97.5% B (elution) ; 27-35 min : 97.5% B (elution) ; 35-44 min : 97.5-100% B (washing) ; 44-45 min : 100-62.5% B (washing) ; 45-55 min : 62.5-100% B (washing) ; 55-65 min : 100-37.5% B (return to initial conditions) ; 65-85 min : 37.5% B. A Pepmap Acclaim 300 column (C18, 3µm particles diameter, 300 Åpores size, 75 µm id x 15 cm column) was used as analytical column with 0.3 µl/min flow rate. A MS full scan was recorded in the so called “enhanced resolution” mode, in the range of 200 to 1600 m/z mass. Maximum accumulation time was set to 200 ms, and ICC target to200000. MS.MS spectra were obtained using the “Xtreme scan” mode with a CID (collision-induced dissociation) fragmentation method (time fragmentation 40 ms). The data were treated by the Data Analysis 4.0 software (Bruker Daltonics). The sequencing of the fengycin peptide cycle was carried out after opening it. Fengycin cycle opening was performed as follow: 50 µL of fengycin extract was evaporated to 10 µL and then supplemented with 10 µL of KOH (2 M). The obtained sample was incubated 1 h at 37°C under mixing. Reaction was stopped by addition of 2 µL formic acid (pH around 3.5). Before LC-MS, 1 µL of sample was diluted 100 times in acetonitrile 30%, formic acid 0.1%. The same LC-MS parameters were used to analyze opened fengycins.

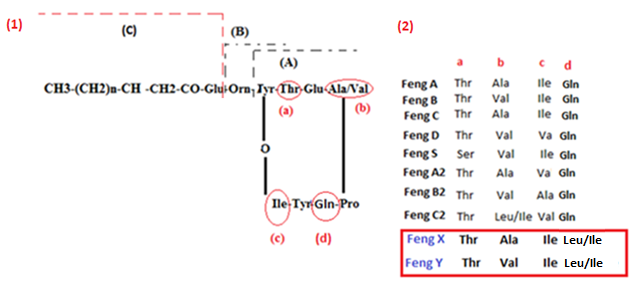
**3- Results and discussion**

**Scheme (a)** shows a fengycin structure which consists of a cyclic peptide with a lactone bond formed between the phenolic -OH of Tyr3 and the C-terminal -COOH. The N-terminus of the decapeptide segment is acylated with a β-hydroxy fatty acid (β-OH FA) which contains 14 to 19 atoms of carbon and may be present as saturated or unsaturated forms. A wide variety of conventional fengycin homologues and new types were detected by HPLC-MS analysis of the *B. amyloliquefaciens* (ET) lipopeptide extracts **(table 1).**

Chromatograms of several peaks consistent with previously reported fengycins are shown in **figure 1.** These peaks may correspond to fengycin A and B [Esumi *et al.,* 2003; Ongena and Jacques, 2008; Stein, 2008; Vater *et al.,* 2002, 2003], fengycin C, D and S (Yu Li *et al.,* 2012) and fengycin A2, B2 and C (Pathak *et al.*, 2012) **(scheme 1).** The 14 Da mass difference observed between successive peaks , suggests that there were several fengycin isomers and homologues with different fatty acid (FA) length (C14-C19). In addition, the observed -2 Da difference suggests the presence of unsaturated FA in several fengycin molecules. Further characterization of these fengycin molecules was carried out by MS. MS (CID) analysis. This technique allows fragmenting the fengycin molecules at different positions giving rise to three main fragments: A, B and C (**scheme a**), under collision induced dissociation (CID) conditions. The intense product ions corresponding to cleavage of Glu1-Orn2 (B) and Orn2-Tyr3 (A) are readily observed and have been used here as diagnostic ions for identifying fengycin variants with potential amino acids replacement within the macrocyclic moiety (sequence tag). On the other hand, the fragment C allows determining the length (C14-C19) and the nature (saturated or unsaturated) of the fatty acid moiety (Scheme a) (FA length tag). Taken together, these tags allows therefore characterizing unambiguously the fengycin variants. The representative MS.MS (CID) spectra in the **figure 2** illustrate the distribution of key product ions (A and B). The product ions at m/z 921.5 and 1080.5 representing fengycin A, while the ions at m/z 949.4 and 1108.5 correspond to fengycin B homologues. Bie *et al.* (2009) detected fengycin A at (A=966.5/B=1080.5) and fengycin B at (A=994.5/B=1108.5, the found difference in the diagnostic ions (A) can be explained by the loss of CO and/ or NH3 fragments. These two sequences tags were detected in the MS.MS analysis of the precursor ions found at the following m/z ratio: 1463,9 ; 1491,9; 1447,8 ; 1461,8; 1476 ; 1489,9; 1506; 1504; 1489,9 and 1518. These peaks correspond to different homologues of fengycin A and/or B, including C20 -2 Da feng A and C18 -2 Da feng B**,** which, to our knowledge, have never been described before **(table 1).** In addition, the mass tags (A=980/B=1094/ C= 382.4), were detected at the peak ion 1476 which may correspond to C16-2 Da fengycin C, D, S or B2 **(table 1).** Interestingly, **figure 3** shows product ions that are not diagnostic of any of the previously reported fengycins. In fact, the peaks that were detected at the following m/z ratio 717.2+; 731.2+; 739. 2+; 724, 2+ and 759, 2+ exhibit on their MS.MS spectra unknown diagnostic product ions at following m/z ratio: **(A= 951/B=1065)** and **(A= 979/B= 1093).** These new diagnostic product ions may only be explained by the substitution of the amino acids composing the lactone cycle by other amino acids and these new fengycin types were named fengycin X and fengycin Y, respectively. Due to the cyclic nature of fengycins, CID of closed forms gives poor information on the amino acids sequence. CID experiments were performed on opened forms, in order to obtain more fragments coming from the peptide part and therefore locate more precisely the modification. Two MS.MS spectra of the open C16 -2 Da forms of fengycin A and fengycin X are presented in the figure 5. It can be seen that most of the sequence is similar in these two fengycin types except for the C-terminal part in which the PQ sequence present in the fengicinA (mass difference of 225.18 Da between b6 and b8 or between y2 and y4 ions) is mutated. In Feng X, the mass difference observed between b6 and b8 (or between y2 and y4) ion is 210.25 Da which could correspond to a mutation of Q to I/L. This mutation would be similar in fengycins Y but compared to fengycins B. Although the new fengycin variants detected in this study are minority compared to conventional forms (106 vs. 107 to8, with similar chromatography yield ionization), as mentioned in the figures 1 and 3, they may modulate the biological activity of the mixture.

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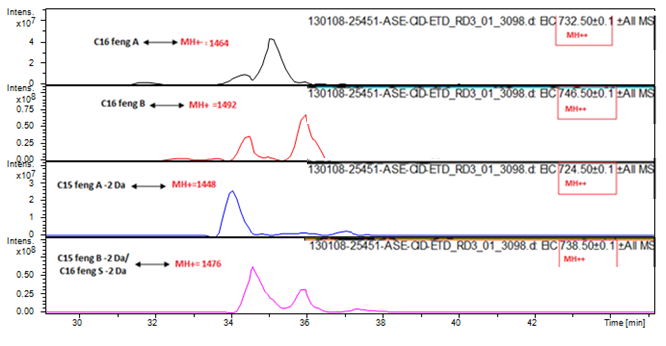


**Scheme A:** Primary structures of fengycins: (1) CID fragmentation scheme, (2) list of identified fengycin, including new variants detected in this study (FengX and FangY).

fengycin A, (2) fengycin B ; (3) fengycin C, (4)fengycin D, (5) fengycin S, (6) fengycin A2, (7) fengycin B2, (8) fengycin C2, (9) fengycin X (10) fengycin Y. Sites of mass spectrometric cleavage with the m/z values for diagnostic product ions (A and B) are indicated.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Retention time | MH++ | MH+ | FA length Tag | Sequence tag +1 | Fengycin homologues |
| 31,5 min | 732,46 | 1463,9 | 384,4 | 1080, 9 | C16 feng A |
| 32,5 min | 746,48 | 1491,9 | 384,4 | 1108, 9 | C16 feng B |
| 33,7 min | 724,5 | 1447,8 | 368,4 | 1080, 9 | Unsaturated  C15 feng A |
| 34,6 min | 731,5 | 1461,8 | 382,5 | 1080, 9 | Unsaturated C16 feng A |
| 35,3 min | 738,5 | 1476 | 368,4 - 382,4 | 1108, 9 /  1094, 9 | Unsaturated C15 feng B / Unsaturated C16 feng S |
| 35,4 min | 745,5 | 1489,9 | 382,4 | 1080, 9  /1108, 9 | Unsaturated C18 feng A / Unsaturated C16 feng B |
| 35,4 min | 717,03 | 1433 | 368,4 | 1065, 9 | Unsaturated C15 feng X |
| 35,8 min | 753,5 | 1506 | 426,5 | 1080, 9 | C19 feng A |
| 36,6 min | 731 | 1461 | 368,4 | 1093, 9 | Unsaturated C16 feng Y |
| 36,9 min | 739 | 1477 | 368 - 382 -396 | 1065, 9/  1093, 9 | Unsaturated C15, C16 and C17 Feng X/ Feng Y |
| 36,9 min | 752,5 | 1504 | 396 | 1080, 9/ 1108, 9 | Unsaturated C19 feng A / unsaturated C17feng B |
| 37 min | 724 | 1447 | 382,4 | 1065, 9 | Unsaturated C16 feng X |
| 38,2 min | 745,5 | 1489,9 | 410,5 | 1080, 9/1108, 9/ | Unsaturated C18 feng A / unsaturated C16feng B |
| 38,3 min | 759,5 | 1518 | 410,5 | 1080, 9/1108, 9 | Unsaturated C20 feng A / Unsaturated C18feng B |

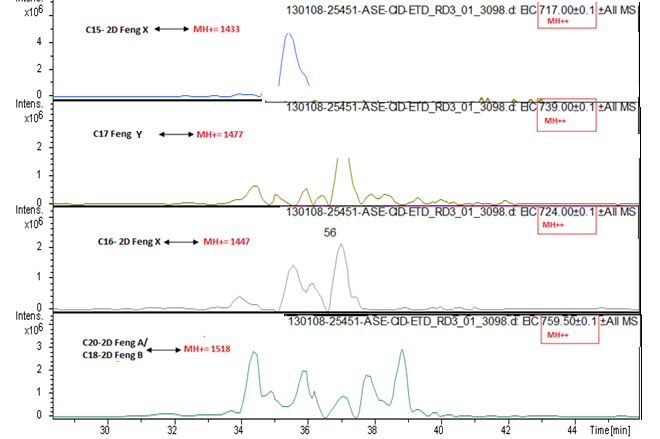
**Table 1:** Fengycin variants produced by *B. amyloliquefaciens* (ET) strain, isolated from a salt lake of Eastern Algeria.



**Figure 1** Some of (ET) conventional fengycin lipopeptides, detected by HPLC- MS analysis: Four kinds of molecular ion peaks are mentioned ([M+H] = 1464, 1492, 1448, and 1476.

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**Figure 2** MS. MS (CID) spectra of conventional fengycin, showing the diagnostic products A, B (tag sequence) and C (FA length tag): (a) fengycin A- C16; (b) fengycin A –C19; (c) fengycin B-C16.



**Figure3** (ET) new fengycin lipopeptides, detected by HPLC- MS analysis: four kinds of molecular ion peaks are mentioned ([MH+] = 1433, 1447 and 1518).

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**Figure 4** MS.MS (CID) spectra of new fengycin variants, showing the diagnostic products A, B (tag sequence) and C (FA length tag): (a) fengycin X- C16-2D; (b) fengycin X –C15-2D; (c) fengycin Y-C15-2D.

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Figure 5. CID spectra of opened forms of fengycins A (a) and X (b) (both C16 -2 Da acyl chain). Mentioned -b- ions are fragments containing the N-terminal part of the peptide while –y- ions are fragments containing the C-terminal part of the peptide (in this case, sequence should be read in the reverse direction).