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Fengycin C Produced by *Bacillus subtilis* EA-CB0015

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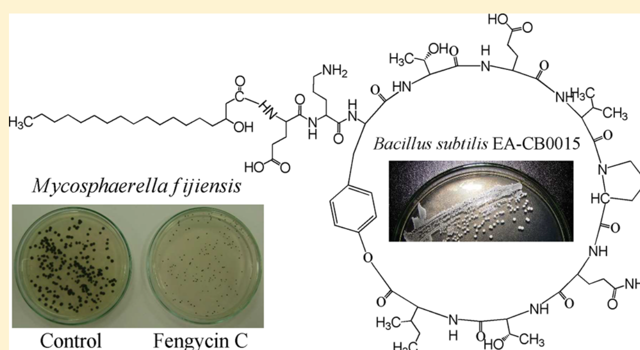
Supporting Information

ABSTRACT: *Bacillus subtilis* EA-CB0015 was isolated from the phyllosphere of a banana plant and tested for its potential to produce bioactive compounds against *Mycosphaerella fijiensis*. Using a dual plate culture technique the cell-free supernatant of *B. subtilis* EA-CB0015 produced inhibition values of 89 ± 1%. The active compounds were purified by solid-phase extraction and HPLC, and their primary structures determined using mass spectrometry and amino acid analysis. A new fengycin isoform, fengycin C, with the amino acid sequence Glu-Orn-Tyr-Thr-Glu-Val-Pro-Gln-Thr-Ile was isolated. The peptidic moiety differs from fengycin B at position 9 and from fengycin A at positions 6 and 9. The β-hydroxy fatty acyl chain is connected to the N-terminal of the decapeptide and can be saturated or unsaturated, ranging from 14 to 18 carbons. The C-terminal residue of the peptidic moiety is linked to the tyrosine residue at position 3, forming the branching point of the acyl peptide and the eight-membered cyclic lactone.



Many antibiotics have been isolated from microorganisms,[1] with *Bacillus* species, actinomycetes, and fungi being important sources. *Bacillus subtilis* is a bacterium capable of growth in many environments and exhibits considerable genomic diversity.[2] Using microarray-based comparative genomic hybridization (M-CGH) of wild strains of *B. subtilis*, it has been found that this diversity is distributed throughout the genome including in the genes that encode components involved in the synthesis of secondary metabolites.[2,3] It might be predicted that bacteria isolated from tropical regions may reveal new features due to their unusual microclimate, culture types, and agriculture practices.

Several *B. subtilis* strains have been collected to study their potential to produce antimicrobial compounds,[3−9] and it was found that an average of about 4−5% of a *B. subtilis* genome is devoted to secondary metabolites production.[3] Among these compounds, lipopeptides of the surfactin, iturin, and fengycin families have been identified as exhibiting a wide antimicrobial spectrum and exceptional surfactant activities.[4−6] These amphiphilic compounds are nonribosomal oligopeptides synthesized by large multienzyme complexes in several *Bacillus* species and share a common cyclic structure including a β-amino or β-hydroxyl fatty acid that is integrated into the peptide moiety.[3] Generally, *Bacillus* species coproduce various families of lipopeptides with different lengths of fatty acid chains, giving rise to different homologues and with different amino acid compositions of the peptidic sequence, providing isoforms leading to a remarkable structural heterogeneity.

The surfactin family consists of a β-hydroxyl fatty acid (C12−C16) linked to the N-terminal amino acid of a heptapeptide to form a cyclic lactone ring structure.[6] They display hemolytic, antiviral, antimycoplasma, and antibacterial activities.[7] Members of the iturin family are also heptapeptides attached to a β-amino fatty acid chain (C14−C17) and display hemolytic and strong *in vitro* antifungal action with limited antibacterial activity.[7,8] The third family compromises fengycins A and B, also referred to as plipastatins. This family consists of a decapeptide with an internal lactone ring in the peptidic moiety and a β-hydroxyl fatty acid chain (C14−C18) as linear, iso, or anteiso forms that may be saturated or unsaturated.[5,9] Fengycins are less hemolytic than iturins and surfactins but retain strong antifungal activity.[7] Other lipopeptides have been discovered, including polymyxins,[10] kurstakin,[11] maltacines,[12] and surfactin-like bamylocin A.[13]

In this study, aerobic endospore-forming bacteria (AEFB) isolated from the phyllosphere of banana plants were analyzed for their ability to inhibit *in vitro* the phytopathogen *Mycosphaerella fijiensis*. Strain EA-CB0015, later identified as *B. subtilis*, which showed the highest inhibition values, was analyzed for the production of antimicrobial compounds. This led to the isolation of a new isoform of fengycin, designated fengycin C,

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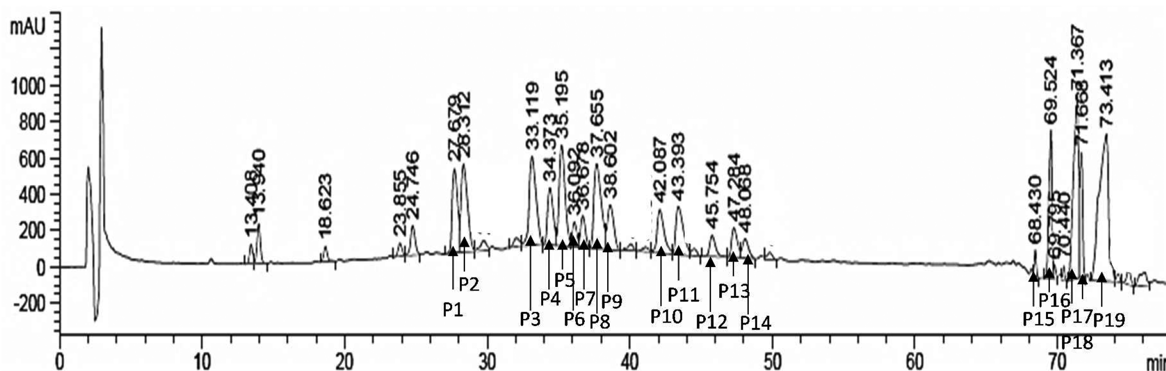


Figure 1. HPLC chromatogram of *Bacillus subtilis* EA-CB0015 compounds contained in the methanol-eluted fraction from a C_{18} SPE cartridge. HPLC peaks P1 to P14 were active against *Mycosphaerella fijiensis*, while peaks P15 to P19 were not active.

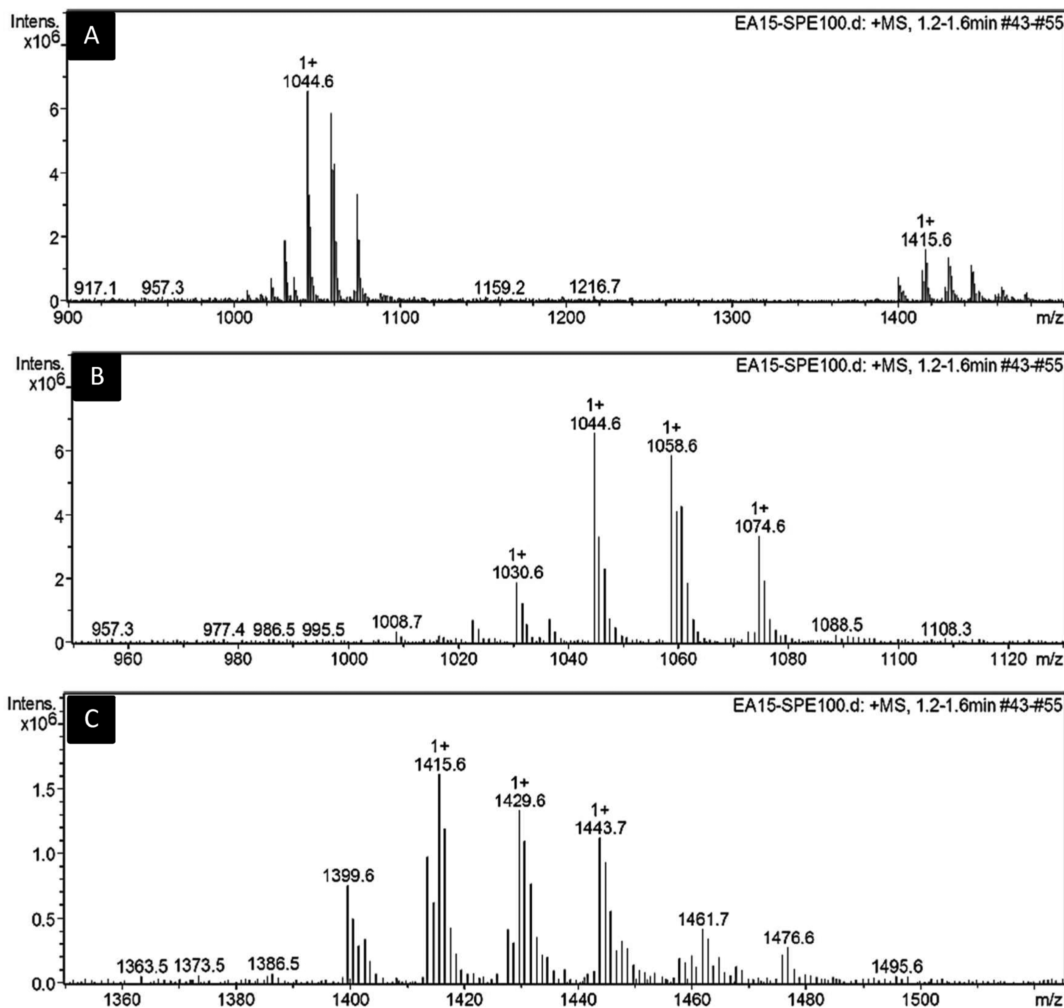


Figure 2. (+) ESI-MS spectrum of methanol-eluted fraction from a C_{18} SPE cartridge. (A) ESI-MS between *m/z* 900 and 1500. (B) Expanded group of ions between *m/z* 950 and 1130. (C) Expanded group of ions from *m/z* 1370 to 1540.

which was composed of several homologues with different fatty acid chains. Here we report the isolation and structural elucidation of these homologues, which are active against *M. fijiensis*.

■ RESULTS AND DISCUSSION

Screening and Identification of Antagonistic Bacteria. For the screening of potential antagonist bacteria to inhibit *M. fijiensis*, 649 AEFBs strains isolated from the phyllosphere of banana plants were evaluated against an inoculum of different strains of *M. fijiensis*. Among these isolates, the cell-free supernatant (CFS) of strain EA-CB0015 showed the strongest activity against *M. fijiensis*, producing inhibition percentages of 89 ± 1% compared with the positive control, *B. subtilis* UA321 (83 ± 2%). Analysis of the 16S rDNA sequence showed that the strain EA-CB0015 has high homology (100%) with *B. subtilis* (GenBank accession number GQ375229) and was characterized

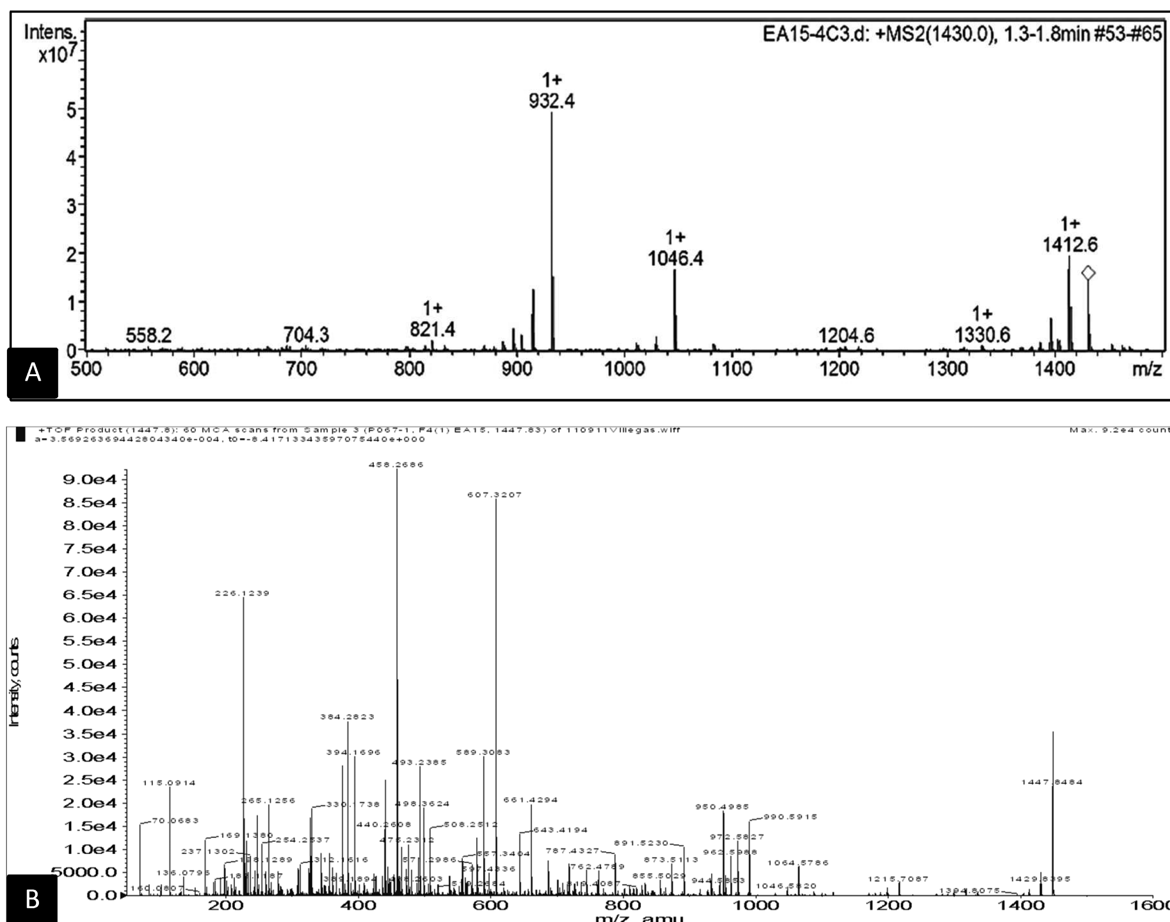


Figure 3. MS/MS spectra of purified lipopeptide P5 before (*m/z* 1429.9) (A) and after (*m/z* 1447.8) (B) hydrolysis of the lactone by 1 M KOH (expanded in the Supporting Information, Figure S2).

as a Gram-positive rod (2.45 ± 0.1 μm long), forming endospores (1.47 ± 0.08 μm) and producing punctiform, opaque white colonies with undulating and rough edges. In the API 50 CHB/E system, the positive reactions were glycerol, L-arabinose, ribose, D-xylose, galactose, glucose, fructose, mannose, mannitol, sorbitol, α-methyl-D-glucoside, *N*-acetyl-glucosamine, amygdalin, arbutine, esculin, salicin, cellobiose, maltose, lactose, sucrose, trehalose, inulin, melezitose, D-raffinose, starch, glycogen, gentiobiose, and D-turanose, and negative reactions were erythritol, D-arabinose, L-xylose, adonitol, sorbose, rhamnosa, dulcitol, inositol, α-methyl-D-mannoside, melibiose, xylitol, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol, glucanate, 2 keto-glucanate, and 5-keto-glucanate.

Purification of Antifungal Compounds. Metabolites produced by *B. subtilis* EA-CB0015 were obtained by eluting the compounds adsorbed by the resin Amberlite XAD16 with methanol. The methanol extract that was active against *M. fijiensis* was further separated using reverse solid-phase extraction (SPE) as described in the Experimental Section. The most active fraction obtained by SPE, eluting with 100% methanol, was then purified on semipreparative reverse-phase high-performance liquid chromatography (HPLC). Fourteen peaks (Figure 1) with statistically significant biological activity were detected eluting between retention times of 26 and 50 min (eluting with 48−60% of acetonitrile−0.1% TFA, solvent B). The peaks eluting between retention times of 69 and 76 min (eluting with 100% B) were not significantly active against *M. fijiensis* (Figure 1).

Structure Analysis. To detect and identify the compounds produced by *B. subtilis* EA-CB0015, the fraction obtained by SPE with 100% methanol was first characterized through ESI-MS direct infusion analysis in positive scan mode (Figure 2). Two clusters of peaks with 14 or 28 Da difference in their molecular ion species were present, revealing two sets of homologous molecules (Figure 2A).

The first set showed three main signals at *m/z* 1030.6, 1044.6, and 1058.6 corresponding to the sodiated molecules $[M + Na]^+$ (Figure 2B). Therefore, the molecular weights of the three molecules were respectively 1007, 1021, and 1035. The sodiated molecules $[M + Na]^+$ at *m/z* 1030, 1044, and 1058 were used as precursor ions for further ESI-MS/MS analysis, and the results showed that the product ions had regularities (Supporting Information, Figure S1) and corresponded to C13, C14, and C15 surfactins.

The second cluster of peaks exhibited signals at *m/z* 1399.6, 1415.6, 1429.6, and 1443.7, corresponding to the protonated molecules $[M + H]^+$ (Figure 2C). These peaks revealed differences of 14 Da, except the ion at *m/z* 1399.6, suggesting a series of homologous molecules differing by one $-CH_2$ and with molecular weights of 1399, 1415, 1429, and 1443 Da, respectively. All precursor ions were used for further ESI-MS/MS analysis, and the results showed identical product ions at *m/z* 1046.4 and 932.4 (Figure 3A). These product ions could be explained as the losses of fatty acid chain-Glu and fatty acid chain-Glu-Orn from the N-terminal region, respectively, characteristic of lipopeptides of the fengycin family. The molecular weights and

Table 1. Precursor Ions, Molecular Weights, and Product Ions of Purified HPLC Fractions[a]

Peaks (Retention time HPLC)	Precursor ions m/z [M+H]$^+$	Product ions m/z	Difference between precursor ion and first product ion (C:D)	Estimated Molecular weight (Da)
P1 (t_R 27.4 – 27.8 min)	1416.2	1046.4, 932.4	369.8 (15:0, Glu)	1415
P2 (t_R 28.1 – 28.8 min)	1416.2	1046.4, 932.4	369.8 (15:0, Glu)	1415
P3 (t_R 32.9 – 33.6 min)	1429.9	1046.4, 932.4	383.5 (16:0, Glu)	1429
P4 (t_R 34.2 – 34.8 min)	1429.9	1046.4, 932.4	383.5 (16:0, Glu)	1429
P5 (t_R 35.0 – 35.4 min)	1429.9	1046.4, 932.4	383.5 (16:0, Glu)	1429
P6 (t_R 36.1 – 36.4 min)	1399.8	1046.4, 932.4	353.4 (14:1, Glu)	1399
P7 (t_R 36.6 – 37.0 min)	1399.8	1046.4, 932.4	353.4 (14:1, Glu)	1399
P8 (t_R 37.4 – 38.1 min)	1444.0	1046.4, 932.4	397.6 (17:0, Glu)	1443
P9 (t_R 38.6 – 39.1 min)	1444.0	1046.4, 932.4	397.6 (17:0, Glu)	1443
P10 (t_R 42.0 – 42.7 min)	1413.8	1046.4, 932.4	367.4 (15:1, Glu)	1413
P11 (t_R 43.2 – 44.1 min)	1413.8	1046.4, 932.4	367.4 (15:1, Glu)	1413
P12 (t_R 45.7 – 46.4 min)	1457.8	1046.4, 932.4	411.4 (18:0, Glu)	1457
P13 (t_R 47.2 – 47.8 min)	1428.0	1046.4, 932.4	381.6 (16:1, Glu)	1427
P14 (t_R 48.1 – 48.8 min)	1428.0	1046.4, 932.4	381.6 (16:1, Glu)	1427

[a]P1 to P14 denote HPLC peaks 1 to 14, respectively. (C:D): C denotes number of carbons and D number of unsaturations. Gray and white: peaks with the same molecular weight.

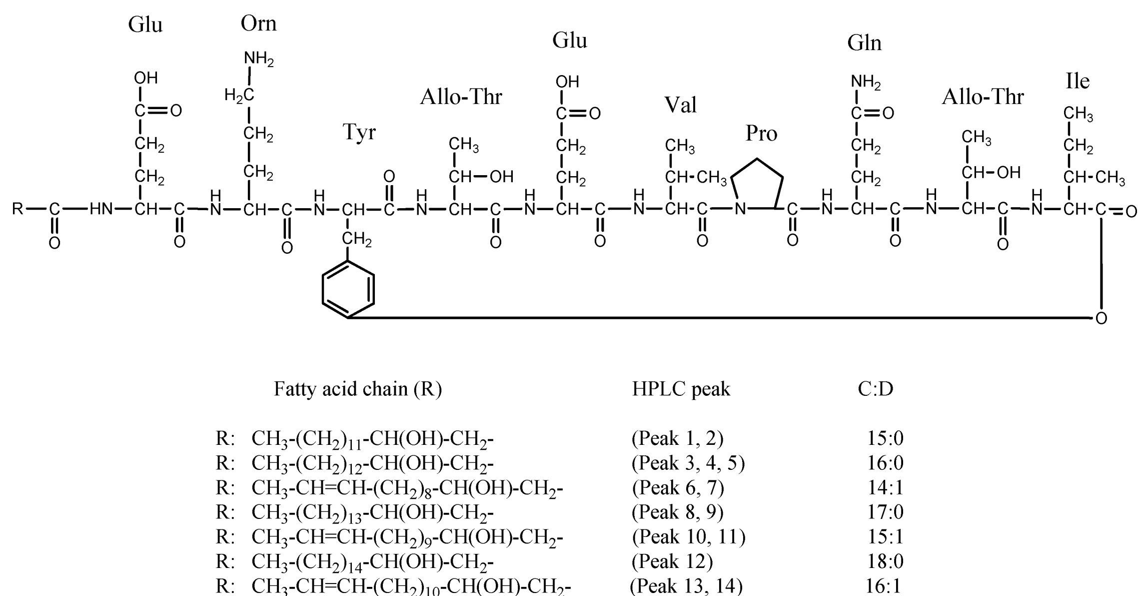
product ions of these molecules are different from those reported for fengycin A (product ions m/z 1080, 966) and fengycin B (product ions m/z 1108, 994)[14,15] and from other *Bacillus* sp. metabolites,[16−18] although its partial sequence (fatty acid-Glu-Orn) is in accordance with the structure of fengycins,[4,14,15] suggesting a difference in the amino acid composition of the peptide and therefore a new isoform of fengycins.

In order to determine if these compounds were responsible for the activity against *M. fijiensis*, the peaks collected from the HPLC (P1 to P19) were characterized through ESI-MS and ESI-MS/MS by direct infusion analysis in positive scan mode. The active peaks (P1 to P14) corresponded to the signals between m/z 1399.6 and 1457.8 of the protonated molecules $[M + H]^+$ (Table 1), while nonactive peaks P15 to P19 corresponded to C12 surfactin ($[M + Na]^+$ 1016.7), C13 surfactin ($[M + Na]^+$ 1030.7), C14 surfactin ($[M + Na]^+$ 1044.6), and C15 surfactin ($[M + Na]^+$ 1058.7) (data not shown).

The bioactive compounds (peaks P1 to P14) (Table 1) could be divided into two groups: the first group, with M_w of 1415, 1429, 1443, and 1457 Da, which differ by 14 Da, and the second group, with M_w of 1399, 1413, and 1427 Da, which also differ by 14 Da. The difference between similar members of these two groups (e.g., 1415 and 1413, 1429 and 1427) is 2 Da, suggesting that the first group (1415, 1429 Da) has a saturated fatty acid chain and the second (1413, 1427 Da) is unsaturated. In addition, more than one peak with different retention times in the HPLC chromatogram gave the same precursor and product ions in the ESI-MS, such as P3, P4, and P5; P6 and P7; P8 and P9; P10 and P11; and P13 and P14 (Table 1), suggesting the presence of isomeric compounds with identical molecular weights but different side chain configurations (iso, anteiso, or linear), as has been encountered for fengycins A and B.[5] These results supported the presence of a new isoform of fengycins, fencycins C.

Elucidation of Fengycins C Structure. Fengycins have an internal lactone ring in the peptidic moiety.[5,9] To identify the location of the lactone ring of fengycins C, peaks 1 to 12 were subjected to alkaline hydrolysis and the hydrolysis products analyzed by ESI-MS and ESI-MS/MS. Each molecule yielded a new product, with protonated masses $[M + H]^+$ of 1435.2 (P1, P2), 1448.9 (P3, P4, P5), 1418.8 (P6, P7), 1463 (P8, P9), 1432.8 (P10, P11), and 1476.8 (P12). The mass gain of 19 Da observed for each product was assigned to hydrolysis of the lactone ring (18 Da). The additional dalton could suggest that a glutamine residue (Gln, 128 Da) in the native lipopeptide was converted to a glutamate residue (Glu, 129) during hydrolysis.[11] This result indicated that each molecule contained a cyclic peptide domain, analogous to previously characterized microbial lipopeptides.[19]

To determine the amino acid sequence of fengycins C, ultrapure samples (peaks 1, 5, 8, and 12) that had been passed three consecutive times through the semipreparative HPLC column were analyzed by MALDI-TOF-MS. The product ions obtained for the precursor ion at m/z 1447.8 (P5) are shown in Figure 3B. The y ion sequence was 1447.8 → 1193.60 (y_{10}) → 1064.6 (y_9) → 950.5 (y_8) → 787.5 (y_7) → 686.4 (y_6) → 557.3 (y_5) → 458.3 (y_4) → 361.2 (y_3) → 233.1 (y_2) →132.1 (y_1), corresponding respectively to the losses of (fatty acid)-Glu-Orn-Tyr-Thr-Glu-Val-Pro-Gln-Thr-(L_{xx}+19). The fragment ion at m/z 1064.6 corresponds to the loss of 383.2 from the $[M + H]^+$ 1446.8 ion, which is exactly the same neutral loss observed in the intact lipopeptide. This result suggests that the lactone was located in the fragment ion of 1046.4, which changes its mass to 1064.6 after hydrolysis of the lactone ring. The b ion series contains the ions at m/z 1429.8 → 1316.7 (b_{10}) → 1215.7 (b_9) → 1087.6 (b_8) → 990.6 (b_7) → 891.5 (b_6) → 762.5 (b_5) → 661.4



	Fatty acid chain (R)	HPLC peak	C:D
R:	CH_3-$(CH_2)_{11}$-CH(OH)-CH_2-	(Peak 1, 2)	15:0
R:	CH_3-$(CH_2)_{12}$-CH(OH)-CH_2-	(Peak 3, 4, 5)	16:0
R:	CH_3-CH=CH-$(CH_2)_8$-CH(OH)-CH_2-	(Peak 6, 7)	14:1
R:	CH_3-$(CH_2)_{13}$-CH(OH)-CH_2-	(Peak 8, 9)	17:0
R:	CH_3-CH=CH-$(CH_2)_9$-CH(OH)-CH_2-	(Peak 10, 11)	15:1
R:	CH_3-$(CH_2)_{14}$-CH(OH)-CH_2-	(Peak 12)	18:0
R:	CH_3-CH=CH-$(CH_2)_{10}$-CH(OH)-CH_2-	(Peak 13, 14)	16:1

Figure 4. Proposed structure of the lipopeptides isolated from *Bacillus subtilis* EA-CB0015. (C:D): C denotes number of carbons and D number of unsaturations.

(b_4) → 498.4 (b_3) → 384.3 (b_2) → 255.2 (b_1), which corresponds to the neutral losses of L_{xx}-Thr-Gln-Pro-Val-Glu-Thr-Tyr-Orn-Glu-(fatty acid) from the N-terminal region, respectively, where L_{xx} could be Leu or Ile. The connection of the two series suggests that the ion at 1447.8 has the structure R_{15}CO-Glu-Orn-Tyr-Thr-Glu-Val-Pro-Gln-Thr-L_{xx}. This structure differs from fengycins B by the presence of threonine instead of tyrosine in position 9, and from fengycins A by the presence of valine and threonine in positions 6 and 9 instead of alanine and tyrosine, respectively. The other precursor ions at m/z 1433.8, 1461.8, and 1475.9 had the same y ions and differ in the b ions by 14 Da between them (Supporting Information, Figure S2).

To confirm these findings, amino acid analysis was carried out on an ultrapure sample that had been passed three consecutive times through a semipreparative HPLC column. The compound P8 with M_w 1443 Da was hydrolyzed with HCl, and its amino acid content analyzed as described in the Experimental Section. The compound contained the amino acid residues (Allo-Thr)-Glu-Pro-Val-Ile-Tyr-Orn with molar ratios of 2:2:1:1:1:1:1.

In conclusion, lipopeptide fengycin C is a new isoform of the fengycin family, which are heptapeptides linked to fatty acid chains (Figure 4). This is the first report elucidating the structure and activity of fengycins against *M. fijiensis*.

■ EXPERIMENTAL SECTION

General Experimental Procedures. The lipopeptides were purified by solid-phase extraction using an Agilent C_{18} SPE column (10 g) and reversed-phase liquid chromatography using an Agilent 1200 instrument equipped with a G1311A quaternary pump (Agilent), G1315B diode array spectrophotometric detector, and Agilent ChemStation software. For routine mass measurements, the samples were analyzed using an Agilent 6300 Series ion trap LC/MS instrument equipped with an ESI source. For high-resolution mass spectra the samples were analyzed on an Applied Biosystems Q-Star XL quadrapole-TOF tandem mass spectrometer.

Microorganisms. *Bacillus subtilis* EA-CB0015 was isolated from the phyllosphere of a banana plant in Urabá (Northeast Colombia, 1352081 N, 1044577 E) in September 2008 and identified by analysis of 16S rDNA gene sequencing (GenBank accession number KC006063, Supporting Information, Table S1). The bacteria were stored in TSB (tripticase soy broth, Merck) with 20% v/v glycerol at −80 °C (Humboldt Institute Collection No. 191) and activated in trypticase soy agar (TSA, Merck) for 48 h at 30 °C before any experimental use. Production of antimicrobial compounds produced by *B. subtilis* EA-CB0015 was evaluated using a medium optimized for lipopeptide production (MOLP).[20]

Isolates EASGK09, EASGK10, EASGK11, and EASGK14 of *M. fijiensis* were obtained from diseased banana leaves cultivar Grand Naine from Urabá (Colombia), following Du Pont methodology.[21] Monosporic, 20 day-old cultures obtained in PDA (potato dextrose agar, Merck) were fragmented and stored.[22] Identification of these strains was carried out using the oligonucleotide primers MF137 (5′GGCGCCCCCGGAGGCCGTCTA 3′) specific for *M. fijiensis* and R635 (5′ GGTCCGTGTTTCAAGACGG 3′),[23] which encode for a preserved region in the 25S subunit of the rDNA. The polymerase chain reaction (PCR) was conducted as previously described,[24] and DNA extraction with the Ultra Clean Microbial Isolation Kit (MoBIO). Fungal activation was performed by placing mycelia fragments into liquid cultures with Sabouraud (Merck) for 10 days at 150 rpm and 30 °C.

Screening of Antagonistic AEFB. The screening of AEFB against *M. fijiensis* was performed to determine the mycelial growth inhibition generated by CFS through a modification of the microplate methodology developed by Peláez et al.[22] The CFS of each AEFB was obtained by centrifuging (3289g, 20 min) and filtering (cellulose acetate membrane filter, 0.45 μm, Startorius Biolab) a 5-day culture in MOLP (140 rpm, 30 °C). Inocula with concentrations between 5×10^2 and 11×10^2 cfu/mL of *M. fijiensis* EASGK09, *M. fijiensis* EASGK10, *M. fijiensis* EASGK11, and *M. fijiensis* EASGK14 in equal proportions were used in this assay and were obtained by the fragmentation method described previously.[22] Each microplate well contained 50 μL of Sabouraud broth, supplemented with chloramphenicol (200 μg/mL) and ampicillin (250 μg/mL), 50 μL of *M. fijiensis* inoculum, and 50 μL of bacterial CFS. The blank contained 50 μL of sterile water, 50 μL of Sabouraud, and 50 μL of TSB. Microplates were incubated for 12 days (darkness, static conditions, 21 ± 3 °C). Mycelial growth was determined by measuring the optical density (OD) in a spectrophotometer (iMark, BIO-RAD) at 595 nm, using four replicas per treatment. Bacteria selected as biologically active were those in which the percentage of CFS inhibition of *M. fijiensis* growth was statistically significantly higher than the CFS of the positive control, *B. subtilis* UA321 (% inhibition = 83 ± 2%).

Cultivation of *Bacillus subtilis* EA-CB0015 Strain and Extraction of Antimicrobial Compounds. The preparation and recovery of antimicrobial compounds was carried out by the modified methodology previously described.[25,26] Briefly, 20 mL of an overnight

culture of *B. subtilis* EA-CB0015 was inoculated in a 500 mL Erlenmeyer flask containing 180 mL of MOLP medium and incubated for 5 days at 30 °C and 140 rpm. After 24 h of incubation 4% Amberlite XAD16 was added to the fermentation. The adsorbent resin was recovered from the culture broths by decantation, transferred onto a column, and washed with 300 mL of distilled water three times. Adsorbed products were subsequently eluted with 200 mL of 100% MeOH, and solvent was removed by evaporation at reduced pressure (−50 psig, 50 °C). After evaporation, approximately 440 mg of solid residue was obtained from a total culture of 200 mL.

The solid residue was suspended in 10 mL of distilled water and applied to a C_{18} cartridge (10 g Varian) from Agilent Technologies (USA). The cartridge was rinsed successively with 80 mL of water, 80 mL of 20% MeOH, 80 mL of 40% MeOH, 80 mL of 70% MeOH, and finally 80 mL of 100% MeOH. The eluates were evaporated at reduced pressure (−50 psig, 50 °C), and the residues were weighed and dissolved in methanol. The different fractions were evaluated against *M. fijiensis*, and the active fractions analyzed by HPLC as described below.

Purification of Lipopeptides. The active fraction obtained with 100% MeOH from the SPE was purified by semipreparative reversed-phase high-pressure liquid chromatography (RP-HPLC) using a Zorbax C_{18} column (250 by 9.5 mm 5 μ, Agilent) connected to an Agilent G1311A quaternary pump. The solvent system consisted of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). Seventy microliters of the sample (50 mg/mL) was injected into the column, and the compounds were eluted by a gradient program developed from 30% to 70% B in 60 min, 70−100% B in 5 min, and 100% B in 5 min at a flow rate of 4 mL/min and UV detection at 214 nm. Peaks with different retention times were collected, evaporated with gaseous N_2, and stored at 4 °C.

Evaluation of SPE and HPLC Fractions against M. fijiensis in Vitro. The activity of the different fractions collected from SPE and HPLC against *M. fijiensis* EAST*K14* was assessed by determining the mycelial growth inhibition in dual plate cultures. Each fraction was suspended in 1 mL or 100 μL of MeOH for SPE or HPLC fractions, respectively. The inoculum of *M. fijiensis* EAST*K14* was obtained by the fragmentation method described previously.[22] Dual plate culture was carried out plating 50 μL of each fraction (or 50 μL of MeOH, absolute control) and 50 μL of *M. fijiensis* inoculum in PDA (Merck) supplemented with 200 μg/mL chloramphenicol and 250 μg/mL ampicillin with two replicates per treatment. Fungal colony diameters were measured after 10 days of incubation (21 ± 3 °C, darkness), and the inhibition percentage for each treatment was calculated considering fungal growth in the absolute control as 100%. Fractions with biological activity were those in which the percentage of inhibition of *M. fijiensis* growth was statistically significant higher than the absolute control.

Mass Spectrometry Analysis. Routine mass spectrometry analyses were carried out on an Agilent 6300 Series ion trap LC/MS instrument equipped with ESI source. Samples (50 μg/mL) dissolved in methanol were infused with a syringe at 150 μL/h flow rate. Ionization parameters were as follows: 300 V capillary exit, +4500 HV capillary, nebulizer 40 psi in positive ion mode. Capillary temperature and sheath gas flow (N_2) were set at 350 °C and 5 L/min, respectively. Data were acquired in positive MS total ion scan mode (mass scan range: m/z 50−2200) and in positive MS/MS product ion scan mode.

High-resolution mass spectrometry analyses were done on an Applied Biosystems Q-Star XL quadrapole-TOF tandem mass spectrometer with cyano-cinnamic acid as matrix in The Protein Facility of the Iowa State University Office of Biotechnology.

Determination of the Lactone Bond. Lipopeptide was dissolved in 1 M potassium hydroxide (KOH) solution and allowed to react overnight at room temperature. Excess KOH was removed by passing the solution thought a C_{18} Omix pipet tip (Varian,100 mg). The trapped sample was washed twice with 100 μL of 0.1% TFA and then eluted three times from the cartridge with 100 μL of methanol. For MS/MS analysis 100 μL of the sample was infused with a syringe to the ion trap as described above.

Amino Acid Analysis. Amino acid analysis of P8 compound was conducted at the AESCL analytical services of Missouri University using the standard method AOAC Official Method 982.30. Briefly, 0.3 mg of the lipopeptide was hydrolyzed with 6 M HCl at 110 °C for 24 h. Free amino acids were separated by ion exchange chromatography using a Hitachi 8800 amino acid analyzer.

Statistical Analysis. Differences in the effects of CFS or SPE and HPLC fractions for the percentage of inhibition were determined by analysis of variance (ANOVA) and with Tukey multiple comparison. The confidence level used for ANOVA analyses was 95%, and they were done using Statgraphics plus 5.1.

■ ASSOCIATED CONTENT

Supporting Information

ESI-MS/MS of precursor ions $[M + Na]^+$ with m/z 1030.7, 1045.1, and 1059.3 from Figure 2. MS/MS spectra of purified lipopeptide P1 (m/z 1433.8), P8 (m/z 1461.8), and P12 (m/z 1475.8) after hydrolysis of the lactone by 1 M KOH. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Peláez, F. *Biochem. Pharmacol.* **2006**, *71*, 981−990.
(2) Earl, A. M.; Losick, R.; Kolter, R. *Trends. Microbiol.* **2008**, *16*, 269−275.
(3) Stein, T. *Mol. Microbiol.* **2005**, *5*, 845−857.
(4) Winkelmann, G.; Allgaier, H.; Lupp, R.; Jung, G. *J. Antibiot. (Tokyo)* **1983**, *36*, 1451−1457.
(5) Vanittanakom, N.; Loeffler, W.; Koch, U.; Jung, G. *J. Antibiot. (Tokyo)* **1986**, *39*, 888−901.
(6) Peypoux, F.; Bonmatin, J. M.; Wallach, J. *Appl. Microbiol. Biotechnol.* **1999**, *51*, 553−563.
(7) Ongena, M.; Jacques, P. *Trends Microbiol.* **2008**, *16*, 115−125.
(8) Delcambe, L.; Peypoux, F.; Besson, F.; Guinand, M.; Michel, G. *Biochem. Soc. Trans.* **1977**, *5*, 1122−1124.
(9) Raaijmakers, J. M.; De Bruijn, I.; Nybroe, O.; Ongena, M. *FEMS Microbiol. Rev.* **2010**, *34*, 1037−1062.
(10) Storm, D. R.; Rosenthal, K. S.; Swanson, P. E. *Annu. Rev. Biochem.* **1977**, *46*, 723−763.
(11) Hathout, Y.; Ho, Y. P.; Ryzhov, V.; Demirev, P.; Fenselau, C. *J. Nat. Prod.* **2000**, *3*, 1492−1496.
(12) Lee, S. C.; Kim, S. H.; Park, I. H.; Chung, S. Y.; Choi, Y. L. *Arch. Microbiol.* **2007**, *188*, 307−312.
(13) Hagelin, G.; Indrevoll, B.; Hoeg-Jensen, T. *Int. J. Mass Spectrom.* **2007**, *268*, 254−264.
(14) Pecci, Y.; Rivardo, F.; Martinotti, M. G.; Allegrone, G. *J. Mass Spectrom.* **2010**, *45*, 772−778.

(15) Wang, J.; Liu, J.; Wang, X.; Yao, J.; Yu, Z. *Lett. Appl. Microbiol.* **2004**, *39*, 98−102.
(16) Hamdache, A.; Lamarti, A.; Aleu, J.; Collado, I. G. *J. Nat. Prod.* **2011**, *74*, 893−899.
(17) Arguelles-Arias, A.; Ongena, M.; Halimi, B.; Lara, Y.; Brans, A.; Joris, B.; Fickers, P. *Microb. Cell Fact.* **2009**, *8*, 63.
(18) Leenders, F.; Stein, T. H.; Kablitz, B.; Franke, P.; Vater, J. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 943−949.
(19) Williams, B. H.; Hathout, Y.; Fenselau, C. *J. Mass Spectrom.* **2002**, *37*, 259−264.
(20) Jacques, P.; Hbid, C.; Destain, J.; Razafindralambo, H.; Paquot, M.; De Pauw, E.; Thonart, P. *Appl. Biochem. Biotechnol.* **1999**, *77*, 223−233.
(21) Du Pont. *Black and yellow Sigatoka: improved identification and management techniques*. Du Pont Latin America: Coral Gables, FL, USA, 1982; p 17.
(22) Peláez, J.; Vásquez, L. E.; Díaz, T. J.; Castañeda, D. A.; Rodríguez, E.; Arango, R. E. *Rev. Fac. Nal. Agric. Medellin* **2006**, *59*, 3425−3433.
(23) Stewart, E. L.; Liu, Z.; Crous, P. W.; Szabo, L. J. *Mycol. Res.* **1999**, *103*, 1491−1499.
(24) Romero, M.; Diaz, T.; Castañeda, D.; Arango, R. *Rev. Fac. Nal. Agric. Medellin* **1999**, *52*, 425−434.
(25) Razafindralambo, H.; Paquot, M.; Baniel, A.; Popineau, Y.; Hbidz, C.; Jacques, P.; Thonart, P. *Food Hydrocolloids* **1997**, *11*, 59−62.
(26) Sasse, F.; Steinmetz, H.; Hofle, G.; Reichenbach, H. *J. Antibiot. (Tokyo)* **1995**, *48*, 21−25.