Biological Control 125 (2018) 20–28

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Lipopeptides from *Bacillus* sp. EA-CB0959: Active metabolites responsible for *in vitro* and *in vivo* control of *Ralstonia solanacearum*

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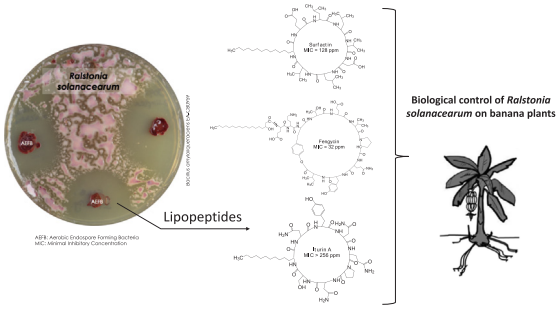
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Antibacterial activity
Fengycins
Operational Group *B. amyloliquefaciens*
Gram-negatives
Ralstonia solanacearum
Biological control

ABSTRACT

Broadening the spectrum of action of microbial bioactive compounds is a priority nowadays. From a collection of 1493 aerobic endospore forming bacteria, 3.1% (47) inhibited *Serratia marcescens* and were highly active against *R. solanacearum*. Thirty-six of these strains were identified as part of the 'Operational Group *B. amyloliquefaciens*', denoting the potential of strains from these species to produce antibacterial substances. Specifically, the strain *Bacillus* sp. EA-CB0959 was selected for further trials. Three families of lipopeptides: surfactins, iturins and fengycins were found as the active compounds produced by this strain. The highest bioactivity, produced by fengycins, had a minimal inhibitory concentration of 32 μg/mL. Treating greenhouse banana plants with a mixed fraction of lipopeptides reduced by 35% the incidence of Moko disease caused by *R. solanacearum*. Here we provide first time evidence of *in vitro* antibacterial activity of purified fengycins and *in vivo* activity of mixed lipopetides against Moko disease in banana plants.

1. Introduction

Several Gram-negative bacteria outstand because of their harmful effects in plants and/or animals, among them *Xanthomonas campestris* (Etchegaray et al., 2008), *Pectobacterium carotovorum* (Byers et al., 2002), *Pseudomonas syringae* (Bais et al., 2004), *Agrobacterium tumefaciens* (Zhang et al. 2002) in agriculture, as well as *Klebsiella pneumoniae* (Navon-Venezia et al., 2017), *Escherichia coli* (Yao et al., 2006),

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https://doi.org/10.1016/j.biocontrol.2018.06.005
Received 10 January 2018; Received in revised form 8 June 2018; Accepted 15 June 2018
Available online 18 June 2018
1049-9644/

Salmonella sp. (Sánchez-Vargas et al., 2011), *Pseudomonas aeruginosa* (Kaye and Pogue, 2015) and *Serratia marcescens* (Moradigaravand et al., 2016) in humans and other animals. Consequently, discovering new active metabolites or broadening the spectrum of existing is a priority nowadays. The stated before, becomes a more pressing issue given the high number of strains developing resistance to traditional antibiotic substances and belonging to the before mentioned and several other microbial species, many of them being plant or animal pathogens. New active metabolites assist against this every-day growing problem, giving a step forward in the battle against deathly infections and narrowing the gap of the antibiotic resistant crisis that the world faces nowadays (WHO, 2014).

Following the same trend, *Ralstonia solanacearum* is one of the most important soil pathogens, causing bacterial wilt disease in over 250 hosts from 54 botanical families in a broad geographic distribution (Elphinstone, 2005). *R. solanacearum* forms a genetically diverse species complex composed of four phylotypes (I–IV) that correspond to evolutionary and geographic origin (Fegan and Prior, 2005). Due to this high genetic variability, persistence in the environment and broad host range, its control becomes difficult to address and ineffective, highlighting the urgent need for development of sustainable and efficient solutions.

As a paradox of nature, antibiotic substances that combat infectious pathogens have been mainly discovered in other microorganisms, specially the domain Bacteria has given the world a vast array of bioactive small molecules useful in medicine and agriculture, positioning itself as a source of innovative antibiotic solutions. Despite this, the number of new approved molecules coming from natural sources, as are microorganisms, has declined as an increased appearance of resistant pathogenic strains is recorded nowadays, which implies the need for discovering new classes of bioactive small molecules (Genilloud, 2014) or broadening the spectrum of available ones. It has been suggested that bacteria still hold great potential as a source of natural products (Traxler and Kolter, 2015) dedicating from 4.0 to 10.9% of its total genetic capacity to the synthesis of secondary metabolites. Today the majority of natural products discovered come from organisms that inhabit the soil, among these actinomycetes (*e.g. Streptomyces*) and *Firmicutes* (*e.g. Bacillus*) are the most important sources (Hamdache et al. 2011; Peláez, 2006). Within *Firmicutes*, several species of *Bacillus* harbor a high capability of producing bioactive molecules, such as peptide antibiotics (Ongena and Jacques, 2008; Raaijmakers et al., 2010).

Among the most largely studied families of natural bioactive compounds are lipopeptides, which represent the family of peptide antibiotics that are synthesized by large nonribosomal peptide synthetases (NRPS) (Marahiel, 2009) and are composed of a hydrocarbon chain linked to a short cyclic oligopeptide. *Bacillus* sp. strains coproduce various families of lipopeptides such as surfactin, iturin and fengycins (Raaijmakers et al., 2010). These lipopeptides have different lengths and composition of fatty acid chains (homologues) and different amino acid compositions of the peptidic sequence (isoforms). 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 (Peypoux et al., 1999). They display hemolytic, antiviral, antimycoplasma and antibacterial activities (Raaijmakers et al., 2010). The antibacterial activities of surfactins have been evaluated against some pathogenic bacteria such as *P. syringae* (Bais et al., 2004) and *X. campestris* (Etchegaray et al., 2008), and only one study has shown antifungal activity against *Magnaporthe grisea* (Tendulkar et al., 2007). The iturin family are heptapeptides attached to a β-amino fatty acid chain (C14-C17) and display hemolytic and strong *in vitro* antifungal action with limited antibacterial activity (Ongena and Jacques, 2008; Raaijmakers et al., 2010; Tanaka et al., 2014) until recently reported by Dimkić et al. (2017), who found an iturin analog being responsible for the inhibition of *X. arboricola* and *P. syringae* phytopathogenic strains. Fengycin are decapeptides with an internal lactone ring in the peptidic moiety and a β-hydroxyl fatty acid chain (C14-C18) (Raaijmakers et al., 2010; Villegas-Escobar et al., 2013) and have shown strong antifungal activity and no antibacterial activity (Raaijmakers et al., 2010).

Lipopeptides are known to act in a synergistic or antagonistic manner as suggested by several studies (Ben Ayed et al., 2017; Ongena et al., 2007; Tao et al., 2011; Zhao et al., 2017). The primary mode of action of lipopeptides usually involves membrane disruption (Peláez, 2006; Peypoux et al., 1999; Tao et al., 2011), but it has also been described that they interact with intracellular structures (Wang and Liang, 2014; Zhang et al., 2013). Their activity has also been shown to be influenced by fatty acid chain length and charge of the peptide moiety (Falardeau et al., 2017; Wise et al., 2014). Therefore, their remarkable structural diversity and their multiple modes of action, might suggest that these metabolites have different natural roles (Raaijmakers et al., 2010), and could have antimicrobial activities not yet described.

Here we report the antagonistic potential of strains belonging to species from the 'Operational Group *B. amyloliquefaciens*' against Gram-negative bacteria, among which *R. solanacearum* outstands as the most sensitive pathogen. We additionally report the isolation and structural elucidation of the compounds responsible for this antibacterial activity, their *in vitro* effects against *R. solanacearum* and *in vivo* effects on Moko disease. This report demonstrates, for the first time, an *in vitro* antibacterial activity of fengycins and a reduced incidence of Moko disease caused by *R. solanacearum*, in greenhouse banana plants treated with a mixed fraction of pure lipopeptides.

2. Materials and methods

2.1. Microorganisms

Aerobic Endospore Forming Bacteria (AEFBs; 1496 isolates, Humboldt Institute Collection No 191) were obtained from the rhizosphere and phyllosphere of banana and plantain plants in Uraba, Antioquia (Ceballos et al., 2012; Posada et al., 2016). *Bacillus* sp. EA-CB0959 was isolated from the phyllosphere of a plantain cv. Harton plant in Urabá, Antioquia (Northeast Colombia, 07°52′25.2″N-76°38′0.26″W) in 2009 (Ceballos et al., 2012), and identified by 16S rDNA gene sequence (GenBank accession no. MH400733). Bacteria were stored in TSB (tripticase soy broth, Merck) with 20% v/v glycerol at −80 °C and activated in TSA (Merck) for 48 h at 30 °C before any experimental use. Production of antimicrobial compounds by *Bacillus* sp. EA-CB0959 was evaluated using a medium optimized for lipopeptide production (MOLP) (Lemessa and Zeller, 2007).

Ralstonia solanacearum EAP-009 and *Serratia marcescens* EAD-005 were isolated from infected corm tissue of a banana plant following a previously described methodology (Álvarez-Restrepo et al., 2008) stored in BG medium (Clough et al., 1994) with 20% glycerol at −80 °C and identified by analysis of 16S rDNA gene sequence (GenBank accession no. KU603426 and KU603427 respectively). Other Gram-negative strains employed to assess *Bacillus* sp. EA-CB0959 bioactivity spectrum were isolated from banana plants infected tissues and identified as *Pseudomonas putida* UA-0095, *Xanthomonas* sp. UA-1539, *Burkholderia cepacia* UA-1541, *Serratia marcescens* UA-1538, donated by Dr. Camilo Ramirez from Universidad de Antioquia. The strain of *Salmonella enterica* ATCC 14028 was part of the biotechnology lab strains collection and *Pectobacterium* spp. strain was donated from the Kolter Lab at Harvard Medical School.

2.2. Screening of antagonist AEFB

The screening of AEFB against *S. marcescens* EAD-005 was performed following a modified methodology previously described (Mora et al., 2015). Briefly, 100 µL of *S. marcescens* or *R. solanacearum* suspension containing 10^6 CFU/mL was spread on BGT agar (BG medium plus 1.6% agar and 0.005% tetrazolium chloride). Afterwards a 5 mm disc of each AEFB, grown in TSA for 48 h at 30 °C, was transferred into

BGT agar and incubated at 22 °C. Inhibition of *S. marcescens* growth was assessed by measuring the radius (mm) of the growth inhibition zone after incubation for 24 or 72 h using 2 replicates per treatment. The AEFB activity against *R. solanacearum* EAP-009, *P. putida* UA-0095, *Xanthomonas* sp. UA-1539, *B. cepacia* UA-1541, *S. marcescens* UA-1538, *S. enterica* ATCC 14028 and *Pectobacterium* sp. followed the same methodology described above, but the assay was performed in BG agar (BGT with no tetrazolium chloride).

2.3. Kinetic analysis of Bacillus sp. EA-CB 0959

Kinetic analysis of strain EA-CB0959 growth and antibacterial activity of the cell-free supernatant (CFS) were performed by inoculating 20 mL of an overnight culture into 500 mL Erlenmeyer flasks containing 180 mL of MOLP medium, incubated at 30 °C and 140 rpm with three replicates. At different time intervals, 2 mL of samples were removed from each Erlenmeyer flask to determine the cell density and the antibacterial activity of the CFS. To determine cell density, the samples obtained were centrifuged at 10.000*g* for 10 min to obtain a pellet and a supernatant. The pellet was washed with distilled water, centrifuged at the same conditions stated above and then suspended in 2 mL of distilled water to determine optical density at 600 nm. The supernatant was filtered (cellulose acetate membrane filter, 0.45 mm, Sartorious Biolab) to obtain the CFS.

2.4. Purification of lipopeptides

Preparation and recovery of antimicrobial compounds was carried out by a methodology previously described (Villegas-Escobar et al., 2013). Briefly, 20 mL of an overnight culture of *Bacillus* sp. EA-CB0959 were inoculated in 500 mL Erlenmeyer flask containing 180 mL of MOLP medium and incubated for 5 days at 30 °C and 140 rpm. After 12 h of incubation, 4% of Amberlite XAD16® (Alfa Aesar) was added to the cell culture. The adsorbent resin was recovered from the culture broths by decantation, transferred into a glass 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 528 mg of solid residue was obtained from a 200 mL total culture.

The solid residue was suspended in 10 mL of distilled water and applied to a solid phase extraction (SPE) 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 60% MeOH, 80 mL of 80% MeOH and finally with 80 mL of 100% MeOH. The elutes were evaporated at reduced pressure (−50 psig, 50 °C), and the residues were weighed and dissolved in MeOH. Fractions were evaluated against *R. solanacearum* and the active fractions analyzed by HPLC.

The active fractions obtained with 80% MeOH and 100% MeOH from the SPE column were purified by reversed-phase high pressure liquid chromatography (RP-HPLC) using an Eclipse XDB C_{18} Column (250 by 4.6 mm, 5 µm, 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). Forty microliters of each sample (50 mg/mL) were injected into the column and the compounds were eluted by a gradient program developed from 41 to 70% B in 52 min; 70–100% B in 13 min and 100% B in 10 min for fraction 100% MeOH and from 20 to 100% B in 25 min and 100% B for 10 min for fraction 80% MeOH (or otherwise stated) at a flow rate of 1 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.

Active fractions were separated by RP-HPLC once more by dissolving the stored material in 45 µL of MeOH and injecting 40 µL into the HPLC column (Eclipse XDB C18). Elution was performed using the same conditions stated above and peaks were collected, evaporated and stored at 4 °C.

2.5. Evaluation of CFS, SPE and HPLC fractions against R. solanacearum

The activity of the CFS or the fractions collected from SPE and HPLC against *R. solanacearum* EAP009, were determined by growth inhibition assays in dual plate cultures. Fractions collected from SPE and HPLC were suspended in 1 mL or 40 µL of MeOH respectively. Dual plate cultures were carried out by adding 40 µL of the CFS or of each fraction on a 6 mm diameter well punched into fresh BGT agar plates seeded with *R. solanacearum* EAP009 strain suspensions (10^6 CFU/mL). Diameters of the growth inhibition zones were measured after incubation periods of 72 h at 22 °C. Addition of 40 µL of MeOH or 40 µL of water were used as absolute controls.

2.6. Mass spectrometry analysis

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 MeOH were infused with a syringe at 150 µL/h flow rate. Ionization parameters were as follow: 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.

2.7. Determination of the lactone bond

Lipopeptides were 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 pipette tip (Varian,100 mg). The trapped sample was washed twice with 100 uL of 0.1% TFA, and then eluted three times from the cartridge with 100 µL of MeOH. For MS/MS analysis, 100 µL of the sample was infused with a syringe to the ion trap as described above.

2.8. Minimal inhibitory concentration (MIC)

The MICs for surfactins, fengycins and iturins were determined by the broth dilution method (Wiegand et al., 2008). Microtiter plates containing 50 µL of the serial diluted lipopeptide dissolved in BG medium were inoculated with 50 µL of *R. solanacearum* EAP-009 to yield an initial concentration of 5×10^5 CFU/mL. Fresh BG medium was used as control. Microtiter plates were incubated at 22 °C and the optical density was determined at 595 nm after 72 h. The assay was performed with 6 replicas per treatment, and repeated two times independently. The MIC was determined as the lower lipopeptide concentration that completely prevented visible growth of *R. solanacearum* after incubation at 22 °C for 72 h.

2.9. Effect of lipopeptides of EA-CB0959 strain on bacterial wilt disease development

Lipopeptides from *Bacillus* sp. EA-CB0959 were applied on banana plants to determine their effect on Moko disease caused by *R. solanacearum*. The lipopeptides mixture was obtained by SPE as stated before, but the cartridge was rinsed successively with 80 mL of water, 80 mL of 50% MeOH and 80 mL of 100% MeOH. This last fraction, which contained all three lipopeptides (verified by mass spectrometry), was evaporated and dissolved in 0.1 M phosphate buffer (pH 7.5).

For this experiment, six weeks old banana plants were infected by injecting 0.3 mL of an inoculum of *R. solanacearum* EAP-009 (1×10^8 CFU/mL) into the base of the pseudostem (2 cm above the soil). Immediately, the plants were injected with 0.3 mL of the lipopeptides (2 mg/mL) in the same position. Plants injected only with *R.*

solanacearum solution and sterile buffer were used as control plants. The experimental unit consisted of one plant per pot (1 kg of non-sterile soil), and 18 plants per treatment were used. Plants were arranged in a complete randomized design, incubated at 22 ± 2 °C, 12 h/12 h photoperiod and the soil was maintained at 60% of water holding capacity in greenhouse conditions. Bacterial wilt disease progression was recorded every 2–3 days for 30 days to determine disease incidence, disease severity and wilting time. To determine disease incidence, the proportion of plants showing symptoms was evaluated 30 days after inoculation. Disease severity was determined only for those plants showing symptoms, by calculating the area under the disease progression curve *(AUDPC)* using Eq. (1).

$$AUDPC = \sum_{i=1}^{n} \left[\frac{x_i + x_{i-1}}{2}\right](t_i - t_{i-1}) \quad (1)$$

where n is the number of times the plants were evaluated, x_i is the wilting ratio at each evaluation time, and $(t_i - t_{i-1})$ is how long wilting lasted (Wicker et al., 2007). Wilting ratio was determined by the number of wilted leaves per total leaves.

Wilting time (t_{50}) was determined as the time when 50% of the plants were wilted, following Eq. (2):

$$t_{50} = \left[\frac{t_{i2} - t_{i1}}{x_{i2} - x_{i1}}\right] * (0.5 - x_{i1}) + t_{i1} \quad (2)$$

where t_{i2} is the day where > 50% of wilting symptoms where recorded for plant i, t_{i1} is the day of measure beforet_{i2}; x_{i1} and x_{i2} is the wilting ratio for day of measure t_{i1} and t_{i2} respectively.

2.10. Statistical analysis

Differences in the effects of CFS, SPE or HPLC fractions for the percentage of inhibition were determined by analysis of variance (ANOVA) and with Tukey multiple comparison using Statgraphics plus 5.1. Three replicates per treatment where used in each trial. The confidence level used for ANOVA analyses was 95%.

For greenhouse trials, differences for each treatment on the two measured variables (*AUDPC* and t_{50}) were also assessed by analysis of variance (ANOVA) and with Tukey multiple comparison using Statgraphics plus 5.1 with 95% confidence level.

3. Results and discussion

3.1. Strains belonging to 'operational Group B. amyloliquefaciens' inhibit gram negative bacterial strains

To determine potential bacteria that inhibit *S. marcescens* EAD005, 1496 AEFBs strains isolated from the phyllosphere and rhizosphere of banana and plantain plants were evaluated *in vitro*. Among these isolates, 3.1% (47 AEFBs) had growth inhibitory effects with an average of 4.2 ± 0.2 mm thickness of inhibition zone in BGT agar. When these 47 AEFBs were evaluated against *R. solanacearum* EAP-009, all had inhibitory effects with growth inhibition zones that ranged from 9.0 to 14.0 mm (Table S1). The wider inhibition zones found in *R. solanacearum* tests suggest that this phytopathogen is more sensitive to the active compounds produced by AEFB than *S. marcescens*, or that the AEFBs produce metabolites that are active only against *R. solanacearum.* Among these isolates, 36 strains were identified by sequencing the 16S rDNA gene sequence showing that all strains had high homology (100%) with the species from 'Operational Group *B. amyloliquefaciens*' (Supporting information Table S1) which is formed by *B. amyloliquefaciens* subsp. *plantarum, B. siamensis*, *B. velezensis* (Fan et al., 2017). Thus, since species resolution was not the focus of this study but their bioactivity instead, all active strains are subsequently called *Bacillus* sp.

Strain EA-CB0959, with growth inhibition zones ranging from of 11.7 ± 0.7 mm against *R. solanacearum*, was selected for further analysis and tested against strains of other gram-negative genera of both clinical and agricultural importance (Figs. S1 and S2). All Gram-negative strains were inhibited by *Bacillus* sp. EA-CB0959 in more or less magnitude, with the strain of *Pectobacterium* spp. presenting the highest inhibition halos (11.2 ± 0.2 mm) after *R. solanacearum* (15.7 ± 0.7 mm), and *P. putida* the lowest one (5.5 ± 0.2 mm).

Different studies have shown the ability of diverse bacterial species such as *Pseudomonas* sp., *Paenibacillus* sp., *Enterobacter* sp., *Bacillus* sp., *Flavobacterium* sp., *Chryseobacterium* sp., *Delftia* sp., to inhibit *R. solanacearum* (Huang et al., 2013; Kheirandish and Harighi, 2015; Lemessa and Zeller, 2007; Mora et al., 2015) whereas few studies have reported novel antibiotic substances from bacteria against other pathogenic Gram-negative species (Wicker et al., 2007).

Additionally, most studies were bioactivity is encountered against *R. solanacearum* or other relevant pathogens do not identify the metabolites that are responsible for this antibacterial activity (Huang et al., 2013; Kheirandish and Harighi, 2015; Mosquera et al., 2014). The findings obtained in this study indicate the ability of bacterial species to antagonize different gram-negative strains, emphasizing on the outstanding potential of the species from 'Operational Group *B. amyloliquefaciens*', which as previously stated, includes the species *B. amyloliquefaciens* subsp. *plantarum, B. velezensis,* and *B. siamensis* (Fan et al., 2017). The study specifies that these species are closely related within the denoted *B. subtilis* Species Complex, based on a phylogenomic analysis using model bacterium FZB42T as the type strain of *Bacillus amyloliquefaciens* subsp. *plantarum*, DSM7T as the type strain of *B. amyloliquefaciens* and 66 additional bacterial genomes from the NCBI data bank. Species from this operational group, specifically *B. amyloliquefaciens*, have been widely studied and reported as a biological control agents of several plant pathogens (Ongena et al., 2007), including fungi and bacteria that affect both banana plants and its fruits during post-harvest (Agrow Biologicals Review, 2017; Alvindia and Natsuaki, 2009), as well as other food-spoiling pathogens such as *B. cereus* and *Salmonella* spp. (Compaore et al., 2013). Besides being an environmentally safe biological agent, it has also become an important active ingredient of bioproducts employed on integrated pest management strategies, because of its great potential for enhancing plant productivity and antagonizing pathogens. Even though *B. amyloliquefaciens* strains have been greatly explored for their biocontrol properties, not many studies available from the literature, focus on their active metabolites and further potential for *in vivo* application against Gram-negative bacteria. An example using a strain of *B. subtilis* is presented by Arroyave-Toro et al. (2017), where the active lipopeptides have a broad antifungal spectrum and can control post-harvest diseases produced by *Botrytis cinerea* and *Colletotrichum acutatum*. There is a need for this kind of studies on strains belonging to 'Operational Group *B. amyloliquefaciens*' in order to create a solid foundation of its mechanism of action for biocontrol and further impacts on soils and other ecosystems when used as a biological control agent.

3.2. The antibacterial activity of the metabolites produced by Bacillus sp. EA-CB0959 is non-growth associated

To determine the optimal harvesting time to obtain the highest antibacterial activity during the fermentation of *Bacillus* sp. EA-CB0959, a kinetic analysis was performed (Fig. S3). Cell culture growth was characterized by an exponential growth that lasted for 10 h and then entered into a stationary phase. Cell culture reached an optical density of 2.0 which was equivalent to 8.4×10^8 CFU/mL after 24 h of incubation. The antibacterial activity of the CFS started to increase at the end of the exponential phase and continues its increase during the stationary phase until it reaches its highest value (9.8 mm of growth inhibition zone) after 36 h of culture, which suggested that the antibacterial metabolites are non-growth associated, as it has been described for lipopeptides of the fengycin and iturin families (Dimkić et al., 2017; Jacques et al., 1999; Mosquera et al., 2014) , whereas

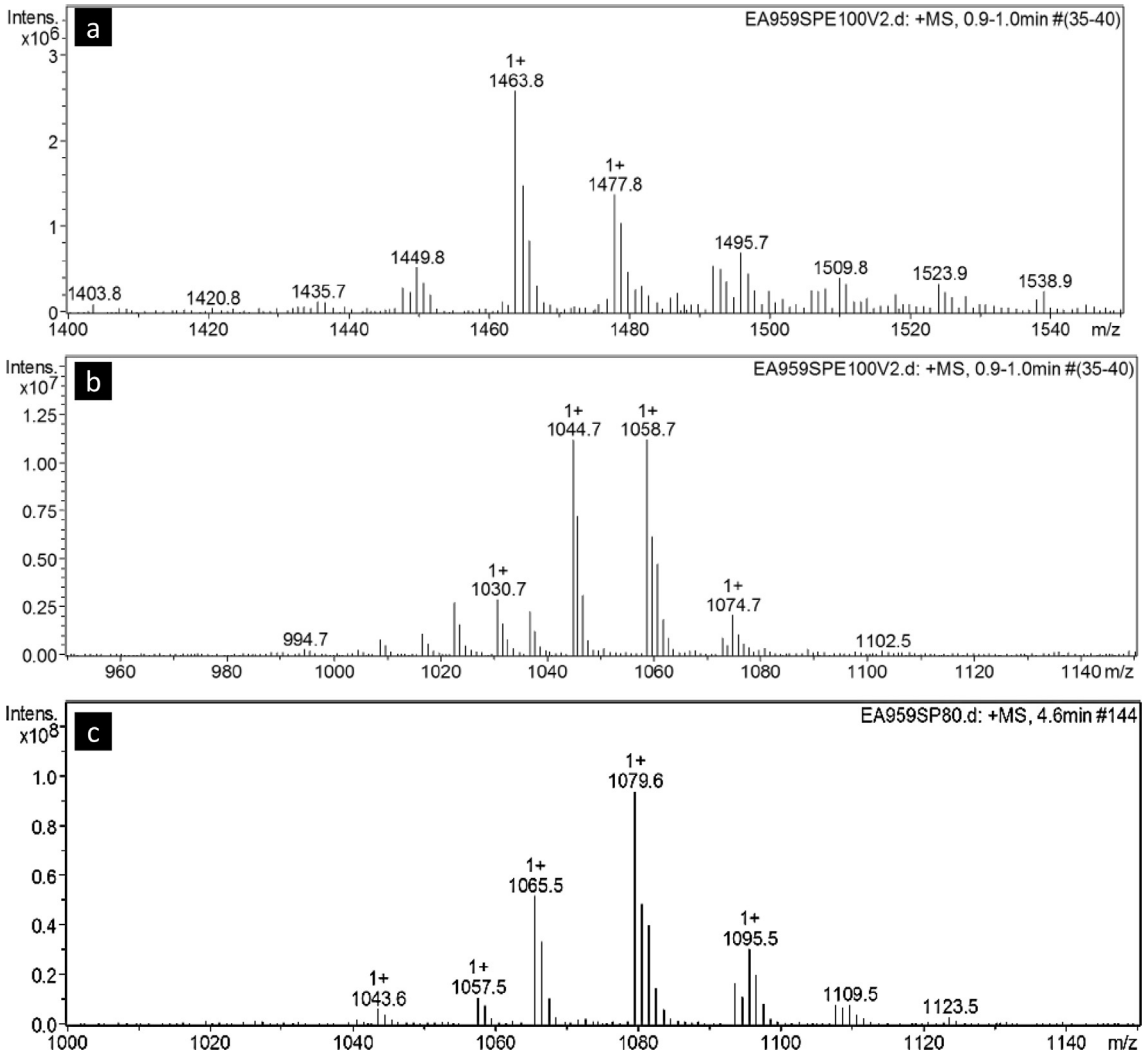

Fig. 1. ESI-MS spectra of HPLC purified fractions. (a) (+) ESI-MS of fraction F1. (b) (+) ESI-MS of fraction F2. (c) (+) ESI-MS spectrum of fraction F3. Fractions were collected from HPLC as described in Fig. S4 from supplementary information.

metabolites such as surfactins and polymixins have been described to be growth associated (Jacques et al., 1999; Paulus and Gray, 1964). To determine which antibacterial compounds were accumulated during the stationary growth phase, the active compounds were purified and identified.

Table 1
Chemical structures of lipopeptides produced by *B. amyloliquefaciens* EA-CB0959 and minimal inhibitory activity against *R. solanacearum* EAP-009.

Peak *m/z*	Compound	[M+X]	Amino acid	Mw (Da)	Chemical structure	MIC (μg/mL)
1043.5 1065.5	C14 iturin A	$[M+H]^+$ $[M+Na]^+$	Asn1	1043	R-CH-CH$_2$-CO→Asn→Tyr→Asn→Gln→Pro→Asn→Ser HN	> 256
1057.6 1079.5 1095.5	C15 iturin A	$[M+H]^+$ $[M+Na]^+$ $[M+K]^+$	Asn1	1057		
1058.7	C15 surfactin	$[M+Na]^+$	Leu7	1035	Val R-CH-CH$_2$-CO→Glu→Leu→Leu→Val→Asp→Leu→Leu O	128
1044.7	C14 surfactin	$[M+Na]^+$	Leu7	1021		
1030.7	C13 surfactin	$[M+Na]^+$	Leu7	1007		
1016.7	C12 surfactin	$[M+Na]^+$	ND	993		
1435.7	C14 fengycin A	$[M+H]^+$	Ala6	1435	Val CH$_3$-(CH$_2$)n-CH-CH$_2$-CO→Glu→Orn→Tyr→Thr→Glu→Ala OH O Ile←Tyr←Gln←Pro	32
1449.8	C15 fengycin A	$[M+H]^+$	Ala6	1449		
1463.8	C16 fengycin A	$[M+H]^+$	Ala6	1463		
1477.8	C17 fengycin A	$[M+H]^+$	Ala6	1477		
1491.8	C16 fengycin B	$[M+H]^+$	Val6	1491		
1505.8	C17 fengycin B	$[M+H]^+$	Val6	1505		

*ND: not determined.

3.3. Three families of lipopeptides are produced by Bacillus sp. EA-CB0959

Metabolites produced by *Bacillus* sp. EA-CB0959 were obtained by eluting the compounds adsorbed by the resin Amberlite XAD16® with MeOH. The MeOH extract that was active against *R. solanacearum,* was further separated using SPE as described in Materials and Methods. Active fractions obtained by SPE, eluting with 80 and 100% MeOH were characterized through HPLC and analyzed by ESI-MS to determine which metabolites were contained in those fractions (Fig. 1; Supporting information, Fig. S4).

The fraction obtained by SPE with 100% MeOH showed two groups of HPLC peaks between the retention times 16–41 min and 60–70 min (Supporting information, Fig. S4A), which were collected (fraction F1 and F2 respectively) and analyzed by ESI-MS. In both fractions (F1 and F2) peaks with 14 or 28 Da difference in their molecular ion species were determined, revealing two sets of homologous molecules (Fig. 1A and B). In fraction F1, the cluster of peaks exhibited signals at *m/z* 1436, 1450, 1464, 1478, 1492 and 1506 corresponding to the protonated molecules $[M+H]^+$ (Fig. 1A). These peaks revealed differences of 14 Da, suggesting a series of homologous molecules differing by one $–CH_2$ and with molecular weights of 1435, 1449, 1463, 1477, 1491 and 1505, in agreement with molecular weights of fengycin A and B (Schell, 2000; Wang et al., 2004). All precursor ions were used for further ESI-MS/MS analysis, and the results showed the appearance of product ions at *m/z* 1080.5 and 966.5 from precursor ions at *m/z* 1435.7, 1449.8, 1478.8; and at *m/z* 1108.6 and 994.5 from precursor ions at 1491.8 and 1506 (Supporting information, Fig. S5). These product ions (*m/z* 1080.5 and 966.5 or *m/z* 1108.6 and 994.5) could be explained as losses of fatty acid chain-Glu and fatty acid chain-Glu-Orn from the N-terminal region, characteristic of lipopeptides fengycin A and B respectively (Wang et al., 2004). The peaks P1, P2 and P3 (Supplementary data S1) were collected and analyzed by ESI-MS corresponding to the signals at *m/z* 1463, 1477 and 1491. To determine exactly the amino acid sequence of the fengycins, purified lipopeptides (P1, P2 and P3) were subjected to alkaline hydrolysis. Each molecule yielded a new product, with protonated masses of 1483, 1496, 1510, respectively (data not shown). The mass gain of 19 Da was assigned to the hydrolysis of the lactone ring and the aminoacid sequence confirmed that these molecules belong to fengycin A and B (Table 1).

The cluster of peaks in fraction F2 showed four main signals at *m/z* 1016.6, 1030.7, 1044.7 and 1058.7 corresponding to the sodiated molecules $[M+Na]^+$ (Fig. 1B). Therefore, the molecular weights of the four molecules were, respectively, 993, 1007, 1021 and 1035. The sodiated molecules 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, Fig. S6) and corresponded to C13, C14 and C15 surfactins (Table 1). The *y* ion sequence for the precursor ion at *m/z* 1044.7 was 1044.7 → 707.5 (y_6) → 594.5 (y_5) → 481.2 (y_4) corresponding respectively to the losses of (fatty acid)-Glu-L_{xx}-L_{xx} from the C terminal region. The *b* ion series contains the ions at *m/z* 1044.7 → 931.7 (b_7) → 818.7 (b_6) → 703.7 (b_5) → 604.7 (b_4) which corresponds to the neutral losses of L_{xx}-L_{xx}-Asp-Val from the N terminal region. The connection of the two series suggests that the ion at 1044.7 has the structure Na^+R_{C14}CO-Glu-L_{xx}-L_{xx}-Val-Asp-L_{xx}-L_{xx}, where L_{xx} could be Leu or Ile. Sodiated molecules at *m/z* 1030.7 and 1058.7 developed the same *y* ions series than ion at *m/z* 1044.7 and the *b* ion series differed on 14 Da from it, confirming the difference of a $–CH_2$ in the side chain. These data were in accordance with the mass spectra of surfactin (Schell, 2000).

The fraction obtained by SPE with 80% MeOH was first purified by HPLC and the fraction F3 collected (Supporting information, Fig. S4B) was analyzed by ESI-MS showing one cluster of peaks between *m/z* values of 1043.6 and 1109.5 (Fig. 1C). Two main signals where detected at *m/z* 1065.5, 1079.6 corresponding to the sodiated molecules $[M+Na]^+$, one signal at *m/z* 1095.5 corresponding to potassium molecule $[M+K]^+$, and two signals at *m/z* 1057.5 and 1043.6 corresponding to protonated molecules $[M+H]^+$ (Fig. 1C). Therefore, the molecular weights of the two molecules were respectively 1043 and 1057 Da. The two protonated molecules were used as precursor ions for further MS/MS analysis (Supporting information, Fig. S7), and the results showed that the product ions had regularities and corresponded to C14 and C15 iturin A. Product ions obtained from the precursor ion at *m/z* 1057.5 showed that the *y* ion sequence was 1057.5 → 846.4 (y_6) → 759.4 (y_5) → 520.3 (y_4) → 406.2 (y_3) corresponding respectively to the losses of (Pro-Asn)-Ser-βAA-Asn. The *b* ion series contains the ions at *m/z* 1058.5 → 929.5 (b_7) → 815.5 (b_6) → 652.4 (b_5) → 538.3 (b_4) which corresponds to the neutral losses of Gln-Asn-Tyr-Asn. The connection of the two series suggests that the ion at 1058.5 has the sequence Pro-(Asn-Ser)-βAA-Asn-Tyr-Asn-Gln, where βAA is theβfatty acid chain of 14 carbons. The other precursor ions at *m/z* 1043.5 had the same *y* ions and differ in the *b* ions by 14 Da between them (data not shown).

3.4. Lipopeptides of the surfactin, iturin and fengycin families inhibit R. solanacearum growth

In order to determine if these compounds were responsible for the activity against *R. solanacearum*, fractions collected from the HPLC as F1, F2 and F3 (Supporting information Fig. S4; now denoted F for fengycins, S for surfactins and I for iturins respectively) were tested individually and in mixtures (I + F, I + S, F + S, and I + F + S) (Fig. 2). Fractions obtained by SPE with 80% and 100% MeOH (SPE80 and SPE100) were used as positive controls and MeOH was used as a

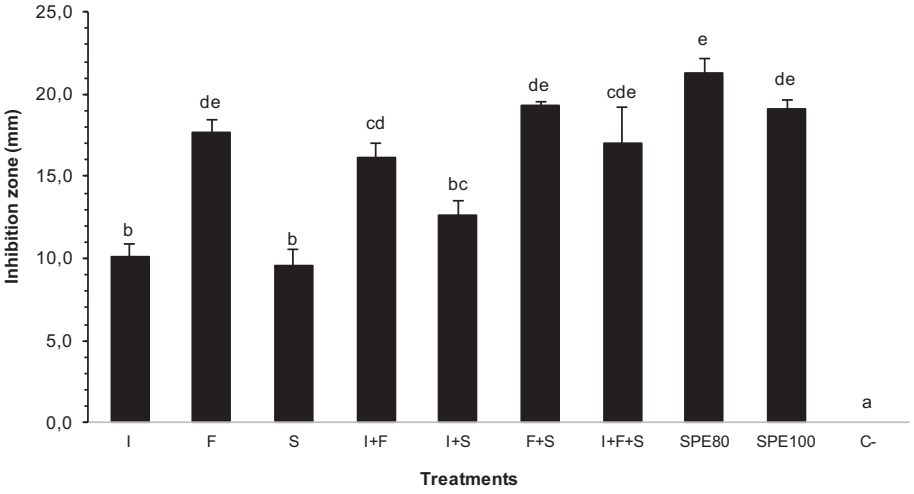

Fig. 2. Effect of lipopeptides on growth inhibition of *R. solanacearum* EAP09. I: iturines, F: fengycins, S: surfactins, SPE80: fraction eluted with 80% methanol from SPE C_{18} cartridge, SPE100: fraction eluted with 100% methanol from SPE C_{18} cartridge. Intervals represent standard errors of the mean (n = 3) and means with the same letter do not differ statistically ($P < 0.05$) by Tukey multiple range tests.

negative control (C-). All fractions inhibited *R. solanacearum* EAP-009 growth with inhibition zones between 9.56 ± 0.95 and 19.33 ± 0.19 mm in BGT agar. Although fractions that contained fengycins (F, I + F, F + S and I + F + S) were the only ones with no significant differences with the positive controls (SPE80 and SPE100), indicating that fengycins are more active against *R. solanacearum* than iturins and surfactins or that the concentration obtained in the fengycin fraction was higher than the ones of iturins and surfactins. Although, the quantity obtained after recovering each fraction (I, S and F) was not significantly different (0.750 ± 0.05; 0.791 ± 0.07; 0.618 ± 0.070 mg), suggesting that fengycins had a lower minimal inhibitory concentration (MIC) than the other two families of lipopeptides. Therefore, MICs of the three families of lipopeptides were determined by the broth dilution method, determining that fengycins homologues had a significantly lower MIC (32 µg/mL) than surfactins and iturins against *R. solanacearum* (MIC of 128 and > 256 µg/mL respectively) (Table 1).

Recently Mora et al. (2015) demonstrated that the antagonistic capacity of plant-associated *Bacillus* against plant pathogenic bacteria is related to the presence of lipopeptide genes and the production of the corresponding lipopeptides (Mora et al. 2015), although they did not determine which of the lipopeptides were responsible of the antibacterial activity. Similarly, Wang and Liang (Wang and Liang, 2014) showed that *B. amyloliquefaciens* strain BZ6-1 had antibacterial activity against *R. solanacearum in vitro*, as well as identifying this strain as capable of producing surfactin and fengycin *in vitro*. Nevertheless, they did not test if the purified lipopeptides where active neither *in vitro* or *in vivo*. From our knowledge, this is the first report that demonstrated antibacterial activity of fengycins, which have been characterized to be highly active against fungi (Raaijmakers et al., 2010; Villegas-Escobar et al., 2013).

Lipopeptides have an amphiphilic structure with a polar head and a hydrophobic chain, property that have been associated with permeabilization of cell membranes (Peláez, 2006). Additionally, the charge and composition of the target cell membrane have shown to be important for lipopeptide activity (Falardeau et al., 2013; Wise et al., 2014) as shown for fengycins which are inhibited by the presence of ergosterol or lipids (phosphatidylglycerol (PG) and phosphatidylethanolamine (PE)) in cell membranes (Fiedler and Heerklotz, 2015; Wise et al., 2014). In another biophysical study on the interaction of the isoform of fengycin C with model dipalmitoyphosphatidylcholine (DPPC) membranes, it was found that fengycin-rich domains, where the surrounding DPPC molecules are highly dehydrated, may well constitute sites of membrane permeabilization leading to a leaky target membrane; these results are a solid support to explain the membrane perturbing action of fengycin, which has been related to its antifungal activity (González-Jaramillo et al., 2017). Furthermore, PE and PG are important constitutes of bacterial cell membranes (Galbraith et al., 1999). Nonetheless, fengycins were active against *R. solanacearum* in this study, suggesting that the mode of action of fengycins may be not solely a consequence of membrane permeabilization but also related to the interaction with other intracelular targets as shown in previus studies (Tao et al., 2011; Zhang et al., 2013).

3.5. Applying a solution of mixed lipopeptides reduces the incidence of Moko disease in banana plants under greenhouse conditions

As lipopeptides from the surfactin, fengycin and iturin families were demonstrated to be responsible for the *in vitro* bioactivity of *Bacillus* sp. EA-CB0959 against *R. solanacearum,* the *in vivo* potential of the lipopeptide mixture for controlling Moko disease on banana plants was evaluated under greenhouse conditions (Table 2; Supporting information Fig. S8). During greenhouse evaluations, wilt incidence of lipopeptide treated plants was significantly lower than the measure registered for control plants (Table 2; Supporting information Fig. S8). Twenty days after inoculation, a total of 94.4% of the control plants showed wilting and chlorosis symptoms characteristics of Moko disease, while only 61.1% of the plants inoculated with the lipopeptide mixture were symptomatic (Table 2). These results indicate that banana plants inoculated with the lipopeptide mixture had less morbidity and a slower onset of symptoms during greenhouse trial. Therefore, the plants with Moko symptoms were evaluated to determine the disease severity. Although, no significant reductions on the disease severity were observed, lipopeptide-treated plants revealed Moko disease symptoms later than control plants and a lower AUDPC value (Table 2) which could be improved by evaluating an optimal dose of application. In studies where it has been reported that *B. amyloliquefaciens* reduces disease caused by *R. solanacearum* or has an *in vivo* effect against other pathogens (Compaore et al., 2013; Wang and Liang, 2014; Wei et al., 2011; Yuliar and Toyota, 2015) the possibility of the produced lipopeptides, playing an important role in the observed effect, is clearly stated. Studies on tomato using a combined application of BCAs, specifically of strains of *B. amyloliquefaciens*, showed an effective suppression of bacterial wilt (BWT), and although not demonstrated, suppression mechanisms were typically attributed to the antibacterial metabolites produced by the BCAs (Wei et al., 2011). This suggestion is exemplified as well by Wang and Liang (2014) on peanuts. These last authors not only identified the strain with highest potential against *R. solanacearum* as *B. amyloliquefaciens* BZ6-1, but also reported a decrease on disease incidence from 84% in controls to 12.1% in peanut seedling treated with a 15 mL suspension of the BCA, with the main antimicrobial substances produced by the strain being confirmed as surfactin and fengycin A homologues by HPLC/MS. Compaore et al. (2013) demonstrated the ability of *B. amyloliquefaciens* subsp. *plantarum* strains to produce several lipopeptide antibiotics that rendered them the potential to control undesired pathogenic bacterial colonization in the fermented food Bikalga.

Table 2
Effect of lipopeptides on the incidence and severity of Moko disease caused by *R. solanacearum* EAP-009 on banana plants.

TREATMENT	Disease incidence	Disease severity	
	%[1]	AUDPC[2]	t_{50}[3]
Lipopeptide mixture	61.1*	5.9 ± 0.8	14.0 ± 5.9
Control	94.4	7.0 ± 0.5	12.9 ± 7.0

[1] Disease incidence determined as the proportion of plants showing symptoms 20 days after inoculation (n = 18).

[2] Means not significantly different at the 95% confidence level ANOVA, *p-value* = 0.1645 (n = 11 lipopeptide mixture, n = 17 control).

[3] Means not significantly different at the 95% confidence level *p-value* = 0.2121 (n = 11 lipopeptide mixture, n = 17 control).

* Statistically significant difference found by Chi-squared test (*P-value* = 0.01151).

Following this idea, the results presented above correspondingly support the indication from this study, of lipopetides produced by strains from 'Operational Group *B. amyloliquefaciens*' having a direct effect on the population of the antagonized pathogen. This effect could be leading to an under-quorum population, avoiding the initiation of quorum sensing. This is the mechanisms employed in many cases, and specifically by *R. solanacearum* (Denny, 2006; Flavier et al., 1997 Flavier et al., 1997; Genin and Denny, 2012; Schell, 2000); by pathogenic bacteria to regulate virulence and pathogenesis (Taga and Bassler, 2003; Waters and Bassler, 2005). The delay in the appearance of disease on treated plants, either with the lipopeptide producing strain or with the pure substances, could be then explained by an under expressed virulent behavior. This extended frametime, for reaching the threshold needed for a pathogen's population to be infective and produce disease, could also be allowing the plant defense mechanisms to detect the pathogen and activate the production of its own resistance tools, as the ethylene-dependent signaling pathway for induced resistance (Hase

et al., 2006; Yuliar and Toyota, 2015; Zhu and Qing, 2004), with this behavior possibly being part of strains from 'Operational Group *B. amyloliquefaciens*' mechanism of action, as it has been demonstrated for other biocontrol agents used against *R. solanacearum,* like *Glomus versiforme* (Zhu and Qing, 2004) and *Phytium oligandrum* (Hase et al., 2006).

These results suggest that the lipopeptide mixture employed in banana plants could be acting directly as an antibacterial compound against *R. solanacearum* reducing its population size and preventing it to reach a quorum that expresses the virulence factors needed to develop Moko disease. This study also exhibits, with no other reports on Moko disease incidence and severity reduction on banana plants using a mixture or purified active metabolites found to the moment, the potential of lipopeptides from *Bacillus* sp. EA-CB0959 to control *R. solanacearum in vivo* as well as other pathogenic gram negative strains *in vitro*, opening a new route for the development of biocontrol treatments for bacterial wilt and therapeutic agents for human/animal pathogens.

4. Conclusions

In this study, we conducted a screening of 1496 AEFB against *S. marcescens* and *R. solanacearum*, a soil borne pathogen of many crops worldwide. Forty seven of the isolates showed inhibiting effects against both strains *in vitro* but largely inhibited *R. solanacearum*. The majority of the active strains belong to species from the 'Operational Group *B. amyloliquefaciens*'. Strain EA-CB0959 was proved active against a broad spectrum of Gram-negative strains, denoting a great potential for species from this operational group for producing antimicrobial compounds against Gram-negative strains from pathogenic species. The main antibacterial activity of strain EA-CB0959 was associated with the lipopeptides surfactin, iturin and fengycin families; being fengycins more active with a MIC of 32 μg/mL and the incidence of Moko disease in banana plants treated with a mixture of purified lipopetides was reduced compared to an untreated control. To our knowledge this is the first time that an antibacterial activity has been associated to fengycins, that pure metabolites from a strain belonging to 'Operational Group *B. amyloliquefaciens*' are directly linked to an *in vitro* bioactivity against Gram-negative pathogens and that *in vivo* application of lipopetides is reported as a biocontrol strategy against Moko disease. Even though further exploration is needed, primarily enhancing the application method when treating plants with pure lipopeptides and elucidating the mechanism of action *in vivo*, these results constitute a first step in order to eventually firmly support the use of lipopeptides as antibiotic substances for controlling diseases produced by Gram-negative pathogens, and specifically, by *R. solanacearum*.

Acknowledgments

The authors are thankful to the Universidad EAFIT, the Association of Banana Producers of Colombia (AUGURA) and Colciencias (contract number 0555-2013) for the financial support of this project. This research was made possible by the Subscribe Contract Number 166 and 139 with the Ministerio de Medio Ambiente y Desarrollo Territorial in the categories "Contrato de Acceso a Recursos Genéticos y Productos Derivados para Investigación Científica" and "Contrato de Acceso a Recursos Genéticos y Productos Derivados con Fines Comerciales" respectively.

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