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CELLULAR AND MOLECULAR BIOLOGY

Purification and characterization of two new antimicrobial molecules produced by an endophytic strain of *Paenibacillus polymyxa*

NADJA F.G. SERRANO, DOUGLAS FERREIRA, EDSON RODRIGUES-FILHO, JOSÉ ANTÓNIO TEIXEIRA, JEAN DANIEL DUBREUIL & CRISTINA P. DE SOUSA

Abstract: An endophytic bacterium inhibiting pathogenic bacteria was isolated and the strain was genetically identified as *Paenibacillus polymyxa*. Biochemical characterization of fermentation broth indicated the presence of peptidic antimicrobial molecules. Liquid-liquid partition resulted in an organic fraction (OF) and an aqueous fraction (AF). OF presented a broad spectrum of activity against a panel of pathogenic bacteria and a fungus whereas the AF was active only against Gram-negative bacteria. AF was sequentially submitted to ion-exchange, desalting and reverse phase (RP) chromatography. A molecule with an RT of 2.45 min exhibited activity against all Gram-negative pathogenic strains tested beside *P. mirabilis*. The primary structure of the molecule, named AMP-Pp, was determined as Gly-Glu-Hyp-Gly-Ala by N-terminal sequencing. The molecular mass and amino acid sequence were confirmed by MS/MS. With a molecular mass of 463 Da, AMP-Pp is one of the smallest active natural peptides reported, yet. RP chromatography of OF resulted in four peaks. The first three peaks corresponded to known antimicrobials. MS analysis of peak 4 revealed the presence of an ion with m/z 3,376.4 Da, whose proposed molecular formula is $C_{182}H_{321}N_{29}O_{29}$. The compound, named polycerradin, showed a spectrum of activity against Gram-positive bacteria, Gram-negative bacteria (beside *P. mirabilis*) and a fungus.

Key words: Antimicrobial peptides, bioassays, endophyte, mass spectrometry, N-terminal sequencing.

INTRODUCTION

Drug resistance in bacteria, the appearance of life-threatening viruses, and the increase in the incidence of fungal infections in the world's population primed researchers to find novel molecules to prevent and treat human diseases (Ratti et al. 2008). Among all known producers of natural products, microorganisms represent a rich source of biologically active metabolites that have wide applications (Gunatilaka 2012). Microorganisms have various niche and among others they can live on and within plant parts. Endophytic microorganisms that colonize internal plant tissues do so without causing negative effects but at the same time produce bioactive compounds. Those are relatively unstudied but could represent a potential source of novel natural antimicrobials.

The Brazilian savannah, known as Cerrado, is the second-richest biome on Earth in terms of biodiversity (Sano et al. 2010). This biome presents a huge concentration of different endemic plants (10,000 plant species) (Myers et al. 2000). In this ecosystem, Brazilian savannah trees are excellent specimens to search for endophytic bacteria producing antimicrobial

compounds (Ratti et al. 2008) and some of these compounds are peptides.

Antimicrobial peptides (AMPs) show many potential advantages as therapeutic drugs such as broad antimicrobial spectrum, quick biocide action and in addition may resist already identified mechanisms of antibiotic resistance (Peschel & Sahl 2006). The aim of this study was to identify new antimicrobial compounds produced by a bacterial endophytic strain isolated from a Brazilian savannah tree and to evaluate their antimicrobial spectrum.

MATERIALS AND METHODS

Microorganisms and growth conditions

Endophytic Bacterium

The endophytic bacterium was isolated from the leaves of *Prunus* spp. (Ratti et al. 2008). The leaves were washed with sterile distilled water and neutral detergent and its surface disinfected with ethanol (70%), sodium hypochlorite (2%), and then rinsed with sterile water. The surface-disinfected leaves were aseptically sectioned into 0.5 cm pieces, distributed onto Petri plates containing growth media (yeast extract agar and peptone agar), and incubated at room temperature for 4 days. From those plates, a colony was selected as it showed antimicrobial effectiveness in a rapid screening test. For long storage period, the bacterial strain was cultivated for 18 h at 30 °C in Yeast Peptone Mannitol medium (YPM: mannitol 25 g, yeast extract 5 g, peptone 3 g and agar 12 g in 1 liter of water) broth and kept frozen with 15% (v/v) glycerol at - 80°C. The inoculum consisted of transfer a single colony by using a platinum handle to a 200 ml flask containing 10 ml YPM. The flasks were incubated under the following conditions: 30 °C/ 180 rpm/ 24 h. Then, 5% (v/v) of the inoculum (optical density 1.00 at A_{600}) was transferred to a 1-liter flask containing 200 ml of YPM. The production medium was incubated under the same temperature and agitation conditions. The stationary phase culture was centrifuged (10,000 g, 15 min) and the supernatant was filtered on a 0.22 µm membrane to obtain the cell-free fermentation broth (named total extract-TE) (Serrano et al. 2012).

Test Strains

The antimicrobial spectrum of TE, fractions and purified molecules was evaluated against a panel of bacteria and fungi described as follows: Gram-negative bacteria - *Alcaligenes faecalis* ATCC 8750, *Enterobacter aerogenes* ATCC 13048, *Escherichia coli* ATCC 25923, *Pasteurella haemolytica* ATCC 33396, *Pseudomonas aeruginosa* ATCC 14207, *Salmonella typhimurium* ATCC 14028, *Serratia marcescens* ATCC 8100, *Shigella sonnei* ATCC 29930, *Yersinia enterocolitica* ATCC 23715. Gram-positive bacteria - *Bacillus subtilis* ATCC 6051, *Listeria monocytogenes* ATCC 15313, *Proteus mirabilis* ATCC 29906, *Staphylococcus aureus* ATCC 25922, *Staphylococcus aureus* Wood 46, *Streptococcus agalactiae* ATCC 13813. Fungus - *Candida albicans* ATCC 10231. All the bacterial strains, including the control *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 strains, respectively, were grown in Mueller Hinton Broth (MHB) at 37 °C for 18-24 h. *Candida albicans* was cultivated using Yeast Extract Peptone (glucose 20 g, 20 g peptone, 10 g yeast extract adjusted to 1 L with deionized water). Bacteria and fungi were from the Culture Collection of the Department of Pathology and Microbiology (Université de Montréal).

Genetic identification of antimicrobial-producing endophytic bacterium

The species level determination of the endophytic isolate was done following 16S rRNA analysis at the Service de diagnostic (Faculté de médecine vétérinaire, Université de Montréal). Extraction of DNA from bacterial isolate was performed using the QIAamp DNA Mini Kit according to the manufacturer's instructions. In the multiplex reaction, 5 µg of the sample containing the DNA template was added to a 15 µl reaction mixture (Qiagen Fast Cycling PCR kit) according to the manufacturer's instructions. The primers used in the reaction were 27F 5'- AGA GTT TGA TCM TGG CTC AG – 3' and 519R 5' – GWA TTA CCG CGG CKG CTG – 3'. The PCR products were purified using a commercial kit (QIAquick PCR purification kit, Qiagen) according to the manufacturer's instructions. Both strands of the purified DNA PCR products were sequenced using the same primer sets with standard automated sequencing methods (FMV Sequencing Laboratory, Bigdye terminator version 3.1, sequencer: AB1 310, Applied Biosystems, Foster City, California, USA). The resulting sequences were analyzed with NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Bioassays

Qualitative tests

Well diffusion assay test was applied as a qualitative assay to characterize the total extract and for the purification of antimicrobial compounds. The microbial inoculum of each strain was prepared according to Clinical and Laboratory Standards Institute (CLSI 2011). The microbial inoculum was spread on Mueller-Hinton agar (MHA) surface by using a sterile swab. Then, the holes on agar were prepared by using a Pasteur pipette, and a volume of 100 µl of the sample was added per hole. Gentamycin, an antibiotic active against Gram-negative bacteria, was included as a control at a final concentration of 10 µg mL^{-1}. Triplicate of each sample were tested. The plates were incubated at 37 °C for 18-24 h.

Quantitative tests

To determine the Minimum Inhibitory Concentration (MIC) of purified molecules, the broth micro-dilution technique was employed. The lyophilized compounds were directly diluted in Mueller-Hinton broth (MHB). The twofold concentrated solutions (2X) were transferred to a 96-well microtiter plate and twofold dilutions ranging from 1,000 – 0.5 µg ml^{-1} were sequentially prepared in MHB, with a final volume of 100 µl. A volume of 1.0 µl of strain inoculum was added to each well (containing $1x10^7$ cells). The sealed plates were incubated at 37 °C for 24 h under gentle agitation at 80 rpm in an orbital shaker. The assays were carried out in triplicate and the MIC value was considered as the lowest concentration for which no microbial growth was observed by the naked eye. The positive and negative growth controls consisted of MHB with and without microbial inoculum, respectively.

Liquid-liquid partition of TE

As a first purification step, a two-phase system (liquid-liquid partition) was done on TE following the methodology described by Folch et al. (1957). Equal volumes of TE and organic solution (chloroform/methanol 2:1, v/v) were mixed with magnetic stirring for 3 min. In sequence, water was added to the mixture (10% of total volume) and kept under constant stirring for 3 min. The mixture was centrifuged (3,500 g / 5 min) for separation of the phases. The organic solvents from both, upper layer (aqueous fraction - AF) and lower layer (organic fraction – OF), were

removed by using a rotary evaporator under vacuum. The AF and OF were submitted to a bioassay against indicator strains.

Purification and characterization of molecules contained in AF

Ion-Exchange Chromatography

The liquid chromatography equipment used was an Akta Purifier10 system (GE Healthcare) and a 1 ml volume Mono Q column (HR 5/5 – Pharmacia). The mobile phase consisted of 20 m mol l^{-1} TRIS HCl, pH 7.5 (buffer A) and 20 m mol l^{-1} TRIS HCl + 1 mol l^{-1} NaCl, pH 7.5 (buffer B), at a flow rate of 0.5 ml min^{-1}. The sample (250 µl AF + 250 µl buffer A) was applied to the column and eluted under a linear gradient from 0 to 100% (v/v) buffer B over 30 min. The elution was monitored at A_{214} and A_{280} and the peaks lyophilized using a speed-vac and re-suspended in 100 µl of buffer A to test the antimicrobial activity against indicator strains.

LC-MS

The fractions obtained from ion-exchange chromatography were analyzed by LC-MS at the Laboratory of Mass Spectrometry and Medical Chemistry (Université de Montréal, Québec, CA). The Thermo Surveyor HPLC system was coupled with a Thermo LCQ Advantage mass spectrometer (San Jose, CA, USA). Data acquisition and analysis were performed using XCalibur 1.4 (San Jose, CA, USA). A gradient mobile phase was used with a microbore column Thermo Biobasic C8 (10 x 1 mm) with particle size of 5 µm. The initial mobile phase condition consisted of acetonitrile and 0.2% (v/v) formic acid in water at a ratio of 5:95 (v/v), respectively. From 0 to 2 min, the ratio was maintained at 5:95 (v/v). From 2 to 10 min, a linear gradient was applied up to a ratio of 70:30 (v/v) and maintained for 4 min. The mobile phase composition ratio was reverted the initial condition and the column was allowed to re-equilibrate for 5 min for a total run time of 19 min. The flow rate was 55 µl min^{-1}.

The mass spectrometer was interfaced with the HPLC system using pneumatic assisted electrospray ionization (ESI+) source. The sheath gas was set to 5 units and the ESI electrode was set to 4000 V. The capillary temperature was set at 300 °C and voltage of 6 V. The mass spectrometer was operated in full scan MS mode from m/z 300-2,000 Da.

Reverse phase chromatography

The fraction obtained from the ion-exchange chromatography was desalted using a 5 ml HiTrap Desalting column (GE Healthcare). A volume of 500 µl of the sample was injected per run and the elution at 0.1 ml min^{-1} was monitored at A_{280} on an Akta Purifier10 system. The desalted fraction was submitted to reverse phase chromatography. The conditions employed were as follows: sample of 100 µl, C_{18} EC column (250 x 4.6 mm, Nucleosil) with 120 Å pore diameter and 5 µm particle size, buffer A (H_2O + 0.1% v/v TFA), buffer B (acetonitrile + 0.1% v/v TFA), elution at 1.0 ml min^{-1} under a linear gradient 0-100% (v/v) B in 30 min with detection at A_{214} (DAD A_{200} - A_{280}) (Liu et al. 2004). The resolved peak (RP-1/AF) was manually collected, concentrated and assayed.

Determination of the primary structure

The sample was solubilized in 30 µl H_2O + 0.1% (v/v) TFA and then adsorbed on glass fiber filter paper. The N-terminal sequence was achieved by using the Procise protein automatic sequencer (Applied Biosystems) at the Laboratory of Mass Spectrometry (Unity of Analytical Services, Institute of Chemical and Biological Technology, University Nova de Lisboa, Portugal). Complementarily, the primary structure of

compound present in RP-1/AF was investigated via tandem mass spectrometry. LTQ Orbitrap Velos ETD (ThermoFisher Scientific) interfaced with EasyLC chromatograph (Proxeon) both controlled by the Thermo Xcalibur 2.1 software (ThermoFisher Scientific). Chromatographic conditions: ReproSilPur C18-AQ (150 x 4mm) column, buffer A (aqueous solution of 0.1% v/v formic acid), buffer B (acetonitrile/water 9:1 v/v + 0.1% v/v formic acid), linear gradient from 0 to 30% v/v buffer B in 60 min, flow rate 0.3 µl min^{-1}. Nanoeletronebulization source (Proxeon) operated in 2.3 kV voltage, temperature of 250 °C. The full MS and MS/MS data were analyzed by software AMDIS version 2.66.

Purification and identification of antimicrobial molecules in OF

Reverse phase chromatography

The OF was fractionated using an Amicon Ultra (Millipore) devices with 10 kDa membrane and the eluted volume was then fractionated using a 1 kDa membrane (3,000 rpm / 20 min). OF fraction smaller than 10 kDa but larger than 1 kDa was subjected to reverse phase chromatography. Chromatographic conditions were as follows: 100 µl of samples, MicroBondapack C18 (300 x 3.9mm) column, mobile phases - buffer A (methanol/water 7:93 v/v) and buffer B (methanol), gradient of 0-40% (v/v) solution B in 30 min. Operating conditions of the equipment (Waters Co. chromatographic system): injector temperature: 4 °C, separation temperature: 28 °C, flow rate: 1.0 ml min^{-1}, elution of the compounds monitored at A_{254} (DAD $A_{215 - 600}$). The peaks RP-1/OF, RP-2/OF, RP-3/OF and RP-4/OF, manually collected, were concentrated in speed-vac and then solubilized in aqueous solution containing 5% (v/v) DMSO for bioassays against the control microorganisms (Tupinamba et al. 2008).

MS analysis

The lyophilized sample (RP-4/OF) was solubilized in methanol and spotted in quintuplets (1.0 µl per spot) on MALDI plates (MALDI-TOF/TOF AutoFlex Speed, Bruker). After evaporation of the solvent, 1.0 µl of HCCA (α-cyano-4-hydroxycinnamic acid) matrix (stock solution 20 mg ml^{-1}) was added over the spots. Ionization was performed in positive mode, and the detection in linear and reflection mode. A 500 Hz laser frequency, 20 kV acceleration voltage and reading at A_{355} were applied. The sum of ten spectra (25 per spectrum) was considered in the elaboration of MS profiles of the samples. The simulation of the molecular formula of the compounds was done with Smart Formula in Flex Analysis software. The MALDI-TOF MS analysis was performed at the Laboratory of Micromolecular Biochemistry of Microorganisms (Department of Chemistry, UFSCar).

RESULTS

Identification of an endophytic microorganism with antimicrobial potential

A bacterium isolated from the leaves of a *Prunus* spp. present in the Brazilian Cerrado was identified by 16S rRNA analysis. A set of primers was used to generate a fragment of ~1500 bp that was further sequenced (Chatellier et al. 1998). The 16S fragments sequenced were compared to sequences in the GenBank database and identified by BLAST 2.2.10 (http://www.ncbi.nlm.nih.gov/BLAST). A similarity score of 99.2% between the 16S rRNA sequence and database sequence indicated that the isolate belonged to *Paenibacillus polymyxa*. It was given the strain designation *P. polymyxa* RNC-D. Its morphology

on YPM agar is shown in Figure 1. The strain was deposited in the culture collection of the Centro pluridisciplinar de pesquisas quimica, biologicas e agricolas of the Universidade Estadual de Campinas, Brazil and its accession number is CBMAI 2314.

Identification of antimicrobial molecules

Cell-free fermentation broth (TE) of *P. polymyxa* RNC-D in YMP inhibited all Gram-negative and Gram-positive test strains and a fungus (Table I). This TE was fractionated following a liquid-liquid phase extraction giving an aqueous (AF) and organic fraction (OF). The AF fraction inhibited all Gram-negative bacteria beside *P. mirabilis* but did not affect the growth of Gram-positive bacteria or *C. albicans*. On the other hand, the OF fraction did inhibit all bacteria tested and *C. albicans* but did not affect the growth of *P. mirabilis*. Gentamycin at a final concentration of 10 μg mL^{-1} did inhibit all Gram-negative bacteria tested (data not shown).

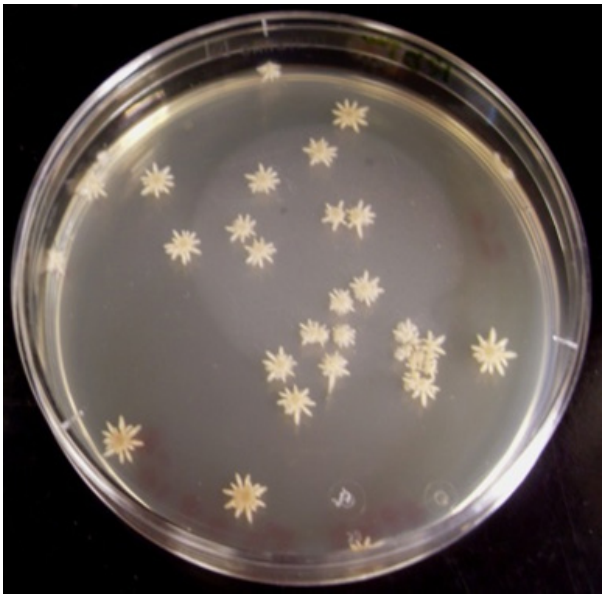

Figure 1. Morphology of *P. polymyxa* RNC-D strain on YPM agar.

AMP-Pp

The molecules contained in the AF were first fractionated using ion-exchange chromatography. The separation profile is shown in Figure 2. All 4 identified peaks (1E-1/AF, IE-2/AF, IE-3/AF and IE-4/AF) were submitted to the bioassay. All fractions inhibited only Gram-negative bacteria beside *P. mirabilis* (Table I-under AMP-Pp).

The fractions were then analyzed by LC-MS to determine the molecular mass of the bioactive compounds. Fractions IE-2/AF, IE-3/AF and IE-4/AF contained Fusaricidin A - 882 Da (Kurusu et al. 1987), Gavaserin – 911 Da (Pichard et al. 1995) and Polymyxin E – 1267 Da (Spinoza et al. 2011), respectively (data not shown). Fraction IE-1/AF was desalted and subsequently submitted to RP chromatography (Figure 3). The compound with an RT 2.45 min exhibited antimicrobial activity against all Gram-negative bacterial indicator strains except *P. mirabilis*. The Gram-positive bacteria and the fungus were not inhibited (Table I).

This molecule was named AMP-Pp and its primary structure was determined by N-terminal sequencing as Gly-Glu-Hyp-Gly-Ala. In addition, the molecular mass and amino acid sequence was confirmed by MS/MS. The spectrum indicates a molecular mass of 463 Da (ion m/z 464) (Figure 4). The peak m/z 446 (464-18) possibly indicates the loss of a water molecule, which is common in peptides having in their structure glutamic acid (Cantu et al. 2008). The m/z 186 peak can be attributed to the dipeptide glycine-glutamic acid and the difference between 446-375 = 71 refers to the alanine residue. Overall, this result corresponds to an unprecedented described molecule.

Table I. Antimicrobial spectrum of TE, AF, OF, AMP-Pp and polycerradin found in the fermentation broth of *P. polymyxa* RNC-D[§].

Test strains	Bioactivity*					
	Extract and fractions			AMP-Pp	Polycerradin	Gentamycin
	TE	AF	OF	MIC**	MIC	MIC
Gram-negative bacteria						
Alcaligenes faecalis ATCC 8750	+	+	+	15.6	3.95	N.D.***
Enterobacter aerogenes ATCC 13048	+	+	+	15.6	3.95	N.D.
Escherichia coli ATCC 25923	+	+	+	7.8	1.97	4.0
Pasteurella haemolytica ATCC 33396	+	+	+	7.8	7.8	N.D.
Pseudomonas aeruginosa ATCC 14207	+	+	+	31.25	15.6	N.D.
Salmonella typhimurium ATCC 14028	+	+	+	31.25	3.95	4.0
Serratia marcescens ATCC 8100	+	+	+	15.6	3.95	N.D.
Shigella sonei ATCC 29930	+	+	+	7.8	7.8	N.D.
Yersinia enterocolitica ATCC 23715	+	+	+	7.8	7.8	N.D.
Proteus mirabilis ATCC 29906	-	-	-	-	-	N.D.
Gram-positive bacteria						
Bacillus subtilis ATCC 6051	+	-	+	-	7.8	N.D.
Listeria monocytogenes ATCC 15313	+	-	+	-	3.95	N.D.
Staphylococcus aureus ATCC 25922	+	-	+	-	3.95	12.5
Staphylococcus aureus Wood 46	+	-	+	-	3.95	N.D.
Streptococcus agalactiae ATCC 13813	+	-	+	-	7.8	N.D.
Fungus						
Candida albicans ATCC 10231	+	-	+	-	3.95	50.0

*** (+) microbial inhibition observed; (-) no inhibition observed. § Gentamycin was included in the study as a reference.**
****Minimal inhibitory concentration (MIC) are in µg/ml. ***N.D., not determined.**

Polycerradin

The RP chromatographic profile of OF fraction between 10 kDa and 1 kDa revealed the presence of major peaks with retention times of 2.7 min, 4.1 min, 6.5 min and 10.4 min (Figure 5). The full MALDI mass spectrum of the peak with an RT of 10.4 is shown in Figure 6. An ion at m/z 3,376.4 Da, was detected. To our knowledge this molecule corresponds to a new molecule never reported in *P. polymyxa*. This novel compound was named polycerradin.

MALDI mass spectra at m/z from 3,375 to 3,386 of polycerradin displayed several peaks relative to the isotopic profile of the molecule (Figure 7). The first peak is composed of C^{12} H^{1} O^{16} N^{14} isotopes. The other peaks are formed by combinations of other isotopes of these atoms. The first peak points to m/z 3,376.4 that was the most abundant molecule (the value is increased by one unit since the analysis was performed in positive mode). The simulation carried out with the SmartFormula (Flex Analysis software) resulted in a proposed molecular formula of $C_{182}H_{321}N_{29}O_{29}$ for polycerradin.

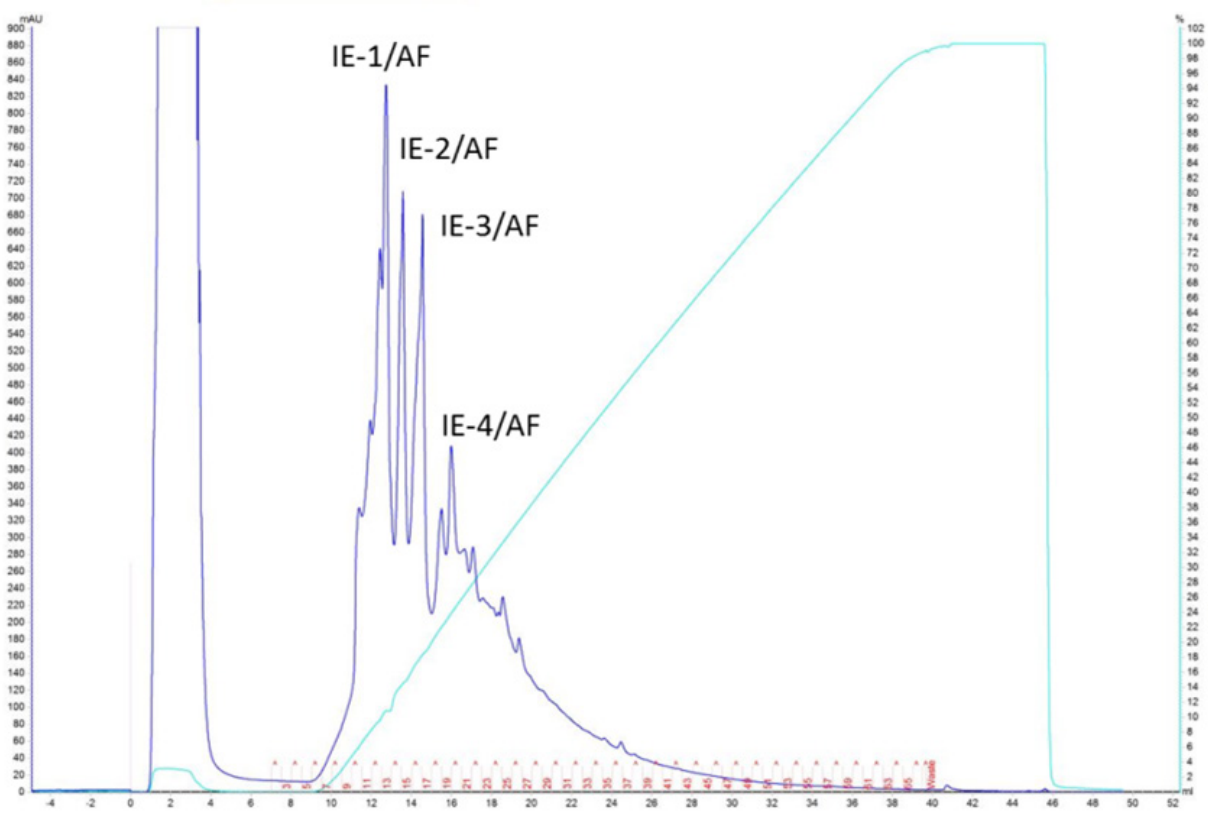

Figure 2. Chromatogram of aqueous fraction (AF) separation using ion-exchange chromatography.

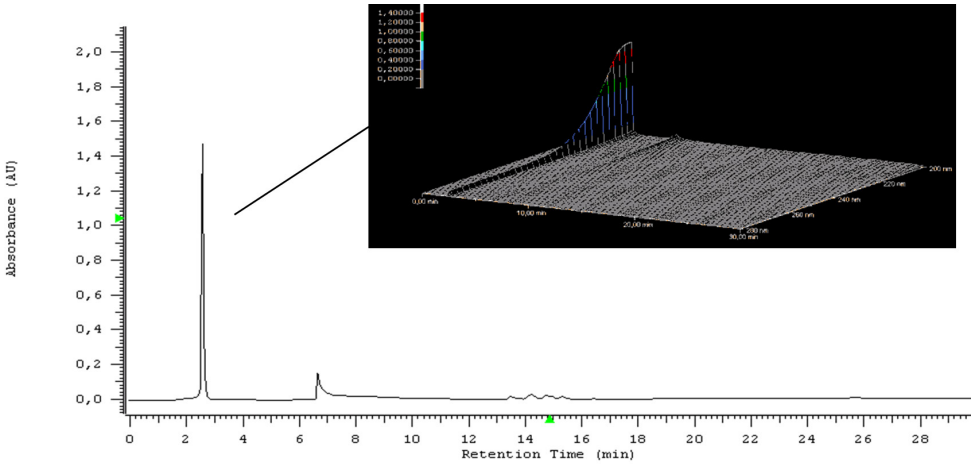

Figure 3. Chromatogram of 1E-1/AF using RP chromatography, UV spectrum (DAD A_{200} - A_{280}).

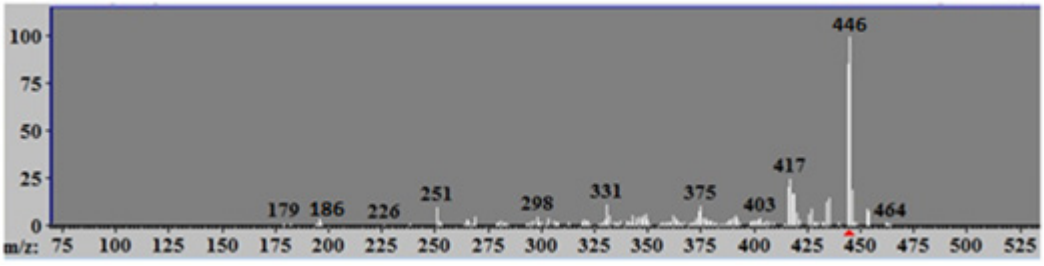

Figure 4. MS/MS of ion *m/z* 464 Da.

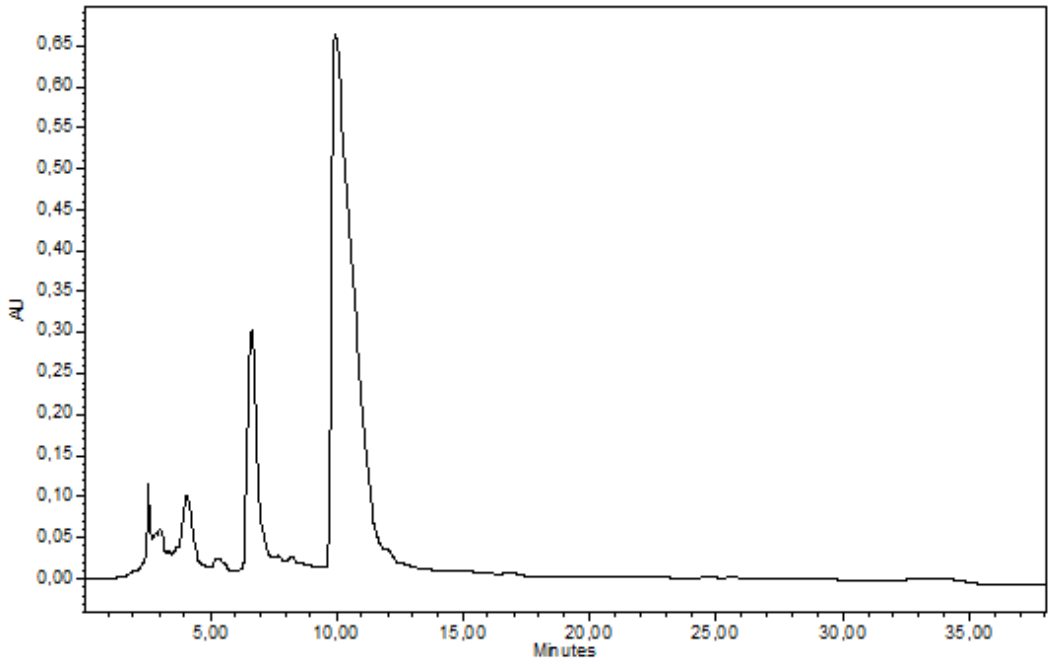

Figure 5. Chromatogram of organic fraction (OF) between 10kDa and 1kDa using RP chromatography.

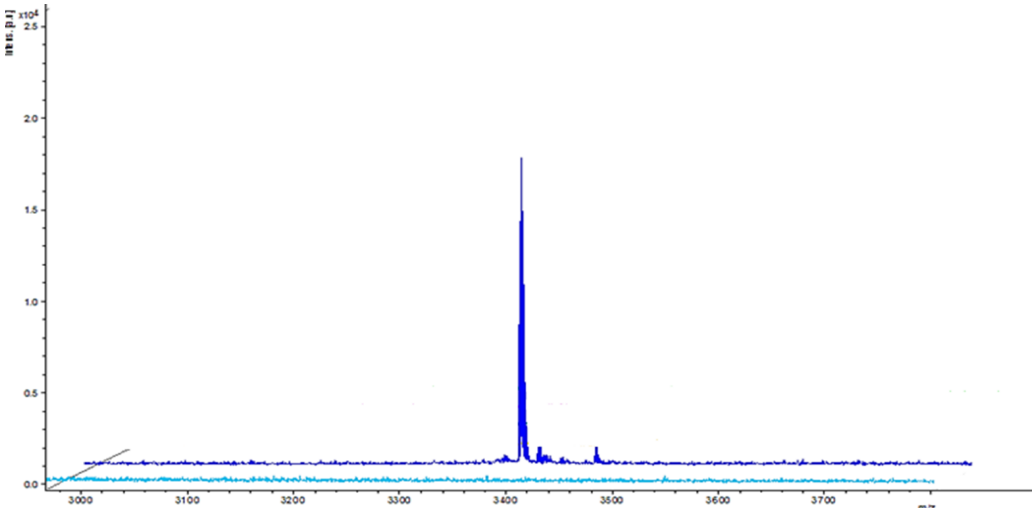

Figure 6. MALDI mass full spectrum of HCCA matrix (light blue line) and the peak with an RT of 10.4 (dark blue line), zoom at *m/z* 3,000 – 3,800.

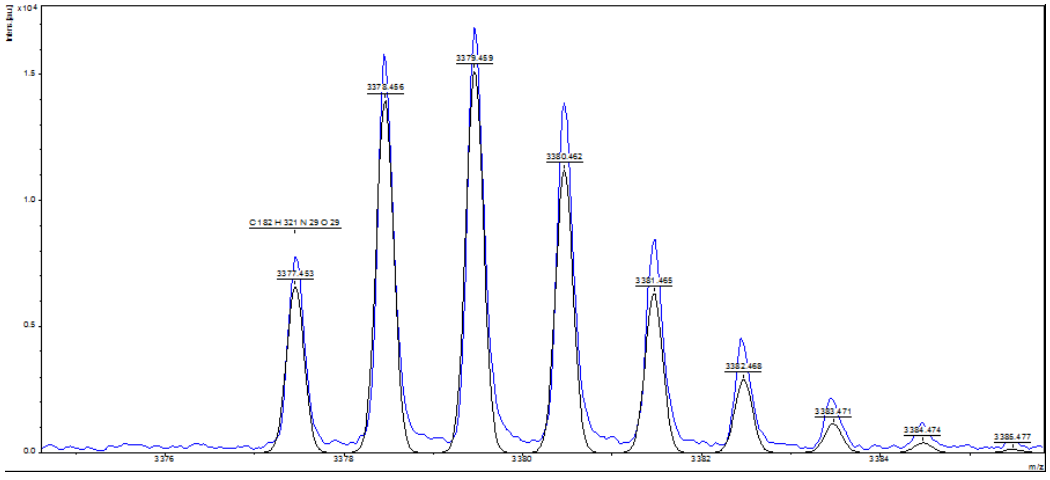

Figure 7. MALDI mass spectrum of polycerradin (zoom *m/z* 3,375 – 3,386). The molecular formula $C_{182}H_{321}N_{29}O_{29}$ corresponds to a simulation of ion *m/z* 3,376.459 made by SmartFormula (Flex Analysis software).

DISCUSSION

P. polymyxa belongs to the group of plant growth-promoting rhizobacteria. Plant growth can rely on the secretion by endophytes of secondary metabolites that antagonize pathogens (Glick 1995). The inoculation of plants with *P. polymyxa* suppresses several phytopathogens (Yuen et al. 1991, Oedjijono et al. 1993) as this bacterium can produce antibacterial compounds (Kurusu et al. 1987, Rosado & Seldin 1993, Pichard et al. 1995, Kajimura & Kaneda 1996). It produces two groups of AMPs comprising molecules such as polymyxins (polymyxin B-MIC, 0.25-2.0 µg/ml for *E. coli*) active against Gram-negative bacteria and a second group includes Fusaricidins A, B, C, D and Fusaricidin analogs able to suppress Gram-positive bacteria and fungi (Kurusu et al. 1987, Rosado & Seldin 1993, Pichard et al. 1995, Kajimura & Kaneda 1996, Beatty & Jensen 2002, Raza et al. 2010). Endophytic *P. polymyxa* strains were isolated from *Stemona japonica* (Lu et al. 2007) and ginseng roots (Cho et al. 2006, 2007), those produced fibrinolytic and hydrolytic enzymes active against phytopathogens as well as antifungal and anti-nematode compounds.

Our study relates to the isolation of an endophytic *P. polymyxa* strain from the leaves of a *Prunus* sp. isolated from the Brazilian Cerrado, showing bioactivity against pathogenic bacteria and a fungus. The antimicrobial spectrum of total extract (TE) produced by *P. polymyxa* strain RNC-D includes Gram-positive, Gram-negative bacteria and one species of fungus. He et al. (2007) have detected antibacterial activity against *E. coli* and *S. aureus* in supernatant produced by *P. polymyxa* OSY-DF when it was cultivated in TSB medium supplemented with 0.6% (w/v) of yeast extract. Likewise, the total extract produced by *P. polymyxa* P13 (Piuri et al. 1998) cultivated in BHI showed antimicrobial activity against *Bacillus cereus*, *Micrococcus luteus* and *E. coli*. Paenibacillin (He et al. 2007) (paenibacillin A-MIC, 0.1-1.56 µM for Gram-positive bacteria) with a molecular mass 2,983.44 Da, active against Gram-positive microorganisms, lost partially its biological activity after treatment with trypsin indicating a peptidic nature. *P. polymyxa* strain P13 was characterized as a polyxin producer (Piuri et al. 1998) a compound with molecular mass of 10 kDa. This compound had bactericidal properties against Gram-positive and was bacteriostatic against Gram-negative bacteria. Polyxin has a proteinaceous nature as its bioactivity was reduced after treatment with proteases.

In terms of molecular size the AMP-Pp identified in this study contains only 5 amino acid residues (Gly-Glu-Hyp-Gly-Ala) and is one of the smallest active natural peptides reported, yet. The lowest known AMP secreted by *P. polymyxa* strain KT-8 is Fusaricidin A with a MW 882 Da (Kurusu et al. 1987). AMPs containing 5 amino acids were also isolated from the worm *Eisenia foetida*, and they were named OEP3121 (Ala-Cys-Ser-Ala-Gly) (Liu et al. 2004), F-1 (Ala-Met-Val-Ser-Ser) and F-2 (Ala-Met-Val-Gly-Thr) (Zhang et al. 2002, Xichun et al. 2002). The hydroxyproline is a non-essential amino acid constituent of glycoproteins and can be found in the plant cell wall. The presence of this amino acid confers greater chemical stability to the molecules. For proline hydroxylation, there is a prolyl hydroxylase recognizing proline as its substrate. According to Cassab (1998), the mandatory condition is that the hydroxylated proline must be next to glycine in the amino-carboxyl sense. The production of antibiotic peptides having in their structures the amino acid hydroxyproline has been reported by Shoji et al. (1992). The substances plusbacin A1, A2, A3, A4 and plusbacin B1, B2, B3 and B4 are produced by *Pseudomonas* spp. Considering that linear peptides are rapidly degraded by

serum proteases and that AMPs with high proline content have low toxicity and high *in vivo* efficacy, Knappe et al. (2014) conducted a modification study of the AMP drosocin. Replacement of proline residues at positions 3, 5, 10 and 14 by trans-4-hydroxyproline resulted in increased antibacterial activity against Gram-negative pathogens *E. coli* and *Klebsiella pneumoniae*. Furthermore, the new Drosocin analogs have an extended half-life, being eight times more stable in the serum of rats when compared to unmodified drosocin.

Two antimicrobials were isolated from *P. polymyxa* OSY-DF: polymyxin E1, which is active against Gram-negative bacteria, and an active compound against Gram-positive, whose molecular mass is 2,983.4 Da. This AMP, named paenibacillin, is active against foodborne bacteria, including *Bacillus* spp., *Clostridium* spp., *Lactobacillus* spp., *Lactococcus lactis*, *S. aureus* and *Streptococcus agalactiae* (MIC, 0.1-1.56 µM for Gram-positive bacteria). Paenibacillin possesses physical-chemical properties of an ideal antimicrobial agent in terms of water solubility, thermal resistance, and acid-alkaline resistance (pH 2.0-9.0). The peptide was defined as a novel lantibiotic (He et al. 2007). The production of bacteriocin by strains of *P. polymyxa* isolated from broiler gut was described by Svetoch et al. (2005). The bacteriocin is active against *Campylobacter jejuni*. The molecular masses, as well as the amino acid sequence, were determined by MALDI-TOF MS as 3,214 Da (compound SRCAM 37) and 3,864 Da (compound SRCAM 602). In our study, a compound with a molecular mass of 3,376.4 Da, was described. Based on the molecular mass and the activity spectrum, we can conclude that polycerradin described in this study is different from the molecule reported by He et al. (2007).

Considering the MIC values observed in our study for AMP-Pp and polycerradin, we can advocate that the relatively low amount of compound needed to inhibit the tested bacterial strains (including *C. albicans* for polycerradin) are within an applicable therapeutic range. The fact that gentamycin inhibited some strains at either higher or similar concentrations than the two new compounds is encouraging. Thus, the low MIC values reported for both new molecules seems rather promising. One last concern will be to check the toxicity, on mammalian cells, of AMP-Pp and polycerradin at high dosage .

CONCLUSIONS

The present study reports the identification of two new AMPs of peptidic nature produced by *P. polymyxa* RNC-D. The compound AMP-Pp (Gly-Glu-Hyp-Gly-Ala) is active against all Gram-negative bacteria tested (except *Proteus mirabilis*). AMP-Pp has the unusual amino acid hydroxyproline in its composition. Considering the bacterial species *P. polymyxa*, the presence of the amino acid hydroxyproline, which confers greater stability to the molecules, was detected for the first time in the peptide discussed here. In terms of molecular size, it can be considered that AMP-Pp which contains only 5 amino acid residues is one of the smallest active natural peptides reported, yet. *P. polymyxa* RNC-D coproduced another molecule never reported before that was named polycerradin. The antimicrobial spectrum of this molecule encompasses a wide range of Gram-negative (beside *Proteus mirabilis*), Gram-positive microorganisms and a fungus. The molecular weight of this molecule is 3,376.4 Da. The structure of this molecule will have to be determined in future studies. These new compounds could represent interesting alternatives to the ever-growing list of antimicrobials for which microbial resistance is observed.

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NADJA F.G. SERRANO[1]
https://orcid.org/0000-0002-8838-7937

DOUGLAS FERREIRA[2]
https://orcid.org/0000-0001-5420-4015

EDSON RODRIGUES-FILHO[3]
https://orcid.org/0000-0003-4305-0326

JOSÉ ANTÓNIO TEIXEIRA[4]
https://orcid.org/0000-0002-4918-3704

JEAN DANIEL DUBREUIL[5]
https://orcid.org/0000-0003-1588-6847

CRISTINA P. DE SOUSA[1]
https://orcid.org/0000-0001-7151-5085

[1]Programa de Pós-Graduação em Biotecnologia, Universidade Federal de São Carlos/UFSCar, Departamento de Morfologia e Patologia, Laboratório de Microbiologia e Biomoléculas, Rod. Washington Luis, Km 235, 13565-905 São Carlos, SP, Brazil

[2]Universidade Federal do Oeste da Bahia, Departamento de Química, Campus Luís Eduardo Magalhães, Rua Professor José Seabra de Lemos, 316, Recanto dos Pássaros, 47808-021 Barreiras, BA, Brazil

[3]UFSCar, Departamento de Química, Laboratório de Bioquímica Micromolecular de Microrganismos, Rod. Washington Luis, Km 235, 13565-905 São Carlos, SP, Brazil

[4]Universidade do Minho, Centro de Engenharia Biológica, Laboratório de Fermentações, Rua da Universidade, 4710-057 Braga, Portugal

[5]Université De Montréal, Faculté de Médecine Vétérinaire, Département de Pathologie et Microbiologie, 3200 Rue Sicotte, J2S 2M2, Saint-Hyacinthe, Québec, Canada

Correspondence to: **Jean Daniel Dubreuil**
E-mail: daniel.dubreuil@umontreal.ca

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by NFGS and DF. ERF, JAT, JDD and CPS supervised both the experimental design and data acquisition. The first draft of the manuscript was written by NFGS and all authors commented and corrected the manuscript. All authors have read and approved the final manuscript.