Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com

Effect of a *Pseudomonas fluorescens* tailocin against phytopathogenic *Xanthomonas* observed by atomic force microscopy

Maricruz Fernandez[a], Agustina Godino[a], Analía Príncipe[a], Gustavo M. Morales[b,*], Sonia Fischer[a,*]

[a] *Departamento de Ciencias Naturales, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto-CONICET, Agencia Postal No. 3, X580BYA Río Cuarto, Córdoba, Argentina*

[b] *Departamento de Química, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto-CONICET, Agencia Postal No. 3, X580BYA Río Cuarto, Córdoba, Argentina*

ARTICLE INFO

Keywords:
Bacteriocin
Pyocin
Tailocin
Atomic force microscopy
Pseudomonas fluorescens
Xanthomonas

ABSTRACT

Phage tail-like bacteriocins, called tailocins, represent a class of protein complexes produced by Gram-negative bacteria. *Pseudomonas fluorescens* SF4c, a strain isolated from wheat rhizosphere, produces a bacteriocin similar to phage-like pyocins of *Pseudomonas aeruginosa*. This tailocin has antimicrobial activity against several phytopathogenic strains of the genus *Xanthomonas* and *Pseudomonas*. In this work, the effect of the SF4c tailocin on the phytopathogenic strain *X. axonopodis pv vesicatoria* Xcv Bv5–4a was analyzed through Atomic Force Microscopy (AFM). We demonstrated that tailocins adhere and cause damage to the cell envelope of strain Xcv Bv54a. This results in a rapid leakage of intracellular materials, with the subsequent decrease of cell volume. Finally, lysis of sensitive bacteria occurs. This study provides, to our knowledge, the first evidence about the effect of a tailocin analyzed by AFM. Further studies are in progress to evaluate the use of SF4c tailocin in the biocontrol of bacterial spot on tomato.

1. Introduction

Bacteriocins are ribosomally encoded peptides or proteins which exhibit high specificity and kill bacteria closely related to the producer strain (Ahmad et al., 2016; Ghequire et al., 2015). The pyocins produced by *Pseudomonas aeruginosa* are the bacteriocins most studied within the genus *Pseudomonas*. S-type pyocins are soluble and protease-sensitive proteins. These antibacterials are secreted as a complex of two proteins consisting of, a larger protein with killing activity and a smaller immunity protein that binds to the C-terminal end of the former and, thus protects the producer cell from its own bacteriocin. The mechanism of action of the S-pyocins is the DNA, tRNA or rRNA degradation in sensitive cells (Dingemans et al., 2016; Elfarash et al., 2012; Michel-Briand and Baysse, 2002). In addition, S5 is the only S-pyocin having a pore-forming activity (Ling et al., 2010). R-type and F-type pyocins are high-molecular-mass protein complexes. These bacteriocins resemble bacteriophage tails and are named as tailocins. The R pyocins are rigid and contractile, whereas the F pyocins are flexible but non-contractile (Ghequire and De Mot, 2014; Michel-Briand and Baysse, 2002). R-type pyocins bind to sensitive cell, form a channel into the bacterial membrane which dissipates the proton potential of the cell and finally, death of microorganism occurs (Ge et al., 2015; Uratani and Hoshino, 1984). F pyocins act by a process similar to R pyocins (Ghequire and De Mot, 2014). In the last years, R-type pyocins from *P. aeruginosa* have attracted attention as a strategy to replace antibiotics. Ritchie et al. (2011) demonstrated that the administration of engineered R-type pyocin can prevent *E. coli* O157:H7-induced diarrhea in a rabbit model. This occurred when the compound was administered either in a prophylactic regimen or after the onset of symptoms.

Recently, the knowledge about bacteriocins produced by plant-associated *Pseudomonas* strains has increased due to the availability of a large amount of genome sequences. Analysis of *Pseudomonas* spp. genomes showed that phage tail-like bacteriocins are also abundant in species other than *P. aeruginos* (Ghequire and De Mot, 2015, 2014; Godino et al., 2016; Ly et al., 2015). However, only few of these bacteriocins have been functionally characterized. In our lab, we have characterized the first tailocin in *Pseudomonas fluorescens* (strain SF4c) (Fischer et al., 2012). In addition, *Pseudomonas putida* strains BW11M1 and RW10S2 also produce R-type tailocin (Ghequire and De Mot,

Abbreviations: AFM, atomic force microscopy; TEM, transmission electron microscopy; PGPR, plant growth-promoting rhizobacteria.

* Corresponding authors.
Email addresses: gmorales@exa.unrc.edu.ar (G.M. Morales); sfischer@exa.unrc.edu.ar (S. Fischer)

http://dx.doi.org/10.1016/j.jbiotec.2017.07.002
Received 5 April 2017; Received in revised form 19 June 2017; Accepted 3 July 2017
Available online xxx
0168-1656/

2015). Recently, R-type syringacin of the phytopathogen *Pseudomonas syringae* pv. *syringae* B728a was functionally characterized (Hockett et al., 2015). Similar tailocins have been also described for several other γ-proteobacterial genera (Jabrane et al., 2002; Ŝmarda and Benada, 2005; Strauch et al., 2001).

Relevant problems in agriculture are bacterial diseases in crop, which cause significant economic losses. *X. axonopodis* pv. *vesicatoria* is the causal agent of bacterial leaf spot, one of the most severe diseases of pepper and tomato, especially in regions with warm and humid climate. Disease symptoms include necrotic lesions on leaves, sepals and fruits (Potnis et al., 2015; Ritchie, 2000). Several studies have shown that bacteriocins are effective against phytopathogenic bacteria. Nevertheless, the use of bacteriocins as biocontrol agents in plants is yet limited. This fact is mainly due to a lack of research about the mechanism of action of these antimicrobials. Such knowledge is a prerequisite for its application in biocontrol (Grinter et al., 2012). In this work, the effect of the SF4c tailocin on *X. axonopodis pv vesicatoria* Xcv Bv5–4a and *P. protegens* CHA0 was analyzed through studies of viability and AFM. Moreover, assays of potassium efflux were performed to confirm that bacteriocin causes damage to the cellular envelope of sensitive bacteria, increasing the permeability of the membrane. Furthermore, we report on the observation of phage tail-like structures corresponding to the tailocins using AFM and Transmission Electron Microscopy (TEM).

2. Materials and methods

2.1. Strains and media

P. fluorescens SF4c and *P. protegens* CHA0 were originally isolated from wheat (Argentina) and tobacco (Switzerland) rhizosphere, respectively (Fischer et al., 2007; Stutz et al., 1986). *X. axonopodis pv vesicatoria* Xcv Bv5–4a was provided by the National Institute of Agricultural Technology (INTA, Bella Vista, Argentina). *Pseudomonas* strains and *X. axonopodis pv vesicatoria* Xcv Bv5–4a were grown at 30 °C in Luria-Bertani (LB) medium and Yeast Extract-Peptone-Dextrose (YPD) medium, respectively. Agar (12%w/v) was used to solidify the media.

2.2. Bacteriocin purification

Saturated cultures of *P. fluorescens* SF4c were diluted 1:100 in LB medium and incubated at 30 °C until they reached an optical density 0.3 at 600 nm (OD_{600}). Mitomycin C was then added to a final concentration of 3 μg/mL and the incubation continued in dark until lysis occurred. Debris was removed by centrifugation at 17,000 × *g* for 1 h. Supernatants were precipitated with ammonium sulfate (60% [wt/vol]) and centrifuged at 17,000 × *g* for 1 h at 4 °C. The pellet was resuspended in 5 mL of TN50 buffer (50 mM NaCl, 10 mM Tris/HCl [pH 7.5]). Tailocin particles were then sedimented at 58,000 × *g* for 1 h at 4 °C and resuspended in 1.5 mL of TN50 buffer (Fischer et al., 2012; Hockett and Baltrus, 2017; Scholl and Martin, 2008). Finally, the suspension containing bacteriocins was filtered through a filter with pore size of 0.2 μm.

Semiquantitative bacteriocin assays were performed through the spot method. The bacteriocin extract was serially diluted in TN50 buffer, and then 5 μL of each dilution was spotted onto lawns of *X. axonopodis pv vesicatoria* Xcv Bv5–4a or *P. protegens* CHA0. Plates were incubated overnight at 30 °C. The tailocin titer was expressed in arbitrary units (AU) per mL, corresponding to the reciprocal of the highest dilution that showed a clear inhibition of the indicator strain (Williams et al., 2008).

2.3. Analysis of the tailocin structure by AFM and TEM

An aliquot of tailocin extract (15 μL) was placed onto the surface of freshly cleaved mica and dried for 1 h at 28 °C. The sample was washed with type I water (ASTM, D1193-06, Elga, Purelab Classic), then dried and mounted for observation. The images were obtained through the use of an atomic force microscope (AFM, Agilent, Technologies, SPM model 5500) working in acoustic mode. AFM probes (Micromasch, HQ:XSC11/A1 BS) with a cantilever resonance frequency and force constant of 155KHz and 7 N/m were used in all the measurements. The diameter of individual bacteriocins (n = 40) was analyzed by using the Gwyddion v2.39 free software for scanning probe microscopy data visualization and analysis (http://gwyddion.net/).

The tailocins were also analyzed by TEM. A drop of the extract containing bacteriocin obtained from cultures treated with mitomycin C, was deposited on collodion-coated 400-mesh copper grids for 5 min, and the excess of the suspended fluid was removed with the help of a filter paper. Finally, the grids were stained negatively with 2% (wt/vol) of phosphotungstic acid and examined with a transmission electron microscope JEM 1200 EX II (JEOL) using an acceleration voltage of 80 kV (Servicio Central de Microscopía Electrónica, Facultad de Ciencias Veterinarias, Universidad Nacional de la Plata, Argentina). The tailocins (n = 33) were visualized and analyzed with ImageJ, an open source image processing program (https://imagej.nih.gov/ij/index.html).

2.4. Atomic force microscopy of bacterial cells treated with tailocins

Cultures of *X. axonopodis pv vesicatoria* Xcv Bv5–4a and *P. protegens* CHA0 were grown to OD_{600} 0.5, harvested by centrifugation (3500 × *g* for 2 min), washed three times and resuspended in water. Afterwards, bacteria were immobilized electrostatically by depositing 20 μL of the bacterial suspension over polyethyleneimine coated slides (Dufrêne, 2008). They were allowed to interact with the substrate for 30 min at 28 °C, and finally rinsed with water. Subsequently, the immobilized cells were treated with bacteriocins (500 AU/mL) and incubated during 15 or 30 min at 28 °C and washed twice with water. The same treatment, replacing the bacteriocin by TN50 buffer, was used as control experiment. The AFM imaging and subsequent analysis were performed as described in the Section 2.3. The height of 160 cells per treatment was measured using Gwyddion v2.39.

2.5. Viability of bacteria treated with tailocin

Overnight cultures of strains Xcv Bv5-4 and CHA0 were diluted in YPD and LB liquid media, respectively. The cultures were grown to OD_{600} 0.5 and then, they were divided into four aliquots. Then, bacteriocins were added to the cultures to a final concentration of 250, 500 or 1000 AU/mL. Instead of bacteriocin, TN50 buffer was used in the control experiments. The samples were taken 30, 60, 120 and 240 min after treatment and the CFU values were determined on solid LB medium (Fischer et al., 2012).

2.6. Potassium ion efflux

Strains Xcv Bv5-4 and CHA0 were grown at 30 °C in YPD and LB liquid medium, respectively, until OD_{600} 0.5. The cells were collected by centrifugation (10,000 × *g* for 15 min) and washed three times with physiological solution (0.9% NaCl). Then, bacterial cells were resupended in the initial volume with physiological solution, and each sample was divided into two aliquots. Bacteriocins (500 AU/mL) were added to one of them and TN50 buffer was added to the other one (negative control). After 30 min of treatment, aliquots (3 mL) of each

culture were centrifuged at 13,000 × *g* for 5 min and the supernatants transferred to other Eppendorf tubes. As positive control, cultures were boiled for 10 min to release intracellular K^+ ions (Strauch et al., 2001). Prior to measurement, the samples were filtered by using a 0.2 μm pore size filter. The concentration of K^+ ions was determined in the supernatants using a single-channel flame photometer (Digital Flame Analyzer Cole-Parmer, model 2655-00). Calibration curves were obtained with potassium standards in the range of 1–10 μg of K^+ per mL. All the reported values of K^+ are the average of three different experiments.

2.7. Statistical analysis

Statistical analyzes were performed by using appropriate parametric and nonparametric procedures with the R software (http://www.r-project.org/). Data were analyzed by one way analysis of variance (ANOVA) and means comparisons among treatments were performed using Tukey's post hoc tests ($P \leq 0.05$) or the Kruskal-Wallis all-pairwise comparison test ($P = 0.05$).

3. Results

3.1. Atomic force and electron microscopy analysis of the SF4c tailocin

Bacteriocins were obtained from *P. fluorescens* SF4c by mitomycin C induction and ammonium sulfate precipitation, and their structure analyzed by AFM and TEM.

Microscopic images revealed the presence of a large number of phage tail-like bacteriocins (Fig. 1). The dimensions of the tailocins were determined from TEM images (Fig. 1A). The average length and diameter of particles was approximately 127 ± 8 nm and 16 ± 2 nm, respectively. These measurements were compared with the dimensions reported for phage tail-like bacteriocins belonging to different bacterial genera. In general, they were in good agreement with the values reported for R-pyocin from *P. aeruginosa* and some other tailocins (Table 1).

The analysis by AFM allowed us to acquire topographical information of the tailocin (Fig. 1B, C). The average diameter of tailocins measured by this technique was approximately 13 ± 3 nm, this agrees with the value measured by TEM.

3.2. Inhibitory activity of the SF4c tailocin against X. axonopodis pv vesicatoria Xcv Bv5–4a and P. protegens CHA0

The activity of tailocins was assayed against *X. axonopodis pv vesicatoria* Xcv Bv5–4a (a phytopathogenic strain) and *P. protegens* CHA0 (a Plant Growth-Promoting Rhizobacteria [PGPR]) through the spot method. Antimicrobial activity against strain Xcv Bv5–4a was 10^4 AU/mL (Fig. 2A), while no growth inhibition was detected against strain CHA0 (Fig. 2B). Furthermore, *X. axonopodis pv vesicatoria* Xcv Bv5–4a and *P. protegens* CHA0 were treated with different tailocin concentrations (250, 500 or 1000 AU/mL) and the number of viable cells after treatment was determined over time. Strain Xcv Bv5–4a was sensitive to all tested tailocin concentrations. A rapid decrease in viability was observed after 30 min of treatment, indicating that the tailocins have a bactericidal effect on strain Xcv Bv5–4a, with a killing rate higher than 97% at 30 min (Fig. 2C). In contrast, the growth of *P. protegens* CHA0 was not affected by any of the tested concentrations of tailocins (Fig. 2D). In addition, the turbidity of the culture of strain Xcv Bv54a decreased after treatment with tailocins, indicating that cells were lysed during the treatment (Fig. 2E). This effect was not observed in strain CHA0 (Fig. 2F).

3.3. Analysis of cell morphology after treatment with tailocins by AFM

To determine the effect of tailocins on bacterial cells, cultures of *X. axonopodis pv vesicatoria* Xcv Bv5–4a and *P. protegens* CHA0 were treated with 500 AU/mL of tailocins during 15 or 30 min. Control experiments were performed by exposing both strains to buffer TN50 during 15 min. Afterwards, the morphology of the cells was studied by AFM. The untreated strain Xcv Bv5-4 presented a regular rod shape with a relatively smooth surface (Fig. 3A). Perturbations on the cellular surface and collapse of the cell apical end were observed after 15 min of incubation with bacteriocin. The density of tailocins visualized on the sensitive bacteria was higher at the pole than in the central part of the cell (Fig. 3B–D).

To quantify changes in the structure and dimensions of the bacteria, the cellular height of 160 bacteria per treatment was measured from the images obtained with AFM. The height of the bacteria decreased significantly after treatment with tailocins, at a height lower than 140 nm compared to the control cells (Fig. 4A–C). This observation suggests a dramatic decrease in the volume of strain Xcv Bv5-4 upon exposure to the tailocin, which is associated to the cellular collapse. Besides, morphological changes in the cells become more evident as the time of incubation with tailocins is longer (Fig. 4D–F). On the contrary, treatment with tailocins had no obvious effects on the morphology of *P. protegens* CHA0 cells. Both, treated and untreated bacteria with tailocins presented similar morphology; a regular rod shape with a relatively smooth surface (Figs. 3E–H and 4J–L). The membrane of examined cell appeared to be undisturbed. In addition, the height distribution measured for this strain did not show significant changes among treatments (Fig. 4G–I). Moreover, tailocins were not visualized neither on the cellular surface of strain CHA0 nor on the polymeric support surface (Fig. 3E–H). This result confirms that the tailocins have no effect on strain CHA0.

3.4. Study of bacterial cell integrity through potassium ion efflux

The leakage of intracellular material may occur through cytoplasmic membranes perturbed by tailocins. To confirm this, the potassium efflux of strains Xcv Bv5–4a and CHA0 was measured after exposing both cells to 500 AU/mL of tailocins. Buffer TN50 was used instead of tailocins as negative control. Heat treatment was used as positive control. In the absence of tailocins (negative control), cells of Xcv Bv5–4a maintained an extracellular concentration of K^+ of 1.5 ppm. The exposure to tailocins during 30 min increased K^+ efflux (approximately 4 ppm) and this occurred faster than for heat-treated cells (2.8 ppm). On the contrary, no significant changes were observed for the tailocin-resistant strain CHA0 (Fig. 5).

4. Discussion

Previously, we reported that antibacterial compounds of *P. fluorescens* SF4c inhibit the growth of several *Pseudomonas* strains isolated from different plants and geographic regions around the world (Fischer et al., 2012). In addition, genetic studies demonstrated that these antimicrobials are similar to phage-like pyocins from *P. aeruginosa,* and their expression is induced by mitomycin C, a DNA damaging agent (Fischer et al., 2012). This work addresses the study of the structure of SF4c tailocin and its effect on phytopathogenic *Xantomonas*. The structure of phage-like bacteriocins has been usually analyzed by TEM (Jabrane et al., 2002; Ŝmarda and Benada, 2005). In this study, the tailocin from *P. fluorescens* SF4c is also investigated by TEM, and the calculated length and diameter are in agreement with the values reported for R-pyocin from *P. aeruginosa* (Ito et al., 1970). It is well known that

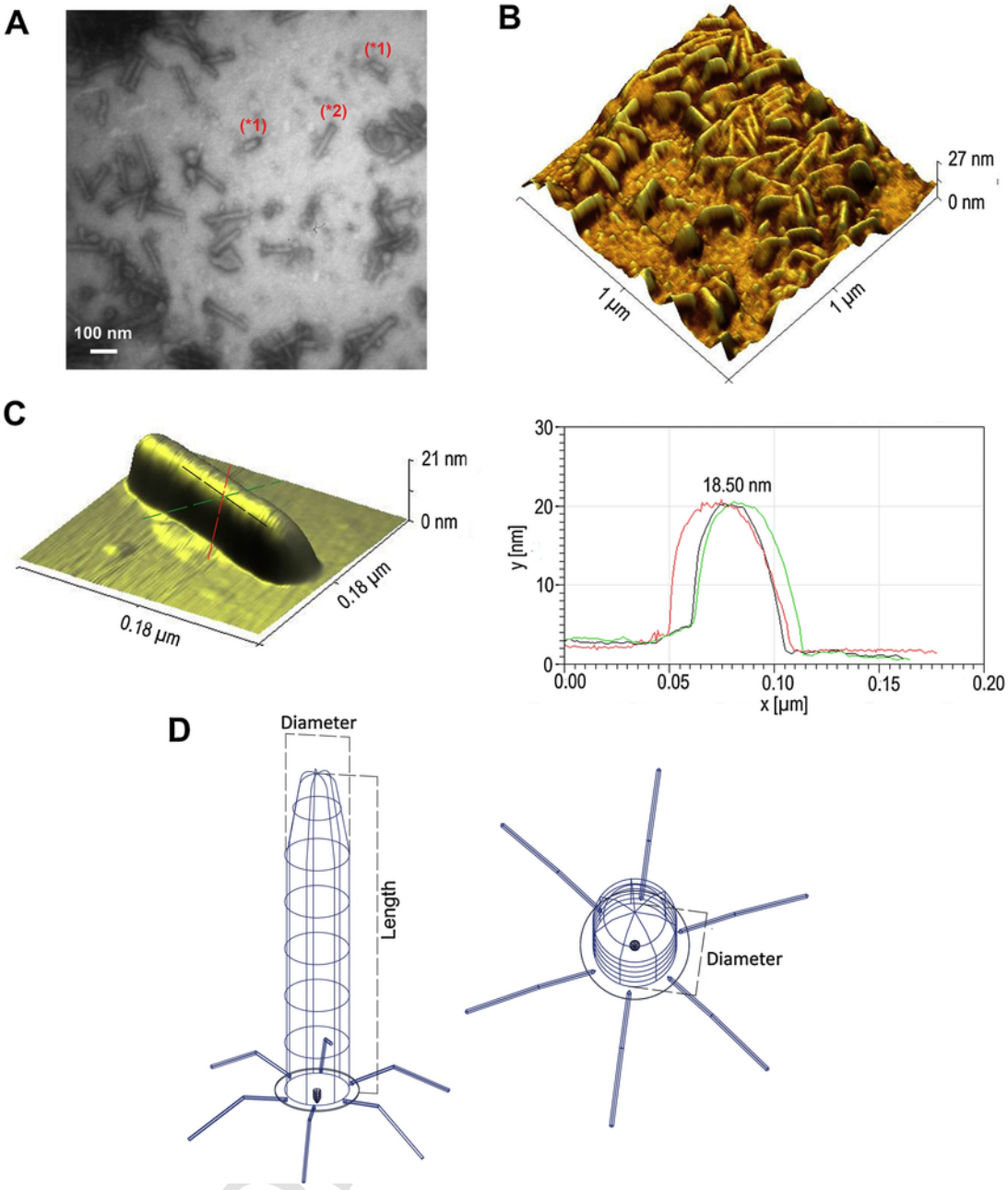

Fig. 1. Phage tail-like bacteriocins of *P. fluorescens* SF4c. (A) Tailocins visualized by TEM. The star symbols (*) indicate contracted structures (*1) and extended structure (*2). The bar indicates 100 nm. (B) AFM phase image of tailocins immobilized on glass. (C) Representative 3D AFM image of a tailocin deposited on mica (left) and the corresponding height line profiles (right) measured along three different directions as indicated by the color lines in the image. (D) Schematic representation of the calculated dimensions in the tailocins by TEM (left schematic) and AFM (right schematic).

biological samples are structurally sensitive to the sample preparation, to vacuum and to electron irradiation required during imaging by TEM. In order to study possible disruptions in the surface topography and dimensions of the SF4c tailocins, an analysis was carried out by using AFM. The diameter of the SF4c tailocin measured by AFM was similar to that calculated by TEM. The comparison between the data obtained with TEM and AFM allows us to conclude that the preparation and instrumental conditions have not substantially affected the tailocin topography.

In addition to displaying antagonistic activity against a select set of pseudomonads (Fischer et al., 2012), screening against a collection of phytopathogenic isolates revealed that SF4c tailocin have inhibitory activity against *X. axonopodis* pv. *vesicatoria* Xcv Bv5–4a. Copper-based bactericides have been applied during several years to control *Xanthomonas* spp; however, they have not been effective because of the emergence of copper-tolerant strains. More recently, nanomaterials have been evaluated to control bacterial spot; for example, TiO_2/Zn-treated tomato plants showed a reduction in disease severity (Paret et al., 2013). Unfortunately, this compound was phytotoxic, thus limiting its commercialization (Potnis et al., 2015). Therefore, the SF4c tailocin could be used in the future as a biocontrol agent of bacterial leaf spot. However, it is necessary to know how these tailocins act on the phytopathogenic bacteria to be considered as a possible microbial control agent.

R-type pyocins from *P. aeruginosa* bind to lipopolysaccharide (LPS) of sensitive cell. An L-rhamnose and two distinct D-glucose residues of

Table 1
Dimensions of contractile tailocins from Gram-negative bacteria measured through the TEM.

Tailocin (producer organism)	Size of the particle extended		Reference
	Length (nm)	Diameter (nm)	
R-type pyocin (*Pseudomonas aeruginosa*)	120–130	15	Ito et al. (1970)
Fonticin (*Pragia fontium* strains 24613, 24647 and 25240)	115 ± 5	20 ± 1	Ŝmarda and Benada (2005)
Aquaticin (*Budvicia aquatica* 24522)	107 ± 7	18 ± 2	Ŝmarda and Benada (2005)
Serracin P (*Serratia plymithicum* J7)	133	16	Jabrane et al. (2002)
Xenorhabdicin (*Xenorhabdus nematophilus* F1)	170	ND	Thaler et al. (1995)
Carotovoricin Er (*Erwinia carotovora* Er)	184 ± 4	22	Nguyen et al. (1999)
Enterocoliticin (*Yersinia enterocolitica* 29930)	80 ± 5	15 ± 1	Strauch et al. (2001)
Phage-like bacteriocin (*Proteus vulgaris*)	128	18	Coetzee et al. (1968)
SF4c tailocin (*Pseudomonas fluorescens* SF4c)	127 ± 8	16 ± 2 (13 ± 3[a])	This study

ND: Not determined.
[a] Diameter of tailocins measured by AFM.

the LPS core are part of the receptor sites for R1, R2, and R5 pyocins, respectively. Therefore, different types of R-pyocins recognize different receptor sites located in the LPS core. In addition, the susceptibility to R-pyocins between different *P. aeruginosa* serotypes depends on the packing density of LPS O-antigen chain, which is a consequence of the sugar constituents. If the receptor sites consist only of these LPS core sugar residues or require other structures is still unknown (Köhler et al., 2010). Once the R pyocin has been adhered to the receptor, form a channel into the bacterial membrane which dissipates the proton potential of the cell and finally, the death of microorganism occurs (Ge et al., 2015; Uratani and Hoshino, 1984).

In this work, AFM was used as a tool to study the effect produced by SF4c tailocins on *X. axonopodis* pv. *vesicatoria* Xcv Bv5–4a. The ability of AFM to monitor superficial alterations in cells has allowed to investigate the mode of action of several antimicrobial agents (Dufrêne, 2014; Longo and Kasas, 2014). This microscopy technique is appropriate to analyze the system maintaining cell viability during the treatment with the tailocins. The AFM images show that SF4c tailocins adhere to the cellular envelope of the sensitive strain Xcv Bv54a, causing changes in its cellular structure with a progressive loss of cellular volume. This is visualized and quantified in the images as a decrease in height. A possible explanation for this observation is that the phage tail-like bacteriocin produced by strain SF4c, like other tailocins, forms pores across the cell envelope. Thereby, those pores cause a rapid leakage of the intracellular materials, with the subsequent decrease of cell volume and finally, the lysis of the sensitive bacteria. Similarly, Su et al. (Su et al., 2012) observed through AFM that *Bacillus subtilis* cells exhibited an increased roughness and showed a decreased cell height upon treatment with the antimicrobial peptide trichokonin VI, thus suggesting that the leakage of intracellular materials could leads to cell death. To confirm if the leakage of the intracellular materials from tailocin-sensitive cells is also operative in the system under study, potassium efflux was measured. The extracellular concentration of K^+ was

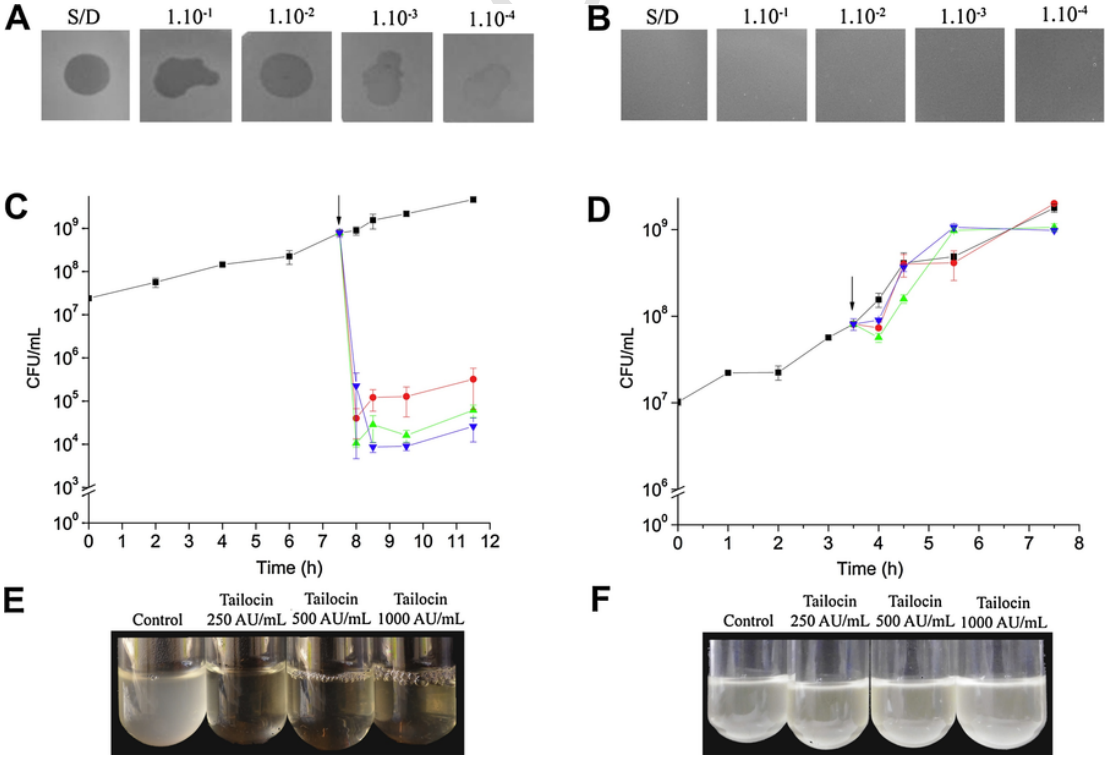

Fig. 2. Effect of the tailocins on strains Xcv Bv5–4a (A, C and E) and CHA0 (B, D and F). Antimicrobial activity of tailocins (A and B). Viability of sensitive (C) and resistant (D) strain after the addition of bacteriocins (↓) at final concentrations of 250 (●), 500 (▲), 1000 (▼) AU/mL or at no addition of tailocins (■). Each point indicates the mean ± SE of three experiments with three samples per experiment. Culture's turbidity after treatment with tailocins (E and F). S/D: undiluted. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

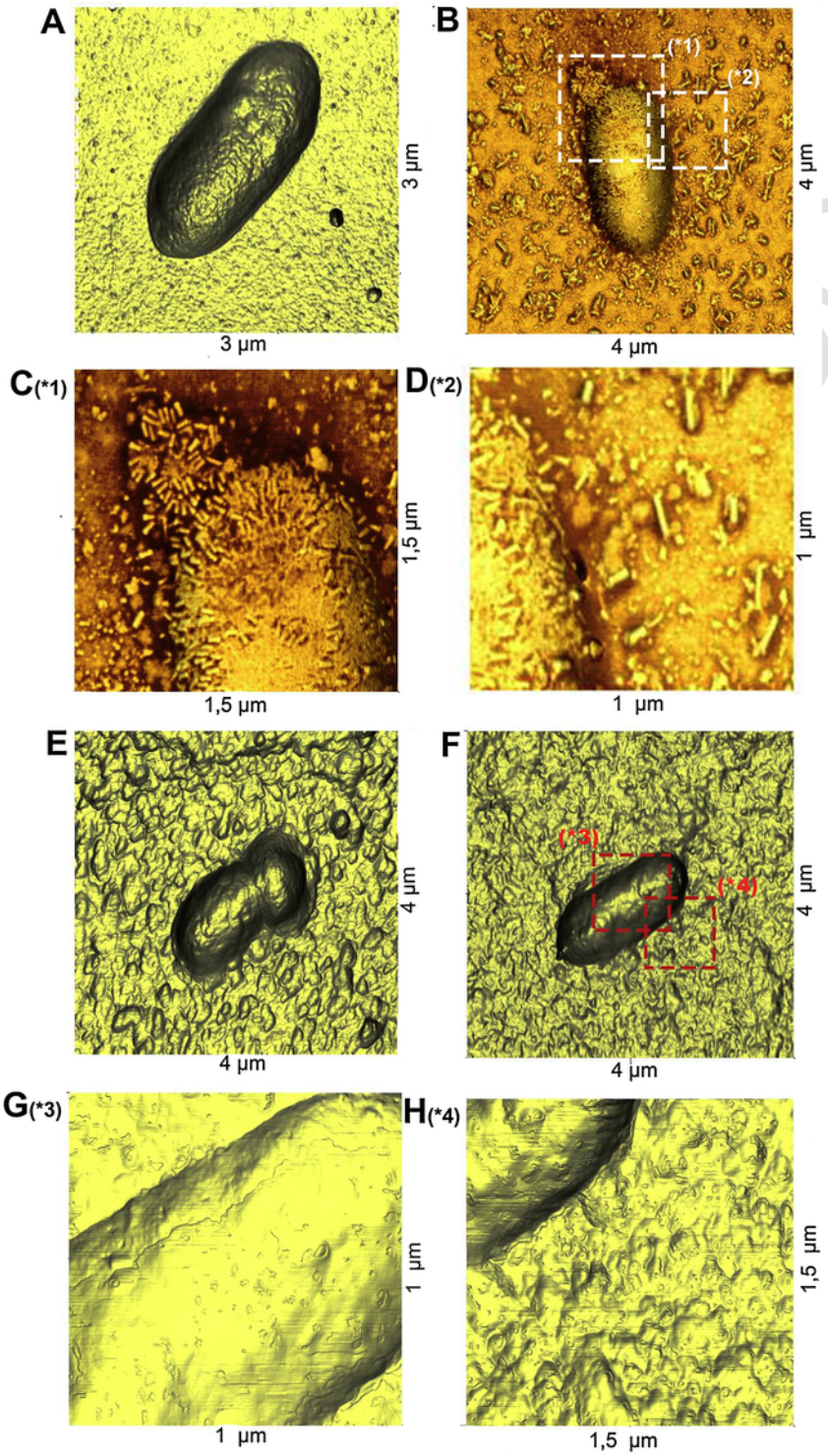

Fig. 3. AFM images of sensitive and resistant strains to tailocins. Topography of a Xcv Bv5-4 cell (A) and a CHA0 cell (E) untreated with tailocins. Phase image of a Xcv Bv5-4 cell (B) and topography of a CHA0 cell (F) treated with tailocins to a final concentration of 500 AU/mL during 15 min. The star symbols (*) indicate magnified regions of the figures B (C*1 and D*2) and F (G*3 and H*4).

higher when strain Xcv Bv54a was treated with tailocin, suggesting an increase in the membrane permeability by pore formation. Besides, the turbidity loss of tailocins-treated Xcv Bv54a cultures demonstrates that the cellular lysis and the death of phytopathogenic bacteria occur.

In this study, we demonstrated that SF4c tailocin was strain-specific; therefore it could be used in the biocontrol of phytopathogenic bacteria without affecting the microbial community, especially the plant growth-promoting rhizobacteria (PGPRs) such as *P. protegens* CHA0. This specificity of SF4c tailocin could be due to differences in the LPS of sensitive and resistant strains. Ghequire et al. (2015) analyzed LPS banding profiles of mutants resistant to R- type tailocins from *P. putida*. Compared to the wild-type, the mutants displayed an altered LPS profile.

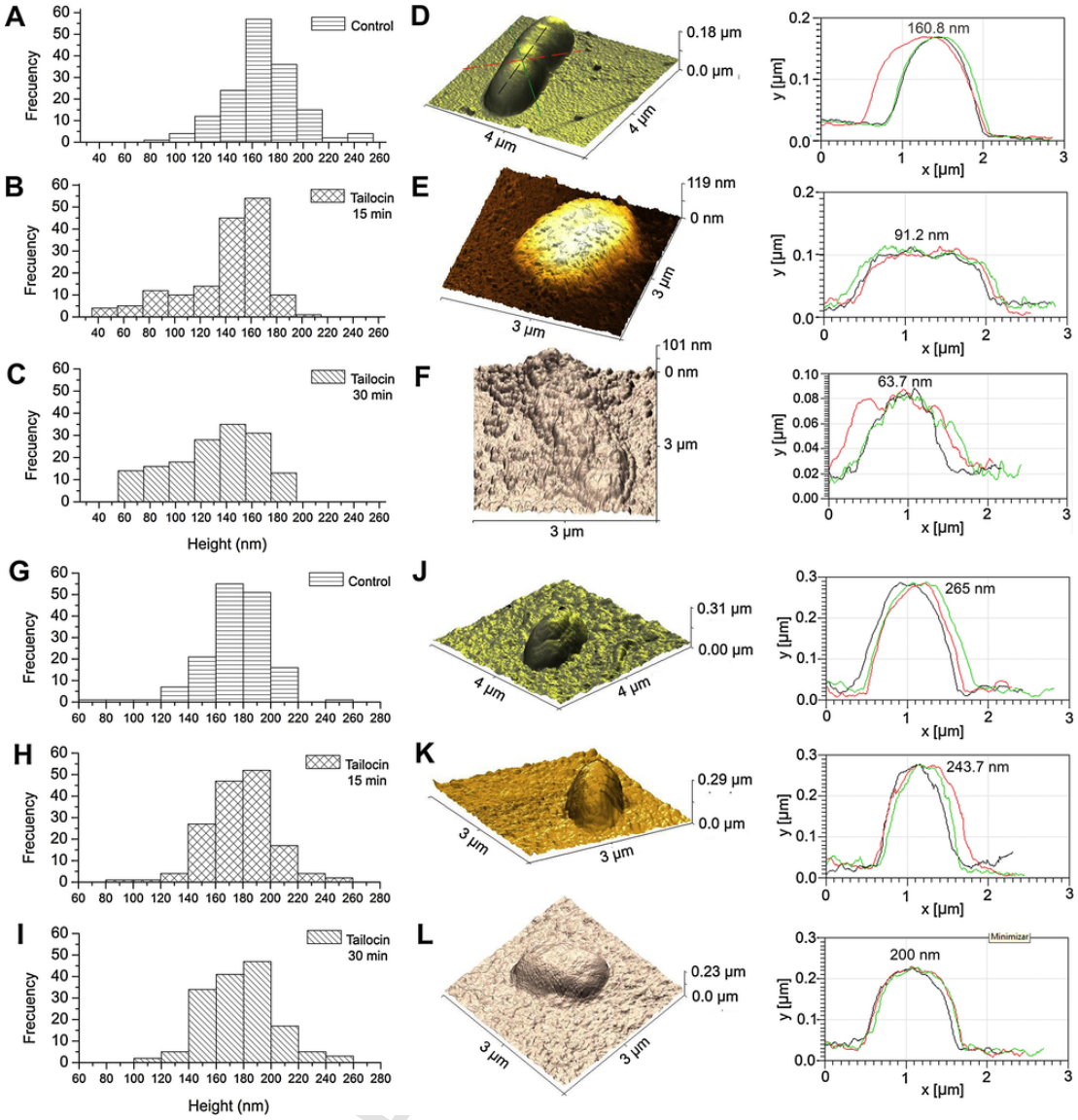

Fig. 4. Analysis of cellular height by AFM. Height distribution histograms of Xcv Bv5–4a cells (A–C) and CHA0 cells (G-I) untreated or treated with tailocins to a final concentration of 500 AU/mL during 15 and 30 min. The treatments in strain Xcv Bv5–4a present a statistically significant difference ($P \leq 0.05$) according to the Tukey's post hoc tests. The treatments in strain CHA0 are not significantly different ($P = 0.05$) according to the Kruskal-Wallis all-pairwise comparison test. Representative 3D AFM image of a Xcv Bv5–4a cell (D–F) and a CHA0 cell (J–L) and the corresponding height line profiles measured along three different directions at the cell as indicated by the color lines in the image.

In conclusion, SF4c tailocins adhere to the cell envelope of Xcv Bv54a and cause damage probably by pore formation, which results in lysis of the cells, a process similar to the bacteriophage infection (Young et al., 2000). SF4c tailocins represent an alternative to antibiotics for their application in the biocontrol of bacterial diseases. This study provides, to the best of our knowledge, the first evidence of the effect a tailocin analyzed by AFM.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

This research was supported by the Agencia Nacional de Promoción Científica y Tecnologica (PICT 1499/12; PICT 0831/14), Secretaría de Ciencia y Técnica de la Universidad Nacional de Río Cuarto and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (11220120100352CO). A. Principe, G.M Morales and S. Fischer are members of the Scientific Researcher Career-CONICET. M. Fernandez and A. Godino are fellowships from CONICET.

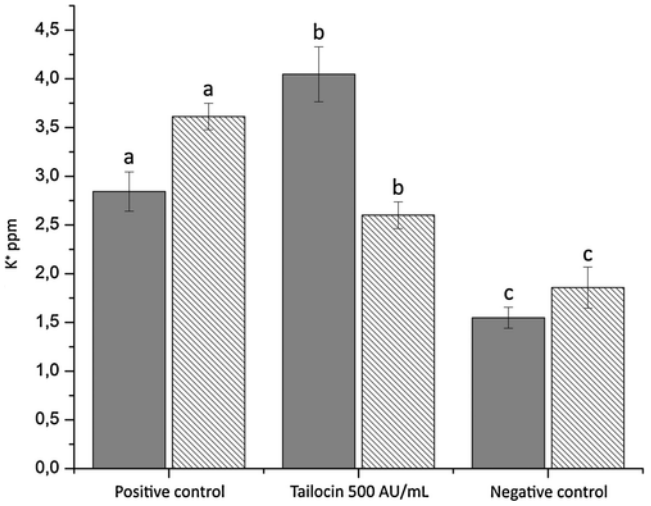

Fig. 5. Potassium efflux of Xcv Bv5–4a (black bars) and CHA0 cells (striped bars) treated with tailocins to a final concentration of 500 AU/mL during 30 min. The graph shows the means ± SE of three independent experiments with three replicates per experiment. Different letters indicate a statistically significant difference ($P \leq 0.05$) when comparing the treatment with tailocins to positive and negative control for each strain.

References

Ahmad, V., Khan, M.S., Jamal, Q.M.S., Alzohairy, M.A., Al Karaawi, M.A., Siddiqui, M.U., 2016. Antimicrobial potential of bacteriocins: in therapy, agriculture and food preservation. Int. J. Antimicrob. Agents 0–10, 1. http://dx.doi.org/10.1016/j.ijantimicag.2016.08.016.

Coetzee, H.L., De Klerk, H.C., Coetzee, J.N., Smit, J.A., 1968. Bacteriophage-tail-like particles associated with intra-species killing of Proteus vulgaris. J. Gen. Virol. 2, 29–36. http://dx.doi.org/10.1099/0022-1317-2-1-29.

Dingemans, J., Ghequire, M.G.K., Craggs, M., De Mot, R., Cornelis, P., 2016. Identification and functional analysis of a bacteriocin, pyocin S6, with ribonuclease activity from a Pseudomonas aeruginosa cystic fibrosis clinical isolate. Microbiol. Open 5, 413–423. http://dx.doi.org/10.1002/mbo3.339.

Dufrêne, Y.F., 2008. Atomic force microscopy and chemical force microscopy of microbial cells. Nat. Protoc. 3, 1132–1138. http://dx.doi.org/10.1038/nprot.2008.101.

Dufrêne, Y.F., 2014. Atomic force microscopy in microbiology: new structural and functional insights into the microbial cell surface. MBio 5, 1–14. http://dx.doi.org/10.1128/mBio.01363-14. Updated.

Elfarash, A., Wei, Q., Cornelis, P., 2012. The soluble pyocins S2 and S4 from Pseudomonas aeruginosa bind to the same FpvAI receptor. Microbiol. Open 1, 268–275. http://dx.doi.org/10.1002/mbo3.27.

Fischer, S.E., Fischer, S.I., Magris, S., Mori, G.B., 2007. Isolation and characterization of bacteria from the rhizosphere of wheat. World J. Microbiol. Biotechnol. 23, 895–903. http://dx.doi.org/10.1007/s11274-006-9312-4.

Fischer, S., Godino, A., Quesada, J.M., Cordero, P., Jofré, E., Mori, G., Espinosa-Urgel, M., 2012. Characterization of a phage-like pyocin from the plant growth-promoting rhizobacterium Pseudomonas fluorescens SF4c. Microbiology 158, 1493–1503. http://dx.doi.org/10.1099/mic.0.056002-0.

Ge, P., Scholl, D., Leiman, P., Yu, X., Miller, J., Hong Zhou, Z., 2015. Atomic structures of a bactericidal contractile nanotube in its pre- and postcontraction states. Nat. Struct. Mol. Biol. 33, 395–401. http://dx.doi.org/10.1038/nbt.3121.ChIP-nexus.

Ghequire, M.G.K., De Mot, R., 2014. Ribosomally encoded antibacterial proteins and peptides from Pseudomonas. FEMS Microbiol. Rev. 38, 523–568. http://dx.doi.org/10.1111/1574-6976.12079.

Ghequire, M.G.K., De Mot, R., 2015. The tailocin tale: peeling off phage tails. Trends Microbiol. 23, 587–590. http://dx.doi.org/10.1016/j.tim.2015.07.011.

Ghequire, M.G.K., Dillen, Y., Lambrichts, I., Proost, P., Wattiez, R., De Mot, R., 2015. Different ancestries of R tailocins in rhizospheric Pseudomonas isolates. Genome Biol. Evol. 7, 2810–2828. http://dx.doi.org/10.1093/gbe/evv184.

Godino, A., Príncipe, A., Fischer, S., 2016. A ptsP deficiency in PGPR Pseudomonas fluorescens SF39a affects bacteriocin production and bacterial fitness in the wheat rhizosphere. Res. Microbiol. 167, 178–189. http://dx.doi.org/10.1016/j.resmic.2015.12.003.

Grinter, R., Milner, J., Walker, D., 2012. Ferredoxin containing bacteriocins suggest a novel mechanism of iron uptake in pectobacterium spp. PLoS One 7, e33033. http://dx.doi.org/10.1371/journal.pone.0033033.

Hockett, K.L., Baltrus, D.A., 2017. Use of the soft-agar overlay technique to screen for bacterially produced inhibitory compounds. J. Vis. Exp. 1–5. http://dx.doi.org/10.3791/55064.

Hockett, K.L., Renner, T., Baltrus, D.A., 2015. Independent co-option of a tailed bacteriophage into a killing complex in Pseudomonas. MBio 6, 1–11. http://dx.doi.org/10.1128/mBio.00452-15.

Ito, S., Kageyama, M., Egami, F., 1970. Isolation and characterization of pyocins from several strains of Pseudomonas aeruginosa. J. Gen. Appl. Microbiol. 16, 205–214.

Jabrane, A., Sabri, A., Compère, P., Jacques, P., Vandenberghe, I., Van Beeumen, J., Thonart, P., 2002. Characterization of serracin P, a phage-tail-like bacteriocin, and its activity against Erwinia amylovora, the fire blight pathogen. Appl. Environ. Microbiol. 68, 5704–5710. http://dx.doi.org/10.1128/AEM.68.11.5704-5710.2002.

Köhler, T., Donner, V., Van Delden, C., 2010. Lipopolysaccharide as shield and receptor for R-pyocin-mediated killing in Pseudomonas aeruginosa. J. Bacteriol. 192, 1921–1928. http://dx.doi.org/10.1128/JB.01459-09.

Ling, H., Saeidi, N., Rasouliha, B.H., Chang, M.W., 2010. A predicted S-type pyocin shows a bactericidal activity against clinical Pseudomonas aeruginosa isolates through membrane damage. FEBS Lett. 584, 3354–3358. http://dx.doi.org/10.1016/j.febslet.2010.06.021.

Longo, G., Kasas, S., 2014. Effects of antibacterial agents and drugs monitored by atomic force microscopy. Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 6, 230–244. http://dx.doi.org/10.1002/wnan.1258.

Ly, L.K., Underwood, G.E., Mccully, L.M., Bitzer, A.S., Godino, A., Bucci, V., Brigham, C.J., Príncipe, A., Fischer, S.E., Silby, W., 2015. Draft genome sequences of Pseudomonas fluorescens strains SF39a and SF4c, potential plant growth promotion and biocontrol agents. Genome Announc. 3, 4–5. http://dx.doi.org/10.1128/genomeA.00219-15.

Michel-Briand, Y., Baysse, C., 2002. The pyocins of Pseudomonas aeruginosa. Biochimie 84, 499–510. http://dx.doi.org/10.1016/S0300-9084(02)01422-0.

Nguyen, A.H., Tomita, T., Hirota, M., Sato, T., Kamio, Y., 1999. A simple purification and morphology and component analyses for Carotovoricin Er, a phage-tail-like bacteriocin fron the plant pathogen Erwinia carotovora Er. Biosci. Biotechnol. Biochem. 63, 1360–1369.

Paret, M.L., Vallad, G.E., Averett, D.R., Jones, J.B., Olson, S.M., 2013. Photocatalysis: effect of light-activated nanoscale formulations of TiO(2) on Xanthomonas perforans and control of bacterial spot of tomato. Phytopathology 103, 228–236. http://dx.doi.org/10.1094/PHYTO-08-12-0183-R.

Potnis, N., Timilsina, S., Strayer, A., Shantharaj, D., Barak, J.D., Paret, M.L., Vallad, G.E., Jones, J.B., 2015. Bacterial spot of tomato and pepper: diverse Xanthomonas species with a wide variety of virulence factors posing a worldwide challenge. Mol. Plant Pathol. 16, 907–920. http://dx.doi.org/10.1111/mpp.12244.

Ritchie, J.M., Greenwich, J.L., Davis, B.M., Bronson, R.T., Gebhart, D., Williams, S.R., Martin, D., Scholl, D., Waldor, M.K., 2011. An Escherichia coli O157-specific engineered pyocin prevents and ameliorates infection by E. coli O157:H7 in an animal model of diarrheal disease. Antimicrob. Agents Chemother. 55, 5469–5474. http://dx.doi.org/10.1128/AAC.05031-11.

Ritchie, D.F., 2000. Bacterial spot of pepper and tomato. Plant Health Instr. http://dx.doi.org/10.1094/phi-i-2000-1027-01.

Scholl, D., Martin, D.W., 2008. Antibacterial efficacy of R-type pyocins towards Pseudomonas aeruginosa in a murine peritonitis model. Antimicrob. Agents Chemother. 52, 1647–1652. http://dx.doi.org/10.1128/AAC.01479-07.

Ŝmarda, J., Benada, O., 2005. Phage tail-like (high-molecular-weight) bacteriocins of Budvicia aquatica and Pragia fontium (Enterobacteriaceae). Appl. Environ. Microbiol. 71, 8970–8973. http://dx.doi.org/10.1128/aem.71.12.8970-8973.2005.

Strauch, E., Kaspar, H., Schaudinn, C., Dersch, P., Madela, K., Gewinner, C., Hertwig, S., Wecke, J., Appel, B., 2001. Characterization of Enterocoliticin, a phage tail-like bacteriocin, and its effect on pathogenic Yersinia enterocolitica strains. Appl. Environ. Microbiol. 67, 5634–5642. http://dx.doi.org/10.1128/AEM.67.12.5634-5642.2001.

Stutz, E., Defago, G., Kern, H., 1986. Naturally occurring fluorescent pseudomonads involved in suppression of black root rot of tobacco. Phytopathology 76, 181–185. http://dx.doi.org/10.1094/Phyto-76-181.

Su, H.N., Chen, Z.H., Song, X.Y., Chen, X.L., Shi, M., Zhou, B.C., Zhao, X., Zhang, Y.Z., 2012. Antimicrobial peptide Trichokonin VI-induced alterations in the morphological and nanomechanical properties of Bacillus subtilis. PLoS One 7, 1–10. http://dx.doi.org/10.1371/journal.pone.0045818.

Thaler, J.O., Baghdiguian, S., Boemare, N., 1995. Purification and characterization of xenorhabdicin a phage tail-like bacteriocin, from the lysogenic strain F1 of Xenorhabdus nematophilus. Appl. Environ. Microbiol. 61, 2049–2052.

Uratani, Y., Hoshino, T., 1984. Pyocin R1 inhibits active transport in Pseudomonas aeruginosa and depolarizes membrane potential. J. Bacteriol. 157, 632–636.

Williams, S.R., Gebhart, D., Martin, D.W., Scholl, D., 2008. Retargeting R-type pyocins to generate novel bactericidal protein complexes. Appl. Environ. Microbiol. 74, 3868–3876. http://dx.doi.org/10.1128/AEM.00141-08.

Young, R., Wang, I., Roof, W.D., 2000. Phages Will Out: Strategies of Host Cell Lysis. 120–128.