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Original article

A *ptsP* deficiency in PGPR *Pseudomonas fluorescens* SF39a affects bacteriocin production and bacterial fitness in the wheat rhizosphere

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Abstract

Pseudomonas fluorescens SF39a is a plant-growth-promoting bacterium isolated from wheat rhizosphere. In this report, we demonstrate that this native strain secretes bacteriocins that inhibit growth of phytopathogenic strains of the genera *Pseudomonas* and *Xanthomonas*. An S-type pyocin gene was detected in the genome of strain SF39a and named *pys*. A non-polar *pys::Km* mutant was constructed. The bacteriocin production was impaired in this mutant.

To identify genes involved in bacteriocin regulation, random transposon mutagenesis was carried out. A miniTn*5*Km1 mutant, called *P. fluorescens* SF39a-451, showed strongly reduced bacteriocin production. This phenotype was caused by inactivation of the *ptsP* gene which encodes a phosphoenolpyruvate phosphotransferase (EI^{Ntr}) of the nitrogen-related phosphotransferase system (PTS^{Ntr}). In addition, this mutant showed a decrease in biofilm formation and protease production, and an increase in surface motility and pyoverdine production compared with the wild-type strain. Moreover, we investigated the ability of strain SF39a-451 to colonize the wheat rhizosphere under greenhouse conditions. Interestingly, the mutant was less competitive than the wild-type strain in the rhizosphere. To our knowledge, this study provides the first evidence of both the relevance of the *ptsP* gene in bacteriocin production and functional characterization of a pyocin S in *P. fluorescens*.

Keywords: Bacteriocin; Regulation; Plant growth-promoting rhizobacterium; Competitiveness; Phosphotransferase system; *Pseudomonas*

1. Introduction

Plant growth-promoting rhizobacteria (PGPR) benefit plants directly by stimulating plant growth, and/or indirectly, through a reduction in the incidence of plant disease. In the rhizosphere, PGPR may compete effectively with native microflora, survive, become established in the plant root and then function under the particular conditions of the ecosystem at hand [1]. Rhizobacteria employ different strategies for competition with other microorganisms from soil; for example, production of antimicrobial compounds such as antibiotics (secondary metabolites; usually broader spectrum) or bacteriocins (ribosomally synthesized proteinaceous substances; usually narrow spectrum) [2]. These antimicrobials are of great interest because they affect bacterial population dynamics and, consequently, survival and virulence [3,4].

One of the most important groups among the PGPR is the genus *Pseudomonas*. The antibiotics produced by *Pseudomonas* spp. have been widely studied. Nevertheless, little is known about the bacteriocins (structure, killing activity, regulatory systems) synthesized by these *Pseudomonas* spp. [5]. For example, lectin-like bacteriocins have been identified in plant-associated *Pseudomonas* strains [6,7]. The first member of this family of antibacterial proteins was identified in *Pseudomonas putida* BW11M1 and called $LlpA_{Bw}$; the crystal structure of $LlpA_{Bw}$ was determined by Ghequire et al. [8]. However, little information is available on the regulation of *llpA* genes. In strain *P. putida* BW11M1, production of $LlpA_{Bw}$ is enhanced upon exposure to UV light [6]. In

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addition, a *recJ* mutant exhibited an overexpression phenotype. In contrast, reduced production was detected upon *recA* inactivation. These different phenotypes in *recA* and *recJ* mutants suggest that two different, but not necessarily independent, stress-responsive regulatory systems are involved in bacteriocin expression. Moreover, a low level of bacteriocin expression was detected in a *spoT* mutant, suggesting that production is also influenced by another global stress response system [9].

Phage tail-like bacteriocins, called tailocins, represent other classes of bacteriocins [5]. We reported that *Pseudomonas fluorescens* SF4c, an isolate from wheat rhizosphere, produces a tailocin (similar to the phage-like pyocin of *Pseudomonas aeruginosa*). The production of this bacteriocin was upregulated by mitomycin C, UV light and hydrogen peroxide, and was downregulated by saline stress [10]. Moreover, analysis of genome sequence data revealed the presence of a variety of tailocins in *Pseudomonas* originating from plant or soil environments [5,11]. Finally, analysis in silico revealed that genes for production of other bacteriocins, for example, S-type pyocin or microcin-like compounds, are present in plant-associated *Pseudomonas* genomes, but functional characterization of these compounds is still lacking [12,13]. Another remaining issue is the identification of environmental factors that regulate bacteriocin production and assessment of their ecological impact [5].

P. fluorescens SF39a is a native PGPR strain isolated from the rhizosphere of wheat (Argentina) [14]. In this work, an S-type pyocin gene was detected in the genome of the strain SF39a and named *pys*. Antimicrobial activity was impaired in a *pys* mutant derivative from strain SF39a. We also report that a mutation in the *ptsP* gene affects bacteriocin and siderophore production, protease activity, biofilm formation and surface motility. Moreover, inactivation of *ptsP* impaired competitiveness of *P. fluorescens* SF39a in the rhizosphere.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

Pseudomonas strains and *Escherichia coli* were grown in Luria–Bertani (LB) at 30 °C or at 37 °C, respectively. When appropriate, the media were supplemented with antibiotics (in μg/ml): ampicillin, 100; chloramphenicol, 30; kanamycin, 25; rifampicin, 50; streptomycin, 100; gentamicin, 20 (for *E. coli*) or 60 (for *Pseudomonas*). The bacterial strains and plasmids used in this study are listed in Table 1.

2.2. Bacteriocin assay

Ten μl of a stationary-phase culture of the producer strain were spotted onto LB agar and incubated overnight at 30 °C. To prevent further cell growth, the plates were then exposed to chloroform vapor (20 min) and overlaid with 3 ml soft agar (0.7% w/v) seeded with 50 μl of a bacterial suspension of the indicator strain. The plates were incubated at 30 °C and bacteriocin production determined by growth inhibition around the colonies [6]. Reference *Pseudomonas* and *Xanthomonas* strains were used as indicator strains (Table 1).

2.3. Bacteriocin crude extracts

The bacteriocin from *P. fluorescens* SF39a was partially purified as described previously [10] with some modifications. Briefly, saturated cultures of *P. fluorescens* SF39a were diluted 1:100 in LB medium and shaken at 150 rpm and 30 °C to OD_{600} 0.3. Then, mitomycin C (final concentration of 6 μg/ml) was added to one of the flasks and the incubation continued overnight with the control flask (no mitomycin C). The samples were centrifuged at 17 000 g for 1 h and the supernatants were divided into two aliquots. One of them was precipitated with ammonium sulfate (60%, w/v) and centrifuged at 17 000 g for 1 h at 4 °C, and the pellet was resuspended in TN50 buffer. The other was concentrated by ultrafiltration through a centrifugal filtration device (Amicon Ultra-15, 100K, Millipore) at 6000 g for 20–25 min at 4 °C. The antimicrobial activity of all samples was assayed by the spot test method against the sensitive strain *P. fluorescens* CTR212. The bacteriocin titer was expressed in arbitrary units (AU)/ml, corresponding to the reciprocal of the highest dilution that showed clear inhibition of the indicator strain [15].

Then, the samples concentrated by ultrafiltration were analyzed by polyacrylamide gel electrophoresis (PAGE) under denaturing [16] or non-denaturing conditions [17]. Gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad). To test for activity, unstained non-denaturing gels were washed with distilled water, placed into Petri dishes containing LB and overlaid with soft agar seeded with the indicator strain. Petri dishes were incubated at 30 °C and tested for growth inhibition zones.

2.4. Sensitivity of bacteriocin to protease and heat

Aliquots of bacteriocin crude extracts were treated with proteinase K (2 mg/ml) at 37 °C for 1 or 3 h. To analyze thermal stability, samples of bacteriocin were heated at 37 °C for 1 or 3 h and, 50 °C, 80 °C and 100 °C for 10 or 20 min. After treatment, the samples were tested for antimicrobial activity against *P. fluorescens* CTR212 [18].

2.5. Molecular biology techniques

Standard techniques were used for plasmid and DNA isolation, restriction enzyme digestion, ligation reactions and agarose gel electrophoresis [19]. For Southern analysis, the Biotin DecaLabel™ DNA Labeling and Biotin Chromogenic Detection kits (Fermentas) were used.

2.6. Bacteriocin prediction and construction of a pyocin mutant

BAGEL3 (web-based bacteriocin mining tool) was used to identify genes encoding bacteriocins in the draft genome of *P. fluorescens* SF39a [20]. In addition, BLAST was performed to

Table 1
Bacterial strains and plasmids used in this study.

Strains or plasmids	Origin or relevant characteristics	Source or reference
Strains		
P. fluorescens SF39a	Isolate from wheat rhizosphere (Argentina)	[14]
P. fluorescens SF4c	Isolate from wheat rhizosphere (Argentina)	[14]
P. putida SF10b	Isolate from wheat rhizosphere (Argentina)	[14]
P. fluorescens CTR212	Soil isolate (France)	[46]
P. fluorescens Pf0-1	Soil isolate (United States)	[47]
P. fluorescens WCS365	Isolate from potato rhizosphere (Netherlands)	[48]
P. protegens CHA0	Isolate from tobacco rhizosphere (Switzerland)	[49]
P. putida KT2440	Plasmid-free derivative of natural isolate mt-2	[50]
P. syringae pv. *syringae* 61	Isolate from wheat (USA)	[51]
P. corrugata NCPPB 2445	Isolate from tomato, pathogen (UK)	[52]
P. corrugata PC5	Isolate from tomato, pathogen (Argentina)	[53]
P. viridiflava Pvalb8	Isolate from basil, pathogen (Argentina)	[53]
X. axonopodis pv. *citri* Xcc991330	Isolate from *C. sinensis*, pathogen	INTA Bella Vista, Argentina
X. axonopodis pv. *vesicatoria* XcvBv54a	Isolate from pepper plants, pathogen	INTA Bella Vista, Argentina
P. fluorescens SF39a-451	*ptsP*::mini-Tn5 Km1 mutant of *P. fluorescens* SF39a, Km^R	This work
P. fluorescens SF39a-451pPtsP	*ptsP*::mini-Tn5 Km1 mutant complemented with pPtsP, Km^R, Gm^R	This work
P. fluorescens SF39a-pys	Non-polar *pys::Km* mutant of *P. fluorescens* SF39a	This work
P. fluorescens 39a-R	Spontaneous rifampicin-resistant mutant derived from *P. fluorescens* SF39a, Rif^R	This work
P. fluorescens SF39a-451-R	Spontaneous rifampicin-resistant mutant derived from *P. fluorescens* SF39a-451, Km^R, Rif^R	This work
E. coli DH5α	endA1 recA1 hsdR17 supE44 thi-1 gyrA96 relA1 Δ(lacZYA-argF)U169 φ80d lacZ Δ M15.	[54]
E. coli CC118λpir	Δ(ara-leu) araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE(Am) recA1 λ pir lisogén.	[21]
E. coli HB101	Pro leu thi lacY endoI- RecA-	[55]
Plasmids		
pUT mini-Tn5 Km1	Suicide plasmid carrying transposon mini-Tn5 Km1, Amp^R, Km^R	[24]
pRK600	ColE1 replicon with RK2 transfer region, helper plasmid, Cm^R	[56]
pBluescript SK	Cloning vector, Amp^R	Stratagene
pBBR1MCS-5	Broad-host-range cloning vector, $P_{(lac)}$, Gm^R	[57]
pBSK451	6-kb *Pst*I fragment containing mini-Tn*5* Km1 insertion and flanking DNA from *P. fluorescens* SF39a-451 cloned into pBluescript SK, Km^R Amp^R	This work
pPtsP	*ptsP* from *P. fluorescens* Pf0-1 cloned into pBBR1MCS-5, Gm^R	This Work
pUC18Not	Cloning vector, pUC18 derivative with polylinker flanked by *Not*I sites	[21]
p34S-Km3	Plasmid carrying a Km^R cassette flanked by duplicated restriction sites	[22]
pKNG101	Suicide vector for gene replacement; Sm^R; *sacB*	[23]
pPYS1	1185 bp *Pst*I/*Bam*HI fragment that includes the 5′ region of *pys* cloned into pUC18Not	This work
pPYS12	1248 bp *Bam*HI/*Kpn*I fragment that includes the 3′ region of *pys* cloned into pPYS1	This work
pPYS12Km	*Bam*HI fragment containing Km^R cassette from p34S-Km3 cloned into pPYS12	This work
pKNGPYS	*Not*I fragment of pPYS12Km cloning into pKNG101	This work

Amp^R, ampicillin resistant; Cm^R, chloramphenicol resistant; Gm^R, gentamicin resistant; Km^R, kanamycin resistant; Rif^R, rifampicin resistant; Sm^R streptomycin resistant.

detect conserved genes (*hol*, *lys*, *prtR*, *mutS* and *recA*−*recX*) of phage tail-like pyocins.

A pyocin mutant derivative of *P. fluorescens* SF39a was constructed by replacement of an internal 1582 bp fragment of *pys* with a Km-resistance cassette. For this step, two fragments were PCR-amplified with the oligonucleotides fw1 [AACTgCAgTACGCCTTCCTCGACCATCAT (*Pst*I restriction site underlined)] and rv1 [CgggATCCAACTTCCTGGCGATCAGGGA (*Bam*HI restriction site underlined)], and fw2 [CgggATCCAAGGCGCATGTTGAAATTCA (*Bam*HI site underlined)] and rv2 [ggggTACCTACACCCTGACGCATTTGCT (*Kpn*I site underlined)]. The PCR products were cloned into pUC18Not [21] to give pPYS1 and pPYS12, respectively. A *Bam*HI fragment carrying a Km-resistance cassette from plasmid p34S-Km3 [22] was then cloned into pPYS12. The resulting plasmid, pPYS12Km, was digested with *Not*I, and the *pys::Km* construct was cloned into pKNG101, a suicide vector unable to replicate in *Pseudomonas* spp. that allows the generation of double recombination events [23]. The resulting plasmid, pKNGPYS, was electroporated into SF39a. Transformants in which the construct was integrated into the chromosome by a single crossover were selected on LB plates supplemented with Km. The second crossover event was selected by plating Km^R clones on medium containing 7% (w/v) sucrose and Km. In addition, the plasmid loss was tested on plates with streptomycin. Km^R Sac^R Sm^S colonies were checked by PCR and Southern hybridization to confirm the replacement of *pys* by *pys*::Km.

2.7. Random mutagenesis

Transposon mutagenesis with mini-Tn*5* Km1 [24], was performed by triparental mating, with *P. fluorescens* SF39a as recipient, *E. coli* CC118λpir (pUT-Km1) as donor and *E. coli* HB101 (RK600) as helper. The transconjugants were selected on medium containing kanamycin and screened to investigate the mutants affected in bacteriocin production. One clone (SF39a-451) was chosen. Chromosomal DNA of mutant *P. fluorescens* SF39a-451 was digested with *PstI* (which has a unique recognition site in mini-Tn*5* Km1) and ligated to pBluescript SK previously digested with *PstI*. Ligation mixes were electroporated into *E. coli* DH5α and plated on LB medium supplemented with ampicillin and kanamycin. The Amp^R Km^R colonies were isolated and one clone containing the recombinant plasmid was used for sequencing (Macrogen Inc.) through the use of primer 5′-AGATCTGATCAAGAGACAG-3′ [25]. The nucleotide sequence obtained was compared with the GenBank database by means of the BLAST algorithm [26].

2.8. Complementation analysis

A fragment containing the coding region of *ptsP*, with its ribosome-binding sequence, was amplified by PCR from *P. fluorescens* Pf0-1 genomic DNA with primers ptsPFw (5′-CggAATTCTCgAgTgTgTTYATYgCCAgC-3′ [*EcoR*I restriction site underlined]) and ptsPRv (5′-CCCAAgCTTggTAAACCggAgTTCgATgg-3′ [*Hind*III restriction site underlined]). The PCR product was purified, restricted with *EcoR*I and *Hind*III and cloned into the pBBR1MCS-5 vector. This construct, pPtsP, was used to transform *P. fluorescens* SF39a-451 by electroporation. The bacteriocin assay was used as a complementation test.

2.9. Phenotypic analyses of mutant P. fluorescens SF39a-451

The biofilm formation ability, pyoverdine and protease production, solubilization of phosphate and motility were tested in the mutant SF39a-451 and wild-type. For the biofilm formation assay, an aliquot of 1.5 µl of overnight cultures grown in LB was transferred into 100 µl of K10T-1 medium in a 96-well plate and grown statically for 12 h at 30 °C. After incubation, 25 µl of 1% solution of crystal violet (CV) was added to each well and the plate was incubated at room temperature for 15 min. Then, the plate was rinsed thoroughly with water and CV-stained biofilm was solubilized in 200 µl of 95% ethanol [27]. The absorbance was determined with a microplate reader at 570 nm (Multiskan™ FC-Thermo Scientific) and the data were normalized to cell density measured at 600 nm.

Pyoverdine production was analyzed on King's B medium. Ten µl of overnight cultures grown in LB were spotted onto agar King's B plates and incubated at 30 °C for 24, 48 and 72 h. Then the plates were observed under UV light. Moreover, pyoverdine levels were quantified spectrophotometrically at OD_{405}. For this, bacteria were grown on King's B liquid medium for 24, 48 or 72 h and the cultures were centrifuged (5 min, 10 000 × g). Then, the pyoverdine levels were measured in the supernatant at OD_{405} [28,29].

Protease assay was performed using a protocol based on that described by Sokol et al. [30]. Ten µl of overnight cultures were spotted onto 3% milk agar plate. The casein degradation zone was measured after 48 h of growth at 30 °C.

Solubilization of phosphate was analyzed by the formation of transparent halos surrounding bacterial colonies on a medium containing insoluble phosphate after 5 days of incubation at 30 °C [31].

Swimming motility and surface motility were evaluated in LB medium supplemented with 0.3% or 0.4% agar, respectively [32,33]. The plates were incubated at 30 °C for 48 h to detect bacterial motility. Moreover, samples for transmission electron microscopy were prepared either from liquid cultures or taken from surface motility plates. In the first case, a drop of an overnight culture in LB medium was placed directly on the grid. In the second case, bacteria from the inoculation point, the middle of the expanding colony or the edge of the expanding colony were gently scraped off and suspended in a drop of physiological solution (0.9% NaCl). In all cases, bacterial suspensions were placed on collodion-coated copper grids (400 mesh) for 5 min. The excess of bacterial suspension was removed and the attached bacteria were stained with 2% phosphotungstic acid for 10 s. Negatively stained cells were visualized with a JEM 1200 EXII transmission electron microscope (JEOL Ltd., Tokyo, Japan) equipped with a Erlangshen ES1000W digital camera (Model 785, Gatan Inc., Pleasanton, CA, USA) (Servicio Central de Microscopía Electrónica de la Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata).

2.10. Rhizosphere colonization and competition

Rhizosphere colonization and competition assays between strains SF39a and SF39a-451 were performed in non-sterile soil. First, the strains were tagged with rifampicin. Spontaneous rifampicin-resistant mutants derived from these strains were isolated on LB medium containing rifampicin (50 µg/ml) and named SF39-R and SF39a-451-R. Studies of growth of these strains were carried out in liquid glucose minimal medium to determine whether spontaneous rifampicin resistant mutants were able to grow the same as their parent strains [34].

Wheat seeds (BIOINTA3004) were surface-sterilized and pregerminated [14]. Germinated seeds were sown into plastic pots containing non-sterile soil and then kept in a greenhouse with light–dark cycles (12 h light, 12 h dark). Plants were irrigated with sterilized water. Uninoculated plants were used as a control.

For production of bacterial inocula, strains SF39a-R and SF39a-451-R were grown in LB broth at 30 °C, 140 rpm until exponential phase $OD_{600} = 1$. Cells were then harvested by centrifugation (10 000 × g for 10 min), washed and resuspended

in physiological solution (0.9% NaCl) at a density of 2×10^8 cfu/ml.

Seven-day-old plants were inoculated with strain SF39-R or SF39a-451-R at a density of 2×10^6 cfu/g soil. In addition, mixed inoculation treatments containing a 1:1 mixture of competing strains were performed (1×10^6 cfu/g soil for each strain).

Plants were harvested at 3 weeks after inoculation and the number of viable cells was determined in the rhizosphere soil. Samples were serially diluted and plated onto LB medium supplemented with dichloran (20 mg/ml) (for fungi inhibition) and rifampicin, or rifampicin and kanamycin, to distinguish between strains in mixed inoculation treatments. The plates were incubated at 30 °C. Viability was expressed as cfu per g of dry soil [34,35]. The experiment was repeated 3 times with $n = 5$.

2.11. Statistical analyses

Data were analyzed by one way analysis of variance (ANOVA) or the Student test when suitable. Differences were considered to be significant at the $P \leq 0.05$ level. Means were compared by Tukey tests (for ANOVA).

3. Results

3.1. Detection of antibacterial compounds in P. fluorescens SF39a

Bacteriocin production by *P. fluorescens* SF39a was analyzed on LB plates against several reference strains of the genera *Pseudomonas* and *Xanthomonas*. From 13 strains tested, only two (*Pseudomonas protegens* CHA0 and *Pseudomonas corrugata* PC5) were not inhibited by strain SF39a (Table 2). In all cases, there was a large inhibition zone surrounding the colonies on lawns of sensitive strains (Figs. 1 and 2).

Table 2
Antibacterial spectrum of *P. fluorescens* SF39a against different *Pseudomonas* and *Xanthomonas* strains.

Indicator strain	Growth inhibition
P. fluorescens CTR212	+
P. fluorescens SF4c	+
P. fluorescens Pf0-1	+
P. fluorescens WCS365	+
P. protegens CHA0	−
P. putida SF10b	+
P. putida KT2440	+
P. syringae pv. *syringae* 61	+
P. corrugata NCPPB 2445	+
P. corrugata PC5	−
P. viridiflava Pvalb8	+
X. axonopodis pv. *citri* Xcc991330	+
X. axonopodis pv. *vesicatoria* XcvBv54a	+

−, No growth inhibition of indicator strain; +, growth inhibition of indicator strain.

Cultures of *P. fluorescens* SF39a were either treated with mitomycin C or untreated (control). Then, bacteriocin crude extracts were obtained from cell-free culture supernatants by precipitation with ammonium sulfate or by ultrafiltration. Bacteriocin production was increased about 100-fold when mitomycin C was added to cultures independently of the extraction method used (10^4 AU/ml for treated culture and 10^2 AU/ml for control) (supplemental Fig. S1). Therefore, this bacteriocin was induced by a DNA damaging agent, suggesting that the SOS response was involved in its expression. Moreover, no antimicrobial activity was obtained in the solution after passage through Amicon 100K. In contrast, bacteriocin activity was retained in the membrane. This suggests that the molecular mass of bacteriocin is greater than 100 kDa.

Crude extracts concentrated by ultrafiltration were analyzed by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions. This analysis resulted in a complex band pattern (supplemental Fig. S1). Then, PAGE under non-denaturing conditions was run in duplicate in the same gel. After electrophoresis, the gel was cut in the half; one of these parts was stained with Coomassie Brilliant Blue R-250 to visualize proteins and the other was used for the antimicrobial activity assay. For this, the gel was overlaid with soft agar seeded with indicator strain CTR212 and incubated overnight at 30 °C. No band with activity against *P. fluorescens* CTR212 could be detected, so there was no correlation between antimicrobial activity observed in the crude extract and the polyacrylamide gel.

In addition, the sensitivity of the bacteriocin crude extract to proteinase K and heat treatment was analyzed. No loss of activity was detected when the samples were incubated at 37 °C for 1 or 3 h. However, the activity was destroyed by heating for 10 min at 50 °C. Moreover, the antibacterial activity was completely lost after proteinase K treatment, indicating its proteinaceous nature (supplemental Fig. S1).

3.2. Bacteriocin prediction

Recently, the genome of *P. fluorescens* SF39a was sequenced [36]. In silico analysis of the draft genome of this strain revealed the presence of genes (*hol* and *lys*) encoding the lytic system, the repressor gene (*prtR*), and structural genes belonging to the tailocin cluster. In *P. fluorescens*, the genes of phage tail-like pyocins are integrated between *mutS* and the *cinA–recA–recX* genes [5,10,11]. These genes were also identified in the genome of strain SF39a (contig 17) and the same genetic organization was observed.

In addition, a gene encoding putative pyocin S with DNase activity was predicted by BAGEL3 in contig 28 (locus tag NX10_RS24625) from the draft genome of *P. fluorescens* SF39a (Fig. 1A). This gene was named *pys*, according to the name of its ortholog in *P. aeruginosa*. The deduced pyocin S protein from *P. fluorescens* SF39a has 635 amino acids and a molecular mass of 68 321.3 Da. Two Pfam domains were found: the S-type pyocin domain (residues 366 to 501) and the DNase/tRNase domain of colicin-like bacteriocin

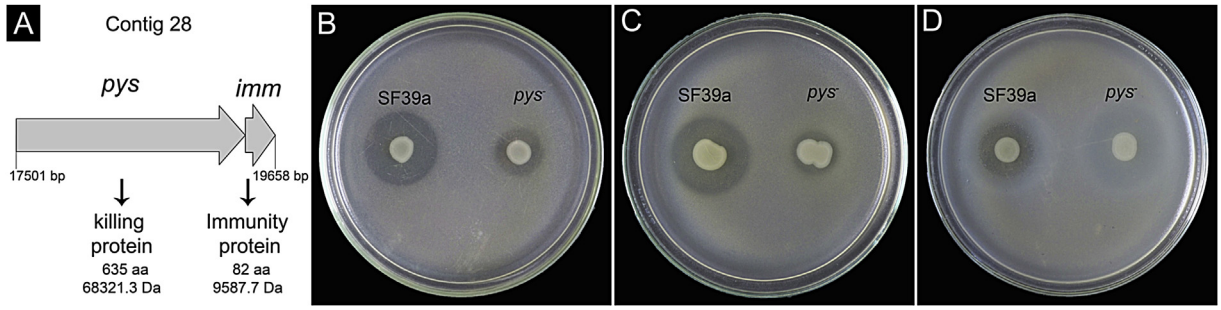

Fig. 1. Schematic representation of the genes encoding a putative pyocin S and immunity protein in strain SF39a (A). Antimicrobial activity of *P. fluorescens* SF39a and *P. fluorescens* SF39a-pys against *P. fluorescens* CTR212 (B), *P. fluorescens* WCS365 (C) and *P. putida* KT2440 (D). SF39a: Wild-type; *pys*⁻: *P. fluorescens* SF39a-pys.

(residues 504 to 634) at the C-terminal. Moreover, this protein showed homology with S-type pyocins from *Pseudomonas* sp. DSM 29165 (64% similarity), *P. fluorescens* Pf0-1(63% similarity), *Pseudomonas syringae* pv. *spinaceae* (60% similarity), *Pseudomonas savastanoi* pv. *glycinea* (56% similarity) and other *Pseudomonas* spp. Furthermore, a putative gene involved in immune function was also detected downstream from the S-type pyocin gene in strain SF39a (Fig. 1A). The predicted immunity protein has 82 amino acids and a molecular mass of 9587.7 Da and it showed strong similarity to bacteriocin immunity proteins from several *Pseudomonas* strains. A colicin immunity protein/pyocin immunity protein domain (residues 1–80) was identified in this putative immunity protein.

Based on observed results, we decided to construct a *pys* mutant derivative of *P. fluorescens* SF39a by replacement of a *pys* fragment with a Km resistance cassette as described in Materials and methods. One double recombinant clone was selected and named *P. fluorescens* SF39a-pys. The double crossover event was confirmed by PCR and Southern blotting in this mutant. Bacteriocin production by the wild-type and *pys* mutant was analyzed on LB plaques against several reference strains. Antimicrobial activity was not completely lost in mutant SF39a-pys. For most of the tested indicator strains, it was possible to observe an inhibition halo around the mutant, although smaller than that produced by the wild-type (Fig. 1B and C). In the case of indicator strain *P. putida* KT2440, strain SF39a produced two inhibition halos, one of them stronger than the other. Mutant SF39a-pys showed only a weak halo against strain KT2440 (Fig. 1D).

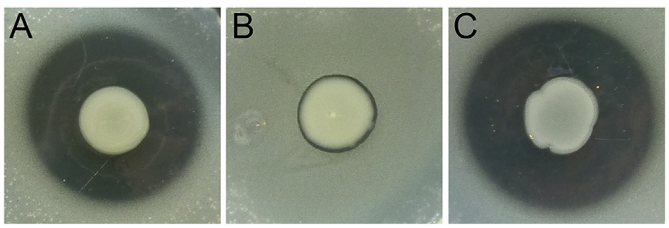

Fig. 2. Antimicrobial activity of *P. fluorescens* SF39a (A), *P. fluorescens* SF39a-451 (B) and *P. fluorescens* SF39a-451pPtsP (C) against *P. fluorescens* CTR212.

3.3. Identification of genes involved in bacteriocin regulation

Mini-Tn*5* Km1 transposon mutagenesis was carried out in *P. fluorescens* SF39a to identify genes involved in bacteriocin regulation. About 1200 mutants were obtained and analyzed by antimicrobial activity against *P. fluorescens* CTR212. None of these clones completely lost their ability to secrete antibacterial compounds. However, one of these mutants (called *P. fluorescens* SF39a-451) produced an inhibition halo smaller than that of the wild-type (Fig. 2). In addition, the mutant grew at the same rate as the wild-type in minimal medium, indicating that the observed decrease in bacteriocin production was not a result of deficiency in growth.

Southern blot was carried out using a Km cassette as the probe. No signal was obtained from chromosomal DNA of the wild-type. In contrast, a single *PstI* fragment of about 6 kb was detected in the mutant, indicating that only a single copy of transposon DNA had been inserted into the chromosome (data not shown).

In mutant *P. fluorescens* SF39a-451, the DNA region interrupted by mini-Tn*5* Km1 was cloned into vector pBluescript SK. Km-resistant transformants were selected and one clone containing recombinant plasmid pBSK451 was sequenced. DNA sequence analysis revealed that the transposon was inserted into an ORF homolog to the *ptsP* gene from several strains of *Pseudomonas*.

The *ptsP* gene encodes a phosphoenolpyruvate-protein phosphotransferase EI^{Ntr}. This protein is a component of the nitrogen-related phosphotransferase system (PTS^{Ntr}), a global regulatory system present in many Gram-negative bacteria. The *ptsP* gene from strain SF39a encodes a predicted protein of 759 amino acids with a molecular mass of 83 387.4 Da, which presents high similarity to its ortholog from *Pseudomonas moraviensis* R28-S (99% identity), *P. fluorescens* Pf0-1 (97% identity), *P. fluorescens* Q8r1-96 (95% identity), *P. fluorescens* F113 (95% identity), *P. fluorescens* Pf-5 (93% identity), *P. protegens* CHA0 (93% identity), *P. savastanoi* pv. *savastanoi* NCPPB 3335 (91% identity) and *P. syringae* pv. *glycinea* B076 (91% identity). The analysis of domain searches in Pfam revealed the presence of four domains: N-terminal (residues 178–302), mobile

(residues 318–399) and TIM barrel (residues 425–715) domains of PEP-utilizing enzymes, and a GAF domain (residues 17–154). Moreover, a conserved PROSITE phosphoenolpyruvate (PEP)-utilizing an enzyme signature (residues 620–638) was found.

The *ptsP* sequence from strain SF39a was deposited in the GenBank nucleotide sequence database under accession number KM948592.

In order to confirm that the altered phenotype in mutant SF39a-451 is due to insertional inactivation of the *ptsP* gene, we performed a heterologous complementation study. A copy of the full-length *ptsP* gene from *P. fluorescens* Pf0-1 was cloned into the pBBR1MCS-5 vector and mobilized into the mutant SF39a-451. Bacteriocin production against *P. fluorescens* CRT212 was restored in this mutant, confirming that *ptsP* gene is involved in regulation of the bacteriocin (Fig. 2C).

3.4. Phenotypic effect

PTS^{Ntr} regulates diverse processes implicated in the metabolism of nitrogen and carbon, and it is essential for virulence in some bacteria [37]. Therefore, relevant traits for bacterial fitness in the rhizosphere were analyzed in mutant SF39a-451.

The formation of multicellular, matrix-encased communities associated with solid surfaces (biofilms) is an intrinsic feature of the bacterial life cycle and a common strategy for microbial persistence in a variety of environments [32]. Biofilm formation ability on microtiter plates was tested in mutant SF39a-451 and compared with the wild-type strain. The amounts of attached cells were quantified after solubilization of crystal violet stain and measurement at OD 570 nm. The results showed that biofilm formation was defective in the mutant (Fig. 3A).

P. fluorescens SF39a produces some compounds involved in the biocontrol and/or promotion of plant growth, for example, the siderophore pyoverdine and lytic enzyme protease. In addition, this strain is also capable of solubilizing phosphate. In this study, production of pyoverdine was analyzed on King's B medium at 24, 48 and 72 h. Production was similar in mutant SF39a-451 and in the wild-type at 24 h. In contrast, inactivation of the *ptsP* gene resulted in overproduction of pyoverdine at 48 and 72 h (Fig. 3B). Moreover, protease activity was tested on a milk agar plate. Fig. 3C shows that protease production was abolished in mutant SF39a-451. Finally, the assay on a medium containing insoluble phosphate demonstrated that both strains, the wild-type and mutant SF39a-451, were able to solubilize phosphate (data not shown). Therefore, system PTS^{Ntr} was not involved in regulation of this trait.

The motility of mutant SF39a-451 was also analyzed, and swimming speed was found to be similar to the wild-type strain (Fig. 4A and D). As expected, no difference in flagellation was observed in electron microscopy (Fig. 4B, C, E and F). These results indicate the presence of functional flagella in mutant SF39a-451. In contrast, this mutant displayed a hypermobile phenotype on LB plates solidified with 0.4% agar compared with the wild-type strain (Fig. 5A). Media that are solidified with agar concentrations above 0.3% exclude swimming motility, and force the bacteria to move over the surface, e.g. swarming motility [38]. Swarming motility is a fast multicellular movement of bacteria across a surface, powered by rotating flagella. In most cases, swarming motility is considered to involve cell differentiation into a hyperflagellated and elongated form [38,39]. Therefore, we decided to clarify whether surface contact induced differentiation in mutant SF39a-451. For this reason, bacteria from three different sites of the moving colony (inoculation point, middle of expanding colony or edge of expanding colony) were analyzed using electron microscopy. No difference in the number of flagella and cell elongation was observed among cells isolated from these sites of the colony (Fig. 5B–D). Therefore, we cannot affirm that the surface motility of the mutant on semisolid agar (0.4%) is swarming motility. However, it could be consistent with sliding motility, "a passive form of surface spreading that involves production of surfactants to reduce surface tension, enabling the colony to spread away from the origin, driven by the outward pressure of cell growth". Sliding is easily mistaken for swarming motility, but unlike this, sliding motility does not require flagella [38,40]. We observed extracellular material around the bacteria isolated from 0.4% agar plates (Fig. 5C–E), whereas such material was absent in bacteria grown in liquid medium (Fig. 4E and F). Extracellular material increased in cells isolated from the edge of the expanding colony (Fig. 5D and E) with respect to those taken from the middle of the expanding colony (Fig. 5C) and the inoculation site (Fig. 5B). This material could act as a surfactant and, in doing so, facilitate spreading over surfaces.

Complementation of mutant SF39a-451 with the *ptsP* gene fully restored biofilm formation and the normal surface motility phenotype. In contrast, protease activity and pyoverdine production could be only partially complemented by plasmid pPtsP in this mutant (Figs. 3 and 5).

3.5. Rhizosphere colonization and competition

For this assay, we first obtained spontaneous rifampicin-resistant mutants (*P. fluorescens* SF39a-R and *P. fluorescens* SF39a-451-R derived from strains SF39a and SF39a-451, respectively). A series of experiments were carried out to determine whether spontaneous rifampicin-resistant mutants were able to grow in the same manner as their parent strains. The number of viable cells of parent strains and spontaneous mutants was similar in glucose minimal medium. Thus, these strains are as competitive in growth as their parent strains, so they could be used in the experiment in wheat rhizosphere.

Wheat rhizosphere colonization and competition assays between strains SF39a and SF39a-451 were performed in non-sterile soil under a greenhouse. For this, wheat seedlings were inoculated with SF39a-R or SF39a-451-R either in a single or mixed inoculation. Three weeks after inoculation, the number

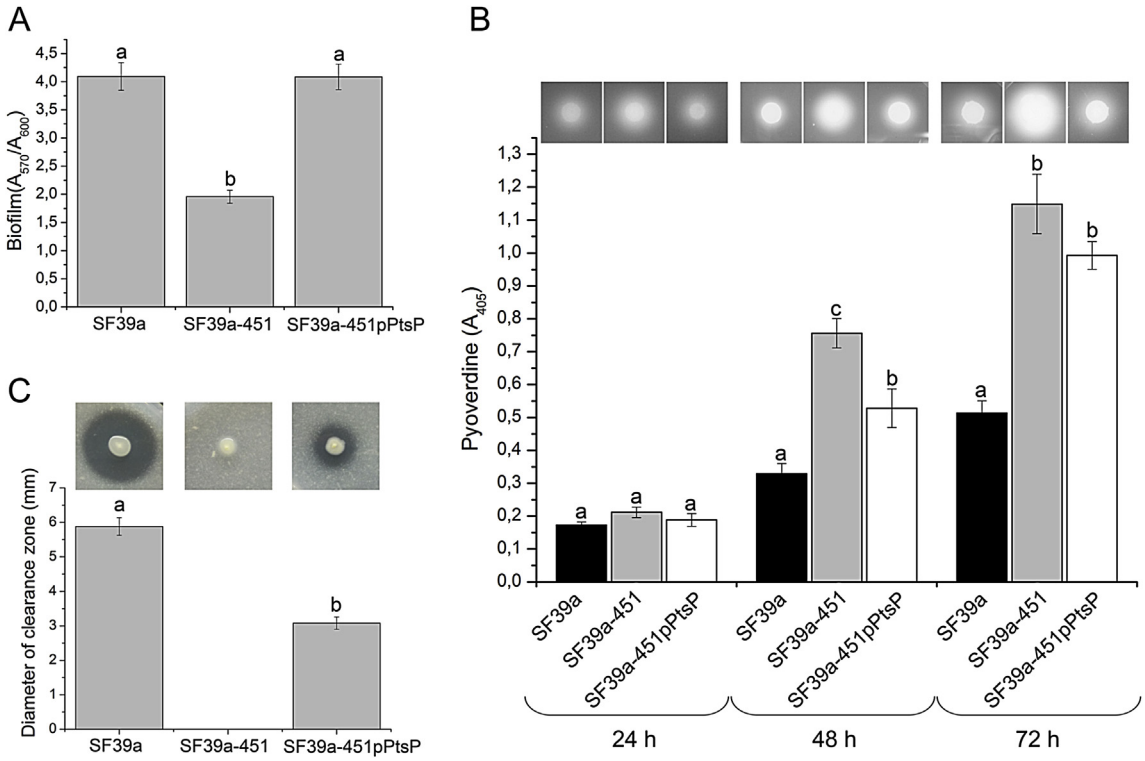

Fig. 3. Phenotypic effects of the *ptsP* mutation in *P. fluorescens* SF39a. Quantitative biofilm assay in 96-well microtiter plates. Biofilm formation was quantified at 570 nm after staining with crystal violet and normalized to cell density measured at 600 nm. The results are means ± SE of ten independent experiments with ten replicates in each (A). Pyoverdine production was quantified spectrophotometrically (OD_{405}) from culture supernatant at 24, 48 and 72 h. Values represent means ± SE of three independent experiments with three replicates per experiment. Images of King's B plates under UV light are shown above the graph (B). Protease production was determined by measuring the casein degradation zone after 48 h of growth. Data are the means ± SE of five independent milk agar plates. Petri dishes exhibiting the degradation zones of the casein are shown above the graph (C). SF39a: wild-type; SF39a-451: *P. fluorescens* SF39a-451; SF39a-451pPtsP: *P. fluorescens* SF39a-451pPtsP. Different letters indicate statistically significant difference ($P \leq 0.05$), comparing wild-type, SF39a-451 and SF39a-451pPtsP each time.

of viable SF39a-R cells in the wheat rhizosphere was 1.25×10^7 cfu/g soil, or 7.64×10^6 cfu/g soil in single or mixed inoculation, respectively. The population density of mutant SF39a-451 was significantly lower than the wild-type in both treatments (Fig. 6), suggesting that inactivation of the *ptsP* gene impaired bacterial competitiveness and fitness in the rhizosphere.

4. Discussion

In this work, we studied bacteriocin production in strain *P. fluorescens* SF39a. Antimicrobial activity against several *Pseudomonas* and *Xanthomonas* strains was detected by the agar spot test. Pyocins S are low-molecular-mass bacteriocins (compared to phage-like pyocin) that diffuse further from the colony than high-molecular weight bacteriocins. In addition, pyocins S have also been reported to be labile to heat and proteinase K [5]. All these properties were also observed with the bacteriocin secreted by strain SF39a.

In *P. aeruginosa*, pyocins S are secreted as binary protein complexes consisting of a large protein that harbors the killing function and a smaller immunity protein that remains tightly bound to the cytotoxic domain of the former [5]. Genes encoding putative pyocin S with DNase activity and an immunity protein were predicted by BAGEL3 in the genome of the native strain, *P. fluorescens* SF39a. The presence of genes encoding pyocins S had been found in several plant-associated *Pseudomonas* strains, but functional characterization is lacking [5]. Therefore, we constructed a *pys* null mutant derivative of *P. fluorescens* SF39a. Bacteriocin production was impaired in this mutant; however, it was not completely abolished. We hypothesize that strain SF39a synthesizes several type of low-molecular-mass bacteriocins, but only one could be detected in silico. It is known that more than one bacteriocin can be produced by one bacterium. Loper et al. [41] analyzed ten genomes of the *P. fluorescens* group and, in each strain, they were able to predict from two to seven bacteriocins. The genomes included genes for many of the structurally diverse bacteriocins known to be produced by *Pseudomonas* spp. These researchers also identified putative novel bacteriocins in the predicted proteomes of *P. fluorescens*.

Knowledge of bacteriocin regulation produced by rhizospheric strains is relevant due to their potential application as biocontrol agents or biofertilizers. When searching for genes involved in bacteriocin regulation from *P. fluorescens* SF39a, we obtained a mini-Tn*5* Km1mutant (strain SF39a-451) with reduced bacteriocin production. The altered phenotype in this mutant was caused by inactivation of the *ptsP* gene, which

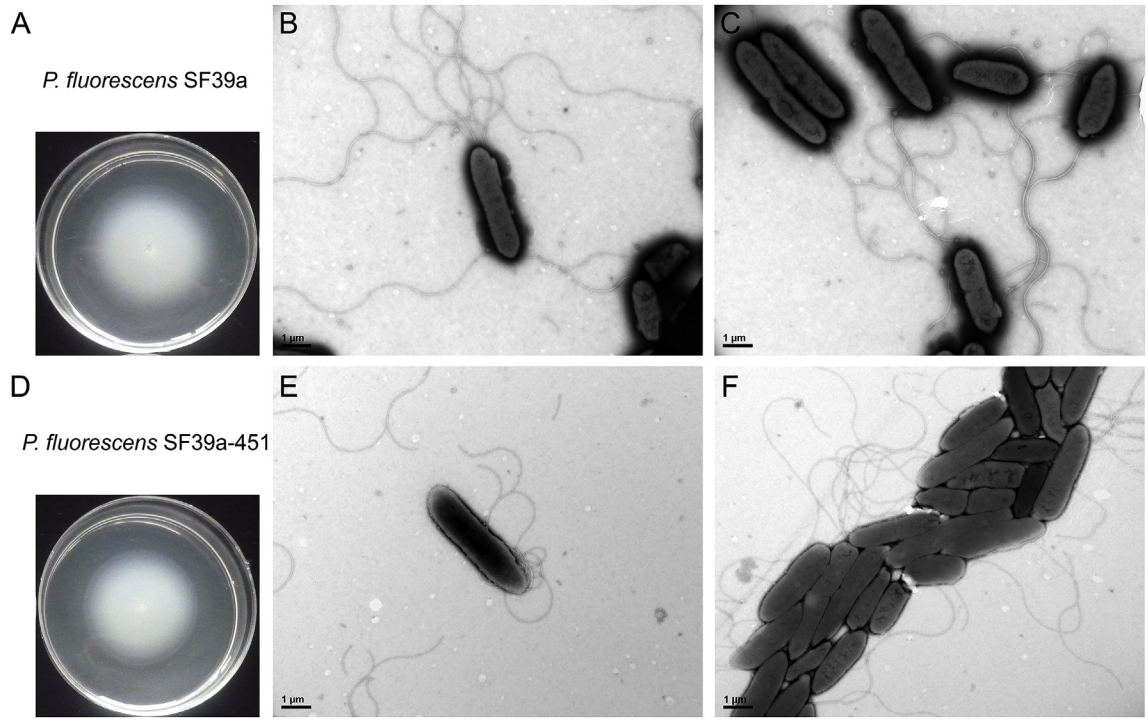

Fig. 4. Swimming motility and observation of bacterial cells by transmission electron microscopy. Swimming motility assay in 0.3% LB agar plates for *P. fluorescens* SF39a (A) and *P. fluorescens* SF39a-451 (D). Images of *P. fluorescens* SF39a (B–C) or *P. fluorescens* SF39a-451 (E–F) by electron microscopy. The bar indicates 1 µm.

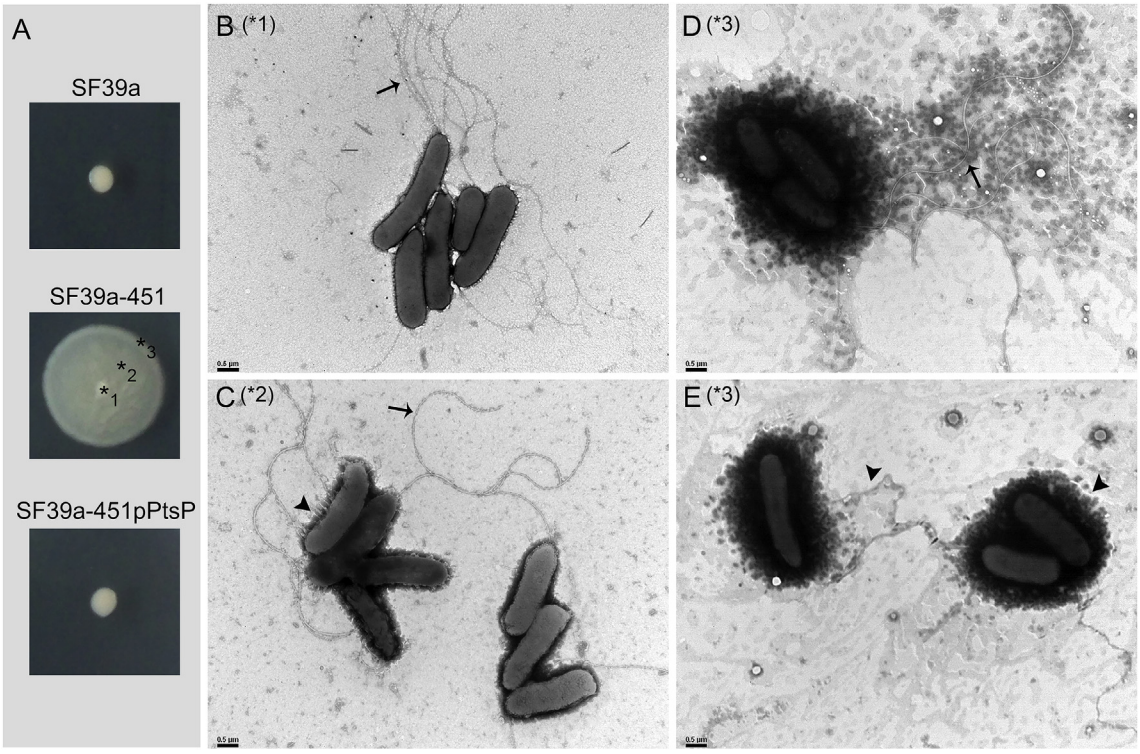

Fig. 5. Surface motility and observation of bacterial cells by transmission electron microscopy. Surface motility phenotypes on 0.4% LB agar plates (A). SF39a: wild-type, SF39a-451: *P. fluorescens* SF39a-451, SF39a-451pPtsP: *P. fluorescens* SF39a-451pPtsP. The star symbols (*) indicate sites from which bacteria were taken for electron microscopy studies. *1: inoculation point; *2: middle of expanding colony; *3: edge of expanding colony. Electron microscopy of *P. fluorescens* SF39a-451 cells taken from: the inoculation point (B), the middle of expanding colony (C) or the edge of expanding colony (D–E). The arrows indicate flagella and the arrowheads show extracellular material. The bar indicates 0.5 µm.

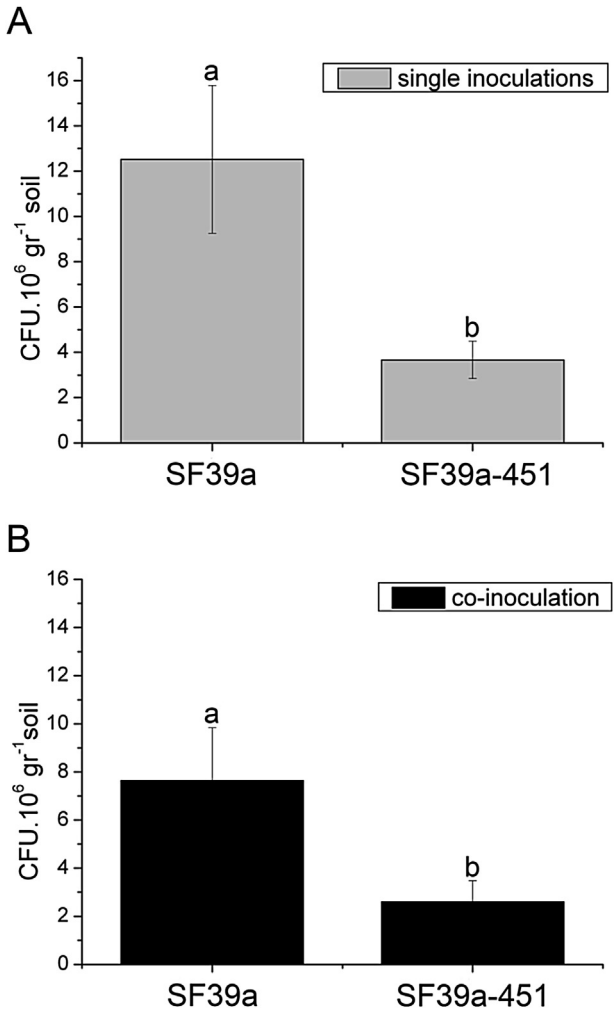

Fig. 6. Wheat rhizosphere colonization by *P. fluorescens* SF39a and *P. fluorescens* SF39a-451. Each strain was introduced into the soil at a final density of 2×10^6 cfu/g soil in single inoculations (A) or 1×10^6 cfu/g soil in mixed inoculations (B). SF39a: wild-type; SF39a-451: *P. fluorescens* SF39a-451. The bars indicate means ± SE of three independent experiments with five replicates per experiment. Different letters indicate significant difference ($P \leq 0.05$).

encodes enzyme EI^{Ntr}. This protein forms part of PTS^{Ntr}. Two general types of PTSs are known: sugar PTS, which is responsible for transport and phosphorylation of a carbohydrate into the cell, and PTS^{Ntr}, which does not transport carbohydrates, but exerts regulatory functions [37]. The fact that a deficiency in the *ptsP* gene causes a reduction in bacteriocin production suggests that the PTS^{Ntr} system is involved in controlling expression of bacteriocins in native strain SF39a. Indeed, De Los Santos et al. [9] inferred that various environmental stress response pathways control expression of a lectin-like bacteriocin produced by *P. putida* BW11M. Those researchers found random mutant derivatives of strain BW11M that showed an altered bacteriocin production phenotype in agar spot assay. Various genes involved in environmental stress response pathways, such as *recA*, *spoT*, *opr*L, were responsible for these altered phenotypes.

On the other hand, we observed that the bacteriocin activity of strain SF39a increases 100 times when the culture of the producer bacterium is induced with mitomycin C, indicating that the SOS response is involved in its expression. The pyocins of *P. aeruginosa* and strain *P. fluorescens* SF4c are known to be activated as part of the response to DNA damage. The presence of a mutagenic agent like mitomycin C or UV light triggers the SOS response, and subsequently, the expression of pyocins genes is activated [5,10]. The SOS system has been well studied in expression of bacteriocins; but to our knowledge, involvement of the PTS^{Ntr} system in bacteriocin regulation has not been reported in none-bacterial genera.

It was discovered that PTS^{Ntr} regulates diverse processes in bacteria; for example, pyocyanin production in *P. aeruginosa* [42] or expression of the alkylresorcinol biosynthetic operon in *Azotobacter vinelandii* [43]. Because PTS^{Ntr} is a global regulatory system, we also studied whether other relevant features, in addition to bacteriocin production, are affected in strain SF39a-451. This mutant showed a decrease in protease activity and biofilm formation, but an increase in siderophore production and surface motility. Similar results were found by Mavrodi et al. [44] in a *ptsP* mutant of *P. fluorescens* Q8r1-96.

Competitiveness is an important feature for the PGPR because they must be able to colonize the root and multiply in microhabitats associated with the root surface, in competition with other microbiota, at least for the time needed to express their plant promotion/protection activities [45]. Bacteriocin, protease and siderophore production, biofilm formation and motility are relevant traits for establishment of the bacteria in the rhizosphere and bacterial performance as PGPR. Therefore, we sought to evaluate the ability of mutant SF39a-451 to colonize the wheat rhizosphere in natural soil. Interestingly, the *ptsP* mutant was less competitive than the wild-type strain and was unable to colonize the wheat rhizosphere as successfully as the wild-type strain.

In conclusion, our results suggest that PTS^{Ntr} controls a plethora of processes that impact the successful performance of the bacterium in the rhizosphere. In addition, to our knowledge, this is the first report to describe the first functionally characterized pyocin S in *P. fluorescens*.

Conflict of interest

There is no conflict of interest for any of the authors.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2015.12.003.

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