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Characterization of a phage-like pyocin from the plant growth-promoting rhizobacterium *Pseudomonas fluorescens* SF4c

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R-type and F-type pyocins are high-molecular-mass bacteriocins produced by *Pseudomonas aeruginosa* that resemble bacteriophage tails. They contain no head structures and no DNA, and are used as defence systems. In this report, we show that *Pseudomonas fluorescens* SF4c, a strain isolated from the wheat rhizosphere, produces a high-molecular-mass bacteriocin which inhibits the growth of closely related bacteria. A mutant deficient in production of this antimicrobial compound was obtained by transposon mutagenesis. Sequence analysis revealed that the transposon had disrupted a gene that we have named *ptm*, since it is homologous to that encoding phage tape-measure protein in *P. fluorescens* Pf0-1, a gene belonging to a prophage similar to phage-like pyocin from *P. aeruginosa* PAO1. In addition, we have identified genes from the SF4c pyocin cluster that encode a lytic system and regulatory genes. We constructed a non-polar *ptm* mutant of *P. fluorescens* SF4c. Heterologous complementation of this mutation restored the production of bacteriocin. Real-time PCR was used to analyse the expression of pyocin under different stress conditions. Bacteriocin was upregulated by mitomycin C, UV light and hydrogen peroxide, and was downregulated by saline stress. This report constitutes, to our knowledge, the first genetic characterization of a phage tail-like bacteriocin in a rhizosphere *Pseudomonas* strain.

INTRODUCTION

The rhizosphere is an environment populated by numerous microbial species. In this niche, bacteria are in constant competition for nutrients and space, and their ability to survive under varying and competitive conditions determines their ecological fitness. Competition strategies include the production of antimicrobial compounds such as antibiotics, bacteriolytic enzymes and bacteriocins (Parret *et al.*, 2003; Holtsmark *et al.*, 2008). Bacteriocins are bacterial proteins or peptides that inhibit the growth of bacteria closely related to the producer strain, while causing little or no harm to the producing cells, thanks to a specific immunity mechanism (Hancock & Chapple, 1999). To date, the pyocins produced by *Pseudomonas aeruginosa* are among the best-known bacteriocins in Gram-negative bacteria. Three different types of pyocins – termed R-, F- and S-type – have been identified, and differ in their morphology and mode of killing. R- and F-type pyocins (high-molecular-mass) resemble bacteriophage tails (the R-type pyocins are non-flexible and contractile, and F-type pyocins are flexible but non-contractile), while the S-type pyocins are smaller proteins, soluble and protease- and heat-sensitive. The R- and F-type pyocins have an ancestral origin in common with the P2 phage and the λ phage, respectively. The gene organization of the R2 and F2 pyocins suggests that they are phage tails that have evolved to become bacteriocins (Nakayama *et al.*, 2000). Pyocin genes are located on the chromosome of *P. aeruginosa*, and their expression can be increased by treatments that cause DNA damage, such as UV irradiation or mitomycin C (Michel-Briand & Baysse, 2002).

In the case of plant-associated pseudomonads, bacteriocin research has been focused on plant-pathogenic strains of

Abbreviation: AU, arbitrary units.

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A supplementary figure, showing the alignment of the predicted amino acid sequence of the *ptm* gene product from *P. fluorescens* SF4c with the PTM protein from *P. fluorescens* Pf0-1, is available with the online version of this paper.

Pseudomonas syringae (Lavermicocca *et al.*, 2002), and little is known about the bacteriocins produced by beneficial *Pseudomonas* strains isolated from the rhizosphere. Parret *et al.* (2003) identified a new type of bacteriocin of 30 kDa (LlpA) secreted by *Pseudomonas* sp. BW11M, a strain isolated from the banana rhizosphere. The bacteriocin LlpA (called putidacin) has similarity to a family of mannose-binding lectins, predominantly found in monocotyledonous plants. Putidacin is active against the rhizosphere strain *Pseudomonas putida* GR12-2R3 as well as against certain phytopathogenic pseudomonads.

In addition, two functional LlpA homologues ($LlpA1_{Pf\text{-}5}$ and $LlpA2_{Pf\text{-}5}$) have been identified in the biocontrol strain *Pseudomonas fluorescens* Pf-5. $LlpA1_{Pf\text{-}5}$ and $LlpA2_{Pf\text{-}5}$ are able to inhibit *P. fluorescens* strains, as well as the related mushroom pathogen *Pseudomonas tolaasii* (Parret *et al.*, 2005).

Although no pyocins have as yet been functionally characterized from *Pseudomonas* species other than *P. aeruginosa*, *in-silico* analysis of genome-sequence data has revealed the presence of a variety of pyocin-like operons in *P. fluorescens*, *P. putida* and *P. syringae* (Parret & De Mot, 2002). Moreover, genomic DNA of different *P. fluorescens* strains has been shown to hybridize with F- and R-pyocin-specific probes (Mavrodi *et al.*, 2009).

P. fluorescens SF4c is a native strain isolated from the rhizosphere of wheat (in Argentina). This strain promotes wheat and tomato growth under greenhouse conditions, and can establish, compete satisfactorily with the indigenous microflora, and persist with significant viable cell numbers in the wheat rhizosphere (Fischer *et al.*, 2007, 2010).

In this work, we report that *P. fluorescens* SF4c produces a high-molecular-mass bacteriocin. In an attempt to determine the genes involved in the synthesis of this bacteriocin, mutants deficient in production of this antimicrobial compound were obtained. We additionally demonstrate that pyocin secreted by strain SF4c is upregulated by mitomycin C, UV light and hydrogen peroxide, and downregulated by saline stress.

METHODS

Strains, plasmids and media. The bacterial strains and plasmids used in this study are listed in Table 1. Cultures were grown in Luria–Bertani (LB) medium at 30 °C for *Pseudomonas* or at 37 °C for *Escherichia coli*. Where indicated, the media were supplemented with antibiotics at the following concentrations (μg ml^{-1}): ampicillin (Amp), 100; chloramphenicol (Cm), 30; kanamycin (Km), 25; streptomycin (Sm), 100; tetracycline (Tet), 10.

Bacteriocin assays. Samples (10 μl) of a stationary phase culture of *P. fluorescens* SF4c were spotted onto LB agar and incubated overnight at 30 °C. To prevent further cell growth, the plates were then exposed to chloroform vapour (20 min) and overlaid with 3 ml soft agar (0.7 %, w/v) seeded with 50 μl of a bacterial suspension of the indicator strains. The plates were incubated at 30 °C and production of bacteriocin was determined by growth inhibition around the colonies (Parret *et al.*, 2003). Reference *Pseudomonas* strains were used as indicator strains (Table 1).

Purification of bacteriocin. Saturated cultures of *P. fluorescens* SF4c were diluted 1 : 100 in LB medium and shaken at 150 r.p.m. and 30 °C to OD_{600} 0.3. Mitomycin C was then added to a final concentration of 3 μg ml^{-1} and the incubation continued in the dark until lysis occurred. DNase I (2 U ml^{-1}) and RNase (10 μg ml^{-1}) were added to reduce the viscosity, followed by 30 min incubation at 37 °C. Debris was removed by centrifugation at 17 000 ***g*** for 1 h. Supernatants were 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 5 ml TN50 buffer [50 mM NaCl, 10 mM Tris/HCl (pH 7.5)]. Pyocin particles were then sedimented at 58 000 ***g*** for 1 h at 4 °C and resuspended in 1.5 ml TN50 buffer (Scholl & Martin, 2008).

Semiquantitative bacteriocin assays were performed by a spot method. Samples were serially diluted in TN50 buffer and 5 μl aliquots spotted onto lawns of *P. fluorescens* CTR212 (indicator bacterium). Plates were incubated overnight at 30 °C. Pyocin titres were expressed in arbitrary units (AU) ml^{-1}, corresponding to the reciprocal of the highest dilution factor that showed a clear inhibition of the indicator strain (Williams *et al.*, 2008).

Sensitivity of bacteriocin to proteolytic enzymes and heat. Aliquots of bacteriocin were treated with trypsin (1 mg ml^{-1}) or proteinase K (10 mg ml^{-1}) at 37 °C for 1 or 24 h. To analyse thermal stability, samples of bacteriocin were heated at 40 °C for 30 min or at 75 °C for 15 min. After treatment, the samples were tested for antimicrobial activity against *P. fluorescens* CTR212 (Lisboa *et al.*, 2006).

Effects of bacteriocin on the viability of *P. fluorescens* CTR212. Overnight cultures of strain CTR212 were diluted in LB broth, grown to OD_{600} 0.5 and divided into two aliquots. The bacteriocin was added to a final concentration of 500 AU ml^{-1} (or buffer alone to the control), and samples were taken after 30, 60, 90, 180 and 360 min. The c.f.u. values were determined on LB plates.

Transposon mutagenesis. Conjugation between recipient *P. fluorescens* SF4c and donor *E. coli* strain S17-1 harbouring the transposon-containing plasmid pSUP102 : : Tn*5*-B20 (Simon *et al.*, 1989) was performed. Transconjugants were selected on medium containing Km and screened for lack of ability to inhibit the growth of *P. fluorescens* CTR212 on plates.

Molecular biology techniques. Standard techniques were used for plasmid and DNA isolation, restriction enzyme digestion, ligation reactions and agarose gel electrophoresis (Sambrook *et al.*, 1989). For Southern analysis, a DIG DNA Labelling and Detection kit (Roche) was used.

Cloning and sequence analysis. Chromosomal DNA of the mutant strain 634 was digested with *Eco*RI (which has a unique recognition site in Tn*5*-B20) and ligated to pBluescript SK previously digested with *Eco*RI. Ligation mixes were electroporated into *E. coli* strain DH5α and plated on LB medium supplemented with Amp and Km. The Amp^R Km^R colonies were isolated and one clone containing the recombinant plasmid (called pSF16) was used for sequencing (Macrogen) through the use of the primer Tn*5* Int (Rossbach *et al.*, 2000). The nucleotide sequence obtained was compared with the GenBank database by means of the BLAST algorithm (Altschul *et al.*, 1997).

Sequencing of *ptm*. Primers PTM-F and PTM-R (Table 2) were designed from the *ptm* gene encoding phage tape-measure protein of

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Origin or relevant characteristics	Reference or source
Strains		
P. fluorescens SF4c	Isolate from wheat rhizosphere (Argentina)	Fischer *et al.* (2007)
P. fluorescens SF39a	Isolate from wheat rhizosphere (Argentina)	Fischer *et al.* (2007)
P. fluorescens WCS365	Isolate from potato rhizosphere (Netherlands)	Geels & Schippers (1983)
P. fluorescens CHA0	Isolate from tobacco rhizosphere (Switzerland)	Stutz *et al.* (1986)
P. fluorescens CTR212	Soil isolate (France)	Latour *et al.* (1996)
P. fluorescens Pf0-1	Soil isolate (USA)	Compeau *et al.* (1988)
P. putida SF10b	Isolate from wheat rhizosphere (Argentina)	Fischer *et al.* (2007)
P. putida KT2440	Plasmid-free derivative of natural isolate mt-2	Franklin *et al.* (1981)
P. syringae pv. *syringae* 61	Isolate from wheat (USA)	Huang *et al.* (1988)
P. corrugata NCPPB 2445	Isolate from tomato, pathogen (UK)	Scarlett *et al.* (1978)
P. corrugata PC5	Isolate from tomato, pathogen (Argentina)	Alippi *et al.* (2003)
P. viridiflava Pvalb8	Isolate from basil, pathogen (Argentina)	Alippi *et al.* (2003)
P. fluorescens 634	*ptm*::Tn*5*-B20 mutant of *P. fluorescens* SF4c	This work
P. fluorescens ptm50	Non-polar *ptm*::Km mutant of *P. fluorescens* SF4c	This work
E. coli DH5α	*endA1 recA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*lacZYA–argF*)*U169* ϕ80d *lacZ* ΔM15	Hanahan (1983)
E. coli S17-1	*recA*, RP4 into chromosome	Simon *et al.* (1983)
Plasmids		
pSUP102-Tn*5*-B20	Suicide plasmid carrying the Tn*5*-*lacZ* transposon Tn*5*-B20	Simon *et al.* (1989)
pBluescript SK	Cloning vector, Amp^R	Stratagene
pFAJ1709	Expression vector, Tet^R	Dombrecht *et al.* (2001)
pSF16	6 kb *Eco*RI fragment containing flanking DNA, IS*50* and Km^R gene from *P. fluorescens* 634 cloned into pBluescript SK, Km^R Amp^R	This work
pPTM28	*ptm* from *P. fluorescens* Pf0-1 cloned into pFAJ1709, Tet^R	This work
pUC18Not	Cloning vector, pUC18 derivative with polylinker flanked by *Not*I sites	Herrero *et al.* (1990)
p34S-Km3	Plasmid carrying a Km^R cassette flanked by duplicated restriction sites	Dennis & Zylstra (1998)
pKNG101	Suicide vector for gene replacement; Sm^R; *sacB*	Kaniga *et al.* (1991)
pRK600	Cm^R; ColE1 replicon with RK2 transfer region, helper plasmid	Finan *et al.* (1986)
pPTM1	719 bp *Bam*HI/*Acc*65I fragment of *ptm* cloned into pUC18Not	This work
pPTM12	633 bp *Acc*65I/*Eco*RI fragment of *ptm* cloned into pPTM1	This work
pPTM12Km	*Acc*65I fragment containing Km^R cassette from p34S-Km3 cloned into pPTM12	This work
pKNGPTM	*Not*I fragment of pPTM12Km cloning into pKNG101	This work

P. fluorescens Pf0-1, and used for PCR amplification of this gene in *P. fluorescens* SF4c. The amplification was carried out using *Pfx* enzyme (Invitrogen). The PCR product was purified with the QIAquick PCR Purification kit (Qiagen) and sequenced by Macrogen through the use of a primer-walking strategy.

PCR and sequencing of genes in the bacteriocin cluster and flanking genes. Primers were designed from conserved regions of holin (*hol*), endolysin (*lys*), a transcriptional regulator (*prtR*), DNA mismatch repair protein MutS (*mutS*), recombinase A (*recA*) and RecA regulator RecX (*recX*) genes from *P. fluorescens* Pf0-1 and/or Pf-5. Primer sequences, annealing temperatures and detected genes are listed in Table 2. The PCR product obtained with genomic DNA of *P. fluorescens* SF4c as template was purified with the QIAquick PCR Purification kit (Qiagen) and sequenced. In addition, an arbitrary PCR was performed to obtain the 5′ or 3′ end of *ptm*, *hol*, *prtR* and *lys* in two rounds of amplification through the use of both an arbitrary and a specific primer, as described by Caetano-Anollés (1993). After the second round of amplification, the PCR products were electrophoresed, and the most intense bands were excised from the gel, purified with a Qiagen Gel Extraction kit and sequenced.

Construction of non-polar *ptm* mutant. A *ptm* mutant derivative of *P. fluorescens* SF4c was constructed by the replacement of an internal 404 bp fragment with a Km-resistance cassette. For this step, two internal fragments of *ptm* were PCR-amplified with the oligonucleotides MUTPTMFW1 [5′-CGGGATCCCAAGGCGGAAA-TCGAGCA-3′ (*Bam*HI restriction site underlined)] and MUTPTMREV1 [5′-CTGGTACCGTTCCAGAACCAGCTTGATCG-3′ (*Acc*65I restriction site underlined)], and MUTPTMFW2 [5′-CTGGTACCTCTGCAGTGC-TTGATGCGGT-3′ (*Acc*65I site underlined)] and MUTPTMREV2 [5′-CGGAATTCCGGGTCCGCGAGTTTTTCT-3′ (*Eco*RI site underlined)]. The PCR products (719 and 633 bp, respectively) were digested with *Bam*HI/*Acc*65I and *Acc*65I/*Eco*RI and cloned into pUC18Not (Herrero *et al.*, 1990) to give pPTM1 and pPTM12, respectively. An *Acc*65I fragment carrying a Km-resistance cassette from plasmid p34S-Km3 (Dennis & Zylstra, 1998) was then cloned into pPTM12. The resulting plasmid, pPTM12Km, was digested with *Not*I, and the *ptm*::*Km* construct was cloned into pKNG101, a suicide vector unable to replicate in *Pseudomonas* spp. that allows the generation of double-recombination events. These double recombinants can be selected after the loss of the Sm-resistance marker and the *sacB* gene (which causes growth inhibition in the presence of 7 % sucrose) contained in plasmid pKNG101 (Kaniga *et al.*, 1991). The resulting plasmid, pKNGPTM, was introduced into *P. fluorescens* SF4c by triparental mating through the use of the helper plasmid RK600. Transformants in which the construct was integrated into the chromosome by a single crossover were selected on minimal medium plates containing citrate and Km.

Table 2. Primers used in this study

Primer	Sequence (5′–3′)	*T* (°C)*	PCR product length (bp)	Detection of:
PTM-F	TGAAAAAGGAGTAGAGCGGC	53	2303/2521†	*ptm*
PTM-R	GCCCAGTGCCATTTGTTGTTT			
HOL-F	ATGACAAACGAGCAACAAGC	52	278	*hol*
HOL-R	TAMAGACCGATGGCGACGT			
LYS-F	CCAAGCGGGCGTTTTTGTT	59	432	*lys*
LYS-R	ATCCGCCGGGTAATGCTGT			
PRTR-F	ATTCTCAGCGGSAAGATCGT	53	614	*prtR*
PRTR-R	TACATGCCCCACCAGAASA			
MUTS-PRTR-F	GTAGAAGTCGCCCATGCGGTA	58	665	5′ Region of *mutS* and 5′ region of *prtR*
MUTS-PRTR-R	GCCAATTCAACCTCGCGAA			
LYS-RECA-F	GACACGAATCGGGGCAATT	58	1668	3′ Region of *lys* and 5′ region of *recA*
LYS-RECA-R	CCATACGCATTACGGCACC			
PRTR-HOL-F	GATTCGTCATCGAAGAGAGCG	58	1661/2240‡	3′ Region of *prtR* and 5′ region of *hol*
PRTR-HOL-R	AGCATGATCGCCGAGACA			
MUTS-F	AACCGCGCGAGATTCTAACA	57	2683	*mutS*
MUTS-R	CGCTTTTGCCCCACACCTT			
RECA-RECX-F	AAATCAGGGGTAGGCGATCC	56	1609	*rec–recX*
RECA-RECX-R	AACAAGCGGYTGATCATSTC			
For arbitrary PCR:				
PTM-2	CCCACCCAAAGTCAAAAC			3′ Region of *ptm*§
LYS-1	AACGCGGCAATACGCTTG			5′ Region of *lys*§
HOL-2	TGCATGATCTGCGGTGTCTC			3′ Region of *hol*§

*Annealing temperature.
†The PCR product is 2303 bp for strain SF4c and 2521 bp for strain Pf0-1.
‡The PCR product is 2240 bp for strain SF4c and 1661 bp for strain Pf0-1.
§Region from *P. fluorescens* SF4c.

The second crossover event was selected by plating Km^R clones on medium containing 7 % (w/v) sucrose and Sm. Km^R Suc^R Sm^S colonies were checked by Southern hybridization, PCR and sequencing to confirm the replacement of *ptm* by *ptm*::*Km*.

Complementation analysis. A fragment containing the coding region of *ptm*, with its ribosome-binding sequence, was amplified by PCR from *P. fluorescens* Pf0-1 genomic DNA with the primers pFAJPTM-F [5′-CCCAAGCTTTGAAAAAGGAGTAGAGCGGC-3′ (*Hin*dIII restriction site underlined)] and pFAJPTM-R [5′-CGGGATCCGCCCAGTGCCATTTGTTGTTT-3′ (*Bam*HI restriction site underlined)]. The PCR product was purified, digested with *Hin*dIII and *Bam*HI, and cloned into the pFAJ1709 vector. This construct, pPTM28, was used to transform *P. fluorescens* ptm50 by electroporation. The bacteriocin assay was used as complementation test.

Expression of pyocin under stress. *P. fluorescens* SF4c was grown overnight at 30 °C in tryptone yeast extract (TY) broth medium with shaking at 200 r.p.m. The culture was then subcultured 1 : 100 into 25 ml TY broth and incubated at 30 °C with shaking at 200 r.p.m. The cultures were either untreated, treated with 1 mM hydrogen peroxide (oxidative stress) or 3 μg mitomycin C ml^{-1}, or exposed to UV light from a UV-B lamp (312 nm, Spectroline EB-280C/FE) for 60 s at the beginning of the exponential phase (OD_{600} 0.6). Finally, the cultures were grown up to early stationary phase (OD_{600} 1.7–1.8) and the cells collected in precooled tubes by centrifugation at 6500 ***g*** (4 °C) for 8 min. The resulting pellets were immediately frozen in liquid nitrogen and preserved at −80 °C. Each treatment was performed in triplicate. In parallel, the overnight culture of strain SF4c was also subcultured 1 : 100 into 25 ml TY broth supplemented with 0.25 or 0.5 M NaCl (saline stress) and then incubated at 30 °C with shaking at 200 r.p.m. At early stationary phase, the cells were collected and the pellet frozen as described above. Both treatments were performed in triplicate.

RNA extraction and preparation of cDNA. Total RNA from frozen pellets of the treated and non-treated bacteria was extracted with TRI Reagent (Ambion) as recommended by the manufacturer, except that the TriPure Isolation Reagent was preheated at 70 °C. The RNA was pretreated with RNase-free DNase I (10 U) (Roche) plus RNaseOUT (40 U) (Invitrogen), followed by a purification with RNeasy columns (Qiagen) and a second DNase I treatment with the Turbo DNA-free kit (Ambion). Reverse transcription reactions on 0.5 μg RNA to generate the corresponding cDNA were performed by means of the SuperScript II Reverse Transcriptase (Invitrogen) with random hexamers as primers according to the protocol supplied.

Quantitative real-time PCR (qRT-PCR). The primers used for real-time PCR analyses were as follows: 5′-GTCAGGGCAAGCAGGCTAAAA-3′ and 5′-CGCAGGCATGACTTTGGCT-3′ for the gene encoding PTM (pyocin); 5′-AAAGCCTGATCCAGCCAT-3′ and 5′-GAAATTCCACCACCCTCTACC-3′ for the gene encoding 16S rRNA. Real-time PCR amplification was carried out on a MyiQ2 system (Bio-Rad) associated with iQ5 Optical System Software (version 2.1.97.1001).

Each 25 μl reaction contained 12.5 μl iQ SYBR Green Supermix [100 mM KCl, 40 mM Tris/HCl, pH 8.4, 0.4 mM of each dNTP, iTaq DNA polymerase (50 U ml^{-1}), 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein and stabilizers (Bio-Rad) (0.4 μM for each primer)] and 2 μl template cDNA (diluted 10- or 1000-fold). Thermal cycling conditions were as follows: one cycle at 95 °C for 10 min and then 40 cycles at 95 °C for 15 s, 68 °C for 30 s and 72 °C for 20 s, with a single fluorescence measurement per cycle according to the manufacturers' recommendations. A final extension cycle (72 °C, 1 min) was performed. The PCR products were 212 and 300 bp for the *ptm* and 16S rRNA genes, respectively. Melt curve analysis was performed by gradually heating the PCR mixture from 55 to 95 °C at a rate of 0.5 °C per 10 s for 80 cycles. The relative expression of each gene was normalized to that of 16S rRNA and the results were analysed by means of the comparative critical threshold ($\Delta\Delta C_t$) method (Pfaffl, 2001).

RESULTS

Bacteriocin activity of *P. fluorescens* SF4c

The antibacterial activity of *P. fluorescens* SF4c was tested against reference strains of the genus *Pseudomonas* (Table 3). Strain SF4c showed a small inhibition zone surrounding the colony on lawns of indicator strains, with a strain-specific rather than species-specific effect. These data suggested that strain SF4c might produce a high-molecular-mass bacteriocin with a limited diffusion in the solid medium. The antimicrobial activity was not affected by treatment with proteinase K or trypsin, and was stable at 40 °C, but was lost after incubation at 75 °C (data not shown).

Purification of bacteriocin and its effect on the viability of *P. fluorescens* CTR212

Bacteriocin was purified from cell-free culture supernatants of *P. fluorescens* SF4c as described in Methods. The production of this antimicrobial compound was increased by about 100-fold when mitomycin C was added to cultures of *P. fluorescens* SF4c in the early exponential growth phase (not shown). Purified bacteriocin was serially diluted and antimicrobial activity was assayed against different reference strains of the genus *Pseudomonas*. The degree of sensitivity of these strains varied, with *P. fluorescens* SF39a, *P. putida* KT2440 and *P. fluorescens* CTR212 being approximately 10^4 times more sensitive than *P. fluorescens* Pf-01, *P. putida* SF10b and *P. syringae* pv. *syringae* 61. No growth inhibition was detected against *Pseudomonas corrugata* PC5, *Pseudomonas viridiflava* Pvalb8, or the *P. fluorescens* strains WCS365 and CHA0 (Table 4), thus confirming the results shown in Table 3. For the sake of comparison, strain Pf0-1 was included in these assays. As shown in Table 4, the antibacterial spectrum of this strain differed from that of SF4c, suggesting that despite the sequence similarity observed in several of their pyocin cluster genes (see below), the bacteriocins produced by these two strains have different specificities.

In order to determine whether the bacteriocin of *P. fluorescens* SF4c has a bactericidal or a bacteriostatic effect, 500 AU bacteriocin ml^{-1} was added to exponential phase cultures of the susceptible indicator strain *P. fluorescens* CTR212, and the number of viable cells was determined over time. An initial rapid decline in viability was observed after 30 min of treatment (Fig. 1), indicating that the bacteriocin has a bactericidal effect on the susceptible cells, with a killing rate higher than 94 % at 60 min.

Genetic characterization of the bacteriocin

Random transposon mutagenesis of *P. fluorescens* SF4c through the use of Tn*5*-B20 was carried out to identify

Table 3. Antibacterial activity of *P. fluorescens* SF4c and bacteriocin mutant ptm50 against different *Pseudomonas* strains

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

Indicator strain	Growth inhibition	
	SF4c	ptm50
P. fluorescens CTR212	+	−
P. fluorescens CHA0	−	−
P. fluorescens Pf0-1	+	+
P. fluorescens WCS365	−	−
P. fluorescens SF39a	+	+
P. putida SF10b	+	+
P. putida KT2440	+	+
P. corrugata NCPPB 2445	+	+
P. corrugata PC5	−	−
P. viridiflava Pvalb8	−	−
P. syringae pv. *syringae* 61	+	+

Table 4. Antibacterial spectra of *P. fluorescens* SF4c, bacteriocin mutant ptm50 and reference strain *P. fluorescens* Pf0-1

–, No growth inhibition of indicator strain. The bacteriocin titre was defined as the reciprocal of the highest dilution factor that showed inhibition of the indicator strain.

Indicator strain	Bacteriocin titre		
	SF4c	ptm50	Pf0-1
P. fluorescens CTR212	10^4	–	–
P. fluorescens CHA0	–	–	–
P. fluorescens Pf0-1	10^1	1	–
P. fluorescens WCS365	–	–	–
P. fluorescens SF4c	–	–	10^3
P. fluorescens SF39a	10^5	10^4	10^4
P. putida SF10b	10^1	10^1	–
P. putida KT2440	10^5	10^4	10^5
P. corrugata NCPPB 2445	1	1	–
P. corrugata PC5	–	–	10^1
P. viridiflava Pvalb8	–	–	–
P. syringae pv. *syringae* 61	10^1	10^1	–

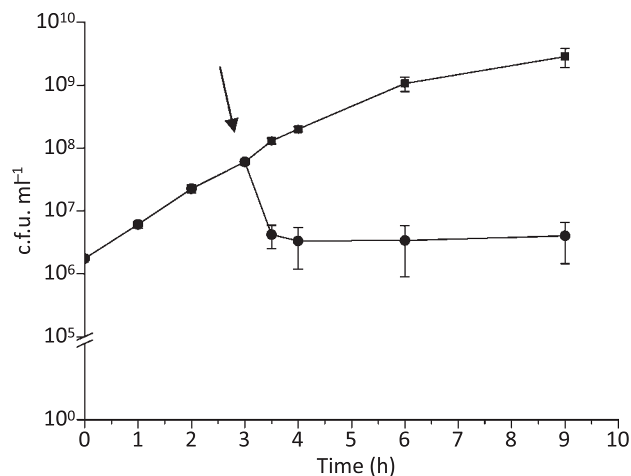

Fig. 1. Effect of bacteriocin on the viability of *P. fluorescens* CTR212. ■, No addition of bacteriocin; ●, addition of bacteriocin at a final concentration of 500 AU ml^{-1}. Each point represents the mean ± SEM of three experiments with three samples per experiment. The arrow indicates when the bacteriocin was added.

genes involved in the production of bacteriocin. Approximately 1000 Km-resistant clones were obtained and screened for lack of ability to inhibit *P. fluorescens* CRT212 (Fig. 2). Three transconjugants that lost their ability to secrete bacteriocins were chosen and Southern hybridization was carried out, with a region of IS*50* being used as a probe. A single *Eco*RI fragment of approximately 6 kb was detected in the three clones, indicating that they had a single transposon insertion. Given the similarity in size of the hybridizing bands, we considered these mutants likely to be siblings and accordingly selected one (designated 634) for further analysis. The DNA region interrupted by Tn*5*-B20 in mutant 634 was cloned into vector pBluescript SK. Km-resistant transformants were selected and one clone containing the recombinant plasmid (pSF16) was sequenced. DNA sequence analysis revealed that the transposon was inserted into an ORF homologous to a gene encoding a putative phage tape-measure protein in *P. fluorescens* Pf0-1. This gene belongs to prophage 01 from Pf0-1 (Fig. 3a) which is similar to the hybrid R2/F2 pyocin locus from *P. aeruginosa* PAO1 (Mavrodi *et al.*, 2009). We have named this gene (locus Pfl01_1148 according to the *P. fluorescens* Pf0-1 genome annotation) *ptm* for phage tape-measure protein.

The complete *ptm* gene (2.5 kb) from the reference strain Pf0-1 and from strain SF4c was amplified through the use of Platinum *Pfx* DNA polymerase. The PCR product of strain SF4c was different in size (2.3 kb) and restriction pattern from that of Pf0-1. Sequence analysis revealed no significant homology at the DNA level with sequences in the databases. The translated sequence did however show a 52 % amino acid similarity to the phage tape-measure protein from *P. fluorescens* Pf0-1 (GenBank accession no YP_346880; see Fig. S1 available with the online version of this paper).

Non-polar *ptm* mutant of *P. fluorescens* SF4c

The *ptm* gene is part of a prophage in *P. fluorescens* Pf0-1, and based on the genetic arrangement of this region, insertion of Tn*5*-B20 into *ptm* could have a polar effect on genes downstream from *ptm*. For this reason, we constructed a *ptm*

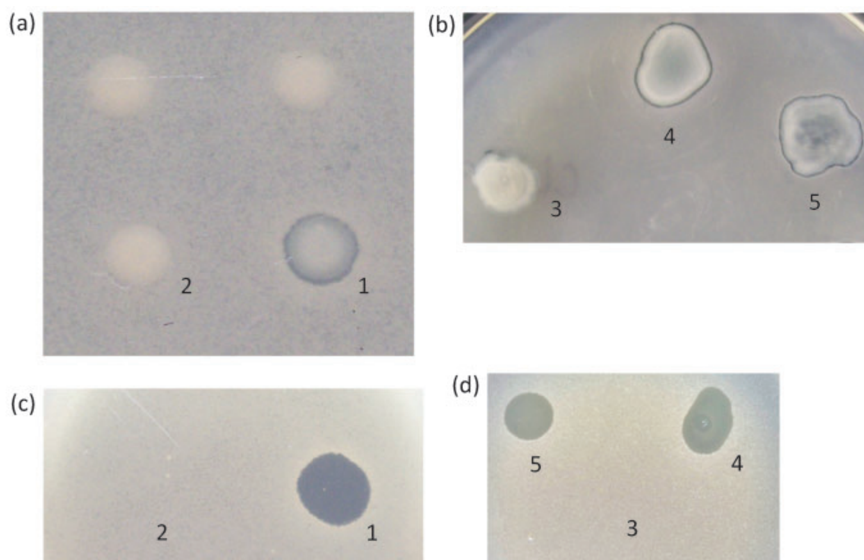

Fig. 2. Antimicrobial activity of *P. fluorescens* SF4c, bacteriocin mutants and purified bacteriocin against *P. fluorescens* CTR212. (a, b) Bacteriocin assays from colonies; (c, d) purified bacteriocin. Wild-type (1, 4); Tn*5*-B20 mutant (2); *ptm* : : Km mutant (3); *ptm* : : Km mutant complemented with plasmid pPTM28 (5).

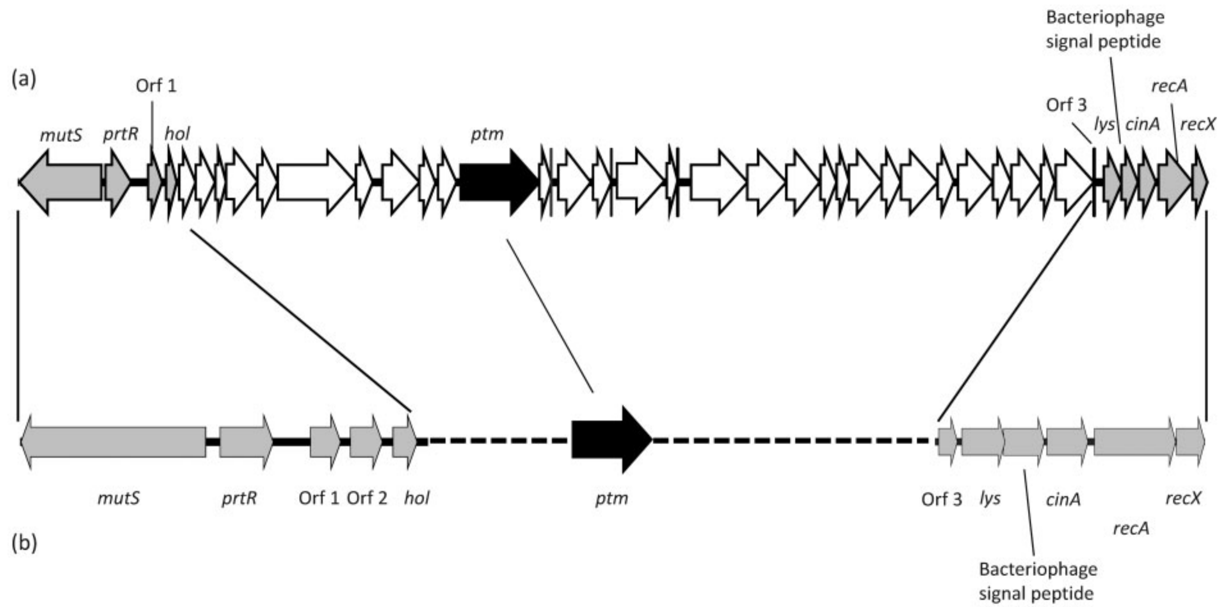

Fig. 3. Scheme of prophage 01 from *P. fluorescens* Pf0-1 (a) and sequenced genes in *P. fluorescens* SF4c (b). The predicted ORFs and their orientations are indicated by arrows. *ptm* is shown in black. Orfs 1, 2 and 3, hypothetical proteins.

null mutant by replacement of the gene with a Km-resistance cassette that had no transcriptional terminators. Clones were analysed by PCR and Southern blotting through the use of a digoxigenin-labelled internal fragment of *ptm* as probe. One clone was selected (ptm50), and the insertion of the Km-resistance cassette 968 bp downstream from the translation initiation site was confirmed by sequencing. Mutant ptm50 did not have antimicrobial activity against *P. fluorescens* CTR212.

Heterologous complementation studies were performed to restore the wild-type phenotype. The *ptm* gene from *P. fluorescens* pf0-1 was cloned into the expression vector pFAJ1709 and the recombinant plasmid (pPTM28) introduced into strain ptm50. This plasmid restored the production of bacteriocin against *P. fluorescens* CRT212 in the *ptm*::Km mutant, indicating that the observed phenotype in the mutant was caused by inactivation of the *ptm* gene (Fig. 2).

The killing spectrum of mutant ptm50 against other *Pseudomonas* reference strains was tested (Tables 3 and 4). The antibacterial spectra of the mutant ptm50 and the wild-type were similar, except that strain SF4c inhibited the growth of *P. fluorescens* CTR212, whereas the mutant did not. The degree of sensitivity of indicator strains, however, varied: *P. fluorescens* strains SF39a and Pf0-1 and *P. putida* KT2440 were 10-fold more sensitive when challenged with SF4c than with ptm50.

Detection of *hol*, *lys, prtR*, *mutS* and *recA–recX* genes

Phage tail-like pyocins contain two highly conserved DNA segments corresponding to the phage repressor (*prtR*) and holin (*hol*) genes as well as the endolysin (*lys*) gene (Mavrodi *et al.*, 2009). The presence of these genes in the genome of *P. fluorescens* SF4c was analysed by PCR amplification through the use of the primers PRT-F/PRT-R, HOL-F/HOL-R and LYS-F/LYS-R, respectively. The predicted 614, 278 and 432 bp fragments were obtained with the DNA of strain Pf0-1 as template, and bands of similar sizes were also obtained with the DNA of SF4c. The PCR products from *P. fluorescens* SF4c were purified, sequenced and analysed by means of ORF Finder. The predicted amino acid sequences in one instance showed a strong similarity to a putative regulatory protein of the cro/cI family (PrtR) from *P. fluorescens* Pf0-1 (99 % similarity), *Pseudomonas* phage DVM-2008 (98 %) and *P. fluorescens* SBW25 (93 %), among others; in the second instance, homology to a holin of pyocin R2_PP from *P. fluorescens* Pf0-1 (100 % similarity), *P. fluorescens* Pf-5 (94 %) and *P. fluorescens* WH6 (93 %); and in the third instance a high similarity to a lytic enzyme from pyocin R2_PP of *P. fluorescens* Pf0-1 (98 % similarity), *P. fluorescens* Pf-5 (91 %) and *P. fluorescens* WH6 (85 %).

In *P. fluorescens* strains Pf-5, Pf0-1, SBW25 and Q8r1-96, the pyocin-like prophages are integrated between the *mutS* and *recA–recX* genes. Therefore, primers MUTS-F/MUTS-R and RECA-RECX-F/RECA-RECX-R were designed on the basis of the conserved region in *Pseudomonas* strains in order to detect the *mutS* and *recA–recX* genes, respectively, in *P. fluorescens* SF4c. Sequencing of the PCR products from strain SF4c enabled the determination of the complete sequences of the *mutS* and *recA* loci along with a partial sequence of the *recX* gene.

Sequences of *hol*, *lys*, *prtR* and *ptm* genes obtained previously did not consist of the complete region. Accordingly, a PCR

with arbitrary primers was performed to obtain the 3′ end for *hol* and *ptm* and 5′ end for *lys*. This procedure also allowed identification of a gene showing 93 % identity to a gene encoding a hypothetical protein from *P. fluorescens* Pf0-1 (locus tag Pfl01_1171), upstream from the *lys* locus.

An amplification of the 3′ region of the *lys* gene from *P. fluorescens* SF4c by PCR with the primers LYS-RECA-F/LYS-RECA-R permitted the identification of the 3′ end of *lys* along with two other loci downstream from *lys* having high identities to genes encoding a probable bacteriophage signal peptide protein (94 %) and competence/damage-inducible protein CinA (96 %) in *P. fluorescens* Pf0-1 (locus tags Pfl01_1173 and Pfl01_1174, respectively).

Primers designed on the basis of inner regions of *prtR* and *hol* from *P. fluorescens* SF4c (PRTR-HOL-F/PRTR-HOL-R) allowed identification of the 3′ and 5′ ends of the respective genes. ORF Finder searching in the region between *prtR* and *hol* revealed two ORFs encoding amino acid sequences that showed 64 and 68 % similarities to a hypothetical phage protein (Pfl01_1136) from *P. fluorescens* Pf0-1. In addition, the primers MUTS-PRTR-F/MUTS-PRTR-R were designed on the basis of the respective inner regions of *prtR* and *mutS*. The PCR product obtained in strain SF4c (665 bp) was sequenced in order to determine the 5′ end of *prtR* (Fig. 3b).

Expression of pyocin

In *P. aeruginosa*, transcription of the pyocin-encoding genes is inducible by DNA damaging agents such as mitomycin C, UV irradiation (Matsui *et al.*, 1993; Michel-Briand & Baysse, 2002), ciprofloxacin (Brazas & Hancock, 2005) and hydrogen peroxide (Chang *et al.*, 2005).

We analysed the influence of environmental stress conditions (saline and oxidative), as well as of UV light or mitomycin C, on the expression of pyocin of *P. fluorescens* SF4c by real-time PCR. A concentration of 500 mM NaCl and 1 mM hydrogen peroxide caused a delayed growth of strain SF4c compared with the growth kinetics under the control conditions. However, bacteria were able to reach the same optical density at the stationary growth phase under control and stress conditions (data not shown).

Primers within one pyocin gene (*ptm*) were designed to allow the evaluation of mRNA levels. The relative expression level of *ptm* was normalized internally to that of the16S rRNA gene. No change in the expression of 16S rRNA was found under different stress conditions. The *ptm* gene showed an increase of expression of approximately 50 and 15-fold when the culture was induced with mitomycin C and UV light, respectively, and by fourfold when cells were grown in the presence of 1 mM hydrogen peroxide with respect to untreated controls. In contrast, the expression of pyocin was downregulated twofold under saline stress (500 mM NaCl). Lower concentrations of NaCl (250 mM) showed no significant effect on bacterial growth and expression of pyocin (Fig. 4).

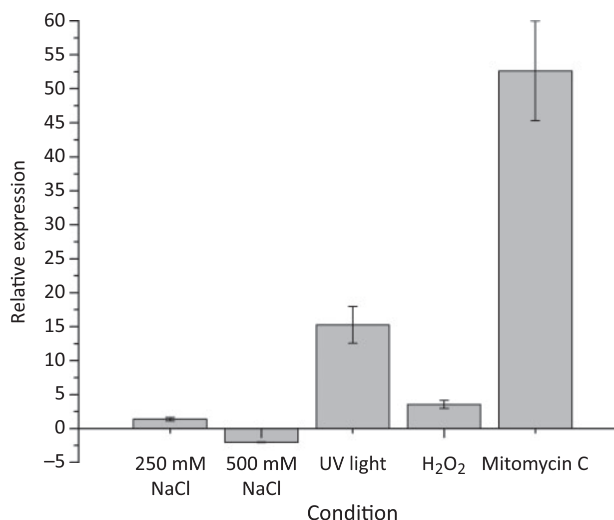

Fig. 4. Expression of pyocin under stress conditions. The relative expression of the *ptm* gene was calculated as fold change of growth in TY broth with 0.25 M NaCl, 0.5 M NaCl, mitomycin C (3 μg ml^{-1}), 1 mM H_2O_2 or exposure to UV light, compared with growth in TY broth without stressing agents. The vertical lines indicate SDs. The results are means from three independent experiments with three replicates in each.

DISCUSSION

A comparative genomic analysis suggested that *P. fluorescens* strains have the potential to produce bacteriocins (Parret & De Mot, 2002; Mavrodi *et al.*, 2009), but so far only one bacteriocin (putidacin) has been genetically characterized in *Pseudomonas* isolated from the rhizosphere, with the mode of action of this bacteriocin differing from that of others described earlier (Parret *et al.*, 2003, 2005). No phage-like bacteriocin, however, had been characterized up to the present in fluorescent *Pseudomonas*. This report shows that *P. fluorescens* SF4c, isolated from wheat rhizosphere, produces a bacteriocin similar to a phage-like pyocin of *P. aeruginosa*. Most phage tail-like bacteriocins have been reported to be resistant to trypsin and to be labile to heat treatment above 50 °C (Strauch *et al.*, 2001), a property that was also observed with the bacteriocin secreted by strain SF4c. The bacteriocin showed antibacterial activity against several *Pseudomonas* strains, some of which might be potential competitors in the rhizosphere, thus suggesting that bacteriocin production is a trait that can influence fitness in that environment. Several genes belonging to a region homologous to that of the putative R- and F-pyocins from *P. fluorescens* Pf0-1 were found in *P. fluorescens* SF4c. For this reason, it is likely that strain SF4c produces both phage-like bacteriocins (R- and F-pyocin).

We have obtained transposon mutants deficient in the production of bacteriocin. One of them was affected in the *ptm* gene. The predicted protein showed homology to the core region of a phage tape-measure protein of the TP901

family from *P. fluorescens* Pf0-1. This region is well conserved among the family. The protein from phage TP901-1 has been characterized as a tail-length tape-measure protein, since a shortened form of the protein leads to phages with proportionately shorter tails (Pedersen *et al.*, 2000).

Orthologues of *ptm* have been found in *P. aeruginosa* strains as structural genes of R-pyocin. In *P. fluorescens* Pf0-1, the *ptm* gene is part of a prophage that is similar to the R2/F2 pyocin from *P. aeruginosa* PAO1. This prophage is located in the *mutS–recA* region, and carries genes for lambda-like and P2-like phage tails, the lytic enzymes holin (*hol*) and endolysin (*lys*), and a gene for a repressor protein (*prtR*). In *P. fluorescens* SBW25, a prophage is present with a similar organization but containing a P2-like tail cluster that is similar to the R-pyocin of *P. aeruginosa*. Related prophages from *P. fluorescens* strains Pf-5 and Q8r1-96 have a myovirus-like tail cluster that is similar to the F-pyocin of *P. aeruginosa* (Nakayama *et al.*, 2000; Mavrodi *et al.*, 2009). Although the complete cluster has not been fully sequenced, our results suggest that the bacteriocin characterized here shows a genetic organization resembling that of the Pf0-1 prophage.

The growth of *P. fluorescens* CTR212 was not affected when challenged with a mutant in the *ptm* gene, indicating that the R-pyocin produced by *P. fluorescens* SF4c is responsible for this antibacterial activity. The antimicrobial activity against other *Pseudomonas* strains, however, did not disappear completely as a consequence of the *ptm* mutation, thus suggesting that another bacteriocin, probably an F-pyocin, is also produced by *P. fluorescens* SF4c. Similar results have been described by Heo *et al.* (2007), who found that the major killing activity of *P. aeruginosa* PA14 culture supernatants toward various clinical isolates disappeared completely after R-pyocin but not F-pyocin mutations.

The lytic system is necessary for a phage-like pyocin to be released from the cells. This lytic system is similar to that of bacteriophages and consists of the *lys* gene, encoding an endolysin with muralytic activities, and the *hol* gene, encoding a holin protein that accumulates in the cytoplasmic membrane and participates in translocation of the endolysin (Nakayama *et al.*, 2000; Michel-Briand & Baysse, 2002). Holins are extremely diverse and can be assigned to one of two classes, based on primary structure analysis. Class I holins are usually 95 residues or longer and have three potential transmembrane domains. Class II holins are usually smaller, 65–95 residues, with only two such domains. Endolysins are also diverse, generally soluble, and with one or more muralytic activities against the three different types of covalent bonds (glycosidic, amide and peptide) of the peptidoglycan polymer that constitutes the cell wall (Young *et al.*, 2000).

The *hol* and *lys* genes were found as part of the *P. fluorescens* SF4c bacteriocin. The *hol* gene product is a 112 aa protein with a hydrophobicity profile similar to that of type II holins – those having two transmembrane regions. In contrast, the *lys* gene product of *P. fluorescens* SF4c is 187 aa long and contains a conserved domain belonging to the lysozyme-like superfamily (cl00222), which includes chitinases of the glycoside hydrolase family.

Pyocins are known to be activated as part of the response to DNA damage. Two genes, *prtR* and *prtN*, regulate pyocin expression (Matsui *et al.*, 1993). Consistent with this, the activated RecA protein cleaves PrtR (the negative regulator) and in this manner enables the expression of the gene that encodes the positive regulator (*prtN*). Subsequently, PrtN activates the expression of the R-, F- and S-type pyocin genes (Michel-Briand & Baysse, 2002). In *P. aeruginosa*, the *prtR* and *prtN* genes are located upstream in the pyocin gene cluster and their transcription is in the opposite direction from the other genes (Nakayama *et al.*, 2000). Nevertheless, in the sequenced genomes of *P. fluorescens* strains (Pf0-1, Pf-5, Q8r1-96 and SBW25), the *prtR* gene is located upstream in the putative pyocin gene cluster and is oriented in the same direction as the other pyocin genes. The same organization is maintained in *P. fluorescens* SF4c. Interestingly, no positive regulator has been found near the pyocin-like prophage cluster in the genomes of *P. fluorescens* strains. This prophage is integrated between *mutS* and the *cinA–recA–recX* genes, which suggests that these elements might be activated during the SOS response (Mavrodi *et al.*, 2009). We confirmed the presence of the *mutS* and *cinA–recA–recX* genes in *P. fluorescens* SF4c. Furthermore, the expression and production of bacteriocin was induced by mitomycin C, a DNA-damaging agent, suggesting that the SOS response is involved in bacteriocin expression.

Bacteria are exposed to different types of stress in the soil, including saline and oxidative stress. Moreover, bacteriocin titres are influenced by environmental conditions (Hurtado *et al.*, 2011). Besides mitomycin C, the bacteriocin produced by *P. fluorescens* SF4c was upregulated by UV light and hydrogen peroxide. These results are in agreement with those reported by Matsui *et al.* (1993) and Chang *et al.* (2005). However, when *P. fluorescens* SF4c grew under saline stress, the expression of pyocin was downregulated. Several studies have demonstrated that NaCl reduces the production of bacteriocin, for example in *Lactobacillus sakei*, *Lactobacillus curvatus* and *Enterococcus faecium* (Leroy & de Vuyst, 1999; Verluyten *et al.*, 2004; Aymerich *et al.*, 2000). Moreover, Hurtado *et al.* (2011) observed changes in bacteriocin expression when *Lactobacillus pentosus* was grown under different NaCl concentrations.

Blázquez *et al.* (2006) demonstrated that ceftazidime, a penicillin binding protein 3 (PBP3) inhibitor, represses the transcription of pyocin genes in *P. aeruginosa*, whereas ciprofloxacin, an inducer of the SOS response, activates expression of pyocin. Those authors speculated that genomes may sense the nature of the signal that induces the SOS system. If the signal indicates DNA damage, then the production of pyocin is induced. If, however, the signal indicates cell wall damage, the transcription of pyocin

genes is decreased to avoid increased damage to the cell wall by lytic enzymes (holin and endolysin). Saline stress is known to affect the components of cell envelopes (Jofré *et al.*, 1998). Therefore, it is possible that the production of pyocin decreases when *P. fluorescens* SF4c is grown under saline stress to prevent cell wall damage.

In conclusion, this report describes, to our knowledge, the first phage-like pyocin from *P. fluorescens* characterized at the genetic level. Bacteriocins are generally associated with bacterial competitiveness within the environment, but this topic is relatively unexplored in soil bacteria. Further studies are in progress to evaluate the ecological role of the bacteriocin secreted by *P. fluorescens* SF4c.

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