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Research Letter

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Pseudomonas fluorescens Pf-5 genome-wide mutant screen for resistance to the antimicrobial peptide alfalfa snakin-1

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One Sentence Summary: A new target of antimicrobial peptide snakin-1.
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ABSTRACT

Snakin-1, a peptide produced by higher plants, has broad-spectrum antibiotic activity, inhibiting organisms ranging from Bacteria to Eukaryotes. However, the mode of action against target organisms is poorly understood. As a first step to elucidate the mechanism, we screened a mutation library of *Pseudomonas fluorescens* Pf-5 in LB and agar medium supplemented with alfalfa snakin-1 (MsSN1). We identified three biofilm formation-related *Pseudomonas* mutants that showed increased resistance to MsSN1. Genetic, physiological and bioinformatics analysis validated the results of the mutant screens, indicating that bacterial adhesion protein lapA is probably the target of MsSN1. Collectively, these findings suggest that snakin-1 acts on microbial adhesion properties.

Key words: innate immunity; antimicrobial peptides; antimicrobial activity; action mechanism

INTRODUCTION

Antimicrobial peptides are part of innate immunity, establishing a first line of defense against pathogen infections. Snakin/GASA peptides, which were initially isolated from potato (Lopez-Solanilla, Garcia-Olmedo and Rodriguez-Palenzuela 1998), are widely distributed among plant species and were found to be active against bacterial, fungal and nematode pathogens (Segura *et al.*, 1999; Berrocal-Lobo *et al.*, 2002; Almasia *et al.*, 2008; Kovalskaya and Hammond 2009; Balaji, Sessa and Smart 2011; Kovalskaya, Zhao and Hammond 2011; Mao *et al.*, 2011), suggesting a critical role of snakin peptides in biotic stress tolerance. Interestingly, phylogenetic and functional analysis of snakin-1 from alfalfa further support that snakin peptides have important and ancestral roles in land plant innate immunity (García *et al.*, 2014).

In recent years, plant antimicrobial peptides snakin-1 and snakin-2 have been the subject of interest as primary candidates for generating broad-spectrum biotic stress tolerance in crops (Kovalskaya *et al.*, 2011; Balaji and Smart 2012; Guzman-Rodriguez *et al.*, 2013; Rong *et al.*, 2013; García *et al.*, 2014; Meiyalaghan *et al.*, 2014; Mohan *et al.*, 2014). Despite the

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importance of roles played by snakin peptides in plant–microbe interactions in nature and the potential of these peptides to improve crop production, the molecular mechanisms underlying its inhibition of microbial cells are poorly understood. Regarding this intriguing topic, pioneer studies demonstrated the *in vitro* aggregation of unrelated bacterial strains under snakin exposure, but this aggregation did not correlate with antimicrobial activity (Segura *et al.*, 1999; Berrocal-Lobo *et al.*, 2002). To explain these results, it has been speculated that snakin peptides could play an important function *in vivo* through a change in adhesion molecules present on the cell surface (Berrocal-Lobo *et al.*, 2002). This attractive idea has been supported by the sequence similarity of snakin peptides to Cys-rich domains of animal proteins that are involved in protein–protein interactions, such as vWF (Shelton-Inloes, Titani and Sadler 1986; Verweij *et al.*, 1986) and MDC proteins (Wolfsberg *et al.*, 1995; Sagane *et al.*, 1998). However, the validation of this hypothesis requires empirical approaches.

Previously, we experimentally demonstrated the antimicrobial activity of alfalfa snakin-1 (MsSN1) against bacterial strain *Pseudomonas fluorescens* Pf-5 both *in vitro* and *in planta* (García *et al.*, 2014). As a strategy for discovering the cellular pathways affected in bacteria by snakin-1, we here screened a genome-wide mutant library of *P. fluorescens* Pf-5 for resistance to MsSN1. Our results indicate that snakin-1 acts on microbial adhesion properties.

MATERIAL AND METHODS

Selection and molecular analysis of MsSN1-resistant *Pseudomonas* mutants

pUC4K mutagenesis was used to generate mutants in *P. fluorescens* Pf-5 (Howell and Stipanovic 1979) as described in Ayub, Tribelli and Lopez (2009). This mobile genetic element, which does not replicate in *Pseudomonas*, was introduced by electroporation into competent cells of *P. fluorescens* Pf-5 (Ayub *et al.*, 2009). Then, the genomic library was screened for the presence of the kanamycin-resistance gene and tolerance to MsSN1. Thus, transformants were selected by plating on LB agar containing 30 μg/ml of kanamycin (Km) and 1 μl/ml of MsSN1 prepared as described by García *et al.* (2014). Five Km^R-$MsSN1^R$ colonies were chosen, and then, molecularly characterized. To study the insertion site of the Km-resistance cassette within mutant strains, a genomic walking assay was performed according to Soto *et al.* (2012) using the following primers: 5′ GTATTGATGTTGGACGAGTCGGAATC 3′ (1a) and 5′ CAGGATCTTGCCATCCTATGGAACTG 3′ (1b) (Fig. S1, Supporting Information). Three non-redundant clones were selected after sequencing and called M1, M2 and M3 (Fig. S1, Supporting Information). Genomic DNA of *P. fluorescens* Pf-5 was partially digested with XhoI and cloned into the mobilizable cosmid pVK102 according to the method of Ayub *et al.* (2006). The genomic library was screened for the presence of the *lapA* gene by complementation analysis using *P. fluorescens* Pf-1 LapA-HA (Monds *et al.*, 2007) as a host. The identity of this fragment was verified by sequencing. The resulting cosmid, named pVK102-lapA, was mutagenized in the vWF domain according to Ayub *et al.* (2009). This novel recombinant cosmid was called pVK102-lapAΔ4411-4536. For complementation analysis, conjugations of *P. fluorescens* Pf-5 and the M1 mutant with *Escherichia coli* S17–1 harboring pVK102-lapA and pVK102-lapAΔ4411-4536 recombinant cosmids were performed on mineral salts medium plates (Schlegel, Lafferty and Krauss 1970) supplemented with octanoate (0.25% w/v), containing tetracycline (5 μg/ml) (Ayub *et al.*, 2006).

Biofilm and surface attachment assays

Pseudomonas fluorescens Pf-5 was statically grown for 6 h in LB medium (Setten *et al.*, 2013) and biomass was stained with 0.1% crystal violet and quantified as described in Monds *et al.* (2007). Values represent the mean ± SD of triplicate measurements. To study the effects of MsSN1 on biofilms, the *P. fluorescens* Pf-5 strain was grown in LB medium supplemented with 10% v/v of physiological (0.9% NaCl) or MsSN1 solutions prepared as described by García *et al.* (2014).

In vitro antimicrobial activity assays

The disk inhibition assays were evaluated as described in Ayub *et al.*, (2004), with very slight modifications. Cultures were performed in 120 ml Erlenmeyer flasks containing 25 ml of LB medium. Bacteria were incubated overnight at 28°C with shaking (250 rpm). Sterile Whatman No. 1 filter disks (5 mm) impregnated with 5 μL of physiological or MsSN1 solutions prepared as described by García *et al.* (2014). These solutions were placed on top of bacteria-seeded plates. Zones of inhibition were measured after incubation at 28°C for 24 h. Significant differences were calculated using one-way ANOVA followed by Tukey test.

Protein structure prediction

The 3D structure of MsSN1 was built based on multiple-threading alignments using the I-TASSER server, setting all the parameters to default values (Roy, Kucukural and Zhang 2010). In the absence of crystallographic structures of both lapA and analogous proteins to be used for homology modeling analysis, its amino acid sequence in the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/cdd), retrieving a von Willebrand factor type A (vWF) domain was analyzed. The vWF domain was homology modeled using the Swiss-Model server, as reported by Mozzicafreddo *et al.* (2009).

Molecular docking analysis between MsSN1 and the vWF domain of lapA

Rigid protein–protein molecular docking between MsSN1 and the vWF domain of lapA homology-modeled was carried out using the ClusPro 2.0 server (Comeau *et al.*, 2004) and setting all the parameters to default values. In particular, MsSN1 (either monomer or dimer) was submitted to the server as ligand, whereas the vWF domain of lapA was submitted as receptor. The MsSN1 dimer was constructed during an early stage, uploading MsSN1 both as ligand and as receptor. The binding affinities (ΔG_{pred} and $K_{d,pred}$) of the most probable (in terms of cluster size) complexes MsSN1 monomer/vWF domain of lapA and MsSN1 dimer/vWF domain of lapA estimated were determined using the FastContact 2.0 server (Camacho and Zhang 2005) and setting all the parameters to default values.

RESULTS AND DISCUSSION

Screening for MsSN1 resistance in *Pseudomonas*

To investigate the action mechanism of snakin peptides, a library of Km-resistant mutants derived from *P. fluorescens* Pf-5 was generated and screened in LB and agar medium supplemented with the MsSN1. Three non-redundant mutants that showed increased tolerance to MsSN1 under *in vitro* conditions

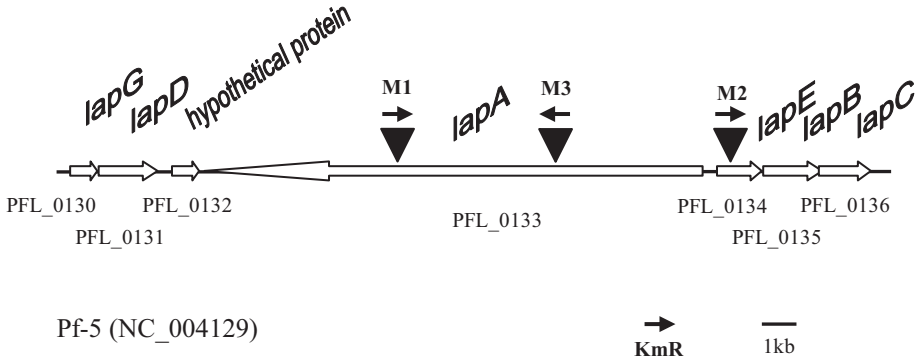

Figure 1. Schematic representation of Km cassette insertions within Lap cluster (*lapGDAEBC*) from *P. fluorescens* Pf-5. Gene disruption by a mobile genetic element containing a Km-resistance cassette (KmR) results in the production of the following recombinant strains: M1 (lapA::KmR), M2 (lapE::KmR) and M3 (lapA::KmR). Black arrows indicate KmR. PFL: *Pseudomonas fluorescens* Pf-5 locus.

were identified. These mutants, called M1–M3, were analyzed by genome walking to characterize the region flanking the insertions. Interestingly, the three insertions were co-localized within the chromosome of *P. fluorescens* Pf-5, specifically in the lap cluster (Fig. 1). Lap is a critical component of biofilm formation by *P. fluorescens* strains. It contains a large adhesion protein (lapA) that is transported to the outer membrane by an ABC transporter (*lapEBC*) (Newell, Monds and O'Toole 2009). Disk inhibition assays showed that contrary to wild-type strain *P. fluorescens* Pf-5, *lapA* (M1 and M3) and *lapE* (M2) mutants are resistant to MsSN1 (Table 1 and Fig. S2, Supporting Information).

Several complementation analyses were performed to confirm whether the resistance of the three mutants to MsSN1 was truly associated with a defect in the *lap* system. Complementation with *lapA* from *P. fluorescens* Pf-5 restored the sensitivity of the M1 mutant to MsSN1 (Table 1). In concordance with the relevance of lapA in the bacterial inhibition process mediated by MsSN1, *P. fluorescens* Pf-5 transformed with the *lapA* gene was more sensitive to MsSN1 than its parental strain *P. fluorescens* Pf-5 (Table 1). Similar to other adhesin proteins involved in cell attachment to surfaces or in cell–cell interactions, lapA has a vWF domain consisting of 126 amino acids within the C-terminal region. To understand the relevance of this functional domain in the antimicrobial activity of MsSN1, wild-type and M1 mutant strains were transformed with the lapA ΔvWF mutant allele (*lapA* without its functional domain) and the recombinant strains were studied. In both strains, the introduction of truncated lapA had no effect on the MsSN1 sensitivity patterns of their parental strains (Table 1), showing the essential role of vWF domain in the bacterial sensitivity to MsSN1. Therefore, these findings suggest that MsSN1 acts on *P. fluorescens* Pf-5 biofilm formation via lapA.

Effect of MsSN1 on *P. fluorescens* Pf-5 biofilm formation and initial surface attachment

Formation of a community of bacterial cells, or a biofilm, is a central component in the fitness of *P. fluorescens* strains (Monds *et al.*, 2007). Thereby, the sensitive phenotype of *P. fluorescens* Pf-5 could be caused by an alteration in the structure of this type of interactions. Thus, the effects of MsSN1 on biofilm formation and initial surface attachment of *P. fluorescens* Pf-5 were determined. After 6 h in static culture, *P. fluorescens* Pf-5 inoculated with MsSN1 showed a hyperadherent biofilm phenotype, accumulating twice as much biomass on the culture well as the same strain without antimicrobial stress (Fig. 2A). These bacteria were analyzed by microscopy under similar, static growth conditions. After a 1 h incubation, irreversibly attached *P. fluorescens* Pf-5 cells treated with MsSN1 covered 4-fold as much of the substratum as the strain under optimal unstressed conditions, suggesting that MsSN1 alters bacterial attachment (Fig. 2B). This phenotype is consistent with previous data demonstrating that bacterial attachment has a direct and proportional stimulatory effect on biofilm formation (Newell *et al.*, 2011).

Molecular docking of the complexes between MsSN1 and the vWF domain of lapA

Considering the evidences of the presence of StSN1 dimers *in vivo* (Nahirñak *et al.*, 2012) and the probable role of lapA in *P. fluorescens* Pf-5 sensitivity to MsSN1, the possibility of the formation of different complexes between lapA and MsSN1 was explored. Interestingly, molecular docking analyses of MsSN1 and the vWF domain of lapA from *P. fluorescens* Pf-5 generated complexes with high affinity, each involving specific

Table 1. Antimicrobial activity of MsSN1 on *P. fluorescens* Pf-5 and recombinant strains derived from this bacterium.

Strain	Relevant characteristic	Zone of inhibition (mm)	Statistical comparations of Pf-5 vs recombinant strains
Pf-5	wild-type	1.76 ± 0.06	—
M1	Pf-5 lapA::km	0	***
M2	Pf-5 lapE::km	0	***
M3	Pf-5 lapA::km	0	***
M1 lapA	M1+ lapA	2.23 ± 0.06	***
M1 lapA ΔvWF	M1+ lapA without vWF domain	0	***
Pf-5 lapA	Pf-5 + lapA	2.30 ± 0.10	***
Pf-5 lapA ΔvWF	Pf-5 + lapA without vWF domain	1.73 ± 0.06	ns

All values are log means ± SEM ($n = 6$). Asterisks indicate a statistically significant difference (Tukey: $^{***}P < 0.001$). ns: no significative.

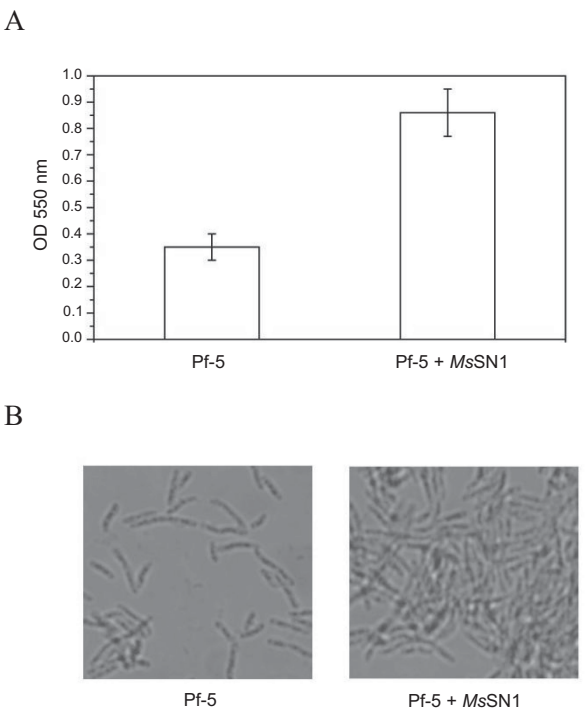

Figure 2. Effect of MsSN1 on *P. fluorescens* Pf-5 adhesion phenotype. Qualitative and quantitative assays comparing (**A**) biofilm formation and (**B**) initial surface attachment of *P. fluorescens* Pf-5 in the presence (right) or absence (left) of MsSN1 in static cultures, respectively.

amino acids (Fig. 3). In particular, the MsSN1 dimer showed an exceptionally high affinity for the vWF domain in terms of the predicted equilibrium dissociation constant ($K_{d,\,pred}$) of 2.92×10^{-14} M, whereas the MsSN1 monomer showed an affinity of 2.32×10^{-8} M. In detail, the complex dimer/vWF domain was characterized by an electrostatic energy of –36.79 kcal/mol, a desolvation free energy of 1.34 kcal/mol and a van der Waals energy of –1484.23 kcal/mol; the complex monomer/vWF domain showed an electrostatic energy of –18.77 kcal/mol, a desolvation free energy of –1.63 kcal/mol and a van der Waals energy of –1015.8 kcal/mol. Both ligands bound the vWF domain in the same region, although the binding interface for the dimer was larger. This different binding mode explained both the higher electrostatic and van der Waals interaction contributions, and the highest affinity of the vWF domain for the dimer. Thus, oligomerization properties of snakin peptides could have an important role in the prevention of microbial infection.

Synthetic peptides, derived from natural peptides such as the human cathelicidin LL-37 (Overhage *et al.*, 2008), the fungal peptide decapeptide KSL (Liu *et al.*, 2011), the bovine peptide indolicidin (de la Fuente-Núñez *et al.*, 2012) and the frog peptide L-K6 (Shang *et al.*, 2014), have been recently identified as biofilm inhibitory compounds. In contrast, previous studies and the results reported here show that snakin peptides exposure markedly increased bacterial aggregation, adhesion and subsequent biofilm formation, suggesting that the inhibitory effect of snakin and other antimicrobial peptides occurs by a different mechanism. The transition between the sessile lifestyle that biofilms represent and a planktonic, motile lifestyle is a universal colonization fitness factor for *Pseudomonas* (Barahona *et al.*,

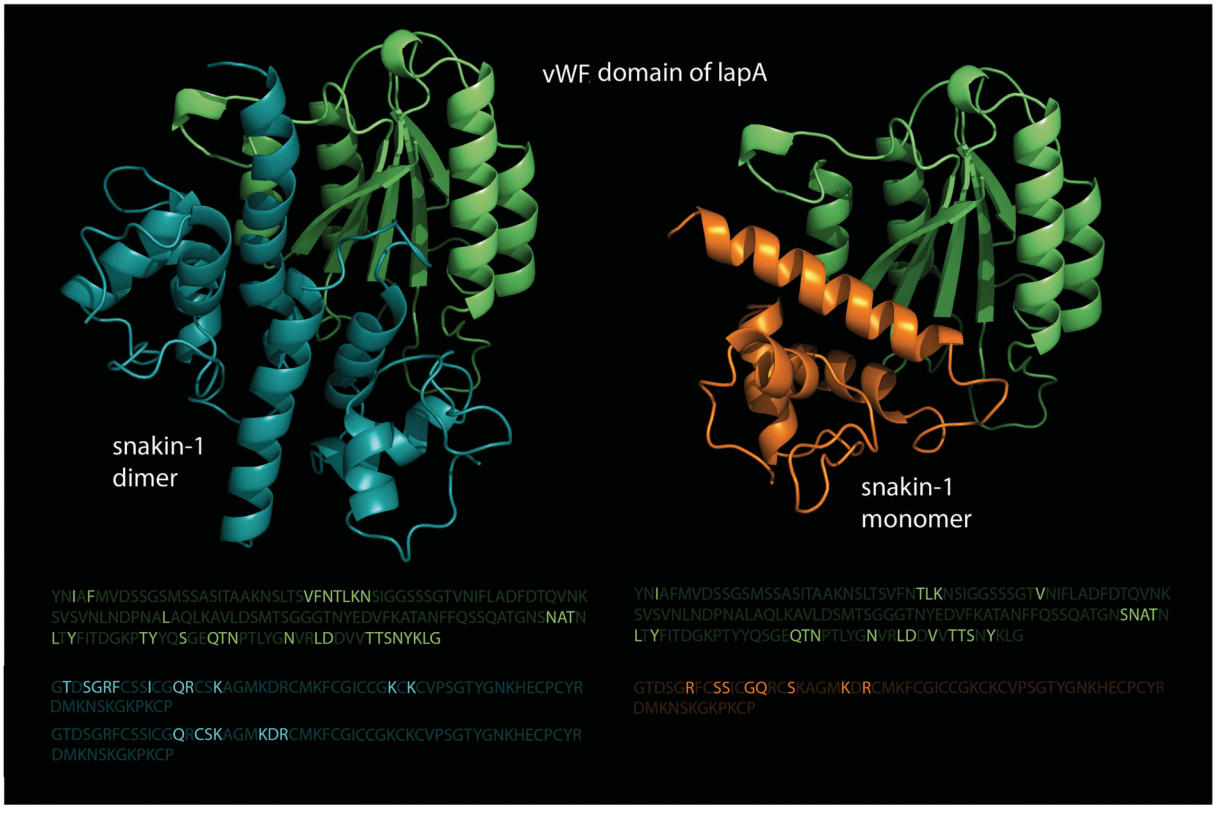

Figure 3. Molecular docking between MsSN1 and vWF domain of lapA from *P. fluorescens* Pf-5. In the docking analysis with the vWF domain of lapA, MsSN1 is presented in dimer (left) or monomer (right) forms. Putative specific amino acids involving in protein–protein interaction are highlighted.

2010; Chua *et al.*, 2014). Therefore, it is possible that prolonged sessile bacterial growth induced by snakin peptides results in lower fitness on potential hosts.

CONCLUSIONS

Adhesion is a universal prerequisite for primary and opportunistic pathogens to efficiently deploy their repertoire of virulence factors and exert effects on host cells. The results presented in this work indicate that snakin peptides can act on microbial adhesion properties. Specifically, our results show that a microbial adhesion protein (lapA) is probably the target of MsSN1. Further studies involving other snakin peptides and microbial strains will show whether the alteration of microbial adhesion properties found in this work is a general mechanism of snakin peptides.

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Conflict of interest statement. None declared.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSLE online.

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