A Type VI Secretion System Is Involved in Pseudomonas fluorescens Bacterial Competition

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Abstract

Protein secretion systems are crucial mediators of bacterial interactions with other organisms. Among them, the type VI secretion system (T6SS) is widespread in Gram-negative bacteria and appears to inject toxins into competitor bacteria and/or eukaryotic cells. Major human pathogens, such as Vibrio cholerae, Burkholderia and Pseudomonas aeruginosa, express T6SSs. Bacteria prevent self-intoxication by their own T6SS toxins by producing immunity proteins, which interact with the cognate toxins. We describe here an environmental P. fluorescens strain, MFE01, displaying an uncommon oversecretion of Hcp (hemolysin-coregulated protein) and VgrG (valine-glycine repeat protein G) into the culture medium. These proteins are characteristic components of a functional T6SS. The aim of this study was to attribute this role to a energy-consuming overexpression of the T6SS. The genome of MFE01 contains at least two hcp genes (hcp1 and hcp2), suggesting that there may be two putative T6SS clusters. Phenotypic studies have shown that MFE01 is avirulent against various eukaryotic cell models (amebas, plant or animal cell models), but has antibacterial activity against a wide range of competitor bacteria, including rhizobacteria and clinical bacteria. Depending on the prey cell, mutagenesis of the hcp2 gene in MFE01 abolishes or reduces this antibacterial killing activity. Moreover, the introduction of T6SS immunity proteins from S. marcescens, which is not killed by MFE01, protects E. coli against MFE01 killing. These findings suggest that the protein encoded by hcp2 is involved in the killing activity of MFE01 mediated by effectors of the T6SS targeting the peptidoglycan of Gram-negative bacteria. Our results indicate that MFE01 can protect potato tubers against Pectobacterium atrosepticum, which causes tuber soft rot. Pseudomonas fluorescens is often described as a major PGPR (plant growth-promoting rhizobacterium), and our results suggest that there may be a connection between the T6SS and the PGPR properties of this bacterium.

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Introduction

Bacteria have developed an arsenal of mechanisms, including secretion systems to enable them to resist the various stresses generated by their environment. Secretion systems are crucial defensive weapons allowing bacteria to persist in an ecological niche or to conquer new one. The most recently described secretion system in the Gram-negative Proteobacteriaceae is the type VI secretion system (T6SS) [1,2]. This protein complex releases virulence factors into the extracellular medium or transports them directly into the target cell. T6SS is a macromolecular machine involved in bacterial virulence and/or interactions with other organisms [3]. T6SS gene clusters differ between bacterial species in terms of gene order and orientation, but all have a conserved group of 13 essential genes, the “core components” [4–6]. Two of the core genes, hcp and vgrG, encode the extracellular part of the secretion machinery. The Hcp and VgrG proteins are released into the culture medium by T6SS activity. This release provides evidence that the T6SS apparatus is functional [7]. The T6SS seems to consist of a needle-like membrane-puncturing device similar to a bacteriophage tail. Hcp and VgrG have secondary structures similar to those of bacteriophage components, with the major phage tail protein and the tail spike phage protein corresponding to Hcp and VgrG, respectively [8,9]. The T6SS is implicated in virulence in some human pathogens, including Vibrio cholerae, Burkholderia pseudomallei, Arthrobacter hydrophila, Acinetobacter baumannii, and Pseudomonas aeruginosa [6,10–15]. However, the contribution of the T6SS to virulence remains unclear.

Several studies have reported that some T6SSs are used to kill competing bacteria [16–18]. Some T6SS toxins or effectors with a peptidoglycan hydrolase structure [19] are responsible for this killing activity. English and coworkers have reported the identification and characterization of two S. marcescens effectors [20] Ssp1 and Ssp2, which have this peptidoglycan hydrolase-like structure [21]. Phospholipase antibacterial effectors were also described [22] and other effectors of unknown activity were identified [17,23,24]. The self-intoxication of bacteria by their own toxins is prevented by the production of immunity proteins, which must interact physically with cognate toxins. Such interactions have been demonstrated in P. aeruginosa (Tsi proteins) and S. marcescens (Rap proteins) [20,25]. The bacteria must come into close contact with prey cells for the T6SS to deliver toxins and exert its antibacterial activity [14,26].

T6SSs are also widespread in many Gram-negative environmental bacteria. Many Pseudomonas fluorescens strains have genes...
encoding T6SS components [27–29]. These genomic and transcriptomic studies have suggested that the T6SS may be involved in interactions of *Pseudomonas fluorescens* with plants.

In this study, we identified an Hcp protein as the major supernatant protein of an environmental strain of *Pseudomonas fluorescens*, MFE01. This indicates that a T6SS of this strain was functional and constitutively overexpressed. We therefore investigated the role of this T6SS overexpression, by phenotypic and genetic studies.

Materials and Methods

Bacterial Strains, Plasmids and Culture Conditions

All strains and plasmids are listed in Table 1. All bacterial strains were grown in LB medium with shaking (180 rpm), except for *Pectobacterium atrosepticum* CFBP 6276, which was grown in PGA minimal medium supplemented with polygalacturonic acid 0.4% (wt/vol) (Sigma-Aldrich, St. Louis). *Pseudomonas fluorescens* strains were grown at 28°C or 37°C, *Pseudomonas aeruginosa* and *E. coli* at 37°C, and *Pectobacterium atrosepticum* was grown at 25°C [30]. When required, media were supplemented with antibiotics: kanamycin (Km), 50 μg/ml (*E. coli* or conjugation) or 100 μg/ml (*P. fluorescens* strains); tetracycline (Tc), 15 μg/ml; rifampicin (Rif), 25 μg/ml.

**Growth Curve and Hcp Secretion Analysis**

*P. fluorescens* strains were cultivated in 25 ml of Luria Bertani medium in a 250 ml Erlenmeyer flask, with shaking at 180 rpm from an OD<sub>580</sub> of 0.06. OD<sub>580</sub> was measured at 45-minute intervals over a nine-hour period. Hcp secretion was assessed by harvesting the supernatants by centrifuging the cultures at 5000 g for 10 minutes at 20°C and passing them through a Millipore membrane with 0.22 μm pores. TCA was added to the supernatant to a final concentration of 10% and the mixture was incubated overnight at 4°C. The supernatant was then centrifuged at 13000 g for 30 minutes at 4°C. The dry pellet was then resuspended in distilled water. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, samples were mixed with an equal volume of 2x Laemmli sample buffer (with β-mercaptoethanol), boiled for 5 min at 100°C and then cooled to room temperature before loading.

Mass spectrometry analysis

Mass spectrometry (MS) analyses were performed in positive ion mode as described by Barbey et al. [31]. Statistical analyses of the sequences were carried out by determining the probability-based on Mowse score with MASCOT software (peptide toleran-

**Table 1. Plasmids and strains included in the study.**

| Strains or plasmids | Relevant characteristics | Reference/source |
|---------------------|--------------------------|------------------|
| **Strains**         |                          |                  |
| *P. fluorescens*    |                          |                  |
| MFE01               | Air isolate, Rif<sup>R</sup> | This study       |
| MFE01<sub>Hcp2</sub> | MFE01 with early stop codon in hcp2 | This study       |
| MFE01<sub>Hcp2,R</sub> | MFE01<sub>Hcp2</sub> revertant with wild-type hcp2 gene in its original chromosomal location | This study       |
| MFE01<sub>Hcp2/hcp2</sub> | MFE01<sub>Hcp2</sub> with pPSV35 carrying the wild-type hcp2 gene | This study       |
| MFN1032             | Clinical strain able to grow at 37°C | [35]             |
| MFP05               | Skin isolate             | LMSM collection  |
| PFD1                | Soil isolate             | [43]             |
| *P. aeruginosa*     |                          |                  |
| H103                | Prototrophic derivative of PAO1 | [37]             |
| PA14                | Clinical isolate         | [32]             |
| *Escherichia coli*  |                          |                  |
| K12                 | General cloning strain   | LMSM collection  |
| DSM<sub>smcr</sub>  |                          | Bethesda Research Laboratories |
| S17.1               | RP4-2-Tc ::Mu aph ::Tn7 recA, Sm<sup>R</sup> donor strain for conjugation | [42]             |
| *Pectobacterium atrosepticum* 6276 | Isolate from Solanum tuberosum | [30]             |
| *Serratia marcescens* |                          |                  |
| *Klebsiella aerogenes* |                          |                  |
| **Vectors**         |                          |                  |
| pME6000             | Replicative plasmid in *Pectobacterium atrosepticum* Tc<sup>R</sup> | [34]             |
| pSMC21              | Replicative in Gram-negative bacteria Km<sup>R</sup>, gfp | [33]             |
| pAKE604             | Conjugal suicide vector for hcp2 gene mutagenesis; Km<sup>R</sup>, sacB | [41]             |
| pSUPROM             | Km<sup>R</sup>, vector for constitutive expression of rap (1a, 1b, 2a or 2b) genes under control of *E. coli* tat promoter | [20]             |
| pPSV35              | *P. aeruginosa oriV, lacIq mob+ PlacUV5, pUC18 MCS, expression vector, Gm<sup>R</sup> | [49]             |
cc = 100 ppm and mass values = MH+). A p-value of less than 0.05 was considered significant. The criteria used to accept protein identification based on peptide mass fingerprinting (PMF) data included the score probability greater than the score threshold 84 (p<0.05), the extent of sequence coverage (minimum 30%) and the number of matched peptides (minimum 8).

Table 2. Oligonucleotides used for this study.

| Primer name | Primer sequence (5’-3’) |
|-------------|------------------------|
| Pf01_2045F  | ACCCGGCAAAACAGGCGCTGA |
| Pf01_2045R  | ACCCGCAGCAGCGGACCCGG |
| Pf01_2328F  | ATACCTGCGCGCCGCTCA |
| Pf01_2328R  | GACCAGGACCCGACATGATC |
| muta1hcp    | CACGGCAGCAGGGCGGTTCA |
| muta2hcp    | ATACCTGCGCGCCGCTCA |
| muta3hcp    | ATACCGTGGACCCGAGGTATA |
| muta4hcp    | CGTTCGGCAGCAGCCGCTCA |
| hcp-vgrF    | ATACCGTGGACCCGAGGTATA |
| hcp-vgrR    | CACGGCAGCAGGGCGGTTCA |
| vgrGqpcF    | TTACGCCCCTCGAGATT |
| vgrGqpcR    | AAGAACAAAGGCTCGGCT |
| 16S F       | CTGTGATCTCCGCGTTTAAC |
| 16S R       | CGGCTGATCTCCGCGTTTAAC |

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Amplification of MFE01 T6SS Genes and Bioinformatics Analysis

For the amplification of Pf01-like hcp genes from P. fluorescens MFE01, we used the oligonucleotide primers Pf01_2045F and Pf01_2045R, and Pf01_2328F and Pf01_2328R (Table 2), designed on the basis of the genomic sequence of Pf01, for standard PCR with P. fluorescens MFE01 genomic DNA. The PCR parameters used were as follows: an annealing temperature of 56°C, an extension time of 25 s and 25 cycles. The polymerase used was the High Fidelity PCR Enzyme (Thermo Scientific). PCR fragments were inserted into the pGEM®-T-easy vector (Promega) and sequenced. Bioinformatics analyses were performed with Blast (NCBI web site).

Antibacterial Competition Assay

Antibacterial competition assays were carried out with a slightly modified version of a protocol described elsewhere [26]. To ensure selection of competitor cells for counting, E. coli K12, P. aeruginosa PA14 [32] and P. fluorescens strains (except MFE01 or MFE01Δhcp2) were transformed with pSMC21 [33] to confer kanamycin resistance and Pectobacterium atrosepticum was transformed with pME6000 to confer tetracycline resistance [34]. S. marcescens was selected by plating on Hektoen medium plates without prior manipulation. The bacterial cells were grown overnight on LB agar plates and resuspended in LB. The OD_{590} was adjusted to 0.5, and the cells were mixed at a ratio of 5:1, P. fluorescens MFE01: target bacteria. 25 μl of this mixture were spotted onto a filter with 0.22 μm pores on a prewarmed LB agar plate, which was then incubated for 4 h at 28°C or 37°C. The bacteria on the filter were resuspended in 1 ml of sterile physiological water and serial dilutions were plated on LB agar supplemented with antibiotic or selective medium. In contact-dependent competition experiments between P. fluorescens MFN1032 [35] and MFE01, the strains were separated by a filter with 0.22 μm pores. 25 μl of a suspension of one strain were spotted onto a filter, which was placed on the surface of the LB agar. A second filter with 0.22 μm pores was placed on top of this spot and 25 μl of the other strain was spotted onto this second filter.

Cytotoxic Assay on Rat Glial Cells

We investigated the cytotoxic activity of P. fluorescens MFE01 with primary cultures of rat glial cells, as previously described [36]. Briefly, rat glial cells, obtained from newborn (24–48 h) rat brain, were grown in DMEM/Ham’s medium (2/1) supplemented with 10% fetal calf serum, 2 mM glutamine, 0.001% insulin, 5 mM HEPES, 0.3% glucose and 1% antibiotic-antimycotic solution (Biowhittaker, Emerainville, France). The cells were layered, at a concentration of 10^5 cells/well, on 24-well plates coated with poly-L-lysine (50 μg.ml^-1) and incubated at 37°C under a humidified atmosphere containing 5% CO₂. Glial cells were allowed to grow for 12 to 16 days before use. Stationary-phase MFE01 cells were harvested by centrifugation at 8000 x g for 5 minutes at room temperature. The bacteria were then resuspended at a density of 10^9 cfu/ml in glial cell culture medium without antibiotics or antimycotics and incubated with the glial cells for 24 h. The concentration of LDH (a marker of necrosis) released by rat glial cells was determined with the Cytotox 96® Enzymatic Assay (Promega, Charbonnieres, France).

Cytotoxicity Assay on Chicory Leaf

Strains were grown overnight, at 28°C for MFE01 or 37°C for P. aeruginosa H103 [37], with shaking (180 rpm). 1 ml of culture
was centrifuged in a benchtop centrifuge and the pellet was resuspended in 1 ml of sterile 10 mM MgSO₄. 10 µl of a suspension with an OD₅₈₀ of 0.1 were injected into the central vein of a chicory leaf, which was then incubated for 24 h at 28°C or 37°C. Soft rot manifested as the appearance of a brown area around the injection.

**Soft-rot Test in Potato Tubers**

*Pectobacterium atrosepticum* 6276 was prepared from stationary-phase cultures grown in PGA minimal medium supplemented with pectolytic polygalacturonic acid 0.4% (wt/vol) (Sigma-Aldrich, St. Louis). Culture of *Pectobacterium* was centrifuged and the pellet was resuspended in sterile physiological water to obtain a suspension of 10⁹ cfu/ml. *P. fluorescens* MFE01 was suspended in 1 ml of sterile water, and the OD₅₈₀ was adjusted to 1. Then *P. fluorescens* MFE01 or MFE01Ahcp2 were mixed with *Pectobacterium* at a ratio of 10:1.

*Solanum tuberosum* cv. Allians tubers were surface-sterilized and infected by the intramedullary injection (at a depth of 1 cm) with 10 µl of the bacterial mix. The inoculated tubers were incubated in a Minitron incubator (Inforis, Massy, France) at 25°C and a relative humidity of 65% ±2%. For each assay, we analyzed 10 tubers, by noting the development of symptoms, by measurements of the diameter of soft rot, seven days after inoculation.

**Dictyostelium discoideum Growth and Predation Assays**

This assay was performed exactly as described by Sperandio et al. [38].

**RNA Extraction, qRT-PCR and Co-transcription Assay**

RNA was extracted by a modified version of the phenol-based extraction procedure described by Grépin et al. [39]. The cells were first lysed with the following lysis buffer: 0.02 M sodium acetate, pH 5.5, 0.5% (w/v) SDS, 1 mM EDTA. The cell lysates obtained were subjected to two consecutive phenol extractions, followed by a chloroform extraction. Total RNA was precipitated in 100% ethanol (2:1, v/v) and 1 M sodium acetate (1:10, v/v) and resuspended in RNase-free water. DNase treatment with RNase-free Ambion® TURBO™ DNase (Life Technologies™) was carried out to remove any contaminating DNA. The quality and concentration of RNA samples were checked by agarose gel electrophoresis and with a Nanodrop spectrophotometer (Bio-Rad Laboratories). The absence of genomic DNA contamination was confirmed by PCR. RT-PCR was performed with 50 ng of RNA as a template, with the Transcripter one-step RT-PCR kit (Roche, Meylan, France), according to the manufacturer’s recommendations. For cotranscription assays, we used the hcp-vgrG primer and hcp-vgrR primers (Table 2) for PCR assays. PCR was carried out in standard conditions, as follows: annealing temperature, 57°C; extension time, 15 s; 25 cycles; polymerase: Phusion® High-Fidelity DNA polymerase (NEB). mRNAs of vgrG were quantified by real-time PCR as described by Guyard-Nicodème and coworkers with minor modifications [40]. Primers (Table 2) were designed with Primer express 3 software and validated by PCR. PCR reactions were performed with the 7500 Fast real-time PCR system (Applied Biosystems). The 15 µL reactions contained 6.5 µL of SYBR Green PCR Master Mix (including AmpliTaq Gold DNA Polymerase, Applied Biosystems), 0.2 µM final of each primer and 13 ng final of cDNAs. PCR conditions as follows: 95°C for 20 sec, 40 cycles at 95°C, 57°C and 72°C for 3 sec, 30 sec and 15 sec respectively, 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec. The relative quantification of the mRNAs was obtained by the comparative CT (2-DDCt) method as described [40].

**Figure 2. *P. fluorescens* MFE01 cytotoxicity toward amebas and animal and plant cells.** (A) Lactate dehydrogenase release was used as a marker of cell lysis. Wild-type MFE01 and glial cells were incubated together, at 37°C, for 4 h and 24 h (N = 3). Error bars indicate the standard error of the mean. The control cells were used to determine the basal rate of lysis of the glial cell culture (Cells control). Cells were incubated with Triton to obtain 100% lysis as a positive control (Cell/triton). Cells/MFE01 corresponds to contact between glial cells and the MFE01 strain for the period indicated (4 h or 24 h). (B) The chicory leaf soft-rot assay was performed with wild-type MFE01 at 28°C (N = 3). “MgSO₄” indicates a representative photograph of the outcome of injecting MgSO₄ into the central vein and is a negative control for chicory leaf soft rot. “H103” shows a representative result for the inoculation of *P. aeruginosa* strain H103 and servers as a positive control for chicory leaf soft rot. “MFE01” shows a representative photograph of the effect on inoculation with *P. fluorescens* MFE01. (C) Approximately 100 D. discoideum AX3 cells were cultured on SM-plates, on a layer of *Klebsiella aerogenes* with or without 10% *Pseudomonas fluorescens* MFE01 or MFN1032. Plates were maintained at 22°C for 5 days. Lysis plaques were counted and compared with those obtained for the negative control for virulence, *Klebsiella aerogenes* (KA:100% of the amebas remain) and the positive control for virulence, *P. fluorescens* MFN1032 (0% of the amebas remain). We calculated ratios of the number of lysis plaques obtained with respect to that for the control *Klebsiella aerogenes*. Data are mean values from three independent experiments (the standard deviation is shown).

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Construction of the hcp2 Mutant of *P. fluorescens* MFE01

A 1.3 kb fragment containing the *hcp2* gene, inserted into pUC19, was obtained from an MFE01 genomic bank. A markerless *hcp2* mutant was constructed by overlap PCR mutagenesis and the conjugative suicide vector pAKE604 [41] was selected on the basis of kanamycin resistance and sucrose sensitivity (Table 1). An early stop codon (TAA) was introduced in the middle of the *hcp2* gene. This was achieved by PCR with the muta1hcp and muta2hcp (<700 bp product) or muta3hcp and muta4hcp (<600 bp product) (Table 2). The PCR products obtained corresponded to the upstream and downstream parts, respectively, of the 1.3 kb genomic MFE01 fragment, each carrying an overlapping sequence at the end including the early stop codon. PCR parameters were as follows: annealing temperature, 53°C; extension time, 35 s; 30 cycles. A third PCR was then carried out in which the overlapping sequences of two first products were hybridized together and the product of polymerization with the muta1hcp and muta4hcp primers was a mutated *hcp2* gene. The PCR parameters were as follows: annealing temperature, 53°C; extension time, 35 s and 30 cycles. Each PCR was performed in standard conditions, with the Phusion® High-Fidelity DNA polymerase (NEB). The PCR fragment obtained was inserted into pAKE604 that had been linearized by digestion with SmaI (NEB). The resulting plasmid, pAKE604Δhcp2, was verified by sequencing and was then transferred into MFE01 by biparental mating. *E. coli* S17-1 [42] containing pAKE604Δhcp2 and recipient MFE01 cells were mixed and spotted onto sterile nitrocellulose filters, which were placed on LB agar plates and incubated overnight at 28°C.

**Figure 3.** *P. fluorescens* MFE01 displays bacterial killing activity. For each assay, *N*= 4, and the error bars represent the standard error of the mean. (A) A quantitative coculture assay was performed for 4 h at 28°C. Different prey cells were incubated with or without *P. fluorescens* MFE01 or MFE01Δhcp2 (Δhcp2), at a ratio of 1:5, respectively. Statistics were done by pairwise strain comparisons (non-parametric Mann-Whitney-two tailed Test): *p*-value <0.05; **p*-value <0.02; ***p*-value <0.002; ns no significant difference. Sma: *Serratia marcescens*. (B) *Pseudomonas fluorescens* MFP05 was incubated with or without wild-type MFE01 (ratio 1:5) for 4 h at 37°C for coculture assays. (C) Quantitative assessment of the MFN1032 population cocultured for 4 h at 28°C with MFE01 or without MFE01. A filter with 0.22 μm pores was placed between MFE01 and MFN1032, to prevent physical contact.

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MFE01 Secretes Hcp-like Protein and VgrG-like Protein

MFE01 is an environmental bacterium with an optimal growth temperature of 30°C that can grow at 37°C. The proteins present in the culture supernatant were precipitated during the early exponential growth phase at 28°C and 37°C. A 20 kDa band was clearly visible after growth at 28°C, but not after growth at 37°C (Fig. 1). This protein was identified by mass spectrometry as an Hcp (hemolysin coregulated protein)-like protein. It matched with WP_003225156 (type VI secretion system effector, hcp1 family from P. fluorescens) with a score of 113, coverage of 48% and a number of peptides of 8.

During the late stationary growth phase at 28°C, an 85 kDa band, absent at 37°C, was identified by mass spectrometry as a VgrG-like protein (Fig. 1). It matched with WP_003225156 (type VI secretion protein Rhs from P. fluorescens) with a score of 103, coverage of 38% and a number of peptides of 23.

The secretion of Hcp and VgrG into the medium has been described as the “hallmark” of a functional T6SS [7]. The putative Hcp is the major protein present in the supernatant from the early exponential growth phase onwards, suggesting an unusual pattern of constitutive T6SS expression at 28°C. We extended our analysis of Hcp secretion to other P. fluorescens strains. Pf01 secreted Hcp, but in smaller amounts. MFP05, an human skin isolate, and MFN1032, a clinical strain, did not secrete Hcp into the supernatant (data not shown). Thus, this over-secretion of Hcp does not appear to be a common feature of the species Pseudomonas fluorescens.

A growth temperature of 37°C corresponds to the regulatory “shutoff” temperature for the T6SS. This “shutoff” temperature has not been definitively determined. This temperature dependence suggests that MFE01 may use T6SS in the environment, but not in association with homoeothermic organisms.

MFE01 has at Least Two hcp-like Genes

PCR primers designed on the basis of the sequence of Pf01 hcp genes [43] were used for the amplification of MFE01 chromosomal DNA (Table 2). Two amplicons, corresponding to the Pf01_2328 (462 bp) and Pf01_2045 (474 bp) primers, were obtained. These two partial sequences were checked by sequencing and subjected to Blast analysis. The gene hcp1 corresponding to the Pf01_2328 primers displayed 95% identity to hcp1 Pf01_2328 (partial sequence hcp1 genBank accession number: KF447439), whereas the gene hcp2 corresponding to the Pf01_2045 primers displayed 97% identity to hcp Pf01_2045 (complete sequence hcp2 genBank accession number: KF447438). Partial hcp1 gene sequence (462 bp) displayed 99% identity with hcp2 gene (516 bp) with a query cover of 89%. Barret and coworkers have suggested that there is a relationship between the P. aeruginosa T6SS locus and VgrGs/Hcps sequences [29], arguing that the P. aeruginosa HSI-II could be linked to virulence in animals and plants, whereas HSI-I mediates interbacterial interactions [44]. Since, HSI-II also has been demonstrated to mediate interbacterial interactions [22]. However, the phylogenetic clusters probably evolved to adapt to various environments, so it is difficult to find a correlation between hcp and vgrG sequences, T6SS clusters and ecological niches [10]. We therefore decided to explore the virulence and interbacterial interactions of MFE01, to determine the role of the T6SS in this strain.

Results and Discussion

P. fluorescens MFE01 Secretes Hcp-like Protein and VgrG-like Protein

Statistical Analysis

Data were analyzed using non-parametric Mann-Whitney Test (two tailed) with GraphPad Prism version 6.0 (La Jolla, CA). A p-value <0.05 was considered to be statistically significant.
**P. fluorescens** MFE01 Displays Antibacterial Activity Rather Than Eukaryotic Virulence

We carried out infection tests with eukaryotic animal and plant cell models. MFE01 cytotoxicity toward glial cells was quantified by measuring lactate dehydrogenase (LDH) release into the culture medium. No significant cytotoxicity was observed after 4 h or 24 h in the MFE01/glial cell contact assay, indicating that MFE01 is avirulent against glial cells (Fig. 2A). The chicory leaf soft-rot test was used to assess MFE01 virulence against plant cells. *P. aeruginosa* H103, the positive control strain for virulence, caused soft rot on chicory leaf. MgSO₄, the negative control, and MFE01 did not induce symptoms on chicory leaf. We can therefore conclude that MFE01 is avirulent toward this plant cell model (Fig. 2B), consistent with the beneficial nature of many strains of this species to plants [45].

We then assessed the ability of *P. fluorescens* MFE01 to resist phagocytic predation by the ameba *Dictyostelium discoideum*. On a layer of *Exoibilla aerogenes* [46] containing 10% MFE01, about a hundred lytic plaques corresponding to zones in which amebas were feeding on bacteria were observed. This suggests that MFE01 does not protect against predation by amebas (Fig. 2C). This absence of virulence in these models is consistent with the avirulent phenotype of the saprophytic members of this species, but not with the results obtained for MFN1032, a clinical isolate of *Pseudomonas fluorescens* that can also grow at 37°C [38].

Given this lack of evidence for MFE01 virulence against various eukaryotic cell models (amebas, plant and glial cells), coupled with several reports of T6SS-dependent antibacterial activity, we investigated whether *P. fluorescens* MFE01 had antibacterial activity. Antibacterial competition assay consists to mix MFE01 and prey bacteria at a ratio of 5:1, respectively, and co-cultured on an agar plate for 4 h. We then counted the number of surviving prey cells. At 28°C, prey bacteria which co-cultivated with MFE01 exhibited a drastic and significant population drop, from three-log loss for *Pseudomonas fluorescens* MFN1032, four-log lost for *P. aeruginosa* PA14, five-log for *E. coli* or *Pseudomonas fluorescens* Pf01 and six-log drop for *P. fluorescens* MFP05 compared to results for cultures without MFE01 (Fig. 3A). *Serratia marcescens* was the only bacterium tested that was resistant to predation by MFE01 at 28°C (Fig. 3A). We concluded that MFE01 had a bacterial killing activity at 28°C.

We investigated whether this killing activity was linked to Hcp secretion, by monitoring antibacterial activity at 37°C (Fig. 3B). *P. fluorescens* MFP05 did not die during coculture at 37°C, while it was the strain most sensitive to MFE01 predation at 28°C. We investigated whether MFE01 killing activity was contact-dependent. A new antibacterial competition assay was carried out at 28°C, without contact between the predator MFE01 and the prey MFN1032 (Fig. 3C). The competitor MFN1032 survived if it was separated from MFE01 by a filter with 0.22 μm pores. In these conditions, no decrease was observed in the recovery of viable *P. fluorescens* MFN1032. MFE01 appeared to lose its killing activity in the absence of close contact. In other species, the antibacterial effect mediated by the T6SS has been shown to be contact-dependent [47], and competition can be abolished by a filter separating the predator and prey, suggesting that MFE01 T6SS is involved in *P. fluorescens* competition.

**The MFE01 T6SS is Involved in Predation on Various Gram-negative Bacteria**

A MFE01 genomic bank was established for analysis of the genomic environment of the *hcp2* gene. Sequencing revealed that there was a *scpG* gene located downstream from *hcp2*. A MFE01Δ*hcp2* mutant was constructed by introducing an early stop codon in the middle of the *hcp2* gene. Wild-type MFE01 and MFE01Δ*hcp2* mutant growth curves were similar, indicating that *hcp2* mutation had no effect on growth kinetics.
However, hcp2 mutation resulted in much lower levels of Hcp protein secretion into the medium than for wild-type MFE01. However, a weak band corresponding to a Hcp-like protein (identified by mass spectrometry) was still visible on the MFE01Δhcp2 strip (matched with gi:398853281, hcp1 family from Pseudomonas Sp. GM 80, score of 108, coverage of 40%, match with 8 peptides) (Fig. 4). This residual Hcp secretion may reflect hcp1 expression. The hcp2 gene seems to be responsible for most of the observed Hcp-like proteins secretion. Mougou and coworkers have reported considerable heterogeneity in Hcp secretion within P. aeruginosa, depending on the hcp gene concerned [2,48].

We investigated the possible role of the hcp2 gene in MFE01 killing activity, by performing competition assays at 28°C with MFE01Δhcp2 (Fig. 3A). During competition with PA14, MFN1032 and PA01, MFE01Δhcp2 reduced significantly prey cell population, but at a significantly lower level than MFE01.

In MPI05 and E.coli competition assay, killing activity was abolished in MFE01Δhcp2, which had no significant effect on the prey cell population (Fig. 3A). Similar findings have also been reported for the T6SSs of V. cholerae [18], A. baumannii [14], B. thailandensis [16] and P. aeruginosa [HSI-4] [17], consistent with a role for the P. fluorescens T6SS in bacterial competition. According to the prey cell, this T6SS seems to act or not in synergy with another unknown mechanism.

We introduced pPSV35 [49], carrying native hcp2, into MFE01Δhcp2, to obtain MFE01Δhcp2/hcp2. The expression of this wild-type hcp2 gene in trans did not restore the wild-type phenotype (data not shown). Carruthers and coworkers encountered the same problem with Acinetobacter baumannii and suggested that it probably resulted from polar effects on downstream genes in the T6SS cluster [14]. Polar effects may be responsible for complementation failure if the vgrG and hcp2 genes are cotranscribed. We tested this hypothesis, by using the hcp-vgrF and hcp-vgrR primers, corresponding to the hcp2 and vgrG regions, respectively, for PCR on cDNA from MFE01 growing exponentially at 28°C. No amplification was detected with these primers, whereas hcp2 was expressed, suggesting that hcp2 and vgrG were not cotranscribed (data not shown). To confirm this result, we used vgrG primers, to quantify transcription of vgrG in MFE01 and MFE01Δhcp2. We observed no significant difference of vgrG mRNA level between these two strains. We then hypothesized that MFE01Δhcp2 might have another mutation outside the hcp2 gene. We checked for the absence of such a mutation in MFE01Δhcp2, by reintroducing the native hcp2 gene at its usual chromosomal location to obtain the revertant strain MFE01Δhcp2-R. Coclusive assays indicate that MFE01Δhcp2-R recovered killing activity, demonstrating that an unidentified mutation was not responsible for complementation failure. We suggest that fine regulation of the dynamics of this overexpressed T6SS occurs during the association between the various constitutive proteins of the T6SS apparatus (Hcp, VgrG, VipA,VipB) and that this regulation is disturbed during the expression of the hcp2 gene in trans.

MFE01 seems to have a large target cell spectrum. Basler and coworkers have shown that the T6SS of prey cells may affect the activation of the predator T6SS in Pseudomonas aeruginosa [50]. We found that expression of a T6SS in PA01, or no expression in MPI03, had no significant impact on the potential activation of killing activity in MFE01. We showed that the hcp genes of MFE01 were similar to PA01 hcp genes, but PA01 was unable to resist MFE01 predation. This suggests that MFE01 is capable of antagonizing PA01 by variation in effector and immunity proteins according to recent works [26,51].

T6SS Immunity Proteins of S. marcescens Protect E. coli Against MFE01

For confirmation of the role of the T6SS in bacterial killing activity, we made use of the resistance of S. marcescens to MFE01. We investigated whether the Rap immunity proteins were responsible for protecting S. marcescens against MFE01, explaining the number of viable S. marcescens cells recovered (Fig. 3A). We used an approach similar to that used by English and coworkers to demonstrate cross-immunity between two effector-immunity (EI) protein families by the introduction of Rap immunity proteins into E. coli [20]. Fig. 5 shows E. coli harboring the pSUPROM vector constitutively expressing the Rap proteins indicated in coculture with wild-type P. fluorescens MFE01.

The expression of Rap1a and Rap1b increased the counts of viable E. coli by two-log with respect to the counts obtained for E.
coli with empty vector. However, Rap2a and Rap2b conferred a resistance similar to that obtained with the empty vector. Although killing activity was only partially suppressed, this experimental approach highlighted the existence of cross-immunity, providing support for a role of hcp2 in MFE01 killing activity mediated by the effector Rap2a. E. coli does not express all the Rap immunity proteins, accounting for the absence of full protection resembling that observed for S. marcescens. According to nomenclature of Russell et al., the S. marcescens toxins SpS1 and SpS2 belong to the Tae4 protein family, and are responsible for hydrolysing peptide crosslinks at the γ-D-glutamyl-miDAP DL-bond [25]. These findings suggest that MFE01 secretes toxins targeting the peptidoglycan of Gram-negative bacteria.

MFE01 Inhibits the Growth of Pectobacterium atrosepticum 6276

As Pseudomonas fluorescens has been described as a major PGPR (plant growth-promoting rhizobacteria) [52], we assessed the killing activity of MFE01 against Pectobacterium atrosepticum 6276, which causes tuber soft rot. MFE01 killed *P. atrosepticum* in vitro (Fig. 6A), decreasing cell counts by five-log whereas MFE01Δhcp2 had no effect on *Pectobacterium atrosepticum*. We assessed the ability of the predator activity of MFE01 to protect tubers against *P. atrosepticum* in planta. MFE01 or MFE01Δhcp2 were mixed with *P. atrosepticum* in a 10:1 ratio and the mixture obtained was then used to inoculate tubers. MFE01 protection was evaluated by determining the presence or absence of tuber soft rot. MFE01 was not pathogenic to tubers and it significantly inhibited the development of soft rot due to *P. atrosepticum*. Conversely, MFE01Δhcp2 did not protect tubers against *P. atrosepticum* (Fig. 6B). This result suggests that the T6SS of MFE01 may be involved in this tuber protection.

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Conclusion

We describe here the first example of a functional T6SS in the environmental *P. fluorescens* strain MFE01. We obtained strong evidence that *P. fluorescens* MFE01 T6SS plays an important role in competition with other bacteria. It is easy to see how this competitive ability would help the bacterium to survive and to prosper in the environmental reservoir or to compete in a new niche. The unusual constitutive production and over-secretion of Hcp in this strain indicates that this T6SS is very important for the fitness of MFE01. The high level of Hcp secretion in this strain suggests that the benefits of this overproduction are greater than the energetic cost, again highlighting the importance of this system. An understanding of this mechanism would facilitate the development of new methods of combating bacterial proliferation, making use of the inhibitory properties reported for the MFE01 strain.

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Author Contributions

Conceived and designed the experiments: AM CB VD. Performed the experiments: AM CB VD DB. Analyzed the data: AM CB VD XL. Wrote the paper: AM VD. Technical and scientific discussion: NO MF XL.
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