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# Characterization of the SPI-1 and Rsp type three secretion systems in *Pseudomonas fluorescens* F113

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## Summary

***Pseudomonas fluorescens* F113 is a plant growth-promoting rhizobacterium (PGPR) isolated from the sugar beet rhizosphere. The recent annotation of the F113 genome sequence has revealed that this strain encodes a wide array of secretion systems, including two complete type three secretion systems (T3SSs) belonging to the Hrp1 and SPI-1 families. While Hrp1 T3SSs are frequently encoded in other *P. fluorescens* strains, the presence of a SPI-1 T3SS in a plant-beneficial bacterial strain was unexpected. In this work, the genetic organization and expression of these two T3SS loci have been analysed by a combination of transcriptional reporter fusions and transcriptome analyses. Overexpression of two transcriptional activators has shown a number of genes encoding putative T3 effectors. In addition, the influence of these two T3SSs during the interaction of *P. fluorescens* F113 with some bacterial predators was also assessed. Our data revealed that the transcriptional activator *hilA* is induced by amoeba and that the SPI-1 T3SS could potentially be involved in resistance to amoeboid grazing.**

## Introduction

Non-flagellar type III secretion systems (T3SS) are nanomachines composed of approximately 25 proteins, encoded in Gram-stain-negative bacteria belonging to the *Proteobacteria*, *Chlamydia* and *Verrucomicrobia* phyla (Cornelis, 2010; Izoré *et al*., 2011; Sait *et al*., 2011; Barret *et al*., 2013). T3SSs are specialized machineries dedicated to the translocation of a wide diversity of Type III effectors (T3Es) from the bacterial cytosol to the eukaryotic host cytoplasm (Dean, 2011). These T3Es could be involved in numerous types of bacterial–host interactions ranging from mutualism to parasitism (Preston, 2007; Coombes, 2009). In contrast to the large diversity observed among T3S effectors, the structural apparatus (or injectisome) itself is highly conserved and has evolved into seven different phylogenetic families: Ysc, Hrp1, Hrp2, SPI-1, SPI-2, Rhizobial and Chlamydial (Pallen *et al*., 2005; Troisfontaines and Cornelis, 2005; Abby and Rocha, 2012).

Modulation of host metabolism by T3Es has been initially studied in plant, animal or human pathogens. However, in the last decade the influence of T3SSs in rhizosphere colonization by plant beneficial bacteria has been investigated in a number of studies. For instance, the protein-coding gene *rscC* involved in the assembly of the Hrp1-like T3SS apparatus of the biocontrol strain *Pseudomonas fluorescens* SBW25 has been shown to be induced in the sugar beet rhizosphere (Rainey, 1999). Subsequent analysis has revealed that the Hrp1-like T3SS of *P. fluorescens* SBW25 (also designated Rsp/Rsc) was able to elicit a hypersensitive response in *Nicotiana clevelandii*, proving its functionality (Preston *et al*., 2001). Hrp1 genes were found to be widespread among a range of plant growth-promoting bacteria (PGPR) belonging to the *P. fluorescens* group (Preston *et al*., 2001; Mazurier *et al*., 2004; Rezzonico *et al*., 2004; Viollet *et al*., 2011). In addition, numerous Hrp1 loci were found in whole or partial genome sequences of strains related to the *P. fluorescens* group (Rezzonico *et al*., 2005; Kimbrel *et al*., 2010; Cusano *et al*., 2011; Mavrodi *et al*., 2011; Ortet *et al*., 2011; Loper *et al*., 2012). The abundance of Hrp1 T3SSs in genomes of strains related to the *P. fluorescens* group, coupled with its expression in different rhizospheres (Jackson *et al*., 2005; Rezzonico *et al*., 2005; Mavrodi *et al*., 2011) implies its ecological significance during 'beneficial plant–bacterial interactions'. Recent evidence suggests that Hrp1 of *P. brassicacearum* Q8r1-96 could be involved in suppression of PAMP-triggered immunity (PTI) through translocation of T3Es related to *P. syringae* strains (Mavrodi *et al*., 2011). Suppression of PTI by T3Es could facilitate colonization of root tissues by Hrp1 positive strains, or alternatively, improve the establishment of ectomycorrhizal symbiosis, a phenomenon observed with the Hrp1 T3SS of the

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mycorrhiza helper bacteria *P. fluorescens* BBc6R8 (Cusano *et al*., 2011). However, these findings are unlikely to be transposed to all Hrp1 positive strains related to the *P. fluorescens* group since the Hrp1 locus and, more importantly the T3E repertoire, vary considerably between strains.

*Pseudomonas fluorescens* F113 is a PGPR strain isolated from the sugar-beet rhizosphere in Ireland (Shanahan *et al*., 1992). Initially this strain was selected and studied because of its capacity to inhibit growth of a range of phytopathogenic bacteria, fungi, oomycetes and nematodes including *Pectobacterium carotovorum* (Cronin *et al*., 1997b), *Fusarium oxysporum* (Barahona *et al*., 2011), *Pythium ultimum* (Fenton *et al*., 1992) and *Globodera* spp. (Cronin *et al*., 1997a). This antimicrobial capacity is strongly linked to the production of a secondary metabolite, 2,4-diacetylphloroglucinol (Fenton *et al*., 1992; Abbas *et al*., 2002). In addition, *P. fluorescens* F113 is also an excellent rhizosphere colonizer of different plant species including wheat (De La Fuente *et al*., 2006), alfalfa (Villacieros *et al*., 2003), and willow (de Carcer *et al*., 2007). For these reasons, F113 is a widely used model strain for studying rhizosphere colonization (Martínez-Granero *et al*., 2006; Barahona *et al*., 2010).

The sequencing of the *P. fluorescens* F113 genome has confirmed the presence of a complete Hrp1 locus (Preston *et al*., 2001), but has also revealed the existence of a T3SS locus related to the SPI-1 family (Redondo-Nieto *et al*., 2012). SPI-1 T3SSs have been mostly found in bacteria associated with mammals (Galan, 1999; Miki *et al*., 2010). However, recent sequencing projects have also identified SPI-1 T3SSs in chromosomes of plant pathogenic bacteria such as *Xanthomonas albilineans* (Marguerettaz *et al*., 2011) and *Pantoea stewartii* (Correa *et al*., 2012). Moreover, some bacterial strains related to the *P. fluorescens* group like *P. fluorescens* HK44 (Chauhan *et al*., 2011) and *P. fluorescens* Q2-87 (Loper *et al*., 2012) also possess the SPI-1 cluster. The presence of SPI-1 T3SSs in commensal rhizospheric bacterial strains raises several questions. First, whether this system is expressed at all and under which conditions it is induced, and then what is its role in the rhizocompetence of these bacterial strains.

In this work two transcriptional activators of Hrp1 and SPI-1 have been identified in *P. fluorescens* F113. Following transcriptome comparisons, a number of F113 genes induced by these two regulators were highlighted. These included genes encoding protein involved in the assembly of the structural apparatus and putative T3Es. In addition, the potential roles of these T3SSs during interactions between F113 and other soil organisms were assessed. Our results demonstrate that the SPI-1 T3SS is induced during F113-amoeba interactions and is involved in resistance to amoeboid predation.

## Results and discussion

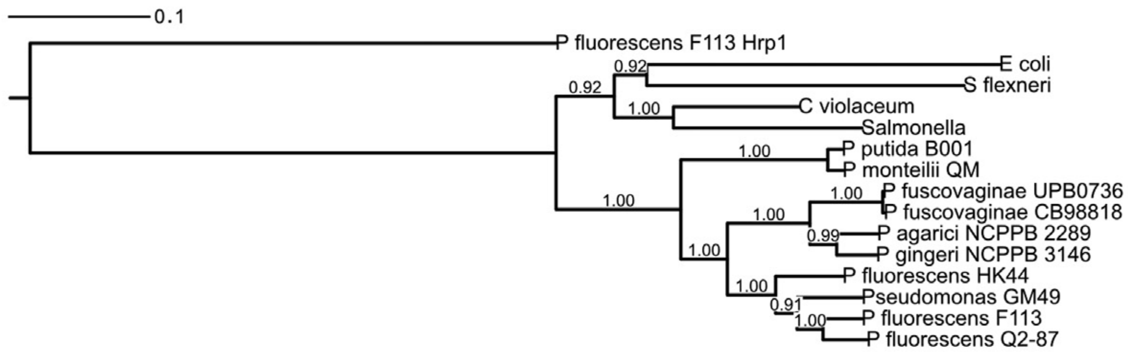
### *Genetic organization of the SPI-1 T3SS in* Pseudomonas

Comparative genomic analysis of the SPI-I clusters encoded in the strains belonging to the *P. fluorescens* group (Figs 1A and S1) indicates a predominantly conserved genetic organization (Fig. 1B). Indeed, all the protein coding genes potentially involved in the assembly of the export apparatus (*spaL*, *spaP*, *spaQ*, *spaR*, *spaS* and *invA*), the basal body (*prgH*, *prgJ*, *prjK* and *invG*), the needle (*prjl*) and the gene encoding a class II chaperone (*sicA*) display more than 60% identity to each other at the amino acid level. However, protein-coding genes involved in the assembly of the cytoplasmic sorting platform (*spaO*, *orgA* and *orgB*) and the putative translocon (*sipB*, *sipC* and *sipD*) are less conserved across the strains related to the *P. fluorescens* group (between 40 and 80% identity). In addition, some variations exist between the *P. fluorescens* SPI-1 clusters. For example, the gene *invB* encoding a putative class IB chaperone (Ehrbar *et al*., 2003) in *P. fluorescens* HK44 and *Pseudomonas* sp. GM49 is not found in SPI-1 clusters of *P. fluorescens* F113 and Q2-87, which could indicate the presence of different T3E repertoires between these strains. Finally, some proteins exhibit low sequence conservation to proteins generally encoded in other SPI-1 clusters from different bacterial species. For instance, the protein-coding gene PSF113_1783 located in place of *invJ* in the SPI-1 cluster of *Salmonella* displays no substantial sequence identity at the amino acid level with InvJ. Since InvJ is involved in controlling needle length (Cornelis, 2006), this might mean that the mechanism for controlling needle length may be different in *P. fluorescens*.

### *RspL and HilA are transcriptional activators of Hrp1 and SPI-1 genes*

Previous studies have shown that the transcriptional activator HilA and the alternative sigma factor RspL induce expression of genes located in the SPI-1 and Hrp1 clusters respectively (Bajaj *et al*., 1995; Jackson *et al*., 2005). To see if these findings were transposable to the SPI-1 and Hrp1 clusters of *P. fluorescens* F113, strains that constitutively expressed either *hilA* (PSF113_1794) or *rspL* (PSF113_5610) were engineered by cloning these genes in the broad host range vector pBBR1MCS5 (Kovach *et al*., 1995) (details on plasmid and mutants constructions are indicated in the experimental procedures provided in *Supporting information*). According to comparative transcriptomic analyses constitutive expression of *hilA* and *rspL* lead to slight transcriptome changes in F113, with 71 (approximately 1% of the whole predicted transcriptome) and 141 (2%) genes

A

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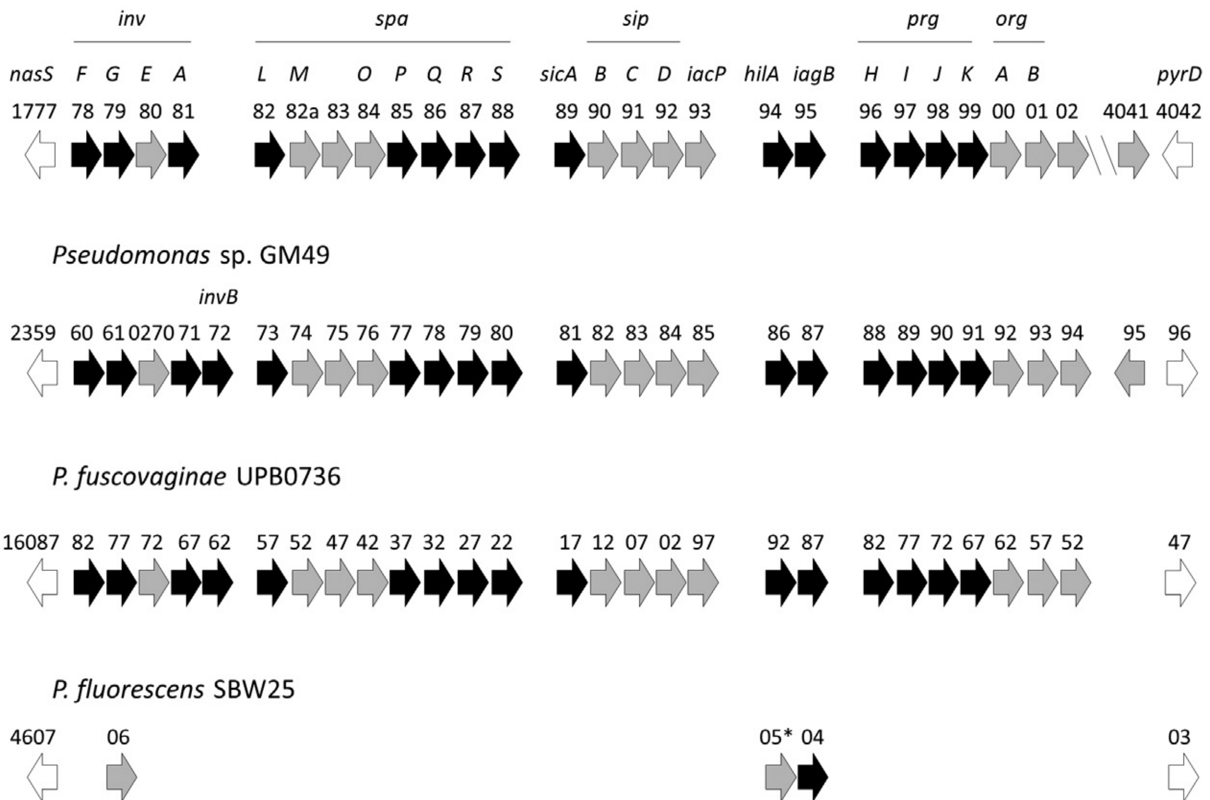


**Fig. 1.** Analysis of the SPI-1 cluster of different bacterial strains related to the *P. fluorescens* group.
A. Phylogenetic distribution of SPI-1 clusters. A distance tree (Maximum likelihood) was calculated from InvA homologues. Only aLRT support values greater than 0.75 (1000 replicates) are displayed.
B. Comparative genomic analysis of the SPI-1 T3SS clusters. Genes encoding T3SSs are represented as block arrows showing the direction of their transcription. Numbers represent the locus identifiers. White arrows represent genes encoding non-T3SS proteins. Grey and black arrows represent T3SS and T3E-related protein-coding genes with less or more than 60% identity at the amino acid level respectively. Asterisks indicate pseudogenes. The SPI-1 cluster of GM49 is located on two separate scaffolds.

**Table 1.** Microarray data validation by qRT-PCR.

| Locus | Gene name | Function | HilA/MCS5 (log2) | | RspL/MCS5 (log2) | |
|---|---|---|---|---|---|---|
| | | | Array | qRT-PCR | Array | qRT-PCR |
| PSF113_0435 | | Chitin binding protein | –2.3 | –0.7 ± 0.6 | –1.3 | n.d. |
| PSF113_1783 | | Type III secretion protein | 6.5 | 6.4 ± 0.3 | – | n.d. |
| PSF113_1794 | *hilA* | Type III secretion transcriptional activator HilA | 6.1 | 10.6 ± 0.4 | – | n.d. |
| PSF113_1799 | *prgK* | Type III secretion protein, PrgK | 5.9 | 8.2 ± 0.3 | – | n.d. |
| PSF113_1802 | | Putative Type III effector | 7.0 | 7.1 ± 0.2 | – | n.d. |
| PSF113_4041 | | Putative Type III effector | 5.3 | 6.3 ± 0.2 | – | n.d. |
| PSF113_3047 | | Non-ribosomal peptide synthetase | 1.5 | 1.6 ± 0.5 | – | n.d. |
| PSF113_4698 | | Transcriptional regulator, PadR family | 6.0 | 5.6 ± 0.3 | – | n.d. |
| PSF113_1126 | *ropAA1* | Type III effector RopAA1 | – | n.d. | 3.3 | 4.5 ± 0.5 |
| PSF113_3486 | *ropAA2* | Putative Type III effector RopAA2 | – | n.d. | – | 0.2 ± 0.1 |
| PSF113_5609 | *rspJ* | Type III secretion protein RspJ | – | n.d. | 3.9 | 5.1 ± 0.6 |
| PSF113_5610 | *rspL* | RNA polymerase sigma-70 factor, ECF subfamily, RspL | – | n.d. | 9.6 | 9.9 ± 0.2 |
| PSF113_5616 | *ropM* | Type III effector RopM | – | n.d. | 4.0 | 3.6 ± 0.2 |
| PSF113_1175 | | Baseplate assembly protein J | – | n.d. | –2.1 | –0.7 ± 0.7 |
| PSF113_4295 | | Phage tail fibre protein | – | n.d. | –8.0 | –12.7 ± 1.4 |
| PSF113_1757 | *pvdH* | Pyoverdin biosynthesis protein PvdH | – | n.d. | –1.8 | 0.1 ± 0.5 |

Microarray data presented here represent only one replicate for each strain. Only $\log_2$ ratios inferior to –1 and superior to 1 are shown in this table. Real-time PCR analyses were performed on some selected differentially expressed genes. Averages and associated standard deviations were calculated from three independent biological replicates, each having three technical replicates. All the values have been transformed to $\log_2$.

differentially regulated in comparison to the strain carrying the empty vector (Fig. S2). Since microarray data presented in this paper represent only one replicate for each strain, selected genes were validated by qRT-PCR on three independent biological replicates.

The transcriptional activator HilA positively regulates the expression of 51 genes, 26 of which are SPI-1 related (Tables 1 and S1). Among these 26 genes, 24 encode regulatory elements or proteins involved in the assembly of the T3SS and two (PSF113_1802 and PSF113_4041) encode proteins with T3 secretion signals. RspL positively regulates the expression of 41 genes, 29 of which are Hrp1 related (Tables 1 and S1). This includes all the genes encoded in the Hrp1 locus with the exception of *rspS*. This is not surprising since RspS orthologues act upstream of RspL orthologues in the regulatory cascades described in *P. syringae* and *Dickeya* sp. (Hutcheson *et al.*, 2001; Tang *et al.*, 2006). Whereas the putative T3Es *ropAA1*, *ropB* and *ropM* are induced by RspL, *ropAA2* is not differentially regulated. In addition to protein-coding genes related to Hrp1, RspL downregulates the expression of 40 genes located in a genomic region (PSF113_4278-4325), which is related to prophage 03 of of *P. protegens* Pf-5 (Mavrodi *et al.*, 2009). The reason for this downregulation is unknown, but interestingly, PrtB, the regulator of pyocin synthesis, is involved in the repression of the Ysc T3SS of *P. aeruginosa* PAO1 under the stress of DNA damage (Wu and Jin, 2005). Therefore, it is tempting to speculate that T3SSs and prophage elements are inversely regulated in *Pseudomonas*.

Twenty-one putative RspL binding sites (Mavrodi *et al.*, 2011) were identified, eight of which are located in intergenic regions (Table S2). The predicted promoter regions of these eight genes/operons were aligned and used to generate a consensus F113 RspL binding site (Fig. 2A). This consensus sequence 5′-YGGAAC-$N_{15–16}$-YCAC-$N_2$-A-3′ is slightly different from the initial *hrp* box 5′-KGGARCY-$N_{15–16}$-CCAC-$N_2$-A-3′ described in *P. syringae* (Zwiesler-Vollick *et al.*, 2002). Among the eight promoters possessing an *rsp* box, six are located upstream of genes upregulated in F113 pBBR-*rspL* (Table S2). Interestingly, the promoter located upstream of *ropAA2* possesses a divergent *rsp* box (Fig. 3A), which might explain why the expression of *ropAA2* is not induced in the strain F113 pBBR1MCS-*rspL*. Although a HilA binding site consisting of two hexamers separated by five nucleotides has been described in *Salmonella typhimurium* (Lostroh *et al.*, 2000) and *Escherichia coli* 042 (Sheikh *et al.*, 2006), no site exhibiting this consensus nucleotide sequence was found in the predicted promoters of SPI-1 genes of *P. fluorescens* F113.

### *GacA regulates hilA expression*

The expression of *hilA* and *rspL* were investigated in different synthetic media using promoter gene fusions. According to these promoter fusion assays, *rspL* is not expressed in Luria–Bertani, while a basal level of expression is observed for *hilA* (Fig. 3B and C). Addition of 10 mM of nitrilotriacetic acid, a di and tri metal cation chelator frequently used for induction of Ysc-T3SS gene

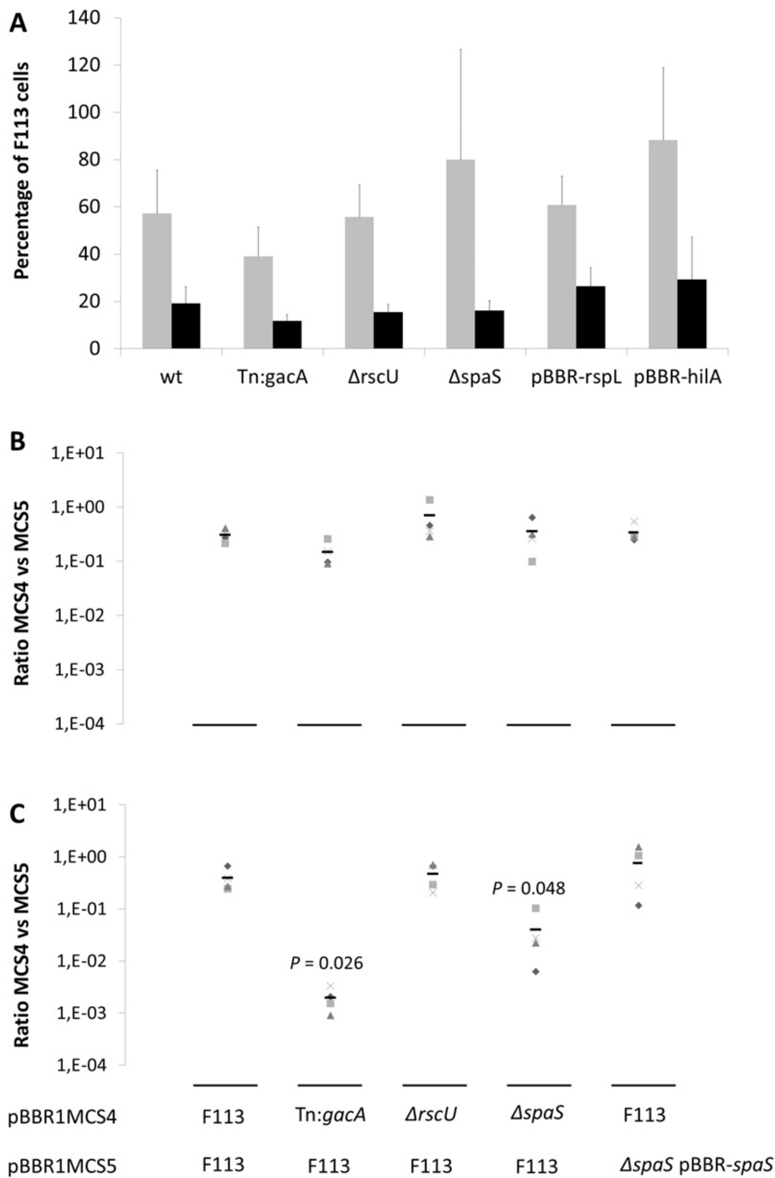


**Fig. 2.** *A. polyphaga–P. fluorescens* F113 interactions.
A. Survival of F113 strains (expressed as percentage) after 24 h (grey bars) and 48 h of co-culture with *A. polyphaga*. Values are mean of three biological replicates. Error bars represent standard errors of the means.
B. Relative fitness of mutant in PAS media.
C. Relative fitness of mutant in PAS media supplemented with amoeba. Fitness is expressed as ratio ($log_{10}$ transformed) between strains carrying the vector pBBR1MCS4 and strains carrying the vector pBBR1MCS5. Experiments have been performed on four independent replicates.

expression in *P. aeruginosa* (Rietsch and Mekalanos, 2006), did not increase *rspL* nor *hilA* promoter activities (Fig. S3). Furthermore, no expression of *hilA* and *rspL* was observed in *hrp*-inducing media (Huynh *et al*., 1989) (data not shown). Since the GacA orthologue of *Salmonella enterica*, SirA, positively regulates *hilA* expression (Altier *et al*., 2000), the *hilA* promoter fusion was introduced into the F113 *gacA* mutant strain FG9 (Delany, 1999; Aarons *et al*., 2000). In LB and LB NTA media, a decrease in *hilA* promoter activity was observed in FG9 in comparison to F113 (Figs 3C and S3). This suggests that GacA is also a positive regulator of *hilA* in *P. fluorescens* F113.

### *The SPI-1 T3SS of* P. fluorescens *F113 is involved in grazing resistance*

To assess the potential role(s) of SPI-1 and Hrp1 T3SSs in interactions with organisms present in the rhizosphere, in-frame deletions of the *spaS* and *hrcU* genes were generated. Indeed mutations of *spaS* and *hrcU* homologues abolish the secretion of needle and translocator subunits, resulting in non-functional T3SSs (reviewed in Izoré *et al*., 2011). The effect of both T3SSs of *P. fluorescens* F113 on the mortality of *Caenorhabditis elegans* was first tested. Under slow-killing conditions (Tan *et al*., 1999), the *gacA* negative strain FG9 is severely defective in its ability to kill

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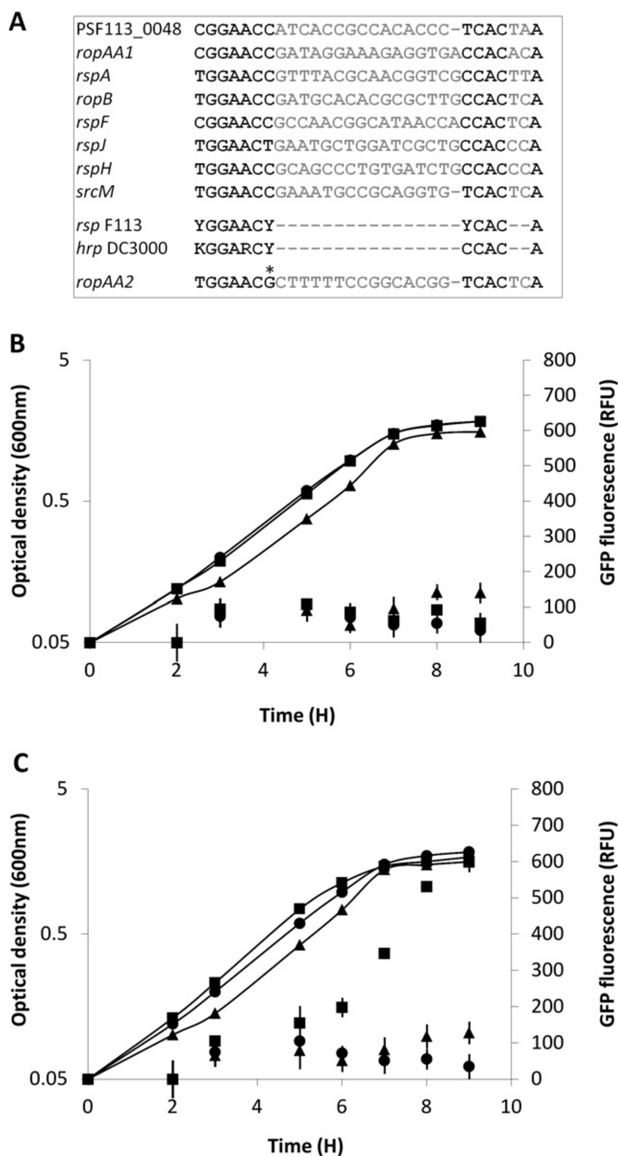


**Fig. 3.** Expression of *hilA* and *rspL* in synthetic media.
A. Consensus '*rsp* box' sequence in *P. fluorescens* F113. The putative RspL binding sites of eight genes/operons were aligned using the CLUSTALW program. A consensus sequence (Rsp_F113) is indicated at the bottom of the alignment. This consensus sequence is slightly different from the Hrp box of DC3000 (Zwiesler-Vollick *et al.*, 2002). The putative promoter of *ropAA2* potentially contains a divergent RspL binding site.
B. Growth curves (principal *y* axis) and relative fluorescence units (secondary *y* axis) were monitored in F113 pPROBE-GT (circle), F113 pPROBE-*rspL* (square) and in FG9 pPROBE-*rspL* (triangle).
C. The same assay was performed with F113 pPROBE-GT (circle), F113 pPROBE-*hilA* (square) and in FG9 pPROBE-*hilA* (triangle). The strain FG9 is a F113 derivative carrying a transposon insertion in *gacA* (Delany, 1999). Strains of *P. fluorescens* F113 carrying *rspL* (B) and *hilA* (C) transcriptional fusions were grown in LB at 30°C shaking. Each value is the mean of three samples obtained from three independent experiments. Error bars represent standard errors of the means.

*C. elegans*, confirming previous results obtained with *P. protegens* CHA0 (Neidig *et al.*, 2011) (Fig. S4A). In addition, worms exposed to the *spaS* mutant exhibited an increase in mortality rate relative to control worms feeding on *P. fluorescens* F113 ($P < 0.0002$) (Fig. S4B). The trans-complemented strain presented a virulence activity comparable to the wild type. Although, the same effect is observed for the *rscU* mutant, this phenotype is not restored by trans-complementation (Fig. S4C). The increase in the mortality rate of *C. elegans* exposed to *spaS* mutants could be explained by abolition of SPI-1 T3Es translocation and subsequent inhibition of defensive reaction in *C. elegans*.

Previous studies have shown that different T3SS families can target a range of unicellular amoebae such as *Dictyostelium discoideum* and *Acanthamoeba castellanii* (Pukatzki *et al.*, 2002; Matz *et al.*, 2011; Sperandio *et al.*, 2012). Since soil bacteria are naturally consumed by protozoan predators (Rosenberg *et al.*, 2009), the potential influence of Hrp1 and SPI-1 was assessed during *P. fluorescens* F113–*Acanthamoeba polyphaga* interactions. To test whether *P. fluorescens* F113 was susceptible to grazing, *A. polyphaga* cells were cocultivated with different F113 strains. After 1 and 2 days of co-culture the relative survival rate of F113 is around 60% and 20% respectively (Fig. 2A). This survival rate is neither significantly different for strains mutated in the *gacA*, *rscU* and *spaS* genes nor for strains overexpressing *hilA* and *rspL*. The result obtained with *gacA* mutant is somewhat surprising since *gacS* mutants of *P. protegens* CHA0 are preferentially grazed by *A. castellanii* (Jousset *et al.*, 2009). A selective feeding assay, in which two bacterial strains were co-inoculated as a mixed population with *A. polyphaga*, was then performed. The bacterial strains were inoculated at a ratio of 1:1 in PAS with or without *A. polyphaga* (Fig. 2B and C). In order to distinguish strains in mixed treatments, the empty vectors pBBR1MCS4 and pBBR1MCS5 were introduced in the different bacterial strains. After 48 h of interaction no difference in fitness was observed between F113 and its isogenic mutants when the bacterial strains were grown in PAS (Fig. 2B). However, when amoebae were present in the media the fitness of the *gacA* mutant was drastically reduced (around 100-fold) in comparison with the wild type (Fig. 2C), confirming previous findings obtained with *gacS* mutant (Jousset *et al.*, 2009). Interestingly, a decrease of fitness was also observed for *spaS* mutant (approximately 25-fold) and was trans-complementable by introduction of *spaS* in the vector pBBR1MCS5 (Fig. 2C). This suggests that the SPI-1 T3SS of *P. fluorescens* F113 might be involved in resistance to grazing. According to our data, the T3SS-mediated resistance of F113 to predation is likely to be efficient only in mixed bacterial population,

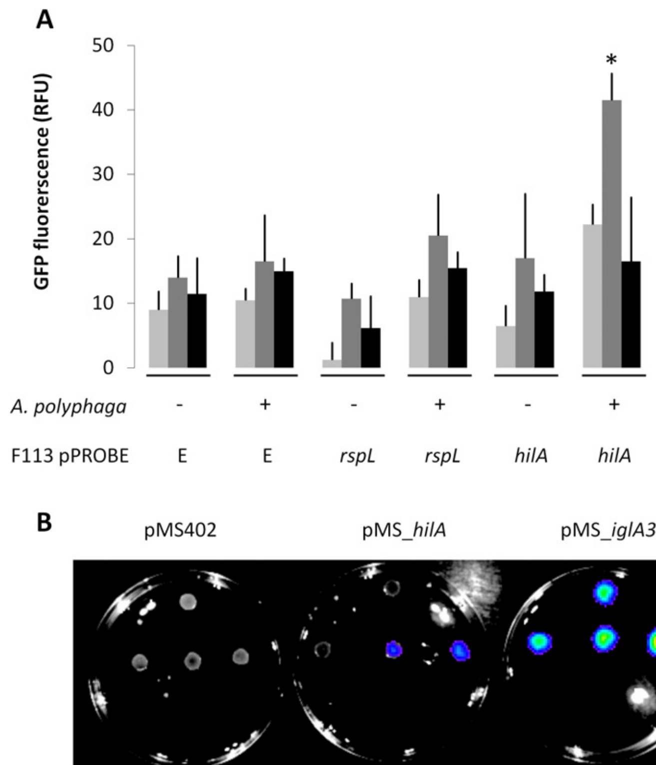


**Fig. 4.** Induction of *hilA* by *A. polyphaga*.
A. Promoter activity of *hilA* during F113–*A. polyphaga* interaction. Expression of *gfp* reporter fusions were monitored from aliquots of 200 μl harvested directly from wells at different time points. Light grey, dark grey and black bars represented 6, 24 and 48 h time-points respectively. Values are means of three biological replicates. Error bars represent standard errors of the means. Asterisks indicate a significant difference in relative fluorescence unit according to unpaired student *t*-test (*P*-value <0.05).
B. Composite photographic and luminescence images of *hilA* and *iglA3* promoter fusions. *Pseudomonas fluorescens* strains carrying the promoterless vector pMS402, pMS-*hilA* or pMS-*iglA3* were spot-inoculated on PYG medium supplemented with trimethoprim 1000. Four spots corresponding to F113 strains without (upper spot) or with amoebae inoculated at a distance of 1 cm (left), 0.5 cm (centre) or in contact (right) with the bacterial spot are presented on each plate. Pictures were taken after 48 h of interaction. The log scales on the right of each image represent luminescence in photons per second per square centimetre per steradian.

such as those found in soil, where SPI-I negative bacteria may be preferentially consumed. This phenomenon is similar to the preferential feeding of bacteriovorus amoebae and nematodes on non-toxic *gacS* mutants (Jousset *et al*., 2009; 2010).

The effect of *A. polyphaga* on the expression of the T3SS transcriptional activators *hilA* and *rspL* was tested using promoter gene fusions. Under the same conditions used for grazing experiments, the activity of the *hilA* promoter is significantly increased in strains inoculated in PAS supplemented with *A. polyphaga* after 24 h of interaction (Fig. 4A). To confirm this finding, a different bioassay was performed with another reporter gene system based on luminescence. In this assay the relative amount of luminescence of F113 strains carrying the empty vector pMS402, pMS-*hilA* or pMS-*iglA3* was compared on a PYG media when amoebae were inoculated at a distance of 1 cm, 0.5 cm or in contact with spot-inoculated bacteria. The promoter of the T6SS structural gene *iglA3*, which belongs to the HSI-3 locus of *P. fluorescens* F113 (Barret *et al*., 2011) was chosen as a positive control of luminescence since expression of this gene is relatively high under *in vitro* conditions (F. Egan, M. Barret and F. O'Gara, unpubl. data). After 48 h luminescence signals were registered using the IVIS 100 Imaging System. While *iglA3* promoter activity is constant under the different conditions tested (presence or absence of *A. polyphaga*), *hilA* promoter activity is increased when the amoebae were present in close contact (Fig. 4B). Altogether, these results suggest that *A. polyphaga* induces *hilA* expression, which in turn probably activates the expression of SPI-1 genes. This suggests that resistance of F113 to predation requires contact between the two organisms.

## Conclusions

*Pseudomonas fluorescens* F113 possesses two T3SSs belonging to the Hrp1 and SPI-1 families. The genetic expression of these two loci is regulated by the alternative sigma factor RspL and the transcriptional activator HilA respectively. While the role of Hrp1 is unknown, SPI-1 seems to be involved in grazing resistance to bacteriovorous protists. Identification and subsequent characterization of T3Es translocated by the SPI-1 T3SS will probably provide useful information about the mechanistic basis of F113 resistance to grazing.

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## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Appendix S1.** Experimental procedures

**Table S1.** Transcriptome profiles of pBBR1MCS-*hilA* and pBBR1MCS-*rspL* strains.

**Table S2.** Prediction of RspL binding site in the genome of *P. fluorescens* F113.

**Table S3.** Bacterial strains and plasmids used.

**Table S4.** Oligonucleotides used.

**Fig. S1.** Distribution of T3SSs in *Pseudomonas* spp. Phylogenomic analysis of 135 *Pseudomonas* genomes was performed using CVTree (Xu and Hao, 2009). *Escherichia coli* K12-DH10B was used as out-group. Strains highlighted in brown are related to the *P. fluorescens* group. Genomes related to *P. protegens* Pf-5, *P. fluorescens* Pf0-1, *P. fluorescens* SBW25, *P. brassicacearum* NFM421 and *P. fluorescens* have been sequence entirely, while genomes of strains belonging to the *P. fluorescens* group are incomplete. The ORFs (open reading frames) PA1703, PSPTO_1402, PSPPH_2520 and PSF113_1781 (COG4789), representing archetypical Ysc, Hrp1, Rhizobial and SPI-1 T3SS proteins, were used as baits in sequential BLASTp and TBLASTn searches to identify T3SS loci in the 135 *Pseudomonas* genomic sequences. Square dots indicate that the T3SS-loci are incomplete.

**Fig. S2.** Genes regulated by RspL and HilA.

A and B. Comparisons of transcripts abundance between F113 carrying pBBR1MCS5-*rspL* or pBBR1MCS5-*hilA* (+) and F113 carrying the empty vector pBBR1MCS5 (–).

C. Plot of F113 pBBR1MCS5-*rspL* versus F113 pBBR1MCS5 average log intensities.

D. Plot of F113 pBBR1MCS5-*hilA* versus F113 pBBR1MCS5 average log intensities.

E. Venn diagram showing the overlap between genes differentially regulated in pBBR1MCS5-*hilA* and pBBR1MCS5-*rspL*.

**Fig. S3.** Promoter fusion activities of *rspL* and *hilA* in LB NTA medium. Strains of *P. fluorescens* F113 carrying *rspL* and *hilA* transcriptional fusions were grown in LB NTA (10 mM) at 30°C shaking. Growth curves (principal *y* axis) and relative fluorescens units (secondary *y* axis) were monitored in F113 pPROBE-GT (blue diamond), F113 pPROBE-*rspL* (red square), F113 pPROBE-*hilA* (green triangle), in FG9 pPROBE-*rspL* (purple arrow) and FG9 pPROBE-*hilA* (black arrow). Each value is the mean of three samples obtained from the same biological replicate.

**Fig. S4.** *P. fluorescens* virulence towards *C. elegans* worms. Slow killing Kaplan–Meier survival plots of worms fed with *P. fluorescens* F113 wt (n = 113), Δ*rscU* ($n = 228$), Δ*rscU* pBBR1-*rscU* ($n = 124$), Δ*spaS* ($n = 158$) or Δ*spaS* pBBR1-*spaS* ($n = 173$). Each value reported for the assay is the mean of measurements of six samples from two independent experiments. Asterisks indicate significant differences ($P < 0.001$) between F113 wild type and the corresponding mutant tested, as assessed by pairwise comparisons (log rank test). The strain FG9 is a F113 derivative carrying a transposon insertion in *gacA* (Delany, 1999).