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Endophytic colonization and biocontrol performance of *Pseudomonas fluorescens* PICF7 in olive (*Olea europaea* L.) are determined neither by pyoverdine production nor swimming motility

M. Mercedes Maldonado-González,[1] Elisabetta Schilirò,[1] Pilar Prieto[2] and Jesús Mercado-Blanco[1]*

Departments of [1]Crop Protection and [2]Plant Breeding, Institute for Sustainable Agriculture, Agencia Estatal Consejo Superior de Investigaciones Científicas (CSIC), Alameda del Obispo s/n, Apartado 4084, E-14080 Córdoba, Spain.

Summary

Pseudomonas fluorescens **PICF7 is an indigenous inhabitant of olive (*Olea europaea* L.) rhizosphere, able to display endophytic lifestyle in roots, to induce a wide range of defence responses upon colonization of this organ and to exert effective biological control against Verticillium wilt of olive (VWO) (*Verticillium dahliae*). We aimed to evaluate the involvement of specific PICF7 phenotypes in olive root colonization and VWO biocontrol effectiveness by generating mutants impaired in swimming motility (*fliI*) or siderophore pyoverdine production (*pvdI*). Besides, the performance of mutants with diminished *in vitro* growth in potato dextrose agar medium (*gltA*) and cysteine (Cys) auxotrophy was also assessed. Results showed that olive root colonization and VWO biocontrol ability of the *fliI*, *pvdI* and *gltA* mutants did not significantly differ from that displayed by the parental strain PICF7. Consequently, altered *in vitro* growth, swimming motility and pyoverdine production contribute neither to PICF7 VWO suppressive effect nor to its colonization ability. In contrast, the Cys auxotroph mutant showed reduced olive root colonization capacity and lost full biocontrol efficacy. Moreover, confocal laser scanning microscopy revealed that all mutants tested were able to endophytically colonize root tissue to the same extent as wild-type PICF7, discarding these traits as relevant for its endophytic lifestyle.**

Received 4 September, 2014; revised 12 November, 2014; accepted 19 November, 2014. *For correspondence. E-mail jesus.mercado@ias.csic.es; Tel. (+34) 957 499 261; Fax (+34) 957 499 252.

Introduction

Verticillium dahliae Kleb. is a soil-borne phytopathogenic fungus causing vascular diseases collectively known as Verticillium wilts in a wide range of plant species (Pegg and Brady, 2002). A susceptible host for *V. dahliae* is olive (*Olea europaea* L.), which is one of the most important woody crops in the Mediterranean Basin. Olive cropping is threatened by diverse (a)biotic menaces of variable importance which highly depend on a number of factors (soil characteristics, climatic conditions, soil resident microbiota, agricultural practices, etc.). One of the most important biotic constraints for olive cultivation is *V. dahliae*. This disease has been steadily spreading in many areas where olive is a relevant commodity, causing great concern to farmers and the olive oil industry (López-Escudero and Mercado-Blanco, 2011).

The effective control of Verticillium wilt of olive (VWO) is very difficult due to several factors (López-Escudero and Mercado-Blanco, 2011). Because of this complex scenario, an integrated disease management strategy has been proposed as the only plausible way to control VWO, combining physical, chemical, biological and agronomical measures. Within this framework, preventive measures (pre-planting) are mostly encouraged, although palliative actions (post-planting) are also needed to limit the expansion of the pathogen or to alleviate losses caused by the disease in established orchards (Tjamos, 1993; López-Escudero and Mercado-Blanco, 2011).

The use of effective biological control agents (BCAs) is a promising tool that can be used both as a preventive and palliative measure. For instance, application of microbial antagonists in pathogen-free certified olive plants during the propagation process at nurseries has been proposed (Tjamos, 1993). There are only few reports on the identification and characterization of potential BCAs against VWO, although mostly preliminary or showing limited success (Müller *et al.*, 2007; Aranda *et al.*, 2011; Sanei and Razavi, 2011; Mercado-Blanco and

López-Escudero, 2012). Moreover, very little is known about the mechanisms involved in biocontrol activity exerted by these microbes.

A well-known effective BCA against VWO is *Pseudomonas fluorescens* PICF7 (Mercado-Blanco *et al*., 2004; Prieto *et al*., 2009). This strain, originally isolated from roots of nursery-propagated olive plants, displays an endophytic lifestyle (Prieto and Mercado-Blanco, 2008; Prieto *et al*., 2009; 2011), produces the siderophore pyoverdine (= pseudobactin) (Pvd) *in vitro* (Mercado-Blanco *et al*., 2004), and induces a broad range of defence responses in both roots (Schilirò *et al*., 2012) and above-ground organs (Gómez-Lama Cabanás *et al*., 2014) of olive. Nevertheless, nothing is known about PICF7 traits involved in both endophytic colonization and biocontrol effectiveness against *V. dahliae*.

Efficient colonization and persistence on/within the targeted plant tissue where biocontrol pseudomonads may deploy its biocontrol activity is essential for successful crop protection (Lugtenberg *et al*., 2001; Mercado-Blanco and Bakker, 2007). While bacterial traits involved in colonization of the rhizosphere and plant root surface have been studied in some detail, little is known about determinants implicated in the active colonization of the root interior by biocontrol endophytic bacteria (Hardoim *et al*., 2008; Reinhold-Hurek and Hurek, 2011). For instance, it is suggested that diverse cell wall-degrading enzymes and motility are involved in the endophytic colonization process (Hallmann *et al*., 1997; Compant *et al*., 2005; Reinhold-Hurek and Hurek, 2011). Bacterial endophytes are adapted to live within a protected niche, less exposed to (a)biotic stresses and relying on a constant source of nutrients provided by the host plant (Bacon and Hinton, 2006). In contrast, they must cope with the defence barriers deployed by the plant to confront this 'non-hostile' colonization (Wang *et al*., 2005; Conn *et al*., 2008; Schilirò *et al*., 2012). Therefore, beneficial bacterial endophytes effective in promoting plant growth and suppressing deleterious microorganisms are an excellent source of biotechnological weapons to be exploited in agro-ecosystems (Mercado-Blanco and Lugtenberg, 2014). For instance, endophytic *Pseudomonas* spp. have proven to provide benefits for the host plant in several cases (Chen *et al*., 1995; Nejad and Johnson, 2000; Kuklinsky-Sobral *et al*., 2004; Prieto *et al*., 2009). As mentioned for the colonization process, biocontrol mechanism(s) deployed by endophytes also remain largely unknown. However, since bacteria able to develop an endophytic lifestyle usually originate from the rhizosphere, it is plausible to assume that their beneficial effects may operate similarly to those described for rhizosphere-associated bacteria (Kloepper and Ryu, 2006; Mercado-Blanco and Lugtenberg, 2014).

The present study is, therefore, aiming to shed light on determinants of PICF7 potentially involved in superficial and endophytic colonization of olive roots and biocontrol activity against *V. dahliae*. We have particularly focused on the implication of motility and siderophore production. Motility is one of the most important traits for efficient rhizosphere colonization by specific *Pseudomonas* spp. strains (Navazo *et al*., 2009). Yet the actual contribution of bacterial motility in rhizosphere (and endophytic) colonization may vary among plant–bacteria interactions. Thus, non-motile derivatives or mutants with reduced motility can be impaired in competitive colonization of the rhizosphere by beneficial bacteria in several plants (Lugtenberg *et al*., 2001; Ormeño-Orrillo *et al*., 2008). In contrast, *P. fluorescens* Q8r1-96 exhibiting decreased motility was not impaired in rhizosphere colonization (Mavrodi *et al*., 2006). Plant beneficial *Pseudomonas* spp. may synthesize a wide diversity of siderophores, iron (Fe^{3+})-chelating compounds frequently related with the biocontrol activity exerted by many strains of this genus (Mercado-Blanco and Bakker, 2007). Nevertheless, the true implication of *Pseudomonas*-produced siderophores in biological control of soil-borne pathogens is a controversial issue (Cornelis and Matthijs, 2002; Weller, 2007; Lemanceau *et al*., 2009; Bakker *et al*., 2014).

In order to elucidate potential mechanisms implicated in olive root colonization (even endophytically) and suppression of VWO by *P. fluorescens* PICF7, a mutant bank of strain PICF7 was generated by random transposon insertion. A set of generated mutants was mainly screened for siderophore production and swimming motility. Selected mutants were further characterized and assessed for their ability to colonize olive root tissues and for their VWO biocontrol performance. Colonization and disease suppression bioassays were performed under non-gnotobiotic conditions, a scenario closer to the environmental conditions where the interaction between olive roots and *P. fluorescens* PICF7 takes place.

Results

Construction of a mutant library of Pseudomonas *fluorescens PICF7*

A total of 9300 *P. fluorescens* PICF7 tetracycline-resistant (Tc^R) colonies were obtained after biparental mating between *Escherichia coli* DH5α (pJQ18) and PICF7 (Table 1). Transposon Tn5-Tc^R insertion frequency was calculated as 2×10^{-5}. The number of mutants thus obtained was theoretically enough to have more than one random insertion every 1 kb throughout the genome of *P. fluorescens* PICF7 (~ 6.1 Mbp) (P.M. Martínez, D. Ruano-Rosa, E. Schilirò, P. Prieto, C. Ramos, P. Rodríguez-Palenzuela and J. Mercado-Blanco,

Table 1. Bacterial strains and plasmids used in this study.

Strains or plasmids	Characteristics	Reference or source
Bacterial strains		
P. fluorescens		
PICF7	Wild-type strain isolated from olive roots	Mercado-Blanco and colleagues (2004)
ME419	PICF7 Tn5 (Tc^R) *in vitro*-reduced growth mutant derivative, GltA$^-$	This work
ME424	PICF7 Tn5 (Tc^R) swimming motility mutant derivative, FliI$^-$	This work
ME589	PICF7 Tn5 (Tc^R) pyoverdin mutant derivative, PvdI$^-$	This work
ME1508	PICF7 Tn5 (Tc^R) Cys auxotroph mutant derivative	This work
PICF7-G	PICF7 (Gm^R) GFP-labelled derivative	This work
ME419-G	ME419 (Tc^R and Gm^R) GFP-labelled derivative	This work
ME424-G	ME424 (Tc^R and Gm^R) GFP-labelled derivative	This work
ME589-G	ME589 (Tc^R and Gm^R) GFP-labelled derivative	This work
ME1508-G	ME1508 (Tc^R and Gm^R) GFP-labelled derivative	This work
Escherichia coli		
S17-1	*thi pro recA hsdR hsdM* RP4-2-Tc,Mu-Km, Tp^RSm^R	Simon and colleagues (1983)
DH5α	*recA1 endA1* Φ80d *lacZ dam-15*	(Clontech)
Plasmids		
pJQ18	pSUP5011 derivative; carries Tn5-Mob-Tc	Alfred Pühler
pLRM1	pBBR1-MCS5 carrying a fusion of the $P_{A1/04/03}$ promoter to the *gfpmut3** gene	Rodríguez-Moreno and colleagues (2009)

GltA, type II citrate synthase; FliI, flagellum-specific ATP synthase; PvdI, putative pyoverdine non-ribosomal peptide synthetase; Tc, tetracycline; Cys, cysteine; Gm, gentamicin; GFP, green fluorescence protein.

unpublished). The percentage of insertions leading to auxotrophy was 1.36%.

Screening and selection of PICF7 mutants affected in swimming motility, siderophore production or antagonism against V. dahliae

Results showed that *P. fluorescens* PICF7 exhibited swimming motility when tested in 'swimming medium' (SM) (Fig. 1). Clear evidence of swarming or twitching motilities was not obtained for strain PICF7 under tested conditions, and thus they were not further investigated. A set of 2000 Tn5-Tc^R insertion mutants were then checked for the loss/alteration in (i) swimming motility, (ii) siderophore production and (iii) *in vitro* antagonism against *V. dahliae* defoliating (D) pathotype. From a preliminary screening, 55 Tc^R mutants affected in one or more of these phenotypes were finally selected for further characterization (Table S1). Screening of this subset of mutants revealed that 13 of them displayed altered swimming motility phenotypes compared with that of the parental strain. Five mutants showed no motility or less than 10% than wild-type PICF7, while nine mutants displayed a reduction ranging from 10% to 70% of the wild-type phenotype (Table S1).

Siderophore production by Tn5-Tc^R insertion mutants was assessed in different media [chrome azurol S (CAS), King's B agar (KBA) and standard succinate medium (SSM)]. A total of 46 mutants were found to exhibit a modified siderophore-producing phenotype depending on the media used. For instance, 10 mutants were completely impaired in Pvd production, since neither green fluorescence nor production of an orange halo around the colonies was found in any of the culturing media tested in comparison to the wild-type phenotype of strain PICF7 (Table S1). Mutants partially altered in siderophore production (i.e. reduced orange halo) were not considered for further characterization in the present study.

The screening for *in vitro* antagonism exerted by Tn5-Tc^R insertion mutants against the *V. dahliae* D isolate V937I (highly virulent) was carried out in potato dextrose agar (PDA) medium. After evaluating a set of 2000 insertion mutants, none of them were inhibiting *V. dahliae* growth inhibition halo, similarly to the phenotype displayed by the parental strain PICF7. During this screening process, one Tn5-Tc^R colony showed reduced growth on PDA (Fig. 1B). This altered phenotype was not observed in KBA, Luria–Bertani agar (LBA) and nutrient agar media (Fig. 1). This mutant was also selected for further characterization (see below).

Identification of genes disrupted in selected PICF7 mutants

Among the 55 pre-selected mutants, 44 were finally confirmed as affected in just one of the phenotypes under study. Localization of the Tn5-Tc^R insertion in these 44 mutants was performed by nested-polymerase chain reaction (PCR) analysis. Nine mutants amplified a single band that was eluted, purified and sequenced. Based on the altered phenotype, the presence of a unique amplicon after nested-PCR, and the disrupted gene identified, four mutants (Fig. 1) were finally selected for evaluation in subsequent olive root colonization and VWO biocontrol

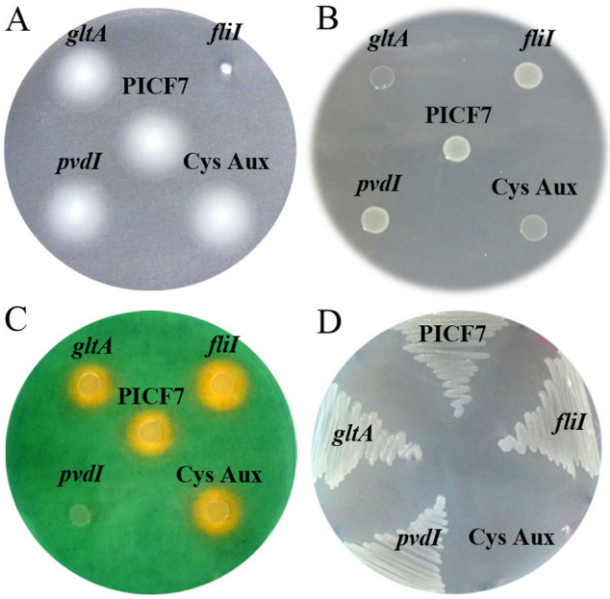

Fig. 1. Phenotypes of *Pseudomonas fluorescens* PICF7 and selected Tn5-Tc^R insertion mutants in SM (A), PDA (B), CAS (C) and SSM (D) agar media. *gltA*, mutant ME419 showing *in vitro* growth delay in PDA; *fliI*, mutant ME424 impaired in swimming motility; *pvdI*, mutant ME589 impaired in pyoverdine (Pvd) production; and Cys Aux, mutant ME1508 (cysteine auxotroph).
A. Individual colonies from overnight cultures of strain PICF7 and mutant derivatives grown on KBA plates were inoculated using a toothpick onto SM and incubated overnight at 25°C. Strains PICF7, *gltA*, *pvdI* and Cys Aux showed swimming motility around the inoculation point but not the *fliI* mutant.
B. Five-microlitre drops of overnight cultures of each strain were deposited over PDA medium and incubated at 25°C for 4 days. Mutant *gltA* showed reduced growth compared with strain PICF7 and other mutants.
C. Five-microlitre drops of fresh cell suspensions of strain PICF7 and its mutants were deposited over CAS medium agar plates and incubated overnight at 25°C. All mutant derivatives but mutant *pvdI* produced similar orange haloes as that observed for wild-type strain PICF7.
D. Growth of PICF7 and its mutant derivatives on SSM after 48 h at 25°C.

bioassays. Mutant ME424 has completely lost swimming motility (Fig. 1), and transposon insertion was located within a putative *fliI* gene homologue, coding for a flagellum-specific ATP synthase (Table 2). Mutant ME589 was totally impaired in Pvd production (no halo on CAS medium and no green fluorescence in KBA/SSM media) (Fig. 1) and harboured the Tn5 insertion in a putative *pvdI* gene homologue that codes for a Pvd non-ribosomal peptide synthetase (Table 2). Mutant ME419, which displayed growth delay in PDA medium (Fig. 1), carries the Tn5-Tc^R insertion in a putative homologue of the *gltA* gene, potentially coding for a type II citrate synthase (Table 2). Finally, mutant ME1508 was randomly selected from auxotroph mutants obtained during the mutagenesis process (Fig. 1). Sequence analysis of adjacent regions to the Tn5-Tc^R insertion in this mutant revealed that the transposon was located in a gene coding for a putative sulfite reductase involved in Cys biosynthesis (Table 2). Indeed, *in vitro* cross-feeding assays showed that addition of L-Cys to SSM medium (up to 20 mg l^{-1}) fully restored ME1508 growth and Pvd production ability in SSM to wild-type PICF7 levels (Table 3). Thus, in addition to *pvdI* (Pvd defective, ME589) and *fliI* (swimming motility defective, ME424) mutants, the *gltA* (reduced *in vitro* growth, ME419) and Cys auxotroph (ME1508) mutants were selected to be included in the *in planta* bioassays as examples of metabolism-altered phenotypes. Thus, *in vitro* growth delay, Cys auxotrophy, siderophore-mediated Fe^{3+} competition and motility were evaluated as per their potential role in rhizosphere competence, olive root colonization and/or biocontrol ability of strain PICF7.

Olive root colonization ability of P. fluorescens *PICF7 mutants*

To determine whether Tc^R mutant derivatives ME424, ME589, ME419 and ME1508 colonize olive roots to the same extent as strain PICF7, three roots per treatment and per biocontrol assay (see below) were examined at the end of the experiments (approximately 110 days after

Table 2. Identification of genes disrupted in selected PICF7 mutants.

Mutant	Amplicon size	Mutant type	Accession number	Closest species/strain	Query cover (%)	E-value	Identity %	Function
ME419	354 bp	Altered *in vitro* growth	AHF49667.1	*Pseudomonas* sp. RM12EL_44B	87	3e-46	74	type II citrate synthase
ME424	426 bp	Swimming motility	WP_010208746.1	*Pseudomonas* sp. R81	84	2e-145	100	Flagellum-specific ATP synthase
ME589	185 bp	Pyoverdine production	1476433	*Pseudomonas fluorescens* A506	49	2e-08	64	Putative pyoverdine non-ribosomal peptide synthetase
ME1508	420 bp	Cysteine auxotroph	WP_012723853.1	*Pseudomonas fluorescens*	99	2e-95	100	Putative sulfite reductase

Disrupted genes identification was carried out by obtaining amplicons of the flanking Tn5 regions in PICF7 Tc^R-mutant derivatives by a combination of arbitrary and nested-PCR, followed by sequence comparison in available databases using the BLASTX programme (see text for details).

Table 3. Bacterial growth (OD_{600}) and pyoverdine production ($OD_{400/600}$) by strain PICF7 and its mutant derivative ME1508 in SSM supplemented with L-Cys.

L-Cys (mg l^{-1})	Bacterial growth (OD_{600})[a]		Pyoverdine ($OD_{400/600}$)[b]	
	PICF7	ME1508	PICF7	ME1508
0	0.40 ± 0.03	0.06 ± 0.01*	3.0 ± 0.15	1.5 ± 0.32*
1	0.44 ± 0.11	0.09 ± 0.01*	2.9 ± 0.11	2.2 ± 0.27*
2	0.43 ± 0.14	0.15 ± 0.02*	2.7 ± 0.11	2.3 ± 0.19*
5	0.46 ± 0.05	0.34 ± 0.03*	2.8 ± 0.17	2.2 ± 0.09*
10	0.38 ± 0.04	0.42 ± 0.15	3.1 ± 0.22	2.5 ± 0.16*
20	0.41 ± 0.03	0.43 ± 0.04	2.9 ± 0.14	2.9 ± 0.20

a. Bacterial growth (OD_{600}) measured 48 h after inoculation of strains in SSM and SSM + L-Cys (see text for details).
b. Production of pyoverdine was calculated according to Djavaheri and colleagues (2012).
Data are means of three replicas from three independent experiments. Means in ME1508 columns followed by an asterisk are significantly different from PICF7 means according to Fisher's protected least significant difference test ($P < 0.05$).

bacterization, DAB). In experiment I, population sizes of introduced bacteria were not significantly different ($P = 0.34$), although mutants always displayed lower population size values compared with strain PICF7 (Table 4). In bioassay II, however, a significantly ($P < 0.05$) lower population size was found for the Cys auxotroph mutant ME1508 compared with that exhibited by strain PICF7 but not with the other mutants (Table 4). Finally, in bioassay III, population sizes of *fliI* and Cys auxotroph mutants were significantly ($P < 0.05$) lower than that of strain PICF7 (Table 4).

Root endophytic colonization ability is not affected in P. fluorescens *PICF7 mutants*

To visualize olive roots surface/inner colonization green fluorescence protein (GFP)-labelled derivatives of PICF7 and the selected Tc^R mutants were generated. Transformation frequency of plasmid pLRM1 ranged from 4.7×10^2 to 7.6×10^3 transformants/μg plasmid DNA. Introduction of plasmid pLRM1 did not affect the behaviour of the mutants, and all selected GFP-labelled clones showed similar phenotypes as their parents (Fig. 1 and Fig. S1). While plasmid pLRM1 revealed as not completely stable neither in PICF7 nor in their mutants (6.14% plasmid loss/generation), the presence of a GFP-labelled bacterial cells within olive root tissues for each strain was

Table 4. Root colonization ability and Verticillium wilt of olive biocontrol performance of *Pseudomonas fluorescens* PICF7 and its Tc^R mutant derivatives.

Experiment[1]	Treatment[2]	Disease assessment[3]			Bacterial population (log_{10} cfu g^{-1} of fresh root)[4]
		SAUDPC	DII	DI (%)	
I	Control	0.36[a]	0.59	93.33	
	PICF7	0.09[c]	0.24	78.57	4.3 ± 0.5[a]
	ME419 (*gltA*)	0.24[abc]	0.44	93.33	2.9 ± 0.8[a]
	ME424 (*fliI*)	0.16[bc]	0.43	84.62	3.3 ± 0.9[a]
	ME589 (*pvdI*)	0.17[bc]	0.33	78.57	3.4 ± 0.7[a]
	ME1508 (Cys Aux)	0.34[ab]	0.51	86.67	3.1 ± 0.3[a]
II	Control	0.28[ab]	0.46	100	
	PICF7	0.24[b]	0.43	80	4.3 ± 0.2[a]
	ME419 (*gltA*)	0.20[b]	0.33	71.43	3.9 ± 0.6[ab]
	ME424 (*fliI*)	0.29[ab]	0.38	84.62	3.5 ± 0.8[ab]
	ME589 (*pvdI*)	0.23[b]	0.35	76.92	3.9 ± 0.5[ab]
	ME1508 (Cys Aux)	0.47[a]	0.53	100	2.9 ± 0.4[b]
III	Control	0.32[a]	0.40	60	
	PICF7	0.05[b]	0.11	43.75	4.1 ± 0.6[a]
	ME419 (*gltA*)	0.15[ab]	0.31	66.67	3.5 ± 0.3[ab]
	ME424 (*fliI*)	0.05[b]	0.13	53.33	3.3 ± 0.2[b]
	ME589 (*pvdI*)[5]	–	–	–	3.5 ± 0.1[ab]
	ME1508 (Cys Aux)	0.10[b]	0.23	71.43	2.6 ± 0.2[c]

1. Three independent experiments were carried out spanning 110 days after inoculation (DAI) (experiment I), 118 DAI (experiment II) and 113 DAI (experiment III).
2. The root system of 3-month-old olive plants were dipped in bacterial suspensions (5.8×10^8–1.7×10^9 cfu ml^{-1}) for 15 min and then transplanted into autoclaved soil for colonization assay, or autoclaved soil artificially infested with 3.2×10^5–2.7×10^6 conidia $g^{-1}$of the defoliating *Verticillium dahliae* isolate V937I for biocontrol assays. Plants were grown in a growth chamber under controlled conditions (see text for details).
3. *SAUDPC*, standardized area under the disease progress curve of *DII* (disease intensity index) plotted over time. *DI*, final disease incidence (percentage of affected plants at the end of the experiment). Means in a column followed by different letters are significantly different according to Fisher's protected least significant difference test ($P < 0.05$).
4. Cell counts of *Pseudomonas* strains were determined on modified King's medium B agar (PICF7) or on modified KBA amended with Tc (Tc^R PICF7 mutant derivatives). Data are means of three root samples (1 g each). Means followed by different letters are significantly different according to Two-sided Dunnett's multiple comparisons with a control (PICF7) at $\alpha = 0.05$.
5. Plants bacterized with ME589 and transplanted to soil infested with *V. dahliae* V937I unexpectedly died. However, plants treated with ME589 alone (9) showed healthy, and therefore were kept and used for viable bacteria counts.

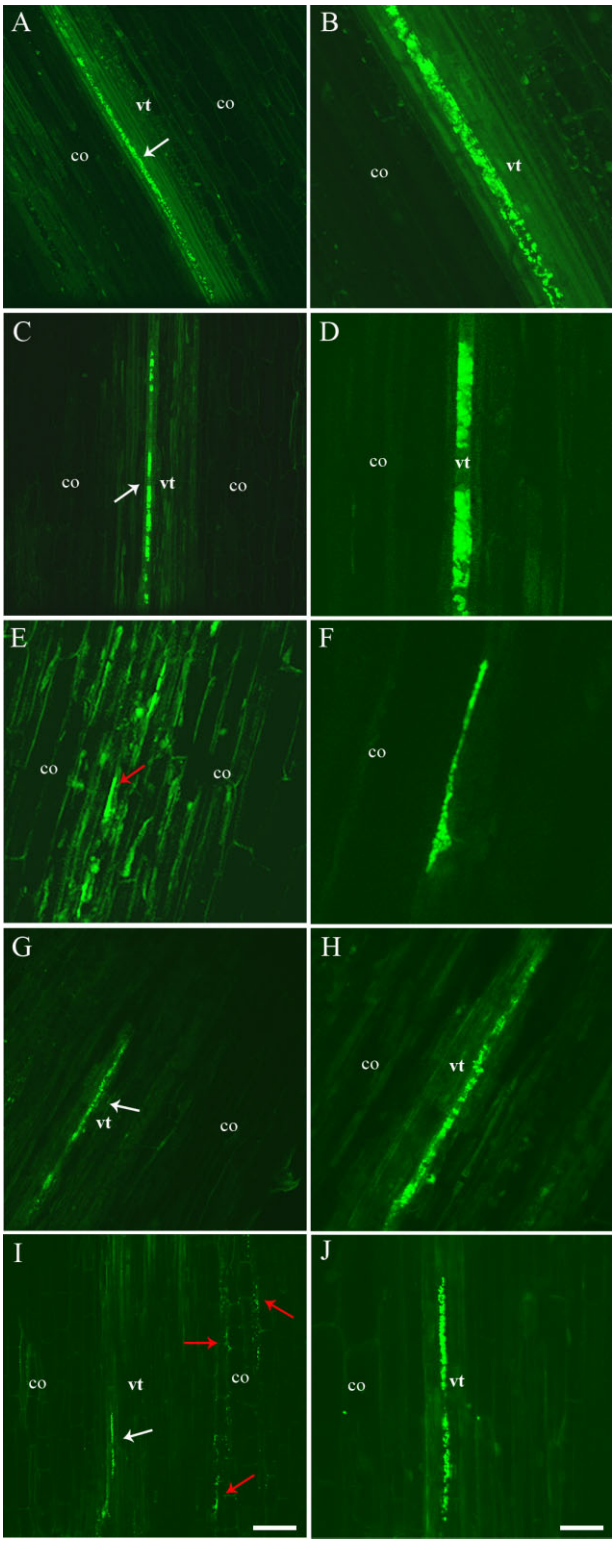

Fig. 2. Confocal laser scanning microscopy images of longitudinal vibratome root sections (40 μm thick) showing localization of *Pseudomonas fluorescens* PICF7 and its Tn5-Tc^R insertion mutant derivatives GFP-labelled. Images were taken from one to two weeks after root-dip bacterization with *Pseudomonas*-GFP. Strain PICF7 (A and B), *gltA* (C and D), *fliI* (E and F), *pvdI* (G and H) and Cys auxotroph (I and J). Pictures show that all strains endophytically colonize olive roots: vascular tissue (white arrows), and intercellular spaces of the cortex (red arrows). Scale bar represents 100 μm in A, C, E, G and I (left panels), and 30 μm in B, D, F, H and J (right panels); co, cortical cells; vt, vascular tissue.

clear (15 DAB). Moreover, population sizes of introduced bacteria at 15 DAB reached similar values for all strains but for mutant ME589-G (*pvdI*), which colonized olive roots/rhizosphere at a significantly ($P < 0.05$) lesser extent (Table S2). Confocal laser scanning microscopy (CLSM) imagery obtained from root tissue samples during 15 DAB showed that PICF7, as well as all mutant strains evaluated, were able to endophytically colonize the root interior predominantly among the intercellular spaces of the root cortex. Remarkably, however, GFP-labelled cells could also be localized within the root vascular system for all strains but for the *fliI* mutant (Fig. 2). Taking into account this finding, an experiment aimed to demonstrate possible active/passive movement of PICF7 cells form root xylem vessels to aerial tissues was carried out. When GFP-tagged PICF7 Gm^R cells were applied to the root system of 'Picual' plants (irrigated three times at days 0, 5 and 10 with a bacterial cells suspension), no tagged bacteria could be retrieved from aerial tissues, indicating that there was no transport to upper parts of the plants through the xylem vessels. In contrast, PICF7 successfully colonized the roots of the examined plants (average population sizes of $\log_{10}$ 5.2 cfu g^{-1} of fresh root tissue).

Biological control activity of strain PICF7 is determined neither by pyoverdine production nor swimming motility

Three independent experiments were carried out to assess biocontrol performance of PICF7 mutants. Results showed, overall, that swimming motility (impaired in mutant ME424) and Pvd production (abolished in mutant ME589) are not needed for the effective biocontrol exerted by strain PICF7 against *V. dahliae* (Table 4). However, differences were found among experiments. Thus, in bioassay I [spanning 110 days after inoculation (DAI)], PICF7-bacterized plants displayed a significant ($P < 0.05$) decrease in the standardized area under the disease progress curve of *DII* plotted over time (days) (*SAUDPC*) in comparison to non-bacterized plants (control). Besides, a decrease in disease intensity index (*DII*) and the final disease incidence (*DI*) values were observed (Table 4). Mutants ME424 and ME589 showed a similar behaviour than the parental strain, and *SAUDPC*

was also significantly ($P < 0.05$) lower than in non-treated plants, although *DII* and *DI* values for ME424-treated and ME589-treated plants were higher than that for PICF7-bacterized plants (Table 4). In contrast, neither *P. fluorescens* PICF7 nor its *pvdI* and *fliI* mutant derivatives displayed biocontrol activity in bioassay II (118 DAI), likely due to a high disease pressure scored in this experiment (i.e. 100% final DI in control plants) (Table 4). Finally, in bioassay III (113 DAI), strain PICF7 and mutant ME424 controlled Verticillium wilt epidemics effectively. Indeed, *SAUDPC* decreased significantly ($P < 0.05$) in both treatments compared with non-bacterized plants (Table 4). The *DII* was also reduced in PICF7-treated and ME424-treated plants in comparison to that observed for control plants. Regarding the final *DI* values, no differences were found between PICF7 and ME424, and both treatments reduced the number of diseased plants at the end of the experiment in comparison to non-bacterized plants (Table 4). It must be mentioned that the effect of ME589 could not be evaluated in this bioassay because most of the *V. dahliae*-inoculated plants in this treatment unexpectedly died after manipulation. In summary, mutations in the putative *pvdI* and *fliI* genes did not seem to affect biocontrol activity in mutants ME589 and ME419 respectively.

Cys auxotrophy in strain PICF7 diminished its VWO biocontrol effectiveness

Besides mutants impaired in swimming motility and Pvd production, two additional mutants, one affected in growth on PDA plates (ME419) and another showing Cys auxotrophy (ME1508), were included in VWO biocontrol experiments. Results also varied among bioassays. Overall, the behaviour of mutant ME419 did not significantly ($P > 0.05$) differ from that of PICF7 for any of the disease parameters analysed in the three experiments (Table 4). Similarly, *SAUDPC*, *DII* and final *DI* from ME419-treated plants were never found to be different from that of non-bacterized plants (Table 4). Therefore, mutation in the putative *gltA* gene did not seem to affect biocontrol activity in mutant ME419, despite of the fact that growth of this mutant *in vitro* was evidently altered (Fig. 1). On the other hand, ME1508 mutant (Cys auxotroph) showed a significantly ($P < 0.05$) higher *SAUDPC* than that scored for PICF7-treated plants in bioassays I and II. Likewise, *DII* (0.51 and 0.53) and final *DI* (86.7% and 100%) were higher in ME1508-treated plants than in PICF7-treated plants (Table 4). This indicated that Cys auxotrophy negatively influenced biocontrol performance in mutant ME1508. However, this mutant behaved similarly to PICF7, ME419 and ME424 in bioassay III, and no significant ($P = 0.29$) differences were scored among treatments (Table 4). It is worth mentioning here that final *DI* in bioassay III was considerably lower in the control (non-bacterized; 60%) treatment than in bioassays I (93.3%) and II (100%).

Discussion

Effective control of VWO is highly difficult by a number of reasons comprehensively reviewed elsewhere, and must thus rely on an integrated disease management strategy with emphasis in pre-planting measures (López-Escudero and Mercado-Blanco, 2011). A promising preventive action is the use of native microbial antagonists able to efficiently colonize the target niche (i.e. soil, rhizosphere, roots, etc.). However, our knowledge on the mechanisms involved in suppression of *V. dahliae* by microbial antagonists is still very limited, not only for the particular case of VWO but also for many crops that can be infected by this pathogen (Alström, 2001; Tjamos *et al*., 2005; Berg *et al*., 2006; Antonopoulos *et al*., 2008; El Hadrami *et al*., 2011; Meschke *et al*., 2012).

One of the best BCA of VWO so far studied is the olive root endophyte *P. fluorescens* PICF7 (Mercado-Blanco *et al*., 2004; Prieto *et al*., 2009; 2011). Strain PICF7 is able to trigger a broad range of defence responses in both root (Schilirò *et al*., 2012) and aerial (Gómez-Lama Cabanás *et al*., 2014) tissues, pointing to a scenario in which VWO biocontrol by PICF7 could be mediated by induced resistance mechanism(s) (Pieterse *et al*., 2014). We have recently shown, however, that the presence of PICF7 cells in root tissues does not suppress olive knot disease in stems caused by *Pseudomonas savastanoi* pv. savastanoi (Maldonado-González *et al*., 2013). Nothing is currently known on which PICF7 traits could be involved in triggering such responses or whether additional biocontrol mechanisms (i.e. antibiosis, competition, etc.) might be effective against *V. dahliae*. Likewise, knowledge on PICF7 phenotypes involved in olive rhizosphere colonization and endophytic lifestyle is absent. Therefore, the objective of the present study was to examine whether selected traits play a role in root colonization and VWO biocontrol abilities exerted by strain PICF7. The approach followed was to generate mutants in specific phenotypes, and to assess their colonization and biocontrol performance *in planta* under non-gnotobiotic conditions. This means that introduced bacteria faced a situation closer to a natural environment (i.e. nursery-produced plants carrying a highly-diverse microbiome) than that found in axenic systems normally used in this type of studies. Besides, this work has been carried out using a woody plant relevant in Mediterranean agro-ecosystems instead of a model plant.

Successful biocontrol of soil-borne phytopathogens by any given BCA must be preceded by the efficient colonization of the target niche (i.e. rhizosphere soil, root

surface, root interior) (Mercado-Blanco and Bakker, 2007). A number of *Pseudomonas* spp. traits involved in rhizosphere and/or root colonization have been studied (Lugtenberg *et al*., 2001), and some have shown as key elements for the subsequent biocontrol efficacy exerted by specific strains (Chin-A-Woeng *et al*., 2000). Motility and chemotaxis are thus considered essential for root colonization by *P. fluorescens* strains (Lugtenberg and Bloemberg, 2004), although flagella-driven motility has been demonstrated to be necessary for root colonization in some cases (Capdevila *et al*., 2004; Martínez-Granero *et al*., 2006) but not in others (Howie *et al*., 1987). The involvement of swimming motility in colonization and biocontrol has been proven for some pseudomonads. Thus, a triple mutant strain (KSW) of *P. fluorescens* F113 affected in *kinB*, *sadB* and *wspR* showed increased swimming motility and rhizosphere colonization ability than that of the wild-type strain, as well as improved biocontrol activity against *Fusarium oxysporum* f.sp. *radicis lycopersici* in tomato (*Solanum lycopersicum* Mill.) and *Phytophthora cactorum* in wild strawberry (*Fragaria vesca* L.). (Barahona *et al*., 2011). Recently, Sang and Kim (2014) have also suggested that biocontrol activity of *Pseudomonas corrugata* CCR04 and CCR80 in pepper (*Capsicum* sp.) plants against the soil-borne oomycete *Phytophthora capsici* can be mediated by successful root colonization through biofilm formation and swimming and swarming motilities. Our results demonstrated that *P. fluorescens* PICF7 displays swimming motility. This trait seems to contribute to the full colonization ability of olive rhizosphere/roots by strain PICF7. Indeed, mutant ME424 always showed lower population sizes than that of the parental strain in all experiments, although it was significantly different only in bioassay III (Table 4). On the contrary, lack of swimming motility did not hinder mutant ME424 to colonize the root interior nor diminished its biocontrol efficiency against *V. dahliae* (see below).

Population sizes associated with roots scored for all mutants tested were always lower, either significantly (i.e. mutant ME1508 in bioassays II and III) or just showing a trend, compared with that observed for strain PICF7 (Table 4). However, this decline in population size over time observed for mutants ME424 and (*flil*) and ME589 (*pvdI*) did not affect their ability to control *V. dahliae*. Therefore, we conclude that neither Pvd production nor swimming motility is implicated in VWO suppression. Regarding ME419 (*gltA*), growth problems observed in PDA medium for this mutant did not affect root colonization ability nor biocontrol performance compared with PICF7. On the contrary, Cys auxotrophy significantly affected the root colonization ability of mutant ME1508, which overall displayed the lowest populations sizes at the end of the bioassays (> 100 DAI). Moreover, mutant ME1508 had lost wild-type VWO biocontrol phenotype. Importance of amino acid synthesis has been earlier shown for the colonization of tomato roots by *P. fluorescens* strain WCS365 (Simons *et al*., 1997). Plant roots produce exudates composed of a broad range of low-molecular (i.e. amino acids, organic acids, sugars, phenolics, etc.) and high-molecular [i.e. mucilage (polysaccharides), proteins, etc.] weight compounds (Bais *et al*., 2006). Soil microorganisms are chemically attracted by root exudates, which serve as an important source of nutrients, including amino acids. Among amino acids synthesized by roots, Cys and cystine (oxidation of two Cys molecules covalently linked via disulfide bond) have been detected in root exudates of several plant species (Gitte *et al*., 1978; Gaworzewska and Carlile, 1982; Dennis *et al*., 2010). A gene coding for a putative sulfite reductase, an enzyme related with Cys metabolism, has been identified in mutant ME1508. Moreover, this mutant was unable to grow in SSM in contrast to nutrient-rich media (i.e. LBA and KBA) where ME1508 grew normally. Amendment of L-Cys to SSM restored the ability of ME1508 to grow and produce Pvd. It is plausible to think that low availability of Cys in the olive rhizosphere makes it ME1508 less efficient in root/rhizosphere colonization, and consequently in VWO suppression effectiveness. Nevertheless, its ability to colonize inner root tissues remained unaffected, in spite of the fact that rhizosphere populations of ME1508 were overall significantly lower than that of the parental strain.

Mechanisms involved in the endophytic colonization of roots by bacteria, including beneficial *Pseudomonas* spp., are mostly unknown (Hardoim *et al*., 2008; Reinhold-Hurek and Hurek, 2011). Recent comparative genomics and bioinformatics approaches may shed light on the identification of specific traits linked to endophytism by beneficial bacteria (Mitter *et al*., 2013; Ali *et al*., 2014). So far, however, only a few bacterial characteristics have been shown, mostly by mutational studies, as truly implicated either on gaining entrance into the root interior, spreading to distant organs, or endurance within plant tissues (Reinhold-Hurek and Hurek, 2011). For instance, a *pilT* mutant of *Azoarcus* sp. BH72 unable of twitching motility was impaired in the endophytic colonization of rice (Böhm *et al*., 2007). Twitching and swarming motilities have not been demonstrated in strain PICF7 under tested conditions. While swimming motility has been shown for PICF7, our results indicate that this phenotype is not relevant for inner colonization of olive roots by strain PICF7. The same accounts for Pvd production, *in vitro* growth delay and Cys auxotrophy. Indeed, all PICF7 mutants tested in this study could be clearly observed colonizing the intercellular spaces of the root cortex. Interestingly enough, root tissue sectioning and CLSM imagery allowed to demonstrate that PICF7, ME419, ME589 and ME1508 were also able to colonize root xylem

vessels, a location not detected in previous studies (Prieto and Mercado-Blanco, 2008; Prieto *et al*., 2009; 2011). However, no evidence of PICF7 movement from roots to above-ground organs using the lumen of the xylem vessels was obtained under experimental conditions used here, corroborating previous results using *in vitro*-propagated olive plants and a root-dip inoculation protocol (Maldonado-González *et al*., 2013).

Pseudomonas spp. produce a large variety of siderophores to cope with iron-limiting conditions (Bultreys, 2007), Pvds being the prevalent class (Meyer, 2000). In addition, many plant beneficial *Pseudomonas* spp. strains produce additional, secondary siderophores (Buysens *et al*., 1996; Mercado-Blanco *et al*., 2001; Loper *et al*., 2012). The involvement of *Pseudomonas*-produced siderophores in biological control is controversial. They may act through competition of Fe^{3+}, thereby limiting its availability for pathogens. Besides, disease suppression mediated by siderophores has been shown to play an important role in some cases (Buysens *et al*., 1996; Audenaert *et al*., 2002), but minor (or no involvement at all) in others (Hamdan *et al*., 1991; Ongena *et al*., 1999; Djavaheri *et al*., 2012). Some *Pseudomonas*-produced siderophores have also been suggested to suppress plant diseases via induction of systemic resistance, although this issue is also controversial (Djavaheri *et al*., 2012; Bakker *et al*., 2014). *Pseudomonas fluorescens* PICF7 produces Pvd (Mercado-Blanco *et al*., 2004; this study), although production of additional, secondary siderophore(s) cannot be completely ruled out according to PICF7 genome data (Martínez *et al*., unpublished). In fact, a large number of PICF7 mutants with altered phenotypes in iron-chelating ability have been generated in this study. However, we focused our attention on a mutant fully impaired in Pvd production (ME589), a phenotype corroborated by cultivation of this mutant in different growing media and by the identification of the gene disrupted in its genome (a putative *pvdI* homologue). Mutant ME589 showed a similar behaviour than PICF7 regarding root colonization ability, endophytism and biocontrol performance. It can, therefore, be concluded that Pvd production does affect neither VWO biocontrol effectiveness nor endophytic colonization by strain PICF7.

Finally, attention should be called here to the frequently observed biocontrol inconsistency/variability, a phenomenon amply referred in the literature (Lindow, 1988; Kraus and Loper, 1992). Biocontrol/colonization assessment carried out in this study showed variable results among bioassays (Table 4), a situation previously found when characterizing biocontrol strains from olive roots, including PICF7 (Mercado-Blanco *et al*., 2004). It is therefore compulsory, in our opinion, to present results from different independent biocontrol assays before to state sound conclusions regarding biocontrol performance of any claimed BCA. This is particularly true when the niche where the host plant, the pathogen, the introduced BCA and the resident microbiota interact is the rhizosphere. This is a complex, highly dynamic scenario where a multiplicity of trophic interactions takes place, thereby influencing the fitness and performance of the BCA (Berg *et al*., 2006; Raaijmakers *et al*., 2009). In our case, this scenario poses the added experimental difficulties of dealing with a nursery-propagated woody host plant used under non-gnotobiotic conditions.

In summary, results presented here shed light, for the first time, on the actual involvement of specific *P. fluorescens* PICF7 phenotypes in olive root colonization and biocontrol against *V. dahliae*. Mutant analysis showed that swimming motility and Pvd production are not implicated in VWO suppression and endophytic behaviour displayed by PICF7. Cys auxotrophy compromised wild-type phenotypes such as VWO control and root colonization ability but did not hinder inner colonization of olive root tissues. The availability of a PICF7 mutant library will allow the screening of more phenotypes in the future, aiming to unravel the underlying mechanisms of PICF7 biocontrol and endophytism in a woody long-living plant such as olive.

Experimental procedures

Bacteria, culturing media and production of bacterial inocula

Pseudomonas fluorescens PICF7 (Mercado-Blanco *et al*., 2004) and several mutants and fluorescently tagged derivatives of this strain were used in this study (Table 1). Tn*5*-Tc^R transposon insertion mutants ME419, ME424, ME589 and ME1508 were constructed by biparental matings (see below). Tc^R and/or Gm^R, GFP-labelled derivatives of strain PICF7, used to monitor olive root colonization by CLSM (see below), were named as PICF7-G, ME419-G, ME424-G, ME589-G and ME1508-G (Table 1). *Pseudomonas* strains were always grown at 25°C in King's medium B (King *et al*., 1954) agar (KBA) plates. When needed, antibiotics were added at the following concentrations (mg l^{-1}): nalidixic acid (Nal), 10; tetracycline (Tc), 20; gentamicin (Gm), 10; ampicillin (Amp), 50; chloramphenicol (Chl), 13; and cycloheximide (Chx), 100. *Escherichia coli* strains (Table 1), S17-1 harbouring the suicide plasmid pJQ18 which contains transposon Tn*5* (Hynes *et al*., 1989) and DH5α harbouring plasmid pLMR1, were grown at 37°C in LB (Miller, 1972) agar (LBA) amended with Tc (20 mg l^{-1}) and Gm (50 mg l^{-1}) respectively. *Pseudomonas* strains inocula were prepared as described in Maldonado-González and colleagues (2013). Bacterial cell densities required for each experiment were determined spectrophotometrically (A600 nm) by building up standard curves and culturing viable cells from serial dilution series onto KBA plates (to count PICF7 wild-type colonies), or KBA plates supplemented with the antibiotics Tc (for Tn*5*-Tc^R derivatives), Gm (for GFP-labelled PICF7 derivative) or Tc plus Gm (for GFP-labelled Tn*5*-Tc^R mutants).

GFP labelling of Pseudomonas

Strain PICF7 and selected Tn5-Tc^R insertion mutants were transformed with plasmid pLRM1 (Gm^R, GFP) (Rodríguez-Moreno *et al*., 2009). Electrocompetent cells of each bacterial strain were transformed with plasmid pLRM1 by electroporation as described by Prieto and Mercado-Blanco (2008). Transformation frequency (transformants/μg plasmid DNA) was calculated for each strain. The presence of plasmid pLMR1 in selected transformed derivatives was further confirmed by plasmid purification (FavorPrep Plasmid DNA Extraction Mini Kit, Ping-Tung, Taiwan) and restriction analysis with EcoRI (New England BioLabs, Beverly, MA). Plasmid pLRM1 stability in each transformed derivative was assessed by continuous growth of exponential-phase cultures kept under vigorous shaking (250 r.p.m.) without antibiotic (Gm) selection pressure for 6 days at 25°C (approximately 120 generations). Serial dilution series of these cultures were plated onto LBA plates every 24 h and incubated at 25°C during 48 h. Subsequently, 100 randomly selected colonies were individually transferred to LB agar plates amended with Gm. The number of Gm^R-resistant colonies for each strain was counted and the percentage of plasmid loss (Gm-sensitive colonies) scored. The rate of plasmid loss/generation was calculated according to Durland and Helinski (1987) equation, which is percent plasmid loss/generation = $[1 - (R_N/R_i)^{1/N}] \times 100$, where R is the frequency of the plasmid-mediated resistance in the population, i is initial, and N is the number of generations elapsed.

Verticillium dahliae *growth conditions, pathogen inoculum production and plant material*

Verticillium dahliae V937I, a highly virulent isolate representative of the D pathotype (Collado-Romero *et al*., 2006), was used in VWO biocontrol assays. Pathogen inoculum was prepared as described in Mercado-Blanco and colleagues (2004). The number of conidia per millilitre was scored in a Neubauer chamber and adjusted to the required working concentration.

Biological control assays were carried out using nursery-produced, 3-month-old olive plants cv. Picual, qualified as highly susceptible to the D pathotype (López-Escudero *et al*., 2004). Plants were originated from two different commercial nurseries located in Córdoba province (Southern Spain). Previous to starting the bioassays, plants were kept at least 1 month within a controlled-growth chamber at 25 ± 1°C with a 14 h photoperiod and a light intensity of 65 μmol m^{-2} $s^{-1}$for acclimatization. Plants used for CLSM were 2-month-old and were acclimated for 2 weeks under the same environmental conditions.

Generation of a Pseudomonas fluorescens *PICF7 mutant bank*

A random transposon insertion mutant bank of *P. fluorescens* PICF7 was generated by using a Tn5 derivative carried in the suicide plasmid pJQ18 (Simon *et al*., 1983) (Table 1) and biparental mating as described in Mercado-Blanco and colleagues (2001). More than 9000 transconjugants were obtained, mixed and cryopreserved in 30% glycerol at −80°C. The percentage of auxotroph mutants in the mutant bank was checked by replicating a fair number (> 500) of Tc^R colonies in both SSM and LBA that were incubated at 25°C during 72 h. Mutants unable to grow in SSM but capable to do so in LBA were considered as auxotrophs, and the percentage of transposon insertions leading to auxotrophy was calculated.

Phenotypic characterization of Pseudomonas fluorescens *PICF7 mutants*

The screening of mutants altered in swimming motility was as follows. In a first round, 2000 Tc^R colonies previously grown overnight on KBA plates (amended with Tc) at 25°C were inoculated (35 mutants/plate) in square SM (1% tryptone, 0.5% NaCl, 0.3% agar) (Déziel *et al*., 2001) agar plates (12 × 12 cm) along with the parental strain PICF7 (used as a control). Swimming motility was revealed after overnight incubation at 25°C. Transconjugants showing altered behaviour (lack, increase or decrease of swimming motility compared with the wild type) were further tested individually (one mutant per plate together with a colony of PICF7) in SM agar. This check step was repeated twice for each mutant. Other types of bacterial motilities, such as swarming (Overhage *et al*., 2007) and twitching (Alit-Susanta and Takikawa, 2006), were also tested in appropriate media. Data on colony diameter of putative mutants were subjected to analysis of variance (ANOVA), and means were compared with that of the parent strain (PICF7) using two-sided Dunnett's multiple comparisons with a control at $P < 0.05$. Mutants displaying a significantly altered swimming motility phenotype were selected for further characterization and stored in 30% glycerol at −80°C.

Two different culturing media were used to screen PICF7 mutants altered in siderophore production. In a first round, a set of 2000 transconjugants were tested in parallel using KBA and the universal siderophore detection medium CAS agar (Schwyn and Neilands, 1987). Production of the major siderophore Pvd in KBA was observed as a green fluorescence when grown colonies were submitted to UV light. Production of iron-chelating compounds in CAS agar plates is revealed by the production of orange haloes around siderophore-producing colonies. A colony of strain PICF7 was included in all plates for comparison. Mutants (Tc^R colonies) previously grown in KBA plates (amended with Tc) (25°C, 24 h) were individually transferred to KBA and CAS plates (49 mutants/plate) without Tc (to allow growth of a colony of PICF7 used as a reference) and incubated at 25°C for 24 h. Absence, decrease or increase of fluorescence under UV irradiation in KBA and orange haloes produced on CAS plates were scored for each single mutant. A preselected set of colonies showing altered siderophore production phenotypes was checked in the same media by plating cell suspensions (5 μl) of each mutant individually, per triplicate, onto CAS and KBA, along with a suspension of *P. fluorescens* PICF7 cells (control). This step was repeated at least twice. Thus, fluorescence in KBA was checked again and the relative halo size [(halo diameter – colony diameter)/halo diameter] produced in CAS media was calculated for each mutant. Relative haloes size data were subjected to

ANOVA and means were compared with strain PICF7 mean using two-sided Dunnett's multiple comparisons with a control at $P < 0.05$.

To screen for *P. fluorescens* PICF7 mutants potentially altered in *in vitro* antagonism against *V. dahliae*, the following experimental approach was carried out. In a first screening round, mutants (2000) and *V. dahliae* isolate V937I were confronted as follows: PDA medium was prepared, cooled down to 45°C and mixed with a conidial suspension of V937I to yield a final concentration of approximately 1×10^4 conidia ml^{-1}. Once agar plates solidified, individual colonies (35 mutants/plate) were inoculated with a sterile toothpick and incubated for 4 days at 28°C. The presence of haloes around bacterial colonies (inhibition or retardation of the fungal growth) was checked periodically. Each plate contained a colony of PICF7 as a reference. Mutants showing a different behaviour compared with that of the parental strain were pre-selected, and the procedure was repeated but each 'candidate' mutant was individually plated as a 5 μl drop of bacterial suspension together with a suspension of PICF7. This check test was repeated twice.

Finally, selected mutants impaired in siderophore production, swimming motility and/or altered behaviour in *in vitro* antagonism assay against *V. dahliae* were further tested for their ability to grow in liquid and solid SSM (pH 7.0) (Meyer and Abdallah, 1978) for 20 h or 48 h respectively. Mutants with the absence or decrease in green fluorescence (Pvd production) or unable to grow in this minimal medium (auxotroph) were selected. This assay was done at least twice for each mutant.

Identification of transposon insertion sites in selected PICF7 mutants

A collection of selected mutants (43) altered in one of the phenotypes mentioned above were analysed to determine the gene disrupted by Tn5 transposon insertion. A combination of arbitrary and nested-PCR was implemented according to Caetano-Anollés (1993). Total DNA of selected mutants was extracted and purified according to i-genomic CTB Extraction Mini Kit (Intron Biotechnology, European Biotech Network, Belgium). A first round of amplification was accomplished by using total DNA from each mutant as a template and two primers, one arbitrary (ARB1; 5′-GGCCACGC GTCGACTAGTACNNNNNNNNNNGATAT-3′) and another specific to the internal right end of the transposon Tn5 (Tn5Ext; 5′-GAACGTTACCATGTTAGGAGGTC-3′). The first PCR round consisted of 5 min at 95°C; 30 cycles of 30 s at 95°C, 30 s at 55°C and 2 min at 72°C, followed by an extension period of 5 min at 72°C. The final volume was 30 μl (1 X *Taq* DNA polymerase buffer, 3 mM MgCl2, 2 mM dNTP, 0.8 μM of each primer, 1 U μl^{-1} of *Taq* DNA polymerase and 0.5 ng of extracted DNA). One microlitre of the previous reaction was submitted to a nested-PCR reaction with specific primers, ARB2 (5′-GGCACGCGTCGACTAGTAC-3′) and Tn5Int (5′-CGGGAAAGGTTCCGTTCAGGACGC-3′), the sequences of which corresponded to the conserved region of ARB1 and to the right end of Tn5Ext respectively. Nested-PCR conditions were as follows: 2 min at 95°C; 30 cycles of 30 s at 95°C, 30 s at 57°C, 2 min at 72°C, followed by 3 min at 72°C. Final volume was 30 μl (1 X Taq DNA polymerase buffer, 3 mM $MgCl_2$, 2 mM dNTP, 0.8 μM of each primer and 1 U μl^{-1} of *Taq* DNA polymerase). Amplicons were electrophoresed in 0.8% agarose gels, and the observed band was extracted from gel and purified (FavorPrep™ GEL/PCR Purification Mini Kit). Adjacent DNA region to the Tn5 insertion site of each selected mutants was sequenced (Sistemas Genómicos S.L., Paterna, Valencia, Spain) using primer Tn5Int. DNA sequences were compared against available databases (GenBank and *Pseudomonas* Genome Database) using the BLASTX and BLASTN programmes (Altschul *et al.*, 1997) available at the NCBI network service, and against the complete PICF7 genome sequence (deposited in GenBank, CP005975) (Martínez *et al.*, unpublished).

L-Cysteine cross-feeding assay

Growth of mutant ME1508 was impaired in SSM and sequence analysis revealed possible auxotrophy in Cys biosynthesis. To check this mutant phenotype, a cross-feeding experiment was carried out. Bacterial suspensions of strain PICF7 (positive control) and mutant ME1508 originating from fresh colonies grown in LBA were prepared in SSM and inoculated (350 μl) in 5 ml of SSM (control) and SSM amended with increasing (1, 2, 5, 10 and 20 mg l^{-1}) concentrations of L-Cys (Sigma-Aldrich, St Louis, MO) (final $OD_{600} = 0.1$). Cultures were grown in an orbital shaker at 25°C for 48 h (150 r.p.m.). Cell growth and restoration of Pvd production by ME1508 in liquid SSM upon L-Cys addition were determined as previously described (Mercado-Blanco *et al.*, 2004). This experiment was repeated twice.

Verticillium wilt of olive biocontrol experiments

Three independent bioassays were conducted to assess the effectiveness of four PICF7 mutants altered in different phenotypes to control VWO. Bioassays were carried according to the procedure described by Mercado-Blanco and colleagues (2004). The carefully washed (tap water) root systems of nursery-produced olive plants (cv. Picual) were dipped in suspensions of each bacterial strain (cell densities ranging from 5.8×10^8 to 1.7×10^9 cfu ml^{-1}, 15 plants) or 10mM $MgSO_4.7H_2O$ (control, 9–12 plants) for 15 min. Then plants were transplanted into clay pots filled with soil mixture (sand/loam, 2:1,vol/vol) thoroughly mixed with a conidia suspension (ranging from 3.2×10^5 to 2.7×10^6 conidia g^{-1} soil) of *V. dahliae* D isolate V937I (Collado-Romero *et al.*, 2006) or distilled sterile water (control treatment). Plants were kept in a growth chamber (conditions describe above) during at least 110 DAI. Disease symptoms, such as defoliation, chlorosis and wilting, were scored along the bioassay using a 0–4 rating scale according to the percentage of affected leaves and twigs (0, no symptom; 1, 1–33%; 2, 34–66%; 3, 67–100%; and 4, dead plant) at weekly intervals after inoculation with the pathogen for the first 2 months, and thereafter every 10 days.

Disease severity data were used to determine the following: (i) a *DII* defined as $DII = (\Sigma Si \times Ni)/(4 \times Nt)$, where *Si* is severity of symptoms, *Ni* is the number of plants with *Si* symptoms severity, and *Nt* the total number of plants; (ii) final *DI* established as the percentage of affected plants at the end of the bioassays; and (iii) *SAUDPC* calculated according to

Campbell and Madden (1990). *SAUDPC* data were subjected to ANOVA, which was calculated using Statistix (NH Analytical Software, Roseville, MN). Treatment means were compared using Fisher's protected least significant difference test at $\alpha = 0.05$.

Olive root colonization ability of PICF7 mutants

To assess whether PICF7 mutant derivatives were affected in their ability to colonize olive roots tissues, even endophytically, two approaches were followed. On the one hand, population size of introduced bacteria was checked on/in root samples from each biocontrol experiment approximately at 110 DAB. To do so, root systems of three plants per treatment were kindly uprooted and immersed in tap water to remove the excess of soil particles at the end of each experiment. Subsequently, plants were deposited over filter paper and the roots were air-dried and weighted. Then, root tissue samples (1 g) of each plant were thoroughly ground using a mortar in 5 ml of 10 mM $MgSO_4 \cdot 7H_2O$, and serial dilutions of root macerates were plated in KBA plates amended with Amp, Chl and Chx (for PICF7 cells counting), plus Tc (for Tc^R mutant cells counting). Plates were incubated at 25°C for 24 h and cfu g^{-1} fresh root tissue was estimated for each strain. Data were subjected to ANOVA and means were compared with strain PICF7 using two-sided Dunnett's multiple comparisons with a control at $\alpha = 0.05$.

On the other hand, to monitor both surface and inner olive root colonization by PICF7 and its Tc^R mutants, GFP (Gm^R) derivatives (see above), vibratome (Vibratome Series 1000plus, TAAB Laboratories Equipment, Aldermaston, UK) root sectioning and CLSM (Axioskop 2 MOT microscope, Carl Zeiss, Jena GmbH, Germany) were used. The Gm^R GFP-labelled mutants were applied to olive root systems as previously indicated for biocontrol assays. One to two weeks after bacteria inoculation, fresh and healthy roots from two–three plants per treatment were manipulated, longitudinally sectioned and visualized by CLSM according to Prieto and Mercado-Blanco (2008). Finally, to count viable cells of each Gm^R GFP-labelled mutant, 1 g from root tissue of each plant analysed by CLSM was ground as mentioned above, and serial dilutions of the macerate were plated onto KBA amended with Amp, Chl, Chx and Gm, and grown at 25°C during 48 h.

In order to assess the possible translocation of *P. fluorescens* PICF7 cells from the roots to aerial tissues of inoculated plants, the following experimental set-up was followed. Two-month-old 'Picual' plants were carefully bacterized, avoiding cross-contamination of above-ground organs, by watering pots with freshly prepared suspensions of the GFP-labelled PICF7 Gm^R derivative (log_{10} 8.8-9.7 cfu ml^{-1}, 120 ml pot^{-1}) three consecutive times (day 0, 5 and 10). Subsequent watering of bacterized plants and that of control, non-bacterized plants, was carried out with tap water. Root and stem samples of two bacterized plants were checked by CLSM (see above) at 14, 21, 60 and 90 DAB. Sampled plants were carefully uprooted, the roots dipped in tap water to remove soil excess and allow to air-dry over filter paper. Each plant was divided into above-ground part and root system. On the one hand, and for assessing PICF7 viable cells, a sample of root tissue (1 g), two segments of the stem (1 cm each) and 4 half-leaves with their petioles originating from the basal zone of each sampled plant were weighted and macerated in 10 ml of 10 mM $MgSO_4.7H_2O$. Serial dilutions of root and basal aerial sections macerates were plated per duplicate in KBA amended with Amp, Chl and Chx (to count indigenous bacteria and PICF7 Gm^R) and with these antibiotics plus Gm (to count PICF7 Gm^R) and incubated at 25°C for 48 h. On the other hand, vibratome sections of roots (longitudinal), stems and petioles (transversal) were analysed by CLSM as mentioned above. Mutants population size data were compared with strain PICF7 using Student's t-test ($\alpha = 0.05$) (Table S2).

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References

Ali, S., Duan, J., Charles, T.C., and Glick, B.R. (2014) A bioinformatics approach to the determination of genes involved in endophytic behavior in *Burkholderia* spp. *J Theor Biol* **343:** 193–198.

Alit-Susanta, W.G.N., and Takikawa, Y. (2006) Phenotypic characterization of *Pseudomonas fluorescens* PfG32R and its spontaneous *gacS* mutants and biocontrol activity against bacterial wilt disease of tomato. *J Gen Plant Pathol* **72:** 168–175.

Alström, S. (2001) Characteristics of bacteria from oilseed rape in relation to their biocontrol activity against *Verticillium dahliae*. *J Phytopathol* **149:** 57–64.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25:** 3389–3402.

Antonopoulos, D.F., Tjamos, S.E., Antoniou, P.P., Rafeletos, P., and Tjamos, E.C. (2008) Effect of *Paenibacillus alvei*, strain K165, on the germination of *Verticillium dahliae* microsclerotia *in planta*. *Biol Control* **46:** 166–170.

Aranda, S., Montes-Borrego, M., Jiménez-Díaz, R.M., and Landa, B.B. (2011) Microbial communities associated with the root system of wild olives (*Olea europaea* L. subsp *europaea* var. *sylvestris*) are good reservoirs of bacteria with antagonistic potential against *Verticillium dahliae*. *Plant Soil* **343:** 329–345.

Audenaert, K., Pattery, T., Cornelis, P., and Höfte, M. (2002) Induction of systemic resistance to *Botrytis cinerea* in tomato by *Pseudomonas aeruginosa* 7NSK2: role of salicylic acid, pyochelin, and pyocyanin. *Mol Plant Microbe Interact* **15:** 1147–1156.

Bacon, C.W., and Hinton, D.M. (2006) Bacterial endophytes: the endophytic niche, its occupants, and its utility. In *Plant-Associated Bacteria*. Gnanamanickam, S.S. (ed.). Dordrecht, The Netherlands: Springer, pp. 155–194.

Bais, H.P., Weir, T.L., Perry, L.G., Gilroy, S., and Vivanco, J.M. (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu Rev Plant Biol* **57:** 233–266.

Bakker, P.A.H.M., Ran, L.X., and Mercado-Blanco, J. (2014) Rhizobacterial salicylate production provokes headaches! *Plant Soil* **382:** 1–16.

Barahona, E., Navazo, A., Martínez-Granero, F., Zea-Bonilla, T., Pérez-Jiménez, R.M., Martín, M., and Rivilla, R. (2011) *Pseudomonas fluorescens* F113 mutant with enhanced competitive colonization ability and improved biocontrol activity against fungal root pathogens. *Appl Environ Microbiol* **77:** 5412–5419.

Berg, G., Opelt, K., Zachow, C., Lottmann, J., Götz, M., Costa, R., and Smalla, K. (2006) The rhizosphere effect on bacteria antagonistic towards the pathogenic fungus *Verticillium* differs depending on plant species and site. *FEMS Microbiol Ecol* **56:** 250–261.

Böhm, M., Hurek, T., and Reinhold-Hurek, B. (2007) Twitching motility is essential for endophytic rice colonization by the N_2-fixing endophyte *Azoarcus* sp. strain BH72. *Mol Plant Microbe Interact* **20:** 526–533.

Bultreys, A. (2007) Siderotyping, a tool to characterize, classify and identify fluorescent *Pseudomonas*. In *Microbial Siderophores*. Varma, A., and Chincholkar, S. (eds). Berlin, Germany: Springer-Verlag, pp. 67–89.

Buysens, S., Heungens, K., Poppe, J., and Höfte, M. (1996) Involvement of pyochelin and pyoverdine in suppression of *Pythium*-induced damping-off of tomato by *Pseudomonas aeruginosa* 7NSK2. *Appl Environ Microbiol* **62:** 865–871.

Caetano-Anollés, G. (1993) Amplifying DNA with arbitrary oligonucleotide primers. *PCR Methods Appl* **3:** 85–92.

Campbell, C.L., and Madden, L.V. (1990) *Introduction to Plant Disease Epidemiology*. New York, NY, USA: John Wiley and Sons.

Capdevila, S., Martínez-Granero, F.M., Sánchez-Contreras, M., Rivilla, R., and Martín, M. (2004) Analysis of *Pseudomonas fluorescens* F113 genes implicated in flagellar filament synthesis and their role in competitive root colonization. *Microbiology* **150:** 3889–3897.

Chen, C., Bauske, E.M., Musson, G., Rodríguez-Kabana, R., and Kloepper, J.W. (1995) Biological control of Fusarium wilt on cotton by use of endophytic bacteria. *Biol Control* **5:** 83–91.

Chin-A-Woeng, T.F.C., Bloemberg, G.V., Mulders, I.H.M., Dekkers, L.C., and Lugtenberg, B.J.J. (2000) Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. *Mol Plant Microbe Interact* **13:** 1340–1345.

Collado-Romero, M., Mercado-Blanco, J., Olivares-García, C., Valverde-Corredor, A., and Jiménez-Díaz, R.M. (2006) Molecular variability within and among *Verticillium dahliae* vegetative compatibility groups determined by fluorescent amplified fragment length polymorphism and polymerase chain reaction markers. *Phytopathology* **96:** 485–495.

Compant, S., Duffy, B., Nowak, J., Clément, C., and Barka, E.A. (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl Environ Microbiol* **71:** 4951–4959.

Conn, V.M., Walker, A.R., and Franco, C.M.M. (2008) Endophytic actinobacteria induce defense pathways in *Arabidopsis thaliana*. *Mol Plant Microbe Interact* **21:** 208–218.

Cornelis, P., and Matthijs, S. (2002) Diversity of siderophore-mediated iron uptake systems in fluorescent pseudomonads: not only pyoverdines. *Environ Microbiol* **4:** 787–798.

Dennis, P.G., Miller, A.J., and Hirsch, P.R. (2010) Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities? *FEMS Microbiol Ecol* **72:** 313–327.

Déziel, E., Comeau, Y., and Villemur, R. (2001) Initiation of biofilm formation by *Pseudomonas aeruginosa* 57RP correlates with emergence of hyperpiliated and highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities. *J Bacteriol* **183:** 1195–1204.

Djavaheri, M., Mercado-Blanco, J., Versluis, C., Meyer, J.M., Van Loon, L.C., and Bakker, P.A.H.M. (2012) Iron-regulated metabolites produced by *Pseudomonas fluorescens* WCS374 are not required for eliciting induced systemic resistance against *Pseudomonas syringae* pv. *tomato* in Arabidopsis. *MicrobiologyOpen* **1:** 311–325.

Durland, R.H., and Helinski, D.R. (1987) The sequence encoding the 43-kilodalton *trfA* protein is required for efficient replication or maintenance of minimal RK2 replicons in *Pseudomonas aeruginosa*. *Plasmid* **18:** 164–169.

El Hadrami, A., Adam, L.R., and Daayf, F. (2011) Biocontrol treatments confer protection against *Verticillium dahliae* infection of potato by inducing antimicrobial metabolites. *Mol Plant Microbe Interact* **24:** 328–335.

Gaworzewska, E.T., and Carlile, M.J. (1982) Positive chemotaxis of *Rhizobium leguminosarum* and other bacteria towards root exudates from legumes and other plants. *J Gen Microbiol* **128:** 1179–1188.

Gitte, R.R., Rai, P.V., and Patil, R.B. (1978) Chemotaxis of *Rhizobium* sp. towards root exudate of *Cicer arietinum* L. *Plant Soil* **50:** 553–566.

Gómez-Lama Cabanás, C., Schilirò, E., Valverde-Corredor, A., and Mercado-Blanco, J. (2014) The biocontrol endophytic bacterium Pseudomonas fluorescens PICF7 induces systemic defense responses in aerial tissues upon colonization of olive roots. *Front Microbiol* **5:** 427. doi:10.3389/fmicb.2014.00427.

Hallmann, J., Quadt-Hallmann, A., Mahafee, W.F., and Kloepper, J.W. (1997) Bacterial endophytes in agricultural crops. *Can J Microbiol* **43:** 895–914.

Hamdan, H., Weller, D.M., and Thomashow, L.S. (1991) Relative importance of fluorescent siderophores and other factors in biological control of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* 2-79 and M4-80R. *Appl Environ Microbiol* **57:** 3270–3277.

Hardoim, P.R., van Overbeek, L.S., and Elsas, J.D.V. (2008) Properties of bacterial endophytes and their proposed role in plant growth. *Trends Microbiol* **16:** 463–471.

Howie, W.J., Cook, R.J., and Weller, D.M. (1987) Effects of soil matric potential and cell motility on wheat root colonization by fluorescent pseudomonads suppressive to take-all. *Phytopathology* **77:** 286–292.

Hynes, M.F., Quandt, J., O'Connell, M.P., and Pühler, A. (1989) Direct selection for curing and deletion of *Rhizobium* plasmids using transposons carrying the *Bacillus subtilis sacB* gene. *Gene* **78:** 111–120.

King, E.O., Ward, M.K., and Raney, D.E. (1954) Two simple media for demonstration of pyocyanin and fluorescein. *J Lab Clin Med* **44:** 301–307.

Kloepper, J.W., and Ryu, C.M. (2006) Bacterial endophytes as elicitors of induced systemic resistance. In *Microbial Root Endophytes*. Schulz, B.J.E., Boyle, C.J.C., and Sieber, T.N. (eds). Berlin, Germany: Springer-Verlag, pp. 33–52.

Kraus, J., and Loper, J.E. (1992) Lack of evidence for a role of antifungal metabolite production by *Pseudomonas fluorescens* Pf-5 in biological control of *Pythium* damping-off of cucumber. *Phytopathology* **82:** 264–271.

Kuklinsky-Sobral, H.L., Araujo, W.L., Mendes, R., Geraldi, I.O., Pizzirani-Kleiner, A.A., and Azevedo, J.L. (2004) Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. *Environ Microbiol* **6:** 1244–1251.

Lemanceau, P., Expert, D., Gaymard, F., Bakker, P.A.H.M., and Briat, J.F. (2009) Role of iron in plant–microbe interactions. *Adv Bot Res* **51:** 491–549.

Lindow, S.E. (1988) Lack of correlation of in vitro antibiosis with antagonism of ice nucleation active bacteria on leaf surfaces by non-ice nucleation active bacteria. *Phytopathology* **78:** 444–450.

López-Escudero, F.J., and Mercado-Blanco, J. (2011) Verticillium wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. *Plant Soil* **344:** 1–50.

López-Escudero, F.J., Del Rio, C., Caballero, J.M., and Blanco-López, M.A. (2004) Evaluation of olive cultivars for resistance to *Verticillium dahliae*. *Eur J Plant Pathol* **110:** 79–85.

Loper, J.E., Hassan, K.A., Mavrodi, D.V., Davis, E.W., II, Lim, C.K., Shaffer, B.T., *et al*. (2012) Comparative genomics of plant-associated *Pseudomonas* spp.: Insights into diversity and inheritance of traits involved in multitrophic interactions. *PLoS Genet* **8:** e1002784.

Lugtenberg, B.J., Dekkers, L., and Bloemberg, G.V. (2001) Molecular determinants of rhizosphere colonization by *Pseudomonas*. *Annu Rev Phytopathol* **39:** 461–490.

Lugtenberg, B.J.J., and Bloemberg, G.V. (2004) Life in the rhizosphere. In *Pseudomonas*, Vol. 1. Ramos, J.L. (ed.). New York, USA: Kluwer Academic/Plenum Publishers, pp. 403–430.

Maldonado-González, M.M., Prieto, P., Ramos, C., and Mercado-Blanco, J. (2013) From the root to the stem: interaction between the biocontrol root endophyte *Pseudomonas fluorescens* PICF7 and the pathogen *Pseudomonas savastanoi* NCPPB 3335 in olive knots. *Microb Biotechnol* **6:** 275–287.

Martínez-Granero, F., Rivilla, R., and Martín, M. (2006) Rhizosphere selection of highly motile phenotypic variants of *Pseudomonas fluorescens* with enhanced competitive colonization ability. *Appl Environ Microbiol* **72:** 3429–3434.

Mavrodi, O.V., Mavrodi, D.V., Park, A.A., Weller, D.M., and Thomashow, L.S. (2006) The role of dsbA in colonization of the wheat rhizosphere by *Pseudomonas fluorescens* Q8r1-96. *Microbiology* **152:** 863–872.

Mercado-Blanco, J., and Bakker, P.A. (2007) Interactions between plants and beneficial *Pseudomonas* spp.: exploiting bacterial traits for crop protection. *Antonie Van Leeuwenhoek* **92:** 367–389.

Mercado-Blanco, J., and López-Escudero, F.J. (2012) Verticillium wilt of olive and its control: the heat is on. *Plant Soil* **355:** 17–21.

Mercado-Blanco, J., and Lugtenberg, B.J.J. (2014) Biotechnological applications of bacterial endophytes. *Curr Biotechnol* **3:** 60–75.

Mercado-Blanco, J., Van der Drift, K.M., Olsson, P.E., Thomas-Oates, J.E., Van Loon, L.C., and Bakker, P.A. (2001) Analysis of the *pmsCEAB* gene cluster involved in biosynthesis of salicylic acid and the siderophore pseudomonine in the biocontrol strain *Pseudomonas fluorescens* WCS374. *J Bacteriol* **183:** 1909–1920.

Mercado-Blanco, J., Rodríguez-Jurado, D., Hervás, A., and Jiménez-Díaz, R.M. (2004) Suppression of Verticillium wilt in olive planting stocks by root-associated fluorescent *Pseudomonas* spp. *Biol Control* **30:** 474–486.

Meschke, H., Walter, S., and Schrempf, H. (2012) Characterization and localization of prodiginines from *Streptomyces lividans* suppressing *Verticillium dahliae* in the absence or presence of *Arabidopsis thaliana*. *Environ Microbiol* **14:** 940–952.

Meyer, J.A., and Abdallah, M.A. (1978) The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. *J Gen Microbiol* **107:** 319–328.

Meyer, J.M. (2000) Pyoverdines: pigments, siderophores and potential taxonomic markers of fluorescent *Pseudomonas* species. *Arch Microbiol* **174:** 135–142.

Miller, J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory.

Mitter, B., Petric, A., Shin, M.W., Chain, P.S.G., Hauberg-Lotte, L., Reinhold-Hurek, B., *et al*. (2013) Comparative genome analysis of Burkholderia phytofirmans PsJN reveals a wide spectrum of endophytic lifestyles based on interaction strategies with host plants. *Front Plant Sci* **4:** 120.

Müller, H., Tejedor-González, E., Mercado-Blanco, J., Rodríguez-Jurado, D., Jiménez-Díaz, R.M., and Berg, G. (2007) Effect of the biological control strain *Serratia plymuthica* HRO-C48 on Verticillium wilt of olive trees cv. Arbequina. In *Fundamental and Practical Approaches to Increase Biocontrol Efficacy*. Elad, Y., Ongena, M., Höfte, M., and Haïssan Jijakli, M. (eds). Dijon, France: IOBC/WPRS, pp. 173–177. IOBC/wprs Bulletin 30.

Navazo, A., Barahona, E., Redondo-Nieto, M., Martínez-Granero, F., Rivilla, R., and Martín, M. (2009) Three independent signalling pathways repress motility in *Pseudomonas fluorescens* F113. *Microb Biotechnol* **2:** 489–498.

Nejad, P., and Johnson, P.A. (2000) Endophytic bacteria induce growth promotion and wilt disease suppression in oilseed rape and tomato. *Biol Control* **18:** 208–215.

Ongena, M., Daayf, F., Jacques, P., Thonart, P., Benhamou, N., Paulitz, T.C., *et al*. (1999) Protection of cucumber

against Pythium root rot by fluorescent pseudomonads: predominant role of induced resistance over siderophores and antibiosis. *Plant Pathol* **48:** 66–76.

Ormeño-Orrillo, E., Rosenblueth, M., Luyten, E., Vanderleyden, J., and Martínez-Romero, E. (2008) Mutations in lipopolysaccharide biosynthetic genes impair maize rhizosphere and root colonization of *Rhizobium tropici* CIAT899. *Environmen microbiol* **10:** 1271–1284.

Overhage, J., Lewenza, S., Marr, A.K., and Hancock, R.E. (2007) Identification of genes involved in swarming motility using a *Pseudomonas aeruginosa* PAO1 mini-Tn5-lux mutant library. *J Bacteriol* **189:** 2164–2169.

Pegg, G.F., and Brady, B.L. (2002) *Verticillium Wilts*. Wallingford, UK: CAB International, pp. 293–340.

Pieterse, C.M.J., Zamioudis, C., Berendsen, R.L., Weller, D.M., Van Wees, S.C.M., and Bakker, P.A.H.M. (2014) Induced systemic resistance by beneficial microbes. *Annu Rev Phytopathol* **52:** 347–375.

Prieto, P., and Mercado-Blanco, J. (2008) Endophytic colonization of olive roots by the biocontrol strain *Pseudomonas fluorescens* PICF7. *FEMS Microbiol Ecol* **64:** 297–306.

Prieto, P., Navarro-Raya, C., Valverde-Corredor, A., Amyotte, S.G., Dobinson, K.F., and Mercado-Blanco, J. (2009) Colonization process of olive tissues by *Verticillium dahliae* and its *in planta* interaction with the biocontrol root endophyte *Pseudomonas fluorescens* PICF7. *Microb Biotechnol* **2:** 499–511.

Prieto, P., Schilirò, E., Maldonado-González, M.M., Valderrama, R., Barroso-Albarracín, J.B., and Mercado-Blanco, J. (2011) Root hairs play a key role in the endophytic colonization of olive roots by *Pseudomonas* spp. with biocontrol activity. *Microbial Ecol* **62:** 435–445.

Raaijmakers, J.M., Paulitz, T.C., Steinberg, C., Alabouvette, C., and Moënne-Loccoz, Y. (2009) The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant Soil* **321:** 341–361.

Reinhold-Hurek, B., and Hurek, T. (2011) Living inside plants: bacterial endophytes. *Curr Opin Plant Biol* **14:** 435–443.

Rodríguez-Moreno, L., Jiménez, A.J., and Ramos, C. (2009) Endopathogenic lifestyle of *Pseudomonas savastanoi* pv. savastanoi in olive knots. *Microb Biotechnol* **2:** 476–488.

Sanei, S.J., and Razavi, S.E. (2011) Suppression of Verticillium wilt of olive by *Pseudomonas fluorescens*. *Am J Exp Agri* **1:** 294–305.

Sang, M.K., and Kim, K.D. (2014) Biocontrol activity and root colonization by *Pseudomonas corrugata* strains CCR04 and CCR80 against Phytophthora blight of pepper. *BioControl* **59:** 437–448.

Schilirò, E., Ferrara, M., Nigro, F., and Mercado-Blanco, J. (2012) Genetic responses induced in olive roots upon colonization by the biocontrol endophytic bacterium *Pseudomonas fluorescens* PICF7. *PLoS ONE* **7:** e48646.

Schwyn, B., and Neilands, J.B. (1987) Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* **160:** 47–56.

Simon, R., Priefer, U., and Pühler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. *Nat Biotechnol* **1:** 784–791.

Simons, M., Permentier, H.P., de Weger, L.A., Wijffelman, C.A., and Lugtenberg, B.J.J. (1997) Amino acid synthesis is necessary for tomato root colonization by *Pseudomonas fluorescens* strain WCS365. *Mol Plant Microbe Interact* **10:** 102–106.

Tjamos, E.C. (1993) Prospects and strategies in controlling Verticillium wilt of olive. *OEPP/EPPO Bull* **23:** 505–512.

Tjamos, S.E., Flemetakis, E., Paplomatas, E.J., and Katinakis, P. (2005) Induction of resistance to *Verticillium dahliae* in *Arabidopsis thaliana* by the biocontrol agent K-165 and pathogenesis-related proteins gene expression. *Mol Plant Microbe Interact* **18:** 555–561.

Wang, Y.Q., Ohara, Y., Nakayashiki, H., Tosa, Y., and Mayama, S. (2005) Microarray analysis of the gene expression profile induced by the endophytic plant growth-promoting rhizobacteria, *Pseudomonas fluorescens* FPT9601-T5 in Arabidopsis. *Mol Plant Microbe Interact* **18:** 385–396.

Weller, D.M. (2007) *Pseudomonas* biocontrol agents of soilborne pathogens: looking back over 30 years. *Phytopathology* **97:** 250–256.

Supporting information

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

Fig. S1. Phenotypes displayed by *Pseudomonas fluorescens* PICF7 mutant derivatives carrying plasmid pLRM1 in SM (up left), PDA (up right), CAS (down left) and SSM (down right) agar media. As observed for strain PICF7 and its Tc^R mutant derivatives (results shown in Fig. 1), the GFP-labelled ME419 mutant (*gltA*-G) displayed growth delay in PDA. The GFP-labelled ME424 mutant (*flil*-G) showed lack of swimming motility in SM. The GFP-labelled ME589 mutant (*pvdI*-G) was impaired in pyoverdine (Pvd) synthesis in CAS. Finally, the GFP-labelled ME1508 mutant (Cys Aux-G) displayed cysteine auxotrophy in SSM.

Table S1. Phenotypes of 55 pre-selected *Pseudomonas fluorescens* PICF7 mutant derivatives.

Table S2. Root colonization ability of GFP-labelled (Gm^R) *Pseudomonas fluorescens* PICF7 derivatives.