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

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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.

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 (Hardoin 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 (Kloeper 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 (Fe³⁺)-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 (Tc⁰) colonies were obtained after biparental mating between Escherichia coli DH5α (pJQ18) and PICF7 (Table 1). Transposon Tn5-Tc⁰ 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, © 2014 Society for Applied Microbiology and John Wiley & Sons Ltd, *Environmental Microbiology*, 17, 3139–3153).
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-TcR 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 pre-screening, 55 TcR 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-TcR 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-TcR 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-TcR 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-TcR 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 ampiclon 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

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

<table>
<thead>
<tr>
<th>Strains or plasmids</th>
<th>Characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. fluorescens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PICF7</td>
<td>Wild-type strain isolated from olive roots</td>
<td>Mercado-Blanco and colleagues (2004)</td>
</tr>
<tr>
<td>ME419</td>
<td>PICF7 Tn5 (TcR) in vitro-reduced growth mutant derivative, GltA-</td>
<td>This work</td>
</tr>
<tr>
<td>ME424</td>
<td>PICF7 Tn5 (TcR) swimming motility mutant derivative, FilL-</td>
<td>This work</td>
</tr>
<tr>
<td>ME589</td>
<td>PICF7 Tn5 (TcR) pyoverdin mutant derivative, PvdI-</td>
<td>This work</td>
</tr>
<tr>
<td>ME1508</td>
<td>PICF7 Tn5 (TcR) Cys auxotroph mutant derivative</td>
<td>This work</td>
</tr>
<tr>
<td>PICF7-G</td>
<td>PICF7 (GmR) GFP-labelled derivative</td>
<td>This work</td>
</tr>
<tr>
<td>ME419-G</td>
<td>ME419 (TcR and GmR) GFP-labelled derivative</td>
<td>This work</td>
</tr>
<tr>
<td>ME424-G</td>
<td>ME424 (TcR and GmR) GFP-labelled derivative</td>
<td>This work</td>
</tr>
<tr>
<td>ME589-G</td>
<td>ME589 (TcR and GmR) GFP-labelled derivative</td>
<td>This work</td>
</tr>
<tr>
<td>ME1508-G</td>
<td>ME1508 (TcR and GmR) GFP-labelled derivative</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17-1</td>
<td>thi pro recA hsdR hsdM RP4-2-Tc,Mu-Km, TpRSmR</td>
<td>Simon and colleagues (1983)</td>
</tr>
<tr>
<td>DH55α</td>
<td>recA1 endA1-Φ80d lacZ dam-15</td>
<td>(Clontech)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pJQ18</td>
<td>pSUP5011 derivative; carries Tn5-Mob-Tc</td>
<td>Alfred Pühler</td>
</tr>
<tr>
<td>pLRM1</td>
<td>pBBR1-MCS5 carrying a fusion of the PA1/04/03 promoter to the gfpmut3* gene</td>
<td>Rodríguez-Moreno and colleagues (2009)</td>
</tr>
</tbody>
</table>

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

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

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).
derivatives.

**Table 3.** Bacterial growth (OD<sub>600</sub>) and pyoverdine production (OD<sub>400/600</sub>) by strain PICF7 and its mutant derivative ME1508 in SSM supplemented with L-Cys.

<table>
<thead>
<tr>
<th>L-Cys (mg l&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>Bacterial growth (OD&lt;sub&gt;600&lt;/sub&gt;)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Pyoverdine (OD&lt;sub&gt;400/600&lt;/sub&gt;)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PICF7</td>
<td>ME1508</td>
</tr>
<tr>
<td></td>
<td>PICF7</td>
<td>ME1508</td>
</tr>
<tr>
<td>0</td>
<td>0.40 ± 0.03</td>
<td>0.06 ± 0.01*</td>
</tr>
<tr>
<td>1</td>
<td>0.06 ± 0.01*</td>
<td>1.5 ± 0.32*</td>
</tr>
<tr>
<td>2</td>
<td>0.34 ± 0.14</td>
<td>2.2 ± 0.19*</td>
</tr>
<tr>
<td>5</td>
<td>0.46 ± 0.05</td>
<td>2.2 ± 0.09*</td>
</tr>
<tr>
<td>10</td>
<td>0.38 ± 0.04</td>
<td>2.5 ± 0.16*</td>
</tr>
<tr>
<td>20</td>
<td>0.41 ± 0.03</td>
<td>2.9 ± 0.20</td>
</tr>
</tbody>
</table>

*a.* Bacterial growth (OD<sub>600</sub>) 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 replicates 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<sup>a</sup> mutants were generated. Transformation frequency of plasmid pLRM1 ranged from 4.7 × 10<sup>5</sup> to 7.6 × 10<sup>6</sup> 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<sup>a</sup> mutant derivatives.

<table>
<thead>
<tr>
<th>Experiment&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Treatment&lt;sup&gt;2&lt;/sup&gt;</th>
<th>SAUDPC</th>
<th>DII</th>
<th>DI (%)</th>
<th>Bacterial population (log&lt;sub&gt;10&lt;/sub&gt; cfu g&lt;sup&gt;−1&lt;/sup&gt; of fresh root)&lt;sup&gt;4&lt;/sup&gt;</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Control</td>
<td>0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.59</td>
<td>93.33</td>
<td>4.3 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>PICF7</td>
<td>0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.24</td>
<td>78.57</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ME419 (gltA)</td>
<td>0.24&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>0.44</td>
<td>93.33</td>
<td>2.9 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>ME424 (fliI)</td>
<td>0.16&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>0.43</td>
<td>84.62</td>
<td>3.3 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>ME589 (pvdI)</td>
<td>0.17cri</td>
<td>0.33</td>
<td>78.57</td>
<td>3.4 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>ME1508 (Cys Aux)</td>
<td>0.34cri</td>
<td>0.51</td>
<td>86.67</td>
<td>3.1 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>II</td>
<td>Control</td>
<td>0.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.46</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PICF7</td>
<td>0.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.43</td>
<td>80</td>
<td>4.2 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>ME419 (gltA)</td>
<td>0.20&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>0.33</td>
<td>71.43</td>
<td>3.9 ± 0.6&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>ME424 (fliI)</td>
<td>0.29&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>0.38</td>
<td>84.62</td>
<td>3.5 ± 0.8&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>ME589 (pvdI)</td>
<td>0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.35</td>
<td>76.92</td>
<td>3.9 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>ME1508 (Cys Aux)</td>
<td>0.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.53</td>
<td>100</td>
<td>2.9 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>III</td>
<td>Control</td>
<td>0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.40</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PICF7</td>
<td>0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11</td>
<td>43.75</td>
<td>4.1 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>ME419 (gltA)</td>
<td>0.15&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.31</td>
<td>66.67</td>
<td>3.5 ± 0.3&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>ME424 (fliI)</td>
<td>0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.13</td>
<td>53.33</td>
<td>3.3 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>ME589 (pvdI)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3.5 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>ME1508 (Cys Aux)</td>
<td>0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.23</td>
<td>71.43</td>
<td>2.6 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> Three independent experiments were carried out spanning 110 days after inoculation (DAI) (experiment I), 118 DAI (experiment II) and 113 DAI (experiment III).

<sup>2</sup> The root system of 3-month-old olive plants were dipped in bacterial suspensions (5.8 × 10<sup>5</sup>–1.7 × 10<sup>6</sup> cfu ml<sup>−1</sup>) for 15 min and then transplanted into autoclaved soil for colonization assay, or autoclaved soil artificially infested with 3.2 × 10<sup>5</sup>–2.7 × 10<sup>6</sup> conidia g<sup>−1</sup> of the defoliating Verticillium dahliae isolate V9371 for biocontrol assays. Plants were grown in a growth chamber under controlled conditions (see text for details).

<sup>3</sup> SAUDPC, standardized area under the disease progress curve (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).

<sup>4</sup> Cell counts of Pseudomonas strains were determined on modified King’s medium B agar (PICF7) or on modified KBA amended with Tc (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 α = 0.05.

<sup>5</sup> Plants bacterized with ME589 and transplanted to soil infested with V. dahliae V9371 unexpectedly died. However, plants treated with ME589 alone (9) showed healthy, and therefore were kept and used for viable bacteria counts.

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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 GmR 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 *fil* 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 *fil* 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 *(fliH)* and ME589 (pvdl) 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 (Hardoi 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). Tn5-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 Tn5 (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 Tn5-Tc\(^{R}\) derivatives), Gm (for GFP-labelled PICF7 derivative) or Tc plus Gm (for GFP-labelled Tn5-Tc\(^{R}\) mutants).

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**GFP labelling of Pseudomonas**

Strain PICF7 and selected Tn5-Tc\(^6\) insertion mutants were transformed with plasmid pLRM1 (Gm\(^\text{R}\), GFP) (Rodríguez-Moreno et al., 2009). Electropotent 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 pLRM1 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 (250 r.p.m.) without antibiotic (Gm) selection pressure for 6 of exponential-phase cultures kept under vigorous shaking transformed derivative was assessed by continuous growth (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\(^\text{R}\)-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 = \([1 - (R/R_i)^{1/N}]\times 100\), 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* V9371, 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\(^\text{R}\) 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\(^\text{R}\) colonies previously grown overnight on KBA plates (amended with Te) 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 Takekawa, 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\(^\text{R}\) colonies) previously grown in KBA plates (amended with Te) (25°C, 24 h) were individually transferred to KBA and CAS plates (49 mutants/plate) without Te (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 pre-selected 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
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 GTGCACTAGTACNNNNNNNNNTI-3′) and another specific to the internal right end of the transposon Tn5 (Tn5Ext; 5′-GAACGTACCATGTTAGGAGGT-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 of 1× Taq DNA polymerase buffer, 3 mM MgCl₂, 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 OD₆₀₀ = 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 × 10⁵ to 1.7 × 10⁹ cfu ml⁻¹, 15 plants) or 10 mM MgSO₄·7H₂O (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 × 10⁵ to 2.7 × 10⁶ conidia g⁻¹ soil) of V. dahliae D isolate V9371 (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 DI defined as $\text{DI} = (\Sigma i \times Ni)/(4 \times Nt)$, 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) $SAU$ 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$_4$·7H$_2$O and serial dilutions of root macerates were plated in KBA plates amended with Amp, Chl and Chx (for PICF7 cells counting), plus Tc (for TcR 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 macerates were plated onto KBA amended with Amp, Chl, Chx and Gm, and grown at 25°C for 24 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. Samples 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$_2$O. 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**


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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:

**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 TcR mutant derivatives (results shown in Fig. 1), the GFP-labelled ME1508 mutant (Cys Aux-G) displayed cysteine auxotrophy in SSM. The GFP-labelled ME424 mutant (Gm R) was impaired in pyoverdine (Pvd) synthesis in CAS. The GFP-labelled ME419 mutant (Pvd-G) was impaired in pyoverdine (Pvd) synthesis in CAS. Finally, the GFP-labelled ME424 mutant (fill-G) showed lack of swimming motility in SM. The GFP-labelled ME589 mutant (pvd-F-G) was impaired in pyoverdine (Pvd) synthesis in CAS. 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.