The c-di-GMP phosphodiesterase BifA is involved in the virulence of bacteria from the *Pseudomonas syringae* complex

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SUMMARY

In a recent screen for novel virulence factors involved in the interaction between *Pseudomonas savastanoi* pv. *savastanoi* and the olive tree, a mutant was selected that contained a transposon insertion in a putative cyclic diguanylate (c-di-GMP) phosphodiesterase-encoding gene. This gene displayed high similarity to *bifA* of *Pseudomonas aeruginosa* and *Pseudomonas putida*. Here, we examined the role of BifA in free-living and virulence-related phenotypes of two bacterial plant pathogens in the *Pseudomonas syringae* complex, the tumour-inducing pathogen of woody hosts, *P. savastanoi* pv. *savastanoi* NCPPB 3335, and the pathogen of tomato and *Arabidopsis*, *P. syringae* pv. *tomato* DC3000. We showed that deletion of the *bifA* gene resulted in decreased swimming motility of both bacteria and inhibited swarming motility of DC3000. In contrast, overexpression of BifA in *P. savastanoi* pv. *savastanoi* had a positive impact on swimming motility and negatively affected biofilm formation. Deletion of *bifA* in NCPPB 3335 and DC3000 resulted in reduced fitness and virulence of the microbes in olive (NCPPB 3335) and tomato (DC3000) plants. In addition, real-time monitoring of olive plants infected with green fluorescent protein (GFP)-tagged *P. savastanoi* cells displayed an altered spatial distribution of mutant Δ *bifA* cells inside olive knots compared with the wild-type strain. All free-living phenotypes that were altered in both Δ *bifA* mutants, as well as the virulence of the NCPPB 3335 Δ *bifA* mutant in olive plants, were fully rescued by complementation with *P. aeruginosa* BifA, whose phosphodiesterase activity has been demonstrated. Thus, these results suggest that *P. syringae* and *P. savastanoi* BifA are also active phosphodiesterases. This first demonstration of the involvement of a putative phosphodiesterase in the virulence of the *P. syringae* complex provides confirmation of the role of c-di-GMP signalling in the virulence of this group of plant pathogens.

Keywords: BifA, c-di-GMP, olive plants, phosphodiesterase, *Pseudomonas savastanoi*, *Pseudomonas syringae*, tomato.

INTRODUCTION

The modified nucleotide cyclic diguanylate (c-di-GMP) is a ubiquitous secondary messenger in bacteria that regulates the transition between planktonic and sessile lifestyles. Thus, high levels of c-di-GMP enhance the production of extracellular matrix components, exopolysaccharides (EPSs) and proteins (Christen *et al.*, 2006; Pérez-Mendoza *et al.*, 2011b; Recouvreur *et al.*, 2008; Solano *et al.*, 2002; Zogaj *et al.*, 2001). c-di-GMP is synthesized from two molecules of guanosine triphosphate (GTP) by diguanylate cyclases (DGCs) with GGDEF catalytic domains (Paul *et al.*, 2004), and is degraded by specific phosphodiesterases (PDEs) with EAL (Christen *et al.*, 2005; Tischler and Camilli, 2005) or HD-GYP (Ryan *et al.*, 2007) domains. The DGC and PDE catalytic domains are frequently associated with different sensory and regulatory domains, so that intracellular levels of c-di-GMP are modulated by various environmental and/or intracellular signals (Mills *et al.*, 2011). Functions regulated by this secondary messenger include biofilm formation and bacterial motility (D'Argenio and Miller, 2004; Dow *et al.*, 2006; Hengge, 2009; Jenal and Malone, 2006; Römling *et al.*, 2013; Tamayo *et al.*, 2007; Yi *et al.*, 2010). In recent years, c-di-GMP has also been shown to be involved in the virulence of animal and plant bacterial pathogens (Hengge, 2009; Kulasakara *et al.*, 2006; Römling *et al.*, 2013; Tamayo *et al.*, 2007). In phytopathogenic bacteria, several proteins have been experimentally shown to function in c-di-GMP signalling. A role in plant infection has been shown for several c-di-GMP-metabolizing proteins and c-di-GMP receptors in *Xanthomonas campestris* (An *et al.*, 2014; Dow *et al.*, 2006; He *et al.*, 2009; McCarthy *et al.*, 2008; Ryan *et al.*, 2006, 2010) and *Xanthomonas oryzae* pv. *oryzicola* (Yang *et al.*, 2012, 2014; Zhang *et al.*, 2013), the PDE proteins EcpB and EcpC of *Dickeya dadantii* (Yi *et al.*, 2010), and the DGC and PDE proteins CgsA and Eal, respectively, of *Xylella fastidiosa* (Chatterjee *et al.*, 2010; de Souza *et al.*, 2013). Other c-di-GMP-metabolizing enzymes have been associated with the

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regulation of surface attachment to plants and other virulence-related phenotypes in *Agrobacterium tumefaciens* (Xu *et al.*, 2013) and *Pectobacterium atrosepticum* (Pérez-Mendoza *et al.*, 2011a, 2011b; Tan *et al.*, 2014), among others. In the *Pseudomonas syringae* complex, which includes some of the most scientifically and economically relevant bacterial plant pathogens (Mansfield *et al.*, 2012), a DGC from the tomato plant pathogen *P. syringae* pv. *tomato* DC3000 has been shown recently to promote plant immune evasion (Engl *et al.*, 2014). In addition, the responses to artificially induced high levels of c-di-GMP have been studied in DC3000, as well as in the bean and olive plant pathogens *P. syringae* pv. *phaseolicola* and *Pseudomonas savastanoi* pv. *savastanoi*, respectively (Pérez-Mendoza *et al.*, 2014). Although elevated intracellular c-di-GMP levels have been associated with several free-living phenotypes in all three pathogens, virulence was clearly affected only in *P. savastanoi* pv. *savastanoi*, the causal agent of olive knot disease (Pérez-Mendoza *et al.*, 2014). In a recent screen for novel virulence factors involved in the interaction between *P. savastanoi* pv. *savastanoi* and olive trees, we identified BifA (Matas *et al.*, 2012), a GGDEF/EAL domain protein that has homologues in *Pseudomonas aeruginosa* (Kuchma *et al.*, 2007) and *Pseudomonas putida* KT2442 (Jiménez-Fernández *et al.*, 2014). In both of these *Pseudomonas* species, BifA possesses an active EAL domain and a non-canonical GGDEF domain (GGDQF), both of which are crucial for the PDE activity required for their function (Jiménez-Fernández *et al.*, 2014; Kuchma *et al.*, 2007). Aside from their domain conservation and similar activity, the set of functions regulated by both proteins seems to be different between these strains. In *P. aeruginosa*, BifA reciprocally regulates biofilm formation and swarming motility, whereas, in *P. putida*, it does not appear to regulate flagella-mediated motility, but rather is a key protein in the starvation-induced dispersal of biofilms (Jiménez-Fernández *et al.*, 2014; Kuchma *et al.*, 2007). Nevertheless, a link between BifA and the virulence of animal- or plant-pathogenic *Pseudomonads* has not yet been established.

The goal of this work was to analyse the role of BifA in the virulence of the *P. syringae* complex. We focused on two strains, *P. savastanoi* pv. *savastanoi* NCPPB 3335, the tumour-inducing pathogen of woody hosts (Ramos *et al.*, 2012), and *P. syringae* pv. *tomato* DC3000, which causes bacterial speck in tomato and *Arabidopsis* (Buell *et al.*, 2003). Our results demonstrate that, in both bacterial pathogens, a functional BifA is required not only for the control of bacterial motility, but also for full fitness and virulence in their respective plant hosts. Furthermore, the overexpression of BifA in *P. savastanoi* negatively impacts biofilm formation whilst positively affecting swimming motility. We also showed that the virulence of a *P. savastanoi* Δ bifA mutant in olive plants could be fully restored by complementation with the *bifA* gene from *P. aeruginosa*, the PDE activity of which has been demonstrated, suggesting that both BifA proteins perform similar functions in both bacterial pathogens.

RESULTS

Conservation of BifA in the *Pseudomonas* genus

A comparative phylogenetic analysis of BifA was carried out using sequences available in the National Center for Biotechnology Information (NCBI) database. The results showed that BifA is widely distributed within the *Pseudomonas* genus and is encoded in the genome of all sequenced *Pseudomonas* strains. The phylogeny of the BifA proteins was largely congruent with the phylogeny of the genus *Pseudomonas* (Yamamoto *et al.*, 2000) and with that of the *P. syringae* complex (Studholme, 2011) deduced from housekeeping genes, suggesting that the *bifA* gene is ancestral within this bacterial group. CLUSTALW2 alignment of the complete amino acid sequences of BifA from *P. savastanoi* pv. *savastanoi* NCPPB 3335 (BifA_{PSV}), *P. syringae* pv. *tomato* DC3000 (BifA_{PTO}), *P. putida* KT2440 (BifA_{PPU}) and *P. aeruginosa* PAO1 (BifA_{PAO}) revealed that BifA_{PSV} and BifA_{PTO} are 97% identical to each other. In addition, BifA_{PTO} and BifA_{PSV} show 87% identity with BifA_{PPU} (93% 'positive'), and 80% and 81% identity with BifA_{PAO} (90% 'positive'), respectively. BifA homologues were also found in other Gammaproteobacteria closely related to the *Pseudomonas* genus, including *Azotobacter vinelandii*, *Hahella chejuensis* and *Marinobacter hydrocarbonoclasticus* (Gauthier *et al.*, 1992; Jeong *et al.*, 2005; Setubal *et al.*, 2009), which showed 60%, 48% and 45% identity with BifA_{PSV}, respectively (Fig. 1A). In addition, conservation of identical EAL (EALX₅₇NX₃₃EX₂₆DX₂₀KX₃₅E) (Rao *et al.*, 2008) and modified GGDEF (GGDQF) domains was observed in all sequenced *Pseudomonas* spp. (Fig. 1B), as well as in *H. chejuensis*. In contrast, *A. vinelandii* and *M. hydrocarbonoclasticus* encoded a different GGDEF-like domain, consisting of GGDRF and GSDQF, respectively. An RXXD allosteric control motif (often referred to as an I-site), which has been shown to provide allosteric control for the activity of several DGCs (Chan *et al.*, 2004; Christen *et al.*, 2006), was not found in any of the *Pseudomonas* BifA homologues.

BifA influences the flagellar motility of both *P. savastanoi* pv. *savastanoi* and *P. syringae* pv. *tomato*

Using gene replacement, we constructed *bifA* deletion mutants for *P. savastanoi* pv. *savastanoi* NCPPB 3335 and *P. syringae* pv. *tomato* DC3000. The loss of *bifA* resulted in decreased swimming motility in both NCPPB 3335 and DC3000 (decreases of approximately 1.3- and 1.5-fold, respectively) (Fig. 2A,B) and an approximately 1.9-fold decrease in DC3000 swarming motility (Fig. 2C,D). Nevertheless, complementation of both Δ bifA mutants with the *bifA* gene from *P. savastanoi* pv. *savastanoi* NCPPB 3335 (plasmid pJB3-*bifA*_{PSV}) or from *P. aeruginosa* PAO1 (plasmid pJB3-*bifA*_{PAO}) (Table 1) resulted in full restoration of both PDE-associated

Fig. 1 Phylogeny and conservation of BifA in the *Pseudomonas* genus. (A) Phylogenetic analysis of BifA from several *Pseudomonas* strains. The tree was constructed using MEGA5 (Tamura *et al.*, 2011) with the neighbour-joining method (Saitou and Nei, 1987), and evolutionary distance units were computed as amino acid substitutions per site. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (10 000 replicates) are shown. The locus tags corresponding to the BifA proteins used in this analysis are shown in Table S2 (see Supporting Information). (B) Conservation of the EAL domain and modified GGDEF (GGDQF) domain of BifA in *P. savastanoi* pv. *savastanoi* NCPPB 3335 (BifA_{PSV}), *P. syringae* pv. *tomato* DC3000 (BifA_{PTO}), *P. putida* KT2440 (BifA_{PDU}) and *P. aeruginosa* PAO1 (BifA_{PAO}). The numbers indicate the positions of the amino acid residues relative to the first methionine of BifA. The grey background indicates the amino acid residues corresponding to the position of the allosteric control RXXD motif present in other GGDEF-containing proteins.

phenotypes (Fig. 2). In addition, to test the importance of the EAL motif of BifA in motility, we generated a BifA_{PSV} mutant allele by changing glutamic acid residue 456 (Glu456) to alanine (Ala) (EAL to AAL). The resulting *bifA* mutant allele (*bifA*_{PSV}AAL) failed to complement the motility defect observed for the Δ *bifA*_{PSV} mutant (Fig. 2A), suggesting a role for this protein in c-di-GMP-mediated signalling pathways regulating flagellar motility. However, overexpression of BifA in NCPPB 3335 was associated with an approximate 1.8-fold increase in swimming motility (Fig. S1A, see Supporting Information), a result expected on the basis of the phenotype observed in the Δ *bifA* mutant of this strain. As reported previously (Pérez-Martínez *et al.*, 2007), *P. savastanoi* pv. *savastanoi* NCPPB 3335 did not display swarming motility under the conditions tested. Thus, the impact of *bifA* deletion or overexpression in NCPPB 3335 could not be evaluated.

Deletion of the *bifA* gene has a positive impact on biofilm formation in *P. aeruginosa* PA14 (Kuchma *et al.*, 2007) and *P. putida* KT2442 (Jiménez-Fernández *et al.*, 2014). Under the

conditions tested, the capacity for biofilm formation of *P. savastanoi* pv. *savastanoi* NCPPB 3335 and *P. syringae* pv. *tomato* DC3000 Δ *bifA* mutants was not altered relative to their respective wild-type strains (data not shown). However, biofilm formation of NCPPB 3335 overexpressing its own BifA ($A_{550\text{ nm}} = 0.20 \pm 0.01$) decreased by approximately three-fold in comparison with the wild-type strain transformed with an empty vector ($A_{550\text{ nm}} = 0.62 \pm 0.04$) (Fig. S1B). Moreover, a decrease in calcofluor (CF) binding was observed in the BifA-overexpressing strain compared with wild-type NCPPB 3335 (Fig. S1C).

The *bifA* gene is involved in *P. savastanoi* and *P. syringae* infections

Fitness attenuation in plant tissues has been associated previously with hypovirulence phenotypes in both *P. savastanoi* pv. *savastanoi* NCPPB 3335 (Matas *et al.*, 2012) and *P. syringae* pv. *tomato* DC3000 (Cunnac *et al.*, 2011; Macho *et al.*, 2007). Mixed

Fig. 2 Swimming and swarming motility of *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335 and *P. syringae* pv. *tomato* DC3000 $\Delta bifA$ mutants. The size of the swimming areas (calculated in mm²) on 20-fold-diluted 0.3% King's B (KB) agar plates of the indicated *P. savastanoi* strains after 72 h at 25 °C (A) or of the indicated *P. syringae* strains after 48 h at 25 °C (B). Quantification (C) and images (D) of the swarming motility of the indicated *P. syringae* strains after 36 h at 25 °C. Bars represent the mean of three biological replicates from two different assays; error bars represent the standard deviation. The same letters indicate not significant differences ($\alpha = 0.05$) using analysis of variance ANOVA. Psv NCPPB 3335 (pJB3), *P. savastanoi* pv. *savastanoi* NCPPB 3335 carrying the empty vector pJB3; $\Delta bifA_{Psv}$ NCPPB 3335 *bifA* mutant carrying pJB3; $\Delta bifA_{Psv}$ pJB3-*bifA*_{Psv} NCPPB 3335 *bifA* mutant complemented with *bifA* from *P. savastanoi* pv. *savastanoi* NCPPB 3335 (*bifA*_{Psv}); $\Delta bifA_{Psv}$ pJB3-*bifA*_{PAO} NCPPB 3335 *bifA* mutant complemented with *bifA* from *P. aeruginosa* PAO1 (*bifA*_{PAO}); $\Delta bifA_{Psv}$ pJB3-*bifA*_{Psv}AAL NCPPB 3335 *bifA* mutant complemented with the *bifA* mutant allele (*bifA*_{Psv}AAL); Pto DC3000 (pJB3), *P. syringae* pv. *tomato* DC3000 carrying the empty vector pJB3; $\Delta bifA_{Pto}$ DC3000 *bifA* mutant carrying pJB3; $\Delta bifA_{Pto}$ pJB3-*bifA*_{Psv} DC3000 *bifA* mutant complemented with *bifA* from *P. savastanoi* pv. *savastanoi* NCPPB 3335 (*bifA*_{Psv}); $\Delta bifA_{Pto}$ pJB3-*bifA*_{PAO} DC3000 *bifA* mutant complemented with *bifA* from *P. aeruginosa* PAO1 (*bifA*_{PAO}).

infections with NCPPB 3335 versus $\Delta bifA_{Psv}$ and DC3000 versus $\Delta bifA_{Pto}$ were prepared to perform competitive assays on *in vitro* micropropagated olive and tomato plants, respectively. The resulting competitive index (CI) values were significantly lower than unity; however, no differences in competence were observed between wild-type and corresponding complemented strains (Fig. 3). These results indicate that $\Delta bifA$ mutants are outcompeted by the respective wild-type strains *in planta*.

The *P. savastanoi* pv. *savastanoi* NCPPB 3335 $\Delta bifA_{Psv}$ mutant was inoculated into *in vitro* micropropagated and lignified olive plants, and the severity of the disease was evaluated after 30 or 90 days, respectively. Knots induced by the $\Delta bifA_{Psv}$ mutant in both plant systems were visibly smaller than those caused by either the wild-type strain or the mutant complemented with its own *bifA* gene. Moreover, plants infected with $\Delta bifA_{Psv}$ complemented with

the *P. aeruginosa* *bifA*_{PAO} gene developed knots similar in size to those induced by the wild-type strain (Fig. 4A,B). Quantification of knot volumes confirmed that the $\Delta bifA_{Psv}$ mutant induced the formation of knots approximately two-fold smaller than those induced by the wild-type or complemented strains on *in vitro* olive plants (Fig. 4C). Lignified olive plants infected with the $\Delta bifA_{Psv}$ mutant showed knots 4.2-fold smaller than those infected with the wild-type strain or the mutant complemented with the *P. aeruginosa* *bifA*_{PAO} gene. However, complementation of the mutant with its own *bifA*_{Psv} gene expressed behind a constitutive promoter (plasmid pJB3-*bifA*_{Psv}) yielded knot volumes higher than those induced by the wild-type strain (Fig. 4D). This result could be caused by the combined effect of the overexpression of BifA_{Psv} in this strain and a better adaptation of this BifA variant to the olive pathogen.

Table 1 Strains and plasmids used in this study.

Strain/plasmid	Relevant characteristics*	Reference or source
Strain		
<i>P. savastanoi</i> pv. <i>savastanoi</i> NCPPB 3335	Wild-type strain	Pérez-Martínez <i>et al.</i> (2007)
$\Delta bifa_{PSV}$	<i>bifA</i> deletion mutant (Km ^R)	This work
<i>P. syringae</i> pv. <i>tomato</i> DC3000	Wild-type strain	Buell <i>et al.</i> (2003)
$\Delta bifa_{PTO}$	<i>bifA</i> deletion mutant (Km ^R)	This work
<i>E. coli</i> DH5 α	<i>F</i> −, ϕ 80 <i>dlacZ</i> M15, (<i>lacZYA-argF</i>) U169, <i>deoR</i> , <i>recA1</i> , <i>endA</i> , <i>hsdR17</i> (<i>rk</i> − <i>mk</i> −), <i>phoA</i> , <i>supE44</i> , <i>thi</i> −1, <i>gyrA96</i> , <i>relA1</i>	Hanahan (1983)
GM2929	<i>F</i> −, <i>ara</i> −14, <i>leuB6</i> , <i>thi</i> −1, <i>tonA31</i> , <i>lacY1</i> , <i>tsx</i> −78, <i>galK2</i> , <i>galT22</i> , <i>glnV44</i> , <i>hisG4</i> , <i>rpsL136</i> , <i>xyl</i> −5, <i>mtl</i> −1, <i>dam13::Tn9</i> , <i>dcm</i> −6, <i>mcrB1</i> , <i>hsdR2</i> , <i>mcrA</i> , <i>recF143</i> (Sp ^R Cm ^R)	Palmer and Marinus (1994)
Plasmids		
pGEM-T <i>easy</i>	Cloning vector containing <i>ori f1</i> and <i>lacZ</i> (Ap ^R)	(Promega, Madison, WI, USA)
pGEM-T-KmFRT- <i>Bam</i> HI	Contains Km ^R from pKD4 (Ap ^R Km ^R)	Ortiz-Martín <i>et al.</i> (2010)
pJB3Tc19	Cloning vector, <i>P</i> _{lac} promoter (Ap ^R Tc ^R)	Blatny <i>et al.</i> (1997)
pLRM1-GFP	pBBR1-MCS5::PA1/04/03-RBSII-GFPmut3*-T0-T1, (Gm ^R)	Rodríguez-Moreno <i>et al.</i> (2009)
pGEM-T- <i>bifA</i> _{PSV}	pGEM-T <i>easy</i> derivative containing the <i>bifA</i> ORF from NCPPB 3335 (Ap ^R)	This work
pGEM-T- <i>bifA</i> _{PAO}	pGEM-T <i>easy</i> derivative containing the <i>bifA</i> ORF from <i>P. aeruginosa</i> PAO1 (Ap ^R)	This work
pGEM-T- <i>bifA</i> _{PSV} AAL	pGEM-T <i>easy</i> derivative containing <i>bifA</i> gene from NCPPB 3335 with a point mutation in the EAL motif (Ap ^R)	This work
pJB3- <i>bifA</i> _{PSV}	pJB3Tc19 derivative carrying the <i>bifA</i> ORF from NCPPB 3335 (Ap ^R Tc ^R)	This work
pJB3- <i>bifA</i> _{PAO}	pJB3Tc19 derivative carrying the <i>bifA</i> ORF from <i>P. aeruginosa</i> PAO1 (Ap ^R Tc ^R)	This work
pJB3- <i>bifA</i> _{PSV} AAL	pJB3Tc19 derivative carrying the <i>bifA</i> ORF from NCPPB 3335 with a point mutation in the EAL motif (Ap ^R Tc ^R)	This work
pGEM-T-K.O <i>bifA</i> _{PSV}	pGEM-T <i>easy</i> derivative, containing 1.2 kb approx. on each side of the <i>bifA</i> gene from NCPPB 3335 (Ap ^R)	This work
pGEM-T-K.O <i>bifA</i> _{PSV} − Km	pGEM-T derivative, containing 1.2 kb approx. on each side of the <i>bifA</i> gene from NCPPB 3335 interrupted by the kanamycin resistance gen <i>nptII</i> (Ap ^R , Km ^R)	This work
pGEM-T-K.O <i>bifA</i> _{PTO}	pGEM-T <i>easy</i> derivative containing 0.5 kb approx. on each side of the <i>bifA</i> gene from DC3000 (Ap ^R)	This work
pGEM-T-K.O <i>bifA</i> _{PTO} − Km	pGEM-T derivative containing 0.5 kb approx. on each side of the <i>bifA</i> gene from DC3000 interrupted by the kanamycin resistance gen <i>nptII</i> (Ap ^R , Km ^R)	This work

*Ap, ampicillin; Cm, chloramphenicol; Gm, gentamycin; Km, kanamycin; ORF, open reading frame; Tc, tetracycline.

To monitor the infection of olive plants by the $\Delta bifa_{PSV}$ mutant in real time, a green fluorescent protein (GFP)-tagged $\Delta bifa_{PSV}$ derivative was constructed by transformation with plasmid pLRM1-GFP (Rodríguez-Moreno *et al.*, 2009). First, in order to determine whether the deletion of *bifA* affects GFP expression or stability, we quantified and compared the GFP fluorescence emitted by GFP-tagged wild-type NCPPB 3335 and $\Delta bifa_{PSV}$ cells during growth in Luria–Bertani (LB) medium. No significant differences in GFP emission were observed between these two strains along the growth curve (Fig. S2, see Supporting Information), suggesting that BifA does not affect the expression or stability of GFP in *P. savastanoi* during growth *in vitro*. Conversely, and although colony-forming unit (CFU) levels in knots induced at 30 days post-inoculation (dpi) by the $\Delta bifa_{PSV}$ mutant were similar to those induced by the wild-type strain (approximately 10⁷ CFU/knot), the GFP fluorescence in knots caused by the mutant was only detected near the stem wounds (Fig. S3, see Supporting Information), suggesting a restricted spatial distribution or a reduction in GFP fluorescence intensity in $\Delta bifa_{PSV}$ mutant cells inside olive knots.

Infection of tomato leaves with wild-type *P. syringae* pv. *tomato* DC3000 caused chlorosis and small necrotic lesions detectable near the infiltration zone at 3–4 dpi. In contrast, the visualization of these symptoms in leaves infected with the $\Delta bifa_{PTO}$ mutant was delayed until 6 dpi (data not shown). At 9 dpi, plants infected with the wild-type strain displayed chlorosis and necrosis of the leaves spanning the entire infiltrated area, as well as the initiation of chlorosis in the non-infiltrated areas. However, plants infected with the $\Delta bifa_{PTO}$ mutant exhibited a clear reduction in both symptoms, which were restricted to the infiltrated areas (Fig. 5A). Quantification of the leaf areas covered by necrotic spots revealed a 3.5-fold reduction in this symptom in plants infected with the $\Delta bifa_{PTO}$ mutant relative to the wild-type (Fig. 5B). Complementation of the $\Delta bifa_{PTO}$ mutant with the *bifA*_{PSV} gene (plasmid pJB3-*bifA*_{PSV}) resulted in a slight, 1.5-fold increase in necrosis and chlorotic symptoms in comparison with the wild-type strain. Indeed, chlorotic lesions of leaves infected with the complemented strain covered almost the entire non-infiltrated areas. Overall, the results obtained in both pathosystems reveal a relevant role for BifA in the virulence of bacteria belonging to the *P. syringae* complex.

Fig. 3 Fitness of *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335 and *P. syringae* pv. *tomato* DC3000 $\Delta bifA$ mutants in plant tissues. (A) Competitive index (CI) assays in olive plants at 30 days post-inoculation (dpi) between an NCPPB 3335 $\Delta bifA$ mutant ($\Delta bifA_{PSV}$) or $\Delta bifA_{PSV}$ complemented with $BifA_{PSV}$ ($\Delta bifA_{PSV}$ pJB3- $bifA_{PSV}$) and the wild-type NCPPB 3335. (B) CI assays in tomato leaves at 5 dpi between a DC3000 $\Delta bifA$ mutant ($\Delta bifA_{PTO}$) or $\Delta bifA_{PTO}$ complemented with $BifA_{PSV}$ ($\Delta bifA_{PSV}$ pJB3- $bifA_{PSV}$) and the wild-type DC3000. Error bars indicate the standard error of the mean of three independent assays. Asterisks indicate indices that deviate significantly from unity. Statistical analyses were performed using Student's *t*-test ($P \leq 0.05$).

DISCUSSION

Bacteria are able to live either as independent planktonic cells or as sessile members of organized biofilms. In the case of bacterial pathogens, the transition between these two lifestyles is crucial for bacterial survival during host invasion and is often associated with virulence (Cotter and Stibitz, 2007). Over the past few years, several c-di-GMP-metabolizing enzymes have been associated with both the transition between acute/planktonic and chronic/biofilm lifestyles and with the virulence of bacterial pathogens (Römling *et al.*, 2013). Although knocking out PDEs has often been associated with a reduction in virulence (Ryan *et al.*, 2007; Yi *et al.*, 2010), and knocking out DGCs is associated with increased virulence (Hisert *et al.*, 2005; Tischler and Camilli, 2005), it is generally accepted that the regulation of virulence involves complex interactions of DGCs, PDEs and c-di-GMP effectors (Römling *et al.*, 2013). Here, we present genetic evidence for the involvement of the putative PDE BifA in the virulence of bacterial phytopathogens belonging to the *P. syringae* complex.

BifA is highly conserved within the *Pseudomonas* genus. This conservation includes the modified GGDEF-like (GGDQF) and EAL domains (Fig. 1), which have been shown to be required for the c-di-GMP PDE activity of BifA in *P. aeruginosa* PA14 (Kuchma *et al.*, 2007) and *P. putida* KT2442 (Jiménez-Fernández *et al.*, 2014). Here, our results suggest that the BifA proteins encoded by *P. savastanoi* pv. *savastanoi* NCPPB 3335 and *P. syringae* pv. *tomato* DC3000 are functional PDEs. Indeed, complementation of NCPPB 3335 and DC3000 $\Delta bifA$ mutants with BifA from *P. aeruginosa* PAO1, which is 100% identical to that of *P. aeruginosa* PA14, resulted in full restoration of the PDE phenotypes associated with the transition between motile and sessile lifestyles (Fig. 2). In addition, complementation of the NCPPB 3335 $\Delta bifA_{PSV}$ mutant with $BifA_{PAO}$ fully restored virulence in olive plants (Fig. 4), suggesting that this protein may have a relevant role not only in the interaction between *P. savastanoi* pv. *savastanoi* and *P. syringae* pv. *tomato* and their respective hosts (Figs 4 and 5), but also in the virulence of other pathogens of the *Pseudomonas* genus, including *P. aeruginosa*.

Virulence-related phenotypes affected by c-di-GMP levels include flagellar motility, EPS production and biofilm formation. With regard to flagellar motility, FleQ and FliC, major regulators of the flagellar regulon and the flagellar structural protein, respectively, have been shown to be required for full *in vivo* fitness and virulence of bacterial pathogens (Rogers *et al.*, 2006; Schulz *et al.*, 2012). BifA has been shown to differentially affect flagellar motility in *P. aeruginosa* PA14 and *P. putida* KT2442. Although deletion of the *bifA* gene in *P. aeruginosa* dramatically affects swarming motility without significantly affecting swimming motility (Kuchma *et al.*, 2007), neither swimming nor swarming assays revealed significant differences between wild-type *P. putida* and its $\Delta bifA$ mutant (Jiménez-Fernández *et al.*, 2014). Here, we report a slight, but significant, reduction in swimming motility for $\Delta bifA$ mutants of both *P. savastanoi* pv. *savastanoi* NCPPB 3335 and *P. syringae* pv. *tomato* DC3000 (Fig. 2). In addition, the swarming motility of DC3000 $\Delta bifA_{PTO}$ is severely affected (Fig. 2). Thus far, conditions to observe swarming motility in *P. savastanoi* pv. *savastanoi* strains have not yet been established (Pérez-Martínez *et al.*, 2007; Pérez-Mendoza *et al.*, 2014). Despite the differences observed between strains, complementation of $\Delta bifA_{PSV}$ and $\Delta bifA_{PTO}$ mutants with either $BifA_{PSV}$ or $BifA_{PAO}$ resulted in full restoration of flagellar motility (Fig. 2). In addition, we show that the restoration of flagellar motility in the $\Delta bifA_{PSV}$ mutant is fully dependent on the integrity of the $BifA_{PSV}$ EAL domain (Fig. 2A). Together, these results suggest the existence of diverse c-di-GMP-mediated signalling pathways regulating flagellar motility in different *Pseudomonas* species. In this model, the different homologues of BifA, despite displaying similar activities, appear to have different relevance to bacterial motility.

Hyperbiofilm phenotypes and increased EPS production have been associated with deletion of the *bifA* gene in both

Fig. 4 Virulence assay of a *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335 *bifA* mutant on olive plants. Knots induced on *in vitro* olive plants by the indicated strains at 30 days post-inoculation (dpi) (A) and in lignified olive plants at 90 dpi (B). Knot volume (mm³) on *in vitro* olive plants (C) and in lignified plants (D) infected with the indicated strains. Bars represent the mean of three biological replicates from two different assays; error bars represent the standard deviation. Letters indicate significant differences ($\alpha = 0.05$) using analysis of variance ANOVA. Psv NCPPB 3335 (pJB3), *P. savastanoi* pv. *savastanoi* NCPPB 3335 carrying the empty vector pJB3; $\Delta bifA_{Psv}$, NCPPB 3335 *bifA* mutant carrying pJB3; $\Delta bifA_{Psv}$ pJB3- $bifA_{Psv}$, NCPPB 3335 *bifA* mutant complemented with BifA from *P. savastanoi* pv. *savastanoi* NCPPB 3335 ($BifA_{Psv}$); $\Delta bifA_{Psv}$ pJB3- $bifA_{PAO}$, NCPPB 3335 *bifA* mutant complemented with BifA from *P. aeruginosa* PAO1 ($BifA_{PAO}$).

P. aeruginosa PA14 (Kuchma *et al.*, 2007) and *P. putida* KT2442 (Jiménez-Fernández *et al.*, 2014). Although biofilm formation and EPS assays showed no significant differences between *P. savastanoi* pv. *savastanoi* NCPPB 3335 and *P. syringae* pv. *tomato* DC3000 $\Delta bifA$ mutants and their corresponding wild-type strains, overexpression of $BifA_{Psv}$ in NCPPB 3335 had a negative impact on biofilm formation and CF binding (Fig. S1). However, a direct role for BifA in the regulation of biofilm-related processes in this pathogen cannot be inferred from these results, as alteration of these phenotypes would also be expected by the

overexpression of other unrelated PDEs or DGCs. In relation to this, an artificial increase in c-di-GMP levels in this pathogen by overexpression of the DGC PleD resulted in enhanced biofilm production (Pérez-Mendoza *et al.*, 2014). BifA has been shown to negatively regulate cellulose biosynthesis in *P. putida* (Jiménez-Fernández *et al.*, 2014) and the production of polysaccharides, dependent on the *pel* locus, in *P. aeruginosa* (Kuchma *et al.*, 2007). Although *pel* homologues are not encoded in the genomes of *P. syringae* and related strains, cellulose biosynthetic genes are found in *P. savastanoi* pv. *savastanoi*

Fig. 5 Hypovirulence of a *Pseudomonas syringae* pv. *tomato* DC3000 *bifA* mutant in tomato plants. (A) Representative images of symptoms induced by the indicated strains in tomato plants var. MoneyMaker at 9 days post-inoculation (dpi). (B) Quantification of necrotic areas in the leaflets shown in (A). The results are the means of nine different leaflets. Error bars represent the standard error. Letters indicate significant differences ($\alpha = 0.05$) using analysis of variance (ANOVA). Pto DC3000 (pJB3), *P. syringae* pv. *tomato* DC3000 carrying the empty vector pJB3; $\Delta bifA_{Pto}$ DC3000 *bifA* mutant carrying pJB3; $\Delta bifA_{Pto}$ pJB3-*bifA*_{PSV} DC3000 *bifA* mutant complemented with BifA from *P. savastanoi* pv. *savastanoi* NCPPB 3335 (BifA_{PSV}).

NCPPB 3335 (Rodríguez-Palenzuela *et al.*, 2010) and *P. syringae* pv. *tomato* DC3000 (Buell *et al.*, 2003), suggesting a possible BifA-mediated regulation of cellulose biosynthesis in these pathogens. In agreement with this hypothesis, a role for c-di-GMP in cellulose production and biofilm formation has been suggested recently for *P. syringae* and related pathogens (Pérez-Mendoza *et al.*, 2014). Furthermore, *P. putida* BifA has been shown to regulate starvation-induced biofilm dispersal and biosynthesis of the adhesin protein LapA (Jiménez-Fernández *et al.*, 2014). However, LapA homologues are not found in the genomes of *P. aeruginosa*, *P. savastanoi* or *P. syringae* strains. The mechanistic basis for the regulation of biofilm dispersal by BifA in these bacterial pathogens thus remains to be elucidated.

Negative regulation of virulence by high c-di-GMP levels in bacterial phytopathogens has been shown for *Erwinia amylovora* (Edmunds *et al.*, 2013), *X. campestris* (Ryan *et al.*, 2006, 2007), *X. oryzae* pv. *oryzicola* (Zhang *et al.*, 2013), *D. dadantii* (Yi *et al.*, 2010) and *Xylella fastidiosa* (de Souza *et al.*, 2013). In agreement with these studies, we show here that the putative PDE BifA positively regulates virulence in bacterial pathogens of the *P. syringae* complex (Figs 4 and 5). Decreased virulence of the

$\Delta bifA_{PSV}$ mutant in olive plants was associated with a reduction in fitness (Fig. 3). Although significant differences in the final population density of the mutant relative to the wild-type strain in olive knots were not observed, epifluorescence microscopy of knot tissues showed that $\Delta bifA_{PSV}$ cells were restricted to the stem wound (Fig. S2). In this sense, a lack of correlation between the cell density inside olive knots and GFP emission has been reported previously for other *P. savastanoi* mutants affected in virulence genes, and has been explained by a spatial distribution of mutant cells inside olive knots totally different from that of wild-type cells (Matas *et al.*, 2012). All of these results also suggest that BifA positively regulates systemic invasion of the host in this pathogen, a phenotype perhaps associated with altered control of the motile-to-sessile switch.

This is the first study to investigate the phenotypic consequences of knocking out a putative PDE in two pathogens of the *P. syringae* complex that induce different symptoms in their plant hosts. The results presented here provide new insights into the diverse processes involved in and affected by c-di-GMP signalling during the interaction of bacterial pathogens with plants.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, media and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335 and *P. syringae* pv. *tomato* DC3000 were grown at 28 °C in LB medium (Miller, 1972) or super-optimal broth (SOB) (Hanahan, 1983). For swimming motility assays, a 20-fold dilution of King's B (KB) medium (King *et al.*, 1954) was used. *Escherichia coli* strains were grown at 37 °C in LB and SOB media. Solid and liquid media were supplemented with the following antibiotics as necessary. For *E. coli*, 100 µg/mL ampicillin (Ap), 50 µg/mL kanamycin (Km), 15 µg/mL tetracycline (Tc) and 10 µg/mL gentamicin (Gm). For NCPPB 3335 and DC3000, 300 µg/mL Ap, 10 µg/mL (NCPPB 3335) or 15 µg/mL (DC3000) Km, 15 µg/mL Tc and 10 µg/mL Gm.

Construction of bacterial strains and plasmids

The oligonucleotides for overexpression and mutant constructs are listed in Table S1 (see Supporting Information). Polymerase chain reaction (PCR) products were cloned into the pGEM-T easy cloning vector (Promega, Madison, WI, USA) and sequenced prior to subcloning into the relevant broad-host-range or suicide vector. The construction of *P. savastanoi* pv. *savastanoi* NCPPB 3335 and *P. syringae* pv. *tomato* DC3000 $\Delta bifA$ mutants was performed using the vectors pGEM-T-*bifA*_{PSV}-Km and pGEM-T-*bifA*_{Pto}-Km, respectively (Table 1). First, DNA fragments of approximately 1.2 kb (NCPPB 3335) or 0.5 kb (DC3000), which corresponded to the 5' and 3' flanking regions of the *bifA* gene, were amplified by three rounds of PCR. The reactions used genomic DNA as a template, as well as primers that included a *Bam*HI site and the T7 primer sequence (Table S1), as

described previously (Zumaquero *et al.*, 2010). The resulting product was A/T cloned into pGEM-T (Table 1) and fully sequenced to discard mutations on flanking genes. Following sequencing, the resulting plasmids were labelled with the *nptII* kanamycin resistance gene, obtained from pGEM-T-KmFRT-BamHI (Table 1), to yield pGEM-T-*bifA*_{psv}-Km and pGEM-T-*bifA*_{ptn}-Km (Table 1). For marker exchange mutagenesis, plasmids were transformed into NCPPB 3335 and DC3000 by electroporation, as described previously (Pérez-Martínez *et al.*, 2007). Transformants were selected on LB medium containing Km, and replica plates of the resulting colonies were made on LB-Ap plates to determine whether each transconjugant underwent plasmid integration (Ap^R) or allelic exchange (Ap^S). Southern blot analyses, using both *nptII* and *bifA* (NCPPB 3335) as probes, were used to confirm that allelic exchange occurred at the single, correct position within the genome.

Overlapping PCR fragments for mutation of the conserved Glu residue of the EAL domain (EAL to AAL) of BifA_{psv} were generated using the primer pairs AER-0004088-F with BifA_{psv}-EALmut-R and BifA_{psv}-EALmut-F with AER-0004088-R (Table S1). The resulting fragments were combined to generate the full-length *bifA*_{psv}-AAL, which was *SacI/XbaI* cloned into plasmid pJB3 yielding pJB3-*bifA*_{psv}-AAL (Table 1).

Motility assays

For swimming motility, bacteria were grown on LB plates for 48 h, resuspended in 10 mM MgCl₂ and adjusted to an optical density at 660 nm (OD_{660 nm}) of 2.0. Aliquots of 2 µL were inoculated in the centre of 0.3% agar plates containing LB (*P. savastanoi* pv. *savastanoi*) or KB medium diluted 20-fold (*P. syringae* pv. *tomato*), and incubated for 72 h at 25 °C. The diameters of the swimming halos were measured after 48 h for *P. syringae* pv. *tomato* strains and after 72 h for *P. savastanoi* pv. *savastanoi* strains. For swarming assays, the cell suspensions were prepared as above, except that the 2-µL aliquots were deposited on the top of a plate supplemented with Tc and incubated at 25 °C. The surface motility was observed after 36 h. Three motility plates were used for each strain, and the experiment was repeated with three independent cultures.

EPS detection and biofilm formation assays

CF binding assays for *P. savastanoi* pv. *savastanoi* were carried out on LB medium supplemented with 200 µg/mL CF and incubated at 25 °C for 48 h.

Biofilm formation by *P. savastanoi* pv. *savastanoi* NCPPB 3335 cells was analysed on glass surfaces using a static microcosm assay, as described previously (Pliego *et al.*, 2008). To quantify biofilms, crystal violet (CV) was used as described by Pérez-Mendoza *et al.* (2011a).

Quantification of green fluorescence

Emission of green fluorescence from GFP-tagged *P. savastanoi* pv. *savastanoi* cells growing on LB cultures was quantified as follows. Two hundred microlitre cell suspensions were harvested at various times and green fluorescence was measured with a fluorometer (model FL600 Fluorescence Microplate Reader; Winooski, Vermont (VT), USA) set at an exci-

tation wavelength of 475 nm with emission detection at 515 nm. The background green autofluorescence of *P. savastanoi* pv. *savastanoi* cells was determined by measurement of the fluorescence emitted by *P. savastanoi* NCPPB 3335 cells grown under the same culture conditions.

Virulence assays in tomato plants

Seeds of *Solanum lycopersicum* var. Moneymaker were germinated and grown in a growth chamber with a 16 h/8 h light/dark photoperiod at 24/18 °C day/night and 70% relative humidity. Bacteria were grown on LB agar plates for 48 h at 28 °C and then resuspended in 10 mM MgCl₂ to an OD_{600 nm} of 0.5, corresponding to 10⁸ CFU/mL. Further serial dilutions were carried out to obtain a suspension of the desired titre for different inoculation dosages. Four- to five-week-old plants were inoculated by pressing the bacterial suspension against leaves with 2-mL syringes lacking a needle to infiltrate the intracellular spaces. Control plants were inoculated with a solution of 10 mM MgCl₂. In all cases, inoculations were carried out in the same half of the leaflet (three inoculations per leaf). This allowed us to use the midrib to differentiate between the infiltrated and non-infiltrated areas. The appearance of disease symptoms was scored at 3, 5, 8 and 10 dpi. Quantification of symptoms was carried out using Visilog 6.3 image analysis software (Noesis, Courtaboeuf, France).

Competition assays were performed by mixing equal volumes of mutant and wild-type cultures with an OD_{660 nm} of 0.5. Tomato plants were inoculated with 5 × 10⁴ CFU/mL of the mixed bacterial suspension described above. Bacteria were recovered from the infected leaves using a 10-mm-diameter cork borer. Five discs (3.9 cm²) per plant were homogenized by mechanical disruption into 1 mL of 10 mM MgCl₂, and then counted by plating serial dilutions on LB with and without Km to select for the mutant and wild-type strains, respectively. At 4 dpi, bacteria were recovered from leaf tissues and plated. CI was calculated as described previously (Macho *et al.*, 2007).

Virulence assay in olive plants

Olive plants derived from seeds germinated *in vitro* (originally collected from a cv. Arbequina plant) were micropropagated, rooted and maintained as described previously (Rodríguez-Moreno *et al.*, 2008, 2009). The stems of micropropagated olive plants were infected with bacterial suspension (c. 10³ CFU) and incubated for 30 days in a growth chamber, as described by Rodríguez-Moreno *et al.* (2008). Bacteria were recovered from the knots using a mortar containing 1 mL of 10 mM MgCl₂, and serial dilutions were plated onto LB (to detect the wild-type), LB-Km (to detect the *bifA* mutant) or LB-Tc (to detect the complemented strain) medium. Population densities were calculated from at least three independent replicates. The morphology of olive plants infected with bacteria was visualized using a stereoscopic microscope (Leica MZ FLIII; Leica Microsystems, Wetzlar, Germany). The knot volume was quantified using a 3D scanner and MiniMagics 2.0 software. Competitive assays between the wild-type and mutant strains were carried out in *in vitro* plants as described previously (Matas *et al.*, 2012; Rodríguez-Moreno *et al.*, 2008). To visualize bacterial infection of tumours, *P. savastanoi* pv. *savastanoi* strains were tagged with GFP using the plasmid pLRM1-GFP, and the knots generated were examined by epifluorescence microscopy as described previously (Rodríguez-Moreno *et al.*, 2009).

The pathogenicity of *P. savastanoi* pv. *savastanoi* strains in 1-year-old olive explants (lignified olive plants) was tested as described previously (Penyalver *et al.*, 2006; Pérez-Martínez *et al.*, 2007). The knot volume was calculated by measuring the length, width and height of the knot with a Vernier calliper (Hosni *et al.*, 2011; Moretti *et al.*, 2008). Statistical analysis was performed by analysis of variance followed by Student's *t*-test ($P \leq 0.05$).

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SUPPORTING INFORMATION

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

Fig. S1 Phenotypes associated with the overexpression of BifA in *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335. (A) Swimming motility of the indicated strains in 0.3% Luria–Bertani (LB) agar at 72 h. (B) Biofilm formation in LB medium under static growth conditions visualized at 24 h by crystal violet staining. (C) Visualization of exopolysaccharide production using calcofluor agar plates. Wild-type, NCPPB 3335 transformed with empty vector pJB3; BifA overexpression, NCPPB 3335 transformed with pJB3-*bifA*_{PSV} (Table 1).

Fig. S2 Green fluorescence emission by green fluorescent protein (GFP)-tagged *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335 and Δ *bifA* mutant (Δ *bifA*_{PSV}) cells grown on Luria–Bertani (LB) medium at 28 °C. (A) Growth curves. (B) Intensity of green fluorescence. The wild-type strain NCPPB 3335 was used as a negative control in this assay.

Fig. S3 Real-time monitoring of green fluorescent protein (GFP)-tagged *Pseudomonas savastanoi* pv. *savastanoi* cells in *in vitro* micropropagated olive plants. Left: images of knots induced by GFP-tagged NCPPB 3335 and a Δ *bifA* mutant (Δ *bifA*_{PSV}) at 30 days post-inoculation (dpi). Right: epifluorescence microscopy images complementary to those on the left.

Table S1 Oligonucleotides used in this study.

Table S2 Locus tag/accession numbers for the BifA proteins used for the construction of the neighbor-joining tree shown in Fig. 1A.