Diguanylate cyclase DgcP is involved in plant and human *Pseudomonas* spp. infections

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Summary

The second messenger cyclic di-GMP controls the transition between different lifestyles in bacterial pathogens. Here, we report the identification of DgcP (diguanylate cyclase conserved in *Pseudomonads*), whose activity in the olive tree pathogen *Pseudomonas savastanoi* pv. *savastanoi* is dependent on the integrity of its GGDEF domain. Furthermore, deletion of the *dgcP* gene revealed that DgcP negatively regulates motility and positively controls biofilm formation in both the olive tree pathogen *P. savastanoi* pv. *savastanoi* and the human opportunistic pathogen *Pseudomonas aeruginosa*. Overexpression of the *dgcP* gene in *P. aeruginosa* PAK led to increased exopolysaccharide production and up-regulation of the type VI secretion system; in turn, it repressed the type III secretion system, which is a hallmark of chronic infections and persistence for *P. aeruginosa*. Deletion of the *dgcP* gene in *P. savastanoi* pv. *savastanoi* NCPPB 3335 and *P. aeruginosa* PAK reduced their virulence in olive plants and in a mouse acute lung injury model, respectively. Our results show that diguanylate cyclase DgcP is a conserved *Pseudomonas* protein with a role in virulence and confirms the existence of common c-di-GMP signalling pathways that are capable of regulating plant and human *Pseudomonas* spp. infections.

Keywords: c-di-GMP/ *Pseudomonas savastanoi*/ *Pseudomonas aeruginosa*/ biofilm formation/ olive/ cystic fibrosis

Introduction

Bacterial pathogens use an extensive arsenal of virulence-related factors to combat host defences and thrive in hostile environments. Although many bacterial virulence factors are host-specific, numerous studies have demonstrated the existence of universal virulence mechanisms against plants, insects and mammals (Finlay and Falkow, 1997). The production of virulence factors common to plant and animal pathogens involves regulatory proteins and networks, of which the response regulator GacA (Kitten et al., 1998; Parkins et al., 2001; Cha et al., 2012) and quorum sensing systems (Hosni et al., 2011; Jimenez et al., 2012) are remarkable examples. Bacterial pathogens share different features, such as efflux pumps to resist the action of drugs and toxic compounds (Rahme et al., 2000) or effectors and toxins delivered through a broad range of protein secretion systems (Hueck, 1998) to manipulate/subvert target cells (Carrion et al., 2012; Jimenez et al., 2012). Recently, several genetic screens have demonstrated that the second messenger cyclic di-GMP (c-di-GMP) is linked to bacterial virulence in numerous animal and plant pathogens (Ryan et al., 2007; Tamayo et al., 2007; Bobrov et al., 2011; Romling et al., 2013). Remarkably, c-di-GMP has been shown to regulate bacterial motility, biofilm formation, exopolysaccharide (EPS) production, the cell cycle, differentiation, and many other processes (D'Argenio and Miller, 2004; Dow et al., 2006; Jenal and Malone, 2006; Tamayo et al., 2007; Hengge, 2009; Yi et al., 2010; Pérez-Mendoza et al., 2015). The enzymes responsible for the synthesis and breakdown of c-di-GMP are diguanylate cyclases (DGCs) and c-di-GMP-specific phosphodiesterases (PDEs), respectively. Proteins containing a GGDEF domain are responsible for c-di-GMP synthesis (Paul et al., 2004), whereas c-di-GMP PDE activity is associated with EAL or HD-GYP domains (Christen et al., 2005; Schmidt et al., 2005; Ryan et al., 2007). Additionally, a RXXD motif located upstream of the GGDEF domain has been shown to provide allosteric control for the activity of several DGCs (Chan et al., 2004; Christen et al., 2006). Recent advances in the bioinformatics analysis of genome sequences have led to the identification of bacterial

enzymes encoding GGDEF, EAL and HY-GYP domains in all major bacterial phyla that recognise c-di-GMP as a universal bacterial second messenger (Romling et al., 2013). The number of genes encoding these enzymes varies from one bacterial genome to another. More than 40 can be found in *Pseudomonas aeruginosa*, which raises the question of whether these enzymes are redundant, have specific roles or targets or are associated with specific environmental conditions.

The role of c-di-GMP in pathogenesis has been extensively studied in a number of animal and human bacterial pathogens, including *Salmonella enterica* (Hisert et al., 2005; Lamprokostopoulou et al., 2010), *Vibrio cholerae* (Tischler and Camilli, 2005), *Yersinia pestis* (Bobrov et al., 2011; Bobrov et al., 2014) and *P. aeruginosa* (Kulasakara et al., 2006; Ryan et al., 2009; Li et al., 2014). *P. aeruginosa* is a versatile Gram-negative bacterium that can be found in various environments, including soil, water and vegetation (Lyczak et al., 2000). Importantly, it is an opportunistic human pathogen that is responsible for numerous nosocomial infections. *P. aeruginosa* chronic infections are fatal in cystic fibrosis patients, where they develop resilient biofilms within the lungs (Singh et al., 2000; Damron and Goldberg, 2012) that are difficult to eradicate by the immune system or classic antibiotic therapy (Costerton et al., 1999; Colvin et al., 2011). Regulation of biofilm formation in *P. aeruginosa* involves c-di-GMP signalling at several stages during the maturation process, from early-stage biofilms (Kuchma et al., 2005) to cell detachment and dispersal (Morgan et al., 2006). A comprehensive analysis of *P. aeruginosa* genes encoding enzymes related to c-di-GMP metabolism revealed that virulence-related phenotypes such as cytotoxicity and biofilm formation are controlled by different DGCs and PDEs (Kulasakara et al., 2006).

The Gac/Rsm pathway is essential for the expression of virulence traits and biofilm formation in *Pseudomonas* species associated with either plant or human infections. For example, the transition between the planktonic and biofilm lifestyles in *P. aeruginosa* involves the sensor kinases LadS (Ventre et al., 2006) and RetS (Goodman et al., 2009), which act antagonistically on the Gac pathway. It was recently shown that

a *retS* mutant displayed a hyperbiofilm phenotype and downregulated the type III secretion system (T3SS), but upregulated the type VI secretion system (T6SS). The RetS regulatory network has been proposed to be connected to the c-di-GMP signalling network because intracellular levels of this second messenger are augmented in the *retS* mutant (Moscoso et al., 2011). In addition to the Gac/Rsm cascade, the transcription factor AmrZ has been shown to limit the intracellular accumulation of c-di-GMP through transcriptional repression of gene(s) encoding a DGC (Jones et al., 2014). These observations contributed to the reconciliation of the roles of both the Gac/Rsm and c-di-GMP pathways in a larger network impacting biofilm formation.

Despite recent advances in understanding the role of c-di-GMP signalling in the pathogenicity and virulence of human and animal pathogens, few studies have been conducted in bacterial phytopathogens. Examples of such phytopathogens include the HD-GYP domain-containing protein RpfG, the c-di-GMP receptor XcCLP from *Xanthomonas campestris* (Dow et al., 2006; Ryan et al., 2006b), the PDE proteins EcpB and EcpC from *Dickeya dadantii* (Yi et al., 2010), the PDE protein PdeR, the degenerate EAL-GGDEF domain protein Filp from *Xanthomonas oryzae* pv. *oryzae* (Yang et al., 2012; Yang et al., 2014) and several c-di-GMP metabolising enzymes involved in the regulation of surface attachment to plant cells in *Agrobacterium tumefaciens* (Xu et al., 2013) and *Pectobacterium atrosepticum* (Pérez-Mendoza et al., 2011b; Pérez-Mendoza et al., 2011a). Moreover, regulation of the T3SS and T6SS by RetS and LadS has also been observed in the bean pathogen *Pseudomonas syringae* B728a (Records and Gross, 2010). Recently, overexpression of the *Caulobacter crescentus* DGC protein PleD* was shown to reduce motility, increase EPS production and enhance biofilm formation in the bacterial phytopathogens *P. syringae* pv. *phaseolicola*, *P. syringae* pv. *tomato* and *Pseudomonas savastanoi* pv. *savastanoi*. However, the development of disease symptoms was only affected by high levels of c-di-GMP upon interaction of *P. savastanoi* pv. *savastanoi* NCPPB 3335 with olive

plants, resulting in the formation of larger knots that displayed reduced necrosis (Pérez-Mendoza et al., 2014). Also related to this bacterium, we have recently reported that PDE BifA is involved in the virulence of this olive pathogen (Aragón et al., 2015). A recent signature-tagged mutagenesis survey of the *P. savastanoi* pv. *savastanoi* NCPPB 3335 genes required for full fitness in olive plants identified a transposon insertion in PSA3335_0619, a gene encoding an endonuclease/exonuclease/phosphatase family protein (EEPP) located upstream of PSA3335_0620 (a GGDEF domain protein-encoding gene) (Matas et al., 2012). However, no direct evidence was reported in this study for the specific involvement of these two genes in the virulence and/or fitness of *P. savastanoi* in olive plants.

The present study focused on the identification of DgcP, a Pseudomonads-specific c-di-GMP DGC that participates in the inverse regulation of swimming motility and biofilm formation in plant and human pathogens. *dgcP* deletion mutants were constructed in *P. aeruginosa* PAK and *P. savastanoi* pv. *savastanoi* NCPPB 3335 and evaluated for their impact on c-di-GMP-related phenotypes and virulence. This work demonstrates that DgcP activity positively regulates biofilm formation and negatively regulates motility. Additionally, we show that the *dgcP* gene is required for the full virulence of *P. aeruginosa* in a mouse model of acute infection and *P. savastanoi* pv. *savastanoi* in olive plants.

Results

*Conservation of the *dgcP* gene-containing cluster in the *Pseudomonas* genus*

In a previous search for attenuated mutants of *P. savastanoi* pv. *savastanoi* NCPPB 3335 in olive plants, we identified a transposon insertion in the PSA3335_0619 gene that encoded a protein from the endonuclease/exonuclease/phosphatase (EEPP) family (Matas et al., 2012). This gene is part of a cluster that also encodes a putative diguanylate cyclase with a GGDEF domain (PSA3335_0620). BLASTP searches with the sequence of this protein revealed that homologs exist only within the *Pseudomonas* genus and include all sequenced *P. syringae* pathovars, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Pseudomonas entomophila*, *P. aeruginosa* and other *Pseudomonas* spp. (Fig. 1A). Thus, we renamed the protein DgcP for “diguanylate cyclase conserved in Pseudomonads”. The DgcP proteins encoded by *P. aeruginosa* strains PAO1, PAK (GenBank accession number KM111506, see Materials and Methods) and PA14 (671-amino acid residues) are almost identical (PAO1 vs. PAK and PAO1 or PAK vs. PA14 possess 100% and 98% sequence similarity, respectively) and show 50% identity to *P. savastanoi* pv. *savastanoi* NCPPB 3335, which is 15 amino acids longer (685-amino acid residues). The sequence similarity between the *P. aeruginosa* and *P. savastanoi* DgcP proteins is most apparent in the 172 amino acid amino-terminal and 320 amino acid carboxy-terminal regions. Additionally, the carboxy-terminal regions of both proteins share a GGEEF motif and an allosteric control RXXD motif (RSDD in *P. savastanoi* pv. *savastanoi* NCPPB 3335 and RQAD *P. aeruginosa* PAO1, PAK and PA14). Moreover, genetic context analysis around the *dgcP* gene in a selection of sequenced *Pseudomonas* spp. genomes revealed a conserved synteny with two other genes located upstream of *dgcP*. One gene encodes a previously described EEPP (PSA3335_0619 and PA5488 in *P. savastanoi* pv. *savastanoi* NCPPB 3335 and *P. aeruginosa* PAO1, respectively) and the other gene encodes a putative disulfide interchange protein (DsbA). Furthermore, the EEPP coding gene overlaps the *dgcP* gene in most sequenced *Pseudomonas* spp. including NCPPB 3335 and PAK,

suggesting that these two genes could be part of the same operon. Additionally, one or two genes encoding a putative cytochrome C were located upstream of the *dsbA* gene in all other *Pseudomonas* spp., with the exception of the strains belonging to the *P. syringae* complex. Finally, a gene encoding a putative N-acetylmuramoyl-L-alanine amidase (*ampDh2*) was found downstream of the *dgcP* gene in all species with the exception of *P. aeruginosa* and *P. fulva*, which encode a MarC transporter and a peptidase family M23 gene, respectively (Fig. 1B).

Overall, cluster analysis based on the amino acid sequence of the DgcP proteins yielded three distinct groups comprised by *P. syringae* and *P. savastanoi* strains (group I), *P. fluorescens*, *P. putida* and *P. entomophila* (group II) and *P. aeruginosa*, *P. stutzeri* and *P. fulva* (group III). Clustering of the DgcP proteins was largely congruent with the phylogeny of the genus *Pseudomonas* (Yamamoto et al., 2000) and the *P. syringae* complex (Studholme, 2011) deduced from housekeeping genes, suggesting that the *dgcP* gene is ancestral within this bacterial group.

The dgcP gene is transcribed within a polycistronic mRNA in both P. savastanoi pv. savastanoi and P. aeruginosa

To determine whether the *dgcP* gene is co-transcribed with the EEPP-encoding gene and *dsbA*, RT-PCR assays were performed using RNA samples isolated from *P. savastanoi* pv. *savastanoi* NCPPB 3335 and *P. aeruginosa* PAK. Amplification of the intergenic regions located between the sequential ORFs (with the exception of the region between *dgcP* and its downstream gene) revealed that the three genes are co-transcribed and suggested the presence of a terminator downstream of *dgcP* in both strains (Fig. 2). The initiation transcription site of the *dsbA-dgcP* operon determined by 5' RACE in NCPPB 3335 and PAK was localised 37 and 48 bp upstream from the first ATG of *dsbA*, respectively. Furthermore, putative σ^{70} promoter sequences were identified upstream of both transcription start sites (Fig. S1) using BPPROM (<http://linux1.softberry.com>).

DgcP is an active diguanylate cyclase

The presence of a GGDEF domain in the DgcP protein suggested that this protein might function as a diguanylate cyclase. To test this hypothesis, a C-terminally His-tagged version of the full-length DgcP protein (DgcP-His6) encoded by *P. savastanoi* pv. *savastanoi* NCPPB 3335 (686 amino acids with a predicted MW of 76 kDa) was overexpressed in *E. coli* BL21. Soluble DgcP-His6 was purified by nickel affinity chromatography (Fig. 3A) and assessed *in vitro* for DGC activity in the presence of 150 μ M GTP (Fig. 3B). As negative controls, we used either purified DgcP-His6 either without substrate or with 2',3'-dideoxyguanosine-5'-triphosphate (ddGTP) instead of GTP or heat-denatured DgcP-His6 with GTP. As a positive control, we used purified PleD*, a mutant form of the polar development regulator PleD that is a well-characterised diguanylate cyclase from *Caulobacter crescentus* (Aldridge et al., 2003; Paul et al., 2004; Pérez-Mendoza et al., 2014). The reaction products were analysed by HPLC-MS/MS. Specific peaks of the c-di-GMP fragments with retention times identical to those of the c-di-GMP standard were observed only in samples containing PleD* or native DgcP plus GTP (Fig. 3B). The DgcP protein produced 0.025 pmol of c-di-GMP/pmol total protein, which was approximately 21-fold less than PleD* (Fig. 3C).

The dgcP gene impacts the motile/sessile lifestyles

It has been previously shown that c-di-GMP is a key second messenger that controls the lifestyle and virulence traits in Gram-negative bacteria. For example, it has been reported that biofilm-related phenotypes such as EPS production and swimming motility are associated with c-di-GMP levels (Merritt et al., 2007; Monds et al., 2007; Pesavento et al., 2008). Considering that the EEPP-encoding gene is co-transcribed with the *dgcP* gene (Fig. 2), we tested the swimming and biofilm formation ability of two *P. savastanoi* pv. *savastanoi* NCPPB 3335 mutant derivatives affected in this gene, a strain containing a transposon insertion in the EEPP-encoding gene (FAM-108) (Matas

et al., 2012) and a mutant encoding a clean deletion of this ORF ($\Delta 0619_{P_{SV}}$). While the transposon mutant FAM-108 showed an increased swimming motility and a reduced ability to form biofilms over glass slides in comparison to the wild-type strain, none of these phenotypes were significantly altered in $\Delta 0619_{P_{SV}}$ (Fig. S2). Furthermore, complementation of FAM-108 with an expression vector encoding the *P. savastanoi* pv. *savastanoi* NCPPB 3335 *dgcP* gene (pJB3-*dgcP*_{PSV}), fully restored the swimming motility of this strain (data not shown), suggesting that the EEPP-encoding gene PSA3335_0619 is not directly involved in the control of this phenotype.

To further validate *in vivo* the *in vitro* demonstrated activity of DgcP, we constructed *P. savastanoi* pv. *savastanoi* NCPPB 3335 and *P. aeruginosa* PAK $\Delta dgcP$ mutants ($\Delta dgcP_{PSV}$ and $\Delta dgcP_{PAK}$, respectively) (Table 1) and strains overexpressing *dgcP*. EPS production was tested using a Congo red (CR) binding assay. No difference in the CR-binding phenotype was observed between the wild-type strains and their respective deletion mutants (data not shown). However, overexpression of the *dgcP* gene from *P. savastanoi* pv. *savastanoi* NCPPB 3335 (pJB3-*dgcP*_{PSV}, Table 1) or *P. aeruginosa* PAK (pJB3-*dgcP*_{PAK}, Table 1) in *P. aeruginosa* PAK resulted in an enhanced CR binding phenotype that was accompanied by the formation of wrinkly colonies (Fig. 4). These two EPS-related phenotypes (CR binding and formation of wrinkly colonies) were more dramatic in the case of PAK overexpressing its own *dgcP*_{PAK} gene. However, the CR binding phenotype of *P. savastanoi* pv. *savastanoi* NCPPB 3335 overexpressing the *dgcP*_{PSV} gene was not significantly different from the wild-type strain (data not shown).

Deletion of the *dgcP* gene in *P. savastanoi* and *P. aeruginosa* resulted in increased swimming motility (~1.4- and ~1.5-fold increase, respectively) (Fig. 5A and 5B) and a ~2.0-fold decrease in biofilm formation (Fig. 5C and 5D). Additionally, complementation of the $\Delta dgcP_{PSV}$ and $\Delta dgcP_{PAK}$ mutants with their corresponding *dgcP* genes and heterologous complementation of the $\Delta dgcP_{PAK}$ mutant with pJB3-*dgcP*_{PSV} resulted in the full restoration of both DGC-associated phenotypes (Fig. 5). The ability of the *P. savastanoi* $\Delta dgcP_{PSV}$ mutant to form biofilms when complemented with pJB3-*dgcP*_{PSV}

was slightly increased compared with the wild-type strain, probably due to the overexpression of the *dgcP* gene from a constitutive promoter in this strain (Fig. 5). Additionally, overexpression of the *dgcP*_{PSV} gene in wild type *P. savastanoi* pv. *savastanoi* NCPPB 3335 and both *dgcP* genes in *P. aeruginosa* PAK was associated with a clear inhibition of swimming motility and a hyperbiofilm phenotype (Figs. S3 and 6 for NCPPB 3335 and PAK, respectively). These results demonstrate that the *dgcP* gene plays a role in swimming motility and biofilm formation in *P. aeruginosa* PAK and *P. savastanoi* pv. *savastanoi* NCPPB 3335. Moreover, the DGC activity of the *P. savastanoi* DgcP protein was able to complement the altered phenotypes of the $\Delta dgcP_{PAK}$ mutant, suggesting the existence of a similar DgcP-mediated signalling pathway in both bacteria.

To analyse the role of DgcP in other *Pseudomonas* species, we also transformed plasmid pJB3-*dgcP*_{PSV} into *Pseudomonas putida* KT2440 (group II DgcP) and *P. syringae* pv. *tomato* DC3000 (group I DgcP). As previously observed in *P. aeruginosa* PAK (group III DgcP) and *P. savastanoi* pv. *savastanoi* NCPPB 3335 (group I DgcP), these two transformants also exhibited an inhibition in swimming motility and a hyperbiofilm phenotype (Fig. S3), suggesting a broad spectrum of action for this DGC protein within the *Pseudomonas* genus.

Role of the dgcP gene in the type III and type VI secretion system switch

Previous studies in *P. aeruginosa* suggested a link between c-di-GMP and certain virulence functions, including a switch from the T3SS to the T6SS and *vice versa* (Moscoso et al., 2011). Thus, the switch between T3SS and T6SS was monitored in *P. aeruginosa* PAK overexpressing either the *dgcP*_{PSV} or the *dgcP*_{PAK} gene. Immunoblot analysis was used to detect PcrV, a structural component of the T3SS needle (Lee et al., 2007), and Hcp1, the main component of the T6SS nanotube (Ballister et al., 2008). A *P. aeruginosa* PAK $\Delta retS$ mutant, which displays an elevation in c-di-GMP levels compared to the wild-type strain (Moscoso et al., 2011), was included as the positive

control. Our data show that Hcp1 is readily detected in the *retS* mutant and in the strains overexpressing either of the DgcP proteins, whereas the parental strain displayed low Hcp1 levels. Conversely, PcrV production was clearly higher in the parental strain than in the *dgcP*-overexpressing strains, which behaved similarly to the *retS* mutant (Fig. 6C). To further analyse the role of DgcP in the T3SS/T6SS switch, we also studied the expression of Hcp1 and PcrV in the $\Delta dgcP_{PAK}$ mutant strain. Immunoblot analysis showed no significant differences in the expression of Hcp1 or PcrV in the $\Delta dgcP_{PAK}$ mutant compared with the wild-type strain (Fig. S4A). Additionally, qRT-PCR assays showed that the expression of the T6SS genes *hcp1* and *vgrG* from *P. aeruginosa* PAK and *P. savastanoi* pv. *savastanoi* NCPPB 3335, respectively, were decreased in the $\Delta dgcP$ mutants compared with their respective wild-type strains (Fig. S4B). However, no significant differences were found in the expression of the T3SS genes *pcrV* (*P. aeruginosa* PAK) or *hrpA*, *hrpL* and *hopBL1* (*P. savastanoi* pv. *savastanoi* NCPPB 3335) in the $\Delta dgcP$ backgrounds under the conditions tested (data not shown). In summary, our results show that the DgcP-mediated modulation of c-di-GMP levels plays a role in the regulation of T6SS-related genes in both *P. aeruginosa* PAK and *P. savastanoi* pv. *savastanoi* NCPPB 3335.

The GGEEF domain of DgcP is required for function

GG(D/E)EF motifs have been shown to be responsible for DGC activity (Romling et al., 2000; Aldridge et al., 2003; Simm et al., 2004). To test the importance of this motif in the functionality of the DgcP protein, we generated a mutant by changing Glu607 to Ala (GGEEF to GGAEF). Then, we evaluated the ability of the resulting *dgcP* mutant allele (*dgcP_{PSV}*GGAEF) to complement the phenotypes altered in the $\Delta dgcP_{PSV}$ mutant. Though the wild type *dgcP_{PSV}*GGEEF allele complemented the mutant phenotypes, expression of the *dgcP_{PSV}*GGAEF allele in the $\Delta dgcP_{PSV}$ mutant failed to restore the wild type biofilm and swimming behaviours (Fig. 5). Therefore, we concluded that the DGC

activity of the *P. savastanoi* pv. *savastanoi* NCPPB 3335 DgcP protein is highly dependent on the presence of an intact GG(D/E)EF domain.

*The *P. savastanoi* pv. *savastanoi* NCPPB 3335 $\Delta dgcP_{PSV}$ mutant is hypovirulent in olive plants*

Because we demonstrated that DgcP is associated with key traits involved in *Pseudomonas* pathogenesis, we analysed the role of the *dgcP_{PSV}* gene in the virulence of *P. savastanoi* pv. *savastanoi* NCPPB 3335 against olive plants. Fitness attenuation and knot size reduction are commonly used to describe hypovirulent mutants in *P. savastanoi* pv. *savastanoi* (Rodríguez-Moreno et al., 2008; Matas et al., 2012). Mixed infections with the wild-type strain NCPPB 3335 and the $\Delta dgcP_{PSV}$ mutant or the complemented strain ($\Delta dgcP_{PSV}$ transformed with plasmid pJB3-*dgcP_{PSV}*) were used to calculate the competitive index (CI) on *in vitro* olive plants at 30 dpi. The CI values obtained for the wild-type strain and the $\Delta dgcP_{PSV}$ mutant were significantly lower than 1 (0.086 ± 0.039), indicating that this mutant was outcompeted by the wild-type strain *in planta*. However, no differences in competence were observed between the wild-type and the complemented strain.

To analyse the role of the *dgcP_{PSV}* gene during the formation of knots on olive plants, we used both *in vitro* micropropagated and lignified one-year-old olive plants infected with approximately 10^4 colony-forming units (CFU); the disease severity was evaluated after 30 or 90 days, respectively. Despite the fact that no difference in the final CFU counts was observed between the wild-type strain and the $\Delta dgcP_{PSV}$ mutant (data not shown), knots induced by the $\Delta dgcP_{PSV}$ mutant in both plant systems were visibly smaller than those developed by the wild-type or the complemented strain. Moreover, expression of the *dgcP_{PSV}*-GGAEF allele in the $\Delta dgcP_{PSV}$ mutant resulted in the induction of knots similar to those induced by the $\Delta dgcP_{PSV}$ mutant (Fig. 7A and 7B). In fact, the volume of the knots developed by the $\Delta dgcP_{PSV}$ mutant or its derivative strain transformed with pJB3-*dgcP_{PSV}*-GGAEF were ~2.5- and 1.8-fold smaller than those

induced by the wild-type strain on *in vitro* and lignified olive plants, respectively (Fig. 7C and 7D). Together, these results demonstrate that the *dgcP_{psv}* gene with an intact GGEEF domain is essential for the full fitness and virulence of *P. savastanoi* pv. *savastanoi* in olive plants.

The P. aeruginosa PAK $\Delta dgcP_{PAK}$ showed decreased virulence in infected mice in an acute lung injury model.

We compared lung injury assessed by the lung permeability index mediated by sub-lethal inocula of either the *P. aeruginosa* PAK, $\Delta dgcP_{PAK}$ or $\Delta dgcP_{PAK}Tn::dgcP_{PAK}$ strains using an *in vivo* infection model in mice. Previously, we demonstrated that the biofilm formation ability of the completed strain $\Delta dgcP_{PAK}Tn::dgcP_{PAK}$ was identical to that of wild type PAK or $\Delta dgcP_{PAK}$ complemented with pJB3-dgcPPAK (data not shown). Mice infected with the $\Delta dgcP_{PAK}$ strain showed decreased lung injury (Fig. 8A), enhanced bacterial clearance (Fig. 8B) and decreased bacterial dissemination (Fig. 8C) compared to those infected with wild-type PAK or the complemented strain ($\Delta dgcP_{PAK}Tn::dgcP_{PAK}$) that expresses a single copy of the *dgcP_{PAK}* gene from its own promoter. Additionally, broncho-alveolar lavage (BAL) cellularity was increased in mice infected with the $\Delta dgcP_{PAK}$ mutant (Fig. 8D), showing a significant increase in lymphocyte counts and a slight but not significant increase in neutrophil and macrophage recruitment (Fig. 8E). Taken together, these results show that the deletion of *dgcP* leads to a reduction in virulence that is reflected by decreased bacterial dissemination, enhanced bacterial clearance and reduced host lung injury.

Discussion

Enzymes involved in the synthesis and degradation of the universal bacterial second messenger c-di-GMP have been identified in all major bacterial phyla (Romling et al., 2013). The genomes of bacterial pathogens encode a variable number of proteins with GGDEF, EAL and HD-GYP domains, ranging from less than 10 (e.g., *Xylella fastidiosa* and *Mycobacterium tuberculosis*) to over 70 in *Vibrio cholerae* (Galperin et al., 2001). The opportunistic human pathogen *P. aeruginosa* and the plant pathogenic bacterium *P. savastanoi* pv. *savastanoi* NCPPB 3335 putatively encode 40 and 30 GGDEF/EAL domain-containing proteins, respectively (Galperin et al., 2001; Rodríguez-Palenzuela et al., 2010). The activity has been clearly demonstrated for the *P. aeruginosa* DGC proteins WspR (Hickman et al., 2005), SadC and RoeA (Merritt et al., 2010; Moscoso et al., 2014) and for the PDE proteins BifA and RocR (Kuchma et al., 2007; Rao et al., 2008). C-di-GMP has been reported to impact biofilm-related phenotypes by controlling the flagellar cascade (Pesavento et al., 2008; Hengge, 2009), the secretion of adhesins (e.g., LapA) (Monds et al., 2007), and the production of EPS (e.g., Pel and Psl) (Merritt et al., 2007). Here, we present biochemical and physiological evidence that DgcP_{PSV}, a GGDEF domain-containing protein encoded in the genome of *P. savastanoi* pv. *savastanoi* NCPPB 3335, possesses DGC activity. Phylogenetic analysis of this novel DGC protein revealed that although DgcP orthologs are widely distributed, they are only found within the *Pseudomonas* genus. Clustering of the DgcP proteins yielded three distinct phylogenetic groups (Fig. 1A). However, the genetic context of the *dgcP* gene was highly conserved among all sequenced *Pseudomonas* spp. (Fig. 1B), suggesting an ancestral origin for this gene within this bacterial group. Furthermore, the *dgcP* gene was shown to be co-transcribed with two other upstream genes (an EEPP-encoding gene and the *dsbA* gene) in both *P. savastanoi* pv. *savastanoi* NCPPB 3335 and *P. aeruginosa* PAK (Figs. 2 and S1). The conservation of these three orthologs in association with their co-transcription as a polycistronic mRNA (Fig. 2) suggests a relevant role for this operon in the physiology of the *Pseudomonas* genus. In fact,

transposon mutants in the *dsbA* gene from *P. aeruginosa* (Ha et al., 2003) and *P. syringae* (Kloek et al., 2000) have been reported to exhibit reduced virulence and altered twitching and swimming motilities, respectively. Additionally, *P. savastanoi* pv. *savastanoi* NCPPB 3333 strain FAM-108, which carries a transposon in PSA3335_0619 (EEPP-encoding gene), showed an increased swimming motility (Fig. S2A), a reduced ability to form biofilms (Fig. S2B) and a reduced virulence in olive plants (Fig. S2C). However, a clean deletion of the PSA3335_0619 gene in NCPPB 3335 did not affect bacterial motility, biofilm formation or virulence of this pathogen in olive plants (Fig. S2), suggesting that the phenotypes observed in the transposon mutant were due to a polar effect on the downstream *dgcP* gene.

In this study, we used clean deletions of the *dgcP* gene to clearly demonstrate its key role in the control of bacterial motility (Figs. 5 and 6), biofilm formation (Figs. 5 and 6), and virulence (Figs. 7 and 8) in *P. aeruginosa* PAK and *P. savastanoi* pv. *savastanoi* NCPPB 3335. Deletion of the *dgcP* gene in both *P. savastanoi* pv. *savastanoi* NCPPB 3335 and *P. aeruginosa* PAK resulted in reduced biofilm formation and increased motility (Fig. 5); both of these phenotypes have been associated with reduced c-di-GMP levels (Simm et al., 2004; Jenal and Malone, 2006; Hengge, 2009). Moreover, a site-directed mutant in the *dgcP*_{PSV} GGDEF domain abolished its ability to complement either of these phenotypes (Fig. 5). This finding is consistent with previous results showing that mutation of the first or second glutamic acid (E) residue of the core GG[D/E]EF motif abrogated the activity of DGC proteins (Kim and McCarter, 2007; De et al., 2008; Merritt et al., 2010). Reduction of EPS production, a phenotype also associated with low levels of c-di-GMP (D'Argenio and Miller, 2004; Merritt et al., 2010; Pérez-Mendoza et al., 2014; Pérez-Mendoza et al., 2015), was not detected using a CR binding assay in either the *P. aeruginosa* PAK or *P. savastanoi* pv. *savastanoi* NCPPB 3335 *dgcP* knock-out mutant. However, overexpression of the *dgcP* gene resulted in increased CR binding associated with wrinkled colony morphology in *P. aeruginosa* (Fig. 4), as expected from the increased Pel production that occurs under

high c-di-GMP conditions in this bacterium (Friedman and Kolter, 2004; Vasseur et al., 2005). In contrast, *P. savastanoi* pv. *savastanoi* NCPPB 3335, which does not encode Pel synthetic genes (Rodríguez-Palenzuela et al., 2010) but does produce cellulose (Ude et al., 2006), did not show an increased CR binding phenotype under *dgcP* overexpression (data not shown). However, the heterologous overexpression of the DGC PleD* in NCPPB 3555 has been reported to increase this EPS-related phenotype (Pérez-Mendoza et al., 2014). Together, these observations suggest that DgcP-mediated modification of c-di-GMP levels influences Pel but not cellulose biosynthesis, opening the possibility that the biofilm phenotype associated with DgcP in both strains could be generated by a common yet undefined effector molecule(s) that is different from Pel and cellulose.

C-di-GMP signalling pathways have been shown to affect virulence in numerous human, animal, and a limited number of plant bacterial pathogens. Although deletion of PDEs generally reduces virulence and deletion of DGCs increases virulence, systematic genetic screens performed in several bacterial pathogens have exposed a more complex picture in which the deletion of specific DGCs and PDEs affects virulence in different ways (Kulasakara et al., 2006; Tamayo et al., 2007; Hengge, 2009; Romling, 2012; Romling et al., 2013). Investigations into the role of c-di-GMP signalling in the virulence of bacterial pathogens belonging to the genus *Pseudomonas* have been mainly focused on *P. aeruginosa*, with little attention paid to *P. syringae* and related plant pathogens. A systematic study of *P. aeruginosa* PA14 DGCs and PDEs showed that transposon mutants affecting PDE proteins (PvrR and RocR) or proteins containing the GGDEF (SadC) or GGDEF/EAL (PA5017 and PA3311) domains resulted in virulence attenuation in a murine thermal injury model (Kulasakara et al., 2006). However, a transposon insertion into PA5487 (the ortholog of *dgcP_{PAK}*) did not abolish the ability of wild-type PA14 to cause lethal infection in mice (Kulasakara et al., 2006). Our study revealed that deletion of this gene in *P. aeruginosa* PAK resulted in a hypovirulent phenotype in a mouse model of acute infection. The observed differences

in the virulence of the PAK and PA14 *dgcP* mutants could be explained by the fact that *P. aeruginosa* PA14 bears a frameshift mutation in *ladS*, the gene encoding the sensor that promotes biofilm formation and represses T3SS expression (Mikkelsen et al., 2011). In addition to the genomics and virulence differences previously observed between these *P. aeruginosa* strains, our virulence assay was not based on mouse survival after bacterial infection, but instead considered several parameters related with acute infection (e.g., lung injury and accumulation of bronchoalveolar lavage (BAL) lymphocytes) (Fig. 8). A reduction in virulence was also observed for the *P. savastanoi* pv. *savastanoi* NCPPB 3335 *dgcP*_{PSV} mutant in olive plants. Although several reports have evidenced the negative regulation of virulence by c-di-GMP in bacterial phytopathogens such as *Erwinia amylovora* (Edmunds et al., 2013), *Xanthomonas campestris* (Ryan et al., 2006a; Ryan et al., 2007; Ryan et al., 2010) or *Dickeya dadantii* (Yi et al., 2010), high c-di-GMP levels increased the virulence of *Xylella fastidiosa* (Chatterjee et al., 2010). Additionally, a recent study showed that the overexpression of DGC PleD* from *C. crescentus* in *P. savastanoi* pv. *savastanoi* NCPPB 3335 resulted in an increased knot size on olive plants (Pérez-Mendoza et al., 2014). Therefore, the observed reduction of virulence for both the *dgcP*_{PAK} and *dgcP*_{PSV} mutants could be related to their reduced ability to form biofilms, a process involved in the infection of mammalian lungs and olive tissues by *P. aeruginosa* (Singh et al., 2000) and *P. savastanoi* pv. *savastanoi* (Rodríguez-Moreno et al., 2009), respectively. It has been proposed that the opposing regulation of virulence by different DGCs is due to temporal and spatial variations in the c-di-GMP levels required during different disease stages (Romling et al., 2013). Moreover, we analysed the impact of DgcP on the switch between the T3SS and T6SS. Although no significant differences in the protein levels of PcrV (T3SS) and Hcp1 (T6SS) were detected in the *P. aeruginosa* PAK $\Delta dgcP$ mutant compared with the wild-type strain (Fig. S4A), a reduction in the transcript levels of T6SS-related genes was detected in the $\Delta dgcP$ mutants of both *P. savastanoi* pv. *savastanoi* NCPPB 3335 and *P. aeruginosa* PAK (Fig. S4B). Moreover,

overexpression of the *dgcP* gene in *P. aeruginosa* PAK repressed the T3SS and induced the T6SS (Fig. 6C). In agreement with these results, high levels of c-di-GMP have been previously related with the T3SS/T6SS switch in *P. aeruginosa* PAK (Moscoso et al., 2011), as well as with T3SS downregulation in *D. dadantii* (Yi et al., 2010), *P. syringae* pv. phaseolicola and *P. syringae* pv. tomato (Pérez-Mendoza et al., 2014). Overexpression of the DGC PleD* in *P. savastanoi* pv. *savastanoi* NCPPB 3335 did not alter the transcription of T3SS genes, although disease features were clearly affected (Pérez-Mendoza et al., 2014). These results suggest that c-di-GMP may regulate the *P. savastanoi* T3SS in a different manner or that the symptomatology observed is a consequence of the regulation of other virulence factor(s) by this second messenger. Moreover, repression of the T3SS and induction of the T6SS have been recently reported in *P. savastanoi* pv. *savastanoi* NCPPB 3335 following the exogenous addition of indole-3-acetic acid (Aragon et al., 2014), a well-studied pathogenicity factor in this bacterial pathogen. Additionally, the T6SS has been shown to be involved in bacterial competition in many instances (Haapalainen et al., 2012; Ma et al., 2014).

In summary, in this work, we identified and characterised a novel Pseudomonads-specific DGC protein and demonstrated its role in biofilm formation and virulence in both the opportunistic human pathogen *P. aeruginosa* and the plant pathogenic bacterium *P. savastanoi* pv. *savastanoi*. Our results with both infection models indicate that biofilm formation is important, as indicated by the virulence attenuation observed for the *dgcP*_{PAK} and *dgcP*_{PSV} mutants. Finally, in light of the broad host range spectrum of DgcP, this DGC protein has emerged as a possible target for the control of *P. aeruginosa*, *P. syringae* and related pathogen infections.

Experimental procedures

Bacterial strains, plasmids, media, and growth conditions

The original bacterial strains and plasmids used in this study are listed in Table 1. *P. savastanoi* pv. *savastanoi* strains were grown at 28°C in Luria-Bertani (LB) medium (Miller, 1972) or super optimal broth (SOB) (Hanahan, 1983). *P. aeruginosa* and *Escherichia coli* strains were grown at 37°C in LB and SOB media. M9 minimal medium (Sambrook and Russell, 2001) was used for the establishment of *P. aeruginosa* biofilms over glass tubes as described below. Solid and liquid media were supplemented when required with the appropriate antibiotics. Antibiotics were used at the following concentrations: For *E. coli*, 100 µg/ml ampicillin (Ap), 50 µg/ml gentamicin (Gm), 50 µg/ml kanamycin (Km), 50 µg/ml streptomycin (Sp), and 15 µg/ml tetracycline (Tc); for *P. savastanoi*, 300 µg/ml ampicillin, 10 µg/ml kanamycin, and 15 µg/ml tetracycline; and for *P. aeruginosa*, 300 µg/ml carbenicillin for maintenance, 500 µg/ml for selection, 100 µg/ml gentamicin, 2 mg/ml streptomycin and 200 µg/ml tetracycline.

Construction of bacterial strains and plasmids

The oligonucleotides used for the overexpression and generation of mutant constructs are listed in Table S1. PCR products were cloned into the pGEM-T easy (Promega, Madison, WI, USA) or pCR2.1 (Invitrogen, Carlsbad, CA, USA) cloning vectors and sequenced; then, the products were subcloned into the relevant broad-host-range or suicide vectors. Construction of the *P. savastanoi* pv. *savastanoi* NCPPB 3335 mutant $\Delta dgcP_{P_{SV}}$ and $\Delta 0619$ mutants was performed using pGEM-T- $\Delta dgcP_{P_{SV}}$ -Km or pGEM-T- $\Delta 0619$ -Km, respectively (Table 1). First, DNA fragments of approximately 1.2 kb corresponding to the 5' and 3' flanking regions of these genes were amplified by three rounds of PCR using NCPPB 3335 genomic DNA as a template. The primers introduced a BamHI site and the T7 primer sequence (Table S1) as previously described (Zumaquero et al., 2010; Matas et al., 2014). The resulting

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

Construction of a clean deletion of the *dgcP* gene in *P. aeruginosa* PAK ($\Delta dgcP_{PAK}$) was performed by marker exchange mutagenesis as previously described (Mikkelsen et al., 2009; Mikkelsen et al., 2013). Briefly, mutator fragments were constructed by PCR amplification of a 500 bp region flanking the gene of interest. DNA fragments were amplified and joined by three rounds of PCR, and the product was cloned into pCR2.1-TA and then sub-cloned into the *P. aeruginosa* suicide vector pKNG101. The first crossover event was selected using Sp, and the second with 5% sucrose. Deletions were confirmed using external primers designed up- or downstream of the mutator fragments. Chromosomal complementation of the $\Delta dgcP_{PAK}$ was performed using the Tn7 transposon vector pME6182. A 125 bp DNA fragment encoding the promoter region of the *dsbA-dgcP* operon, located upstream of the *dsbA* gene, was ligated to a DNA fragment encoding the complete *dgcP_{PAK}* ORF and cloned into plasmid pME6182, yielding pME6182-*dgcP_{PAK}* (Table 1). pME6182-*dgcP_{PAK}* was transformed in the *P. aeruginosa* PAK $\Delta dgcP_{PAK}$ mutant, and the selected transconjugants in LB-Gm yielded the complemented strain $\Delta dgcP_{PAK}$ Tn7::*dgcP_{PAK}*. Insertion of the Tn7 transposon into the *glmS* gene was verified using the primers Tn7-glmS-PAO1 and Tn7R-109 (Table S1).

The His-tagged construct of the DgcP protein from *P. savastanoi* pv. *savastanoi* NCPPB 3335 was generated by PCR amplification of the wild type template and subcloning into the pET28a expression vector for C-terminal His-tagging (Table 1). Site-directed mutagenesis was performed on constructs in pGEM-T using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene, USA) following the supplier's instructions.

Sequencing of the P. aeruginosa PAK dgcP gene-containing cluster

The complete *P. aeruginosa* PAK *dgcP* gene-containing cluster (*dsbA-dgcP* operon) was PCR amplified using total DNA from *P. aeruginosa* and sequenced using the following primers: i) Promoter PA5489 F-HindIII; ii) DS PA5487 R-KpnI; iii) PA5487 R-1174 and iv) PA5488 F- 34 (Table S1). The sequence of the total 3,811-bp fragment was deposited in GenBank under accession number KM111506.

Motility assays

For swimming motility assays, *P. savastanoi* pv. *savastanoi* NCPPB 3335 cells were grown on LB plates for 48 h, resuspended in 10 mM MgCl₂ and adjusted to an OD₆₆₀ of 2.0. Two µl aliquots were inoculated onto the centre of 0.3% agar LB plates with Tc and incubated for 72 h at 25°C. The diameters of the swimming halos were measured after 24, 48, and 72 h. For *P. aeruginosa* PAK, 0.5 µl of an overnight bacterial culture was spotted onto LB plates (0.25% agar), and the halo diameters were measured after incubation for 24 h at 30°C. Three motility plates were used for each strain, and the experiment was repeated with three independent cultures.

Biofilm assays

Biofilm formation by *P. savastanoi* pv. *savastanoi* NCPPB 3335 and *P. syringae* pv. tomato DC3000 was analysed on glass surfaces using a static microcosm assay

(Pliego et al., 2008). Static microcosms were established using 50 ml polypropylene Falcon tubes containing 15 ml of LB-Tc medium with a sterile object slide (25 mm x 15 mm x 1.5 mm) placed inside. Overnight LB cultures were used to inoculate microcosms to densities of approximately 10^7 cfu/ml ($OD_{600} = 0.1$). The microcosms were incubated for 24 and 48 h in a vibration-free environment at 28°C with lids loosely in place. To quantify the biofilms, 500 μ l of 0.1% crystal violet (CV) was added to each object slide and left to stain for 30 min. The CV was removed by aspiration, and each object slide was washed carefully three times with deionised water. CV adhered to the slide was removed by pipetting 1 ml of 70% of ethanol and quantified by measurement of the A_{595} (Pérez-Mendoza et al., 2011b).

For *P. aeruginosa* PAK and *P. putida* KT2440, visualisation of biofilm formation was performed in 14 ml borosilicate tubes. Briefly, LB (3 ml) supplemented with the appropriate antibiotics was inoculated to a final OD_{600} of 0.1 and incubated at 37°C. Biofilms were stained with 0.1% CV, and the tubes were washed with water to remove unbound dye. Quantification of biofilm formation was performed in 24-well polystyrene microtitre plates. M9 medium (1 ml per well) supplemented with the appropriate antibiotics was inoculated to a final OD_{600} of 0.01 and incubated at 37°C with shaking for 6, 8 and 24 h. The biofilms were stained and washed as described above, and the CV was solubilised in 96% ethanol before measuring the A_{600} . Three independent experimental repetitions were performed for each biofilm assay.

Exopolysaccharide detection with Congo red staining

For *P. savastanoi* pv. *savastanoi* NCPPB 3335, the CR-binding assay was performed on LB medium supplemented with CR (50 μ g/ml) and incubated at 25°C for 48 h. For *P. aeruginosa* PAK, CR staining assay was performed on tryptone (10 g/l) agar (1%) plates supplemented with 40 μ g/ml CR and 20 μ g/ml Coomassie brilliant blue. A total of 5 μ l of an overnight bacterial culture was spotted over the tryptone plates and incubated at 30°C for 24 h.

Western blots

Cultures were inoculated into LB medium to an OD₆₀₀ of 0.1 and incubated at 37°C with shaking for 6 h. Whole-cell lysates were loaded (0.1 OD₆₀₀ equivalent unit) on SDS-PAGE, and then the proteins were transferred onto nitrocellulose membranes. The primary antibodies anti-Hcp1 and anti-PcrV were used at dilutions of 1:500 and 1:1000, respectively. The secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG) was used at a 1:5000 dilution. Visualisation was achieved using the SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo) and a LAS3000 Imaging System (Fuji). Three independent experimental repetitions were performed for each Western blot.

DgcP and PleD expression, purification and enzymatic assays*

E. coli cells carrying the respective expression plasmid were grown at 30°C in 2-litre Erlenmeyer flasks containing 1 litre of 2xYT medium (Sambrook and Russell, 2001) supplemented with Km. For DgcP and PleD* production, protein expression was induced at an OD₆₆₀ of 0.5 to 0.6 by adding isopropyl 1-thio-β-D-galactopyranoside to a 1 mM final concentration and incubating for 3 h at 22°C. After harvesting by centrifugation, the resulting pellet was resuspended in 30 ml of buffer A (20 mM Tris-HCl [pH 6.4], 20 mM NaCl, 5% [vol/vol] glycerol, 0.1 mM EDTA, and protease inhibitor mixture) (Complete; Diagnostic GmbH, Mannheim, Germany) and broken by treatment with lysozyme and using a French press at 20 µg/ml. Following centrifugation at 13,000×g for 60 min, the supernatant was loaded onto Ni-NTA affinity resin (Qiagen), washed with buffer A, and eluted with an imidazole gradient. The elution fractions were examined for purity by SDS-PAGE, and fractions containing the proteins were pooled. The proteins were extensively dialysed against 500 mM NaCl, 50 mM Tris-HCl pH 8.0, 5 mM EDTA, 5 mM β-mercaptoethanol, 5 mM dithiothreitol, and 10% glycerol and stored at -70°C. Protein concentrations were determined using the Bio-Rad Protein

Assay kit. DGC assays were adapted from procedures described previously (Tran et al., 2011). *C. crescentus* PleD* (Paul et al., 2007) was used as a positive control. The standard reaction mixtures with purified protein (DgcP or PleD*) contained 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM MgCl₂, and 5% glycerol in a 300 µl volume and were started by adding different concentrations (100-250 µM) of GTP. Purified DgcP without substrate, denaturated protein and reactions using ddGTP as the substrate were used as the negative controls. The reactions were incubated at 30°C for 2 h. Cyclic-di-GMP production was analysed by reversed phase-coupled high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) as previously described (Pérez-Mendoza et al., 2014).

Quantitative RT-PCR assays

Quantitative real-time PCR (qRT-PCR) assays in *P. savastanoi* pv. *savastanoi* NCPPB 3335 were performed as previously described (Aragon et al., 2014; Matas et al., 2014). RNA extraction was performed after a 6 h incubation in the Hrp-inducing medium (Huynh et al., 1989). For *P. aeruginosa* PAK, RNA extractions were performed under the same conditions described above for the Western blot assays. The cells were pelleted and processed for RNA isolation using the TriPure Isolation Reagent (Roche Applied Science, Mannheim, Germany) as described previously (Matas et al., 2014). cDNA synthesis and qRT-PCR assays were performed as described by Matas et al. (2014). Transcriptional data were normalised to the housekeeping genes *gyrA* and *proC* for *P. savastanoi* pv. *savastanoi* NCPPB 3335 and PAK, respectively. The qRT-PCR reactions were performed in triplicate. cDNA synthesis and qRT-PCR were performed as described above.

Plant infection and isolation of bacteria from olive knots

Olive plants derived from seeds germinated *in vitro* (originally collected from a cv Arbequina plant) were micropropagated, rooted and maintained as previously described (Rodríguez-Moreno et al., 2008; Rodríguez-Moreno et al., 2009).

Micropropagated olive plants were infected in the stem wound with a bacterial suspension (c. 10^3 CFU) and incubated for 30 days in a growth chamber as described previously (Rodríguez-Moreno et al., 2008). Bacteria were recovered from the knots using a mortar and pestle containing 1 ml of 10 mM $MgCl_2$, and serial dilutions were plated onto LB medium (to detect the wild type), LB-Km medium (to detect the mutants) or LB-Tc medium (to detect the complemented strains). Population densities were calculated from at least three independent replicates. The morphology of the olive plants infected with bacteria was visualised using a stereoscopic microscope (Leica MZ FLIII; Leica Microsystems, Wetzlar, Germany). Knot volumes were quantified using a 3D scanner and the MiniMagics 2.0 software. Competitive indices (CI) were performed with *in vitro*-grown olive plants as described previously (Rodríguez-Moreno et al., 2008; Matas et al., 2012).

The pathogenicity of *P. savastanoi* pv. *savastanoi* NCPPB 3335 isolates in 1-year-old olive explants (lignified plants) was analysed as previously described (Penyalver et al., 2006; Pérez-Martínez et al., 2007). Morphological changes scored at 90 dpi were captured with a high-resolution digital camera Canon D6200 (Canon Corporation, Tokyo, Japan). The knot volume was calculated by measuring the length, width (subtracting the stem diameter measured above and under the knot from that measured below the knot) and height of the knot with a Vernier calliper (Moretti et al., 2008; Hosni et al., 2011). Statistical data analysis was performed by ANOVA test ($P \leq 0.05$).

Acute lung injury model

Age- and gender-matched animals in the C57BL/6J background were provided free access to a standard laboratory chow diet in a half-day light cycle exposure and temperature-controlled Specified-Pathogen Free (SPF) environment as determined by the FELASA recommendations. The acute respiratory tract infection model was induced by intranasal instillation of *P. aeruginosa*. C57BL6/J mice were lightly

anesthetised with inhaled sevoflurane (Forene Abbott, Queensborough, Kent, United Kingdom), and 50 µl of the bacterial solution was then administered intranasally (5×10^7 CFU of each strain). All of the mice were euthanised at 24 h. For bacterial culture, the mouse lungs were homogenised in sterile containers with sterile isotonic saline. Lung and spleen homogenates were sequentially diluted and cultured on bromocresol purple agar plates for 24 h to assess the bacterial load. Transvascular transport of albumin-FITC was used to study endothelial permeability in mouse lungs to assess the alveolar capillary permeability. For bronchoalveolar lavage (BAL), lungs from each experimental group were washed with a total of 1.5 ml of sterile phosphate-buffered saline (PBS). The recovered lavage fluid was pooled and centrifuged (200 x g for 10 min), and the cellular pellet was washed twice with PBS. BAL samples were filtered and immediately frozen at -80°C after collection for cytokine measurement using an ELISA kit (R&D systems, Minneapolis, MN, USA). Cell monolayers were prepared with a cytocentrifuge and stained with Wright-Giemsa stain. Cellular types were obtained by counting 200 cells/sample (Leica DM100) and expressing each cell type as a percentage of the total number counted. Statistical analysis was performed using the Prism 6 software (GraphPad). One-way analysis of variance (ANOVA) followed by multiple comparison tests or a *t*-test was used for all comparisons. Significance was accepted at $p < 0.05$.

Ethics Statement

Information regarding all animal experiments was submitted to the French Ministry of Research and received the reference number 00481.01 for the protocol entitled "Study of immunity in a murine acute lung aggression model mediated by *P. aeruginosa*". In compliance with French animal care and use in investigational research guidelines, the mice were sacrificed by lethal intraperitoneal injection of 0.3 ml of 5.47% pentobarbital (Laboratoire CEVA, Libourne, France).

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

Strains/Plasmids	Relevant characteristics ^a	Reference or source
Strains		
<i>P. savastanoi</i> pv. <i>savastanoi</i>		
NCPPB 3335	Wild-type strain	(Pérez-Martínez et al., 2007)
FAM-108	Signature-tagged mutagenesis (STM) mutant disrupted in the PSA3335_0619 gene	(Matas et al., 2012)
$\Delta dgcP_{Psv}$	Deletion <i>dgcP</i> (PSA3335_0620) mutant (Km ^R)	This work
$\Delta 0619$	Deletion of the <i>EEPP</i> -encoding gene PSA3335_0619 (Km ^R)	This work
<i>P. aeruginosa</i>		
PAK	Wild-type strain	S. Lory
$\Delta dgcP_{PAK}$	<i>dgcP</i> _{PAK} (PAK) mutant (Km ^R)	This work
$\Delta dgcP_{PAK}$ Tn:: <i>dgcP</i> _{PAK}	Chromosomal complemented <i>dgcP</i> _{PAK} (PAK) mutant (Km ^R Gm ^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-1</i> , <i>gyrA96</i> , <i>relA1</i> (Nal ^R).	(Hanahan, 1983)
GM2929	<i>F</i> -, <i>ara-14</i> , <i>leuB6</i> , <i>thi-1</i> , <i>tonA31</i> , <i>lacY1</i> , <i>tsx-78</i> , <i>galK2</i> , <i>galT22</i> , <i>glnV44</i> , <i>hisG4</i> , <i>rpsL136</i> , <i>xyl-5</i> , <i>mtl-1</i> , <i>dam13::Tn9</i> , <i>dcm-6</i> , <i>mcrB1</i> , <i>hsdR2</i> , <i>mcrA</i> , <i>recF143</i> (Sp ^R Cm ^R).	(Palmer and Marinus, 1994)
TOP10	<i>F</i> - <i>mcrA</i> , Δ (<i>mrr-hsdRMS-mcrBC</i>), Φ 80 <i>lacZ</i> Δ M15, <i>lacX74</i> , <i>recA1</i> , <i>araD139</i> , Δ (<i>ara leu</i>) 7697, <i>galU</i> , <i>endA1</i> , <i>nupG</i> (Sp ^R).	(Invitrogen,USA)
OmniMAX	<i>F'</i> <i>proAB</i> +, <i>lacIq</i> , <i>lacZ</i> Δ M15, Δ (<i>ccdAB</i>), <i>mcrA</i> , Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80(<i>lacZ</i>) Δ M15, Δ (<i>lacZYA-argF</i>), U169, <i>endA1</i> , <i>recA1</i> , <i>supE44</i> , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i> , <i>tonA</i> , <i>panD</i> (Tc ^R).	(Invitrogen,USA)
BL21	<i>F</i> -, <i>ompT</i> , <i>gal</i> , <i>dcm</i> , <i>lon</i> , <i>hsdSB</i> (<i>rB</i> - <i>mB</i> -), λ (<i>DE3</i> [<i>lacI</i> , <i>lacUV5-T7</i> , <i>gene 1</i> , <i>ind1</i> , <i>sam7</i> , <i>nin5</i>])	(Novagen,USA)
Original Plasmids		
pGEM-T easy	Cloning vector containing ori f1 and <i>lacZ</i> (Ap ^R)	(Promega, USA)
pCR2.1	TA cloning, <i>lacZ</i> α , ColE1, ori f1. (Ap ^R Km ^R)	(Invitrogen,USA)
pGEM-T- KmFRT-BamHI	Contains Km ^R from pKD4 (Ap ^R Km ^R)	(Ortíz-Martín et al., 2010)
pJB3Tc19	Cloning vector, P _{lac} promoter (Ap ^R Tc ^R)	(Blatny et al., 1997)
pET28a(+)	T7 promoter based <i>E. coli</i> expression vector (Km ^R)	(Novagen,USA)
pKNG110	oriR6K, oriTRK2, mobRK2, sacBR ⁺ (Sm ^R)	(Kaniga et al., 1991)
pME6182	Mini-Tn7 gene delivery vector based on pME3280, <i>HindIII</i> - <i>SmaI</i> - <i>KpnI</i> - <i>NcoI</i> - <i>SphI</i> MCS, ColE1 replicon (Gm ^R Ap ^R)	C. Reimann

pGEM-T- $\Delta dgcP_{P_{SV}}$	pGEM-T easy derivative containing <i>dgcP</i> from NCPPB 3335 (Ap ^R)	This work
pCR2.1- <i>dgcP</i> _{PAK}	pCR2.1 derivative containing <i>dgcP</i> from PAK (Km ^R)	This work
pJB3- <i>dgcP</i> _{P_{SV}}	pJB3Tc19 derivative carrying <i>dgcP</i> gene from NCPPB 3335 (Tc ^R Ap ^R)	This work
pJB3- <i>dgcP</i> _{P_{SV}} GGAEF	pJB3Tc19 derivative carrying <i>dgcP</i> gene from NCPPB 3335 with a point mutation in the canonical GGDEF motif (Tc ^R Ap ^R)	This work
pJB3- <i>dgcP</i> _{PAK}	pJB3Tc19 derivative carrying <i>dgcP</i> gene from PAK (Tc ^R Ap ^R)	This work
pGEM-T- $\Delta dgcP_{P_{SV}}$	pGEM-T easy derivative, contains 1.2 kb approx. on each side of the <i>dgcP</i> (PSA3335_0620) gene from NCPPB 3335 (Ap ^R)	This work
pGEM-T- $\Delta 0619$	pGEM-T easy derivative, contains 1.2 kb approx. on each side of the PSA3335_0619 gene from NCPPB 3335 (Ap ^R)	This work
pGEM-T- $\Delta dgcP_{P_{SV}}$ -Km	pGEM-T derivative, contains 1.2 kb approx. on each side of the <i>dgcP</i> gene from NCPPB 3335 interrupted by the kanamycin resistance gene <i>nptII</i> (Ap ^R Km ^R)	This work
pGEM-T- $\Delta 0619$ -Km	pGEM-T derivative, contains 1.2 kb approx. on each side of the PSA3335_0619 gene from NCPPB 3335 interrupted by the kanamycin resistance gene <i>nptII</i> (Ap ^R Km ^R)	This work
pCR2.1- $\Delta dgcP_{PAK}$	pCR2.1 derivative, contains 0.5 kb approx. on each side of the <i>dgcP</i> gene from PAK (Km ^R)	This work
pNG101- $\Delta dgcP_{PAK}$	pKNG101 derivative, contains 0.5 kb approx. on each side of the <i>dgcP</i> gene from PAK cloned <i>SpeI</i> - <i>Apal</i> (Sp ^R).	This work
pET28a- <i>dgcP</i> _{P_{SV}}	pET28a(+) derivative carrying <i>dgcP</i> gene from NCPPB 3335 (Km ^R)	This work
pGEM-T- <i>dgcP</i> _{PAK}	pGEM-T easy derivative containing the <i>P. aeruginosa</i> PAK <i>dgcP</i> gene under the control of the <i>dsbA-dgcP</i> operon promoter region (Ap ^R)	This work
pME6182- <i>dgcP</i> _{PAK}	pEM6182 derivative containing the <i>P. aeruginosa</i> PAK <i>dgcP</i> gene under the control of the <i>dsbA-dgcP</i> operon promoter region (Gm ^R)	This work

^a Ap, ampicillin; Cm, chloramphenicol; Gm, gentamycin; Km, kanamycin; Sp, spectinomycin and Tc, tetracycline

Table S1. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5' to 3') ^{a,b}
AER-0002141 RACE-R1	CCAGGTCAAAGCGGTACTTGCCATT
AER-0002141 RACE-R2	GTCGGACGGCAGTTTTTCAATCCAC
US AER-0002141-F	AGAGGTGTCGGTTTCGTTTG
AER-0002141-R-134	GCTCGACGACTTCGATCTTG
AER-0002141 F-461	ACGTTCTGACTCTTTCGCAGT
AER-0002142 R-216	ATCAGATCGCCGATTTTCTG
AER-0002142 F- 616	TCTGATGGGCGACATGAATA
AER-0002143 R-231	TCCAGTACGGCTTTTTTCGAG
AER-0002143 F-1881	GTCTGACACTGCTCGACGAA
AER-0002144 R-140	GAAGCTGGAGGGATTTTTCC
PA5489 RACE-R1	ATTCCTTGCCGGCGGTATAGTCGTC
PA5489 RACE-R2	GCTTTCCAGGGTCAGGAACATCTGC
US PA5489- F	TACTTCGCCAGCCAGAAGAT
US PA5489- R	GGCAGCCATACCAGAACAGT
PA5489-5488 F	CAGATGGAAAAGGCCAAGAA
PA5489-5488 R	GCTGATACCGACCTGGATGT
PA5488-5487 F	CCGTCGATCTTCTGAAAAC
PA5488-5487 R	AGCTCCTTCATGCACTGGTC
DS PA5487 F	CCTGTCCGTTCCATTTCAAG
DS PA5487 R	GTGAGGAAGAGCAGCAGCA
TA AER-0002143 F-444	TCGTATGAAGTCCTCACC
AER-0002142 R-37	CCCTATAGTGAGTCGGATCCTTCATCGATCCATGATT
AER-0002143 F-13	GGATCCGACTCACTATAGGGGGTTGATCACACCCCGT
AER-0002143 R-1012	CAGGCTGGCATGGGTCAG
AER-0002141 F-543	CATGATCGTCAATGGCAAG
AER-0002143 R-46	CCCTATAGTGAGTCGGATCCTTCCTTGAGGTA
TD-AER-0002143 F-30	GGATCCGACTCACTATAGGGGACCTGAACGAGCAGC
TD-AER-0002144 R-196	CCTTCGTGCAGGTCGGTC
TA AER-0002141 F-444	TCGTATGAAGTCCTCACC
AER-0002142 R-37	CCCTATAGTGAGTCGGATCCTTCATCGATCCATGATT
AER-0002143 F-13	GGATCCGACTCACTATAGGGGGTTGATCACACCCCGT
AER-0002143 R-1012	CAGGCTGGCATGGGTCAG
GGDEF2143-F	CGGTTTGGCGGTGAGGCATTTGTCATGCTG
GGDEF2143-R	CAGCATGACAAATTCCTCAGCGCCAAACCG
AER-0002143 F-18	AACCAACATATGAGCGACGACGCGGAG
AER-0002143 R-2041	AACCAACTCGAGTCAGCCTTGTTTCGACCCTG
TA-PA5487 F-458	ACCCTGCTGGAAGATCATC
PA5487 R-161	CCCTATAGTGAGTCGGATCCCGCAGCTCCTTCATGCA
PA5487 F-1983	GGATCCGACTCACTATAGGGCGCTGTATCGAGCCAA
TD-PA5487 R-500	GTGGTGCGCAGGGTGATC
EP PA5487 mutant F	TGGTACCAGCAGCTCAACC
EP PA5487 mutant R	GCAGGTTGTCCAACAGCATA
PA5487 F-1265	TATACCGACGTGCTGGACAA
PA5487 R-1419	GACACCTCCTGTTTCGTGCTC
Promoter PA5489 F- <i>HindIII</i> *	AAGCTTTGATCGCTGCGTAGCACGG
Promoter PA5489 R- <i>EcoRI</i>	GAATTCGCCCGGTCAAGTTTCGACCG
UP 5487F- <i>EcoRI</i>	GAATTCATCTGACCACATCCTGCTC
DS PA5487 R- <i>KpnI</i> *	GGTACCGGGACAGCCGGTCATTCTAC
Tn7- <i>GlmS</i> -PAO1	CTCAAGTCGAACCTGCAGGAAGTC
Tn7-R-109	CAGCATAACTGGACTGATTTTCAG
PA5487 R-1174*	GTTGAGTTGCCGCAGGTATT

PA5488 F- 34*

Hcp1-F

Hcp1-R

ProC F-289

ProC R-417

VgrG F-524

VgrG R-697

gyrA-F

gyrA-R

AGCGTGAACCTCGGGTCG

GGACCTGTCGTTACCAAGT

GGACACCAGGACTTCCTTCA

GCAACTGATCGTCTCCATCG

TTCGTCTGTCAGCCACAG

CGTCTGCTGGAAGAAGAAGG

CGTCTGAACCGCCTATTTTCC

GACGAGCTGAAGCAGTCCTACC

TTCCAGTCGTTACCCAGCTCG

^aRestriction sites generated in the oligonucleotide sequence are underlined.

^bF and R, forward and reverse primer, respectively. Numbers included after F and R in primers names correspond to the hybridisation position of the 3' end of the primer in the corresponding ORF sequence.

*Primers used for the amplification and the sequencing of the *dsbA-dgcP* operon from *P. aeruginosa* PAK.

Table S2. Locus tag for DgcP proteins used construct the NJ tree shown in Fig. 1A.

Strains	Locus Tag
<i>P. savastanoi</i> pv. <i>savastanoi</i> NCPPB 3335	PSA3335_0620
<i>P. syringae</i> Cit7	PSYCIT7_11999
<i>P. syringae</i> pv. <i>aceris</i> M302273PT	PSYAR_16805
<i>P. syringae</i> pv. <i>actinidae</i> M302091	PSYAC_13693
<i>P. syringae</i> pv. <i>aesculi</i> NCPPB3681	PsyrpaN_010100023083
<i>P. syringae</i> pv. <i>aptata</i> DSM 50252	PSYAP_11255
<i>P. syringae</i> pv. <i>glycinea</i> race4	PsgRace4_02295
<i>P. syringae</i> pv. <i>japonica</i> M301072PT	PSYJA_29888
<i>P. syringae</i> pv. <i>lachrymans</i> M301315	PLA107_17437
<i>P. syringae</i> pv. <i>maculicola</i> ES4326	PMA4326_01170
<i>P. syringae</i> pv. <i>mori</i> 301020	PSYMO_20118
<i>P. syringae</i> pv. <i>morsprunorum</i> M302280PT	PSYMP_07510
<i>P. syringae</i> pv. <i>oryzae</i> 1_6	Psyrpo1_010100003834
<i>P. syringae</i> pv. <i>phaseolicola</i> 1448A	PSPPH_0255
<i>P. syringae</i> pv. <i>syringae</i> 642	Psyrps6_010100021588
<i>P. syringae</i> pv. <i>syringae</i> B728a	Psyr_0266
<i>P. syringae</i> pv. <i>tabaci</i> ATCC 11528	PsyrptA_020100000998
<i>P. syringae</i> pv. <i>tomato</i> DC3000	PSPTO_0339
<i>Pseudomonas aeruginosa</i> PA7	PSPA7_6286
<i>Pseudomonas aeruginosa</i> PAO1	PA5487
<i>Pseudomonas aeruginosa</i> PA14	PA14_72420
<i>Pseudomonas aeruginosa</i> 39016	PA39016_003370047
<i>Pseudomonas entomophila</i> L48	PSEEN0083
<i>Pseudomonas fluorescens</i> Pf-5	PFL_0087
<i>Pseudomonas fluorescens</i> PfO-1	PfIO1_0050
<i>Pseudomonas fluorescens</i> SBW25	PFLU_0085
<i>Pseudomonas fluorescens</i> WH6	PFWH6_0084
<i>Pseudomonas fulva</i> 12-X	Psefu_0269
<i>Pseudomonas mendocina</i> NK-01	MDS_0176
<i>Pseudomonas putida</i> GB-1	PputGB1_0144
<i>Pseudomonas putida</i> F1	Pput_0146
<i>Pseudomonas putida</i> GB-1	PputGB1_0144
<i>Pseudomonas putida</i> KT2440	PP_0129
<i>Pseudomonas putida</i> S16	PPS_0094
<i>Pseudomonas putida</i> W619	PputW619_5099
<i>Pseudomonas stutzeri</i> A1501	PST_4057
<i>Pseudomonas stutzeri</i> DSM4166	PSTAA_4207
<i>Thalassolituus oleivorans</i> MIL-1	TOL_0074

Titles and figure legends

Fig. 1. Phylogeny and genetic organisation of the *dgcP* gene cluster in *Pseudomonas*.

A. Phylogenetic analysis of DgcP proteins 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 distances were computed in units of 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.

Thalassolituus oleivorans MIL-1 was used as the outgroup outside the *Pseudomonas* genus. The locus tags corresponding to the DgcP proteins used in this analysis are shown in Table S2.

B. Schematic map of the *dgcP* gene cluster and the genetic context in the *Pseudomonas* genus. Red arrows indicate the *dgcP* gene cluster; downstream, the grey arrow indicates a gene encoding an antibiotic transporter (*marC*), the orange arrow a peptidase M23-encoding gene, and the blue arrows an N-acetylmuramoyl-L-alanine amidase gene (*ampDh2*). Upstream, yellow arrows indicate cytochrome C-encoding genes, the white arrow indicates a gene encoding a surface antigen of 17KDa, and the green arrows indicate a gene encoding a GTP-binding protein.

Fig. 2. Transcriptional analysis of the *dsbA-dgcP* operon in *P. savastanoi* pv. *savastanoi* NCPPB 3335 and *P. aeruginosa* PAK.

A and C. Localisation of primers used for RT-PCR in *P. savastanoi* pv. *savastanoi* NCPPB 3335 (A) and *P. aeruginosa* PAK (C).

B and D. Gel electrophoresis (1.5% agarose) of RT-PCR amplicons obtained for NCPPB 3335 (B) and *P. aeruginosa* PAK (D). C+, RT-PCR amplification reaction from total DNA of NCPPB 3335 or PAK (positive control); C-, negative control using RNA as the template; RT, amplification using cDNA synthesised from RNA samples. L, molecular weight DNA marker (DNA ladder, Promega Co.). The primer pairs used are detailed in Table S1.

Fig. 3. Purification and demonstration of the activity of the DgcP protein.

A. Lane 1, protein molecular weight marker SDS7 (Sigma-Aldrich); lane 2, SDS-PAGE of the purified DgcP protein with a predicted molecular weight of 76 KDa.

B. Mass spectroscopy chromatograms of c-di-GMP produced by 10 μ M of DgcP (DgcP activity) and 4 μ M PleD* from *C. crescentus* (positive control, PleD* activity). C-di-GMP, chromatogram obtained for synthetic c-di-GMP (20 nM); denatured protein, chromatogram obtained for heat-denatured DgcP (negative control). The three peaks observed in the chromatograms correspond to the three specific ions resulting from the fragmentation of c-di-GMP: m/z 152 (1, red), 248 (3, blue) and 540 (2, green).

C. c-di-GMP produced by the activity of DgcP and PleD* quantified by determining the peak areas (B) using a standard curve of synthetic c-di-GMP (2-2000 nM). Average production of c-di-GMP is indicated as pmol of c-di-GMP/pmol of protein and was calculated using three different amounts of protein.

Fig. 4. Exopolysaccharide production in *P. aeruginosa* PAK overexpressing the *dgcP* gene. Colony morphology in Congo red agar plates of *P. aeruginosa* PAK transformants with the *dgcP* gene from *P. aeruginosa* PAK (*dgcP*_{PAK}) or *P. savastanoi* pv. *savastanoi* NCPPB 3335 (*dgcP*_{PSV}).

Fig. 5. Swimming motility and biofilm formation of *P. aeruginosa* PAK and *P. savastanoi* pv. *savastanoi* NCPPB 3335 Δ *dgcP* mutants.

A-B. Area of the swimming halos (mm²) produced by *P. savastanoi* pv. *savastanoi* NCPPB 3335 (A) and *P. aeruginosa* PAK (B) at 72 h (0.3% agar LB plates) and 24 h (0.25% agar LB plates), respectively.

C-D. Quantification of biofilm formation by Δ *dgcP* mutants of NCPPB 3335 (*dgcP*_{PSV}) over glass slides (C) and PAK (*dgcP*_{PAK}) in borosilicate glass tubes (D) using crystal violet. Bars represent the means of three biological replicates, and the error bars

represent the standard deviation from the average. The same letters indicate differences that were not significant ($\alpha=0.05$) using the ANOVA statistical test. pJB3, control empty plasmid; *dgcP_{PSV}*, NCPPB 3335 *dgcP* wild-type gene encoding a GGEEF motif; *dgcP_{PSV}-GGAEF*, NCPPB 3335 mutant of the *dgcP* gene encoding a GGAEF motif; *dgcP_{PAK}*, PAK *dgcP* gene.

Fig. 6. Phenotypes of *P. aeruginosa* PAK associated with increased c-di-GMP levels due to the overexpression of the *dgcP* gene from *P. aeruginosa* PAK (*dgcP_{PAK}*) or *P. savastanoi* pv. *savastanoi* NCPPB 3335 (*dgcP_{PSV}*).

- A. Swimming motility in LB 0.25% agar.
- B. Quantification of crystal violet-stained biofilms under static growth conditions.
- C. Western blot analysis using antibodies directed against PcrV (T3SS effector) and Hcp1 (T6SS structural component).

Fig. 7. Virulence assay of the *P. savastanoi* pv. *savastanoi* NCPPB 3335 Δ *dgcP_{PSV}* mutant on olive plants. A-B. Knots induced on *in vitro* (30 dpi) (A) or lignified (90 dpi) olive plants (B) by the indicated strains.

C-D. Knot volume (mm³) induced by the indicated strains on *in vitro* olive plants (C) and lignified plants (D). Bars represent the means of three biological replicates from two different assays; error bars represent the standard deviation from the average. The same letters indicate differences that were not significant ($\alpha=0.05$) using the ANOVA statistical test. (from the left to the right): pJB3, control empty plasmid; *dgcP_{PSV}*, NCPPB 3335 *dgcP* wild type gene encoding a GGEEF motif; *dgcP_{PSV}-GGDAF*, NCPPB 3335 mutant of the *dgcP* gene encoding a GGAEF motif.

Fig. 8. The *P. aeruginosa* PAK Δ *dgcP_{PAK}* mutant is hypovirulent in mice. Wild-type mice 24 h after infection with either PAK, Δ *dgcP_{PAK}* or Δ *dgcP_{PAK}*Tn::*dgcP_{PAK}* (n=6 per group).

- A. Lung injury index assessed by alveolar capillary barrier permeability.
- B. Bacterial burden in the lungs after 24 hours of culture.
- C. Bacterial dissemination assessed by spleen homogenates after culture.
- D. Bronchoalveolar lavage cellularity.
- E. Cell count from bronchoalveolar lavage. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ error bars represent $\pm$ s.d. (n=6). PAK, *P. aeruginosa* PAK.

Supplemental figure legends

Fig. S1. Identification of the transcription start site of the *dsbA-dgcP* operon in *P. aeruginosa* PAK and *P. savastanoi* pv. *savastanoi* NCPPB 3335. The +1 positions in NCPPB 3335 (A) and PAK (B) were determined by 5' RACE and subsequent sequencing. The coding sequence of the *dsbA* genes is shadowed. The transcription start sites for NCPPB 3335 ($P_{P_{SV}}$) and PAK (P_{PAK}) are shown in bold font. The putative promoters identified using BPROM (<http://linux1.softberry.com>) and the sequence of the primers used for 5' RACE are underlined. The sequences corresponding to the -35 and -10 promoter boxes are shadowed in grey.

Fig S2. Phenotypic characterisation of the *P. savastanoi* pv. *savastanoi* NCPPB 3335 mutants affected in the *dsbA-dgcP* operon. FAM108 contains a transposon in the PSA3335_0619 gene (Matas et al., 2012); $\Delta 0619_{P_{SV}}$, deletion mutant of the PSA3335_0619 gene.

- A. Swimming motility in LB 0.3% agar plates after incubation for 72 hours.
- B. Biofilm formation over glass slides after 24 h of incubation under static conditions.
- C. Virulence assay of *P. savastanoi* pv. *savastanoi* NCPPB 3335 strains FAM-108 and $\Delta 0619$ on olive plants. Knots induced on *in vitro* olive plants at 30 dpi.

Fig. S3. Phenotypes associated with the overexpression of the *dgcP_{PSV}* gene in *Pseudomonas* strains.

- A. Swimming motility (LB 0.3% agar supplemented with tetracycline) of *P. savastanoi* pv. *savastanoi* NCPPB 3335, *P. syringae* pv. tomato DC3000 and *P. putida* KT2440 transformed with pJB3 (empty vector) or pJB3-*dgcP_{PSV}* at 72, 48 and 24 h, respectively.
- B. Quantification of the biofilms in the strains shown above using crystal violet (CV). The bars represent the mean values from 3 independent cultures $\pm$ standard deviations. The asterisks indicate values that were significantly different between both

strains. Statistical analyses were performed using the Student's t-test with a threshold of $P = 0.05$.

Fig S4. Impact of DgcP on the switch between the T3SS and T6SS in *P. aeruginosa* PAK and *P. savastanoi* pv. *savastanoi* NCPPB 3335.

A. Western blot analysis of PcrV (T3SS effector) and Hcp1 (T6SS structural component) in *P. aeruginosa* PAK. $\Delta dgcP_{PAK}$ and $\Delta retS$, clean deletion mutants of the *dgcP* and *retS* genes, respectively.

B. Quantitative reverse-transcription polymerase chain reaction (q-RT-PCR) with the indicated T6SS-related genes in *P. savastanoi* pv. *savastanoi* NCPPB 3335 $\Delta dgcP_{Psv}$ versus wild type NCPPB 3335 and *P. aeruginosa* $\Delta dgcP_{PAK}$ versus wild type PAK. The fold-change was calculated after normalisation using housekeeping genes as an internal control. Error bars represent standard deviations. Asterisks indicate significant differences ($P=0.05$) between the values obtained for the different conditions.

EMI_12856_F1

EMI_12856_F2

EMI_12856_F3

EMI_12856_F4

EMI_12856_F5

EMI_12856_F6

EMI_12856_F7

EMI_12856_F8