Environmental Microbiology (2009) **11**(5), 1126–1136 doi:10.1111/j.1462-2920.2008.01842.x

HD-GYP domain proteins regulate biofilm formation and virulence in *Pseudomonas aeruginosa*

Robert P. Ryan,[1] Jean Lucey,[1] Karen O'Donovan,[1] Yvonne McCarthy,[1] Liang Yang,[2] Tim Tolker-Nielsen[2] and J. Maxwell Dow[1]*

[1]*BIOMERIT Research Centre, Department of Microbiology, BioSciences Institute, National University of Ireland, Cork, Ireland.*

[2]*Center for Biomedical Microbiology, BioCentrum-DTU, Technical University of Denmark, DK-2800 Lyngby, Denmark.*

Summary

HD-GYP is a protein domain involved in the hydrolysis of the bacterial second messenger cyclic-di-GMP. The genome of the human pathogen *Pseudomonas aeruginosa* PAO1 encodes two proteins (PA4108, PA4781) with an HD-GYP domain and a third protein, PA2572, which contains a domain with variant key residues (YN-GYP). Here we have investigated the role of these proteins in biofilm formation, virulence factor synthesis and virulence of *P. aeruginosa*. Mutation of *PA4108* and *PA4781* led to an increase in the level of cyclic-di-GMP in *P. aeruginosa*, consistent with the predicted activity of the encoded proteins as cyclic-di-GMP phosphodiesterases. Mutation of both genes led to reduced swarming motility but had differing effects on production of the virulence factors pyocyanin, pyoverdin and ExoS. Mutation of *PA2572* had no effect on cyclic-di-GMP levels and did not influence swarming motility. However, PA2572 had a negative influence on swarming that was cryptic and was revealed only after removal of an uncharacterized C-terminal domain. Mutation of *PA4108*, *PA4781* and *PA2572* had distinct effects on biofilm formation and architecture of *P. aeruginosa*. All three proteins contributed to virulence of *P. aeruginosa* to larvae of the Greater Wax moth *Galleria mellonella*.

Introduction

The nucleotide cyclic di-GMP [bis-(3′-5′)-cyclic di-guanosine monophosphate] is a second messenger in bacteria that regulates a range of functions including developmental transitions, polysaccharide synthesis, adhesion, biofilm formation, the synthesis of virulence factors and virulence in bacterial pathogens (Römling *et al*., 2005; Jenal and Malone, 2006; Ryan *et al*., 2006a; Cotter and Stibitz, 2007). The level of cyclic di-GMP in bacterial cells is influenced by both synthesis and degradation. The GGDEF protein domain synthesizes cyclic di-GMP, whereas EAL and HD-GYP domains are involved in cyclic di-GMP hydrolysis (Paul *et al*., 2004; Christen *et al*., 2005; Ryjenkov *et al*., 2005; Schmidt *et al*., 2005; Tamayo *et al*., 2005; Ryan *et al*., 2006b). GGDEF and EAL domains were first linked to cyclic di-GMP turnover through studies of the regulation of cellulose synthesis in *Gluconacetobacter xylinus* (Tal *et al*., 1998). Subsequently, a number of reports have described the role of GGDEF/EAL domain proteins in the control of different cellular functions in diverse bacteria and the enzymatic activity of the two domains has been established. In contrast, comparatively little is known about the role of HD-GYP domain proteins in bacteria, although bioinformatic studies indicate that genes encoding proteins with this domain occur in many genomic sequences representing all major classes of bacteria (Galperin, 2005).

Studies of the function and role of the HD-GYP domain have thus far focused on the regulatory protein RpfG from the plant pathogen *Xanthomonas campestris*. RpfG is required for full virulence of *X. campestris* and acts to positively regulate the synthesis of virulence factors such as extracellular enzymes and extracellular polysaccharide, positively regulate motility, but negatively regulate adherence and biofilm formation (Slater *et al*., 2000; Dow *et al*., 2003; Crossman and Dow, 2004; Dow *et al*., 2006; Ryan *et al*., 2007). The isolated HD-GYP domain of RpfG can hydrolyse cyclic di-GMP to GMP via a linear intermediate (Ryan *et al*., 2006b). Mutation of the HD residues comprising the presumed catalytic diad of the HD-GYP domain abolishes both the regulatory activity and enzymatic activity against cyclic di-GMP (Ryan *et al*., 2006b).

The work in this paper had the overall aim of broadening our understanding of the cellular function of HD-GYP domain proteins by examination of their role in the human pathogen *Pseudomonas aeruginosa*. The *P. aeruginosa* genome encodes two proteins (PA4108, PA4781) with an HD-GYP domain and a third protein PA2572, which contains a variant HD-GYP domain (specifically YN-GYP). It is not known whether all three proteins are capable of

Received 4 September, 2008; accepted 5 November, 2008. *For correspondence. E-mail m.dow@ucc.ie; Tel. (+353) 21 4901316; Fax (+353) 21 4275934.

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cyclic di-GMP degradation. The expression of *PA4108*, *PA4781* and *PA2572* is substantially increased during co-culture of *P. aeruginosa* with human epithelial cells (Ichikawa *et al*., 2000; Chugani and Greenberg, 2007) and *PA2572* and *PA4781* are induced by mucopurulent fluid from cystic fibrosis patients (Wolfgang *et al*., 2004). These observations raise the question as to whether these proteins act to regulate factors associated with the virulence of *P. aeruginosa*, an issue we address here. Our findings indicate that PA4108 and PA4781 are cyclic di-GMP phosphodiesterases that regulate the synthesis and/or secretion of the virulence determinants pyocyanin, pyoverdin and ExoS as well as having a positive effect on swarming. In contrast, PA2572, which is inactive in cyclic di-GMP hydrolysis, has a cryptic negative influence on swarming. Mutations of *PA4108*, *PA4781* and *PA2572* have distinct effects on the synthesis of different virulence determinants and on biofilm formation and architecture but all mutants have reduced virulence to larvae of the Greater Wax Moth *Galleria mellonella*.

Results

Domain organization of PA2572, PA4108 and PA4781

The HD-GYP domain in PA2572 and PA4781 is found in association with an N-terminal CheY-like response receiver (REC) regulatory domain. PA2572 additionally has an uncharacterized C-terminal extension that is absent from PA4781 and from RpfG of *X. campestris*. PA4108 has an HD-GYP domain with additional N-terminal and C-terminal domains that are uncharacterized. The presence of the REC domain suggests that PA2572 and PA4781 are elements within two component regulatory systems. Although *PA2572* is adjacent to a gene (*PA2571*) encoding a sensor kinase, no gene encoding a sensor kinase is found within the genomic vicinity of *PA4781*, which appears therefore to be an 'orphan' regulator.

Analysis of the enzymatic activities of the HD-GYP domains of PA2572, PA4108 and PA4781 by expression in Xanthomonas campestris

As outlined in *Introduction*, mutation of *rpfG* in *X. campestris* leads to a substantial reduction in the synthesis of the extracellular enzymes endoglucanase, endomannanase and protease. Expression of the isolated HD-GYP domain of RpfG in the *rpfG* mutant background restores enzyme production to near wild-type levels. This effect, which is also seen with this domain carrying a C-terminal His6 tag, depends upon the HD diad and the ability to degrade cyclic di-GMP (Ryan *et al*., 2006b). Expression in the *X. campestris rpfG* mutant was used to analyse the enzymatic activities of the isolated HD-GYP domains of PA2572, PA4108 and PA4781 that had C-terminal His6 tags. HD-GYP domains from both PA4108 and PA4781 were able to restore production of endoglucanase, endomannanase and protease towards wild-type levels (Fig. 1A–C). In contrast, no effect was seen with the YN-GYP domain of PA2572 (Fig. 1A–C). Western analysis with antisera against the His6 tag showed that all the domains were expressed (Fig. 1D).

The effects of expression of the various HD-GYP domains on the cellular level of cyclic di-GMP in *X. campestris* were also determined. The levels of the nucleotide were elevated in the *rpfG* mutant compared with the wild-type *X. campestris* strain 8004 (Fig. 2; Fig. S1). Expression of the HD-GYP domains of RpfG, PA4108 and PA4781 caused a reduction in the level, although expression of the YN-GYP domain of PA2572 had no effect (Fig. 2). The absence of any effect with the YN-GYP variant domain suggested that this does not have any enzymatic activity against cyclic di-GMP. This contention was further examined by assessment of the effects of alteration of the conserved H231 and D232 residues of RpfG to Y and N residues respectively. These changes (H231Y, D232N) were introduced by site-directed mutagenic PCR into the construct expressing HD-GYPHis6 (see *Experimental procedures*). Western analysis with antisera against the His6 tag showed that the variant protein was expressed to the same level as the wild-type (Fig. S2). However, these alterations in the HD diad eliminated the regulatory activity of the HD-GYPHis6 domain in restoring extracellular enzyme synthesis (Fig. S2).

Taken together, these findings suggest that the HD-GYP domains of PA4108 and PA4781 have cyclic di-GMP phosphodiesterase activity but that the YN-GYP domain of PA2572 is inactive. Unfortunately, it was not possible to purify sufficient protein to examine these proposed enzymatic activities directly.

Phenotypic effects of mutation of genes encoding HD-GYP domain proteins in Pseudomonas aeruginosa

Specific disruption of *PA2572*, *PA4108* and *PA4781* was carried out using pEX18-Gm as described in *Experimental procedures*. The mutant strains were tested for the cellular level of cyclic di-GMP and for production of factors or phenotypes that have been associated with *P. aeruginosa* virulence. Mutation of *PA2572*, *PA4108* and *PA4781* had no effect on growth in minimal M9 medium or complex LB medium. Mutation of *PA4108* and *PA4781* gave rise to increased levels of cyclic di-GMP (Fig. 3), consistent with the role of the HD-GYP domain in cyclic di-GMP degradation. In contrast, there was no discernible

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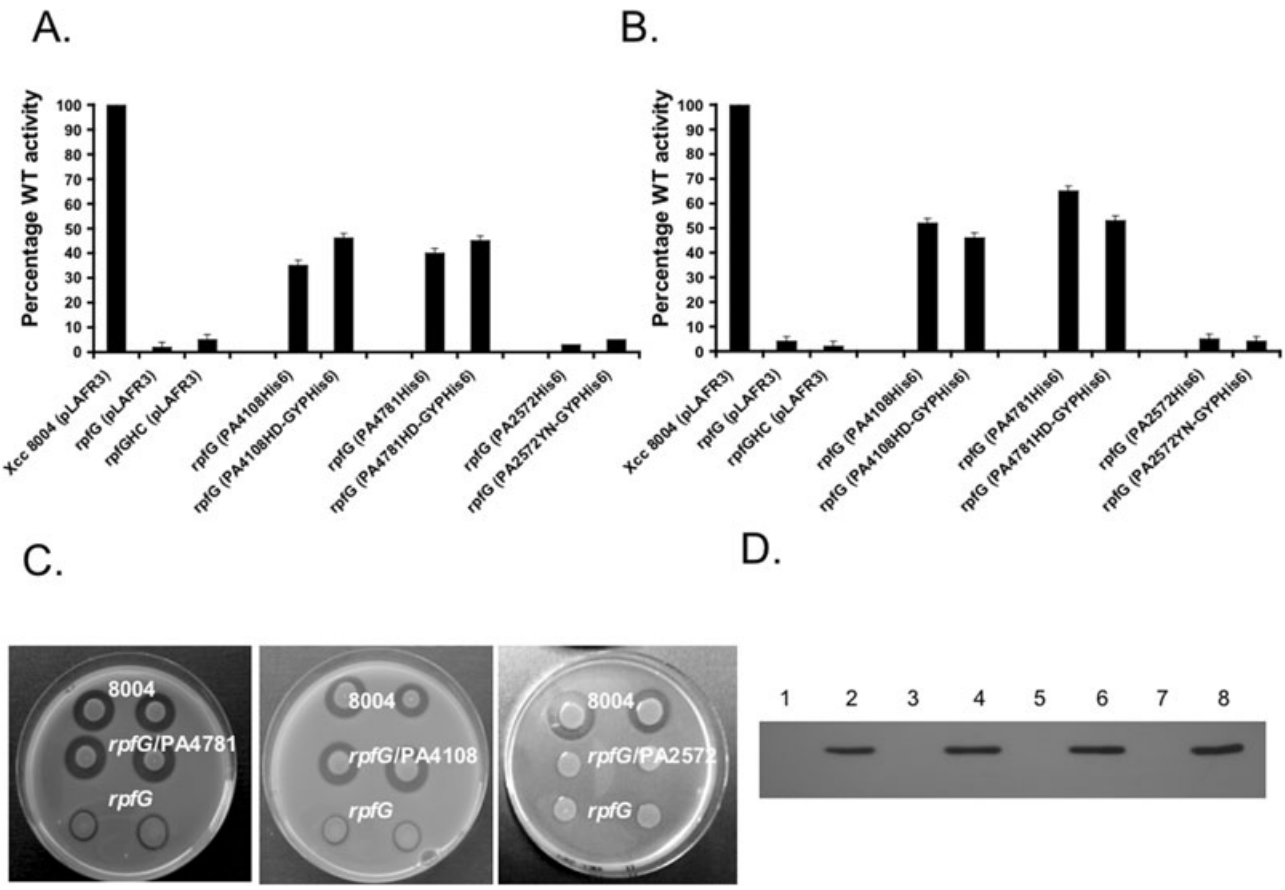

Fig. 1. Analysis of the ability of the HD-GYP domains of PA2572, PA4108 and PA4781 to regulate of production of extracellular enzymes in *X. campestris*. DNA encoding the different HD-GYP domains with a C-terminal His6 tag was cloned into pLAFR3 and constructs introduced into the *rpfG* mutant of *X. campestris*. The relative level of mannanase (A) and endoglucanase (B) in culture supernatants of different bacterial strains was measured after growth in NYGB medium to an OD at 600 nm of 2.0 and compared with that of the wild-type *X. campestris* strain 8004. Values given are the means and standard errors of triplicate measurements. (C) The relative level of protease production was assessed by zones of clearing around bacterial colonies grown on skimmed milk agar plates. The HD-GYP domain of PA4108 and PA4781 but not PA2572 restored production of the three enzyme activities to near levels of the *X. campestris* wild-type strain 8004. Western blot analysis with an anti-His6 antiserum (D) showed that all protein domains were expressed in *X. campestris*. Lanes: 1, *X. campestris* wild-type strain 8004 carrying pLAFR3; 2, *X. campestris rpfG* mutant expressing RpfG HD-GYPHis6 [*rpfG*(RpfG HD-GYP)]; 3, *rpfG* (pLAFR3); 4, *rpfG* (PA2572 HD-GYPHis6); 5, *rpfG* (pLAFR3); 6, *rpfG* (PA4781 HD-GYPHis6); 7, *rpfG* (pLAFR3); 8, *rpfG* (PA4108 HD-GYPHis6).

difference in the level of cyclic di-GMP between the PA2572 mutant and the wild-type (Fig. 3). Introduction of the *PA4108* and *PA4781* genes cloned in pBBRMCS1-Cm into the appropriate mutants reduced cyclic di-GMP levels to wild-type (Fig. 3).

Mutation of *PA4108* and *PA4781* led to reduction in both swarming and twitching motility (Fig. 4C and data not shown), whereas mutation of *PA2572* had no effect on either form of motility. All strains showed wild-type swimming motility. Introduction of *PA4108* and *PA4781* genes into the appropriate mutants restored wild-type swarming and twitching motility (Fig. 4C and data not shown).

Mutation of *PA4108*, *PA4781* and *PA2572* had different effects on the production of the phenazine, pyocyanin, the siderophore pyoverdine and rhamnolipid (Fig. 4; Fig. S3). Mutation of *PA4781* led to an increase in pyoverdine production, whereas mutation of *PA2572* led to a reduction and mutation of *PA4108* had no significant effect (Fig. 4A). In contrast, only mutation of *PA4108* had a significant effect on pyocyanin production, which was reduced compared with wild-type (Fig. 4B). The production of rhamnolipid was elevated in the *PA2572* mutant but was at wild-type level in both the *PA4108* and *PA4781* mutant strains (Fig. S3). Mutation of *PA4108* but not *PA2572* or *PA4781* led to reduction in the extracellular levels of the Type III-secreted effector ExoS (Fig. 5A). Introduction of the cloned PA4108 into the PA4108 mutant restored ExoS levels to wild-type (Fig. 5B). Overall, these results indicated that PA2572, PA4108 and PA4781 have distinct regulatory effects on production of certain virulence factors in *P. aeruginosa*, although PA4108 and PA4781 have the same influence on motility.

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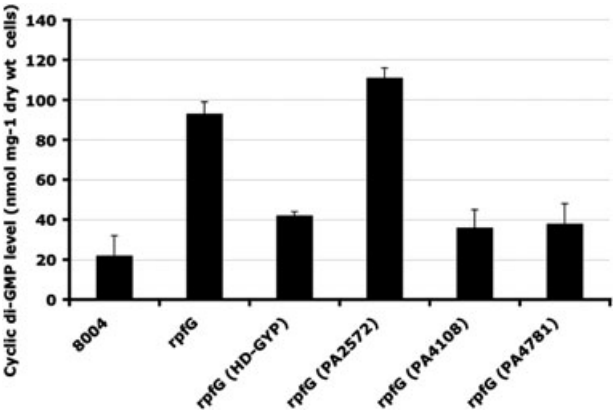

Fig. 2. Analysis of the cyclic di-GMP phosphodiesterase activity of the HD-GYP domains of PA2572, PA4108 and PA4781 by expression in the *rpfG* mutant of *X. campestris*. Relative levels of cyclic di-GMP in the *rpfG* mutant of *X. campestris* expressing HD-GYP domains from different *P. aeruginosa* proteins are compared with those in the *rpfG* mutant expressing the HD-GYP domain of RpfG, the *rpfG* mutant alone and wild-type *X. campestris* strain 8004. Values given are the mean and standard deviation of triplicate measurements.

Mutations of genes encoding different HD-GYP domain proteins have distinct effects on biofilm formation in Pseudomonas aeruginosa

The effect of mutation of *PA4108*, *PA4781* and *PA2572* on biofilm formation in *P. aeruginosa* was assessed using bacteria growing in flow cells (see *Experimental procedures*). In FAB medium, wild-type *P. aeruginosa* developed characteristic mushroom structures with a stalk and cap (Fig. 6A). The *PA2572* mutant formed similar structures, but unlike the wild-type, had central hollowing of the stalk with a region that was devoid of bacteria, as seen by staining with either the live stain Syto9 (Fig. 6B) or dead stain propidium iodide (data not shown). The *PA4108* mutant produced a flatter biofilm than wild-type with smaller mushroom structures and reduced biomass (Fig. 6C; Fig. S4E). The *PA4781* mutant produced a biofilm with little heterogeneity that covered a large percentage of the surface and encompassed a few mushroom-like structures (Fig. 6D). Introduction of cloned *PA2572*, *PA4108* or *PA4781* genes into the appropriate mutant strains restored the formation of mushroom structures similar to those of the wild-type in each case (Fig. S4). These differences in architecture of the biofilms formed by the different mutants provide further support for the notion that PA2572, PA4108 and PA4781 have distinct regulatory actions.

Mutation of genes encoding HD-GYP domain proteins influences virulence of Pseudomonas aeruginosa

The virulence of different strains of *P. aeruginosa* to larvae of *G. mellonella* were assessed as described in *Experimental procedures*, using 10 larvae at each inoculum level. The minimum inoculum of the wild-type required to kill all 10 larvae was 12 $\pm$ 3 cfu ($n = 9$). For strains with mutation of *PA2572*, *PA4108* and *PA4781*, a higher level of bacteria was required to kill all 10 larvae, indicating a reduction in virulence. The minimum inoculum level (cfu) was 1000 for the *PA2572* mutant, 1100 for the *PA4108* mutant and 745 for the *PA4781* mutant. Complemented *PA2572*, PA4108 and *PA4781* strains had wild-type levels of virulence, with minimum inoculum level between 10 and 15 cfu. All values are the means of quadruplicate measurements. The whole experiment was repeated twice with essentially the same result. These findings indicated that all three HD-GYP domain proteins contribute to virulence in the *Galleria* model.

A cryptic regulatory activity of the YN-GYP domain protein PA2572

In some cases functions of domains within modular proteins may be cryptic and may only be revealed by analysis of the phenotypes of multiple transposon insertions in each gene or by the generation of specific deletion mutants (Kulesekara *et al*., 2006; Tarutina *et al*., 2006). A preliminary indication of such a cryptic activity of the YN-GYP domain of PA2572 was provided by the analysis of the motility phenotypes of four mutants with transposon insertions in different sites in *PA2572* (Jacobs *et al*., 2003; Fig. 7A, which is a gene of 1344 nt. Although transposon insertions at positions 113, 615 and 928 did not influence swarming or twitching, the insertion at position 1107 (in strain 16841) led to a reduction in both types of motility (Fig. 7B and C; Fig. S5). All transposon mutants showed wild-type swimming motility, however (Fig. S5). The inser-

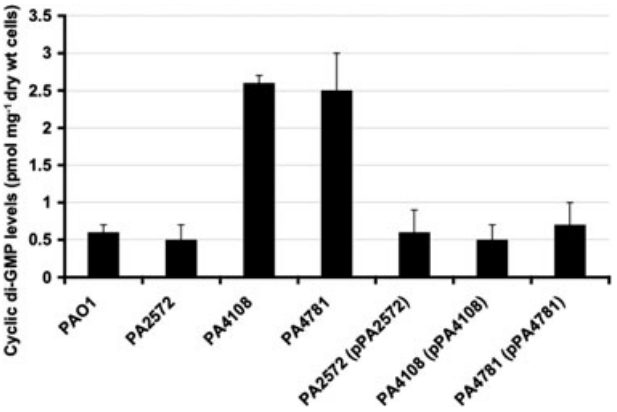

Fig. 3. Effects of mutation of *PA2572*, *PA4108* and *PA4781* on cyclic di-GMP level in *P. aeruginosa*. Elevated levels of extractable nucleotide were seen after mutation of *PA4108* and *PA4781*, but not *PA2572*. Introduction of the cloned *PA4108* and *PA4781* genes into the appropriate mutants reduced cyclic di-GMP levels to wild-type.

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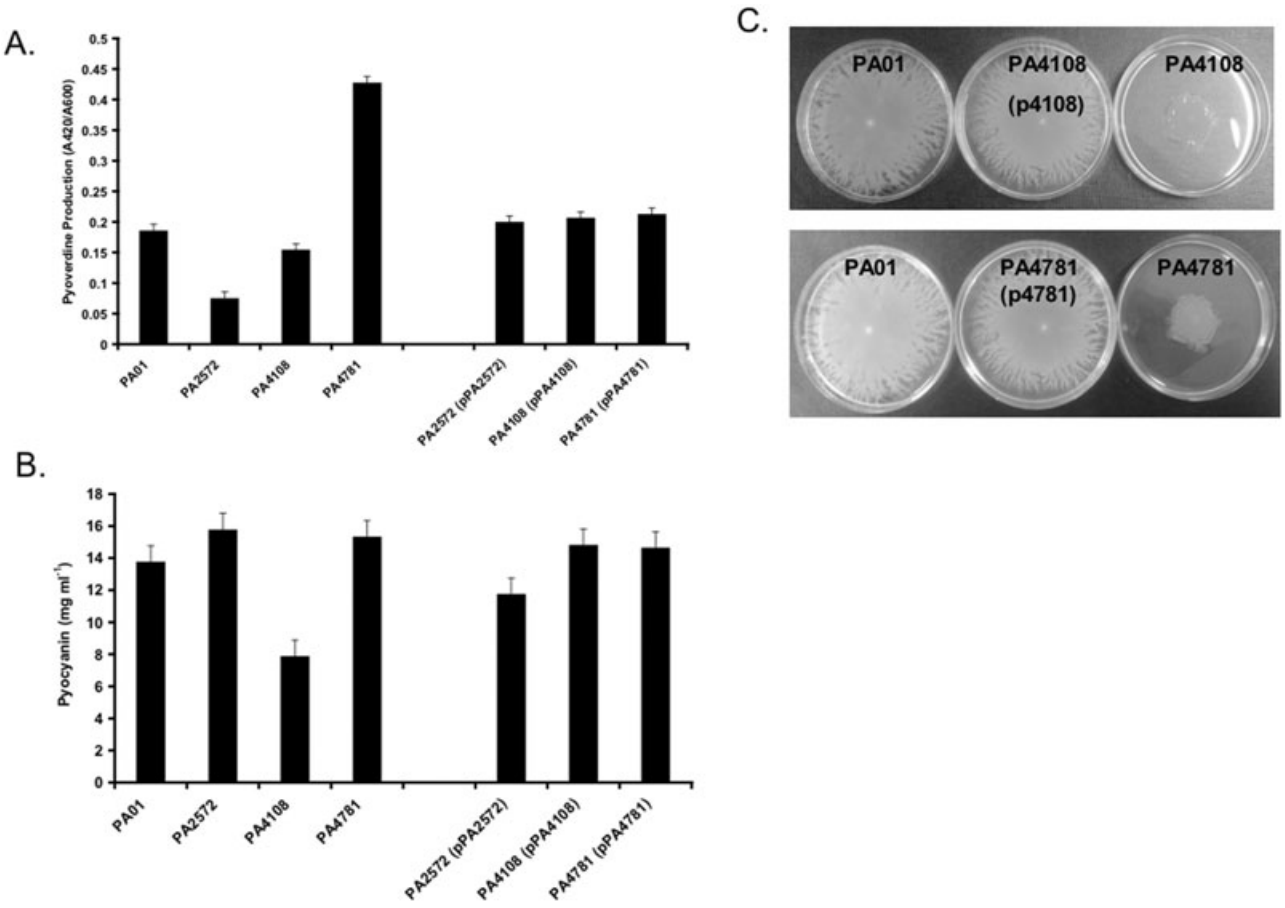

Fig. 4. Mutation of genes encoding different HD-GYP domain proteins has different phenotypic effects in *P. aeruginosa* PAO1. (A) Pyoverdine production; (B) pyocyanin production; and (C) swarming motility on 0.5% Eiken agar. Mutation of *PA2572* did not influence swarming (not shown). The phenotypic effects of mutation could be reversed by introduction of the cloned genes into the appropriate mutants.

tion affecting swarming and twitching is within the region of *PA2572* encoding the uncharacterized C-terminal extension. Introduction of the wild-type *PA2572* gene cloned in pBBRMCS5-GmR into strain 16841 did not restore wild-type motility (data not shown). These findings suggested that truncation of PA2572 had revealed a dominant negative regulatory activity of the protein on *P. aeruginosa* twitching and swarming motility. Although the insertion in strain 16841 led to a reduction in motility, there was no effect on the production of pyocyanin, pyoverdin or rhamnolipid (data not shown).

The YN-GYP domain has a dominant negative effect on swarming motility

The hypothesis that the truncated PA2572 has a dominant negative effect on motility was further tested by experiments in which the effects of expression of a truncated protein with only REC and YN-GYP domains (encoded by nucleotides 1–1110) were examined. Constructs expressing a protein with both REC and YN-GYP domains cloned in pBBRMCS5-GmR or the REC and YN-GYP domains with a His6 tag cloned into pLAFR3 (see *Experimental procedures*) were introduced into the wild-type strains mPAO1 and PAO1. This resulted in a negative effect on swarming and twitching in both strains (Fig. 8). These effects were also seen when the construct cloned in pBBRMCS5-Gm was introduced into strain 8166 (Fig. S6), which has a transposon insertion at position 113 of *PA2572* (Fig. 7A). In contrast, expression of either the REC domain alone, the YN-GYP domain with the C-terminal extension (both with His6 tags) or of the YN-GYP domain alone had no effect on swarming motility (Fig. S7A and data not shown). Western analysis with antisera against the His6 tag showed that the different proteins were however expressed (Fig. S7B). Taken together, these results support the view that PA2572 has a cryptic dominant negative effect on motility and indicate that this action requires both the REC and YN-GYP domains.

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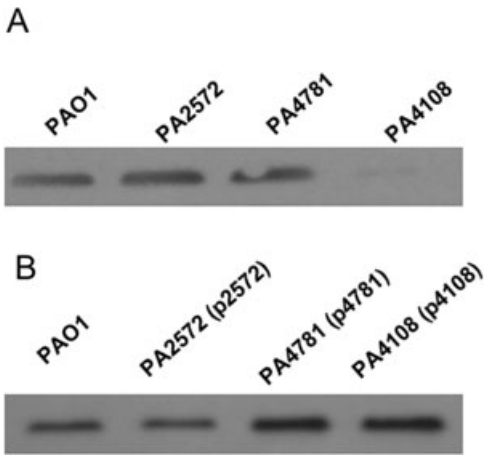

Fig. 5. Effects of mutation of genes encoding HD-GYP domain proteins on production of extracellular type III secreted effector ExoS.
A. Immunoblot of extracellular proteins of *P. aeruginosa* strains separated by SDS-polyacrylamide gel and probed with antisera to ExoS. The *PA4108* mutant shows significantly reduced ExoS level.
B. ExoS levels in extracellular protein preparations from complemented mutants. Strains were grown in the presence of the calcium chelator nitrilotriacetic acid and equivalent amounts of protein (10 μg) were loaded in each lane.

Discussion

HD-GYP domains proteins regulate virulence factor synthesis in Pseudomonas aeruginosa

The work in this paper provides evidence that the HD-GYP domain proteins PA4108 and PA4781 are cyclic di-GMP phosphodiesterases that act in regulation of motility, synthesis of virulence determinants and biofilm architecture in *P. aeruginosa*. Mutation of either *PA4108* or *PA4781* leads to a reduction in motility associated with elevated cellular levels of cyclic di-GMP. Observations from a range of bacteria have similarly linked lower levels of the nucleotide with motile behaviour and higher cellular levels with sessile behaviour and biofilm formation (Simm *et al*., 2004). This apparently common action of PA4108 and PA4781 in regulation of motility contrasts with distinct effects of mutation of *PA4108* and *PA4781* on biofilm architecture and the synthesis of virulence determinants; mutation of *PA4108* has different effects to mutation of *PA4781* on the synthesis of pyoverdin and pyocyanin and only mutation of *PA4108* influences the levels of the type III-secreted effector ExoS. The mechanistic basis for these apparently diverse actions of HD-GYP domain proteins remains obscure.

An enzymatically inactive HD-GYP domain has a regulatory role

A number of lines of evidence indicate that the YN-GYP domain of PA2572 has no activity as a cyclic di-GMP phosphodiesterase. Expression of PA2572, the YN-GYP domain attached to the uncharacterized C-terminal domain or the isolated YN-GYP domain did not restore extracellular enzyme activity to the *rpfG* mutant of

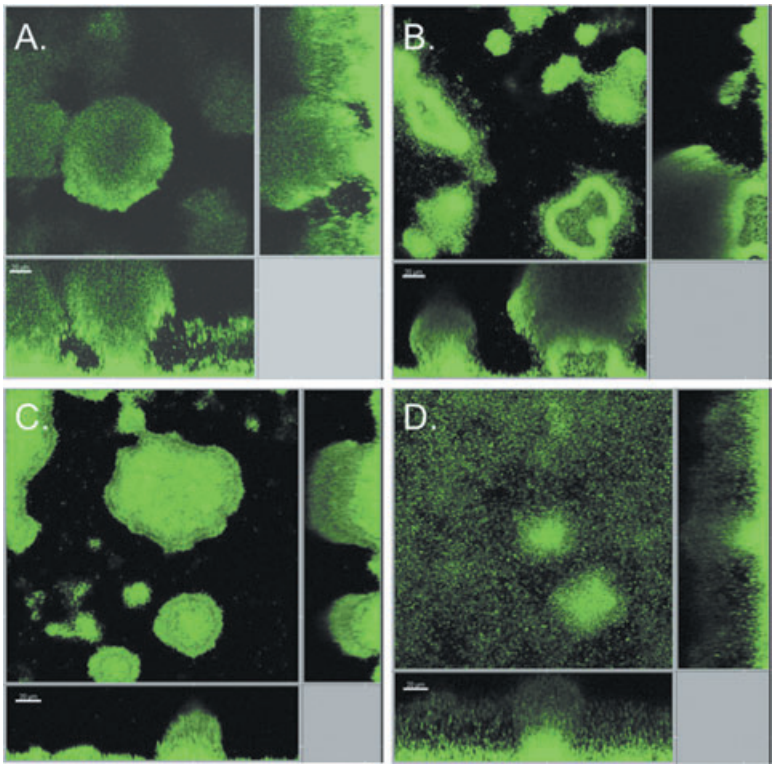

Fig. 6. Effects of mutation of genes encoding HD-GYP domain proteins on biofilm architecture in *P. aeruginosa*. Images are of 4-day-old biofilms in flow cells in FAB medium. (A) *P. aeruginosa* PAO1 wild-type; (B) *PA2572* mutant; (C) *PA4108* mutant; and (D) *PA4781* mutant. For these experiments, *P. aeruginosa* was visualized with Syto9, as described in *Experimental procedures*. Scale bars, 20 μm.

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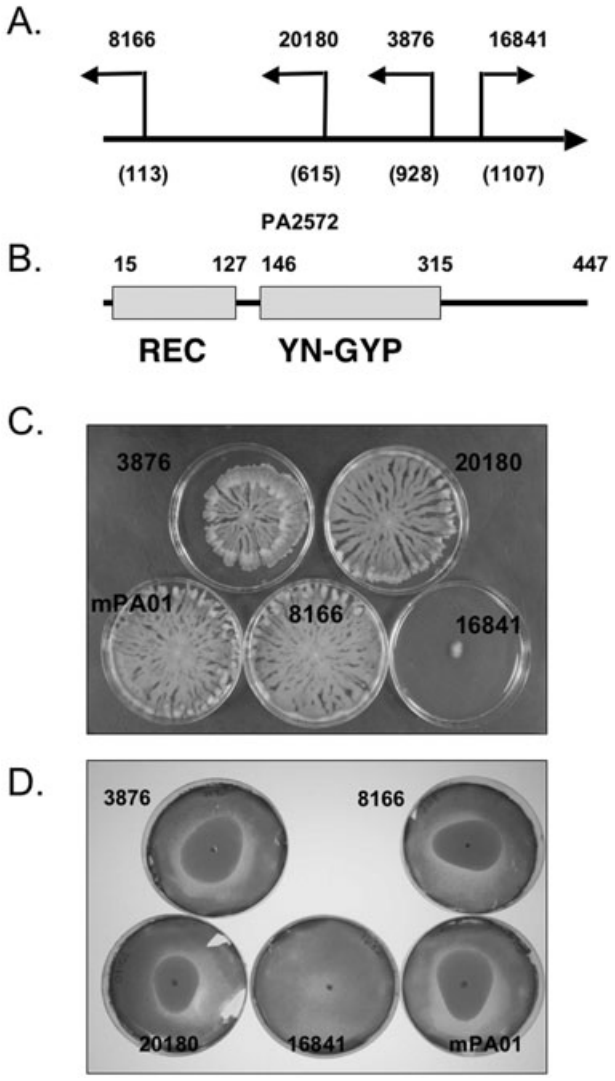

Fig. 7. Motility phenotypes of *P. aeruginosa* mPAO1 (Jacobs *et al*., 2003) and derived mutants with transposon insertion at different locations within *PA2572*.
A. Map of the locations of the transposon insertions within the *PA2572* gene, nucleotide positions given in brackets. Arrows indicate orientations of the outward facing neomycin phosphotransferase promoter of the transposon. These strains, constructed by Jacobs and colleagues (2003), were obtained from the University of Washington Genome Centre (http://www.gs.washington.edu/labs/manoil).
B. Predicted domain organization of PA2572 comprising REC, YN-GYP and the uncharacterized C-terminal domain, with amino acid positions indicated.
C. Swarming motility of mPAO1 and mutants with transposon insertions in different positions within *PA2572*.
D. Twitching motility of mPAO1 and mutants with transposon insertions in different positions within *PA2572*.

X. campestris and had no effect on the levels of cyclic di-GMP. Furthermore, the H231Y, D232N alteration in the presumed catalytic diad of the HD-GYP domain of RpfG abolished the regulatory effect on extracellular enzyme synthesis in *X. campestris*. Nevertheless, mutation of *PA2572* influenced biofilm architecture and caused overproduction of rhamnolipid. Boles and colleagues (2005) have demonstrated that rhamnolipids mediate formation of hollows or cavities within the centre of *P. aeruginosa* biofilms and have proposed that this is associated with the process of bacterial detachment. It is therefore plausible that the hollowing of the stalk of the mushroom produced by the *PA2572* mutant is a consequence of the overproduction of rhamnolipid by this strain.

Further to this, a cryptic regulatory role for the YN-GYP domain on swarming was revealed through the use of multiple transposon mutants within *PA2572* and by expression of the different domain combinations in the wild-type *P. aeruginosa*. Although the complete loss of PA2572 has no apparent effect on swarming, the loss of the C-terminal domain results in a protein with a dominant

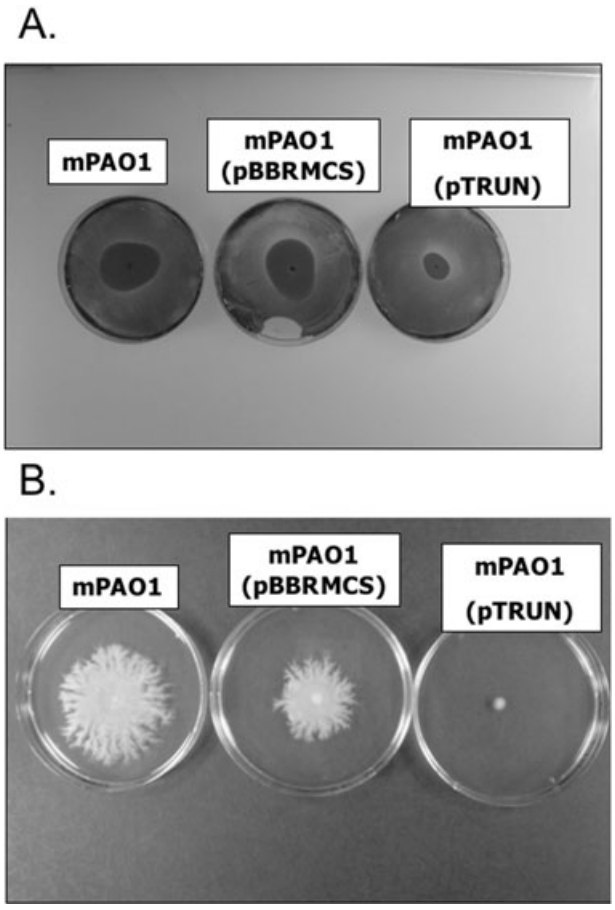

Fig. 8. Motility of wild-type *P. aeruginosa* is influenced by expression of PA2572 with a C-terminal truncation. Twitching (A) and swarming motility (B) were assessed in mPAO1, mPAO1 carrying the cloning vector pBBRMCS5-GmR and mPAO1 carrying a construct comprising nucleotides 1–1110 of *PA2572* (labelled as pTRUN), which expresses the C-terminally truncated PA2572 protein. A similar reduction in swarming was seen in *P. aeruginosa* PAO1 carrying a construct expressing the C-terminally truncated PA2572 protein with a His6 tag, cloned in pLAFR3.

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negative effect. This construct also had no enzymatic activity against cyclic di-GMP as assessed by heterologous expression in *X. campestris*. As pointed out by Kulesekara and colleagues (2006), analysis of the phenotypic effects of expressing such truncated proteins may or may not represent regulatory functions that occur during the biological function of the full-length protein. The mechanism(s) by which the YN-GYP domain exerts these effects are unknown, but it is plausible that this involves protein–protein interactions. Yeast two-hybrid analysis has revealed physical interactions between the HD-GYP domain of RpfG from *Xanthomonas axonopodis* pv. *citri* and a range of proteins including several with a GGDEF domain as well as other regulators (Andrade *et al*., 2006). Although the biological relevance of such interactions has yet to be tested, these observations suggest a scenario whereby the YN-GYP domain could modulate the activity of a subset of GGDEF domain proteins or other regulators (Andrade *et al*., 2006) with a consequent negative effect on swarming. It is also possible that the YN-GYP domain interferes with the action of the HD-GYP domain proteins PA4108 and PA4718, which have opposite effects on swarming to (truncated) PA2572.

Conservation of the C-terminal domain of PA2572 in other HD-GYP domain proteins

Our results are consistent with a model in which the C-terminal domain of PA2572 acts to inhibit the function of the YN-GYP domain in regulation of swarming, although they give no clues as to the mechanism of inhibition or how this is relieved to allow the proposed action of the domain. Examination of the databases using BLASTP indicated that proteins with the same domain structure as PA2572, including the C-terminal domain, occur in a wide range of bacteria including other *Pseudomonas* spp., *Burkholderia* spp. and *Vibrio cholerae*. Many of these proteins have variations in the HD motif that include HG, HN, HE and HQ in addition to YN, which appears to be restricted to the pseudomonads. It remains to be determined if these proteins with alteration of the HD diad have enzymatic activity against cyclic di-GMP.

The finding that an enzymatically inactive HD-GYP domain can have a regulatory activity has parallels in studies of GGDEF and EAL domains, where variations in the amino acids within the GGDEF and EAL motifs occur naturally in some proteins. Domains with sequences that are divergent from the consensus may have lost enzymatic activity, but may still play a regulatory role. In the GGDEF-EAL domain proteins CC3396 from *Caulobacter crescentus* and FimX from *P. aeruginosa*, binding of GTP to an enzymatically inactive GGDEF domain acts to regulate the activity of the EAL domain in cyclic di-GMP hydrolysis (Christen *et al*., 2005; Kazmierczak *et al*., 2006). Similar considerations may apply to inactive EAL domains (Schmidt *et al*., 2005), which perhaps can bind cyclic di-GMP. As well as intramolecular effects, enzymatically inactive EAL or GGDEF domains could conceivably regulate cellular processes through intermolecular interactions with other proteins or other molecules, in a fashion influenced by the binding of cyclic di-GMP or GTP.

HD-GYP domain proteins regulate virulence of Pseudomonas aeruginosa

Mutation of *PA2572*, *PA4108* and *PA4781* led to a reduction in bacterial virulence in the larvae of *G. mellonella*. In *P. aeruginosa* strain PA14, the type III-secreted effectors ExoT and ExoU have a significant role in *G. mellonella* killing, although translocation of ExoT or ExoU alone is sufficient to give near wild-type levels of killing. (*P. aeruginosa* PA14 has no *exoS* gene). As far as we are aware, the role of ExoS in virulence of *P. aeruginosa* PAO1 to *G. mellonella* has not yet been established. Although mutation of *PA4108* leads to a reduction in the levels of ExoS we cannot infer a causal relationship to the reduction of killing; the processes that contribute to virulence that are regulated by the HD-GYP domain proteins in bacteria within the host are unknown.

These observations add to a body of work that indicates a role for proteins with GGDEF and/or EAL domain, and by inference cyclic di-GMP, in the regulation of virulence and virulence factor synthesis in *P. aeruginosa* as well as in a number of human, animal and plant pathogens including *Vibrio cholerae*, *Salmonella* spp. and *Xanthomonas campestris* (Hisert *et al*., 2005; Tischler and Camilli, 2005; Kulesekara *et al*., 2006; Ryan *et al*., 2007). As with *P. aeruginosa*, the genomes of many of these bacteria encode between 1 and 4 HD-GYP domain proteins, although they are absent in *Salmonella* spp. and *E. coli* and more highly represented in *Vibrio* spp., where *V. cholerae* has 9 and *V. vulnificus* has 15. HD-GYP domain proteins are also found in a number of other bacterial pathogens of animals including *Clostridium perfringens*, *Bordetella bronchiseptica* and *Treponema denticola*. Whether these proteins play a significant role in the virulence of these diverse pathogenic bacteria remains to be determined.

Experimental procedures

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table S1. *Pseudomonas aeruginosa* strains were routinely cultured in Luria–Bertani (LB) broth. *Xanthomonas campestris* strains were routinely grown in NYGB medium, which comprises Bacteriological Peptone (Oxoid, Basingstoke, UK), 5 g l^{-1}; yeast extract (Difco), 3 g l^{-1} and glycerol,

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20 g l^{-1}. Antibiotics rifampicin (Rif), tetracycline (Tc), chloramphenicol (Cm) and gentamycin (Gm) were added where appropriate.

Construction and complementation of PA2572*,* PA4108 *and* PA4781 *mutants*

Mutants were created by the disruption of gene with the use of the suicide plasmid pEX18Gm (see Table S1). Briefly, an internal fragment of each of the genes was amplified using the primers detailed in Table S2 and was cloned into the TOPO (Invitrogen) vector. This fragment was excised with EcoRI and ligated into the suicide plasmid pEX18Gm cut with the same enzyme. This construct was introduced into PAO1 by electroporation. Mutants were selected by plating on LB agar medium containing gentamicin (20 μg ml^{-1}). Candidate strains were analysed by colony PCR using the primers detailed in Table S2 to confirm disruption of the *PA2572*, *PA4108* and *PA4781* gene by integration of the suicide vector. Complementing clones were constructed in pBBRMCS3 using DNA fragments amplified using primers detailed in Table S2.

Expression of the HD-GYP domains of P. aeruginosa *proteins in* Xanthomonas campestris

DNA fragments encoding the HD-GYP domain from PA2572, PA4108 and PA4781 with a C-terminal His6-tag were amplified by PCR by using chromosomal DNA of *P. aeruginosa* strain PAO1, as template with primers carrying restriction sites for BamHI and HindIII (see Table S2). These amplified fragments were cloned into the TOPO (Invitrogen) vector. The appropriate DNA fragments were excised from these constructs using BamHI and HindIII and ligated into pLAFR3 that had been cut with the same enzymes so that expression is under control of the *lac* promoter of the vector. Constructs were introduced into *X. campestris* wild-type or *rpfG* mutant by triparental mating using the helper plasmid pRK2073. Procedures used for Western analysis to examine protein expression levels using an anti-His6 antibody are described in Supporting Information.

Phenotypic analyses

Methods used for assay of extracellular enzyme activities in *X. campestris*, and for assays in of motility, pyoverdine, pyocyanin, rhamnolipid and ExoS production in *P. aeruginosa* are given in Supporting Information. Virulence of *P. aeruginosa* to larvae of *G. mellonella* was tested as described previously (Miyata *et al*., 2003). Bacterial suspensions from a dilution series were inoculated into larvae, with 10 larvae used for each inoculum level. The minimum inoculum required to kill all 10 larvae was determined for each strain. Additional details are given in the Supporting Information.

Cultivation and imaging of biofilms

Biofilms were grown in flow chambers with individual channel dimensions of $1 \times 4 \times 40$ mm. The flow system was assembled and prepared as described by Sternberg and Tolker-Nielsen (2005). The flow chambers were inoculated by injecting 350 μl of overnight culture diluted to an OD_{600} of 0.01 into each flow channel with a syringe. After inoculation, flow channels were left without flow for 1 h, after which medium flow at a linear rate of 0.2 mm s^{-1} was started using a Watson Marlow 205S peristaltic pump.

All microscopic observations and image acquisitions were performed on an LSM510 confocal laser scanning microscope (CLSM) (Carl Zeiss, Jena, Germany) equipped with detectors and filter sets for monitoring Syto9 fluorescence. Simulated three-dimensional images, shadow projections and sections were generated using the Imaris software package (Bitplane AG, Zürich, Switzerland).

Detection and quantification of cyclic di-GMP

The method for detection and quantification of cyclic di-GMP from *X. campestris* or *P. aeruginosa* strains was adapted from Simm and colleagues (2004; 2005). Details are given in Supporting Information.

Acknowledgements

We thank Susan Lynch and Dara Frank for providing monoclonal antibodies to ExoS, and Roger Simm and Ute Römling for technical advice on the measurement of cyclic di-GMP and for helpful discussions. We are grateful to Susan Joyce for guidance with the *Galleria* virulence assays. This work has been supported by grants from Science Foundation of Ireland and Enterprise Ireland (to J.M.D.), the Royal Irish Academy (to R.P.P.) and the Danish Natural Science Research Council (to T.T.-N.), which are gratefully acknowledged.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Analysis of the cyclic di-GMP phosphodiesterase activity of the HD-GYP domains of PA2572, PA4108 and PA4781 by expression in the *rpfG* mutant of *X. campestris*. (A and B) Representative HPLC chromatograms showing detection of cyclic di-GMP in extracts from (A) *X. campestris* wild-type strain 8004 and (B) the *rpfG* mutant. The peak indicated by the arrow is cyclic di-GMP.

Fig. S2. Mutation of the signature H and D residues of the HD-GYP domain of RpfG to Y and N respectively, abolishes

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the regulatory function on extracellular enzyme synthesis. Unlike the wild-type HD-GYP domain, an H231Y D232N variant (labelled YN-GYP) carrying a C terminal His6 tag, is unable to restore production of mannanase (A) and endoglucanase (B) to the *rpfG* mutant of *X. campestris*. Values given are the means and standard errors of triplicate measurements. Variants with the single alteration H231Y or D232N also lost the regulatory ability (data not shown). (C) Western blot analysis with an anti-His6 antiserum shows that all variant proteins are expressed in *X. campestris.* Lanes: 1, *rpfG* (pLAFR3); 2, *rpfG* (HD-GYPHis6); 3, *rpfG* (YD-GYPHis6); 4, *rpfG* (YN-GYPHis6).

Fig. S3. Mutation of *PA2752* but not *PA4108* or *PA4781* has an effect on the production of rhamnolipid in culture supernatants of *Pseudomonas aeruginosa*. The level of rhamnolipid in culture supernatants of strains grown in PPGAS medium was measured as described by Boles and colleagues (2005). Complementation of the *PA2572* mutant reduces rhamnolipid production back to wild-type levels.

Fig. S4. Biofilm architectures of complemented *PA2572*, *PA4108* and *PA4781* mutants are similar to the wild-type *P. aeruginosa*. Images are of 4-day-old biofilms in flow cells in FAB medium. (A) *P. aeruginosa* PAO1 wild-type; (B) complemented *PA2572* mutant; (C) complemented *PA4108* mutant; and (D) complemented *PA4781* mutant. For these experiments, *P. aeruginosa* was visualized with Syto9, as described in *Experimental procedures*. Scale bars = 20 μm. (E) Biomass of biofilms produced by different strains of *P. aeruginosa* as measured using COMSTAT. Values are means ± SD of 13 measurements.

Fig. S5. Motility phenotypes of *P. aeruginosa* mPAO1 and derived mutants with transposon insertion at different locations within *PA2572*, which has 1344 nt. (A and B) Swimming behaviour of the mutants is indistinguishable from the wild-type mPAO1 on (A) Eiken medium with 0.3% agar or (B) CAA medium with 0.3% agar. (C) Swarming motility on CAA medium solidified with 0.6% agar at pH 7.4 is affected only in strain 16841, which has the transposon insertion located at nt 1107. (D) Twitching motility on CAA medium solidified with 0.5% agar at pH 7.4 is also only affected in strain 16841.

Fig. S6. The negative influence of expression of PA2572 with a Cterminal truncation on swarming motility is also seen in a *PA2572* mutant background. Swarming motility (A and B) and swimming motility (C and D) were assessed in the *P. aeruginosa* wild-type mPA01 and mutant strain 8166, these strains carrying the cloning vector pBBRMCS5 (labelled as vector) and these strains carrying a construct expressing the C-terminally truncated PA2572 protein (labelled as Insert). Introduction of pTRUN into mPA01 and strain 8166 influences swarming motility over that seen with the vector control (A and B) but has no effect on swimming compared with a vector control (C and D). Note that the pBBRMCS5 vector alone has an influence on both forms of motility. Shown are representatives of swimming and swarming plates after 18 h of incubation.

Fig. S7. Expression of the isolated REC domain of PA2572 in mPAO1 does not influence swarming motility. (A) The REC domain of PA2572 expressed using pBBRMCS-5 does not give the swarming-negative phenotype in a *P. aeruginosa* mPAO1 or in strain 8166 (shown). Introduction of the REC domain with a His6 tag expressed using pLAFR3 gave the same result. This contrasts with the effects seen with expression of REC-YN-GYP (the truncated PA2572) using pTRUN, which gives substantially reduced swarming (see Fig. S5). (B) Western blot analysis with an anti-His6 antiserum shows that both the REC domain and REC-YN-GYP domain proteins are expressed in mPAO1. Lanes: 1, mPAO1 (pBBRMCS); 2, mPAO1 (pLAFR3); 3, mPAO1 (pRECHis6); 4, mPAO1 (pTRUNHis6).

Table S1. Strains and plasmids.

Table S2. Primers used in this work.

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