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Genetic dissection of a motility-associated c-di-GMP signalling protein of *Pseudomonas putida*

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Summary

Lack of the *Pseudomonas putida* PP2258 protein or its overexpression results in defective motility on solid media. The PP2258 protein is tripartite, possessing a PAS domain linked to two domains associated with turnover of c-di-GMP – a cyclic nucleotide that controls the switch between motile and sessile lifestyles. The second messenger c-di-GMP is produced by diguanylate cyclases and degraded by phosphodiesterases containing GGDEF and EAL or HD-GYP domains respectively. It is common for enzymes involved in c-di-GMP signalling to contain two domains with potentially opposing c-di-GMP turnover activities; however, usually one is degenerate and has been adopted to serve regulatory functions. Only a few proteins have previously been found to have dual enzymatic activities – being capable of both synthesizing and hydrolysing c-di-GMP. Here, using truncated and mutant derivatives of PP2258, we show that despite a lack of complete consensus in either the GGDEF or EAL motifs, the two c-di-GMP turnover domains can function independently of each other, and that the diguanylate cyclase activity is regulated by an inhibitory I-site within its GGDEF domain. Thus, motility-associated PP2258 can be added to the short list of bifunctional c-di-GMP signalling proteins.

Introduction

Bacterial flagella-driven motility is important for establishing bacteria–host interactions involved in virulence and symbiotic/mutualistic associations, and for directed movement (taxis) towards environmental niches favourable for energy generation. *Pseudomonas putida* KT2440 possess a bundle of ~6 polar-located flagella that are used in taxis responses through chemical and oxygen gradient (Sarand *et al*., 2008; Osterberg *et al*., 2010). These responses are mediated via 27 potential receptors, three of which (Aer1 to Aer3) are similar to inner-membrane anchored Aer-receptor of *Escherichia coli* (Sarand *et al*., 2008). Analysis of the function of the three Aer-receptors of *P. putida* KT2440 serendipitously uncovered an important motility-associated role for the PP2258 protein encoded downstream of the *aer1* gene in a bi-cistronic operon (Sarand *et al*., 2008). Lack of the PP2258 protein renders *P. putida* defective in motility on solid media.

The PP2258 protein has a tripartite domain structure with central- and carboxy-terminal domains bearing signature motifs indicative of roles in turnover of bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP). C-di-GMP was first identified as a regulator of cellulose synthase in *Gluconacetobacter xylinus* (Ross *et al*., 1987). Since then, c-di-GMP has been found in a wide range of bacteria species where it is involved in controlling many adaptive processes, including the major bacterial lifestyle switch from motile to sessile modes of growth (Schirmer and Jenal, 2009).

The cellular levels of c-di-GMP are controlled by two types of enzymes: diguanylate cyclases (DGCs) and phosphodiesterases (PDEs). C-di-GMP is produced from GTP by DGC homodimers that have the GGDEF signature motif in their active site (A-site) (Paul *et al*., 2004; Ryjenkov *et al*., 2005; Wassmann *et al*., 2007). Some DGCs contain an inhibitory site (I-site) – recognized by an RXXD motif – that can allosterically hinder c-di-GMP synthesis (Chan *et al*., 2004; Christen *et al*., 2006). Degradation of c-di-GMP is performed by PDEs that either contain an EAL or HD-GYP domain. PDEs with an EAL domain degrade c-di-GMP to the linear pGpG (Christen *et al*., 2005; Schmidt *et al*., 2005), while HD-GYP domain enzymes takes the degradation a step further and converts it to GMP (Ryan *et al*., 2006).

Most GGDEF and EAL/HD-GYP domains are coupled to signal input domains that directly or indirectly sense a diverse array of external or internal signals (e.g. lights,

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oxygen and cellular energy status), to modulate the c-di-GMP levels in bacteria. One domain that is frequently found at the amino-termini of DGCs and PDEs is the Per-ARNT-Sim (PAS) domain. This type of domain is involved in recognition of many different types of signals, which often trigger changes of the oligomeric state of the protein (reviewed in Moglich *et al*., 2009).

Although GGDEF and EAL domains have opposing activities, they are often found coupled together in the same protein. In most of these composite proteins, one or both of the domains are degenerate and have instead taken on new, non-enzymatic, regulatory functions (reviewed in Schirmer and Jenal, 2009). To our knowledge, only five proteins have been shown to have both DGC and PDE activity: *Rhodobacter sphaeroides* BphG1 (Tarutina *et al*., 2006), *Vibrio parahaemolyticus* ScrC (Ferreira *et al*., 2008), *Mycobacterium smegmatis* MSDGC-1 (Kumar and Chatterji, 2008), *M. tuberculosis* MtbDGC (Gupta *et al*., 2010) and *Legionella pneumophila* Lpl0329 (Levet-Paulo *et al*., 2011).

The *P. putida* PP2258 protein has a PAS-GGDEF-EAL domain architecture. Thus, the motility defect associated with loss of this protein is likely to involve c-di-GMP dependent signalling (Sarand *et al*., 2008). However, neither the GGDEF nor the EAL domain of PP2258 has a complete consensus signature motif (SGDEF and EGL respectively). Therefore, the aim of this work was to determine which, if any, of the two domains is enzymatically active. To analyse this we made use of structural modelling and a genetic approach using mutated and/or truncated variants of PP2258. We found that PP2258 does have the potential to both synthesize and degrade c-di-GMP and can thereby be added to the short list of bifunctional enzymes involved in c-di-GMP turnover. Furthermore, in contrast to some other bifunctional proteins (e.g. MSDGC-1; Kumar and Chatterji, 2008), the two c-di-GMP turnover domains of PP2258 can be physically uncoupled and still retain their cognate activities. However, the GGDEF domain requires the amino-terminal PAS domain for the truncate to remain stable and, therefore, for its activity.

Results and discussion

All three domains of PP2258 contain conserved features

The PP2258 PAS-GGDEF-EAL domain protein is one of 16 *P. putida* KT2240 proteins that contain both a GGDEF and an EAL domain and thus potentially dual enzymatic properties (Fig. S1). Neither the GGDEF nor the EAL domain of PP2258 has a complete consensus motif, being SGDEF and EGL respectively. To gain insights into potential functionality, each of the three domains of PP2258 was independently modelled on known structures. The PAS domain (Fig. 1, left) was modelled using the counterpart domain of the NifL from *Azotobacter vinelandii* (PDB code 2GJ3). The resulting model suggests that it has an amino-terminal dimerization helix that could, by analogy to the NifL-PAS, mediate dimer formation (Key

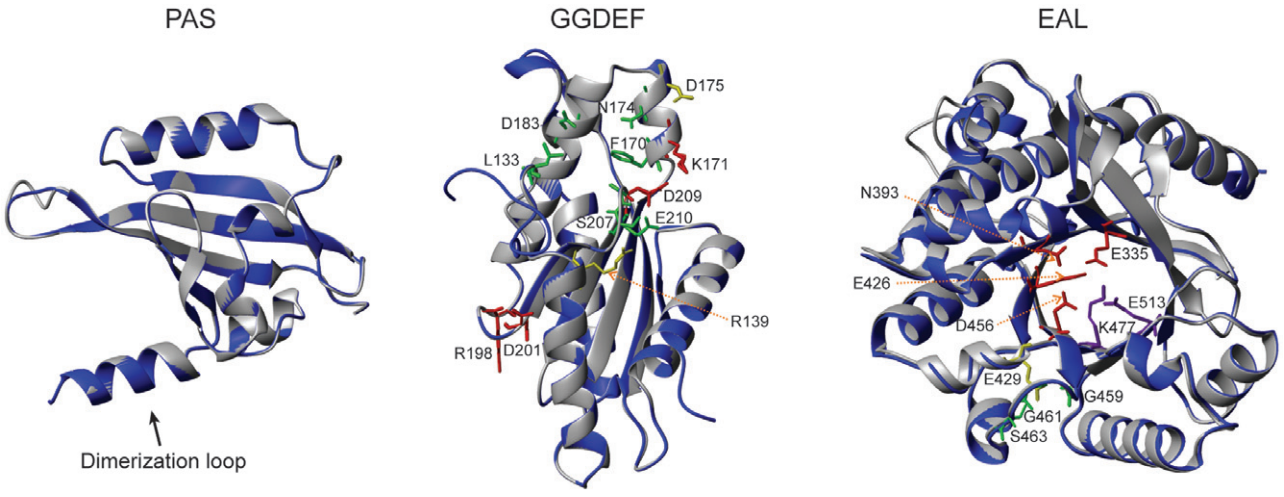

Fig. 1. Structural models of the three domains of PP2258. Structural models of the individual domains of PP2258 (grey) were generated using the SWISS-model program (Arnold *et al*., 2006; Kiefer *et al*., 2009) and are shown superimposed on the corresponding structures (blue) upon which it was modelled. Colour key for highlighted residues within the GGDEF domain is: red – catalytically important (A-site residues K171, D209; and I-site residues R198, 201D), green – substrate binding (L133, F170, N174, D183, S207, E210), yellow – stabilization of the GGDEF dimer (R139, D175). Colour key for highlighted residues within the EAL domain is: red – Mg^{2+} coordinating (E335, N393, E426, D456), yellow – indirect role in Mg^{2+} binding (E429), violet – structurally or catalytically important (K477, E513), green – part of the structurally important loop6 (G459, G461, S463), see text for details.

et al., 2007) – a property that is required for DGC activity of GGDEF domains.

A model of the PP2258 GGDEF domain was generated using the GGDEF domain of *Caulobacter crescentus* PleD (PDB code 1W25A; Fig. 1, centre). All residues in the PleD GGDEF domain that interact with GTP and discriminate it from ATP are present in the PP2258 counterpart [L294, F331, N335, D344, L347 and G369 in PleD; L133, F170, N174, D183, L186 and G208 in PP2258 (Chan *et al*., 2004)]. For GTP to be converted to c-di-GMP, a dimer of two GGDEF domains is needed to create the active site (Wassmann *et al*., 2007). In PleD this is suggested to be facilitated by D336 and R300 (Chan *et al*., 2004). PP2258 D175 and R139 align with these two residues and could thus serve the same function. PleD residue E370 is thought to function as a general base for deprotonating GTP, with K332 stabilizing the transition state (Chan *et al*., 2004). In the PP2258 model, D209 – which should also be able to act as a base – superimposes on PleD E370, while K171 aligns with PleD K332, suggesting conservation of this feature. A final key aspect of the PP2258 model is correct alignment of the RXXD inhibitory site (I-site) motif (R198, D201 in PP2258). Hence, the model suggests that the PP2258 GGDEF domain would be functional in c-di-GMP production, with its activity controlled by feedback inhibition through a regulatory I-site.

The PP2258 EGL domain was modelled using the EAL domain of TBD1265 from *Thiobacillus denitrificans* (PDB code 2R6O; Fig. 1, right). The catalytically active EAL domain of *P. aeruginosa* RocR has also been modelled using TBD1265 as template. Alanine substitution mutagenesis of RocR identified seven residues important for its PDE activity, including key residues for binding of Mg^{2+} that is required for enzymatic activity (Rao *et al*., 2008). Appropriately spaced equivalents for all of these residues are present within the PP2258 EGL domain (E175, N233, E265, E268, D295, K316 and E352 in RocR, E335, N393, E426, E429, D456, K477 and E513 in PP2258). In light of this, it appears likely that E335 and E426 of PP2258 could mediate Mg^{2+} binding with K477 being involved in correct positioning of these two residues. In RocR, E352 functions as the general base catalyst, generating the nucleophilic attack involved in degrading c-di-GMP to 5′-pGpG (Rao *et al*., 2008), a process that could potentially be performed by E513 of PP2258. Loop6, located between the β6 strand and the α6 helix, is a structurally and functionally important feature of catalytically active EAL domains [consensus DFG(A/T)GYSS; Rao *et al*., 2008]. Loop6 is conserved in the PP2258 EGL domain (DFGTGYSS; residues 457–464) and could be stabilized by E429 – the corresponding residue of E268 that serves this function in RocR (Rao *et al*., 2008; 2009). The conservation of key structural features suggested to us that the PP2258 EGL domain would likely constitute an active PDE.

Phenotypic effects of altered c-di-GMP levels in P. putida

The PP2258 null derivative of *P. putida* KT2701 (*ΔPP2258*::Km) exhibits an impaired motility phenotype on soft agar motility plates (Sarand *et al*., 2008). Therefore, we first ascertained the motility phenotypes of KT2701on 0.3% soft agar plates upon artificially increasing or decreasing c-di-GMP levels via expression of previously characterized c-di-GMP turnover proteins. To generate a strain with elevated c-di-GMP levels, a plasmid expressing a constitutively active version of the DGC PleD (PleD*) was introduced. This strain exhibited reduced motility, indicative of high c-di-GMP levels, as compared with *P. putida* KT2701 harbouring a cognate vector control (Fig. 2A, white bars). Conversely, expression of the PDE YhjH from a plasmid resulted in enhanced motility indicative of reduced c-di-GMP levels (Fig. 2A, grey bars). Hence, altering the c-di-GMP levels in *P. putida* has the same effect on its motility as it has been found for other species (Simm *et al*., 2004). These results suggest that reduced motility associated with lack of PP2258 is likely a consequence of elevated c-di-GMP, which in turn implies that under these conditions, PP2258 predominantly functions as PDE.

Overexpression of PP2258 results in c-di-GMP production

We next examined how the cellular levels of c-di-GMP were affected by altered expression of full-length PP2258. To this end, PP2258 was expressed from the $lacI^Q/P_{tac}$ promoter on a 16–20 copy number vector in a PP2258 null derivative of *P. putida* KT2701 (*ΔPP2258*::Km; Sarand *et al*., 2008). Because the $lacI^Q/P_{tac}$ promoter is leaky, bacteria containing the $lacI^Q/P_{tac}$-*PP2258* expression plasmid always produce some PP2258 protein even in the absence of the inducer, while the vector control counterpart always reflects the PP2258 null phenotype.

The phenotypic effects of increasing expression of PP2258 on motility (Fig. 2B, insert) were analysed alongside the resulting cellular levels of c-di-GMP (Fig. 2B, graph) as determined by two dimensional thin layer chromatography (2D-TLC, Fig. 2C). Consistent with previous findings (Sarand *et al*., 2008), the PP2258 null strain carrying the vector control plasmid showed impaired motility as compared with wild-type *P. putida* KT2701, while the presence of the $lacI^Q/P_{tac}$-PP2258 expression plasmid in the absence of IPTG induction restored motility to the wild-type level. However, IPTG-induced expression of

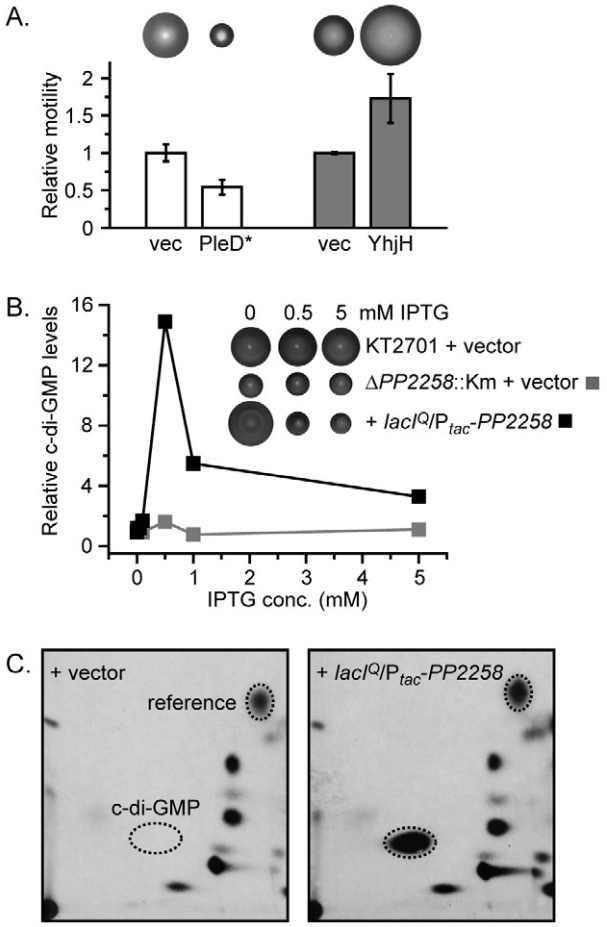

Fig. 2. Altered c-di-GMP levels and motility of *P. putida.* Motility of strains harbouring expression plasmids (Table S1) was assessed after overnight growth at 30°C on soft agar plates containing 0.3% (w/v) agar in either Luria–Bertani broth (LB, AppliChem) or minimal MOPS medium (Bochner and Ames, 1982) supplemented with 0.4% glucose and micronutrients as previously described (Sarand *et al.*, 2008).
A. Motility of *P. putida* KT2701 harbouring *bona fide* c-di-GMP turnover enzymes on soft agar LB plates. White bars: KT2701 harbouring either a P_{tac} driven expression plasmid for the PDE PleD* (pRP65) or its cognate vector control (vec; pBBR1MCS-3); Grey bars: KT2701 harbouring either an IPTG inducible *lacI*^{Q}/P_{tac} expression plasmid for the DGC YhjH (pVI2197), or its corresponding vector (vec; pVI520). For strains harbouring pVI2197 or pVI520, 0.5 mM IPTG was included to induce expression. Sizes of swim-rings used to determine relative motility were normalized against the vector control strains. Inserts above the graph are representatives of each strain.
B. Graphed values of the relative cellular levels of c-di-GMP (determined as in C) in *P. putida* KT2701-*ΔPP2258*::Km expressing PP2258 from the IPTG inducible *lacI*^{Q}/P_{tac} promoter of pVI817 (black squares) or harbouring a corresponding vector control (pVI520; grey squares). Levels of c-di-GMP are shown with those of the pVI817 containing strain in the absence of IPTG set as 1. Bacteria were cultured at 30°C in MOPS minimal glucose media containing 0.5 mM phosphate and ^{32}Pi, supplemented with the indicated concentrations of IPTG. The insert shows the motility of the two strains on 0.3% soft agar MOPS minimal glucose medium with 0, 0.5 or 5 mM IPTG induction as compared with that of KT2701 harbouring the vector control pVI520.
C. Example images of 2D-TLC plates for quantification of c-di-GMP in extracts from the two strains in B with 0.5 mM IPTG induction. Nucleotides were extracted and separated using NH_4HCO_3 (0.2 M, pH 7.8) for the first dimension and KH_2PO_4 (1.5 M, pH 3.65) for the second dimension. Dotted circles indicate the locations of c-di-GMP (R_f 0.2 $\pm$ 0.02 for the first dimension, 0.34 $\pm$ 0.03 for the second dimension) and the reference spot to which c-di-GMP levels were normalized (Tischler and Camilli, 2004; Kim and McCarter, 2007; Ferreira *et al.*, 2008).

PP2258 did not further enhance motility, rather the bacteria became less motile (Fig. 2B, insert). Hence both lack of PP2258 or its overexpression result in defective motility indicative of elevated c-di-GMP levels.

Quantitatively, c-di-GMP levels increased as induction of PP2258 expression increased, but only up to a certain point. A peak was reached with 0.5 mM IPTG induction (Fig. 2B, graph). Further induction of PP2258 resulted in lower cellular c-di-GMP levels. Even though the levels of c-di-GMP are lower with 5 mM than with 0.5 mM IPTG induction, the motility phenotypes were similar. This phenocopy is probably because these artificially manipulated c-di-GMP levels are still far above the maximum threshold level that the bacteria can sense. Taken together, the results in Fig. 2A and B suggest that PP2258 functions predominantly as PDE when expressed at low levels (uninduced), while moderated elevated expression levels (0.5 mM IPTG induction) causes a shift in activity to a predominant DGC activity. Further elevation of PP2258 levels above those induced by 0.5 mM IPTG appears detrimental, and could potentially be attributable to intracellular precipitation of PP2258 and/or triggering of malfunctioning of other c-di-GMP turnover enzymes.

The active and inhibitory sites of the PP2258 GGDEF domain are functional

Even though the PP2258 GGDEF domain lacks the complete consensus sequence in its active site (A-site), the data above suggests that it has retained DGC activity and potentially has a functional I-site as predicted by structural modelling. To test these possibilities, we generated independent alanine substitution mutants of the A-site (SGDEF to SAAAF) and the I-site (RGGD to AGGA) within the context of the full-length protein. As for the wild-type, the mutant derivatives were placed under control of the *lacI*^{Q}/P_{tac} promoter on a 16–20 copy number vector and the intracellular c-di-GMP levels produced in the PP2258 null strain upon induction with 0.5 mM IPTG were then determined.

The A-site mutant produced less c-di-GMP than the wild-type protein, but its activity was not fully abolished (compare 1 and 2, Fig. 3A). Consistent with impaired

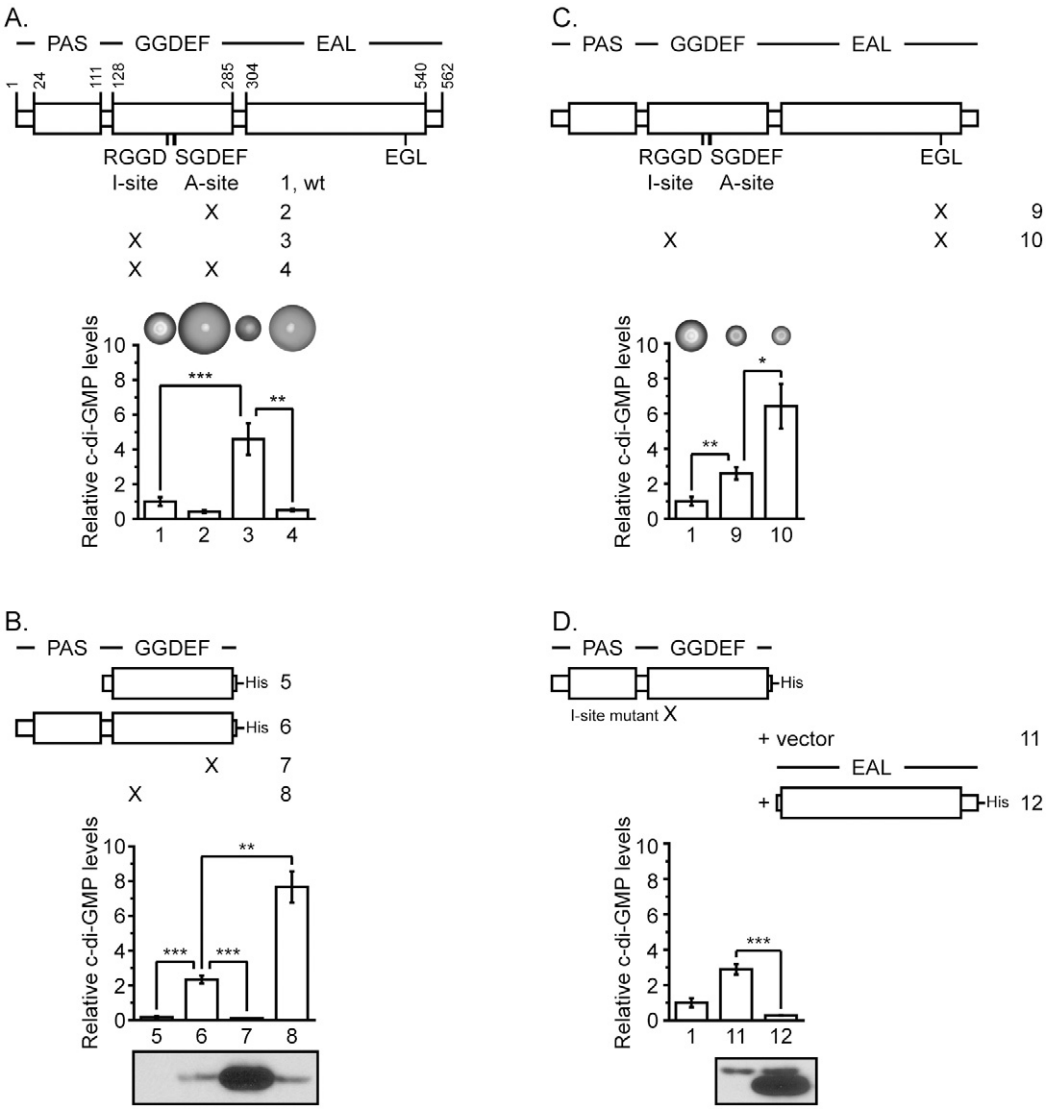

Fig. 3. Catalytic activities of PP2258.

A. The upper schematic illustrates the three domains of PP2258 with X indicating alanine substitutions of the A-site (to SAAAF) and/or the I-site (to AGGA). 1, pVI817 – PP2258 wild-type (wt); 2, pVI1040 – A-site mutant derivative; 3, pVI1041 – I-site mutant derivative; 4, pVI1042 – A/I-site double mutant derivative. The graph shows relative cellular levels of c-di-GMP produced by these PP2258 variants when their expression from the *lacI*^{Q}/P$_{tac}$ promoter is induced with 0.5 mM IPTG in *P. putida* KT2701*ΔPP2258*::Km. The c-di-GMP levels were normalized with that produced by wild-type PP2258 set as 1. The graphed values are averages ± SE of at least 3 different experiments. *P*-values from statistical *t*-test are shown for relevant comparisons (**$P < 0.01$, ***$P < 0.001$). Inserts above the graph show motility of the strains on 0.3% soft agar LB plates containing 0.5 mM IPTG.

B. As under (A), but with plasmid expressing His-tagged PP2258 truncated derivatives: 5, pVI1043 – the PP2258 GGDEF domain alone; 6, pVI1044 – a wild-type PAS-GGDEF truncate; 7, pVI1045 – a PAS-GGDEF truncate with the A-site substitution; 8, pVI1046 – a PAS-GGDEF truncate with the I-site substitution. The graphed values are normalized to that produced by wild-type PP2258 as under (A). The insert under the graph shows Western analysis of His-tagged PP2258 truncates present in 7.5 μg of crude extract from the same cells revealed using Tetra·His mouse monoclonal antibodies (QIAexpress, Qiagen).

C. As under A but with X indicating alanine substitutions of the I-site (to AGGA) and/or the motif of the EAL domain to AGA as indicated: 1, pVI817 – PP2258 wild-type (wt); 9, pVI1047 – EGL motif mutant; 10, pVI1048 – double I-site and EGL motif mutant. Inserts above the graph show relative motility of the strains on 0.3% soft agar LB plates containing 0.5 mM IPTG.

D. Relative cellular levels of c-di-GMP produced in KT2701 expressing 1, pVI817 – PP2258 wild-type (wt); 11, pVI1050 and pVI520 – a PAS-GGDEF derivative with the I-site substitution carried on a low copy plasmid (~ 3 copies per cell) with a vector control; 12, pVI1050 and pVI1049 – the PAS-GGDEF I-site derivative with the EAL domain expressed as an independent protein. Expression of both the PAS-GGDEF I-site derivative and the EAL domain protein are controlled by the *lacI*^{Q}/P$_{tac}$ systems of their cognate vectors and were induced with 0.5 mM IPTG. The values obtained were treated as in (A). The insert under the graph shows Western analysis of His-tagged proteins in 20 μg of crude extract from the same cells.

DGC activity and reduced cellular c-di-GMP levels by the A-site mutant, motility was enhanced as compared with expression of the wild-type protein (see insert in Fig. 3A). In contrast, the I-site mutant produced ~ 4.5-fold higher intracellular levels than the wild-type counterpart and elicited the anticipated concomitant reduction in motility (compare 1 and 3, Fig. 3A). To confirm that the I-site directly inhibited activity of the A-site, both mutations were combined in full-length PP2258. As would be predicted by I-site inhibition of A-site activity, the double mutant produced reduced c-di-GMP levels and a motility phenotype similar to those of the A-site mutant rather than the greatly elevated levels observed with the I-site mutant (Fig. 3A).

PP2258 DGC activity requires the amino-terminal PAS domain

To further confirm the control of the A-site DGC activity by the I-site of the PP2258 GGDEF domain, carboxy-terminal His-tagged truncated versions of PP2258 were generated and analysed as above. When expressed in isolation, the GGDEF domain was found to be unstable as no protein of the expected size could be detected in crude extracts (see 5, insert in Fig. 3B). However, when coexpressed in its native configuration with its amino-terminal PAS domain, the resulting truncate was readily detectable and produced intracellular c-di-GMP levels ~ 2-fold higher than expression of the intact protein (see 6, Fig. 3B). Based on the structural predictions, it appears likely that PAS domain-mediated dimerization is needed for protein stability and consequently for detectable *in vivo* activity of the PP2258 GGDEF domain. Therefore, we analysed the effects of the A- and I-site mutations in the context of PAS-GGDEF truncates. Despite greatly higher expression levels, the A-site mutant did not result in elevated c-di-GMP levels (compare 6 and 7 in Fig. 3B), confirming the need for the integrity of this motif for c-di-GMP production. In contrast, the I-site mutant truncate was produced at comparable levels as the wild-type truncate, but produced ~ 3-fold higher cellular levels of c-di-GMP than its wild-type counterpart. Taken together, the results in Figs 2 and 3 demonstrate that the PP2258 SGDEF A-site is functionally capable of synthesizing c-di-GMP when associated with the PAS domain, and that its activity is inhibited through its I-site.

PP2258, like the ECA3270 DGC of *Pectobacterium atrosepticum*, contains a serine at the first position of the GGDEF signature motif (SGDEF). *In silico* analysis by Perez-Mendoza and colleagues (2011) revealed that serine is the second most common residue at this position in potential DGCs of many different bacterial species. Our finding that PP2258 can function as an active DGC supports the idea that many SGDEF motif-containing proteins are likely to be able to synthesize c-di-GMP. Perez-Mendoza and colleagues (2011) also speculated that the serine within the SGDEF motif might be the target for modifications such as phosphorylation that could provide a mean of regulating the DGC activities of this type of proteins. However, to our knowledge no verified examples of this type of regulation exist to date.

PP2258 exhibits PDE activity

Like the SGDEF motif of PP2258, the EAL domain also naturally contains a non-consensus substitution of it signature motif to EGL. However, only the glutamine of the EAL motif is absolutely required for PDE activity and possession of a full consensus motif is not predictive of activity (Rao *et al*., 2008 and references therein). For example, the *P. putida* RUP4959 protein contains a consensus EAL motif but does not exhibit PDE activity (Matilla *et al*., 2011), most probably because it lacks the critical loop6 of active PDEs. To test the prediction from structural modelling of PP2258 that its carboxy-terminal EGL domain could function as a PDE, we took a similar genetic approach as described above for the GGDEF domain.

Within the context of full-length PP2258, alanine substitutions of the EAL motif (EGL to AGA) resulted in ~ 3-fold higher intracellular c-di-GMP levels upon induction with 0.5 mM IPTG than the wild-type protein (compare 1 and 2, Fig. 3C), suggesting that PDE activity of this domain indeed actively counteracts c-di-GMP production of the PP2258 GGDEF domain. To further test this idea, we combined the regulatory I-site mutation (RGGD to AGGA) – which results in ~ 4.5-fold higher intracellular c-di-GMP levels than the wild-type counterpart (Fig. 3A) – with the EGL to AGA substitution. The resulting double mutant produced ~ 6.5-fold higher c-di-GMP levels than those found for the wild-type protein (compare 1 and 3, Fig. 3C), which is indicative of an additive effect of the two mutations. As previously, the motility phenotypes of these strains correlated with the intracellular levels of c-di-GMP, with elevated levels resulting in markedly reduced motility (see inserts in Fig. 3C). These data provides support for the idea that negative regulation of c-di-GMP production through I-site inhibition of DGC activity and active degradation of c-di-GMP through PDE activity of the EGL domain of PP2258 both contribute to the net level of c-di-GMP produced upon overexpression of this protein.

The PP2258 EGL domain is active as an independent polypeptide

To test whether the PP2258 EGL domain could be physically uncoupled and still retain its apparent PDE activity, we generated a carboxy-terminal His-tagged truncated version of PP2258 that lacked both its PAS and GGDEF

domains. Consistent with a capacity to mediate an independent PDE activity, induced expression (0.5 mM IPTG) of the PP2258 EAL – like expression of the *bona fide* PDE YhjH – increases motility of the *P. putida* KT2701 PP2258 null strain (data not shown). The levels of c-di-GMP in *P. putida* and its PP2258 null derivative are extremely low. Therefore, to be able to directly assay PDE activity of the EAL domain, we generated a strain with artificially elevated c-di-GMP levels. For this we used *lacI*^{Q}/P$_{tac}$ controlled expression of the I-site mutant PAS-GGDEF-His truncate carried on a low copy number plasmid (~ 3 copies per cell) in *P. putida* KT2701. Induced expression (0.5 mM IPTG) of this derivate resulted in ~ 3-fold higher levels of c-di-GMP as compared with when intact wild-type PP2258 is expressed on a 16–20 copy number plasmid (compare 1 and 4, Fig. 3D). Coexpression of the EGL-His truncate did not affect the levels of the I-site mutant PAS-GGDEF-His truncate but did result in 10-fold lower c-di-GMP levels (compare 4 and 5, Fig. 3D). Thus, these data strongly support the notion that the PP2258 EGL domain can function independently to degrade c-di-GMP through an innate PDE activity. We were unable to use the cognate AGA mutant derivative as a control in this experiment as the mutation rendered the truncate unstable and consequently undetectable by Western analysis (data not shown).

Transcription of the aer1-PP2258 operon is not controlled by altered c-di-GMP levels

We have previously shown that the *PP2258* gene is co-transcribed with an upstream gene – *aer1* that encodes a polar localized receptor – and that transcription of this operon is mediated by σ^{70} RNA-polymerase (RNAP) (Sarand *et al*., 2008; Osterberg *et al*., 2010). Expression of some DGCs and PDEs is regulated via c-di-GMP dependent riboswitches within the 5′ UTRs (Sudarsan *et al*., 2008). Such potential regulation would be abolished in our genetic analysis of PP2258, since expression was controlled through the heterologous *lacI*^{Q}/P$_{tac}$ promoter. Therefore, we set out to determine if the regulatory region of the *aer1-PP2258* operon encoded a 5′ UTR and if so, if it constituted a c-di-GMP responsive element.

We first mapped the σ^{70}-promoter to a region between 153 and 196 bp upstream of the *aer1-PP2258* operon by using a series of plasmids bearing deleted and/or mutated regulatory regions in *in vitro* transcription assays with *P. putida* σ^{70}-RNAP (Fig. S2A and B). The location of the σ^{70}-dependent promoter was subsequently verified *in vivo* using the same series of regulatory regions controlling transcription of the *Vibrio harveyi luxAB* genes (Figs S2C and S3). This analysis revealed that the regulatory region of the *aer1-PP2258* operon indeed encompasses a 5′ UTR. And further, that DNA upstream of the identified promoter influenced promoter output, suggesting positive regulation by some unknown factor through this DNA region (Fig. S2).

Having established the presence of a 5′ UTR, we next examined the possibility that this region might respond to changes in c-di-GMP levels. To this end we monitored transcription using the reporter plasmids that differ only in respect to the presence or absence of DNA encoding the 5′ UTR. The reporter plasmids were introduced into strains with highly elevated or native c-di-GMP levels – namely KT2701 harbouring the *lacI*^{Q}/P$_{tac}$ PAS-GGDEF-His I-site mutant truncate expression plasmid that greatly elevates c-di-GMP levels (see 4 in Fig. 3D), or KT2701 harbouring a cognate vector control. However, no significant difference in transcription was detected (Figs S2C and S3). These data lead us to conclude that neither the *aer1-PP2258* operon promoter nor the 5′ UTR of the operon mediates c-di-GMP responsive alterations at the level of transcription.

Control of the enzymatic activities of dual functional c-di-GMP turnover proteins

The results described in the preceding sections show that the tri-domain motility-associated PP2258 protein displays both DGC and PDE activities. Moreover, the data support the idea that the amino-terminal PAS domain is required for stability of the central domain and consequently for cognate DGC activity that is controlled by feedback inhibition through an I-site, while the carboxy-terminal domain can function as a PDE even when expressed as an independent polypeptide (Figs 2 and 3). Based on structural modelling and identification of conserved dimerization helix within the PAS domain, we suggest that the physical continuity with the PAS domain is required to form a stable dimer that is obligatory for DGC activity.

To our knowledge, only five other proteins have been shown to possess both DGC and PDE activities. In most cases, these proteins show signal-responsive control of one of their two opposing activities. The first one discovered – BphG1 of *Rhodobacter sphaeroides* – is a bacteriophytochrome (Tarutina *et al*., 2006). Within the BphG1 protein, the EAL domain exhibits light-independent PDE activity; however, in the presence of red/far red light, BphG1 is cleaved and the DGC activity of the PAS-GAF-PHY coupled GGDEF domain becomes activated (Tarutina *et al*., 2006). ScrC, another dual enzymatic protein, regulates swarming in *Vibrio parahaemolyticus*. ScrC produces c-di-GMP in the absence of a quorum sensing signal and switches activity to become a PDE at high cell densities when the signal accumulates (Ferreira *et al*., 2008; Trimble and McCarter, 2011). *Mycobacterium*

smegmatis MSDGC-1, *M. tuberculosis* MtbDGC and *Legionella pneumophila* Lpl0329 are also dual enzymatic proteins (Kumar and Chatterji, 2008; Gupta *et al*., 2010; Levet-Paulo *et al*., 2011). *In vitro*, all three proteins show simultaneous DGC and PDE activities, however, their activities appear to be regulated *in vivo*. MSDGC-1 and MtbDGC are highly homologous GAF-GGDEF-EAL domain proteins. MSDGC-1 has been shown to be important for survival during starvation and its GAF domain is required for this activity (Kumar and Chatterji, 2008). Lpl0329 possess a response regulator domain and its DGC activity is inhibited upon phosphorylation by the histidine kinase Lpl0330 (Levet-Paulo *et al*., 2011).

The mechanism by which the opposing activities of PP2258 are controlled is as yet unknown but is likely to involve its PAS domain. Although highly speculative, we can envisage two non-mutually exclusive scenarios for control. In the first, the PAS domain may directly mediate signal-responsive dimerization to allow a switch from a constitutive monomeric PDE to dual PDE/DGC or predominant DGC enzymatic activity. A second plausible scenario invokes a similar signal responsiveness switch signalled through the Aer1 receptor that is co-transcribed with PP2258.

Concluding remarks

Both lack of and overexpression of PP2258 result in motility defects of *P. putida* (Fig. 2B), indicating a physiological role for PP2258 in controlling motility. Signalling via c-di-GMP from PP2258 could potentially affect motility through a number of different routes. However, one likely candidate route is through a homologue of the YcgR protein of *E. coli* and *Salmonella enterica* – a protein that upon binding c-di-GMP acts like a hand-break on the flagella motor (Fang and Gomelsky, 2010; Paul *et al*., 2010). YcgR was first discovered through its ability to restore (at least in part) the motility inhibition phenotype seen in an *S. enterica* strain with elevated c-di-GMP levels (Ryjenkov *et al*., 2006). *P. putida* encodes a protein called PP4397 that has 22% and 24% identity to *E. coli* and *S. enterica* YcgR respectively. Structural studies of PP4397 have demonstrated that it undergoes a dimer to monomer transition when binding two molecules of c-di-GMP per PP4397 monomer (Ko *et al*., 2010). Our current dissection of the enzymatic activities of PP2258 should greatly facilitate future work to determine if this protein is the YcgR counterpart of *P. putida* and the link between PP2258 c-di-GMP signal and altered motility.

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Supporting information

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

Fig. S1. Schematic representation of the GGDEF, EAL and HD-GYP domain proteins in *P. putida* KT2440. Data extracted from http://www.ncbi.nlm.nih.gov/Complete_Genomes/SignalCensus.html (Galperin *et al*., 2010).

Fig. S2. Transcription from the *aer1-PP2258* promoter is unaffected by elevated c-di-GMP.

A. Schematic representation of the region upstream of the *aer1-PP2258* operon with the intergenic region, but not the genes, drawn to scale. The DNA sequence of the operon promoter region is given at the top with the –35 (TTTATT, consensus TTGACA) and –10 (TAGAAT, consensus TATAAT) motifs highlighted in bold. The shuffled –10 sequence (aAagtT) present in pVI1057 and pVI1058 is indicated by an asterisk (*). The DNA regions 1–5 show the extent of the intergenic DNA present in *in vitro* transcription templates and luciferase transcriptional reporter plasmids used in B and C.

B. *In vitro* multiple round transcription assay with 25 nM σ^{70}-RNAP and supercoiled plasmid templates (10 nM) carrying different regions upstream of *aer1-PP2258* (see A). Assays were performed as described in Bernardo and colleagues (2006) using T-buffer (35 mM Tris-Ac pH 7.9, 70 mM KAc, 5 mM $MgAc_2$, 20 mM NH_4Ac, 1 mM DTT and 0.275 mg ml^{-1} BSA). Transcripts produced from the *aer1-PP2258* promoter are indicated by black arrows – upper arrow for region 1, lower arrow for regions 2–5. The upper grey arrow indicates the transcript from a control plasmid, pVI948, that carries the σ^{70}-dependent Pr promoter with 8 nucleotides (+8)

downstream of the transcriptional start, while the lower grey arrow indicates the transcripts produced from the vector located RNA1 promoter, which is present on all templates and serves as a loading control.

C. *In vivo* luciferase plate assays with *P. putida* strains harbouring transcriptional reporter plasmids with the promoterless *luxAB* genes under control of the different *aer1-PP2258* upstream regions (see A). Cells were grown overnight at 30^{O}C on LB plates containing carbenicillin and the results recorded on X-ray film after addition of decanal (100 μl of 1:200 dilution) to the lid of inverted plates. The left-hand image shows luciferase activity of KT2701 harbouring the indicated reporter plasmid or the cognate pVI928 vector control (vec.). The right hand image shows luciferase activity of the same strains that also harbour either the *lacI*^{Q}/P$_{tac}$-PAS-GGDEF I-site mutant expression plasmid pVI1059 (+) or its cognate vector control pVI898 (–). Expression from the *lacI*^{Q}/P$_{tac}$ promoter of pVI1059 (and hence elevated intracellular levels of c-di-GMP) was induced by including 0.5 mM IPTG in the medium.

Fig. S3. Transcription from the *aer1-PP2258* regulatory region is unresponsive to c-di-GMP.

A. Schematic representation of the region upstream of the *aer1-PP2258* operon within luciferase transcriptional reporters, as in Fig. S2.

B–D. The graphs show growth (open symbols) and luciferase activity profiles (closed symbols) of LB-cultured *P. putida* KT2701 (native c-di-GMP levels; B), its PP2258 null counterpart (C), or KT2701 expressing the PAS-GGDEF I-site mutant from plasmid pVI1059 (highly elevated c-di-GMP levels; D). Resident reporter plasmids present in the three strains are as indicated by the symbol code in (A). Cells were grown at 30°C to exponential phase prior to a second dilution and initiation of the experiment. Light emission from 100 μl of whole cells, after addition of 1:2000 dilution of decanal, was measured using an Infinite M200 (Tecan) luminomiter. Specific activity is expressed as relative luciferase units per OD_{600} of 1.0. Data are the average of at least two independent cultures for each strain ± standard errors.

Table S1. Plasmids used in this study.