Comprehensive overexpression analysis of cyclic-di-GMP signalling proteins in the phytopathogen Pectobacterium atrosepticum reveals diverse effects on motility and virulence phenotypes


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Received 9 January 2014
Accepted 12 April 2014

Bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) is a ubiquitous bacterial signalling molecule produced by diguanylate cyclases of the GGDEF-domain family. Elevated c-di-GMP levels or increased GGDEF protein expression is frequently associated with the onset of sessility and biofilm formation in numerous bacterial species. Conversely, phosphodiesterase-dependent diminution of c-di-GMP levels by EAL- and HD-GYP-domain proteins is often accompanied by increased motility and virulence. In this study, we individually overexpressed 23 predicted GGDEF, EAL or HD-GYP-domain proteins encoded by the phytopathogen Pectobacterium atrosepticum strain SCRI1043. MS-based detection of c-di-GMP and 5′-phosphoguanylyl-(3′-5′)-guanosine in these strains revealed that overexpression of most genes promoted modest 1–10-fold changes in cellular levels of c-di-GMP, with the exception of the GGDEF-domain proteins ECA0659 and ECA3374, which induced 1290- and 7660-fold increases, respectively. Overexpression of most EAL domain proteins increased motility, while overexpression of most GGDEF domain proteins reduced motility and increased poly-β-1,6-N-acetyl-D-glucosamine-dependent flocculation. In contrast to domain-based predictions, overexpression of the EAL protein ECA3549 or the HD-GYP protein ECA3548 increased c-di-GMP concentrations and reduced motility. Most overexpression constructs altered the levels of secreted cellulases, pectinases and proteases, confirming c-di-GMP regulation of virulence in Pe. atrosepticum. However, there was no apparent correlation between virulence-factor induction and the domain class expressed or cellular c-di-GMP levels, suggesting that regulation was in response to specific effectors within the network, rather than total c-di-GMP concentration. Finally, we demonstrated that the cellular localization patterns vary considerably for GGDEF/EAL/HD-GYP proteins, indicating it is a likely factor restricting specific interactions within the c-di-GMP network.

INTRODUCTION

Bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) is an intracellular signalling molecule that was initially identified over 25 years ago through its ability to control bacterial cellulose biosynthesis (Ross et al., 1987). Since then, c-di-GMP has been discovered throughout eubacteria and found to act as a secondary messenger controlling diverse phenotypes, such as biofilm formation, virulence, motility and pathogenesis (Hengge, 2009; Mills et al., 2011; Sondermann et al., 2012; Wolfe & Visick, 2008; Yi et al., 2010). Numerous studies have shown that increases in c-di-GMP production correlate with biofilm formation and a sessile lifestyle, while reduced c-di-GMP concentrations promote motility and virulence-factor production. However, there appears to be significant complexity in the control of both intracellular c-di-GMP availability and the response by downstream effectors.

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Abbreviations: c-di-GMP, bis-(3′-5′)-cyclic dimeric guanosine monophosphate; Cel, cellulase; DGC, diguanylate cyclase; IS, internal standard; PDE, phosphodiesterase; Pel, pectate lyase; pGpG, 5′-phosphoguanylyl-(3′-5′)-guanosine; PNAG, poly-β-1,6-N-acetyl-D-glucosamine; Prt, protease; TM, transmembrane.

Two supplementary tables and two supplementary figures are available with the online version of this paper.
It is common to find numerous, seemingly redundant, c-di-GMP synthesis and degradation proteins in bacteria. Diguanylate cyclases (DGCs) synthesize c-di-GMP from two molecules of GTP and contain a consensus [S/G][D/E]RxxD motif/catalytic active site (A-site) along with a second RxxD motif product inhibition site (I-site) that allows c-di-GMP binding to regulate the active domain (Chan et al., 2004; Christen et al., 2006; Pérez-Mendoza et al., 2011a). Studies of well-characterized DGCs, such as PdeD from Caulobacter crescentus and WspR from Pseudomonas aeruginosa, demonstrated that DGCs could be activated, via their N-terminal sensor domains, to form dimers (De et al., 2009; Paul et al., 2007). N-terminal sensor domains may be also important for the correct localization, protein–protein interactions and consequent physiological functions of DGCs (Güvener & Harwood, 2007).

Breakdown of c-di-GMP occurs via two classes of c-di-GMP-specific phosphodiesterases (PDEs) containing either an EAL catalytic site, or an HD-GYP motif. Active EAL domains are characterized by the presence of seven key residues and a consensus loop 6, found to be important for stabilization of the catalytic domain (Rao et al., 2008, 2009). These active EAL domains require Mg\(^{2+}\) or Mn\(^{2+}\) to catalyse the degradation of c-di-GMP to a linear intermediate, 5′-phosphoguananylyl-(3′-5′)-guanosine (pGpG). EAL domains perform this reaction more efficiently than the second reaction converting pGpG into two molecules of GMP (Rao et al., 2008; Ross et al., 1987; Schmidt et al., 2005). Thus, for all EAL domain proteins currently examined, pGpG is the major product of this reaction in vivo. Structural studies of EAL domain proteins, such as RocR, from Ps. aeruginosa, demonstrate that these proteins likely function as homodimers using a two-metal mode of catalysis (Barends et al., 2009; Kotaka et al., 2009; Minasov et al., 2009; Tchigvintsev et al., 2010). Additionally, work examining the Ps. aeruginosa GGDEF-EAL domain protein FimX demonstrated structurally and experimentally that the affinity for pGpG by the EAL domain was significantly less than its affinity for c-di-GMP (Robert-Paganin et al., 2012). This provides further evidence that while EAL domains can catalyse both steps in the breakdown of c-di-GMP into two molecules of GTP, this may not happen in vivo.

In contrast, PDEs that contain the less prevalent HD-GYP motif catalyse the two-step reaction, converting c-di-GMP into two molecules of GMP (Galperin et al., 2001). Recent work on the HD-GYP containing protein PmGH from the marine bacterium Persephonella marina solved the structure of PmG bound to either c-di-GMP or the terminal reaction product GMP. Within the c-di-GMP binding site, a trinuclear Fe centre was identified and was shown to be important for the recognition and catalysis of c-di-GMP. The structural information from this study also suggested how this two-step reaction proceeds sequentially (Bellini et al., 2014). Other work on the HD-GYP protein RpfG, from Xanthomonas campestris, the best characterized protein of this type, demonstrated that it requires the HD dyad for catalysis, whereas the GYP residues may only be important for protein–protein interactions with DGCs (Andrade et al., 2006; Ryan et al., 2006, 2010).

Composite proteins containing both a GGDEF and an EAL domain have also been identified, although typically only one domain is functional. However, the degenerate domain may still provide allosteric control, as demonstrated by CC3396 from C. crescentus, where the degenerate GGDEF domain can bind GTP and stimulate the EAL domain (Christen et al., 2005). Two reports of bi-functional tandem GGDEF-EAL domain containing proteins have been described in Legionella pneumophila and Mycobacterium smegmatis. These proteins were shown to both deplete GTP and produce pGpG, demonstrating their ability to perform both DGC and PDE catalytic functions (Bharati et al., 2012; Levet-Paulo et al., 2011).

In addition to the known enzymes with defined catalytic functions, various c-di-GMP effector proteins are also present within bacteria. These include proteins containing PilZ domains, or even degenerate GGDEF/EAL domains, which have been found to bind c-di-GMP and regulate related downstream processes such as motility and virulence (Amikam & Galperin, 2006; McCarthy et al., 2008; Navarro et al., 2009; Newell et al., 2009; Pratt et al., 2007; Ryjenkov et al., 2006). Besides mediating protein–protein interactions, c-di-GMP has been shown also to regulate protein translation through two classes of c-di-GMP riboswitches (Smith et al., 2011; Sudarsan et al., 2008).

The abundance of c-di-GMP metabolism proteins found in many strains suggests that titration of c-di-GMP levels could act as a physiological nexus to integrate numerous environmental signals to control common sets and/or subsets of genes required for particular cell fates. The complexity of the c-di-GMP network has led researchers to hypothesize that temporal and/or spatial control of c-di-GMP concentration, and specific interaction of c-di-GMP proteins with their targets, must occur. In support of this view, cellular localization has been found to be critical for facilitating the protein–protein interactions of several c-di-GMP metabolism proteins (Güvener & Harwood, 2007; Huang et al., 2003; Ryan et al., 2010). Similarly, localization of the DGC PopA from C. crescentus to the old cell pole is dependent on c-di-GMP binding. When correctly localized, PopA recruits CtrA, an important regulator of cell cycle progression, to the old cell pole, ultimately leading to its degradation (Duerig et al., 2009). A recent study of cellular c-di-GMP levels in Ps. aeruginosa has shown that heterogeneity within a single cell is promoted by the PDE Pch after cell division. This in turn leads to asymmetrical inheritance of the chemotaxis machinery (Kulasekara et al., 2013). Therefore, discreet spatial and temporal pockets of changing c-di-GMP concentrations may mask the phenotypes caused by certain DGCs or PDEs when analysed on a global scale (Somerfeldt et al., 2009; Tuckerman et al., 2009).

Pectobacterium atrosepticum is a phytopathogenic enterobacterium (taxonomically related to Escherichia coli and...
Salmonella typhimurium) that causes soft rot of potato tubers and blackleg disease in potato stems. The genomically sequenced strain SCRI1043 has been shown to cause disease via the maceration of plant tissue through the secretion of plant cell wall degrading enzymes such as pectate lyases (Pels) and cellulases (Cels) through the type II secretion system (Bell et al., 2004; Cooper & Salmond, 1993; Delepaire & Wandersman, 1991; Hinton et al., 1989; Reeves et al., 1993). In addition, Pe. atrosepticum is multi-flagellated, but contains a frameshift mutation in bcsA that encodes a protein required for cellulose biosynthesis, and so it does not typically form cellulose-dependent biofilms under standard laboratory conditions (Bell et al., 2004).

Recently, Pérez-Mendoza and colleagues demonstrated that Pe. atrosepticum could be induced to flocculate through c-di-GMP-dependent production of the exopolysaccharide adhesin poly-β-1,6-N-acetyl-d-glucosamine (PNAG) (Itoh et al., 2008; Pérez-Mendoza et al., 2011b; Tagliabue et al., 2010). PNAG (and the associated flocculation phenotype) was expressed upon induction of a heterologous, constitutively active PleD, revealing that Pe. atrosepticum has the capacity for c-di-GMP regulation of a previously cryptic phenotype (Pérez-Mendoza et al., 2011a, b).

In this study, we attempted to gain a broad overview of the c-di-GMP network in Pe. atrosepticum through bioinformatic identification and individual overexpression of all predicted c-di-GMP proteins in the wild-type background. For each overexpression construct, assessment of impacts on c-di-GMP metabolism was carried out via MS. Analysis of downstream phenotypes, such as virulence-factor production, flocculation, adhesion, motility and pathogenesis, were also measured. This revealed that the c-di-GMP metabolism proteins encoded by Pe. atrosepticum were capable of modulating a wide range of c-di-GMP concentrations from 0.34 pmol (mg wet weight of bacteria)−1 to 6936 pmol (mg wet weight of bacteria)−1. The changes in concentrations were broadly in line with the notion that increased c-di-GMP correlates with sessility. However, we were also able to define proteins that altered phenotypes in a highly specific way, without necessarily altering intracellular concentrations of c-di-GMP, suggesting that protein–protein interactions or spatial positioning of the proteins were likely to be more important than gross c-di-GMP concentration. Finally, we demonstrated the specific subcellular localization of several of these proteins to the membrane and cell poles.

METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table S1 (available in the online Supplementary Material). All assays were carried out on biological triplicates of wild-type Pe. atrosepticum, or derivatives of Pe. atrosepticum overexpressing the respective plasmids. Strains were grown at 25°C with the appropriate antibiotics and/or inducers as specified (pBAD30 derivatives supplemented with ampicillin at 100 μg ml−1 and arabinose at 0.02%; pJRGFUS derivatives supplemented with ampicillin at 100 μg ml−1 and IPTG at 1 mM). Overnight cultures (inoculants) were grown in 5 ml Luria–Bertani (LB) broth, and liquid exoenzyme cultures were subinoculated at a starting OD600 of 0.05 in 25 ml Pel minimal media [PMM: 40 mM K2HPO4, 15 mM KH2PO4, 0.1% (NH4)2SO4, 0.4 mM MgSO4, 0.5% (v/v) glycerol and 0.5% polygalacturonic acid (Sigma)] and grown for 18 h to induce production of plant cell wall degrading enzymes.

Construction of strains and plasmids. The overexpression constructs were created using the pBAD30 vector (Guzman et al., 1995) and the genes of interest were cloned as XbaI-bluunt fragments from the Pe. atrosepticum genome using the respective primers (Table S2). The pJRGFUS GFP vector was created using a pQE80-oriT vector backbone and a GFP-encoding gene amplified from pGReENTIR using primers GFP5′-LinkerHindIII and GFP_fusionvector3′ to incorporate the synthetic linker ‘EASKEAAS’, modified from Araji et al. (2001). The GFP plus linker product was then amplified using GFP_fusion_vector5′ and GFP_fusionvector3′, and cloned as an EcoRI-bluunt fragment into pQE80-oriT using EcoRI and HindIII restriction sites. GFP-tagged constructs were created using the pJRGFUS vector and the genes of interest were cloned from the Pe. atrosepticum genome using the respective primers (Table S2).

Bioinformatics analysis. Pe. atrosepticum homologues of c-di-GMP metabolism proteins were identified from example proteins PleD (accession no. AAA87378.1), RocR (accession no. AAG67334.1) and RpfG (accession no. CAP51495.1) using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the Pe. atrosepticum genome (taxonomy ID 218491). The conserved domain database (CDD) was used to assign predicted domains to the identified protein sequences (Marchler-Bauer et al., 2011). The presence of transmembrane (TM) helices were predicted by running the Pe. atrosepticum protein sequences through TMHMM2.0 (Krogh et al., 2001) and corroborating the results using TMpred (Hofmann & Stoffel, 1993) and DAS (Cserzo et al., 1997).

Intracellular nucleotide quantification. Pe. atrosepticum containing the desired plasmids was grown and induced on LB agar plates, without salt, for 48 h. The resultant lawn of cells was recovered by resuspension in 1 ml 50 mM Tris-buffered saline pH 7.5, the cells pelleted by centrifugation, 16,000 g for 5 min, and the wet weight determined. The number of cells (mg of wet weight)−1 was determined using parallel cultures of Pe. atrosepticum carrying pBAD30-based vectors grown and resuspended under the same conditions. The cell numbers were not found to vary depending on the particular plasmid and a mean measurement of 4 × 109 cells (mg wet weight)−1 was used for all further calculations. The c-di-AMP (Biolog) internal standard (IS) was added to each sample before the cells were lysed via boiling in pH 7.5 50 mM Tris-buffered saline. Nucleotides were then repeatedly extracted using ice-cold 65% ethanol (method adapted from Amikam et al., 1995). Samples were filtered (0.22 μm pore size) then lyophilized and reconstituted in a final volume of 200 μl 20 mM ammonium acetate, giving an IS concentration of 500 nM, assuming no loss. Injection volumes of 10 μl were loaded onto a high strength silica T3 column (Waters; dimensions 100 × 2.1 mm with a particle size of 1.8 μm) to separate individual nucleotides. Samples were then parsed into a Quattro Premiere XE tandem mass spectrometer (Waters) with an initial solution of 97% buffer A:3% buffer B and a final solution of 88% buffer A:12% buffer B (buffer A: 20 mM NH4HCO3 in water; buffer B: neat acetonitrile). All nucleotides were detected in positive ionization mode. Levels of c-di-AMP were detected at a parent ion of 659 m/z and a daughter ion of 136 m/z, corresponding to adenosine. Levels of c-di-GMP were detected at a parent ion of 691 m/z and a daughter ion of 152 m/z, corresponding to guanosine. Finally, pGpG levels were detected at a parent ion of 709 m/z and a daughter ion of 152 m/z corresponding to guanosine.
The mass spectrometer parameters were as follows: source temperature 150 °C; desolvation temperature 350 °C; multiplier 650 V. MS/MS was performed using nitrogen as the collision gas. The following collision energies were applied to all samples: capillary voltage 3.5 kV, collision voltage 25 V. Measurements were then normalized with respect to the c-di-AMP IS and the initial wet weight of cells to give a final pmol mg⁻¹ level of c-di-GMP.

**Attachment assay.** The protocol used was adapted from Christensen et al. (1985). Briefly, *Pe. atrosepticum* containing the desired plasmids were subinoculated to a starting OD₆₀₀ of 0.2 into LB broth and grown, statically, in microtiter plates for 4 days. Attachment was then visualized using 0.1 % crystal violet and detected at 595 nm. Parallel cultures were grown to determine the OD₆₀₀ of the static samples.

**Plate phenotypic assays.** *Pe. atrosepticum* cells containing the desired plasmids were grown overnight then diluted to an OD₆₀₀ of 0.05 and spotted onto the respective assay plates. Halo diameter measurements were calculated using the Fiji Is Just ImageJ software. All values were expressed as a fold-change (log₂) relative to the appropriate vector control.

Motility plates (0.5 % NaCl, 0.3 % agar, 1 % tryptone) were incubated at 25 °C for 18 h before halo sizes were measured, and Prt plates (1.3 % nutrient broth, 3 % gelatin, 1.6 % agar) were incubated at 25 °C for 48 h before visualization using 4 M ammonium sulphate.

**Liquid phenotypic assays.** *Pe. atrosepticum* cells containing the desired plasmids were grown overnight then diluted to a starting OD₆₀₀ of 0.05 in PMM. Cultures were sampled to determine their final OD₆₀₀, and stationary phase samples were taken, pelleted and the supernatants used for the exoenzyme assays. Pel and Cel activities were measured as detailed in Coulthurst et al. (2006). All values were expressed as a fold-change (log₂) relative to the appropriate vector control.

**Potato tuber experiments.** *Pe. atrosepticum* cells containing the desired plasmids were grown overnight and normalized so that 100 cells were inoculated per strain. Maris Piper potato tubers were surface sterilized (1 % Virkon, 5 min) and washed before inoculation. Following inoculation of 100 cells, inoculum sites were sealed and the tubers wrapped in repeating layers of moistened paper towel and cling film before incubation at 25 °C for 3 days. Following incubation, the rot at each inoculation site was measured. All values were normalized against wild-type *Pe. atrosepticum* carrying a vector control and then converted to log₂. Where images were taken, the area of rot at inoculation sites was stained using iodine to obtain better contrast.

**Fluorescence microscopy.** Samples were grown as overnight cultures, without induction, before subculturing 200 μl in 5 ml fresh LB broth containing ampicillin and IPTG, for 5 h. Induced samples were spotted onto poly-1-lysine coated glass slides (VWR International), pre-prepared with a 1 % agarose pad, before visualization. An Olympus BX51 microscope was used with a ×100 oil immersion lens and a U-MNB2 GFP filter (Olympus; 470–490 nm excitation, 520 nm emission and 500 nm dichromatic filters). Images were captured using a QI CAM FAST 1394 camera controlled using QCapture Pro software (QImaging). QCapture Pro was also used to analyse images.

## RESULTS

**Identification of putative c-di-GMP metabolism proteins**

BLASTP was used, along with information from the representative proteins PleD, RocR and RpfG, to identify all proteins in the *Pe. atrosepticum* genome sequence (accession no. BX950851) that contain the GGDEF, EAL and HD-GYP domains, respectively (Aldridge et al., 2003; Rao et al., 2008; Ryan et al., 2006). These searches identified 23 putative c-di-GMP metabolism proteins encoded by the genome of *Pe. atrosepticum*, which were then annotated for predicted TM regions and conserved domains, shown in Fig. 1.

Of the 23 proteins identified, 12 were predicted to contain a catalytically conserved GG(D/E)EF domain, although two GGDEF domain proteins (ECA1086 and ECA3199) lacked the consensus I-site motif, RxxD. In addition, only one DGC (ECA1086) appeared to contain a lone GGDEF domain, lacking TM helices, sensor domains or a consensus I-site. Of the predicted catalytically active DGCs, ECA2433 also contained a conserved EAL domain. However, the remaining three predicted dual GGDEF-EAL domain-containing proteins lacked the catalytic residues required for c-di-GMP synthesis. Furthermore, both GGDEF and EAL domains appeared to be degenerate in ECA0266 and ECA3264 – homologues of PigX from *Serratia* sp. ATCC 39006 (CsrD in *E. coli*) and LapD from *Pseudomonas fluorescens*, respectively. Both ECA0266 and ECA3264 also lack the canonical active (A) site and inhibitory (I) site found in GGDEF domain-containing proteins. However, despite their lack of catalytic c-di-GMP metabolism domains, PigX and LapD have well characterized roles in pathogenicity and virulence (Fineran et al., 2007; Hinsa & O’Toole, 2006).

Five proteins were predicted to contain functional EAL domains, including the two dual GGDEF-EAL domain proteins ECA2433 and ECA2008. In contrast to the GGDEF domain proteins, none of the predicted EAL-only proteins were found coupled to sensor domains and only half were predicted to contain TM helices.

Eleven of the putative DGCs were predicted to contain N-terminal TM helices and/or sensor domains. The dual GGDEF-EAL domain proteins and half of the predicted DGCs also contained possible sensor domains. These included PAS, GAF and globin domains, which have been predicted to sense oxygen, the redox state of the cell and, potentially, other small ligands (Aravind & Ponting, 1997; Hou et al., 2001).

Finally, a single HD-GYP-domain protein was identified that carried a non-canonical HT-GYP motif and a predicted N-terminal PilZ domain. The only two structures for this family of proteins have carried either an HD-GYP or HD-G.P domain, as such it is unclear what the effect of a non-canonical HT-GYP domain, as such it is unclear what the effect of a non-canonical HT-GYP domain, respectively (Bellini et al., 2014; Lovering et al., 2011). PilZ domains have been demonstrated to bind c-di-GMP and act as receptor domains on several proteins (Pratt et al., 2007; Ryjenkov et al., 2006; Zorraquino et al., 2013; Guzzo et al., 2013). Interestingly, ECA3548 was found to be divergently transcribed from the gene encoding an EAL domain protein, ECA3549.
**MS quantification of the intracellular c-di-GMP and pGpG concentrations**

We sought to elucidate the role of each gene identified in the previous section in c-di-GMP metabolism. Previous investigations have demonstrated that the expression levels of GGDEF/EAL/HD-GYP-encoding genes are often low or cryptic and that alterations in cellular levels of c-di-GMP in mutant strains can be hard to detect (Solano et al., 2009). Therefore, we employed an overexpression approach to help identify potential phenotypes and enzymic activities controlled by the GGDEF, EAL and HD-GYP domain-encoding genes in the previous section. Each gene was cloned into pBAD30 under the control of an arabinose-inducible promoter and introduced into wild-type *P. atrosepticum*. The cells were grown on solid media under inducing conditions, harvested and subjected to nucleotide extraction by ethanol.

The concentrations of c-di-GMP, and the breakdown product pGpG, produced from each engineered construct are shown in Table 1. The basal level of c-di-GMP in *P. atrosepticum* was found to be approximately 1 pmol (mg wet weight of bacteria)$^{-1}$, which equates to approximately 1300 molecules per cell, assuming an estimated $4 \times 10^8$ cells (mg of wet weight)$^{-1}$. This is higher than the level of c-di-GMP predicted in other bacteria such as *S. typhimurium* and *E. coli* (100–200 molecules), suggesting that *P. atrosepticum* may require or respond to a higher concentration of c-di-GMP for cellular processes than either *S. typhimurium* or *E. coli* under these lab conditions (Simm et al., 2009).

Induction of the majority of predicted DGCs (8 out of 12) led to a significant increase in cellular c-di-GMP levels, suggesting these proteins likely contributed enzymically to the production of c-di-GMP. Strikingly, ECA3374 and ECA0659 increased c-di-GMP concentrations into the millimolar range, to approximately $1 \times 10^7$ molecules per cell. This correlated with a substantial increase in levels of the pGpG breakdown product, indicating that mechanisms...
must be present in the cell to maintain steady-state levels of the two molecules. However, three predicted DGCs (ECA1086, ECA1453 and ECA1681) did not significantly affect c-di-GMP levels. Instead, induction of (ECA1086, ECA1453 and ECA1681) did not significantly alter c-di-GMP concentrations. Similarly, c-di-GMP levels were not significantly altered after over-expression of four of the six predicted PDEs. However, the predicted bi-functional ECA2433 did not show a significant increase in c-di-GMP levels. Surprisingly, induction of ECA3548 and ECA3549 caused a detectable increase in c-di-GMP levels. This is despite neither protein having predicted DGC activity, with ECA3549 predicted to contain a functional EAL domain and ECA3548 predicted to contain an HT-GYP domain.

Effects of the predicted c-di-GMP metabolism proteins on the motility and attachment of Pe. atrosepticum

Pe. atrosepticum does not typically form cell aggregates or biofilms under standard laboratory conditions, although recent studies found that the bacterium has a cryptic capacity for cellular attachment (flocculation), mediated by PNAG exopolysaccharide adhesion (Pérez-Mendoza et al., 2011a, b). It was observed that overexpression of certain native Pe. atrosepticum DGCs could induce flocculation (Fig. 2). In particular, induction of ECA3374 caused the greatest increase in attachment, correlating with the significant increase in c-di-GMP levels. However, this was not mirrored in the degree of motility repression observed. Instead, induction of ECA3851 caused a loss of motility, despite having only a small effect on c-di-GMP levels. In addition, induction of ECA3199 showed a significant increase in both detected c-di-GMP levels and motility. Furthermore, although induction of ECA0659 causes a substantial increase in cellular c-di-GMP levels, no significant increase in attachment was observed.

Induction of flocculation-causing genes, ECA3374 and ECA3886, in a PNAG-negative background did not promote flocculation, although similar increases in c-di-GMP levels were detected (Fig. 3a, c). Moreover, motility and virulence-factor production were repressed (Fig. 3b), demonstrating that the decreased virulence and motility seen in Fig. 2 were not artefacts caused by cell adhesion.

Overexpression of predicted PDEs, ECA3548 and ECA3549, which led to increased c-di-GMP levels, decreased motility.

<table>
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<tr>
<th>Gene</th>
<th>Domains</th>
<th>c-di-GMP Level (pmol mg⁻¹)</th>
<th>SE</th>
<th>x^t</th>
<th>pGpG Level (pmol mg⁻¹)</th>
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<td>pBAD</td>
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<td>48.47*</td>
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<tr>
<td>ECA3886</td>
<td>GGDEF (A + I +)</td>
<td>8.44*</td>
<td>± 1.02</td>
<td>9.3</td>
<td>9.01</td>
<td>± 0.764</td>
<td>1.4</td>
</tr>
<tr>
<td>ECA4026</td>
<td>FSDS (A-I-)-EMM</td>
<td>1.00</td>
<td>± 0.123</td>
<td>1.1</td>
<td>10.29</td>
<td>± 0.713</td>
<td>1.2</td>
</tr>
<tr>
<td>ECA2008</td>
<td>AVGFR (A-I-)-EAL</td>
<td>0.34‡</td>
<td>± 0.0441</td>
<td>-2.7</td>
<td>9.69</td>
<td>± 1.51</td>
<td>1.3</td>
</tr>
<tr>
<td>ECA2433</td>
<td>GGDEF (A + I +)-EAL</td>
<td>2.36</td>
<td>± 0.553</td>
<td>2.6</td>
<td>10.13</td>
<td>± 1.09</td>
<td>1.2</td>
</tr>
<tr>
<td>ECA0046</td>
<td>ETL</td>
<td>0.49</td>
<td>± 0.178</td>
<td>-1.9</td>
<td>11.57</td>
<td>± 0.24</td>
<td>1.1</td>
</tr>
<tr>
<td>ECA1081</td>
<td>EAL</td>
<td>2.07</td>
<td>± 0.436</td>
<td>2.3</td>
<td>15.23</td>
<td>± 2.47</td>
<td>1.2</td>
</tr>
<tr>
<td>ECA2091</td>
<td>EAL</td>
<td>1.01</td>
<td>± 0.227</td>
<td>1.1</td>
<td>9.29</td>
<td>± 0.783</td>
<td>1.4</td>
</tr>
<tr>
<td>ECA2497</td>
<td>ELL</td>
<td>1.40</td>
<td>± 0.277</td>
<td>1.5</td>
<td>8.47‡</td>
<td>± 0.281</td>
<td>1.5</td>
</tr>
<tr>
<td>ECA3549</td>
<td>EAL</td>
<td>7.01*</td>
<td>± 0.712</td>
<td>7.7</td>
<td>12.83</td>
<td>± 0.547</td>
<td>1.0</td>
</tr>
<tr>
<td>ECA3548</td>
<td>HT-GYP</td>
<td>3.83*</td>
<td>± 0.542</td>
<td>4.2</td>
<td>9.56</td>
<td>± 0.865</td>
<td>1.3</td>
</tr>
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</table>

The levels of c-di-GMP and pGpG are expressed in pmol mg⁻¹ indicating the quantity of nucleotides (mg of original sample pellet wet weight)⁻¹. x^t denotes the fold-change difference in levels after induction of the respective c-di-GMP metabolism genes compared to the vector control levels. The ± value denotes the SE for each construct (n=3). The significance values were calculated using a standard two-sample equal variance Student’s t-test with a two-tailed distribution; †, P<0.05; *, P<0.01; ‡, P<0.001. All values are to three significant figures.
In contrast, overexpression of other proteins containing predicted functional EAL domains (including the dual domain proteins ECA2008 and ECA2433) increased motility, as expected.

Interestingly, ECA0266 overexpression significantly increased motility, despite containing degenerate domains. In contrast, no effect on motility was observed upon induction of the homologue, PigX, in *Serratia* 39006 (Fineran et al., 2007).

Similarly, expression of ECA3264 decreased motility. This correlates with another study wherein the homologue of ECA3264, LapD, was shown to be required for biofilm formation in *Ps. fluorescens* (Newell et al., 2009).

**Effects of the predicted c-di-GMP metabolism proteins on virulence in Pe. atrosepticum**

The primary virulence determinants for plant pathogenicity were altered by induction of the predicted c-di-GMP metabolism proteins in *Pe. atrosepticum* (Fig. 4). The production of Pels, Cels and Prts was normalized against the wild-type strain expressing the vector control and represented as a log$_2$ ± se. Therefore, a value of 0 indicated no difference observed between the vector control and a strain expressing a protein of interest.

Induction of all predicted DGCs caused a decrease in Pel activity (Fig. 4a). An increase in Cel activity was observed upon induction of four proteins: ECA1086, ECA1453, ECA1681, ECA2840, ECA3148, ECA3199, ECA3270, ECA3374, ECA3625, ECA3851, ECA3886; DGCs and PDEs – ECA0266, ECA2008, ECA2433, ECA3264; PDEs – ECA0046, ECA1081, ECA2091, ECA2497, ECA3271, ECA3549, ECA3548.

The virulence phenotypes observed after induction of genes encoding the four composite GGDEF-EAL domain-containing proteins are shown in Fig. 4b. Correlating with the decrease in c-di-GMP levels and the increase in motility, induction of *ECA2008* led to an overall increase in virulence-factor production. However, overexpression of the putative bi-functional ECA2433 caused an increase in Prt activity, but decreased Pel and Cel activities. The largest difference in expression of virulence determinants was observed upon overexpression of ECA0266. Production of Pel and Cel were reduced over fourfold in this strain.

Changes in virulence-factor production caused by induction of the seven genes encoding predicted PDEs were observed (Fig. 4c). Unlike the predicted DGCs, the phenotypic
profiles observed after overexpression of the predicted PDEs were more variable, and did not indicate a strict relationship between putative PDE function and virulence. Overexpressing five of the seven PDE constructs caused an increase in motility, as expected. Furthermore, expression of six out of seven constructs led to decreased Pel activity, and only induction of three PDE genes caused an increase in Cel activities. Only overexpression of ECA3549 led to significant decreases for all three virulence determinants. Induction of ECA2091 was the only predicted PDE gene to manifest the expected increased motility and virulence phenotypes. Although no significant change in c-di-GMP levels were detected in this strain (Table 1).

To investigate any changes in virulence determinants further, potato tuber assays were carried out using strains expressing ECA2008 or ECA3851. The tuber rot levels were increased threefold for strains overexpressing ECA2008 and decreased when overexpressing ECA3851 (Fig. S1). These results were consistent with our earlier observations (Figs 2 and 4).

Localization of c-di-GMP metabolism proteins in *Pe. atrosepticum*

Studies have shown that some c-di-GMP metabolism proteins require a specific subcellular location in order to regulate cell behaviour (Güvener & Harwood, 2007; Ryan et al., 2010). To investigate this, a subset of predicted *Pe. atrosepticum* c-di-GMP metabolism proteins was tagged with a C-terminal GFP moiety and expressed to visualize their distribution in the wild-type cell (Fig. 5).

GFP fusions to the four proteins not predicted to contain TM helices (ECA2433, ECA3548, ECA3549 and ECA3625) were uniformly distributed throughout the cytosol (Fig. 5a). In contrast, proteins predicted to contain TM helices (ECA0266, ECA2840, ECA3851 and ECA3886) localized to the cell membrane (Fig. 5b). Furthermore, ECA0266-GFP localized to the cell poles and in a speckled pattern around the cell. ECA3851-GFP localized specifically at the old cell poles (Fig. 5b). In addition, expression of the C-terminal GFP tag caused cells overexpressing ECA3851-GFP to induce cell flocculation (Fig. 6).

**GFP-tagged ECA3851 specifically localizes to the cell poles and induces flocculation due to an increase in c-di-GMP levels**

To determine what components of ECA3851 contribute to the flocculation and localization of the protein, the GGDEF domain was mutated to a degenerate GAAAF motif. Overexpression of the GAAAF mutant protein caused an increase in virulence phenotypes relative to the wild-type ECA3851 (Fig. S2). Similarly, overexpression of
GAAAF-GFP caused a lack of cell aggregation, demonstrating that flocculation was related to the DGC activity of ECA3851 (Fig. 6). However, the GAAAF-GFP construct still maintained polar localization, suggesting that distribution (and presumably consequent protein–protein interactions) were not determined by the same determinants as flocculation (Fig. 6). Levels of intracellular c-di-GMP concentrations were also measured and ECA3851-GFP produced approximately 15 times more c-di-GMP than the non-tagged or GAAAF mutant constructs (Fig. 6, Table 1). This change in the amount of c-di-GMP may be caused by the different plasmid system used to express the GFP-tagged protein (pQE80 for GFP-tagged constructs compared with pBAD for non-tagged proteins) or the GFP tag may have altered the stability of the protein.

**DISCUSSION**

The metabolism network controlling the levels of the ubiquitous signalling molecule, c-di-GMP, in *Pe. atrosepticum* were probed in this study. The overexpression experiments were designed specifically to let the proteins perturb natural cellular c-di-GMP concentrations, allowing these experiments to identify cryptic phenotypes that might not be observed during a mutational analysis.

Overall, we found a significant modulation of c-di-GMP levels upon induction of a few genes. Notably, induction of ECA3374 upregulated intracellular c-di-GMP 7660-fold and overexpression of ECA0659 resulted in a 1290-fold increase (Table 1). However, induction of predicted c-di-GMP metabolism proteins did not always alter levels of c-di-GMP. In contrast, virulence and/or motility of *Pe. atrosepticum* was affected by overexpression of all c-di-GMP metabolism proteins. These modulations of cellular behaviours may be due to the presence of TM helices and sensor domains within the majority of the predicted c-di-GMP metabolism proteins, suggesting some involvement of these proteins in the transduction of physiological or environmental signals.

Generally, there was a correlation between increased cellular c-di-GMP levels and a reduction in the motility and virulence of *Pe. atrosepticum*. This was especially evident for the predicted DGC ECA3374 that, when overexpressed, caused *Pe. atrosepticum* to flocculate. Flocculation is a
where more controlled expression may operate. In *Pe. atrosepticum*, flocculation can be induced by expression of PleD*, a constitutively active DGC, indicating that high intracellular levels of c-di-GMP activate this phenotype (Pérez-Mendoza et al., 2011b). Furthermore, we were surprised to note that overexpression of ECA3374 also led to a significant increase in cellular levels of pGpG, the c-di-GMP breakdown product, suggesting that expression or activity of cellular PDEs may have been induced by the large increase in c-di-GMP availability.

However, overexpression of many c-di-GMP metabolism proteins did not provide a clear correlation between c-di-GMP levels and virulence/motility outputs, and this might suggest that c-di-GMP independently regulates the different pathways. Furthermore, induction of ECA1453 caused no detectable effect on c-di-GMP levels, but showed both an increase in attachment and motility. These results suggest that although motility and biofilm formation are thought to be reciprocally regulated, they may be controlled by two or more distinct pathways in *Pe. atrosepticum*. This also reinforces the notion of a nonlinear, and non-obvious, relationship between c-di-GMP concentrations and virulence in this plant pathogen.

This study also found that detected levels of pGpG were rarely altered by the different overexpression constructs and implies that the breakdown of pGpG could be maintained by ancillary factors. Little is known about the enzymes that catalyse the conversion of pGpG to GMP, although the decrease in pGpG seen after induction of ECA1453, ECA1681 and ECA2497 may suggest a role for the products of these genes in the activation of the hydrolysis pathway. This poses a question as to what those other proteins and signals might be, and how they relate to the overall c-di-GMP metabolism network. In addition, the general lack of Cel and Prt activity increase caused by overexpression of the majority of the predicted PDEs is suggestive of a more complex regulatory network governing the phenotypes than strict changes in c-di-GMP concentrations. Instead, specific protein–protein interactions may be more important for control of particular products.

The varying motility and virulence phenotypes associated with the predicted dual functional GGDEF-EAL domain protein, ECA2433, indicate that the GGDEF and EAL domains may individually interact with, or be activated by, subsets of proteins involved in different cellular processes. The other three dual-domain-containing proteins contain predicted degenerate GGDEF-domains, suggesting that the GGDEF domains have evolved a non-catalytic function, possibly involving allosteric control of the adjacent domain(s), c-di-GMP binding or interaction with other factors (Christen et al., 2005).

Interestingly, the different virulence phenotypes observed with ECA1453, ECA0266 and ECA3624 expression (compared to their well-characterized homologues in other bacterial species: AdrA – *S. typhimurium*, PigX – *Serratia* 39006 and LapD – *Ps. fluorescens*, respectively) demonstrates
that homologous proteins cannot be assumed to have similar functions across different organisms. This serves as a salutary reminder that every organism and protein has to be investigated independently.

In the case of ECA3548 (whose closest homologue is present in the halophilic bacterium *Chromohalobacter salexigens* DSM 3043) and its divergently transcribed neighbour, ECA3549, it is possible the unexpected increases in c-di-GMP concentrations detected and phenotypes observed after induction may be caused by some interplay, whether transcriptionally or translationally, between the two. However, the localization patterns of the two proteins did not immediately suggest any potential interaction partners.

In contrast, the speckled and potentially helical patterns seen after overexpression of ECA0266-GFP could indicate interactions with the peritrichous flagellar machinery and the cell-shape determination complex, MreBCD (Sourjik & Berg, 2000; Jones et al., 2001; Kruse et al., 2005). The latter possibility is a seductive idea because ECA0266 and mreBCD are co-located in the *Pe. atrosepticum* genome.

A specific localization pattern was also observed after overexpression of ECA3851-GFP, which was seen to localize to the old cell poles (Fig. 5b). This could suggest an involvement in cell division, although it is also possible that ECA3851 may interact with chemotaxis clusters, given the drastic reduction in motility upon induction of the non-tagged construct (Smith & Hoover, 2009). Mutation of the catalytic GGDEF motif to GAAAF increased motility to wild-type levels, and abolished flocculation (Figs 6 & S2). Localization of ECA3851-GAAAF and ECA3851-GGDEF were the same. This indicated that the preferential old-cell pole localization of ECA3851 may be important for the repression of motility. In contrast, a functional GGDEF motif, and the consequent increase in c-di-GMP levels was required for induction of flocculation.

Overall, this study indicates that the complete c-di-GMP metabolism network in *Pectobacterium* does not merely follow a linear input/output relationship as suggested in many other studies. Therefore, further investigations into non-canonical behaviours are required. Mutation of non-canonical domains to their canonical domain or to alanines would provide further information about the precise activity and localization of each component of the regulatory network. Further localization studies will also be required to dissect the role of GGDEF, EAL and HD-GYP domains in both localization patterns and c-di-GMP metabolism. Additionally, genome-wide studies analysing global transcriptional changes associated with the c-di-GMP signalling network will likely reveal new phenotypes, beyond the virulence-associated phenotypes assayed in this study, affected by this global signalling system. These future studies may provide a deeper insight into the diversity of mechanisms of action and why they have evolved to perform different physiological functions in different bacteria.

**ACKNOWLEDGEMENTS**

We thank the Biotechnology and Biological Sciences Research Council for support in the GPCS lab (BBSRC Award No. RG6324). H. T. was supported by the BBSRC and the James Hutton Institute. Work in the JLG lab is supported by the Medical Research Council (MC_UP_A90_1006) and J. A. W. was funded by the EU Framework 7 grant INHERITANCE. We thank Alison Rawlinson for technical support and Miguel Matilla-Vasquez for critical review of the manuscript. We acknowledge the generous gift of pBAD30-ECA3264, pBAD30-ECA3270 and pBAD30-ECA3271 plasmids from Daniel Perez-Mendoza.

**REFERENCES**


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Edited by: D. Kelly