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Mini-Review

Cyclic di-GMP as a bacterial second messenger

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Environmental signals trigger changes in the bacterial cell surface, including changes in exopolysaccharides and proteinaceous appendages that ultimately favour bacterial persistence and proliferation. Such adaptations are regulated in diverse bacteria by proteins with GGDEF and EAL domains. These proteins are predicted to regulate cell surface adhesiveness by controlling the level of a second messenger, the cyclic dinucleotide c-di-GMP. Genetic evidence suggests that the GGDEF domain acts as a nucleotide cyclase for c-di-GMP synthesis while the EAL domain is a good candidate for the opposing activity, a phosphodiesterase for c-di-GMP degradation.

Introduction

Bacteria modify their cell surface in response to environmental cues. These changes can facilitate either dispersion to a new environment or adhesion to a surface, including aggregation with members of their own or other species. The particular outcome is often determined by changes in exopolysaccharides and proteinaceous appendages. The bacterium *Gluconacetobacter xylinus* (formerly *Acetobacter xylinum*) produces an extracellular matrix of cellulose (a polymer of glucose with β-1,4 linkages) whose quantity and purity has stimulated the search for industrial applications (Ross *et al.*, 1991). Cellulose production in *G. xylinus* is regulated by the cyclic dinucleotide c-di-GMP, and proteins with GGDEF and EAL domains control the intracellular level of this signal (Tal *et al.*, 1998). Analysis of bacterial genome sequences suggests that such proteins constitute a widespread and mostly uncharacterized signalling system (Galperin *et al.*, 2001). For members of this protein family that have been characterized, regulation of bacterial cell surface adhesiveness is a unifying theme (Table 1). Given the economy of bacterial physiology, a likely basis for such regulation is signalling by c-di-GMP.

c-di-GMP is a second messenger in *G. xylinus*

Cells of *G. xylinus*, in colonies and in pellicles in static liquid culture, are encased in a matrix of cellulose (Sowden & Colvin, 1978; Ross *et al.*, 1991). Biochemical studies revealed that a cyclic dinucleotide, c-di-GMP, is an allosteric activator of the membrane-bound cellulose synthase complex (Ross *et al.*, 1987). The levels of this dinucleotide are controlled by two opposing activities (Fig. 1): a nucleotide cyclase activity for c-di-GMP synthesis and a phosphodiesterase activity for c-di-GMP degradation. A c-di-GMP binding protein provides another layer of regulation (Ross *et al.*, 1991). All three of these regulatory elements are membrane-associated and predicted to form part of the cellulose synthase complex (Ross *et al.*, 1991; Kimura *et al.*, 2001).

Genetic studies linked c-di-GMP synthesis and degradation to the activity of six proteins encoded by three *pdeA* genes and three *dgc* genes (Tal *et al.*, 1998). Each of these proteins has three amino acid domains: a GGDEF domain, an EAL domain, and a PAS domain (Table 1). Sequence analysis suggests that the GGDEF domain acts as the cyclase (Fig. 1; Galperin *et al.*, 2001; Pei & Grishin, 2001). The EAL domain, by virtue of its association with the GGDEF domain, is therefore a good candidate for the phosphodiesterase activity (Fig. 1; Galperin *et al.*, 2001). The linking of these opposing activities in a single protein would enable c-di-GMP accumulation to be finely tuned. Such tight regulation may enable these proteins to serve as molecular pacemakers to coordinate the production of individual cellulose fibres, each polymerized from multiple discrete membrane complexes along the length of the cell (Ross *et al.*, 1991). The PAS domain in the PDEA1 protein was shown to be a haem-binding oxygen sensor (Chang *et al.*, 2001). Thus, for regulation of cellulose production in the obligate aerobe *G. xylinus*, oxygen is the first messenger while c-di-GMP is the second messenger (Chang *et al.*, 2001).

Proteins with GGDEF and EAL domains implicate c-di-GMP signalling in diverse bacteria

The GGDEF domain was first identified in PleD (Table 1), a global regulatory protein controlling the transition between a free-swimming and an attached phase of the life cycle of the aquatic bacterium *Caulobacter crescentus* (Hecht & Newton, 1995; Aldridge *et al.*, 2003). Subsequently, it was noted that individual bacterial genomes encode numerous proteins with a GGDEF domain, although Gram-positive bacteria tend to have fewer than Gram-negative bacteria (Galperin *et al.*, 2001; Pei & Grishin, 2001). *Pseudomonas aeruginosa* PAO1 has 33 such proteins, *Escherichia coli* K-12 has 19, and *Vibrio cholerae* O1 biotype El Tor has 41. These proteins often have an EAL domain, a motif first

Table 1. Selected bacterial regulatory proteins with a GGDEF or EAL domain

Protein	Organism	Domain organization*	Associated phenotype/function	Reference
HmsT	*Yersinia pestis*	GGDEF	Autoaggregation	Jones *et al.* (1999)
PleD	*Caulobacter crescentus*	CheY-GGDEF	Attachment/dispersal	Aldridge *et al.* (2003)
WspR	*Pseudomonas fluorescens*	CheY-GGDEF	Wrinkled colonies	Spiers *et al.* (2003)
WspR	*Pseudomonas aeruginosa*	CheY-GGDEF	Wrinkled colonies	D'Argenio *et al.* (2002)
ActA	*Myxococcus xanthus*	CheY-GGDEF	Fruiting body formation	Gronewold & Kaiser (2001)
CelR2	*Rhizobium leguminosarum* bv. trifolii	CheY-GGDEF	Cellulose production	Ausmees *et al.* (1999)
AdrA	*Salmonella enterica* bv. Typhimurium	TM-GGDEF	Rdar colonies	Römling *et al.* (2000)
ScrC	*Vibrio parahaemolyticus*	GGDEF-EAL	Rugose colonies	Boles & McCarter (2002)
MbaA	*Vibrio cholerae*	GGDEF-EAL	Biofilm matrix	Bomchil *et al.* (2003)
DGC1, 2, 3	*Gluconacetobacter xylinus*	PAS-GGDEF-EAL	Cellulose production	Tal *et al.* (1998)
PDEA1, 2, 3	*Gluconacetobacter xylinus*	PAS-GGDEF-EAL	Cellulose production	Tal *et al.* (1998)
RocS	*Vibrio cholerae*	PAS-GGDEF-EAL	Rugose colonies	Rashid *et al.* (2003)
FimX	*Pseudomonas aeruginosa*	CheY-PAS-GGDEF-EAL	Twitching motility	Huang *et al.* (2003)
VieA	*Vibrio cholerae*	CheY-EAL-HTH	Cholera toxin production	Tischler *et al.* (2002)
PvrR	*Pseudomonas aeruginosa*	CheY-EAL	Repression of autoaggregation	Drenkard & Ausubel (2002)
BvgR	*Bordetella pertussis*	EAL	Repression of virulence	Merkel *et al.* (1998)

*Protein domains (Galperin *et al.*, 2001) include GGDEF and EAL (named for conserved amino acids); PAS (named for founding members of the protein family); helix–turn–helix (HTH); transmembrane (TM) domains (indicated where four or more are predicted); and the CheY phosphorylation receiver domain. Only one CheY domain is indicated for proteins (including PleD and VieA) predicted to contain two such domains, one of which may be non-functional (Galperin *et al.*, 2001).

delineated in BvgR (Table 1), a repressor of virulence gene expression in *Bordetella pertussis*.

Proteins with a GGDEF domain often also have well-recognized regulatory motifs, such as a CheY phosphorylation receiver domain or a PAS domain, a potential oxygen sensor (Galperin *et al.*, 2001; Pei & Grishin, 2001). These proteins therefore appear to constitute a complex and widespread regulatory system whose function, based on studies with *G. xylinus*, is to control levels of the c-di-GMP second messenger (Fig. 1). The polar localization discovered for one such protein, FimX (Table 1), may reflect a general theme: localized amplification or quenching of the c-di-GMP signal by individual regulators.

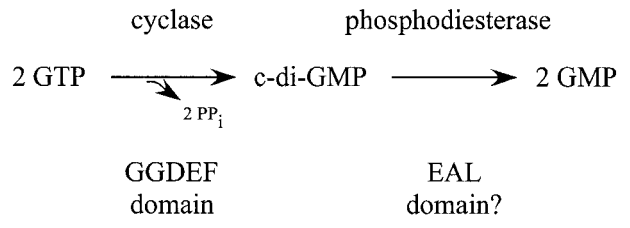

Fig. 1. Synthesis and degradation of cyclic diguanosine monophosphate, based on studies with *G. xylinus* (Ross *et al.*, 1987). Synthesis of c-di-GMP from two molecules of GTP is predicted to occur in two steps (with pppGpG as intermediate, and each step releasing pyrophosphate that is ultimately hydrolysed to inorganic phosphate). Degradation of c-di-GMP to two molecules of 5′ GMP is also predicted to occur in two steps, with a linear dinucleotide (pGpG) intermediate. The correlation of the GGDEF domain with cyclase activity is based on genetic evidence; the correlation of the EAL domain with phosphodiesterase activity is more tentative and is based on the co-occurrence of GGDEF and EAL domains in regulatory proteins that control c-di-GMP synthesis and degradation (Galperin *et al.*, 2001).

Although biochemical evidence for a widespread role for c-di-GMP is still lacking, genetic evidence is steadily accumulating (Table 1). Three proteins with GGDEF domains (*Rhizobium leguminosarum* CelR2, *G. xylinus* DGC1, and the arbitrarily chosen *E. coli* YhcK) conferred cellulose-dependent phenotypes when expressed in both *R. leguminosarum* and *Agrobacterium tumefaciens*, suggesting that they had activated the endogenous cellulose synthases by increasing the level of c-di-GMP (Ausmees *et al.*, 2001). Similarly, *Pseudomonas fluorescens* WspR was functional in *P. aeruginosa* and *C. crescentus*, and *C. crescentus* PleD was functional when its GGDEF domain was swapped with that of *P. fluorescens* WspR (D'Argenio *et al.*, 2002; Aldridge *et al.*, 2003). The c-di-GMP binding protein also appears to be broadly distributed in bacteria. Gene clusters encoding both a cellulose synthase enzyme and a homologue of the *G. xylinus* c-di-GMP binding protein are present not only in bacteria known to be cellulose producers but also in members of the genus *Burkholderia* and *Ralstonia* (Römling, 2002).

Furthermore, genetic data suggest that c-di-GMP metabolism is integrated into cellular physiology in consistent

ways. *In silico* analysis of bacterial genomes noted multiple instances in which a gene predicted to encode a haem-dependent sensor of gases (oxygen or nitric oxide) is found in a putative operon with a gene encoding a GGDEF domain (Iyer *et al.*, 2003). Suggesting coordinate regulation of c-di-GMP metabolism with the BvgAS two-component signalling systems, the *bvgR* gene encoding an EAL domain (Table 1) is part of the *bvgSAR* locus in *Bordetella pertussis* (Merkel *et al.*, 1998). Although the genes have been shuffled, equivalent clustering (that includes genes predicted to encode a two-component signalling system) is found in *V. cholerae* as *vieSAB* (Table 1; Tischler *et al.*, 2002) and in *P. aeruginosa* as PA3946-PA3948, this latter locus identified by a mutation with a pleiotropic phenotype (Gallagher & Manoil, 2001).

c-di-GMP as a conserved regulator of bacterial cell surface adhesiveness

Extracellular cellulose production by *G. xylinus* results in colonies with a rough surface, and aggregation of cells into a thick pellicle in static liquid culture (Sowden & Colvin, 1978; Cook & Colvin, 1980). Colony morphology and pellicle formation thus represent readily visible phenotypes associated with extracellular matrix production. Genetic analysis of these traits in diverse bacteria has consistently identified a regulatory role for proteins with a GGDEF domain (Table 1).

Spontaneous mutants of *P. fluorescens*, selected to grow in liquid culture preferentially as a pellicle, formed wrinkled colonies on agar surfaces (Rainey & Travisano, 1998). These aggregative properties required an extracellular matrix composed of an acetylated cellulose-like exopolysaccharide as well as an undetermined proteinaceous component (Spiers *et al.*, 2003). Grown on a low-osmolarity agar medium, cells of *P. aeruginosa* also formed wrinkled colonies (Friedman & Kolter, 2004). This trait, as well as robust pellicle formation, required a glucose-rich exopolysaccharide. The *cupA* locus, encoding a putative fimbrial adhesin, contributed to pellicle strength. Both of these extracellular matrix components were also detected in studies of *P. aeruginosa* biofilm formation (Vallet *et al.*, 2001; Wozniak *et al.*, 2003). The wrinkled colony morphotype in both *P. fluorescens* and *P. aeruginosa* was linked to the activity of the GGDEF-type response regulator WspR (Table 1). The opposing activities predicted for the GGDEF and EAL domains (Fig. 1) are consistent with the fact that in *P. aeruginosa*, constitutive activation of WspR caused autoaggregation (D'Argenio *et al.*, 2002) while expression of PvrR (Table 1), an EAL-type response regulator, suppressed autoaggregation (Drenkard & Ausubel, 2002).

Salmonella spp. after prolonged incubation at temperatures below 37 °C typically form colonies with a distinctive morphology variously referred to as rdar (Congo-red binding, dry and rough), convoluted, and rugose (Allen-Vercoe *et al.*, 1997; Anriany *et al.*, 2001; Zogaj *et al.*, 2001). This morphotype is found in other genera of the family *Enterobacteriacae*, but is absent in some laboratory strains, including *E. coli* K-12 (Zogaj *et al.*, 2001, 2003). Further suggesting that passaging of strains in the laboratory can have unintended consequences, an ATCC stock culture of a *Salmonella* strain was found to be a mixture of cells in which the rdar morphotype was either temperature dependent or constitutive (Römling *et al.*, 1998). In *Salmonella enterica* serovar Typhimurium and *E. coli*, colonies with the rdar morphotype were found to consist of cells linked by an extracellular matrix of cellulose together with thin, aggregative fimbriae also called curli in *E. coli* and Tafi or SEF17 in *Salmonella* spp. (Zogaj *et al.*, 2001; Solano *et al.*, 2002). The rdar morphotype in *Salmonella* spp. and *E. coli* requires the GGDEF-type regulator AdrA (Table 1).

In liquid culture with limiting nutrients, static incubation of various epidemic *V. cholerae* strains resulted in the accumulation of spontaneous mutants that formed wrinkled colonies (the rugose morphotype) distinct from the smooth colonies of the parental strains (Wai *et al.*, 1998; Yildiz & Schoolnik, 1999; Ali *et al.*, 2002). These variants had enhanced pellicle formation and overproduced an exopolysaccharide that appeared to vary between strains but in *V. cholerae* O1 El Tor consisted of a polymer mainly of glucose and galactose. Exopolysaccharides in the rugose morphotype (as well as those required for the rdar morphotype of *S. enterica* biovar Typhimurium) were associated with enhanced resistance to chlorine, demonstrating that the phenotype has implications that extend beyond growth in the laboratory (Yildiz & Schoolnik, 1999; Solano *et al.*, 2002). In *Vibrio parahaemolyticus*, overproduction of an exopolysaccharide was also associated with rugose colony morphology (Boles & McCarter, 2002). The rugose morphology of *V. cholerae* and *V. parahaemolyticus* was linked to the activity of RocS and ScrC, respectively, proteins that each have a GGDEF domain (Table 1). Increased biofilm matrix in *V. cholerae* was also correlated with loss of MbaA (Table 1) and with increased expression of gene VCA0074 encoding a protein with a GGDEF domain (Zhu & Mekalanos, 2003).

Cells of the plague bacterium *Yersinia pestis* autoaggregate during liquid culture, and when incubated on an agar surface at temperatures below 37 °C, form colonies that bind Congo red dye (Hinnebusch *et al.*, 1996; Hare & McDonough, 1999; Jones *et al.*, 1999). These traits require the GGDEF-type regulator HmsT (Table 1), and increase transmission of *Y. pestis* by the flea vector. Autoaggregating bacterial cells block the foregut of the flea, leading the starving flea to feed more frequently and thereby enhancing plague transmission. For such bacterial aggregates that contain a mixture of species, the close cell contacts can facilitate interspecies horizontal transfer of antibiotic resistance genes (Hinnebusch *et al.*, 2002).

The genetic basis of autoaggregation and wrinkled colony formation implicates c-di-GMP signalling (Table 1). As in *G. xylinus*, c-di-GMP could be acting as a second messenger that activates production of exopolysaccharides

(and possibly other components of the cell surface). Mutations affecting nucleotide or phosphate metabolism would be expected to perturb c-di-GMP metabolism (Fig. 1), thereby causing changes in bacterial cell surface adhesiveness. Indeed, inactivation of *V. cholerae cytR*, encoding a regulator of nucleoside scavenging, resulted in the formation of colonies with a rugose morphology (Haugo & Watnick, 2002). More unexpectedly, inactivation of *Salmonella pnp*, encoding polynucleotide phosphorylase involved in mRNA degradation and virulence, resulted in loss of the rdar colony morphology (Clements *et al.*, 2002). Conversely, perturbation of c-di-GMP metabolism may have global physiological consequences. Inactivation of a *Burkholderia cepacia* gene encoding a protein with a PAS/GGDEF/EAL domain organization resulted in a defect not only in biofilm formation but also in quorum sensing signal synthesis (Huber *et al.*, 2002).

c-di-GMP as a conserved element of bacterial cell-to-cell signalling

Proteins with a GGDEF domain regulate the production of an extracellular matrix that has been noted for *G. xylinus* as well as for *S. enterica* biovar Typhimurium and *V. parahaemolyticus* to mediate a stable array of cells with a strikingly ordered pattern (Sowden & Colvin, 1978; Enos-Berlage & McCarter, 2000; Zogaj *et al.*, 2001). Such alignment of cells could facilitate a variety of intercellular signals. Indeed, studies with *Myxococcus xanthus* provide genetic evidence that the intracellular c-di-GMP signal may be translated into an intercellular signal. The *M. xanthus* GGDEF-type response regulator ActA (Table 1) controls the production of C-signal, a cell surface-associated protein required for starvation-induced aggregation into a fruiting body. The bacterial extracellular matrix can further align the fates of individual cells (Rainey & Rainey, 2003). In *M. xanthus*, the Type IV pili on one cell interact with the extracellular matrix on adjacent cells (Li *et al.*, 2003). An equivalent phenomenon may occur in *P. aeruginosa* (and include a role for c-di-GMP signalling) based on the domain organization of FimX (Table 1) and its involvement in Type IV pilus-mediated twitching motility.

c-di-GMP as a conserved regulator of bacterial adhesion to plant and animal surfaces

The bacterial extracellular matrix also plays a role in interactions of bacterial cells with plant and animal cells. The cellulose-producer *G. xylinus* grows on decaying plant material, and bacterial cellulose contributes to the initial attachment to plants of *Rhizobium* spp. and *A. tumefaciens* (Williams & Cannon, 1989; Ross *et al.*, 1991; Römling, 2002). For *Salmonella* spp. that have a niche in the intestinal tract of animals, cellulose production may facilitate adhesion during host colonization or during transmission to a new host (with unfortunate consequences in the human food chain). In *Yersinia* spp., HmsT-regulated cell surface properties mediate attachment of bacterial aggregates to *Caenorhabditis elegans*, a nematode worm that eats soil bacteria (Darby *et al.*, 2002; Joshua *et al.*, 2003). Such attachment can block worm feeding, but may also serve to disseminate the bacteria. In all of these bacteria, extracellular matrix production has been linked to the activity of GGDEF-type regulators (Table 1), therefore suggesting a conserved ecological role for c-di-GMP signalling. As noted for the role of *P. fluorescens* cellulose production during rhizosphere and phyllosphere colonization, however, the importance of bacterial cell–cell interactions may be difficult to separate from the importance of bacterium–host interactions (Gal *et al.*, 2003).

Conclusion

Studies of cellulose production in the bacterium *G. xylinus* have had a great impact. They enabled the first identification of plant cellulose synthase genes, and they led to the discovery of the c-di-GMP intracellular signal (Römling, 2002). Proteins with GGDEF and EAL domains are found in diverse bacteria (Table 1), and signalling by c-di-GMP is likely to be a conserved physiological basis for their activities. In larger organisms, cGMP signalling regulates aggregative behaviours such as social feeding in times of stress (Sokolowski, 2002). Thus, the regulation of bacterial cell surface adhesiveness by the second messenger c-di-GMP may be a unifying theme with ramifications that extend beyond the microbial world.

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