Cyclic di-GMP as a bacterial second messenger

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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 PDE1 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...
Since 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. aeruginosa 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 andRalstonia (Römling, 2002).

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

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.

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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 occurrence of GGDEF and EAL domains in regulatory proteins that control c-di-GMP synthesis and degradation (Galperin et al., 2001).
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 morphology 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-Vерcoe et al., 1997; Aniriany et al., 2001; Zogaj et al., 2001). This morphotype is found in other genera of the family Enterobacteriaceae, 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 fimbiae 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
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 pilus 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.

References


