Quorum sensing and pathogenesis: role of small signalling molecules in bacterial persistence

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The pathogenesis of *Mycobacterium tuberculosis* is associated with its ability to survive inside the human host and the bacteria use a variety of mechanism to evade the host's defence. A clearer understanding of the host–pathogen interaction is needed to follow the pathogenicity and virulence. Recent advances in the study of inter and intra-cellular communication in bacteria had prompted us to study the role of quorum sensing in bacterial survival and pathogenicity. The cell–cell communication in bacteria (quorum sensing) is mediated through the exchange of small molecules called as autoinducers that allow bacteria to modulate their gene expression in response to change in cell-population density. It is a coordinated response that confers multicellularity to a bacterial population in response to stress from external environment. Quorum sensing molecules are the global regulators and regulate a wide range of physiological processes including biofilm formation, motility, cell differentiation, long-term survival and many others. Many bacterial pathogens require quorum sensing to produce the virulence factors in response to host–pathogen interaction. Here, we summarize our current understanding on small molecule signalling and their role in the bacterial persistence. New discoveries in these areas have enriched our knowledge on intracellular signalling and their role in the long-term survival of mycobacteria under nutrient starvation.

Keywords: Bacterial pathogenesis, c-di-GMP signalling, quorum sensing, second messenger, tuberculosis.

Introduction

MYCOBACTERIUM TUBERCULOSIS, the causative agent of tuberculosis (TB), was discovered by Robert Koch in 1882 and is still a leading cause of mortality worldwide. The condition is worsening with the emergence of totally drug-resistant tuberculosis (TDR-TB), an incurable form of tuberculosis, and in patients co-infected with tuberculosis and HIV (human immunodeficiency virus). Antibiotics do not create drug-resistance in bacteria, but they create a selective pressure on bacteria for resistance to occur and an antibiotic resistant bacterium emerges. The emergence of antibiotic-resistant bacteria is the result of evolution and is a continuous process. Thus, there will always be a need of new drugs and targets to fight efficiently against the rapid spreading of multi-drug resistant tuberculosis strains. Recent advances in molecular biological techniques have brought to light that quorum sensing, two-component signal transduction, cell division machinery, isoprenoid biosynthesis, and fatty acid biosynthesis are few of the most important targets for drug development.

Organisms have the ability to sense and respond to the environmental fluctuations to ensure their survival by regulating cellular metabolism, which is known as signal transduction. Signal transduction mechanism helps cells to detect and amplify the extra cellular signals, which are received by them and converted into cellular process. The

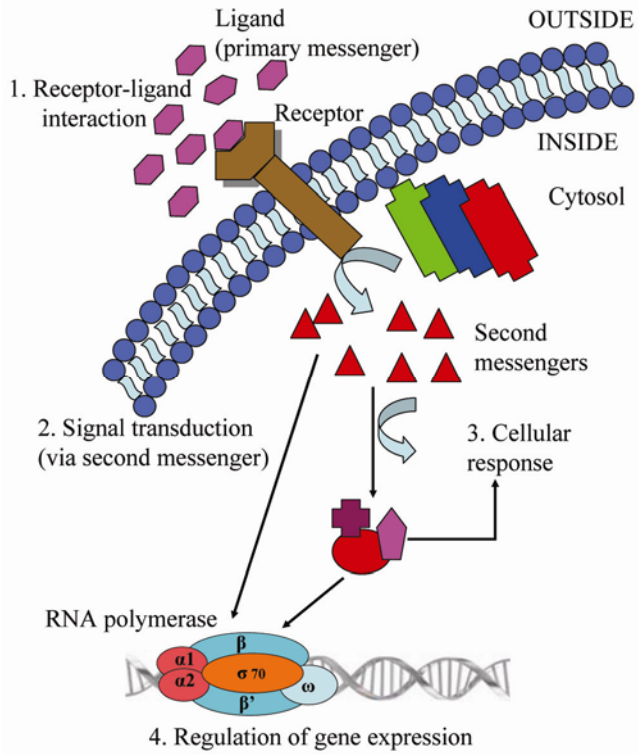

Figure 1. Schematic representation of a signal transduction pathway in bacteria.

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general mechanism of signal transduction is shown in Figure 1. The extracellular signals are unique chemical substances, which may be amino acids derivatives, small peptides, nucleotides and proteins. Change in the environmental condition will trigger the secretion of small chemicals (ligands or primary messengers) from inside to outside of the cell. On account of the binding of a ligand or primary messenger to a receptor second messengers are produced and relay the signal to the effector to carry out the change in the cellular metabolism. There are several bacterial receptors with histidine kinase effector domains in which extracellular sensor domain is involved in the catalysis of synthesis and hydrolysis of cyclic nucleotides, like adenylyl cyclase (AC), diguanylate cyclase (DGC) and phosphodiesterases (PDE)[1]. The conformational change in the receptor due to ligand–receptor interaction often stimulates the enzymes within a cell that produces multiple products or small chemicals/second messengers, such as cAMP, cGMP, ppGpp, c-di-AMP or c-di-GMP. and triggers intracellular signalling cascades. These second messengers either bind to the targets/effectors, which will regulate the cellular functions, or RNA polymerase to regulate the specific gene expression[2–6]. The signal transduction cascades control the bacterial motility, biofilm formation, secretary system, chemotaxis, expression of gene regulation and also determine the virulence of pathogenic bacteria[1]. The study of molecular mechanisms of external signal detection by bacterial transduction system and their regulatory effects on the cellular metabolism is the most important problem in the current practice of medicine. This review will focus on quorum sensing and specifically c-di-GMP signalling and their role in pathogenic relationship with different bacterial species.

Quorum sensing in bacteria

Quorum sensing is a process of cell-to-cell communication in bacteria and mediated by small molecules, known as autoinducers. Bacteria can monitor the presence of same or other bacteria in their environment by producing and responding to these autoinducers[7]. A threshold concentration of autoinducer is necessary to detect and respond to this signal by altering their gene expression in the bacteria[7,8]. Thus, bacteria living in isolation confer multicellularity by responding to autoinducers and quorum-sensing controlled behaviors and are noticeable only when they are performed in a group[9–11]. The classical example of quorum sensing is the regulation of bioluminescence in *Vibrio fischeri* and *Vibrio harveyi*[12,13]. The other quorum sensing-mediated phenomena are biofilm formation, sporulation, virulence factor expression, conjugation, pigment production, etc.[9,10].

In Gram-negative bacteria, LuxI and LuxR regulatory proteins are involved in quorum sensing. The LuxI-like proteins are responsible for the biosynthesis of specific signalling molecule, acylated homoserine lactone (HSL) known as an autoinducer (Figure 2). When the concentration of autoinducers increases to a critical threshold level, the LuxR-like protein bind cognate HSL autoinducers and LuxR-autoinducer complexes specifically activate the gene transcription. The LuxI and LuxR mediated signalling system are reported in *V. fischeri*, *Pseudomonas aeruginosa*, *Agrobacterium tumefaciens*, *Erwinia carotovora*, etc.[14–16]. On the other hand, Gram-positive bacteria use modified oligopeptides (Figure 2) as autoinducers and regulate a variety of functions in response to increase in cell-population density[17–20]. The autoinducing polypeptides (AIPs) are produced in the cytoplasm as precursor peptides and are subsequently cleaved and modified before secreting outside the cell via a dedicated ATP-binding cassette (ABC) transporter. After secretion, these AIPs interact specifically with a membrane bound two-component sensor kinase and stimulate the kinase activity of the sensor kinase protein that results in the phosphorylation of its response regulator protein. The phosphorylated response regulator protein binds DNA and alters the transcription of the target genes[21]. The virulence factor expression in *Staphylococcus aureus*[22] and *Enterococcus faecalis*[23], sporulation in *Bacillus subtilis*[24,25] and competence for DNA uptake in *Streptococcus pneumoniae*[26] are the typical examples of AIPs-mediated quorum-sensing systems.

Cyclic nucleotides signalling and their role in bacteria

In unicellular organisms, signal transduction mechanism demonstrated that the 'coded' information in the form of quorum sensing molecules (autoinducers) must be 'decoded' to sense and bring appropriate changes in their environment by expression of target genes. Thus, inter- and intra-cellular signalling must be integrated. The quorum sensing signalling system includes the diffusion of autoinducers across the membrane and that is followed by the phosphorelay cascade from the membrane bound receptors. These membrane bound receptors are integrated with the second-messenger system. Both, mono (cAMP and cGMP) and di-cyclic or modified nucleotide (ppGpp, c-di-GMP and c-di-AMP) based second messengers (Figure 3) play a key role in relaying the signals received from the receptor (on the surface) to the target molecule in the cell. These nucleotide-based second messengers regulate the different processes in various bacterial systems, but they follow a common principle of second messenger signalling, as depicted in Figure 4.

Cyclic AMP signalling

Cyclic AMP is a well-characterized second messenger in both prokaryotes and eukaryotes[27–31]. It was first

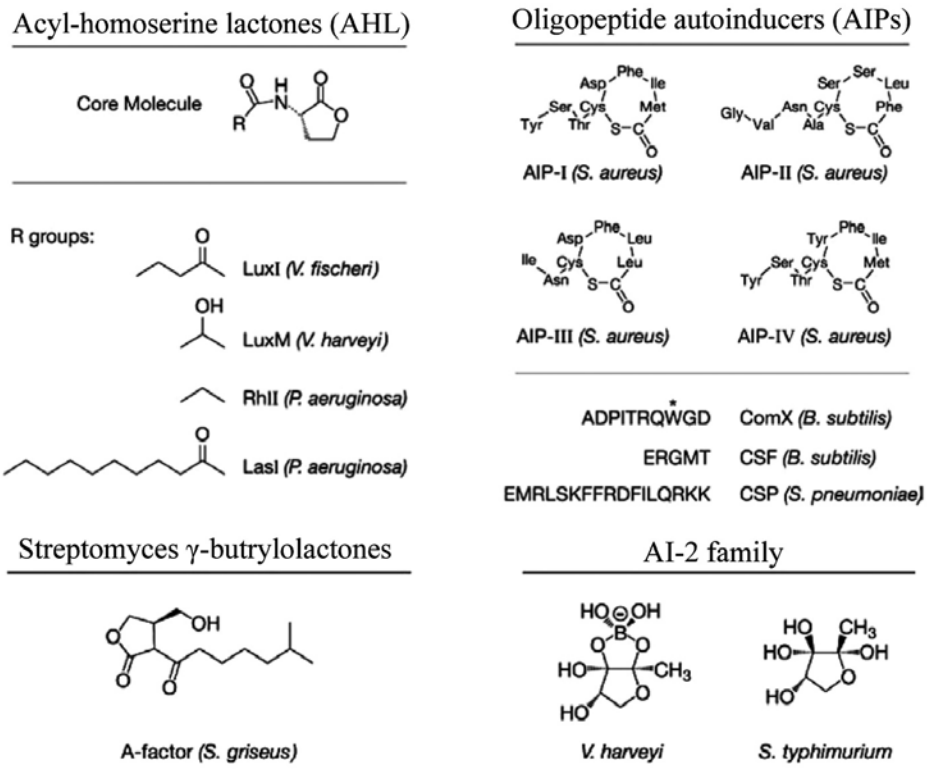

Figure 2. Quorum sensing molecules in bacteria. [This picture was adapted from Waters and Bassler, *Annu. Rev. Cell Dev. Biol.*, 2005, **21**, 319–346.]

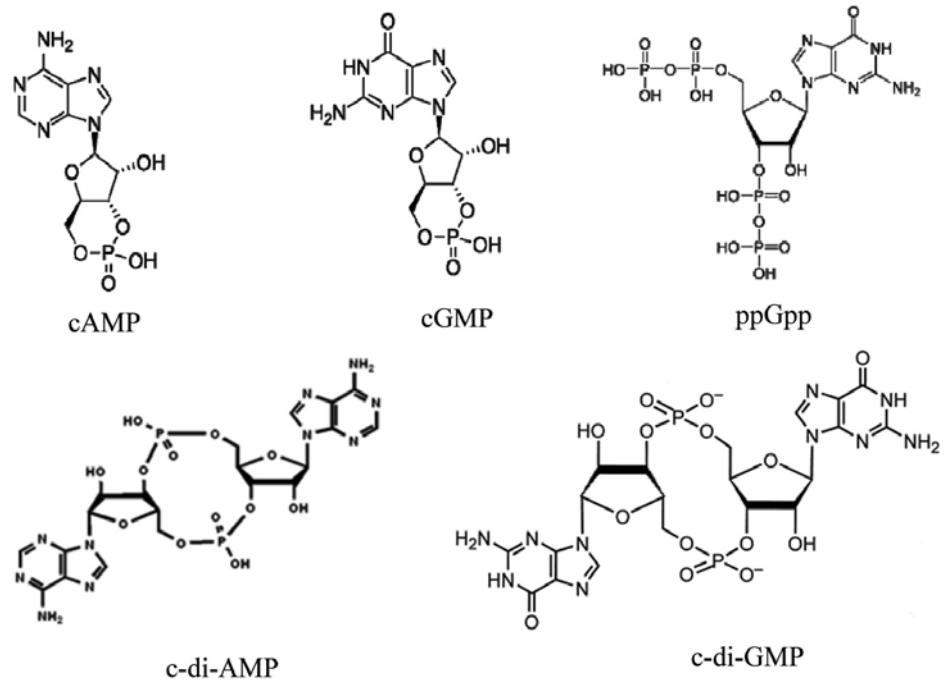

Figure 3. Intracellular mono- and di-cyclic nucleotides signalling molecules in bacteria.

discovered by Earl Sutherland in 1957 during the studies of the mechanism of hyperglycaemic action of epinephrine and glucagon[32]. Cyclic AMP synthesis can be catalysed by adenylate cyclase (encoded by the *cya* gene) from ATP during carbon starvation in bacteria and further degraded to AMP by the cAMP specific phosphodiesterase, *CpdA* (Figure 4 *a*). Cyclic AMP directly or indirectly regulates a variety of cellular functions such as

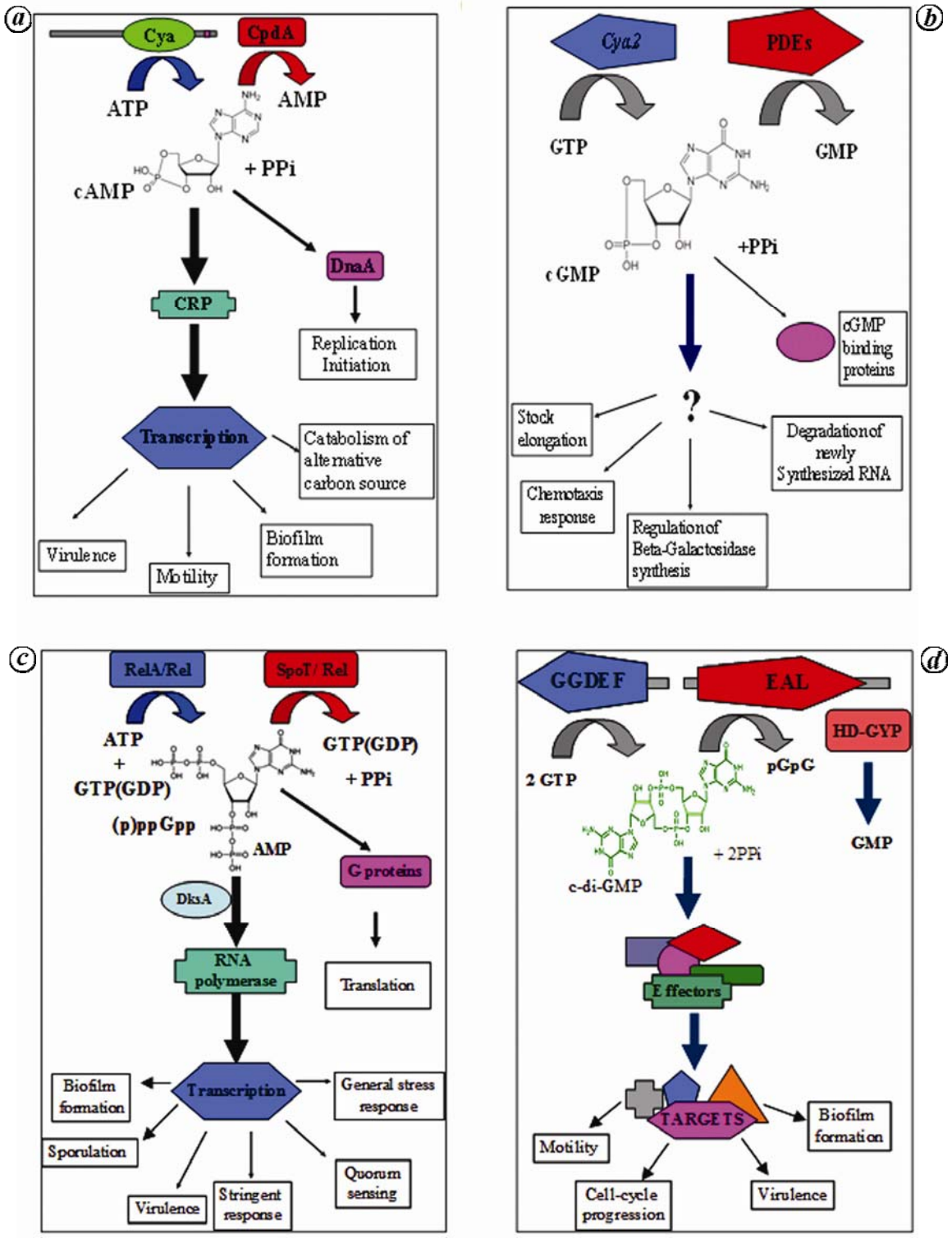

Figure 4. A schematic representation of second messenger signalling modules in bacteria. Cyclic AMP, cyclic GMP, ppGpp and c-di-GMP signalling modules have been shown in (***a***), (***b***), (***c***) and (***d***) respectively. [This picture was adapted and modified from Pesavento and Hegge, *Curr. Opin. Microbiol.*, 2009, **12**, 170–176.]

flagellum biosynthesis, motility, biofilm formation and virulence[29,33–36]. The cAMP binds to the cAMP receptor protein (CRP) and the resulting cAMP–CRP complex binds to target DNA sequences that regulates the transcription of specific genes[37]. It has also been reported that cAMP binds to DnaA, a replication initiation protein, which promotes rapid reactivation of inactive ADP-bound DnaA by the removal of bound ADP[38].

In mycobacteria, cAMP was identified during mid-1970s in both the pathogenic and non-pathogenic species. The pathogenic strain of mycobacteria generally contains multiple copies of adenylate cyclases, for example in *M. tuberculosis* there are 17 adenylate cyclases (ACs) and this feature is shared by *M. avium* (12 ACs) and *M. marinum* (31 ACs). The cellular concentration of cAMP in mycobacteria was found to be 100–200-fold higher than that of *E. coli* (grown in glycerol). Cyclic AMP was also detected in the extracellular secretion (10–20 μM) from *M. smegmatis* and other mycobacterial species[39–41]. Although the role of multiple copies of ACs in *M. tuberculosis* is not well understood especially with respect to physiology and pathology, the high level of cAMP in mycobacteria and mutational studies on Rv3676 protein suggest that cAMP controls the expression of genes required for *in vivo* survival and persistence[42]. Furthermore, cAMP has been linked to virulence of enterobacteria such as *Vibrio vulnificus* and *Yersinia enterocolitica* and is thought to facilitate secretion of virulence factors[43,44]. In Gram-positive bacteria, cAMP also appears to play diverse roles, for example, in the pH-homeostasis of *Streptomyces coelicolor* and in the virulence of *M. tuberculosis*[45,46].

Cyclic GMP signalling

Signal transduction via the second messenger cGMP has been widely studied in eukaryotes, but there are few reports on cGMP signalling in prokaryotes. The presence of cGMP in *E. coli* cells had first been described in 1974 and the level of cGMP was two-fold less than that of cAMP. The cellular levels of cAMP and cGMP depend on the growth phases and change in the external environmental condition. The *E. coli* cells growing in glucose containing media show ~10-fold decrease in cGMP, whereas cAMP level was 10-fold high during the entry into stationary phase of growth, which indicate that cGMP and cAMP signalling are playing a crucial role in the physiology of bacteria[47]. It has been reported earlier that cGMP is involved in the intra-cellular signalling during chemotactic response in *E. coli*[48]. Cyclic AMP and cGMP signalling work in a similar fashion. The cGMP synthesis is catalysed by GTP via the *Cya* gene product and degraded to GMP via cGMP specific PDEs, as shown in Figure 4 *b* (ref. 49).

The presence of cGMP, guanylate cyclase (GC) and cGMP-PDE has been reported in *M. smegmatis* in 1984 and cGMP specific PDE (Rv0805) has been well characterized[50,51]. cGMP was extracted from the *M. smegmatis* cells growing in liquid cultures and 5-fold decreased level of cGMP was observed in the logarithmic phase and it stayed low in the stationary phase of growth. Moreover, it was also shown that glucose starvation for 2 h increases the cGMP level up to 2-fold in the logarithmic phase, but again 5-fold decrease was observed in the late stationary phase cells[50]. The genome of *M. tuberculosis* contains 15 genes encoding class III AC/GC catalytic domains[52] and 10 gene products were catalytically active when expressed in *E. coli*. Eight out of 10 cyclases were specific adenylate cyclases, while two of them, Rv0386 and Rv1900c, show both ACs with 20% and 7% GC side-activity respectively[53,54]. The binding targets are not known in cGMP signalling, except in *C. crescentus*[55]. Recently, cGMP is shown to be involved in the cyst development in bacteria *Rhodospirillum centenum*[56].

The physiological significance of cGMP is difficult to estimate, as there are very few reports on cGMP in mycobacteria and in other bacteria. The data available on the cGMP signalling to date show that cGMP is the important second messenger with respect to stationary phase of growth and low level of cGMP in the cells might be the signal for the bacteria to slow down the physiological activities to survive under starvation in the stationary phase.

(p)ppGpp or ppGpp signalling

The (p)ppGpp is a well-characterized second messengers in bacteria[27,57–60]. The pppGpp and/or ppGpp are synthesized by ribosome associated proteins, Rel or Rel-A, which transfer the PPi moiety from ATP to the 3′ end of GTP or GDP respectively[57,61]. The functional significance of (p)ppGpp has been studied extensively[62–64]. It has been reported in earlier studies that under nutrient starvation, *M. smegmatis* show accumulation of the stringent factor ppGpp (refs 59 and 65), thereby indicating a link between persistence and the stringent response. The binding of ppGpp to RNA polymerase down-regulates the rRNA synthesis and up-regulates the protein degradation and amino acid biosynthesis as a consequence of stringent response, as depicted in Figure 4 *c* (refs 60, 66 and 67).

We have reported earlier that the ppGpp signalling is involved in the long-term survival of *M. smegmatis* under condition of nutritional starvation. The percentage survival of *relA*/*spoT* mutant was compared with that of wild-type *M. smegmatis* using CFU assay grown in carbon-limiting condition (0.02% glucose) and monitored up to 15 days. Figure 5 shows that within 15 days of its entry into stationary phase, the numbers of viable bacteria in the mutant strains were 10-fold lesser than those of wild-type. The deletion mutant of *rel* (Δ*relA*/*spoT* strain) in *M. tuberculosis* was also generated showing the absence of ppGpp and the knockout strains showed the diminished and sluggish growth profile when compared with that of the wild-type[59]. The stringent response utilizes (p)ppGpp as a signalling molecule to control bacterial gene expression involved in long-term survival under starvation conditions. The nutrient depletion activates the stringent pathway and helps the bacterium to minimize energy con-

sumption and survive the stressful condition[68]. Recently, another ppGpp synthase with RNase H activity from *M. smegmatis* has been reported in the literature[69].

Cyclic di-AMP signalling

Cyclic di-AMP [*bis*-(3′–5′)-cyclic dimeric adenosine monophosphate], a putative second messenger was first identified in *B. subtilis* (Gram-positive) in 2008 during the crystallization studies of the DNA integrity scanning protein, DisA. The protein, DisA catalyses the synthesis of c-di-AMP at its DAC (diadenylate cyclase) domain[70]. The DAC domain containing proteins are found in many other bacteria (predominantly in Gram-positive) and archaea. Many bacterial species harbour only one DAC domain protein and null mutant of the single copy of DAC appears to be lethal in *Listeria monocytogenes*, *S. aureus*, *S. pneumoniae*, *Mycoplasma pulmonis* and *Mycoplasma genitalium*[71]. The genome of *B. subtilis* encodes three DAC-containing proteins, DisA, YbbP and YojJ. It has been reported that the single mutants of these DAC proteins are viable, but the double mutant lacking both DisA and YbbP is non-viable[71]. GdpP (homolog of YybT) is a protein with PDE activity and degrades c-di-AMP to pApA. It has been reported that the overproduction of GdpP greatly enhances the sensitivity of *B. subtilis* cells to β-lactam antibiotics[71,72]. More recently, mutational studies in the PDE gene of *S. aureus* show the increased amount of cross-linked peptidoglycans, which play important roles in controlling bacterial cell size and envelope[73]. In another study, c-di-AMP was found to be secreted by the bacterial pathogen *L. monocytogenes* and could stimulate an IFN-β-mediated host immune response and overexpression of the diadenylate cyclase dacA (lmo2120). This resulted in elevated levels of the host response during infection.

However, the regulatory functions and mechanisms of c-di-AMP signalling in mycobacteria remain to be explored. But, a disA homolog that can synthesize c-di-AMP exists in almost all mycobacterial genomes with the exception of *M. leprae*[70]. In *M. tuberculosis* Rv3586, a DisA homolog is a functional DAC that can convert ATP or ADP to c-di-AMP[74]. Moreover, the first c-di-AMP receptor regulator, DarR (encoded by Ms5346) in *M. smegmatis* has been identified recently that specifically associates with c-di-AMP and negatively regulates the expression of its target genes[75]. Thus, c-di-AMP represents a putative bacterial secondary signalling molecule that triggers a cytosolic pathway of innate immunity and is predicted to be present in a wide variety of bacteria and archaea[76]. The essential roles of c-di-AMP are not well understood, but recent results suggest that c-di-AMP may act as either intracellular or extracellular signal to participate in regulating bacterial physiology, pathogenesis and directly or indirectly involved in peptidoglycan homeostasis[71,73].

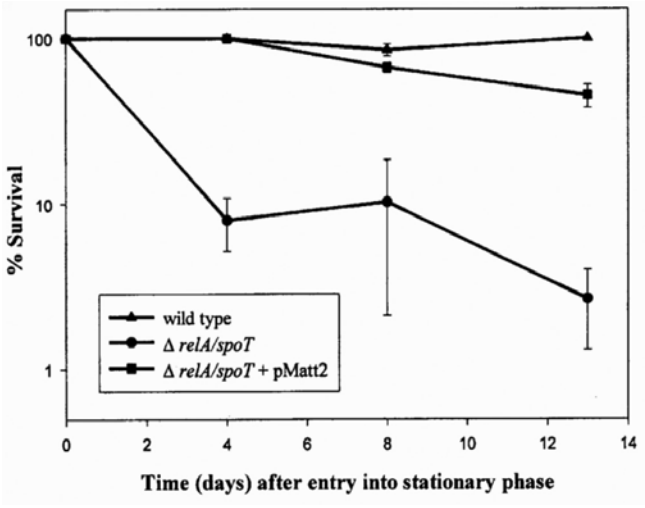

Figure 5. Role of ppGpp signalling in the long-term survival of *Mycobacterium smegmatis*. CFUs of the cultures were obtained at different time intervals after their entry into stationary phase and monitored up to 13 days. It can be noticed that the percentage survival of the Δ*relA*/*SpoT* strain was decreased up to 90% in just 4 days, when compared with that of wild type and there was further decrease in the number of viable cells if monitored for longer period of time. The complemented strain Δ*relA*/*SpoT* + pMatt2 (containing *rel* gene) shows the reversal of the phenomenon and behave like wild type. [This picture was adapted from Mathew *et al*., *Curr. Sci.*, 2004, **86**, 149–153.]

Cyclic di-GMP signalling

The novel second messenger c-di-GMP [*bis*-(3′–5′)-cyclic dimeric guanosine monophosphate] has emerged as a ubiquitous second messenger in prokaryotes. It was first discovered by Benziman and co-workers in *Gluconacetobacter xylinus* as an allosteric activator of cellulose synthase[77,78]. The synthesis of c-di-GMP is carried out by DGCs from two molecules of GTP, whereas PDEs hydrolyse c-di-GMP into pGpG or GMP, as shown in Figures 4 *d* and 6 *b* (refs 79–81). The level of c-di-GMP within a cell is maintained by a balance between DGC and PDE activities. The GGDEF domain protein is 170 amino-acid long and the domain name originates from the conserved amino acid motif GGDEF (Gly–Gly–Asp–Glu–Phe)[82], and Mg^{++} is required for the c-di-GMP synthesis. Similarly, the EAL domain protein is 240 amino-acids long and the domain name originates from the conserved amino acid motif EAL (Glu–Ala–Leu). The hydrolysis of c-di-GMP requires either Mg^{++} or Mn^{++}, and is inhibited by Ca^{++} and Zn^{++} (refs 83–87). C-di-GMP signalling regulates bacterial life-style transition, biofilm formation, motility, virulence, cell division and many other biological functions[88,89]. An increased level of c-di-GMP has been found to stimulate matrix (exopolysaccharide/cellulose) production and hence biofilm formation. It also regulates the transition between motile and sessile forms[90]. Various

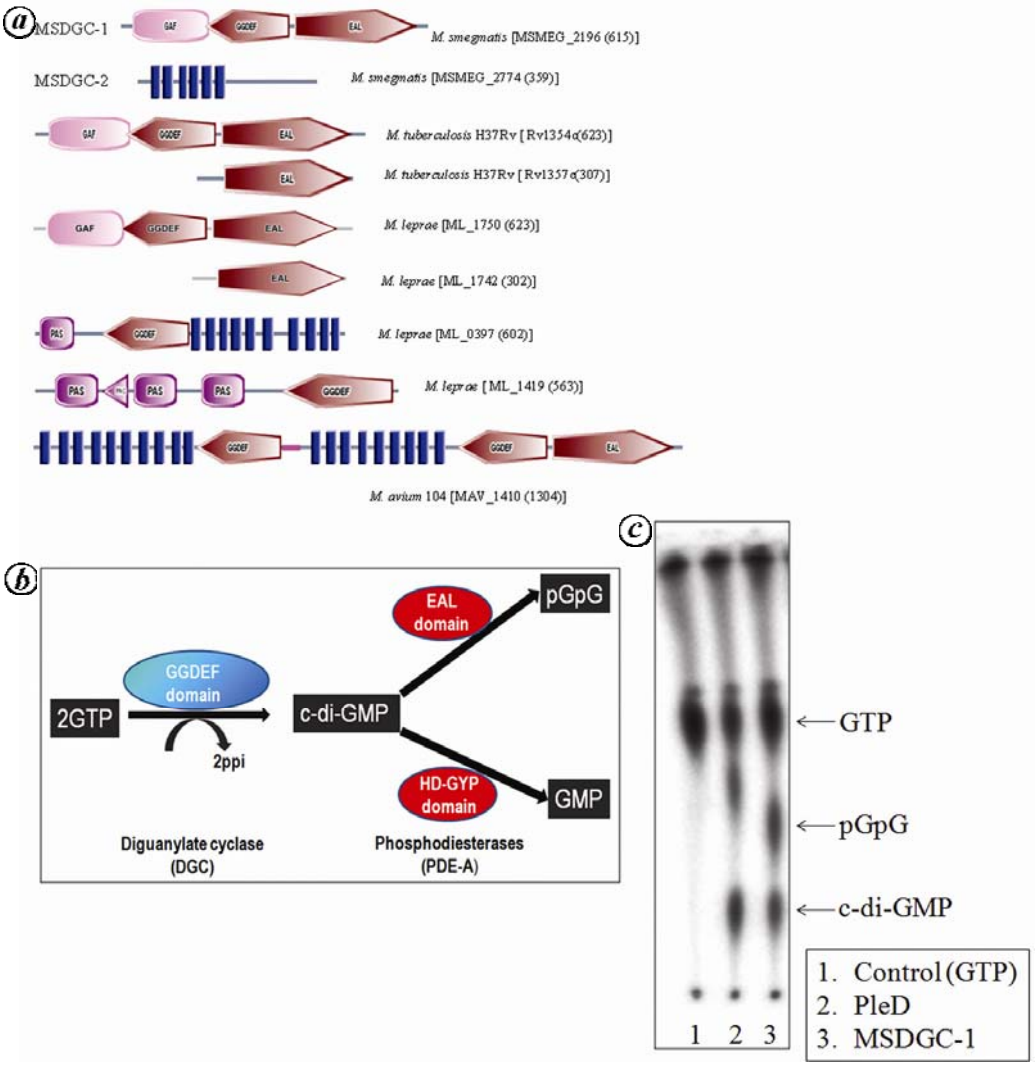

Figure 6 *a–c*. Evidence of c-di-GMP in mycobacteria. ***a***, Domain architecture of the various mycobacterial GGDEF-EAL domain containing proteins. The vertical bar shows the trans-membrane helix. The numbers in parentheses show the number of amino acids in the protein. ***b***, Cartoon representation showing the synthesis and hydrolysis of c-di-GMP. ***c***, MSDGC-1 is a bifunctional protein with DGC and PDE-A activities *in vitro*. The c-di-GMP is a condensation product of 2 GTP molecules by DGC and can be further degraded to pGpG or GMP by c-di-GMP PDE.

studies have demonstrated a clear link between the level of c-di-GMP and alterations in cell-surface properties, motility and biofilm formation in bacteria[79,91–95].

Multiplicity of GGDEF and EAL domain proteins in the prokaryotic organism: The GGDEF and EAL domain proteins are present in most sequenced genomes from the entire bacterial kingdom. The GGDEF and EAL domain proteins are well characterized, but there are few reports available that characterize another metal dependent PDE domain called as HD-GYP. It has been observed that Gram-negative bacteria, especially the Gamma proteobacteria have greater number of GGDEF/EAL domain proteins than Gram-positive bacteria. Comparative analysis of the complete genome sequences shows the presence of multiple DGCs and PDEs in a single bacterial species which generates new flexibility of signalling, but raises the questions about their specificity. The genome of *E. coli* K-12 encodes for 19 proteins with GGDEF and 17 with EAL domains, whereas *B. subtilis* encodes for 4 GGDEF and 3 EAL domains respectively. Moreover, the genome of *V. vulnificus* encodes for 66 proteins with GGDEF and 33 with EAL domains[96]. Interestingly, several GGDEF and EAL domain proteins with degenerate active site motifs have also been reported that do not possess DGC and PDE activities, but they function in the regulatory circuits that do not involve c-di-GMP[20].

GGDEF and EAL domain proteins in mycobacteria: The GGDEF and EAL domains are highly abundant in

mycobacterial species and ~99 sequences containing either or both GGDEF, EAL and HD-GYP domains were found[96]. Interestingly, most mycobacterial species appear to have at least one protein with GAF-GGDEF-EAL domain organization (Figure 6 *a*). However, *M. gilvum* PYR-GCK and *M. vanbaalenii* PYR-1 have the highest number of these proteins among mycobacteria, i.e. 25 and 16 respectively and few mycobacterial species appear to have a HD-GYP domain for c-di-GMP degradation. The genome of *M. smegmatis* encodes two genes *MSMEG_2196* (*msdgc-1*) and *MSMEG_2774* (*msdgc-2*). The MSDGC-1 protein shows the presence of GAF, GGDEF and EAL domains in a tandem arrangement, whereas the MSDGC-2 shows the presence of only GGDEF domain with degenerate motif (SDSEF) at the C-terminal and transmembrane helix at the N-terminal. Interestingly, the genome of *M. tuberculosis* H37Rv shows the presence of one GAF-GGDEF-EAL and one EAL domain proteins. The MSDGC-1 protein is a bifunctional protein and showed both c-di-GMP synthesis and hydrolysis activities (pGpG formation) *in vitro* (Figure 6 *c*). A reaction mixture containing PleD protein, which is a known DGC protein of *C. crescentus* was used as a positive control and shows only c-di-GMP synthesis activity[81,97].

Physiological implications of c-di-GMP signalling in the bacterial physiology

Cyclic di-GMP signalling and regulation of biofilm formation

A biofilm is an aggregate of bacteria and are formed to protect the bacteria from the host-defence, antibiotics and harsh environmental condition. The formation of biofilm is a quorum sensing mediated phenomenon and thought to be the primary cause for antibiotic resistance during persistent infection. Thus, the studies on biofilms will explore the possibilities for the development of novel antimicrobial approaches of great interest to the scientific, medical and agriculture communities. It has been proposed recently that modulating levels of c-di-GMP regulates biofilms formation in various bacterial species.

Cyclic di-GMP and biofilm formation in V. cholerae: *V. cholerae* is a Gram-negative bacterium and is ubiquitous in aquatic ecosystems. Many species of *Vibrio* are free living; a small group can form pathogenic or symbiotic interaction with eukaryotic host and causes the acute intestinal infection cholera. *V. cholerae* genome represents the highest number of GGDEF-EAL domain containing proteins with 31 GGDEF, 10 GGDEF-EAL, 12 EAL, 9 HD-GYP, 5 PilZ and 2 riboswitch[96]. Increased level of c-di-GMP in the cell activates the genes required for biofilm formation in *V. cholerae*[92]. It has been shown that VieA, with EAL domain regulates the intracellular concentration of c-di-GMP that represses transcription of *vps* (Vibrio exopolysaccharide synthesis) genes involved in biofilm formation[92].

Cyclic di-GMP and biofilm formation in P. aeruginosa: *P. aeruginosa*, an opportunistic pathogen is capable of coordinated group behavior that includes biofilm formation, swarming motility, which is linked to the persistence of these bacteria. These group behaviour is regulated by both AHLs quorum sensing systems and intracellular level of c-di-GMP[27,98–101]. The biofilm dispersal in *P. aeruginosa* can be triggered by various environmental factors such as availability of nutrients and iron, and now has been linked with c-di-GMP signalling[90,102–105]. In *P. aeruginosa* genome there are 17 GGDEF, 16 GGDEF-EAL, 5 EAL, 3 HD-GYP and 8 PilZ domains that regulate the level of c-di-GMP and are involved in the regulation of various function of the bacterial physiology[96]. Recently, a new factor nitric oxide (NO) has been identified as an important factor in the regulation of bacterial biofilm dispersal and exogenous addition of nanomolar concentration of NO stimulates motility and biofilm dispersal[106,107]. More recently, it has been shown in *P. fluorescens* Pf0-1 that biofilm formation is regulated by the amount of inorganic phosphate (pi) available in the environment. It has been reported that the activation of Pho regulon in phosphate limiting condition resulted in the high expression of a c-di-GMP phosphodiesterase RapA, which lowers the c-di-GMP level and inhibits the secretion of LapA, required for the biofilm formation.

Cyclic di-GMP and biofilm formation in S. aureus: *S. aureus* is an important pathogen for both human and animals and its antibiotic resistance is a public health concern. *S. aureus* plays a major role in creating surgical site infection and is the second most common cause for nosocomial bacteremia[108]. Biofilm formation is a key factor in the establishment and persistence of staphylococcal infections in humans and animals. The COG database (Clusters of Orthologous Groups of proteins) shows the presence of only one functional GGDEF (COG3887). However, another copy of gene was also noticed with modified GGDEF motif (COG2199) in the database[109]. It has been reported that the treatment of *S. aureus* with chemically synthesized c-di-GMP resulted in the inhibition of cell–cell adhesive interaction in liquid medium with concomitant decrease in biofilm formation. It has been proposed that c-di-GMP can be used either alone or in combination with other antimicrobial agents for the prevention of biofilms, and the control and treatment of infection.

Cyclic di-GMP signalling is not involved in the biofilm formation in M. smegmatis: The GGDEF-EAL domains are conserved in mycobacterial species and there is at least one copy of the GGDEF and/or EAL domain present in their genome. We have shown that the protein MSDGC-1 is responsible for *in vivo* cellular concentra-

tion of c-di-GMP and MSDGC-1 deleted mutant (DGCKO) is a c-di-GMP^0 strain that does not show the change in the biofilm formation or maturation. The sliding motility shown by DGCKO also remained unaltered in comparison to that of the wild type. It can be concluded from the mutational studies that c-di-GMP signalling is not involved in the surface regulated phenomena such as regulation of biofilm and sliding motility in mycobacteria, especially in *M. smegmatis* under enriched condition[97].

Cyclic di-GMP and regulation of virulence gene expression

Cyclic di-GMP and virulence gene expression in V. cholerae: *V. cholerae* is ubiquitous in aquatic environment and pathogenic life style begins with the transition from the aquatic life style to human host. The role of c-di-GMP signalling, with respect to pathogenesis is best understood in *V. cholerae* and it has been shown that biofilm formation and virulence gene expression are inversely related phenomena. In *V. cholerae*, the low level of c-di-GMP increases the expression of virulence genes while increased level of c-di-GMP activates the genes required for biofilm formation[93]. In human, *V. cholerae* cause disease by colonizing the small intestine and secreting cholera toxin (CT), an ADP-ribosylating toxin that elicits profuse watery diarrhoea[110]. The expression of CT and the toxin-coregulated pilus (TCP) are controlled by a complex network of transcriptional activators[111]. The *vieSAB* operon encodes for the VieSA putative two-component signal transduction system containing the *VieA* response regulator (PDE-A). The *VieB* was induced during infection of infant mice and *vieS* was identified as a positive regulator of CT[112,113]. VieA is required for the expression of *toxT*, which encodes for a transcriptional activator of toxin coregulated pili and CT[114–116]. The low-level of c-di-GMP allows the dispersion from the biofilm and maximal expression of virulence genes. Upon entry of *V. cholerae* into the human host, the intracellular level of c-di-GMP decreases by induction of the PDE or repression of the DGC activities. It was shown that an unknown extracellular signal, present in the small intestine, activates the VieS sensor. This leads to the autophosphorylation of VieS and subsequent phosphotransfer to the dual-function protein VieA. The phosphorylated and activated protein VieA activates the transcription of *vieSA*. This results in higher VieA (PDE-A) and reduced level of c-di-GMP in the cell that results in high expression of the virulence factor. The *V. cholerae* inevitably involve dissemination of bacteria back into the environment and thus the c-di-GMP level increases at the later stage of infection and turns off the virulence gene expression[117–119]. The regulation of virulence with respect to c-di-GMP has also been studied in many other bacteria including *Legionella pneumophila*, *Salmonella typhimurium*, *Brucella melitensis*, *Bordetella pertussis* and *V. vulnificus*. These studies include genetic screens or transcriptional profiling, which show that c-di-GMP represses virulence gene expression[120–123]. Thus, the pathogenicity of bacteria involves a tight regulation of c-di-GMP and a complex interaction between DGC, PDE and the regulatory targets of c-di-GMP.

Cyclic di-GMP signalling and virulence gene expression in Salmonella: *Salmonella enterica* var. typhimurium (*Stm*) is a facultative intracellular pathogen that causes typhoid fever in the human host. The mechanism of infection in mice has been studied intensively as a model system for typhoid fever and host–pathogen interactions. It has been reported that cdgR of *Salmonella* is required for the bacterium to survive inside host phagocyte. The cdgR is a c-di-GMP PDE and disruption of the gene decreases the bacterial resistance against H_2O_2 and accelerates bacterial killing of macrophages. Here the c-di-GMP signalling is not only involved in the bacterial biofilm formation and cellulose synthesis, but also regulates host–pathogen interaction that involves antioxidant defence and cytotoxicity[124].

Cyclic di-GMP and the bacterial motility

Cyclic di-GMP controls the bacterial flagellar motor direction and speed: Cyclic di-GMP has emerged as a key player in the decision between motile and sessile forms of bacterial life and has been reported in many reviews[95,125]. The role of c-di-GMP in bacterial swimming has been reported recently. It was shown that c-di-GMP binding protein YcgR of *E. coli* interacts with the flagellar rotor proteins FliM and FliG, and controls the flagellar motor direction and speed in a c-di-GMP dependent fashion. YcgR is a PilZ domain protein and binds c-di-GMP[126]. The rotary flagellar motor contains two major complexes of stator and rotor. The MotA and MotB form a ring of stationary complexes called stator and FliG, FliM and FliN form a rotor to rotate the flagella. The level of c-di-GMP is controlled by DGC and PDE YhjH. During exponential growth, the PDE activity is favoured to keep the c-di-GMP level low resulting into fast motor speed. During starvation, level of c-di-GMP increases and as a result c-di-GMP binds and activates the protein YcgR, which in turn binds to motor directly and slows down its speed[73]. It has been reported earlier that in response to changing environment, the level of c-di-GMP inversely regulates the cell motility and cell surface adhesions. The c-di-GMP binding proteins DgrA and DgrB are the part of signal transduction pathways and interfere with flagellar function in response to high-level of c-di-GMP. DgrA is a member of new family of c-di-GMP receptors and c-di-GMP binds to DgrA, which interacts with FliM and controls flagellar motor function in *Caulobacter crescentus*[127].

Cyclic di-GMP regulates the transition from swarmer to stalked cell in C. crescentus: *C. crescentus* is an asymmetrically dividing bacterium, which produces a non-motile stalked cell and a flagellated swarmer cell at the end of each cell cycle[95,128]. At the time of cell differentiation from swarmer to stacked cell, it was noticed that DGC (PleD) was specifically localized at the differentiating pole to maintain the high-level of c-di-GMP, which favours the holdfast formation and ejection of flagellum[81,113,128]. The progression of the cell cycle is dependent on GGDEF and c-di-GMP effector protein PopA[95]. The protein PopA sequesters CtrA to this pole[129]. To retain PopA and sequester CtrA to the stalked pole, c-di-GMP binding to PopA is essential. Thus, the cell cycle and the spatial localization and action of GGDEF and EAL domain proteins are tightly regulated. DGC (PleD) and PDE (TipF) are directed to the cell poles and are active in a cell cycle-dependent manner.

Role of cyclic di-GMP signalling in the bacterial survival

Cyclic di-GMP signalling is necessary for long-term survival in mycobacteria: To study the physiological role and implication of c-di-GMP signalling in mycobacteria, the gene responsible for c-di-GMP turnover (*msdgc-1*) was disrupted and the number of viable cells was counted using CFUs assay under nutrient starvation condition (0.02% glucose) and compared with that of the wild-type strain. It was found that the *msdgc-1* knockout strain (DGCKO) had a reduced viability of more than 50% within 5 days of its entry into stationary phase as compared to that of the wild-type, followed by further decrease in the viable cells when monitored for longer period of time up to 20 days (Figure 7). The complemented strain (DGCCO) was able to survive under the condition of carbon starvation similar to that of the wild-type strain, which suggests that this effect was not due to the polar effect upon the disruption of *msdgc-1* gene[97]. Thus, c-di-GMP signalling is involved in the long-term survival of *M. smegmatis*. It would be worth to mention here that this characteristic of c-di-GMP^0 strain of *M. smegmatis* is similar to that of the cells devoid of ppGpp, another second messenger[68].

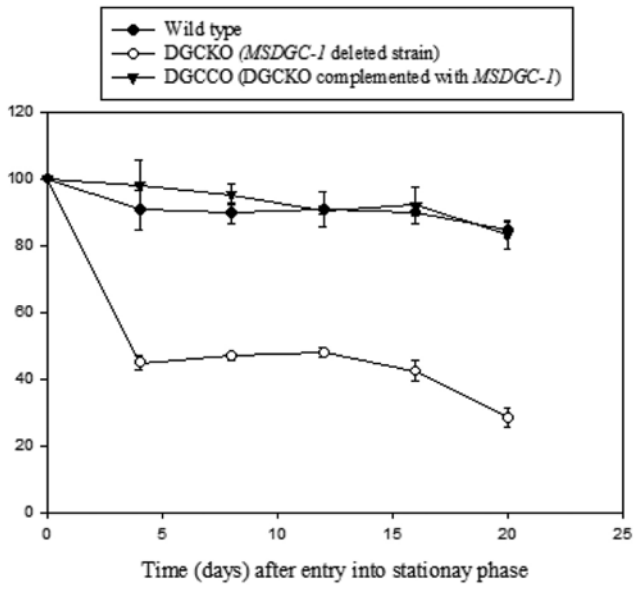

Figure 7. Role of c-di-GMP signalling in the long-term survival of *M. smegmatis*. CFUs of the cultures were obtained at different time intervals after their entry into stationary phase and monitored up to 20 days. It can be noticed that the survival of the DGCKO strain was decreased up to 50% in just 5 days, when compared with that of wild type and there was further decrease in the viable cells if monitored for longer time. The complemented strain DGCCO shows the reversal of the phenomenon and behaves like wild-type. [This picture was adapted from Bharati *et al.*, *Microbiology*, 2012, **158**, 1415–1427.]

Cyclic di-GMP is essential for the survival of the lyme disease spirochete in ticks: *Borrelia burgdorferi* is a spirochete that causes lyme disease, the most prevalent vector-borne infection in the United States[130]. The enzootic cycle of the pathogen involves both a mammalian host and an ixodes tick vector. *B. burgdorferi* has two sets of two-component systems, Hk1-Rrp1 and Hk2-Rrp2, and its genome encodes a single copy of the DGC gene (*rrp1*), which is responsible for c-di-GMP synthesis. It has been found that the Rrp1-deficient mutants remain infectious in the mammalian host but cannot survive in the tick vector. Moreover, the microarray data suggested that abrogation of Rrp1 down-regulated the metabolism of the cell and transport of glycerol. Thus, c-di-GMP is essential for spirochete survival in the tick vector, although it was not required for the mammalian infection.

Cyclic di-GMP in eukaryotes

C-di-GMP triggers stalk cell differentiation in Dictyostelium: Cyclic di-GMP has been identified as a ubiquitous second messenger in prokaryotic organism to date. The first report which describes the c-di-GMP signalling in eukaryotes showed the presence of DGCs protein, DgcA in *D. discoideum amoeba*, which indicates that c-di-GMP signalling is not confined to prokaryotes only. Interestingly, DGC was detected in all major groups of *Dictyostelia*. It has been observed that the DgcA produces c-di-GMP in a GTP-dependent manner and was expressed at the slug tip that is responsible for stalk cell differentiation. Moreover, disruption of the DgcA gene blocked the transition from slug migration to fructification and the expression of stalk genes[131].

Cyclic di-GMP inhibits human colon cancer cell prolixferation: Recently, it has been shown that c-di-GMP has drug-like properties in *S. aureus* and treatment with c-di-

GMP (200 μM) inhibits the adherence of *S. aureus* to human epithelial HeLa cells[132]. In another study, it was shown that low concentration of c-di-GMP does not alter the basal level of cell proliferation, but high-levels of c-di-GMP arrest both basal and growth factor-stimulated proliferation of human colon cancer cells in H508 cell lines[133]. The cell cycle arrest has also been shown by another group and they observed that treatment of 50 μM c-di-GMP with Jurkat cell lines stall the cell cycle at S-phase and finally decreased the rate of cell division[134]. Thus, c-di-GMP could be used clinically for the development of novel antimicrobial and cancer therapeutic agents.

Conclusion and perspectives

In recent years, quorum sensing along with mono- and di-cyclic nucleotides (cAMP, cGMP, ppGpp, c-di-AMP and c-di-GMP) have been recognized as key players in the regulation of life-style switches, motility, virulence factor secretion and survival strategy of the bacteria. The stationary phase of mycobacteria is important with respect to its survival and pathogenicity. The important challenge today is to understand the stationary phase both from the phenotypic alteration and regulation of the gene expression at various stages of growth. Although c-di-GMP signalling has been established in bacteria and the presence of c-di-GMP with its specific receptor in eukaryotes has also been reported, its role is not completely understood with respect to the host immune system. Because, approximately 75% bacterial infections are caused due to their biofilm-forming ability, which is a deleterious outcome of quorum sensing. Enormous efforts have been made to disrupt such biofilms, in particular during the development and identification of antibiotics and other small molecules. Some of these antibiotics are tobramycin, colistin and novobiocin among others and small molecules like D-amino acids, 2-amino imidazoles, etc.[135,136]. We believe that future research will establish a connection among all second messengers within a network which in turn will help in designing specific new class of antibiotics.

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