Chapter 21
Cyclic di-GMP Signaling in Extreme Acidophilic Bacteria

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Abstract Extreme acidophilic bacteria are a phylogenetically diverse group of microorganisms that grow optimally at pH values below 3. They thrive in natural or man-made environments where life is challenged by extreme acidity, low availability of organic matter, and high concentrations of heavy metals. Most acidophilic bacteria are chemolitho(auto)trophs, obtaining energy from the oxidation of metal sulfides, one of the most abundant mineral classes on earth. Bacterial attachment on mineral surface and the subsequent biofilm development plays critical role in mineral dissolution, which is directly related with ecologic phenomena and biotechnological applications, such as acid mine drainage, biogeochemical cycles, and bioleaching processes. In contrast to well-studied neutrophilic bacterial strains, the understanding of cyclic di-GMP signaling in extreme acidophilic bacteria is still incipient. However, significant progress has been made in the last several years through global genomic analysis on acidophilic communities, and genetic work on species belonging to the most iconic acidophilic genus, *Acidithiobacillus*. This chapter presents an overview of molecular insights into cyclic di-GMP signaling obtained from *At. ferrooxidans*, *At. caldus*, and *At. thiooxidans*. In addition, it describes the cyclic di-GMP signaling network as a widespread but highly diverse mechanism used by acidophilic bacteria to transduce environmental signals into

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biofilm-related responses mainly driven by cyclic di-GMP effector proteins involved in swarming motility and the production of exopolymeric substances.

Keywords *Acidithiobacillus* · Acidophilic bacteria · Biofilm · Cyclic di-GMP pathway · Extremophile

21.1 Acidophilic Microorganisms

Acidophilic microorganisms are defined as those that have pH optima below 7. They have been classified as acid-tolerant ($7 \geq \text{pH} \geq 5$), moderate acidophiles ($5 > \text{pH} > 3$), and extreme acidophiles ($\text{pH} \leq 3$) [1]. Acidophilic microorganisms exist in natural acidic environments such as acid sulfate soils, volcanic and geothermal areas where sulfur gases occur in association with water vapor (solfataras), hydrogen sulfide caves, as well as man-made environments such as biomining operations and bioreactors for wastewaters treatment. These environments are characterized by low pH, high metal, and low nutritional conditions, which result in a relatively low phylogenetic diversity of microorganisms [2], dominated by chemolithoautotrophic bacteria and archaea capable of oxidizing inorganic electron donors, generally ferrous iron and reduced inorganic sulfur compounds (RISCs), coupled to the reduction of oxygen or ferric iron. Chemolithoautotrophic microorganisms provide organic materials to some acidophilic mixotrophs and heterotrophs, such as bacteria belonging to *Alicyclobacillus* and *Acidiphilium* genus, respectively, that in turn, consume these compounds potentially toxic for chemolithoautotrophs maintaining the community structure [3]. Along with pH, a key physical–chemical parameter for the occurrence of particular microorganism is temperature, ranging from near to zero in acid mine drainages to near to 90 °C at solfataric springs. While most of the extreme thermophilic acidophiles correspond to archaea, mesophilic and moderate thermophilic bacteria dominate acidic environments below 50 °C.

Molecular mechanisms that allow the recognition of environmental cues and the coordination of suitable responses are important to survive in these challenging environments. Recently, through bioinformatics analysis, we have described components of different nucleotide second messenger-based signaling in extreme acidophilic bacteria of the *Acidithiobacillus* genus [4]. An extended analysis, comprising 201 prokaryotic genomes [106 Bacteria (38 completes, 36 drafts >91% completeness and 16S sequence complete predicted), 95 Archaea (44 completes, 35 draft)] from extreme, moderate and acid-tolerant acidophiles, gave us a general scenery of nucleotide transduction pathways in acidophilic microorganism (Fig. 21.1).

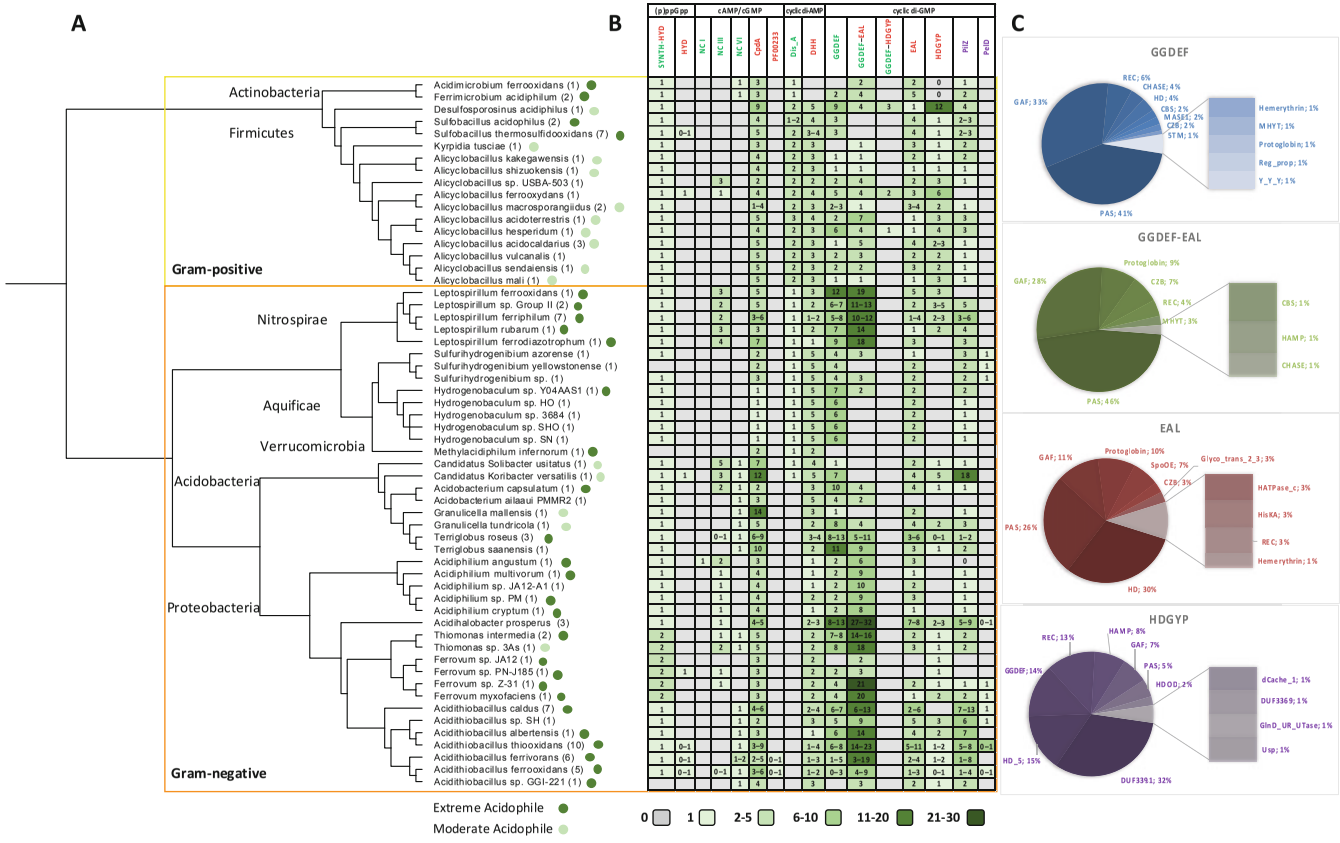

Fig. 21.1 Distribution of proteins related with nucleotide second messenger systems in acidophilic bacteria**.** (**a**) Phylogenetic tree of acidophilic bacterial genus based on 16S rRNA. The number of sequenced genomes from different strains belonging to the same species is indicated in brackets. (**b**) Number of second messenger-related proteins in each acidophilic species. Proteins or domains involves in synthesis, degradation, and transduction of signaling nucleotides are written in green, red, and purple letters, respectively. SYNTH, RelA_SpoT (synthesis domain); HYD, HD (Hydrolase Domain); NC, Nucleotidyl cyclase. (**c**) Proportion of partner domains associated with the identified cyclic di-GMP metabolizing domains (synthesis: GGDEF; degradation: EAL and HD-GYP)

21.2 General Overview of Nucleotide Second Messenger Metabolism in Acidophilic Microorganisms

Among the most important signaling nucleotides are guanosine 3′,5′-bispyrophosphate (ppGpp) and guanosine 3′-diphosphate, 5′-triphosphate (pppGpp), known as (p)ppGpp [5], cyclic adenosine 3′,5′-monophosphate (cAMP) [6], cyclic dimeric guanosine 3′,5′-monophosphate (cyclic di-GMP) [7], and cyclic dimeric adenosine 3′,5′-monophosphate (cyclic di-AMP) [8]. (p)ppGpp, cAMP, cyclic di-GMP, and cAMP are synthetized, respectively, by (p)ppGpp synthetases, adenylyl cyclases (ACs), diguanylate cyclases (DGCs), and diadenylate cyclases (DACs). Based on amino acid sequences, nucleotidyl cyclases (NCs) have been classified into six classes (I–VI) [9]. Class III is the most diverse group of cyclases, including ACs and DGCs, while NCs belonging to classes I, II, IV, V, and VI are all AC enzymes.

The repertoire of enzymes related with the metabolism of nucleotide second messengers is very different between acidophilic Bacteria and Archaea. Just a few enzymes for the synthesis and degradation of signaling nucleotides are encoded in acidophilic archaeal genomes. Genes coding for (p)ppGpp metabolizing enzymes are mainly absent in the Archaea domain, excepting a few genes likely acquired by horizontal gene transfer from Bacteria [10]. Indeed, acidophilic Archaea have no genes for (p)ppGpp metabolism (personal communication), and therefore, they probably do not use this signaling molecule. A public database of cyclic di-GMP related proteins (http://www.ncbi.nlm.nih.gov/Complete_Genomes/c-di-GMP.html), shows that three out of 105 archaeal genomes only encode one protein containing the GGDEF domain related to cyclic di-GMP synthesis. On the other hand, the acidophilic archaeal genome of *Candidatus Micrarchaeum acidiphilum* ARMAN-2 encodes a predicted inactive GGDEF domain containing protein because it does not possess the key amino acids for catalytic activity neither for cyclic di-GMP binding at the inhibition sites location. Then, altogether this data indicate that acidophilic archaea do not use cyclic di-GMP either. Moreover, besides moderate acidophilic marine archaea belonging to the *Aciduliprofundum* genus, no other acidophilic archaea possess homologues of DAC for synthesis of cyclic di-AMP. The most common component found in 80% of the acidophilic archaeal genomes is a single putative AC belonging to class IV NCs. These proteins are formed by a single CYTH domain whose name was coined from the first two identified members, the CyaB adenylyl cyclase from *Aeromonas hydrophila* and the human thiamine triphosphatase (ThTPase).

Only few bacterial members of class IV ACs have been biochemically characterized so far. CyaB (*A. hydrophila)* and YpAC-IV (*Yersinia pestis*), two bacterial homologues of archaeal CYTH-containing ACs, show optimum activity at 65 °C and 50 °C, respectively [11, 12].

In acidophilic bacteria, cAMP synthesis could be achieved by CyaB orthologs (class IV NC), as well as through class III NCs. In Betaproteobacteria and Acidithiobacillia classes, the CYTH domain of ACs is fused with a “conserved histidine alpha-helical domain” (CHAD), and therefore, probably are able to interact

with polyphosphate (polyP) [13], a key player in metal resistance/tolerance of acidophilic bacteria [14]. Notably, *Candidatus Koribacter, Candidatus Soilbacter,* and *Leptospirillum* class III NCs possess extracellular or membrane sensory domains like CHASE, dCache, or HAMP, suggesting that cAMP synthesis may be regulated by direct sensing of extracellular signals such as cytokinin-like adenine derivatives or peptides [15], polyamines [16], and also by signals not determined yet [17] that may be inherent to its acidophile habitat. To date, just one acidophilic bacterial genome code for one protein belonging to class I NC (Anthrax_toxA), *Acidiphilium angustum* ATCC 35903. The presence of a canonical secretion signal indicates that this protein may be secreted, suggesting an extracellular role, as it happens in class I NCs from other bacteria. The outcome of cAMP signaling may be the regulation of gene expression through direct binding to transcription regulator proteins such as well-known CRP (cAMP receptor protein) [18]. Class II, V, and VI NCs were not found in acidophilic bacterial genomes.

Thirteen out of twenty acidophilic bacterial genera encode DACs for cyclic di-AMP synthesis. Putative DACs are present in every Gram-positive acidophilic bacterium but are absent in 7 out of 13 Gram-negative genera, including *Acidithiobacillus*. Acidophilic bacteria possess one or two copies of DAC genes in their chromosomes. The DAC encoded as a single copy corresponds to a DacZ ortholog, a cytoplasmic protein with a stand-alone DisA_N domain generally found in Archaea [8]. Instead, when two DAC encoding genes are present in a single genome, one of them code for an orthologue of the membrane protein DacA, while the second one is a *Thermotoga maritima* DisA ortholog (DisA-DisA_linker-HhH) which couple chromosome integrity state with cyclic di-AMP synthesis [19]. Except those involved in cyclic di-AMP synthesis, none homologs genes coding for enzymes that catalyze the synthesis of other signaling nucleotides have been identified in the extremely acidophilic methanotroph *Methylacidiphilum infernorum* V4, suggesting that cyclic di-AMP pathway could be the exclusive nucleotide signaling system in this microorganism.

Excepting *M. infernorum* V4, every acidophilic bacterial genus encodes proteins for (p)ppGpp and cyclic di-GMP metabolism. All acidophilic bacteria encode one long (p)ppGpp synthetase with a classical domain configuration: synthesis (RelA_SpoT, pfam04607), hydrolysis (HD, pfam13328), TGS (ThrRS, GTPase, and SpoT), ZFD (zinc-finger domain), and RRM (RNA recognition motif) domains [10, 20]. The conservation of these accessory domains suggests that (p)ppGpp synthesis could be activated by amino acid and/or fatty acid starvation as it occurs in well-studied model microorganisms [20, 21]. As it occurs in non-acidophilic bacteria (e.g., *E. coli*), (p)ppGpp signaling may work through global control of gene expression by direct binding to the RNA polymerase omega subunit (RpoZ), whose gene product is encoded together with (p)ppGpp synthase gene by the same operon in many acidophilic bacteria. On the other hand, the multiplicity of DGCs and phosphodiesterases (PDEs) enzymes involved in cyclic di-GMP synthesis and degradation, respectively, present in acidophilic bacteria suggests that these microorganisms probably integrate a wide range of signals into cyclic di-GMP pathway for targeting different cellular functions.

21.3 Cyclic di-GMP in Acidophilic Bacteria

Globally, acidophilic bacteria encode near to the same amount of putative DGC proteins (481 GGDEF domains) and PDEs (446) including EAL (304) and HD_GYP (142) domains. However, the distribution of cyclic di-GMP metabolism related genes is not balanced in individual chromosomes, some of them having more DGCs than PDEs, while in others the opposite distribution occurs. GGDEF and HDGYP containing proteins possess sensor domains in almost the half of cases, 47% and 42%, respectively, meanwhile only one fifth (21%) of EAL PDEs does.

As shown in Fig. 21.1c, most common partners domains in GGDEF-containing proteins are PAS (Period circadian protein, Aryl hydrocarbon receptor nuclear translocator protein and Single-minded protein) and GAF (cGMP-specific phosphodiesterases, Adenylyl cyclases, and FhlA), followed from afar by other domains such as REC (response regulator receiver domain) and Protoglobin [or globin-coupled sensor (GCS)]. On the other hand, most partner domains present in EAL and HD-GYP putative PDEs belong to metal-dependent phosphohydrolase [HD (histidine and aspartate)] family. Recently, the regulation of DGC/PDE activities through the binding of small nucleotides (such as cAMP, GDP) to GAF domain have been characterized [22, 23], suggesting that the GAF domain may establish a functional link between signaling pathways based in different nucleotides. PAS and GCS domains are able to sense two key environmental factors for energetic requirements of chemolithoautotrophic acidophilic bacteria: redox potential [24] and O_2 [25, 26]. Energetically, molecular oxygen is the most favorable electron acceptor during RISCs and iron oxidation, while redox potential, which is conditioned by ferrous/ferric iron ratios, drives the composition of the community [27]. In some acidophilic bacteria, such as *Acidithiobacillus* and *Leptospirillum*, REC-GGDEF-EAL proteins are encoded together with a histidine kinase in a characteristic configuration of a two-component system [28]. In these cases, phosphorylated and unphosphorylated state of REC domain may modulate the rates of cyclic di-GMP synthesis or degradation by these enzymes. Since acidophilic bacteria thrive in metal-rich environments, it is interesting to note that signaling transduction through chemoreception of metals like zinc may perform through CZB (*C*hemoreceptor *Z*n-*B*inding) domain coupled to GGDEF-EAL (7%), GGDEF-only (2%), or EAL-only proteins (3%).

The output of cyclic di-GMP signaling in acidophilic bacteria appears to mainly occur by PilZ receptor proteins. Excepting for *M. infernorum* V4, every acidophilic bacterial genera [19] encode at least one PilZ domain protein (Fig. 21.1b). However, there is no evident correlation between the quantity of PilZ proteins and cyclic di-GMP turnover proteins. This could be explained by the presence of other cyclic di-GMP receptor proteins in acidophilic bacteria such as FleQ, PelD and catalytically degenerate and inactive GGDEF and EAL domains also able to bind cyclic di-GMP allosterically (personal communication). PilZ domain occurs alone or in conjunction with other domains in the same polypeptide chain. The most frequent configuration is PilZ domain alone, but it includes proteins long enough to contain non-characterized domains. These proteins are generally related with the formation of type 4 pili

(T4P) and twitching motility [29], which has been implicated in irreversible attachment to surfaces, microcolony grouping, and structural development of biofilm [30, 31]. Common partner domains of PilZ proteins of acidophiles are YcgR (pfam07317) and Glycosyl_transferase_2 (pfam00535), which are related with flagellar-based motility [32] and synthesis of different types of exopolysaccharide (EPS) [33, 34], respectively. Among the later, the most recognizable PilZ proteins correspond to A subunit of bacterial cellulose synthase (BcsA), which in some acidophiles is fused with BcsB, forming a fused membrane protein predicted to produce a cellulose-like EPS. An interesting subgroup of enhancer-binding proteins (EBPs) containing PilZ domains are present in the "professional" iron oxidizer genus, *Leptospirillum*, which have been related with lipopolysaccharide and flagellar biosynthesis [35]. Besides PilZ, a few acidophilic bacteria encode PelD-like effector proteins (see below).

21.4 Cyclic di-GMP Pathway in *Acidithiobacillus*

Acidithiobacillus genus members are the most studied acidophilic microorganism. They are chemolithoautotrophic Gram-negative bacteria involved in bio-oxidation of metal sulfides in natural and mining environments. *Acidithiobacillus* obtain energy from the oxidation of RISCs, producing sulfuric acid as a byproduct. Therefore, all of them are also extreme acidophiles, with optimal growing at pH values below 3. However, there are some important differences among *Acidithiobacillus* species. Besides RISCs, *At. ferrooxidans*, *At. ferrivorans*, *At. ferridurans*, and *At. ferriphilus* catalyze the oxidation of ferrous to ferric iron (Fe^{3+}). While most of acidithiobacilli are mesophilic, showing an optimum growth temperature near to 30 °C, *At. ferrivorans* has psychrotolerant characteristics, being able to grow at 4 °C [36]. On the other hand, *At. caldus* is the only moderate thermophilic species of the genus, growing between 30 and 50 °C [37]. Several acidithiobacilli are able to express a single polar flagellum for swimming and/or swarming motility. However, strains of *At. albertensis* express a tuft of polar flagella [38], meanwhile, some *At. ferrooxidans* strains have no genes required for production and functioning of the flagellum machinery [39]. These variations may be very important in the colonization and dominance at different micro-niches in natural environments, as well as in bioleaching operations, since bio-oxidation activity depends largely on the physiological state of the cell, which in turn is intimately associated with the different bacterial lifestyles such as the single-cell planktonic state and multicellular biofilms. The attachment of acidithiobacilli to the substrate surface and the subsequent biofilm development (Fig. 21.2) plays essential role in bio-oxidation processes for the acidithiobacilli. This is due to the creation of a reaction space that concentrates

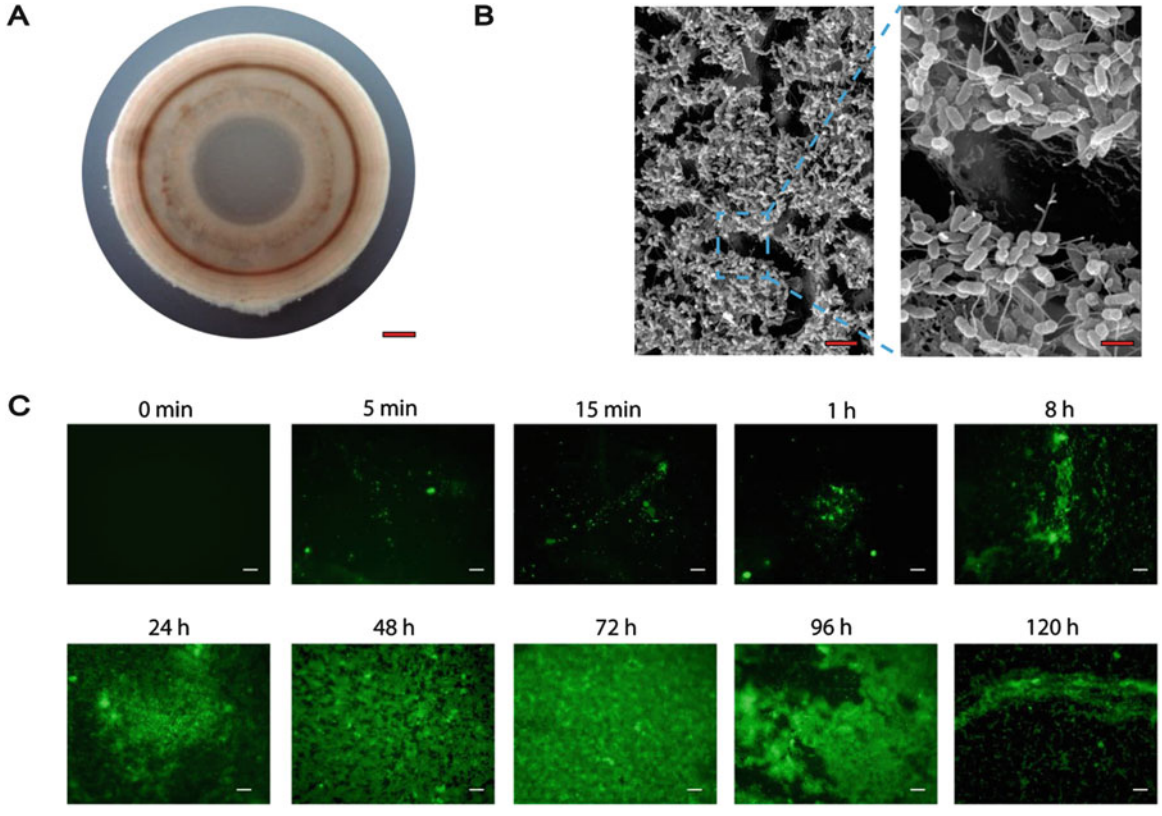

Fig. 21.2 *Acidithiobacillus caldus* biofilms. (**a**) Macrobiofilm on tetrathionate plate obtained from a 3 µl-culture inoculum. Red bar, 1 mm. (**b**) SEM imaging of a 48 h old biofilm developed on elemental sulfur surface. Zoom (right panel) is pointing out the network of filamentous projection connecting biofilm cells. Red bar, 5 µm (left) and 1 µm (right). (**c**) Fluorescence microscopy (Concanavalin A-FitC) of biofilm dynamics. Sulfur surface is quickly colonized by cells that develop a flat biofilm during 72 h. Then, dispersion of cells conforming the biofilm structure begins (96 h). White bar, 10 µm

leaching chemicals at the cell/mineral interface, accelerating the leaching activity [40, 41].

Recently, some DGCs and cyclic di-GMP receptor proteins of *Acidithiobacillus* type strains of *At. ferrooxidans* (ATCC 23270) [42], *At. thiooxidans* (ATCC 19377) [43, 44], and *At. caldus* (ATCC 51756) [45, 46] have been characterized experimentally, validating the functionality of a cyclic di-GMP based signaling in these microorganisms and its relationship with the classical motility and biofilm phenotypes.

The number and the nature of cyclic di-GMP related proteins among *Acidithiobacillus* species differ notably, ranging from near to 40 metabolism proteins in *At. thiooxidans* to just 5 (4 GGDEF-EAL, 1 EAL) proteins in both collection strains of *At. ferrooxidans* (ATCC 23270 and ATCC 53993) (Fig. 21.1). This variability is widespread in acidophile bacteria and it is not related with genome size (Fig. 21.3). In part, this asymmetry could be explained by the presence of transferable genetic elements that contain cyclic di-GMP related genes. Different plasmids and mobile genetic elements containing GGDEF/EAL and PilZ genes have been identified in complete genomes of *Acidithiobacillus* species, suggesting that they constitute a coherent cyclic di-GMP control module [4]. For instance, the integrative and conjugative element ICEAca$_{TY}$2, widely present in *At. caldus* genomes, carries genes predicted to encode cyclic di-GMP metabolism enzymes (*dgc1879*, *pde1853*), and cyclic di-GMP effector proteins (*pilz1908, ycgR* and *fleQ*). Besides the inherently biofilm-related functions encode in ICEs, such as assembly factors of T4P and the conjugation machinery, ICEAca$_{TY}$2 encodes accessory genes for assembly and functioning of flagellar apparatus. Such elements are potential direct or indirect targets of cyclic di-GMP signaling as it has been observed in other bacteria [47].

Compared with other *Acidithiobacillus* species, the cyclic di-GMP signaling pathway in *At. ferrooxidans* ATCC 23270 has the lowest complexity and comprise four GGDEF-EAL, one single EAL, and only two PilZ proteins. Moreover, the single EAL protein and one of the two PilZ proteins do not possess key amino acid motifs for cyclic di-GMP interaction. The use of different electron donors, such as sulfur and iron, supports the expression of all four GGDEF-EAL domain proteins. Furthermore, induction of the cellulose-production phenotype in heterologous complementation assays suggests a net DGC activity of these enzymes [42]. Notably, three GGDEF-EAL proteins, as well as the second PilZ protein are encoded on the ICEAfe$_{TY}$2 (also present in *At. ferrooxidans* ATCC 53993). These four genes are clustered in a cyclic di-GMP genetic hot spot together with Tra conjugation and transposase genes, while the fourth GGDEF-EAL protein is encoded next to von Willebrand factor type A domain-containing protein. The genetic contexts strongly suggest the association of the cyclic di-GMP pathway with cellular attachment and clustering. Consequently, cyclic di-GMP levels in biofilm cells are near to 10 times higher than planktonic cells harvested from the same culture [42].

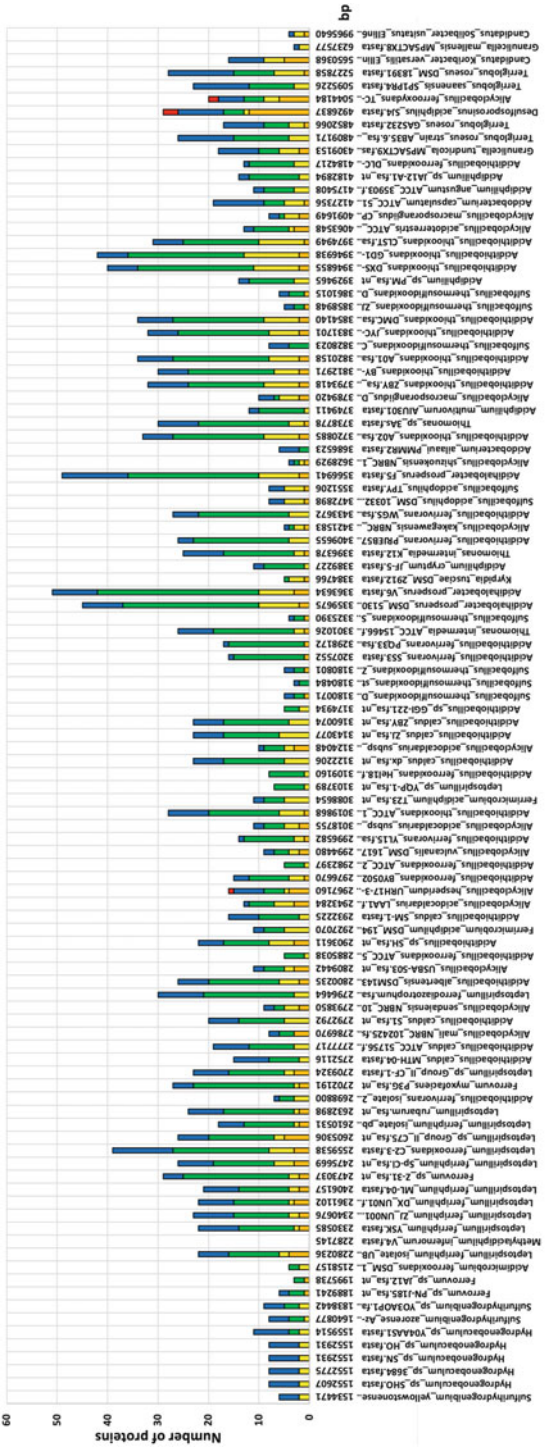

Fig. 21.3 Distribution of cyclic di-GMP turnover proteins in genomes from acidophilic bacteria. Acidophilic bacterial strains are organized by genome size (pb) and the corresponding number of predicted cyclic di-GMP proteins are showed

The bioinformatic analysis of *At. caldus* ATCC 51756 genome allowed the identification of 18 genes related to cyclic di-GMP turnover (9 single GGDEF, 6 GGDEF-EAL, and 3 single EAL) and 10 putative cyclic di-GMP effector proteins (9 PilZ and 1 PelD). Based on key amino acid conservation and cellulose-production phenotype assays in *E. coli* and *Salmonella* Typhimurium, the single GGDEF protein ACAty1319 was identified as a functional DGC in this bacterium and selected for mutagenesis experiment [46].

A null-mutant strain ΔACAty1319 was developed by using a suicide vector harboring a kanamycin cassette and both 5′ and 3′ ends of ACAty1319 encoding gene [46]. Then, by comparing mutant and wild-type strains it was reported that this DGC is mainly responsible for 85–93% of the global cyclic di-GMP intracellular levels and plays significant roles on (1) early stages of biofilm development (Fig. 21.4a) and (2) swarming motility (Fig. 21.4b). The immediate gene context of ACAty1319 that contains *fliL*, *motA*, and *motB* orthologous suggests that it may affect flagellar motor performance [46].

The *At. thiooxidans* ATCC 19377 strain encodes an extended cyclic di-GMP signaling network with 25 cyclic di-GMP metabolism genes (9 GGDEF, 12 GGDEF-EAL, 3 EAL, 1 HD-GYP) plus 9 PilZ and 1 PelD. Most of GGDEF-containing proteins were characterized as functional DGCs through induction of the rdar (rough, dry and red) biofilm morphotype in *S*. Typhimurium and high intracellular levels of cyclic di-GMP were reported in attached cells [44]. Like *At. caldus*, *At. thiooxidans* encode a PelD cyclic di-GMP effector in a complete *pel* operon (Fig. 21.5a) [48, 49] that includes an additional gene, *wcaG* [uridine diphosphate (UDP)-glucose-4-epimerase], downstream *pelG* gene and it is probably involved in the synthesis of PEL exopolysaccharide precursor. Transcription levels of *pelA*, *pelD*, and *wcaG* genes increase in *At. thiooxidans* biofilm cells. In cells attached to sulfur surface, the deletion of *pelD* gene induces a decrease of EPS production (sixfold less of total carbohydrates fraction), and an increase of the total protein fraction. SEM imaging reveals that Δ*pelD* null-mutant cells attached to sulfur overexpress a filamentous structure (Fig. 21.5b) that could have a proteinaceous nature [44]. Intriguingly, Pel biosynthesis operon does not fallow the phylogenetic distribution, and are not carried on an apparent mobile genetic element. The Pel exopolysaccharide cluster is present in *Sulfurihydrogenibium* [moderate acidophile (pH 6) thermophilic (68 °C)], *Acidihalobacter prosperus* [extreme acidophilic (pH 2) mesophile (37 °C)], *Ferrovum myxofaciens* [moderate acidophile (pH 2.5–4.8) mesophile (30 °C)], *At. caldus* [extreme acidophilic (pH 2.5), moderate thermophilic (45 °C)] and *At. thiooxidans* [extreme acidophilic (pH 2.5), mesophile (30 °C)]. Interestingly, PelB, a component that spans the periplasm and the outer membrane, seems to be the most variable element of the Pel biosynthesis gene products, probably due to adaptation to different extracellular conditions (Fig. 21.5a).

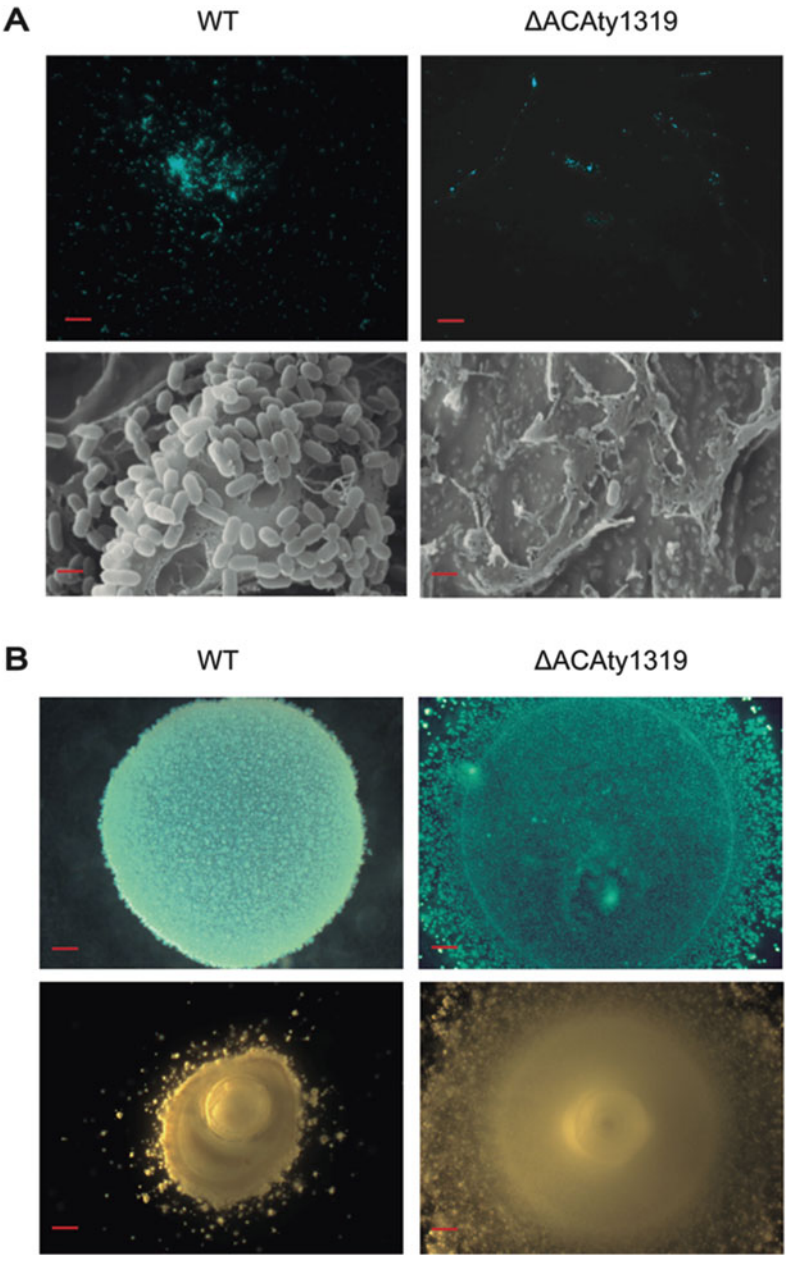

Fig. 21.4 Attachment (**a**) and motility (**b**) phenotypes in *At. caldus* ATCC 51756 wild type (WT) and DGC null-mutant (ΔACAty1319) strains. Attached cells on elemental sulfur were visualized by fluorescence microscopy (upper panel **a**) and SEM (bottom panel **a**). Swarming motility patterns were observed on semi-solid media [phytagel 0.2%, pH 4.7 (upper panel **b**), and phytagel 0.1% pH 2.5 (bottom panel **b**)] with tetrathionate as an energy source

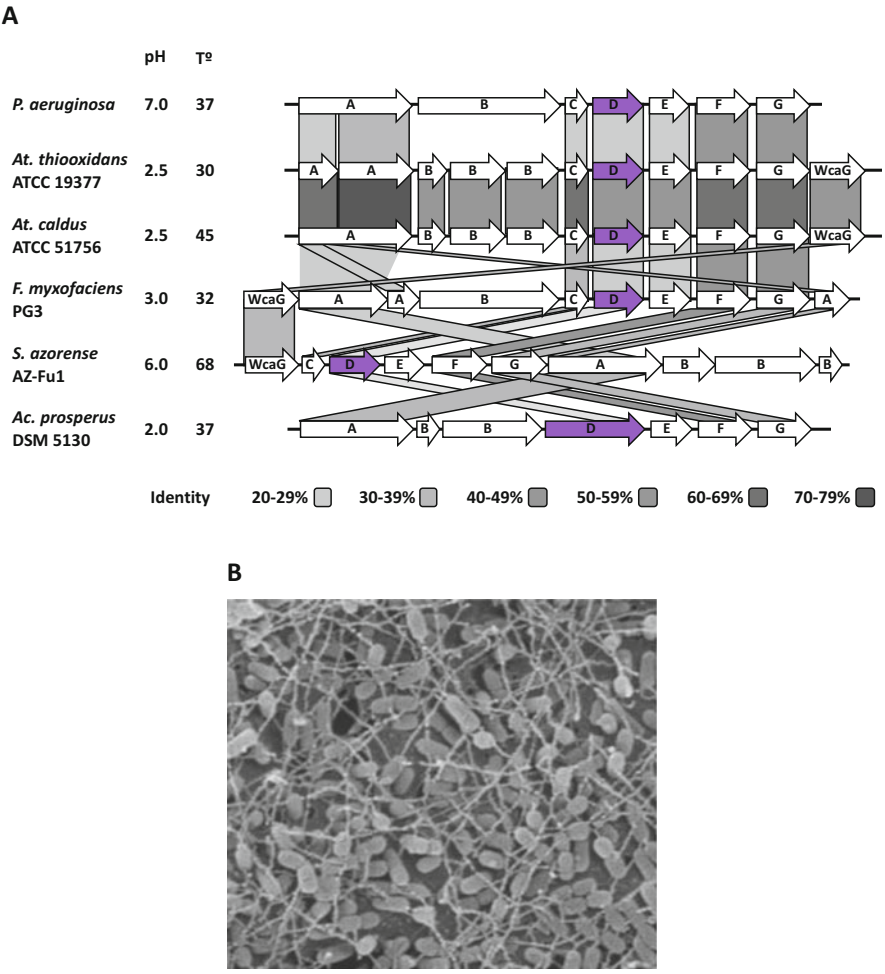

Fig. 21.5 (**a**) Organization and conservation of the *pel* gene clusters in acidophilic bacteria. Optima growth pH and temperature for *pel* operon containing acidophilic bacteria are noted. *Pel* operon structure from *P. aeruginosa* PA14 is presented as a reference. The percentage of identity between adjacent gene clusters is represented as gray bars. *PelD* gene is depicted in purple. Note that *wcaG* is part of *pel* gene cluster in several acidophiles. (**b**) SEM imaging of biofilm structure produced by the *At. thiooxidans* Δ*pelD* null-mutant strain overexpressing a filamentous structure

21.5 Concluding Remarks

Signal transduction mechanisms are key molecular processes that allow diverse microorganisms to adapt and respond to their surroundings. This is particularly important in extremophilic microorganisms which thrive in very harsh environments. As pointed out in this chapter, acidophilic microorganisms have the

capability to create signaling circuits based on diverse nucleotide second messengers. Most acidophilic Archaea probably use cAMP-based signaling, meanwhile several acidophilic bacteria use cAMP, cyclic di-AMP, (p)ppGpp, and cyclic di-GMP, with the last two being the most prevalent second messenger signaling molecules. In acidophilic bacteria, (p)ppGpp and cAMP seem to regulate gene expression in response to starvation and polyP-related signal, respectively. On the other hand, cyclic di-GMP signaling integrates several input signals through different metabolism components. DGCs from acidophilic bacteria detect environmental signals through protein domains related with critical cues for chemolithoautotrophic bacteria which dedicate large quantity of energy to carbon fixation. Oxidation of RISCs and iron II offers little amount of energy, leaving oxygen as the most favorable electron acceptor [27]. Besides, the redox potential and consequently the energy output has a tremendous impact on the growth rate of acidophilic bacteria such as *Acidithiobacillus* and *Leptospirillum* due their different iron oxidizing machinery [27]. Then, DGCs containing domains able to sense redox potential and/or oxygen, such as PAS and protoglobin may be vital to transduce these cues to a particular response, such as biofilm formation on an oxidizable substrate. On the other hand, metal sensing through CZB domain appears as an important cue for regulation of acidophiles DGCs, probably impeding biofilm formation through DGC activity inhibition, as has been shown for other bacteria [50]. Besides, acidophiles DGCs couple GGDEF domain with poorly characterized N-terminal signaling domains involved in the perception of signals at extracellular (7TMR-DISMED2 and dCache_1), membrane (CHASE, MASE1 and MHYT), and periplasmic (Reg_prop and Y_Y_Y) level. They may help to transduce specific signals from acidic ecological niche and then have to be targeted for further studies.

Key behaviors for energy acquisition, such as attachment and subsequent biofilm formation, may be regulated by cyclic di-GMP through flagellar motility inhibition and EPS synthesis, specially, cellulose-like and PEL expolysaccharides, which in turn is achieved through effector proteins such as PelD and PilZ domains containing proteins.

The physiological characterization of cyclic di-GMP pathway in acidophilic bacteria is still incipient but the current knowledge obtained from bioinformatics analysis and biological experiments in *Acidithiobacillus* species introduces some clues about the regulation of biofilm formation in this kind of microorganisms. Thus, it opens new ways to regulate this key physiological behavior for industrial or environmental applications, either to increase precious metals release from leaching ores or to control unwanted acid production in natural habitats.

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