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ORIGINAL ARTICLE

The extremophile *Acidithiobacillus ferrooxidans* possesses a c-di-GMP signalling pathway that could play a significant role during bioleaching of minerals

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Acidithiobacillus ferrooxidans, biofilm, bioleaching, c-di-GMP, extremophile.

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Abstract

Aims: The primary goal of this study was to characterize the existence of a functional c-di-GMP pathway in the bioleaching bacterium *Acidithiobacillus ferrooxidans*.

Methods and Results: A bioinformatic search revealed that the genome sequence of *At. ferrooxidans* ATCC 23270 codes for several proteins involved in the c-di-GMP pathway, including diguanylate cyclases (DGC), phosphodiesterases and PilZ effector proteins. Overexpression in *Escherichia coli* demonstrated that four *At. ferrooxidans* genes code for proteins containing GGDEF/EAL domains with functional DGC activity. MS/MS analysis allowed the identification of c-di-GMP in nucleotide preparations obtained from *At. ferrooxidans* cells. In addition, c-di-GMP levels in cells grown on the surface of solid energetic substrates such as sulfur prills or pyrite were higher than those measured in ferrous iron planktonic cells.

Conclusions: *At. ferrooxidans* possesses a functional c-di-GMP pathway that could play a key role in *At. ferrooxidans* biofilm formation during bioleaching processes.

Significance and Impact of the Study: This is the first global study about the c-di-GMP pathway in an acidophilic bacterium of great interest for the biomining industry. It opens a new way to explore the regulation of biofilm formation by biomining micro-organisms during the bioleaching process.

Introduction

Acidithiobacillus ferrooxidans is an acidophilic Gram-negative bacterium whose energetic metabolism is responsible for the solubilization of metals present in ores, and therefore, the micro-organisms are directly involved in biomining. Adherence of *At. ferrooxidans* to the mineral surface and subsequent biofilm formation can improve the leaching efficiency (Schippers and Sand 1999; Rohwerder *et al.* 2003). Therefore, there is great interest in understanding the molecular mechanisms involved in biofilm formation by this acidophilic bacterium. *At. ferrooxidans* forms a monolayer biofilm composed by bacterial cells embedded in an exopolysaccharide (EPS) matrix whose role is fundamental in creating a special microenvironment that favours mineral dissolution by oxidation (Harneit *et al.* 2006; Sand and Gehrke 2006).

C-di-GMP has been recognized as an important bacterial second messenger involved in the regulation of several physiological processes (Hengge 2009; Romling and Simm 2009; Schirmer and Jenal 2009). This messenger plays a central role in controlling surface attachment in many bacterial systems by regulating the transition from planktonic to biofilm lifestyles through the control of EPS production, biosynthesis of pili and flagellum and flagellar motor speed (Garcia *et al.* 2004; Hickman *et al.* 2005;

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Thormann *et al.* 2006; Armitage and Berry 2010). C-di-GMP is synthesized by diguanylate cyclase (DGC) and degraded by phosphodiesterase (PDE) enzymes. DGC proteins are characterized by a GGDEF domain whereas PDE proteins can present EAL or HD-GYP domains. Five classes of c-di-GMP effector proteins capable of binding c-di-GMP molecules including PilZ domain have been identified until now (Newell *et al.* 2009; Schirmer and Jenal 2009; Tao *et al.* 2010).

During the course of identifying molecular mechanism that regulates biofilm formation in extremophilic bacteria involved in bioleaching processes, we previously reported the existence of a functional quorum-sensing system in *At. ferrooxidans* (Farah *et al.* 2005; Ruiz *et al.* 2008). Here, we present the first evidence for a functional c-di-GMP pathway in the acidophilic bacteria *At. ferrooxidans*. The intracellular levels of c-di-GMP increase in this microorganism during its biofilm lifestyle. These results in turn strongly suggest a key role for these signalling molecules during bacteria–mineral interactions.

Materials and Methods

Bacterial strains and growth conditions

This study was carried out with the *At. ferrooxidans* type strain ATCC 23270. It was grown as described previously in ferrous iron-containing modified 9K medium (M9K) at pH 1·5 or in DSMZ medium 71 containing 20 mmol l^{-1} thiosulfate at pH 4·4 and in elemental sulfur prills (Ramirez *et al.* 2004). Pyrite and a copper concentrate were kindly provided by W. Sand and C. Demergasso, respectively, and were used to 50 g l^{-1} in modified 9K medium (M9K) at pH 1·5 (Demergasso *et al.* 2005; Ruiz *et al.* 2008).

Bioinformatic tools

Protein domains involved in c-di-GMP pathway (EAL, GGDEF, HD-GYP and PilZ) were used as queries to search in the genome sequence of *At. ferrooxidans* (http://cmr.tigr.org/tigr-scripts/CMR/shared/Genomes.cgi) by using Artemis software (Sanger Institute Pathogen Sequencing Unit http://www.sanger.ac.uk/resources/software/artemis/). The potential candidate proteins identified with Artemis were used to formulate a BlastP search at the nonredundant NCBI database (http://www.ncbi.nlm.nih.gov).

C-di-GMP purification from *At. ferrooxidans*

Total nucleotides were extracted from *At. ferrooxidans* with organic solvents as described previously (Hisert *et al.* 2005). Cellular pellets obtained by centrifugation were washed twice with acidified water at pH 2·0 (H_2SO_4) followed by a wash with sodium citrate (pH 7·0). Washed cells were then resuspended in 1 ml of 50 mmol l^{-1} Tris–HCl, pH 8·5 and disrupted by sonication on ice. Afterwards, lysates were centrifugated at 15 800 ***g*** for 30 min at 4°C, and the supernatants were saved. Subsequently, one volume of supernatant was combined with one volume of chloroform, four volumes of methanol and three volumes of nanopure water. Finally, after pelleting proteins and nucleic acids by centrifugation, the aqueous phase rich in nucleotides was evaporated under vacuum and stored at −80°C.

At. ferrooxidans cells present in biofilms formed on sulfur prills, pyrite or a copper concentrate, were separated from planktonic cells by centrifugation at low speed (3000 ***g***). The colonized solid substrates were then washed three times with fresh M9K medium to remove the remaining planktonic cells. Colonized solid substrates were then incubated with fresh M9K medium supplemented with 0·05% of Triton X-100 for 5 min at 20°C and vortexed to release adhered cells that were recovered by centrifugation. Finally, cell pellets obtained from colonized solid substrates were processed as indicated above.

Detection and quantification of c-di-GMP by mass spectrometric analysis

Measurements were performed as previously described (Waters *et al.* 2008). Synthetic c-di-GMP was prepared in 80% acetonitrile and 20 mmol l^{-1} ammonium acetate. Nucleotidic extracts from *At. ferrooxidans* and *Escherichia coli* were diluted 1 : 10 in 80% acetonitrile per 20 mmol l^{-1} ammonium acetate. The spectrograms were obtained with an electrospray-ionization (ESI) ion-trap (IT) mass spectrometer (MS) Esquire 4000 ESI-IT (Bruker Daltonics, Inc., MA, USA). Synthetic c-di-GMP and samples were loaded directly into the ESI-IT-MS. The flux was 2·5 μl min^{-1}. The nebulization was performed at 300°C, 10·0 psi and 5 l min^{-1}. Samples were analysed in negative-ion detection mode. Based on (i) the *m/z* 689 [M-H]- of the molecular ion and (ii) *m/z* signals 344 and 538 obtained by MS/MS fragmentation pattern of the molecular ion, synthetic c-di-GMP was used as a reference to identify c-di-GMP in the nucleotidic extracts obtained from the cells. To quantify c-di-GMP levels in nucleotide extracts from *At. ferrooxidans*, samples were analysed by HPLC (Agilent 1100, Agilent Technologies) coupled to mass spectrometry. A C18 column (150 × 1·0 mm; 4 μm and 90 Å) (Jupiter-Proteo, Phenomenex Inc., CA, USA) was directly connected to the MS. HPLC separations were performed with a 10 mmol l^{-1} tributylamine, 15 mmol l^{-1} acetic acid and 3% methanol solution and a methanol gradient.

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Molecular cloning of *At. ferrooxidans* genes coding for proteins with GGDEF/EAL domains

Genomic DNA from *At. ferrooxidans* strain was used as template for PCR amplification. *AFE0053, AFE1360, AFE1373* and *AFE1379* genes were amplified by PCR with primers containing insert restriction sites for cloning purposes. PCR products were cloned in pGEM-T plasmids (Promega). Recombinant plasmids were transformed into *E. coli* JM109 strain, purified and digested with restriction enzymes. Released DNA fragments containing the different AFE genes were cloned into the *NheI/Bam*HI or *NdeI/Bam*HI sites of the expression vector pET-21b(+) (Novagen). All plasmid constructions were checked by automatic DNA sequencing (Macrogen, Korea) and transformed into *E. coli* BL21 (DE3) strain.

Determination of c-di-GMP synthesis in recombinant *E. coli* strains overexpressing *At. ferrooxidans* genes coding for putative DGC/PDE proteins

Cellulose biosynthesis was determined in *E. coli* BL21(DE3) and TOP10 strains overexpressing *At. ferrooxidans* genes coding for proteins with GGDEF domains. Quantitative assays based on the binding of Congo red (CR) to cellulose (Spiers *et al.* 2003; Lee *et al.* 2007) were used to detect the cellulose produced by *E. coli*. As a positive control, an *E. coli* strain overexpressing the DGC PleD was used (Christen *et al.* 2005). Three μl of an overnight culture of each strain was spotted onto LB agar plates with no NaCl and supplemented with ampicillin (100 μg ml^{-1}) and with or without IPTG (1 mmol l^{-1}). Plates were incubated for 24 h at 37°C. Bacteria contained in each spot were collected with a spatula, resuspended in 1 ml of 1% tryptone supplemented with CR (50 μg ml^{-1}) and incubated at 37°C for 90 min with shaking (150 rev min^{-1}). After centrifugation, the amount of cell-bound CR was quantified by comparing values of absorbance at 490 nm of CR in standard solutions and cell-free supernatants (remaining CR). Results obtained were normalized by the total protein content obtained from each cellular pellet.

Results

At. ferrooxidans possesses all the molecular components of the c-di-GMP pathway

Bioinformatic analysis of the genomic sequence of *At. ferrooxidans* allowed identification of five open reading frames (ORFs) encoding for metabolic enzymes of the c-di-GMP pathway, DGCs and PDEs (Table 1). Four of them (AFE0053, AFE1360, AFE 1373 and AFE1379) coded for proteins with both GGDEF and EAL domains while one (AFE1852) coded for a protein containing a single EAL domain. The four proteins having GGDEF/EAL domains also contained sensing domains including PAS and GAF that are involved in signal transduction (Romling *et al.* 2005). As reported for most bacteria (Romling *et al.* 2005), the GGDEF/EAL and EAL proteins from *At. ferrooxidans* can be predicted as mainly cytosolic (SubCell 1.0 Server, Technical University of Denmark). Sequence alignments revealed that all the GGDEF domains could be active and possess the characteristic motifs involved in allosteric feedback inhibition by c-di-GMP binding (Fig. S1) (Schirmer and Jenal 2009). In addition, amino acid sequence analysis of the five EAL domains (Fig. S2) showed that the eight essential residues involved in the coordination of c-di-GMP and Mg^{2+} into the active site and in assisting the role of the catalytic water molecule were conserved (Fig. S2) (Tchigvintsev *et al.* 2010). However, Thr and Asn residues located at position 546 instead of Glu strongly suggested that EAL domains respectively from AFE0053 and AFE1379 should be inactive or less efficient (Tchigvintsev *et al.* 2010).

Finally, two ORFs (AFE1172 and AFE1374) coding for putative c-di-GMP effector proteins with a PilZ domain were also identified (Table 1), indicating that downstream signalling may also occur in *At. ferrooxidans*.

Table 1 Identification of proteins possibly involved in the c-di-GMP pathway of *Acidithiobacillus ferrooxidans*

Gene	Identified domains	Predicted function
AFE0053	GGDEF/EAL	Diguanylate cyclase (DGC) with sensor domain (841 aa)
AFE1360	GGDEF/EAL	DGC /phosphodiesterase (PDE) with sensor domain (709 aa)
AFE1373	GGDEF/EAL	DGC /PDE (665 aa)
AFE1379	GGDEF/EAL	DGC /with sensor domains (1057 aa)
AFE1852	EAL	PDE (228 aa)
AFE1172	PilZ	PilZ effector (118 aa)
AFE1374	PilZ	PilZ effector (185 aa)

Secondary messenger c-di-GMP is present in *At. ferrooxidans*

The 344 *m/z* and 538 *m/z* MS/MS signals characteristic of c-di-GMP fragments (Fig. S3A) were identified in nucleotide extracts from *At. ferrooxidans* cells grown in thiosulphate medium (Fig. S3B), demonstrating that *At. ferrooxidans* synthesizes c-di-GMP molecules. Therefore, this bacterium possesses a functional DGC activity that could be associated with one or even several of the four proteins with GGDEF domains.

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All four *At. ferrooxidans* proteins with GGDEF⁄EAL domains have a DGC activity in *E. coli*

RT-PCR measurements revealed that AFE0053, AFE1360, AFE1373 and AFE1379 were transcribed (not shown). Thus, all genes encoding for both GGDEF⁄EAL domains were cloned and overexpressed in *E. coli,* and the four recombinant strains were tested for the cellulose-production phenotype, which is related to the intracellular levels of c-di-GMP, c-di-GMP synthesis and PDE activity.

On agar plates supplemented with CR, *E. coli* strains transformed with a plasmid harbouring a gene encoding for a DGC enzyme developed colonies with a characteristic red colour because of the binding of CR to cellulose (Spiers *et al.* 2003; Lee *et al.* 2007). Under our experimental conditions, no change was observed in the recombinant strain harbouring *At. ferrooxidans AFE0053* gene compared with the negative control (pET-21b) (Fig. 1a). However, the levels of CR-binding after IPTG induction increased 2-fold in strains harbouring *At. ferrooxidans* ORFs *AFE1360* and *AFE1373* (Fig. 1a), strongly suggesting that both genes code for DGC enzymes. As cloning of AFE1379 gene into pET-21b failed, it was cloned into the overexpression vector pBADTOPO and transformed into the *E. coli* TOP10 strain. To compare the result obtained from the pBADTOPO::AFE1379 construction with the previous results, genes AFE0053 and AFE1360 were also cloned in this overexpression vector. Results obtained with the pBADTOPO::AFE0053 and pBADTOPO::AFE1360 constructs (Fig. 1b) were similar to those previously seen when using pET21b (Fig. 1a). On the other hand, the strain harbouring the plasmid pBADTOPO::AFE1379 gave no differences compared with the control (Fig. 1b). C-di-GMP levels strongly increased in the four recombinant strains compared with those of control strains (Table 2; Fig. S4). These results strongly suggest that AFE0053, AFE1360, AFE1373 and AFE1379 code for active DGC proteins with different functional specificities resulting on CR-binding differences.

Apparent role of c-di-GMP pathway in the regulation of biofilm formation in *At. ferrooxidans*

C-di-GMP levels were determined in plancktonic and attached sulfur-grown bacterial subpopulations present in the same culture (Fig. 2a) and in *At. ferrooxidans* cells grown in different substrates (Fig. 2b). C-di-GMP levels increased 11-fold in cells conforming a biofilm on sulfur prills (568 ± 199·3 ng c-di-GMP per mg protein) compared with those of planktonic cells (56·4 ± 20·1 ng c-di-GMP per mg protein) harvested from the same culture (Fig. 2a). In agreement with this result, c-di-GMP levels were higher also (from 25- to 190-fold) in attached cells grown on solid substrates than in plancktonic cells grown on soluble substrate such as iron sulphate (Fig. 2b). These results strongly suggest that the c-di-GMP pathway is involved in biofilm formation in *At. ferrooxidans*.

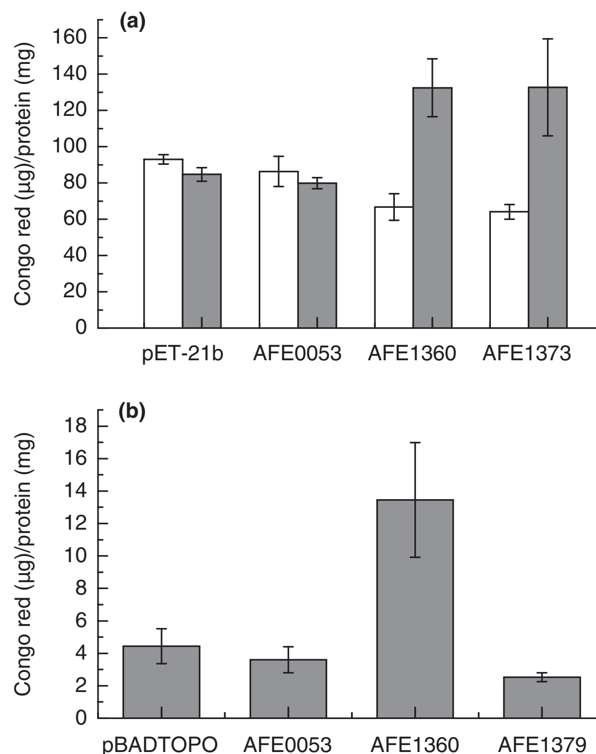

Figure 1 Phenotypic characterization of *Escherichia coli* BL21 (DE3) strain overexpressing genes coding proteins with GGDEF⁄EAL domains from *Acidithiobacillus ferrooxidans*. (a) Quantification of Congo red (CR)-binding to different *E. coli* BL21(DE3) strains expressing the genes coding for diguanylate cyclases (DGC)⁄ phosphodiesterase (PDE). White bars (without IPTG), black bars (with IPTG). (b) Quantitative analysis of CR-binding to *E. coli* TOP10 strains expressing *At. ferrooxidans* proteins with DGC⁄PDE domains cloned in pBADTOPO vector and induced by arabinose. As negative control pBADTOPO without insert was used.

Table 2 Relative quantities of *m⁄z* 344 signal evaluated by MS⁄MS analysis of nucleotide extracts from induced *Escherichia coli* strains transformed with *Acidithiobacillus ferrooxidans* genes cloned in expression vectors pET-21b or pBADTOPO

	C-di-GMP relative quantity
pET21b⁄BL21+ I	1
AFE0053::pET21b⁄BL21+ I	50
AFE1360::pET21b⁄BL21+ I	12
AFE1373::pET21b⁄BL21+ I	45
pBADTOPO⁄TOP10+ A	1 (ND)
AFE1379::pBADTOPO⁄TOP10+ A	>145

I, IPTG; A, arabinose; ND, not detected.

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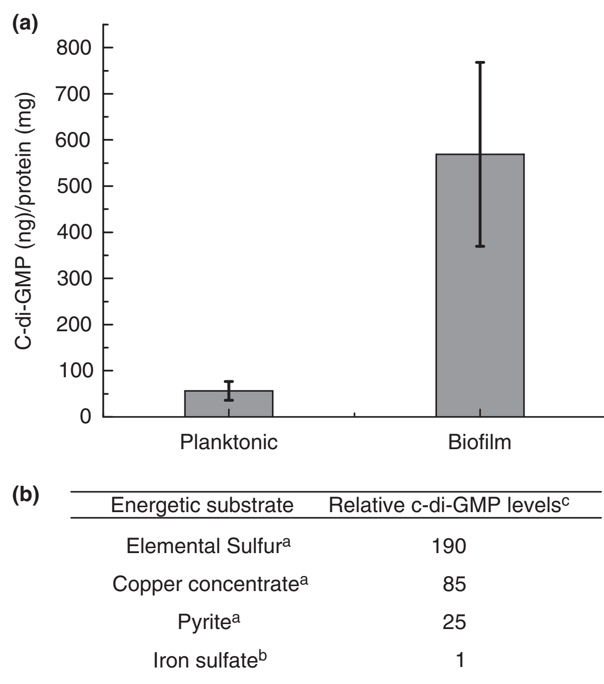

Energetic substrate	Relative c-di-GMP levels[c]
Elemental Sulfur[a]	190
Copper concentrate[a]	85
Pyrite[a]	25
Iron sulfate[b]	1

Figure 2 Analysis of c-di-GMP levels in *Acidithiobacillus ferrooxidans* by HPLC coupled to mass spectrometry. (a) *At. ferrooxidans* cells grown on sulfur (prills) medium. (b) *At. ferrooxidans* cells grown with different energy substrates. [a]Solid substrate, [b]Soluble substrate. Only attached cells were analysed when solid substrates were used. [c]Relative fold numbers are related to the basal level obtained in iron sulfate soluble substrate.

Discussion

Development of prokaryotic biofilms on mineral surfaces can contribute to enhanced leaching activities. This could be advantageous in biomining operations, but it is strongly negative for the environment in case of acid mine drainage generation (AMD). Therefore, studies to define new strategies to control biofilm formation are highly relevant (Farah *et al.* 2005; Ruiz *et al.* 2008). Except for our preliminary reports (Ruiz *et al.* 2007; Castro *et al.* 2009), no data were available for the c-di-GMP pathway in acidophilic leaching bacteria until now.

With only five ORFs involved in c-di-GMP metabolism, *At. ferrooxidans* genome, this signalling pathway in this bacterium has a lower complexity than that in several other bacterial species (Romling *et al.* 2005; Simm *et al.* 2007). In addition, as the four *At. ferrooxidans* proteins with GGDEF/EAL domains were able to synthesize c-di-GMP in *E. coli* recombinant strains (Fig. S4) and no change in PDE activity was detected (not shown), the PDE activity in *At. ferrooxidans* could be exclusively generated by the protein containing a single EAL domain (AFE1852).

The increased levels of c-di-GMP in cells adhered to solid energetic substrates compared with those in planktonic cells clearly indicate a role for the c-di-GMP pathway in biofilm formation by *At. ferrooxidans*. This is in agreement with previous results that by transcriptomic analysis revealed that proteins with GGDEF/EAL domains are preferentially expressed in the biofilm state of another Gram-negative iron-oxidizing bacteria (*Leptospirillum* spp.) living together with *At. ferrooxidans* in streamers (macroscopic biofilms) (Moreno-Paz *et al.* 2010). On the other hand, previous reports concluded that QS is also involved in regulation of biofilm formation by *At. ferrooxidans* (Farah *et al.* 2005; Rivas *et al.* 2005; Ruiz *et al.* 2008). All these results suggest that as it occurs in other Gram-negative bacteria (Ueda and Wood 2009), the QS system and the c-di-GMP pathway should be connected allowing *At. ferrooxidans* to switch from planktonic to biofilm lifestyle based on the integration of both environmental signals and cell density.

Twitching motility mediated by type IV pili and flagellar motility are regulated by proteins with the PilZ domain. In addition, twitching motility is required for the development of a biofilm structure (Kazmierczak *et al.* 2006; Guzzo *et al.* 2009; Paul *et al.* 2010). Results reported here allowed the identification of two putative c-di-GMP effector proteins with a PilZ domain. As conserved *fla* and *che* genes coding for flagella and chemotaxis functions respectively have not been identified in the genome of *At. ferrooxidans* (Valdes *et al.* 2008), c-di-GMP signalling pathway in this bacteria may be involved only in the regulation of twitching motility, controlling biofilm formation and mineral colonization.

Finally, this is the first global study on the c-di-GMP pathway in an acidophilic bacterium of great interest for the biomining industry. It opens a new way to explore molecular adaptation mechanisms in biomining microorganisms, especially on the regulation of the way of life of *At. ferrooxidans* in a biofilm state.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Sequence alignments of the four GGDEF domains from *Acidithiobacillus ferrooxidans* with known GGDEF domains. Essential amino acid residues (Schirmer and Jenal 2009) for diguanylate cyclases activity (blue stars) and c-di-GMP feedback inhibition (green and red stars) are noted. The figure was produced with ESPript (Gouet *et al.* 1999).

Figure S2 Sequence alignments of the five EAL domains from *Acidithiobacillus ferrooxidans* with TBD1265 from *Thiobacillus denitrificans*. Residue numbers and secondary structure at the top correspond to TBD1265. Partly and highly conserved residues are indicated by grey and black boxes, respectively. Essential residues for catalysis and structure of the active site (Tchigvintsev *et al.* 2010) are noted by blue stars. E546 strongly involved in phosphodiesterase activity (Tchigvintsev *et al.* 2010) is indicated by black dot. The figure was produced with ESPript (Gouet *et al.* 1999).

Figure S3 Production of c-di-GMP by *Acidithiobacillus ferrooxidans* cells grown in thiosulfate. (A) Synthetic c-di-GMP spectrograms. MS spectrogram shows a strong signal, 689 *m/z* ([M-H]-) (black diamond) that is equivalent to 690 g mol^{-1} of nominal mass. The 689 *m/z* signal was fragmented, and several specific signals (▼) were obtained out of which 344 *m/z* and 538 *m/z* were the strongest. (B) Spectrograms of nucleotides extracted from *At. ferrooxidans*. The second MS-step allowed the identification of the specific c-di-GMP fragments *m/z* 344 (343,9) and *m/z* 538.

Figure S4 HPLC–MS/MS analysis of nucleotidic extracts from *Escherichia coli* strains overexpressing *Acidithiobacillus ferrooxidans* GGDEF/EAL proteins. Higher levels of the specific fragments 344 *m/z* and 538 *m/z* (▼) were identified in all recombinant strains (C-E, G) compared with those in the controls (A, B and F).

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