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Diguanylate Cyclase Null Mutant Reveals That C-Di-GMP Pathway Regulates the Motility and Adherence of the Extremophile Bacterium *Acidithiobacillus caldus*

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Abstract

An understanding of biofilm formation is relevant to the design of biological strategies to improve the efficiency of the bioleaching process and to prevent environmental damages caused by acid mine/rock drainage. For this reason, our laboratory is focused on the characterization of the molecular mechanisms involved in biofilm formation in different biomining bacteria. In many bacteria, the intracellular levels of c-di-GMP molecules regulate the transition from the motile planktonic state to sessile community-based behaviors, such as biofilm development, through different kinds of effectors. Thus, we recently started a study of the c-di-GMP pathway in several biomining bacteria including *Acidithiobacillus caldus*. C-di-GMP molecules are synthesized by diguanylate cyclases (DGCs) and degraded by phosphodiesterases (PDEs). We previously reported the existence of intermediates involved in c-di-GMP pathway from different *Acidithiobacillus* species. Here, we report our work related to *At. caldus* ATCC 51756. We identified several putative-ORFs encoding DGC and PDE and effector proteins. By using total RNA extracted from *At. caldus* cells and RT-PCR, we demonstrated that these genes are expressed. We also demonstrated the presence of c-di-GMP by mass spectrometry and showed that genes for several of the DGC enzymes were functional by heterologous genetic complementation in *Salmonella enterica serovar* Typhimurium mutants. Moreover, we developed a DGC defective mutant strain (Δ*c1319*) that strongly indicated that the c-di-GMP pathway regulates the swarming motility and adherence to sulfur surfaces by *At. caldus*. Together, our results revealed that *At. caldus* possesses a functional c-di-GMP pathway which could be significant for ores colonization during the bioleaching process.

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Introduction

Acidithiobacillus caldus is an acidophilic, Gram-negative bacterium which gains energy by oxidation of elemental sulfur and reduced inorganic sulfur compounds [1]. Unlike others members of the *Acidithiobacillus* genus (*At. ferrooxidans*, *At. thiooxidans*, *At. albertensis*, *At. ferrivorans* and *At. ferridurans*), *At. caldus* is moderately thermophilic [1]. It has been isolated from many commercial biomining plants [2–5], together with the iron oxidizing bacteria belonging to the genus *Leptospirillum*, and dominates bacterial populations in processes operating within the temperature range of 35–50°C [2]. In bioleaching processes, ore dissolution can be achieved by two pathways, in which iron and sulfur oxidizing bacteria have specific roles [6–8]. As with other bioleaching bacteria, most *At. caldus* cells appear to attach to mineral surfaces [9]. In related bacterial species, such as *At. ferrooxidans* and *At. thiooxidans*, it has been shown that cell attachment to ore is determined by the presence of extracellular polymeric substances (EPS). EPS production by *At. ferrooxidans* has been directly correlated with bioleaching rates [10]. Even though a chemotactic response to sulfur gradients has been suggested [9], molecular mechanisms involved in the transition from planktonic to attached state by *At. caldus* are still unknown.

Quorum sensing (QS) and the cyclic diguanilate (c-di-GMP) pathway are the two main signalling mechanisms involved in the regulation of biofilm formation by bacteria. QS is a cell-to-cell signalling system mostly mediated by N-acyl homoserine lactones (AHL) that allows the coordination of bacterial behaviour in a cell-density-dependent manner. A functional QS system has been reported in the acidophilic bacterium *At. ferrooxidans* [11–12]. In this biomining bacterium, AHL-signaling molecules have been recently linked to exopolysaccharide (EPS) production and biofilm formation [13–14].

C-di-GMP is a second messenger mostly used by bacteria [15–19]. The characteristics of the phenotypes regulated by c-di-GMP signalling are still being discovered, however, it is well established that its main role is to control the switch between motile planktonic and biofilm-associated 'lifestyles' [20–22]. C-di-GMP is synthesized from two GTP molecules by DGCs and degraded by PDEs [23]. DGC activity is associated with GGDEF protein domains [24–25] while PDE activity is linked to two structurally unrelated domains named EAL [26] and HD-GYP [27]. To prevent GTP wastage, DGC activity is under non-competitive product inhibition [28–29]. The primary inhibition site (Ip) is composed of a RxxD motif [30–31]. Bacterial responses to intracellular levels of c-di-GMP are mediated by c-di-GMP effectors. To date, two kinds of c-di-GMP effectors have been identified: c-di-GMP binding proteins and RNA riboswitches [17, 32]. Most of the c-di-GMP effector proteins control biofilm development by stimulating EPS biosynthesis [33–40] and inhibiting bacterial motility devices [41–45]. PilZ protein domains are widespread and are the main characterized c-di-GMP effector-domain family [33, 46–48]. Proteins with a PilZ domain regulate several phenotypes such as flagellar motility, twitching motility mediated by type IV pili and synthesis of EPS. Some c-di-GMP effector proteins with a degenerated GGDEF domain use the RxxD motif from the Ip to bind c-di-GMP [35, 49–50]. Conserved global bacterial regulators control the expression of many GGDEF, EAL, and HD-GYP domain-type genes in response to external signals, linking c-di-GMP signalling not only with motility and biofilm phenotypes, but also with bacterial metabolism [51].

A functional c-di-GMP pathway has been recently reported in *At. ferrooxidans* [52]. An increase in c-di-GMP levels resulted in *At. ferrooxidans* cells adhering to solid energy-providing substrates more effectively, indicating a role in biofilm formation and suggesting that the QS and the c-di-GMP pathways could be connected in this microorganism as it is in other Gram-negative bacteria [52].

Most of the data regarding molecular mechanisms involved in EPS production and biofilm formation by bioleaching bacteria are from just one mesophilic, iron- and sulfur- oxidizing bacterium, *At. ferrooxidans*. Sulfur oxidizing species such as *At. thiooxidans* and *At. caldus* are fundamental players within the bioleaching community. In addition, as a moderate thermophile, *At. caldus* is of special interest. As no genes involved in known QS systems have, however, been identified by analysing the *At. caldus* genome sequences [53], we focused on the characterization of the c-di-GMP pathway in this sulfur-oxidizing specie to develop a global view on biofilm formation by the *Acidithiobacillus* genus. We have previously suggested that *At. caldus* could have a functional c-di-GMP signalling pathway [54]. We report here the initial characterization of several *At. caldus* DGC enzymes, by heterologous complementation experiments in *Escherichia coli* and *Salmonella* Typhimurium. An *At. caldus* mutant strain lacking the DGC ACAty_C1319 was constructed and a comparative analysis of motility and adherence phenotypes of the mutant and wild type strains was carried out by motility assays on semi-solid medium and microscopy observations. In this work, we provide the first evidence that it is possible to genetically manipulate key bioleaching phenotypes such as motility in *At. caldus*.

Materials and Methods

Bacterial Strains, Plasmids and Primers

Bacterial strains, plasmids and primers used in this work are listed in Table 1. *At. caldus* ATCC 51756 was grown in mineral salt liquid medium (MSM) [6] at pH 4.5 when thiosulfate (0.5 mg/ml) or tetrathionate (2 mg/ml) was used as energy source, while the pH was 2.5 when elemental sulfur (1 mg/ml) was used. Cultures were incubated in shake flasks at 45°C and 100 rpm. Growth curves were performed by a daily cell counting using a Petroff-Hausser chamber. Solid medium was obtained by adding Phytagel (Sigma-Aldrich) 0.9% (wt/vol) in tetrathionate liquid medium. Kanamycin (150 μg/ml final concentration) was added to liquid and solid media when selection was required. The mating medium was obtained by adding yeast extract 0.05% (wt/vol) to solid medium [55].

Bioinformatic tools

Genome sequence and predicted proteome of *At. caldus* ATCC 23270 (GenBank CP005986.1, CP005989.1, CP005988.1, CP005987) was obtained from the NCBI website (http://www.ncbi.nlm.nih.gov). Hidden Markov models (HMMs) of GGDEF, EAL, HD, and PilZ domains were obtained from Trust Sanger Institute server, Pfam. Searches were performed by HMMER 2.3.2 (http://hmmer.janelia.org), using HMMs as input and the *At. caldus* ATCC 51756 proteome as the database. HD-GYP domains were finally defined by the identification of the GYP signature motif inside HD positive results. To search for PelD, FleQ and Clp homologues in *At. caldus*, amino acid sequences (NCBI accession N° AAG06449.1, AAY47667 and AAC37124.1) were used as query in a BlastP analysis (http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi). Sequence analysis was performed by using Artemis software (Sanger Institute Pathogen Sequencing Unit). Potential candidate proteins identified with Artemis were used to formulate a BlastP search of the non-redundant database at NCBI (www.ncbi.nlm.nih.gov). Further characterization of putative proteins was performed employing bioinformatic tools in the Expert Protein Analysis System portal (http://www.expasy.org/). Transmembrane domain predictions were done by SubCell (http://www.cbs.dtu.dk/services/SubCell/abstract.php).

Table 1. Bacterial strains, plasmids and primers used in this study.

	Description	Referencee
Bacterial strains		
Acidithiobacillus caldus		
ATCC 51756	Wild type	1
Δc1319	ATCC 51756 ac*aty*_C1319::Km^r	This study
Salmonella Typhimurium		
UMR1	ATCC 14028–1 s Nal^r	53
AdrA1f	UMR1 adrA101::MudJ	54
MAE282	UMR1 STM1703::Cm^r	51
Escherichia coli		
MG1655	Standard reference strain F$^-$, λ$^-$, rph-1[a]	
AM70	MG1655 Δ*csgA*::cat[a]	50
LMG194	F$^-$ Δ*lac*X74 *gal*E*thirps*LΔ*pho*A (Pvu II) Δ*ara*714 *leu*::Tn*10*	Invitrogen
HB101	F$^-$ (*mcrC-mrr*) *hsdS20* (rB$^-$mB$^-$) *recA13 ara-14 proA2 lacY1*Δ$^-$ *galK2 rpsL20*(Sm^r) *Xyl-5 mtl-1 leuB6 thi-1 supE44*	58
Plasmids		
pBAD24	Arabinose-regulated expression vector, Amp^r	55
pBAD24AdrA	pBAD24::*adrA*	This study
pBAD24_C1319	pBAD24::*acaty_c1319*	This study
pBAD24_C1325	pBAD24::*acaty_c1325*	This study
pBAD24_C1328	pBAD24::*acaty_c1328*	This study
pTOPO	Control vector allowing direct cloning of PCR products	Invitrogen
pTOPOAdrA$_{wt}$	*adrA* gene cloned as PCR product into pTOPO vector	50
pTOPOYdaM	*ydaM* gene cloned as PCR product into pTOPO vector	50
pTOPOWspR	*wspR* gene cloned as PCR product into pTOPO vector	50
pTOPO_C1013	*acaty_c1013* gene cloned as PCR product into pTOPO vector	This study
pTOPO_C1184	*acaty_c1184* gene cloned as PCR product into pTOPO vector	This study
pTOPO_C1319	*acaty_c1319* gene cloned as PCR product into pTOPO vector	This study
pTOPO_C1325	*acaty_c1325* gene cloned as PCR product into pTOPO vector	This study
pTOPO_C1328	*acaty_c1328* gene cloned as PCR product into pTOPO vector	This study
pTOPO_C1360	*acaty_c1960* gene cloned as PCR product into pTOPO vector	This study
pGEM-T	Ap^r; T-tailed PCR product cloning vector	Promega
pOT	Apr; the 557-bp origin of transfer region from pR388 cloned into the *Eco*RI site of pUC19	58
pOT_*acaty_c1319*	Ap^r; A 2.2 kb fragment containing *acaty_c1319* cloned into *Xba*I site of pOT	This study
pOT_MUT_*acaty_c1319*::Km	Ap^{r}Km^r; the kanamycin resistance cassette from Tn*5* cloned into blunted, *Stu*I-*Bst*XI sites internal to *acaty_c1319* gene on pOT_ *acaty_c1319*	This study
pR388	TraWIncWTp^r Su^r	58
Primers		
MUT_*acaty_c1319*_F	GCTCTAGAGCTTTCGCGATGGATACAGC	
MUT_*acaty_c1319*_R	GCTCTAGAGCGACGAGTTCAGACAGGGC	
*acaty_c1013*_F	ACCTCTAGATAAGGAGGCGCGCTGATGAACCTTGGCGGA	
*acaty_c1013*_R	ACCAAGCTTCA*ATGATGATGATGATGATGATGATG*GGGGTAACTGGGCAAGAAC	
*acaty_c1184*_F	ACCTCTAGATAAGGAGGGCCTGCGATGCGCAAATCCGAT	
*acaty_c1184*_R	ACCGTCGACCA*ATGATGATGATGATGATGATGATG*CGAGGGTAATCCTTGGGAA	
*acaty_c1319*_F	ACCTCTAGATAAGGAGGTTGGAACATGGTCCGAACCAAA	
*acaty_c1319*_R	ACCAAGCTTCA*ATGATGATGATGATGATGATGATG*GCCCATGGAATAGCTGACT	
*acaty_c1325*_F	ACCTCTAGATAAGGAGGTCTCGACATGCGCTCCGATTCC	

(*Continued*)

Table 1. (*Continued*)

	Description	Referencee
*acaty_c1325*_R	ACCAAGCTTCA*ATGATGATGATGATGATGATGATG*GCAAATGCTCATACACCAA	
*acaty_c1328*_F	ACCTCTAGATAAGGAGGGCAAAGGATGACCGAAGCTCAG	
*acaty_c1328*_R	ACCAAGCTTCA*ATGATGATGATGATGATGATGATG*AACATGATCTCGGATCGCT	
*acaty_c1960*_F	ACCGTCGACTAAGGAGGTGGGCGCATGGCCAACTGTCTG	
*acaty_c1960*_R	ACCAAGCTTCA*ATGATGATGATGATGATGATGATG*GCCATGGATGTCTAGATCT	

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RNA extraction

At. caldus ATCC 51756 strain was grown in tetrathionate media as described above. Cells were harvested at late exponential phase and washed with acid water (pH 2.0). This was followed by alkaline lysis and RNA extraction by a modified hot-phenol method [56]. Briefly, two successive extractions with acid phenol (60°C), acid phenol/chloroform and chloroform were performed. RNA was precipitated overnight with 0.1 V of sodium acetate (3M) and 1 V of absolute ethanol. After centrifugation, RNA pellets were washed twice with ethanol at 70% and then treated with DNase I (Roche). Total RNA obtained was checked by 2% agarose gel electrophoresis and quantified by spectrometry.

C-di-GMP extraction

Cells of *Salmonella* from 5 ml of overnight cultures were collected by centrifugation. Supernatants were removed, and cellular pellets were washed twice with sterile water. Bacterial cells were then inoculated in M9 medium using glycerol (1%) as an energetic source, and immediately induced with L- arabinose (0.2%) for 4 hours. Cells were collected by centrifugation and were resuspended in 0.7 ml of sonication buffer 50 mM Tris, pH 8.5. After sonication, cell debris were removed by centrifugation and c-di-GMP was extracted as described previously [57]. Supernatants were acidified with 0.35 ml of 1.2 M $HClO_4$ (final concentration of 0.4 M) and then neutralized with 0.3 ml of 1.6 M K_2CO_3 on ice for 15 min before centrifugation at 12,000 × g for 10 min. New supernatants were filtered and concentrated at 60°C for 1 h by speedvac. For *At. caldus* cells, 400 ml of sulfur grown cultures were harvested by centrifugation and cells were washed twice with acidic water. Then, nucleotide extractions from *At. caldus* cells were performed as described above.

C-di-GMP detection and quantification

In order to measure c-di-GMP levels in *S.* Typhimurium recombinant strains overexpressing *At. caldus* genes *acaty_c1319*, *acaty_c1325* and *acaty_c1328*, 20 µL of nucleotidic extracts were injected into an HPLC system equipped with a diode-array detector. A 15-cm Supelcosil LC-18-DB, 3 µm particle size, reversed phase column was used. Elution was at room temperature (22°C) with a flow rate of 0.5 ml/min, starting with 10 min in buffer A (120 mM KH_2PO_4, pH 6.0), followed by a linear gradient rising to 20% methanol in 10 min, ending with 10 min in buffer A. C-di-GMP was determined by co-elution and identical UV absorption spectra with a c-di-GMP standard (Biolog, Bremen, Germany). C-di-GMP concentration was calculated based on a calibration curve ($y = 4.1204x$, $R^2 = 0.967$).

To increase the sensitivity of our measurements, nucleotidic extracts from *At. caldus* were analyzed by HPLC coupled to a mass spectrometer. Separation was performed on an Agilent 1290 HPLC System using a 2.0 × 50 mm Keystone Scientific Nucleosil C18 RP column (Sigma-Aldrich) at the Center for Metabolomics and Mass Spectrometry of The Scripps Research

Institute, California, EE.UU. Briefly, nucleotidic extracts were dried by lyophilization and then solubilized in 10 mM ammonium acetate. HPLC separations were performed with a 10 mM ammonium acetate solution and a methanol gradient. The injection volume was 5 μL and the flow rate was 0.4 mL/min throughout the chromatographic run. The detection of c-di-GMP was performed on an Agilent 6490 triple quadrupole mass spectrometer equipped with an electro spray ionization source using selected reaction monitoring analysis in a positive ionization mode. Therefore, transitions m/z 691>152 and 691>135 were used for quantification and signal specificity, respectively.

Molecular cloning of *At. caldus* genes encoding proteins with GGDEF domains

Genomic DNA from *At. caldus* strain ATCC 51756 was used as template for PCR experiments. The *acaty_c1013*, *acaty_c1184*, *acaty_c1319*, *acaty_c1325 acaty_c1328* and *acaty_c1960* genes encoding proteins with single DGC domains were amplified using specific primers containing restriction sites for cloning purposes (Table 1). The PCR products were separated by electrophoresis and directly purified from agarose gel with the specific Wizard kit (Promega), and cloned independently in pGEM-T plasmids (Promega). The recombinant plasmids were transformed into *E. coli* strain LMG194. The purified recombinant plasmids obtained by Miniprep Wizard kit (Promega) were digested with suitable restriction endonucleases. DNA-restriction fragments containing *At. caldus* genes were separated by electrophoresis, recovered from agarose gel and cloned into pTOPO (*acaty_c1013*, *acaty_c1184*, *acaty_c1319*, *acaty_c1325 acaty_c1328* and *acaty_c1960*) and pBAD24 (*acaty_c1319*, *acaty_c1325*, *acaty_c1328*) vectors. The pTOPO and pBAD24 recombinant plasmids harbouring *At. caldus* genes were transformed into the *E. coli* AM70 and *Salmonella enterica* serovar Typhimurium AdrA1f strains, respectively.

Congo red binding assay

S. Typhimurium and *E. coli* cells were grown 48 h at 28°C on LB agar plates without salt and supplemented with Congo Red (CR) (40 μg/ml) and Coomassie brilliant blue G-250 (10 μg/ml). L(+)-arabinose (0.2%) and IPTG (1 mM) were used to overexpress *At. caldus* genes cloned in pBAD24 and pTOPO, respectively. Ampicillin (100 μg/ml), chloramphenicol (20 μg/ml) and kanamycin (30 μg/ml) were added to selective media.

Construction of an *acaty_c1319* suicide plasmid

A suicide plasmid was constructed as previously described [55]. A 2.2 kb fragment containing the *acaty_c1319* gene was amplified by the PCR using specific primers with *Xba*I restriction sites. This fragment was pre-cloned in pGEM-T Easy (Promega) and excised by *Xba*I digestion. In parallel, the Ori-T containing vector pOT (56) was digested with *Xba*I. Digested pOT and *Xba*I-fragments were ligated by T4 ligase (Promega) to give pOT_*acaty_c1319*. This construct was digested with *Stu*I and *Bst*XI, removing almost the entire GGDEF domain, and the ends were filled in using T4 DNA polymerase (Fermentas). A 1.45 kb *Hin*dIII-*Sma* fragment from pSKM2 [55] carrying the kanamycin resistance cassette from Tn*5* was blunt-end cloned into the blunted sites of pOT_*acaty_c1319* to give pOT_MUT_*acaty_c1319*::Km. For conjugation experiments, pOT_MUT_*acaty_c1319*::Km was transformed into *E. coli* HB101 cells that contained pR388 by using kanamycin and trimethoprim resistance selection. Then, the mobilizable suicide vector was transferred from *E. coli* HB101-R388 to *At. caldus* by conjugation.

Conjugation

At. caldus ATCC 51756 was cultured in thiosulfate liquid medium at 37°C for 5 days. *E. coli* HB101 cells were pre-adapted overnight in 50 ml of thiosulfate medium pH 4.5 supplemented with yeast extract 0.05% (wt/vol) at 37°C. Fifty ml of *E. coli* HB101 containing the suicide plasmid, and 500 ml of *At. caldus* were collected by centrifugation and washed twice with mineral salts medium (MSM). *E. coli* HB101 (pOT_MUT_*acaty_c1319*::Km) and *At. caldus* cells were resuspended separately in 250 µl of MSM and mixed 1:1. 100 µl of the mix was spread onto a Supor 0.2 µm filter (PALL Gelman Laboratory) and placed on the mating medium. After 5 days of incubation at 37°C, cells were harvested by scraping growth from the filters with a loop, washed twice in mineral salts solution and inoculated into 500 ml of thiosulfate liquid medium supplemented with kanamycin (150 µg/ml). Cultures were incubated at 37°C for seven days in shake flasks with aeration. Cells were diluted appropriately, plated on solid thiosulfate media with kanamycin (150 µg/ml), and cultivated for 6 days until the colonies appeared.

Screening for single or double crossover mutants

To assess whether they were single or double crossovers, screening of transconjugants was done by colony and Southern blot hybridizations [55]. Kanamycin resistant colonies of *At. caldus* obtained from conjugation assays were replicated on selective solid media and incubated at 37°C for 6 days before blots were performed. Hybridization was under stringent conditions with a probe specific for the kanamycin resistance cassette [55]. Following this step, hybridized blots were stripped of the kanamycin probe and re-probed with a 520-bp *acaty_c1319* internal fragment (GGDEF probe). Both probes were labelled by PCR with digoxigenin according to the manufacturer's recommendations (Roche). Signals obtained with both probes were considered as single-crossover mutants, while signals obtained only with the kanamycin probe corresponded to double-crossover mutants.

Phenotypic assays in *At. caldus*

Wild type and Δ*c1319* strains of *At. caldus* were grown using sulfur coupons as an energy source. Attachment was evaluated indirectly by counting of planktonic cells. In parallel, sulfur coupons were fixed and prepared for scanning electron microscopy (SEM) and fluorescence microscopy (FM) as described by González et al. [13]. For FM visualizing, adhered cells were fixed in 4% p-formaldehyde and stained with 4,6-diamidino-2-phenylindole (DAPI) at 1 mg/mL. Finally, coupons were washed in sterile water, dried at room temperature and imaged. Two coupons and five fields per coupon were observed for each sample. To perform the motility assay, 10 µl of supernatant from cultures extracted during exponential phase were spotted on semi-solid media (phytagel 0.2%, pH 4.7 with tetrathionate as an energy source). Motility was evaluated by measuring the area occupied by bacterial cells after incubation for 7 days at 45°C.

DNA manipulations and sequencing

Plasmid preparation, restriction endonuclease digestions, gel electrophoresis, ligations, and Southern blot/colony hybridizations were performed using standard methods [58]. All the plasmid constructs were checked by automatic DNA sequencing (MACROGEN, Korea).

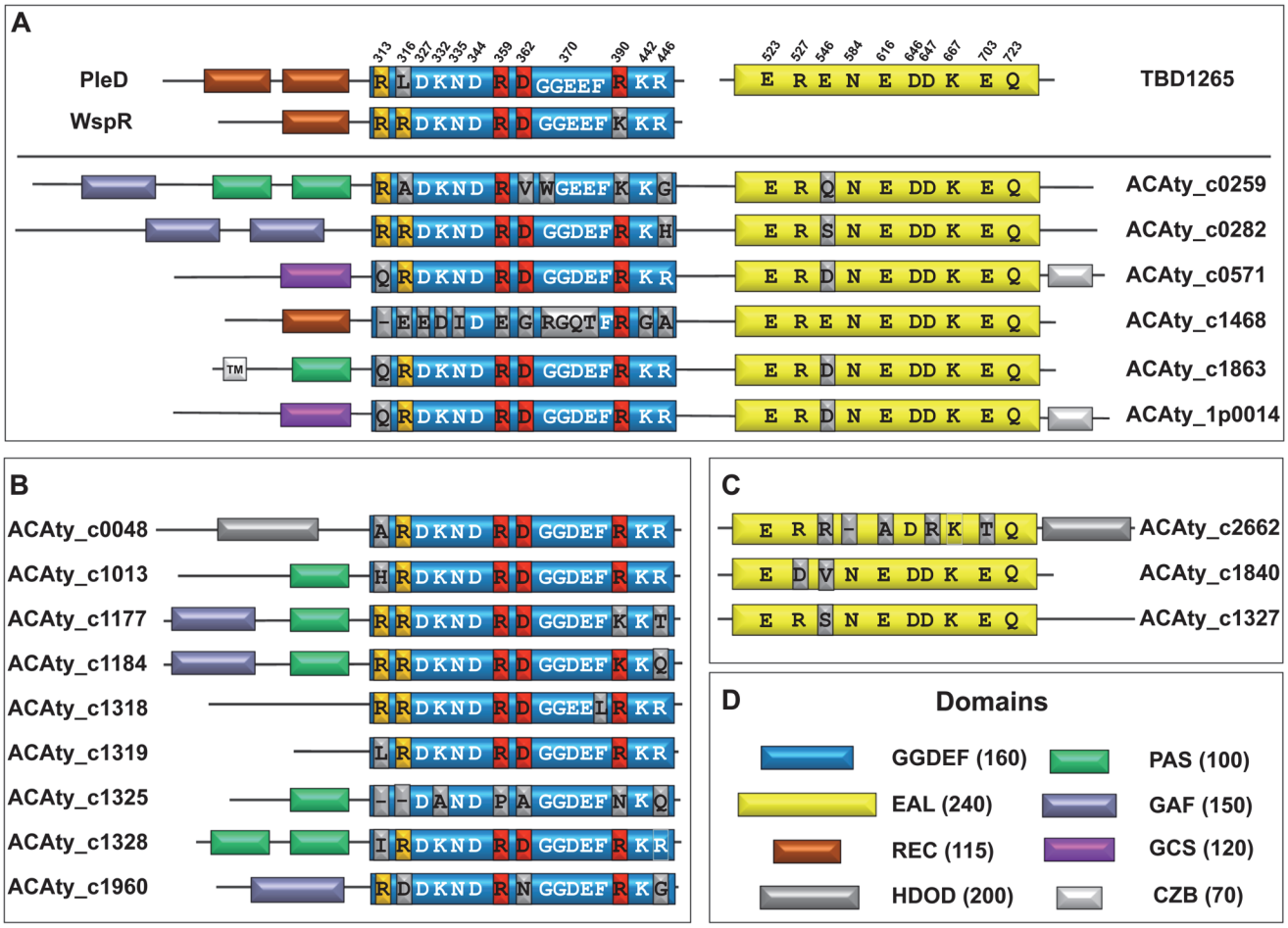

Fig 1. Domain organization and key amino acids of *At. caldus* proteins involved in c-di-GMP metabolism. Schematic representations of (**A**) GGDEF/EAL-, (**B**) single GGDEF- and (**C**) single EAL-domain containing proteins from *At. caldus* ATCC 51756. Color codes for the different domains are noted (**D**). Numbers in brackets indicate the average number of amino acids for each domain. GGDEF domains (blue) are compared to GGDEF domains of PleD and WspR from *C. crescentus* and *P. aeruginosa*, respectively (A, upper, left). EAL domains (yellow) are compared to the EAL domain of TBD1265 from *T. denitrificans* (A, upper, right). Numbers above the domains indicate key residues involved in DGC and PDE activities. Primary inhibition site (Ip) and secondary inhibition site (Is) residues of GGDEF domains are depicted in red and orange, respectively. Non-conserved amino acids are shown in gray. Domains are not drawn to scale. TM, transmembrane segment.

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Results

In silico identification of essential genetic components for a functional c-di-GMP signalling pathway in *At. caldus*

The bioinformatic analysis of the *At. caldus* ATCC 51756 genomic sequence allowed the identification of eighteen open reading frames (ORFs) related to c-di-GMP metabolism. Nine of them encode proteins with a single GGDEF domain (ACAty_c0048, ACAty_c1013, ACAty_c1177, ACAty_c1184, ACAty_c1318, ACAty_c1319, ACAty_c1325, ACAty_c1328, ACAty_c1960), three with a single EAL domain (ACAty_c1327, ACAty_c1840 y ACAty_c2662), and six with both GGEDEF and EAL domains (ACAty$_{1p}$0014, ACAty_c0259, ACAty_c0282, ACAty_c0571, ACAty_c1468, ACAty_c1863) (Fig. 1). Sensor domains PAS (Period circadian protein, Aryl hydrocarbon receptor nuclear translocator protein and Single-minded protein), GAF (cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA), REC (Response regulator receiver domain), Protoglobin and CZB (Chemoreceptor zinc-binding domain) have also been predicted

Table 2. C-di-GMP genes identified from available *Acidithiobacillus* genomes.

	At. ferrooxidans		*At. thioxidans*	*At. caldus*		*At. ferrivorans*
	ATCC 23270	**ATCC 53993**	**ATCC 19377**	**ATCC 51756**	**SM-1**	**SS3**
GGDEF	N.D.	1	9	9	8	3
EAL	1	1	3	3	3	N.D.
GGDEF-EAL	4	3	12	6	6	15
HD-GYP	N.D.	N.D.	1	N.D.	N.D.	1
PilZ	2	2	9[a]	9[a]	6[a]	6[a]
PelD	N.D.	N.D.	1	1	1	N.D.

N.D., Not Detected.

a, BcsA subunit of cellulose synthase is one of the PilZ domains.

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for most of these proteins. All these sensor domains have been reported to play an important regulatory role in c-di-GMP signalling [25, 59–68]. No HD-GYP domains were identified from the *At. caldus* genome suggesting that PDE activity in *At. caldus* is likely to be generated by proteins containing only an EAL domain. On the other hand, nine ORFs encoding putative c-di-GMP effector proteins with a PilZ domain, and one encoding a PelD-like effector have been identified (Table 2). In addition, ACAty_c1318 could act as an effector protein since it possesses a conserved RxxD motif and an altered GGDEF motif (Fig. 1), where a phenylalanine substitution for leucine (GGDEF→GGDEL) should inhibit its DGC activity completely as occurs in the DGC WspR from *P. aeruginosa* [69]. Except for one of the PilZ effectors, in our experimental conditions RT-PCR assays revealed that all these c-di-GMP related ORFs were transcribed (S1 Fig.).

Detection of c-di-GMP in wild type strain ATCC 51756

To determine the presence of DGC activity, nucleotide extracts from *At. caldus* were analyzed by electrospray Ionization-Ion Trap mass spectrometry (ESI-IT). As described by Ruiz et al. (2012), negative polarity analysis allowed the identification of 344 m/z and 538 m/z MS/MS signals characteristic of c-di-GMP fragments (results not shown). This indicated that *At. caldus* possesses functional DGC activity as suggested by the previous identification of fifteen proteins with GGDEF domains.

Analysis of the DGC activity of *At. caldus* proteins a with single GGDEF domain

It is well established that production of curli fibers and cellulose depends on c-di-GMP biosynthesis mediated by specific DGCs YdaM (curli fibers) in *E. coli* [70–71] and AdrA (cellulose) in *S.* Typhimurium [72–73]. Based on the presence of key amino acids [29, 31, 69], eleven GGDEF domains (ACAty$_{1P}$0014, ACAty_c0048, ACAty_c0282, ACAty_c0571, ACAty_c1013, ACAty_c1177, ACAty_c1184, ACAty_c1319, ACAty_c1328, ACAty_c1863, ACAty_c1960) from *At. caldus* were predicted as active DGC while four (ACAty_c0259, ACAty_c1318, ACAty_c1325, ACAty_c1468) should be inactive DGC. To gain insight into c-di-GMP synthesis by *At. caldus*, six proteins with a single GGDEF domain (ACAty_c1013, ACAty_c1184, ACAty_c1319, ACAty_c1325, ACAty_c1328, ACAty_c1960) were assessed for DGC activity through Congo Red (CR) phenotype assays. CR is a diazo-dye with strong affinity for amyloid fibers, such as curli fibers produced by Enterobacteria and for polysaccharides such as cellulose. Genes *acaty_c1013*, *acaty_c1184*, *acaty_c1319*, *acaty_c1325*, *acaty_c1328* and *acaty_c1960*, were

cloned and overexpressed in an *E. coli* AM70 strain. The different recombinant strains were tested for cellulose-production phenotype (Fig. 2). Positive results were obtained for *acaty_c1184*, *acaty_c1319*, *acaty_c1328* and *acaty_c1960* genes indicating that they code for functional DGC enzymes. An intermediate result was obtained with the *acaty_c1013* gene and the *E. coli* reporter system. Under our experimental conditions, no significant change was observed in the *E. coli* recombinant strains harbouring *At. caldus* gene *acaty_c1325* compared to the vector control without insert. This is in agreement with bioinformatic predictions that revealed the loss of two key amino acids involved in catalysis and GTP binding in the GGDEF domains of ACAty_c1325. The c-di-GMP signalling pathway has been associated with specific and compartmentalized metabolic activity [15, 74, 75]. To exclude the possibility that the negative result obtained with *acaty_c1325* was due to the reporter system, *acaty_c1325* was also cloned and overexpressed in *S.* Typhimurium AdrA1f (Fig. 3) to assess the multicellular red, dry and rough (rdar) morphotype which is also expressed upon the presence of a DGC activity [76]. In addition, to compare to the result obtained in the *E. coli* reporter system, *S.* Typhimurium recombinant strains harbouring *acaty_c1319* and *acaty_c1328* genes were also constructed (Fig. 3). In agreement with the results obtained with *E. coli*, recombinant strains *S.* Typhimurium *acaty_c1325* also failed to restore rdar morphotype while *acaty_c1328* and *acaty_c1319* genes did so partially and completely, respectively. Similar results were confirmed in calcofluor (CF) binding assays, which monitors the expression of cellulose (S2 Fig.). Altogether, these results indicate that *acaty_c1325* codes for an inactive DGC. As neither partner domains nor inhibition sites were identified in the GGDEF domain of ACAty_c1325, its function is still uncertain. Recently, the crystal structure of XCC4471^{GGDEF} from *X. campestris* showed that a dimer of GGDEF domains was joined through c-di-GMP dimer molecules located at the active sites [77]. The GGDEF domain of ACAty_c1325 could therefore still play a functional role as a c-di-GMP effector domain.

Construction of a Δ*c1319* null mutant

In both reporter systems, the highest positive results were obtained with the *acaty_c1319* gene (Fig. 4), indicating that this may encode a key DGC enzyme for *At. caldus*. No genetic transfert techniques are currently available in *Acidithiobacillus* sp. However, two recent reports described the construction of a specific null mutant gene in *At. caldus* [55, 78]. To assess whether this DGC enzyme is related to a specific phenotype in *At. caldus*, the construction of a *At. caldus* Δ*c1319* null mutant strain was challenged by using a marker exchange strategy as described by Van Zyl et al. [55]. Recombinant colonies were checked for single or double crossover events (S3 Fig., A) by colony blot hybridization (S3 Fig., B). From eighty colonies giving a positive result with the specific kanamycin probe, twenty-one gave a negative result with the specific internal GGDEF probe (S3 Fig., B) suggesting that these colonies were true double-crossover mutants. To confirm the correct location of the DNA exchange, Southern blot and PCR analysis were performed using genomic DNA from wild type as well as two potential double-crossover *At. caldus* clones (called Δ*c1319*_6 and Δ*c1319*_10) and the specific kanamycin probe (S3 Fig., C). Two specific restriction enzyme-digested DNA fragments (7.2 kb and 8.0 kb for *Nhe*I and *Eco*RI, respectively) corresponding to a double-crossover event were obtained for both mutated clones, while a negative result was observed with DNA from the wild type strain (S3 Fig., A and C). In addition, due to the replacement of a small piece of the *acaty_c1319* gene by the kanamycin resistance gene, PCR allowed the amplification of 2.8 kb DNA fragments from both mutated clones compared to the 1.8 kb DNA-fragment obtained from the wild type strain (not shown).

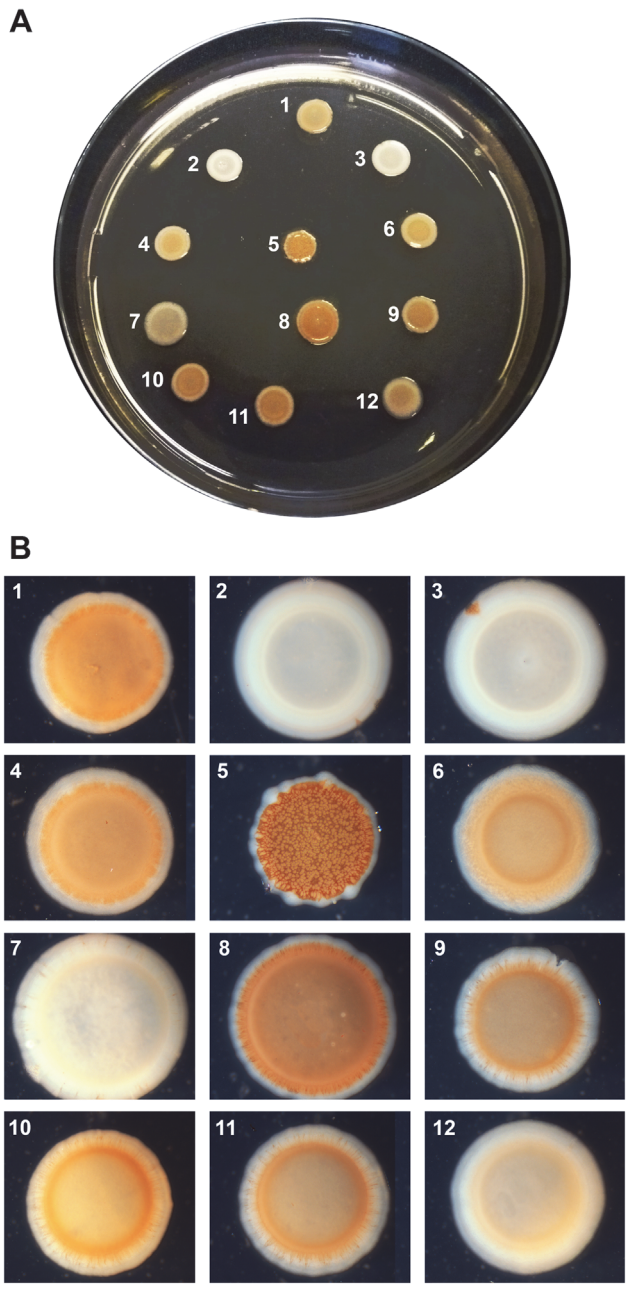

Fig 2. Heterologous complementation analysis in E. coli of the diguanylate cyclase activity from single GGDEF domains encoded by At. caldus genes. The ability of GGDEF-domain proteins to synthesize c-di-GMP and to induce a Red phenotype on a Congo red agar plate (**A**) by the *E. coli* MG1655 wild type strain (1), AM70 diguanylate cyclase null-mutant strain (2) and *E. coli* AM70 strains transformed with pTOPO2.1 vector carrying different *At. caldus* genes [*acaty_c1325* (7), *acaty_cC1319* (8), *acaty_c1328* (9), *acaty_c1960* (10), *acaty_c1184* (11), and *acaty_c1013* (12)] encoding GGDEF single domains were compared. A negative vector control without DNA insert (3) and three different diguanylate cyclase-carrying strains [YdaM (4), AdrA (5) or WspR (6)] were also analyzed. 10X magnification of each drop are shown in (**B**).

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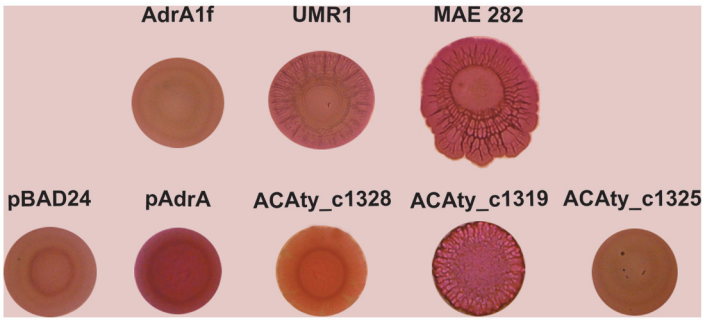

Fig 3. Heterologous complementation of Salmonella Typhimurium AdrAf1 strain by GGDEF-proteins ACAty_c1319, ACAty_c1325 and ACAty_c1328 from At. caldus. *S*. Typhimurium AdrA1f [Adr (DGC) null mutant] was complemented with pBAD24 plasmids harboring *At*. *caldus* genes coding for ACAty_c1319, ACAty_c1325 and ACAty_c1328 proteins. rdar morphotype was analyzed on Congo red agar plates and compared to wild type (UMR1), positive control (pAdrA), negative control (pBAD24 without insert) and a phosphodiesterase null mutant (MAE 282) strains.

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Comparative analysis of wild type and Δc1319 strains

Growth. No differences were observed when comparing the growth curves of wild type and both Δc1319 mutants in tetrathionate liquid media during 4 days (not shown).

C-di-GMP levels. The levels of c-di-GMP were decreased by 13.75-fold in the Δc1319_6 mutant (0,36 ± 0,10 pmol of c-di-GMP per mg protein) compared to the wild type strain (4,95 ± 2,05 pmol of c-di-GMP per mg protein) as indicated in Fig. 5. A similar decrease (6.6-fold) was observed with the Δc1319_10 mutant. Thus, in our experimental conditions the DGC ACAty_c1319 was responsible for 85% to 93% of total c-di-GMP.

Motility. As no motility assays have been previously described for any *Acidithiobacillus* species, a specific semi-solid medium including phytagel and tetrathionate as gelling agent and energy source, respectively, was developed to analyse *At*. *caldus* motility. Results clearly indicated that the Δc1319 mutants have a higher motility than the wild type strain (Fig. 6).

Attachment to sulfur coupons. Direct cell counting of liquid supernatants from cultures with elemental sulfur as energy source revealed that Δc1319_6 and Δc1319_10 planktonic cell numbers were higher compared with the wild type strain (Fig. 7A). To assess if these planktonic-cell number differences were related to the ability to attach on sulfur coupons, fluorescence and scanning electron microscopy analyses were performed (Fig. 7B). Similar results were obtained with both Δc1319 mutants. Both microscopy analyses clearly revealed that wild type strain (Fig. 7B, upper) was capable to develop microcolonies on sulfur coupons while it was possible to image only few isolated cells of mutant strains (Fig. 7B, lower). These results strongly suggested that the ability to attach on sulfur coupons by Δc1319 cells is lower compared with wild type cells.

Discussion

In this study a bioinformatic, biochemical and genetic characterization of the c-di-GMP pathway of the acidophile *At*. *caldus* has been carried out. This is the first time that a DGC mutant strain of a bioleaching bacterium has been made and analysed.

An understanding of the number of genes involved in the c-di-GMP metabolism and their role in various environments faced by different bacteria is still lacking. In *Vibrio cholerae* in which c-di-GMP plays a key role in regulating changes in gene expression that occur during the shift from aquatic to host environments, 61 proteins (12 EAL, 30 GGDEF, 9 HD-GYP, and 10 GGDEF-EAL) related to c-di-GMP metabolism have been identified [79–80]. On the other

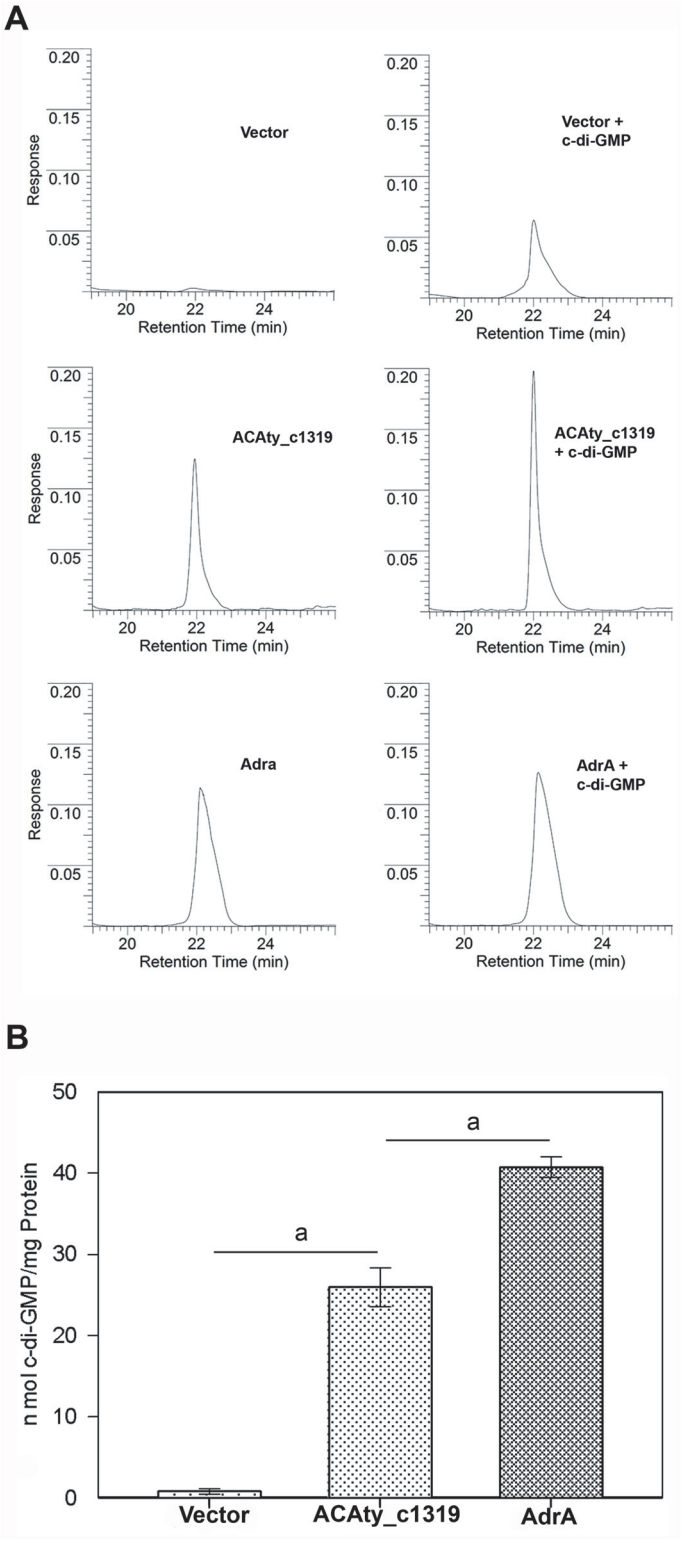

Fig 4. ACAty_c1319 is a functional diguanylate cyclase (DGC) enzyme. A. Cell extracts from recombinant strains of *S*. Typhimurium AdrA1f transformed with the pBAD24 plasmid vector without DNA insert, or harboring *At*. *caldus acaty_c1319* or *adrA* genes were supplemented with 2 nmol of synthetic c-di-GMP (**right panel**) or not (**left panel**) and analyzed by HPLC. **B**. HPLC quantifications of c-di-GMP intracellular levels. Values with standard deviations were obtained from triplicates. a, Statistical significance was determined by ANOVA ($p < 0.05$).

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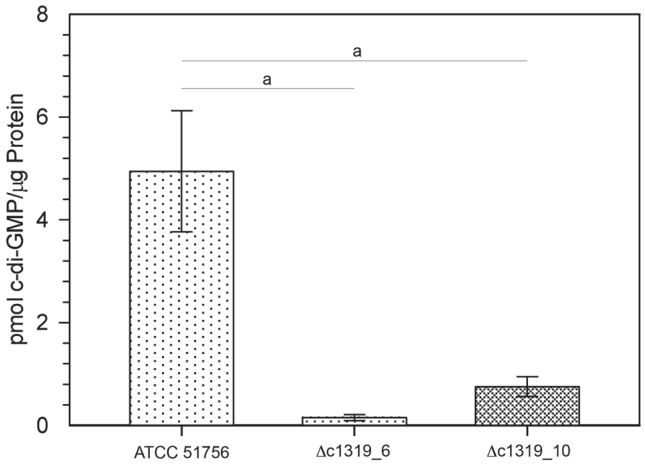

Fig 5. HPLC-MS/MS quantification of c-di-GMP intracellular levels in the *At. caldus* Δc1319 mutants. Values with standard deviations were obtained from triplicates. a, Statistical significance was determined by ANOVA ($p < 0.05$).

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extreme, *Mycobacterium smegmatis* has a single bifunctional protein with both GGDEF and EAL domains that plays a central role in long-term survival under conditions of nutritional starvation [81–82]. In this study, bioinformatic, biochemical and genetic strategies have been developed to characterize the c-di-GMP pathway of the acidophilic bacterium *At. caldus*. Eighteen ORFs (9 GGDEF, 3 GGDEF/EAL, 6 EAL) related to the metabolism of c-di-GMP have been identified. This number is higher than in *At. ferrooxidans* ATCC 23270 in which only five ORFs have been identified [52]. As a comparative analysis of genome sequence was aimed at predicting behavioural properties [83], we also searched for GGDEF and EAL domain proteins in other available genome sequences from *Acidithiobacillus* species (*At. caldus* SM-1, *At. ferrooxidans* ATCC 53993, *At. thiooxidans* ATCC 19377, *At. ferrivorans*). Our results revealed that the c-di-GMP metabolism pattern differs not only among species but also between strains from the same specie (Table 2). These results suggested that the complexity of c-di-GMP metabolism cannot be directly associated with the acidophilic ecological niche. However, proteins with single GGDEF and EAL domains and the PelD effector (Table 2) suggest that the global organization of the c-di-GMP pathway in sulfur-oxidizing *Acidithiobacillus* species is different to iron-sulphur species. Interestingly, in the course of identification of molecular mechanisms involved in biofilm formation in bioleaching bacterial species a canonical quorum sensing (QS) system has been identified in the iron-sulfur oxidizing specie *At. ferrooxidans* [11] while in both sulfur-oxidizing bacteria, specific genes related to QS are absent [53].

As a chemolithoautotrophic bacterium, *At. caldus* may require large quantities of energy to support carbon fixation, whereas molecular oxygen is the most suitable electron acceptor [84]. As found in other Gram-negative bacteria [78], in addition to their DGC and PDE domains, *At. caldus* enzymes involved in c-di-GMP metabolism possess sensor domains located in their N-terminal regions (Fig. 1). Interestingly most of them are PAS, GAF, and Protoglobin domains that are related to oxygen sensing and have been reported to regulate DGC or PDE activity in other bacteria [59–62, 65, 67, 68]. Hence, molecular oxygen availability may be an important stimulus to control intracellular levels of c-di-GMP in *At. caldus*.

Comparative assays between the wild type strain and the Δc1319 strain revealed that the presence of DGC ACAty_c1319 inhibits flagellar motility and is required for attachment on solid substrates (Figs. 6, 7). In the course of attempting to understand how both phenotypes

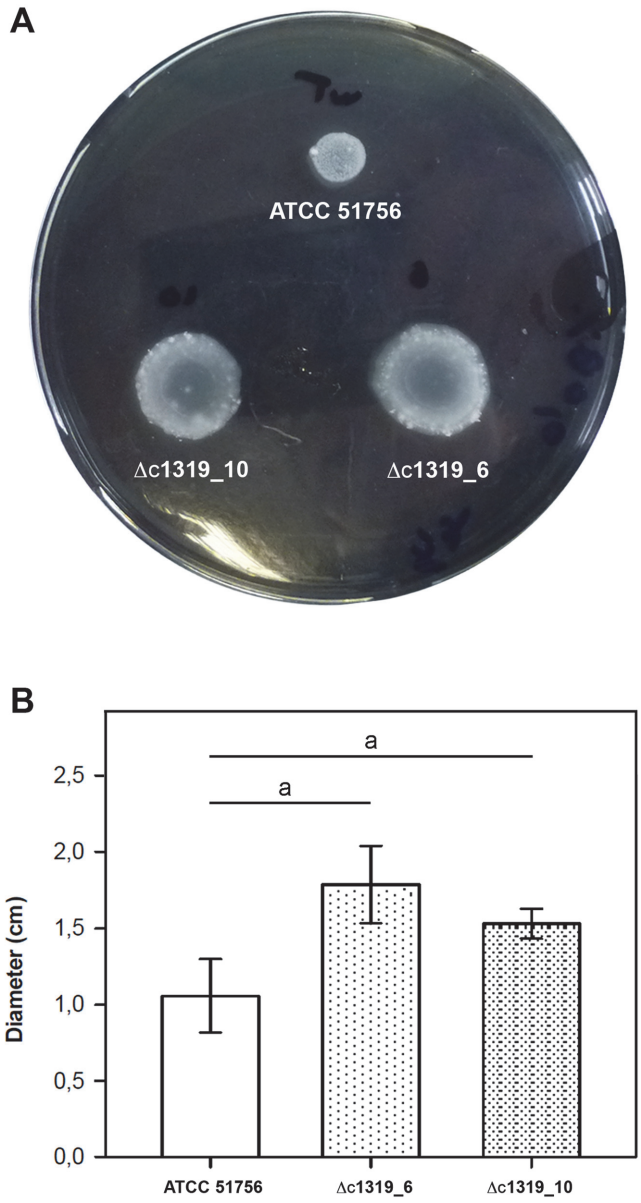

Fig 6. C-di-GMP modulates motility behavior in *At. caldus*. A, Motility behavior of *At. caldus* Δc1319 mutants on tetrathionate plates compared with wild type strain. **B**, Diameters of motility zones of *At. caldus* wild type ATCC 51756 and mutant Δc1319 strains after 96 h of incubation at 45°C. The error bars indicate standard deviations obtained from three independent trials. a, Statistical significance was determined by ANOVA ($p < 0.05$).

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could be regulated, several key c-di-GMP effectors have been singled out. Nine genes encoding proteins containing PilZ domains were identified in *At. caldus* ATCC 51756 (Table 2), seven of which have key amino acids for c-di-GMP binding. Among them, are four proteins related to type IV pili (T4P) assembly and one with significant similarity (27%) to YcgR. T4P has been shown to be involved in twitching motility in several bacterial species [85] whereas the binding of c-di-GMP by the YcgR protein family down-regulates the flagellar motor through interactions with flagellum components MotA and FliL located in the inner membrane of different Gram-negative bacteria [42–45]. Thus the location of orthologous flagellar genes *fliL*, *motA*,

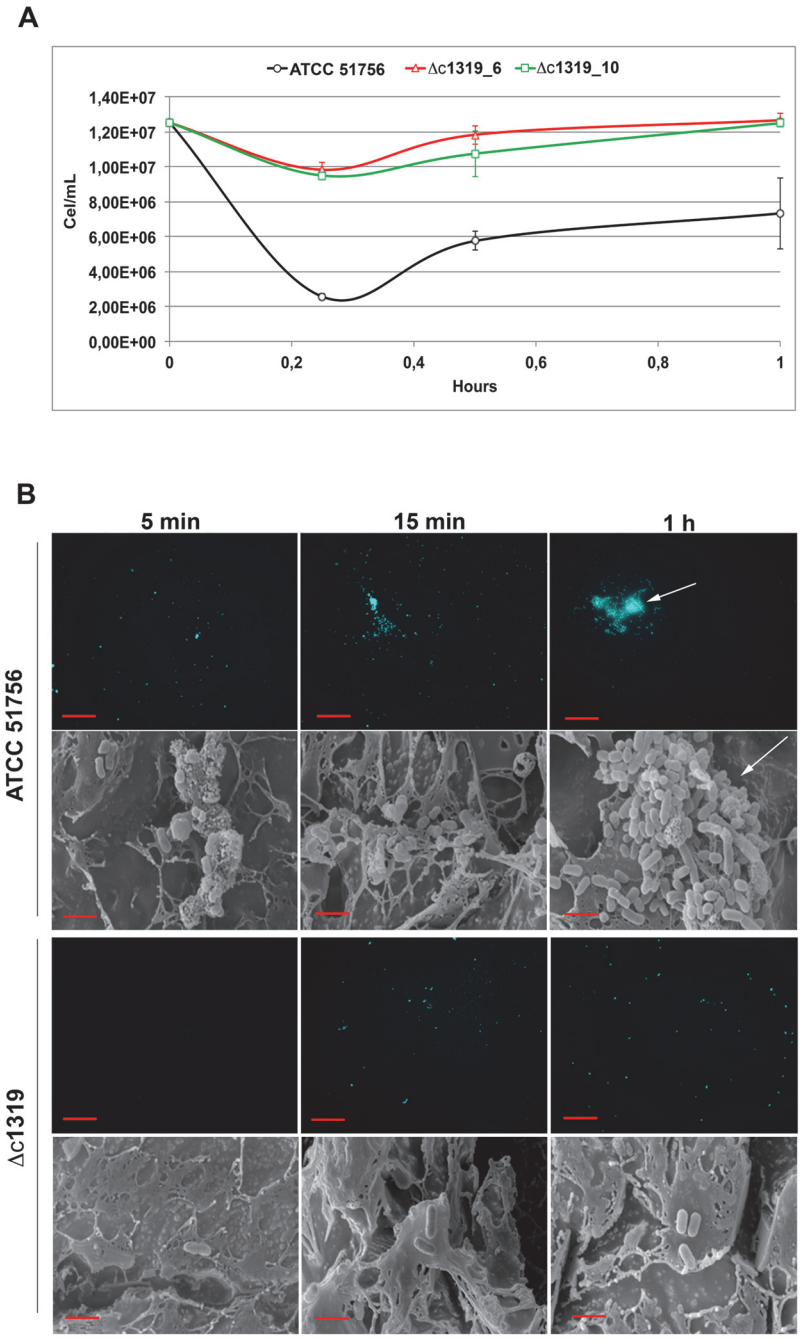

Fig 7. The ability to attach on sulfur coupons by At. caldus cells is decreased in Δc1319 mutant strains. A. Indirect determination of cell attachment by counting of remaining planktonic cells. **B**. Microscopy analysis of cell attachment on sulfur coupons by *At*. *caldus* wild type (upper) and Δc1319 mutant (lower). Sulfur coupons were retrieved from cultures at different times and visualized by scanning electron microscopy (SEM; 20 000X) and fluorescence microscopy (FM; 400X). As described by González et al. [13], cells attached on sulfur coupons were previously stained with 4′,6-diamidino-2-phenylindole (DAPI) for FM visualizing. Microcolonies are indicated by white arrows. Size bars represent 10 μM (FM) and 1 μM (SEM).

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and *motB* upstream of *acaty_c1319* gene (S4 Fig.) strongly suggests that DGC ACAty_c1319 is involved in flagella functioning [44–45]. On the other side and immediately downstream of *acaty_c1319*, the *acaty_c1318* gene is predicted to encode a putative c-di-GMP effector protein with an inactive GGDEF domain. However, further studies are necessary to determine if FliL, MotAB, *ACAty_c1318* and *ACAty_c1319* are part of the same c-di-GMP regulatory module [15]. Regarding biofilm formation and EPS synthesis, orthologues of BcsA-BcsB (S5 Fig.) and PelD (S6 Fig.) effector proteins have been also identified. BcsA possesses a PilZ domain and as a subunit of the bacterial cellulose synthase [86] is responsible as a BcsA-BcsB complex for the synthesis of cellulose, an EPS frequently found in biofilms. PelD belongs to the Pel machinery and regulates PEL polysaccharide synthesis [35, 87]. As a complete *pel* operon including genes from *pelA to pelG* has been identified in *At. caldus* (S6 Fig.) and in this predicted PelD-like protein all of the key amino acids for c-di-GMP binding are conserved, we hypothesize that the synthesis of the Pel EPS occurs in *At. caldus* in a similar way that *P. aeruginosa*. In addition, a gene coding for an UDP-glucose 4-epimerase has also been identified at the 3' end of this *pel* operon. This could be involved in the synthesis of PEL exopolysaccharide precursor. Moreover, it appears that such *pel*-like EPS operons are present only in the sulfur-oxidizing species *At. caldus* and *At. thiooxidans* while *bscAB* operon is present in both sulfur-oxidizing species and the iron-oxidizing species *At. ferrivorans* (Table 2).

Taken together these results demonstrate a specific role for the c-di-GMP pathway and Pel EPS for biofilm formation by a sulfur-oxidizing *Acidithiobacillus* species and comprise the first steps in deciphering the amazing cell network generated by bacterial acidophilic cells adhered to solid energetic substrate (S7 Fig.). However further genetic and biochemical studies are still necessary to understand if all these filaments protruding from cell bodies act cooperatively during cell attachment on solid energetic substrate and to determine if *At. caldus* is capable to produce specific bacterial adhesives such as the holdfast described in *Caulobacter crescentus* [88].

Supporting Information

S1 Fig. Expression analysis of *At. caldus* genes involved in c-di-GMP metabolism and signal transduction. Total RNA was extracted from *At. caldus* cells grown on elemental sulphur. All cDNA were synthesized from 1 μg of purified RNA by reverse transcriptase (+) and used as template for a conventional PCR with specific primers to amplify a 200–250 bp DNA fragment corresponding to the c-di-GMP synthesis (GGDEF), degradation (EAL) and signal transduction (PilZ and PelD) domains. (-) control without reverse transcriptase. The 200 bp fragment-size of DNA ladder is indicated by a red dot. The *acaty_c1319* gene encoding a DGC functional enzyme is indicated by a box.
(TIFF)

S2 Fig. Heterologous complementation of S. Typhimurium AdrA1f strain by GGDEF-proteins ACAty_c1328, ACAty_c1319 and ACAty_c1325 from At. caldus. *S. typhimurium* AdrA1f [Adra (DGC) null mutant] was complemented with pBAD24 plasmids harboring *At. caldus* genes coding for ACAty_c1328, ACAty_c1319 and ACAty_c1325 GGDEF-proteins. Cellulose synthesis phenotype was analyzed by monitoring fluorescence intensity on Calcofluor (CF) agar plates compared to wild type (UMR1), positive control (pAdrA), negative control (pBAD24 without insert) and a phosphodiesterase null mutant (MAE 282) strains. The binding of CF to extracellular matrix was evident from the fluorescence intensity emitted under U.V. light.
(TIFF)

S3 Fig. Confirmation of Δc1319 mutants by hybridization analysis. A, Schematic representation of the different possibilities for single and double recombination events of pOT_MUT_*aca_1319*::Kan suicide plasmid into the *At. caldus* genome. *Eco*RI and *Nhe*I restriction enzyme sites are given for comparison with results obtained by Southern blot analysis. Red boxes indicate locations of specific probes 1 and 2. **B**, Colony blot analysis. **C**, Southern blot analysis. Genomic DNA from wild type (WT) and Δ*1319_6* and Δ*1319_10* mutant strains of *At. caldus* was purified and digested by *Eco*RI and *Nhe*I. DNA fragments were separated by agarose gel electrophoresis and then transferred to a nitrocellulose membrane for hybridization with probe 2 (kanamycin) labelled with digoxigenin. Positive signals were obtained for clones 6 (lanes 5–7) and 10 (lanes 8–10) of Δ*1319* mutant strains while no signals were detected for WT strain (lanes 2–4). The sizes of positive *Eco*RI and *Nhe*I DNA fragments (lanes 6, 7, 9 and 10) correspond to double crossover events. DNA of the pOT_MUT_*acaty_c1319*:Kan plasmid employed for the mutagenesis was used as a positive control for hybridization (lanes 11 and 12). **ND**, not-digested. **MW**, Molecular weight.
(TIFF)

S4 Fig. Genomic context of acaty_1319 gene from At. caldus ATCC 51756. Color code: gene coding for proteins with a predicted functional GGDEF (blue), EAL (yellow) and no functional GGDEF (cyan) domains and proteins involved in flagellar motility (green).
(TIFF)

S5 Fig. Genomic organization of putative cellulose synthase operon from At. caldus ATCC 51756. Amino acid sequences of BcsA-BcsB subunits forming the catalytic core of cellulose synthase from *Glucoacetobacter xylinus* E25 and *At. caldus* have been compared. Percentage identity (I) and similarity (S) are noted. UGPU, UTP-glucose-1-phosphate uridylyltransferase.
(TIF)

S6 Fig. Pel-like operon in At. caldus ATCC 51756. Amino acid sequences of the different proteins belonging to the *pel* operon from *At. caldus* and *P. aeruginosa* have been compared. Percentage identity (I) and similarity (S) are noted.
(TIF)

S7 Fig. Biofilm network mediated by filament projections emerging from At. caldus cells grown on a sulfur coupon. Cells are able to directly contact other cells through filament projections that look like to holdfasts described in *Caulobacter crescentus* [88]. In several cases, connections appear to be achieved by more than one filament (blue arrows). Red arrows indicate putative sensing points between cooperative filaments from different cells and/or breaking points in several filaments. A putative holdfast-like extremity of a stalk protruding from the cell body is indicated by a yellow arrow.
(TIF)

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Author Contributions

Conceived and designed the experiments: MC NG. Performed the experiments: MC LR SMD. Analyzed the data: MC SMD DER NG. Contributed reagents/materials/analysis tools: DER NG. Wrote the paper: MC SMD DER NG.

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