 doi:10.20944/preprints201807.0520.v1

Article

The Response of *Cupriavidus Metallidurans* CH34 to Cadmium Involves a Decrease in Intracellular Levels of C-Di-GMP Probably Mediated by a Novel Metal Regulated Phosphodiesterase That Inhibits the Biofilm Lifestyle

Pablo Alviz-Gazitua [1,2,3], Sebastián Fuentes [4], Luis Rojas [2,5], Raymond J. Turner [6], Michael Seeger [2,*] and Nicolás Guiliani [1,*]

1. Bacterial Communication Laboratory, Biology Department, Faculty of Sciences, Universidad de Chile, Ñuñoa, Santiago, Chile. alviz.pablo@gmail.com
2. Molecular Microbiology and Environmental Biotechnology Laboratory, Chemistry Department & Center of Biotechnology, Universidad Técnica Federico Santa María, Valparaíso, Chile.
3. PhD program of Microbiology, Faculty of Sciences, Universidad de Chile, Ñuñoa, Santiago, Chile.
4. Microbial Ecology of Extreme Systems Laboratory, Biological Sciences Faculty, Pontificia Universidad Católica de Chile, Santiago, Chile.
5. Chemistry Department, Universidad Católica del Norte, Avenida Angamos 0610, Antofagasta CL-1240000, Chile
6. Biofilm Research Group, Department of Biological Sciences, University of Calgary, Calgary, Canada.

* Correspondence: michael.seeger@gmail.com, michael.seeger@usm.cl; Tel.: +56-322654223 & nguilian@uchile.cl; Tel.: +56-229787241. These authors contributed equally to this work.

Abstract: Cadmium is a highly toxic heavy metal for biological systems. *Cupriavidus metallidurans* CH34 is a model strain for heavy metal resistance and bioremediation. The aim of this study was to determine the role of the c-di-GMP pathway in the *C. metallidurans* CH34 response to cadmium in both planktonic and biofilm cells. Increasing cadmium concentrations correlates with an inhibition of biofilm formation and EPS production in *C. metallidurans* cells. Planktonic and biofilm cells showed similar tolerance to cadmium. During exposure to cadmium an acute decrease of c-di-GMP levels in planktonic and biofilm cells was observed. Transcription analysis by RT-qPCR showed that cadmium induced in planktonic cells and strongly induced in biofilm cells the expression of the *urf2* gene and the mercuric reductase encoding *merA* gene, which belong to the Tn*501*/Tn*21 mer* operon. After exposure to cadmium the *cadA* gene involved in cadmium resistance was equally upregulated in both lifestyles. Bioinformatic analysis and null mutant complementation assays indicated that the protein encoded by the *urf2* gene is a functional phosphodiesterase involved in the c-di-GMP metabolism. We propose to rename the *urf2* gene as *mrp* gene for metal regulated phosphodiesterase. An increase of the second messenger c-di-GMP content by the heterologous expression of the constitutively active diguanylate cyclase PleD* correlated with an increase in biofilm formation and cadmium susceptibility. These results indicate that the response to cadmium in *C. metallidurans* CH34 involves a decrease in c-di-GMP content that inhibits the biofilm lifestyle.

Keywords: c-di-GMP; *Cupriavidus metallidurans;* cadmium; phosphodiesterase; biofilm; *urf2* gene; *mer* gene; PleD.

 doi:10.20944/preprints201807.0520.v1

1. Introduction

Cadmium is a highly toxic heavy metal for biological systems. Cadmium induces indirectly oxidative stress, disturbs manganese acquisition and depletes the cellular reduced thiols pool [1-3]. This heavy metal is also carcinogenic [4]. Global cadmium production has risen >1,000 fold in the last century due to its incorporation to diverse manufacture applications [1]. The Word Health Organization has included cadmium in the top ten of the "Chemicals of major Public Health concern" list [4].

The remediation of heavy metals in polluted waters and soils is a major challenge for sustainable development. Microorganisms are used to immobilize and/or transform heavy metals into less toxic forms [5,6]. Heavy metal bioremediation strategies are commonly carried out in fixed bed reactors wherein the formation of attached multicellular communities – i.e. biofilm – is crucial. Biofilm cells produce extracellular polymeric substances (EPS) such as polysaccharides, lipids, proteins and extracellular DNA, providing different functional groups that may bind heavy metal ions via passive biosorption processes that immobilize heavy metals and protect cells [7]. Biofilms are also involved in bioreduction processes trough electrically conductive components [8-10]. Biofilms has been described as an advantageous lifestyle promoted by toxic heavy metals or compounds, which allow the selection of resistant cells [7]. However, in contrast to the bacterial response to antibiotics, planktonic and biofilm cells are in most cases almost equally susceptible to heavy metal oxyanions and cations [8,11]. Consequently, the minimum biofilm-eradication concentration (MBEC) is often not higher than the minimum bactericidal concentration (MBC) observed under planktonic growth conditions. Specific bacterial biofilms are even more susceptible to oxyanions than planktonic cells. The effect of the heavy metals on biofilm depend on the bacterial strain, the metal and the culture medium [11–13].

Biofilm formation in response to environmental conditions is mediated by the second messenger bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) [14]. Intracellular c-di-GMP content depends on the balance between the enzymatic activities of diguanylate cyclases (DGCs) and phosphodiesterases (PDEs) that catalyze the formation and the degradation of c-di-GMP, respectively. DGC activity depends on the characteristic protein domain GGDEF [15], whereas PDE activity is mediated by the two motifs EAL and HD-GYP [16,17]. In addition to the catalytic motifs, DGCs and PDEs contain accessory motifs responsible for the regulation of the activity under different conditions. At high intracellular c-di-GMP content, it interacts with a broad spectra of c-di-GMP binding effectors that can be riboswitches and a wide diversity of proteins. The effectors include degenerate GGDEF/EAL domain proteins [18,19] and PilZ domain proteins [20]. The c-di-GMP binding with effectors triggers a reduction of cell motility and an increase of EPS matrix synthesis, which promote biofilm formation [18,19,21]. However, the link between the c-di-GMP signaling pathway, bacterial lifestyle and heavy metal tolerance has not been explored.

Cupravidus metallidurans CH34 is a model heavy metal-resistant bacterium that has been used for bioremediation, recovery and reduction of heavy metals [22–24]. *C. metallidurans* CH34

 doi:10.20944/preprints201807.0520.v1

harbors numerous heavy metal resistance mechanisms, including complexation, efflux systems, reduction and reductive precipitation, which enable cell detoxification and survival [25-27]. A high number of CH34 genes confer tolerance to high concentrations of heavy metals, including cadmium [25]. Nevertheless, molecular mechanisms driving the bacterial lifestyle in presence of heavy metals have not been studied. The aim of this study was to determine the role of the c-di-GMP pathway in the *C. metallidurans* CH34 response to cadmium in both planktonic and biofilm cells.

2. Materials and Methods

2.1 Organisms, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *C. metallidurans* strains were cultured in low phosphate Tris-buffered mineral salts (LPTMS) broth (Tris 6.06 g L^{-1}, NaCl 4.68 g L^{-1}, NH_4Cl 1.07 g L^{-1}, KCl 1.49 g L^{-1}, $Na_2SO_4 \cdot 10H_2O$ 0.98 g L^{-1}, $MgCl_2$ $6H_2O$ 0.2 g L^{-1}, $CaCl_2 \cdot 2H_20$ 0.03 g L^{-1}, Na_2HPO_4 0.085 g L^{-1}, Fe(III)(NH_4) citrate 0.005 g L^{-1}, succinate 0.3 %, and 1 mL L^{-1} of SL7 trace solution of Biebl and Pfennig [22]). *Escherichia coli* and *Pseudomonas aeruginosa* strains were cultured in LB broth (yeast extract 5 g L^{-1}, peptone 10 g L^{-1}, NaCl 5 g L^{-1}). Plasmids pJB3Tc19 and pJB:*pleD** [28] were donated by Juan San Juan research group (EEZ-CSIC, Granada, Spain).

2.2 Biofilm formation assay

C. metallidurans CH34 cell attachment in cadmium presence was assessed by an adapted protocol developed to monitor initial stages of biofilm formation [29]. Briefly, a stationary culture in LPTMS broth at 30 °C and 150 rpm was centrifuged and the cells were suspended in fresh LPTMS broth to an optical density at 600 nm of 1.0. This culture was distributed into a 96-well microtiter plate by placing 200 µL per well. A concentrated cadmium solution was further added to reach different concentrations. After 15 h of incubation at 30 °C in static condition, planktonic cells were removed. Wells were washed with sterile distilled water and the biofilm adhered to the bottom was stained with crystal violet (200 µL per well, 0.1 % solution for 15 min). Plate was air-dried for 16 h and the stained biomass was suspended in 200 µL of acetic acid 30%. Absorbance of this suspension was measured either at 550 or 595 nm. This value was normalized by the OD_{600} of the planktonic fraction removed previously. Viability of the each planktonic cell fraction was quantified by serial dilution and plating by triplicate on tryptic soy agar (15 g L^{-1} pancreatic digest of casein, 5 g L^{-1} peptic digest of soybean meal, 5 g L^{-1} sodium chloride, 15 g L^{-1} agar), where the colony forming units (CFU) were counted.

Table 1. Plasmids and strains used in this study

Plasmids	Description	Reference
pJBTc19	Ap^r, Tc^r; expression vector	[28]

pJBTc19:*urf2.2*	Ap^r, Tc^r; pJBTc19 derivate bearing a 1423 bp *Xba*I/*Eco*RI fragment containing *RMET_RS31035*	This work
pJBTc19:*pleD**	Ap^r, Tc^r; pJBTc19 derivate bearing a 1423 bp *Xba*I/*Eco*RI fragment containing *pleD**	[28]

Strains	Description	Reference
C. metallidurans CH34	Heavy metal multiresistant strain	[22]
CH34 pJBTc19	CH34 strain transformed with an empty vector	This work
CH34 pJBTc19:*urf2.2*	CH34 strain transformed with pJBTc19:*urf2.2* construct	This work
CH34 pJBTc19:*pleD**	CH34 strain transformed with pJBTc19:*pleD** construct	This work
P. aeruginosa PAO1	Opportunistic pathogen	[30]
Δ*rocR* pJBTc19	*roc*R null mutant PAO1 strain transformed with pJBTc19	This work
Δ*rocR* pJBTc19:*urf2.2*	*roc*R null mutant PAO1 strain transformed with pJBTc19:*urf2.2*	This work

2.3 Qualitative assay for extracellular polymeric substances

The assessment of cadmium effect on EPS synthesis by *C. metallidurans* CH34 macrocolonies was carried out in salt free LB agar with Congo Red 50 μg mL^{-1} and Coomassie Blue 10 μg mL^{-1} [31]. Plates were incubated for five days at room temperature and visualized with an Olympus MVX10 lens.

2.4 Analysis of biofilm and planktonic lifestyles of C. metallidurans CH34

For comparisons between biofilm and planktonic lifestyles, two aliquots of an LPTMS broth culture (10^8 cells mL^{-1}) were taken in parallel. For biofilm growth, 1 mL was vacuum filtered through a 0.2-μm polycarbonate filter (Whatman) and the filter was placed on LPTMS agar 0.8 % with the cell filtrate facing upwards. The second 1-mL aliquot was inoculated into 9 mL of fresh LPTMS broth for planktonic growth. Both were incubated at 30 °C, without shaking and at 150 rpm, respectively. This procedure synchronized both lifestyles cultures starting from the same growth phase and biomass. After 13 h at 30 °C, cultures were exposed to 2 mM cadmium (Figure S1), either by translating the filter to a new LPTMS agar with cadmium (biofilm cells) or by directly adding a cadmium concentrated solution (planktonic cells). After 45 min incubation in presence of cadmium, cells were processed for c-di-GMP determination or qRT-PCR.

2.5 Extraction and quantification of c-di-GMP

After incubation in presence or absence of cadmium, 9 of the 10 mL of planktonic culture were collected, leaving 1 mL for total protein content determination. To discard sample processing bias, biofilm cells were suspended in a mixture of 10 mL of LPTMS, separating a 1-mL aliquot for total protein content determination. Cells in suspension were collected by centrifugation at 1,920 x g for 10 min at 4 °C. As suggested by the quantification service provider, pellets were suspended in 300 μL of extraction buffer (acetonitrile:methanol:water,

2:2:1) into 2-mL micro tube vials and incubated 10 min at 95 °C followed by cooling on ice. Cells lysates were centrifuged at 16,000 x g for 10 min at 4 °C and supernatants were transferred into new microtube vials. Extractions were repeteated twice omiting the heat incubation step. Combined supernatants of 3 extraction steps (about 700 µL) were stored at -20 °C overnight. Prepitation of remaining cellular debris was made by centrifugation at 16,000 x g for 10 min at 4 °C. Finally, supernatants were evaporated to dryness in Speed-Vac at room temperature. Nucleotide extraction from planktonic cells of *P. aeruginosa* strains followed the same procedure.

Quantification of c-di-GMP was achieved by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) at BIOLOG Life-Sciences Institute (Bremen, Germany).

2.6 Susceptibility Tests in Calgary Biofilm Device

C. metallidurans cultures in the Calgary Biofilm Device were performed according to indications elsewhere specified [32]. Briefly, colonies grown in LPTMS agar during 48 h incubation at 30 °C were suspended in NaCl 0.9% to reach a turbidity equivalent to McFarland standard 1.0 (Thermo R20421). The suspension was diluted 15 times in LPTMS broth, loaded into the Calgary Biofilm Device (Innovotech) and incubated for 24 h at 150 rpm and 30 °C. Biofilms adhered to the lid pegs were separated from the planktonic fraction that stayed into the well. Cadmium susceptibility was determined by CFU counting in tryptic soy agar. MBC and MBEC were defined as the cadmium concentration killing 99.9 % of the original population [7], starting from the fitted exponential decay curve achieved by non-linear regression in Prism v.5 (GraphPad software, San Diego-CA, USA). Determinations were carried out in two independent experiments, each in triplicate.

2.7 Functional prediction of genes encoding DGCs, PDEs and PilZ domain effectors in C. metallidurans CH34 genome

A list of genes coding for diguanylate cyclases (DGCs) and phosphodiesterases (PDEs) in *C. metallidurans* CH34 genome were retrieved from the NCBI website http://www.ncbi.nlm.nih.gov/Complete_Genomes/c-di-GMP.html, according to NCBI annotations. GGDEF, EAL and HD-GYP domain proteins coding genes were analysed. The catalytic functionality of each protein was evaluated by analyzing the conservation of the active amino acid residues in domain sequence motifs: residues Asp327, Lys332, Asn335, Asp344, Asp370, Lys442 and Arg446 for GGDEF; residues Glu523, Arg527, Glu546, Asn584, Glu616, Asp646, Asp647, Glu703 and Gln723 for EAL, and residues His183, Asp184, His212, His237, Glu (His)238, Asn(Glu)265, Asn (Arg)269 for HD-GYP, using as reference functional domains from PleD (GGDEF) of *Caulobacter crescentus* [15], TBD_1265 (EAL) of *Thiobacillus denitrificans* [16] and Bd1817 (HD-GYP) from *Bdellovibrio bacteriovorus* [17], respectively.

The c-di-GMP binding capability of the PilZ domains of *C. metallidurans* CH34 was predicted based on the conservation of the "c-di-GMP switch" (RxxxR & D/NzSxxG), which undergoes the conformational change that wraps the c-di-GMP molecule [20]. The PilZ domain

that integrates PA4608 of *P. aeruginosa* was used as active form model [21], whereas the PilZ domain present in XC1028 of *Xanthomonas campestris* was used as inactive form reference [33]. The search of c-di-GMP effectors related to *Burkholderia cenocepacia* Bcam1349 [34] and *P. aeruginosa* PelD in *C. metallidurans* CH34 genome was assessed by local alignment tools and pairwise sequence alignment.

Motif conservation in GGDEF and EAL domains was determined by multiple alignments in Clustal W [35], whereas motif conservation in PilZ domain was determined in multiple alignment by MAFFT method [36]. Motif conservation was visualized using Jalview software (http://www.jalview.org). Orthology inference was achieved through sequence similarity searching using BlastP algorithm [37] and confirmed by conservation of domains architectures obtained from NCBI´s CDART tool [38].

2.8 RNA extraction and reverse transcription

After incubation of cells in presence or absence of cadmium, 10 mL planktonic culture was mixed with 2 mL "stop solution" (water-saturated phenol 5 % (pH < 7.0), ethanol 95 %) [39,40]. To discard sample processing bias, biofilm cells were suspended in a mixture of 10 mL LPTMS and 2 mL stop solution. For both samples, cells were then collected by centrifugation at 1,920 x g for 10 min at 4 °C and suspended in 700 µL of lysis buffer (sodium acetate 20 mM, EDTA 1 mM, SDS 0.5%, β-mercaptoethanol 1%, pH 5.5) and incubated 5 min at room temperature. Preheated acid phenol (700 µL) was added, mixed by inversion, and incubated 10 min at room temperature. Mixtures were centrifuged at 16,000 x g for 5 min at 4 °C. The aqueous phase was mixed with 1 volume of acid phenol:chloroform:isoamyl alcohol (25:24:1)and incubated for 5 min at room temperature . The mixture was centrifuged at 16,000 x g for 5 min at 4 °C and the aqueous phase was mixed with 1 volume chloroform, incubated 5 min at room temperature, and centrifuged at 16,000 x g for 5 min at 4 °C. The resulting aqueous phase was mixed with 1 volume isopropanol and 0.1 volume sodium acetate 3 M (pH 5.2). The mixture was incubated overnight at -20 °C. RNA was precipitated through centrifugation at 16,000 x g for 30 min at 4 °C and washed twice with ethanol 70 % (v/v). RNA samples were air dried, suspended in nuclease free water, and treated with TURBO DNAse (AM1907) following manufacturer's instructions. Absence of genomic DNA was controlled by PCR of the the *cadA* gene with the same primers used for qPCR (Table S1) Reverse transcription reactions were carried out using 500 ng of RNA with the ImProm II Reverse Transcription system (Promega A3800) with random primers, following manufacturer's instructions.

2.9 Quantitative polymerase chain reaction of cDNA

Quantitative PCR was carried out with the Fast SYBRGreen Master Mix (Applied Biosystems, 4385612) in a qPCR Step One machine (Applied Biosystems, 271003314). Primers (Table S1) were used at 0.5 µM each. PCR conditions included a hot start step at 95 °C for 20 s, followed by 40 cycles of two steps amplification (95 °C for 3 s, 60 °C for 30 s). For each condition, three independent experiments each with at least two technical replicates

were conducted. Bacteria incubated in absence of cadmium was used as the control condition. Ct values were normalized to expression of housekeeping *rpoZ* and *gyrB* genes as described [41]. Fold changes were assessed by one sample t test. Fold changes outside of [+1,-1] range (P-values below 0.05) were significant.

2.10 Construction of recombinant C. metallidurans and P. aeruginosa strains

Cloning procedures were performed with *E. coli* DH5α and SL17.1 λ pir strains, high fidelity DNA polymerase (Invitrogen 1253216), fast digest restriction enzymes (Thermo Scientific), and T4 DNA ligase (Promega M1801), according to standard protocols provided by the manufacturers. Primer sequences for cloning are shown in Table S1.

To characterize the function of the *urf*2.2 gene product, the *urf*2.2 gene (*RMET_RS31035*) was overexpressed in *C. metallidurans* and *P. aeruginosa*. Briefly, *urf2.2* was amplified from CH34 genomic DNA using an *Eco*RI restriction site flanked forward primer and a *Hin*dIII site flanked reverse primer. This amplicon was subcloned into pGEM-T vector (Promega A1360) through T4 ligase, following manufacturer's instructions. The recombinant vector was introduced into *E. coli* strain DH5α by electrotransformation. Transformed *E. coli* was cultured overnight in LB broth with ampicillin 100 μg mL^{-1} at 37 °C. The *urf*2.2 insert was recovered by plasmid miniprep and *Eco*RI/*Hin*dIII double digestion, and was further ligated into the *Eco*RI/*Hin*dIII digested pJBTc19 vector and electrotransformed into *E. coli* SL17.1 λ pir donor strain. The donor strain *E. coli* SL17.1 λ pir pJBTc19:*urf*2.2 was grown in LB broth with ampicillin 100 μg mL^{-1} and tetracycline 15 μg mL^{-1}. *C. metallidurans* CH34 conjugation transfer was achieved by mixing cell suspensions of donor and recipient strains through vacuum filtration over a 0.2-μm polycarbonate filter and incubated in nutrient agar (beef extract 1 g L^{-1}, yeast extract 2 g L^{-1}, peptone 5 g L^{-1}, NaCl 5 g L^{-1}) for 4 days at 30 °C. The biomass grown over the filter was spread on LPTMS agar supplemented with ampicillin 100 μg mL^{-1}, tetracycline 15 μg mL^{-1}, and kanamycin 1 mg mL^{-1} to kill the donor *E. coli* strain. On the other hand, *P. aeruginosa* was transformed by electroporation and selection on LB agar supplemented with ampicillin 100 μg mL^{-1}, tetracycline 15 μg mL^{-1}. All transformations were confirmed by double *Eco*RI/*Hin*dIII digestion of plasmid extraction products.

3. Results

3.1 Cadmium exerts inhibition of biofilm formation through a decay in c-di-GMP levels

Cell attachment assays show that cadmium exerts a dose dependent inhibition of early stages of biofilm formation in *C. metallidurans* CH34 (Figure 1A). Cell viability (as shown by CFU/mL) was not affected by the cadmium concentration range used, thus discarding a bias due to cell death (Figure 1A). At increasing cadmium concentrations, *C. metallidurans* macrocolonies on plates showed decreasing levels of EPS, which was observed by Congo Red staining (Figure 1B). A colorless extracellular matrix was observed at 8 mM cadmium. To assess the role of the c-di-GMP pathway in these responses, nucleotide extracts were obtained from planktonic and biofilm cells after exposure to 2 mM cadmium during 45 min. Cadmium

 doi:10.20944/preprints201807.0520.v1

decreased c-di-GMP levels in cells of both lifestyles (Figure 1C). However, cadmium exerted a stronger c-di-GMP level decrease in planktonic cells, triggering an 8-fold drop of c-di-GMP concentration compared to control cells.

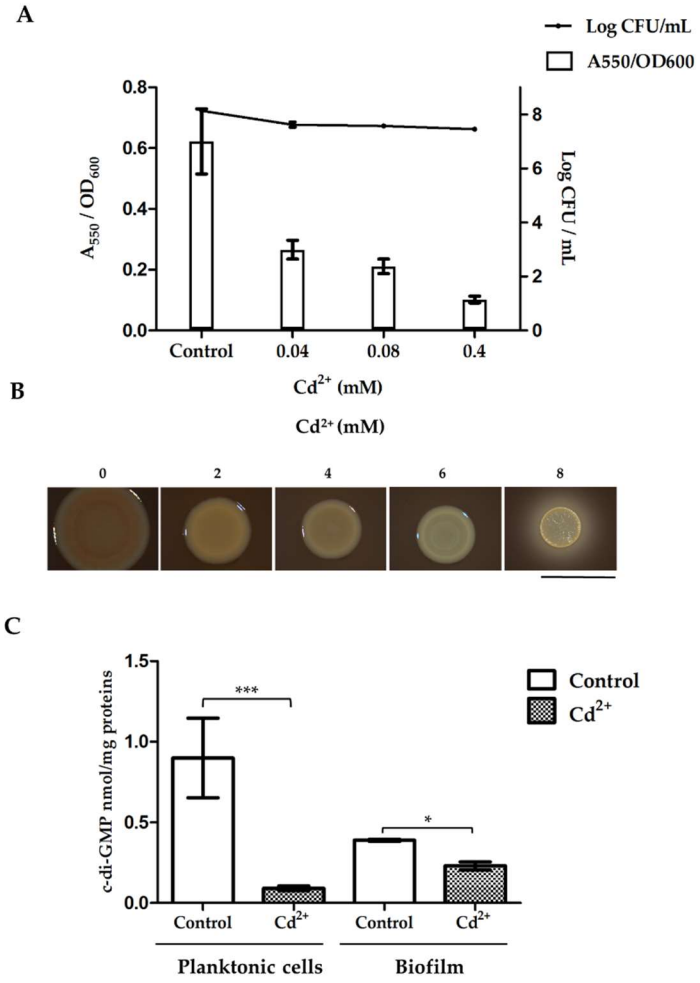

Figure 1. Effect of cadmium on *C. metallidurans* CH34 biofilm formation and c-di-GMP levels. A, Cell attachment and cell viability after 6 h of incubation in LPTMS broth at 30 °C. The means of three independent experiments and standard deviations are shown. **B**, Macrocolonies grown in Congo Red LB agar plates at different cadmium concentrations. Images were taken after five days of incubation at room temperature. The bar represents 1 cm. **C**, c-di-GMP levels after 2 mM cadmium exposure during 45 min in LPTMS broth (planktonic) or agar (biofilm). The c-di-GMP concentration was normalized respect to total protein content in each sample. The means of three independent experiments and standard deviations are shown. Significant differences assessed by *t* test: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).

3.2 Planktonic and biofilm cells have similar tolerance to cadmium

The susceptibility to cadmium of *C. metallidurans* CH34 planktonic and biofilm cells was determined due to the cadmium effect favouring the planktonic lifestyle. For microtiter determinations of

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 26 July 2018 doi:10.20944/preprints201807.0520.v1

antimicrobial susceptibility, the Calgary Biofilm Device was used, which allows batch culture of biofilms on peg lids [39]. Microbicidal endpoints were determined quantitatively using viable cell counting following a MBEC assay (Figure S2) [21,53]. Planktonic and biofilm cells showed similar tolerance to cadmium (Figure 2).

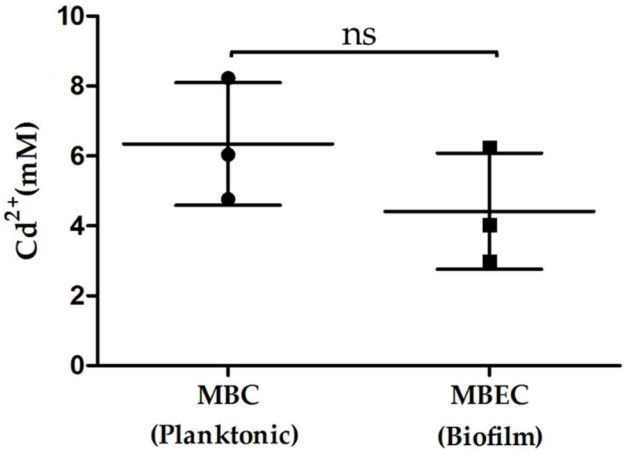

Figure 2. Cadmium susceptibility of *C. metallidurans* CH34 planktonic and biofilm cells. Values were determined by calculation in two independent kill curves experiments in triplicate. The mean of the cadmium concentration that kill 99.9% of cells is plotted. Dots and squares represent the interpolated values in the each kill curve. Significant differences assessed by *t* test: ns, non-significant.

3.3 C. metallidurans CH34 genome encodes functional DGCs and PDEs

Genes encoding putative DGC and PDE domain-containing proteins in the *C. metallidurans* CH34 genome were searched due to the putative role of c-di-GMP in the cadmium response. To evaluate a role in the c-di-GMP metabolism, the functionality of proteins with GGDEF, EAL and HD-GYP domains encoded in the genome of *C. metallidurans* CH34 were predicted through the analysis of the conservation of amino acid determinants for the catalytic activity (Figures S3, S4 and S5) [15-17]. *C. metallidurans* CH34 genome encodes 18 different GGDEF domain proteins, 10 EAL domain proteins, 12 GGDEF/EAL hybrid proteins, and 2 HD-GYP domain proteins. Since the functionality of all these CH34 proteins was unknown, the function of each protein was predicted based on conservation of at least 50% of the active-site motifs defined by crystallography [15–17] (Tables S2, S3 and S4). In our study, proteins with conservation of at least 50% amino acid determinants for the catalytic activity were predicted to be active proteins, whereas proteins with conservation of less than 50% amino acid determinants for the catalytic activity were predicted to be non-active. Seventeen of 18 annotated GGDEF domain proteins were predicted to possess DGC enzymatic activity. On the other hand, 9 of 10 annotated EAL domain proteins were predicted to have PDE enzymatic activity, whereas the *RMET_RS19345* gene product was predicted to be non-active.

Among the 12 hybrid proteins, the GGDEF domain of *RMET_RS19120* gene product was predicted to be non-active. In addition, both GGDEF and EAL domains of *RMET_RS24490* and *RMET_RS29670* gene products were also predicted to be non-active. The *RMET_RS29670* gene product showed partial

conservation in primary inhibition site motif, indicating c-di-GMP binding capabilities (Figure S5, Table S4). The EAL domain of two hybrid proteins (*RMET_RS24490* and *RMET_RS29670*) was predicted to be non-active (Table S4). Finally, the 2 loci encoding HD-GYP domain PDEs (*RMET_RS09120* and *RMET_RS20080*) were predicted to be non-active and were thus not further analyzed (Figure S5).

In summary, thirty six genes coding for proteins with catalytic active domains in the c-di-GMP metabolism were found in the *C. metallidurans* CH34 genome. For these genes, accessory domains and orthology analyses were performed to select a subset of genes for further studies (Table S5).

An accessory domain analysis was performed with the proteins predicted to have catalytic active c-di-GMP domains (Figure 3). The cytoplasmic HAMP domain, important for receptor signal(s) transduction [42], and the extracellular CACHE sensor domains [43] were encoded exclusively in DGCs. The ionic strength sensitive domain CBS [44] was located exclusively in three hybrid proteins, including an uncommon localization between EAL and GGDEF domains in *RMET_RS24295* gene product. An uncharacterized domain DUF3030 was observed in carboxy terminals of the EAL domain of the *RMET_RS30310* and *RMET_RS31035* genes proteins (Figure 3).

Five and four accessory domains were identified in single proteins, three of them in hybrid proteins. In DGC proteins, a Tsr_Tar chemoreceptor domain [45] was observed in the *RMET_RS08460* gene product, a nucleotide binding GAF domain [46] in the *RMET_RS19900* gene product and a histidine kinase domain HisK in the *RMET_RS28515* gene product. The oxidative stress sensitive domain CSS identified in the EAL domain *RMET_RS05485* and *RMET_RS26980* genes products [47]. On the other hand, an integral membrane domain associated to gaseous ligand sensing (MHYT) [48] was identified in the hybrid protein *RMET_RS22980*, and a membrane-anchoring domain MASE1 [49] in the hybrid protein *RMET_RS02985*.

Other accessory domains were transversally distributed between different proteins of c-di-GMP metabolism. For example, the phosphorylation receiver domains (REC) were identified in DGC *RMET_RS20545* and in PDE *RMET_RS20720* gene products. The gaseous ligand binding domain PAS [50] was observed in five DGCs (*RMET_RS04365, RMET_RS08460, RMET_RS11180*, *RMET_RS28515* and *RMET_RS29325*) and three hybrid proteins (*RMET_RS02985*, *RMET_RS11280* and *RMET_RS28625*) (Figure 3).

A gene orthology analysis in *E. coli* and *P. aeruginosa* genomes was performed. Based in sequence similarity and architectural domain profiles, nine CH34 orthologous DGC genes were identified in *E. coli* and *P. aeruginosa* (Table S5). Three DGCs (*RMET_RS09375*, *RMET_RS11270* and *RMET_RS19900*) showed high identity with YedQ (27%) [51], TpbB (55%) [52] and YeaP (41%) [53], respectively. All aforementioned gene products are enzymes that promote EPS production and surface adherence. The analyses of the PDEs revealed that, the CH34 *RMET_RS05485* and *RMET_RS26980* gene products possess high identity (33-34%) to the PDE YjcC that is involved in the oxidative stress response and motility in *K. pneumoniae* [47], whereas the *RMET_RS20720* gene product showed high identity (30%) to RocR, which is involved in biofilm formation and virulence gene expression in *P. aeruginosa* [30]. Finally, the hybrid *RMET_RS22980* gene product showed high identity (65%) to the bifunctional enzyme MucR, which is involved in biofilm dispersion in *P. aeruginosa* [54].

 doi:10.20944/preprints201807.0520.v1

The genomic context of c-di-GMP pathway genes and genes related to heavy metal resistance, motility and biofilm formation was analyzed. Five c-di-GMP pathway genes were located in plasmids associated to heavy metal resistance. The *RMET_RS30470* gene that encodes a bifunctional DGC/PDE was located in the genomic *cop-sil-nre-ncc* genes island, which codes for the heavy metal resistance machinery in pMOL30 [55]. In addition, the *RMET_RS29670* gene that codes a predicted non-active protein is located in *C. metallidurans* CH34 plasmid pMOL30, but outside of the heavy metal resistance genomic island [55]. The PDE encoding *RMET_RS30310* gene is located in the pMOL30 Tn*4378 mer* operon between the *merE* gene and a gene encoding a recombinase, overlapping their ORFs in ninety four bases (Figure S6). An identical context shows the PDE encoding *RMET_RS31035* gene in the pMOL28 Tn4380 *mer* operon. Both EAL domain PDEs showed 95.1% sequence identity. The *RMET_RS30310* and *RMET_RS31035* genes are annotated as *urf2* (for *u*nkown *r*elated *f*unction) genes.

 doi:10.20944/preprints201807.0520.v1

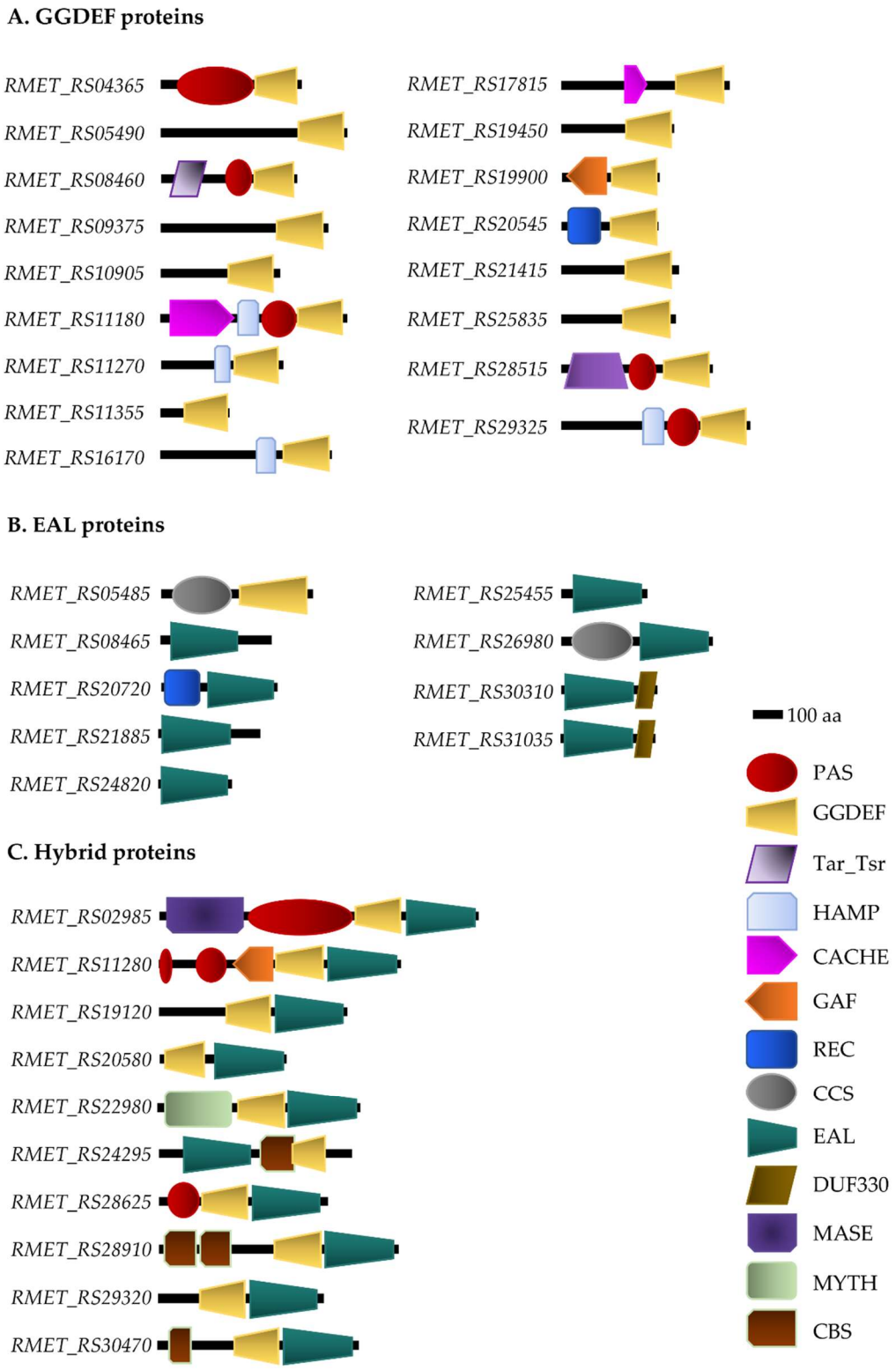

Figure 3. Domain-level depiction of c-di-GMP metabolism proteins from *C. metallidurans* CH34. Proteins with GGDEF/EAL domains that are catalytic active according to bioinformatic analyses are shown.

Some c-di-GMP pathway genes identified are associated with biofilm formation. The *RMET_RS11355* gene is located downstream to an operon gene organization that is associated with cellulose synthesis (Figure S6). The *RMET_RS20545* gene is located downstream to a gene cluster associated to chemotaxis response, and the *RMET_RS25455* gene is located upstream to a fimbrial synthesis gene cluster (Figure S6).

3.4 C. metallidurans genome encodes protein effectors of c-di-GMP pathway

C. metallidurans CH34 possesses four genes (*RMET_RS05745*, *RMET_RS08705*, *RMET_RS09140* and *RMET_RS11325*) encoding PilZ domain proteins. The PilZ domain protein encoded in *RMET_RS09140* gene was discarded as c-di-GMP effector due to the absence of c-di-GMP binding motif (Figure S7). The hybrid GGDEF/EAL domains protein encoded by the *RMET_RS29670* gene that was predicted to be catalytically non active, was included as potential effector of c-di-GMP pathway in *C. metallidurans* CH34 (Table S4). Local and pairwise sequence alignment determined the CH34 genes encoding orthologous proteins of the c-di-GMP effectors Bcam1349 (*RMET_RS18290)*, which is the transcription factor that regulates biofilm formation in *B. cenocepacia* (Figure S8) and PelD (*RMET_RS RS21490)*, which is a degenerate DGC that regulates Pel cationic exopolysaccharide synthesis in *P. aeruginosa* (Figure S9).

3.5 Cadmium induce the expression of genes coding for PDE and heavy metal resistance genes

A subset of 19 genes coding DGCs and PDEs were selected based on three criteria: i) genes coding for accessory domains associated to extracellular signals sensing, ii) genomic context associated to biofilm, motility or heavy metal resistance, and iii) orthology with *E. coli* or *P. aeruginosa* genes that encode enzymes of the c-di-GMP metabolism. Thus, the transcription levels of these genes (8 DGCs, 5 PDEs and 6 hybrids) were quantified after 45 min exposure to 2 mM cadmium in both biofilm and planktonic cells (Figure 4A). In addition, 6 transcripts of c-di-GMP effectors were also quantified: three transcript encoding PilZ domain proteins that conserves the "c-di-GMP swicht" binding motif (*RMET_RS05745, RMET_RS08705* and *RMET_RS11325*), one transcript encoding a degenerate hybrid GGDEF/EAL domain protein (*RMET_RS29670*), one transcript encoding for a PelD-like c-di-GMP effector (*RMET_RS21490*) and one transcript (*RMET_RS18290)* that encodes a protein with high identity (53%) to Bcam1349, which is a c-di-GMP-dependent transcription factor in *Burkholderia cenocepacia* [34] (Figure 4B).

In both lifestyles, most of the selected transcripts (DGCs, PDEs and hybrids) showed a non-significant abundance fold change in response to cadmium (Figure 4A). In planktonic cells two exceptions were observed. The *RMET_RS11180* gene that encodes a DGC that possesses HAMP and CACHE extracellular signal sensing domains was strongly downregulated (~5 fold). The *urf2* genes expression was upregulated (~3 fold). Primers amplified the two *urf2* genes transcripts, hence this value is the sum of *urf2.1* and *urf2.2* transcripts.

After biofilm cells exposure to cadmium, a higher number of transcripts showed a significant fold change. The *urf2* gene transcription increased very strongly (~36 fold) in biofilms (Figure 4A). The transcription of two hybrids proteins were significantly downregulated by cadmium: *RMET_RS02985* (~6 fold) that encodes to an integral membrane protein with a MASE1 domain, and *RMET_RS28910* (~4

fold) that encodes to a protein with two CBS domains. The *RMET_RS11180* gene transcription was also significantly downregulated (~3 fold), which was also observed in planktonic cells.

Almost all effector transcripts assessed were significantly downregulated after cadmium exposure, in higher magnitude in biofilm cells (Figure 4B). Only the *RMET_RS08705* and *RMET_RS29670* transcripts were non-significantly regulated in planktonic conditions.

In addition, the transcription of two heavy metal resistance genes was analyzed. The transcription of the *cadA* gene that encodes a Cd^{2+}/ATPase protein transporter involved in cadmium resistance was monitored. As expected, after exposure to cadmium the *cadA* gene was strongly upregulated in both lifestyles at similar levels (~10 fold) (Figure 4C). The effect of cadmium on the expression of the *merA* genes that encodes a mercuric reductase and belongs to the *mer* operons Tn***4378*** and Tn***4380***, which also harbor the *urf2* genes, was also analyzed. The primers used amplify both *mer*A transcripts. Interestingly, cadmium induced the transcription of the *merA* genes in both lifestyles (Figure 4C), suggesting an induction of mercury resistance by cadmium. The upregulation by cadmium of the *merA* genes was much stronger in biofilms (121 fold) than in planktonic cells (14 fold).

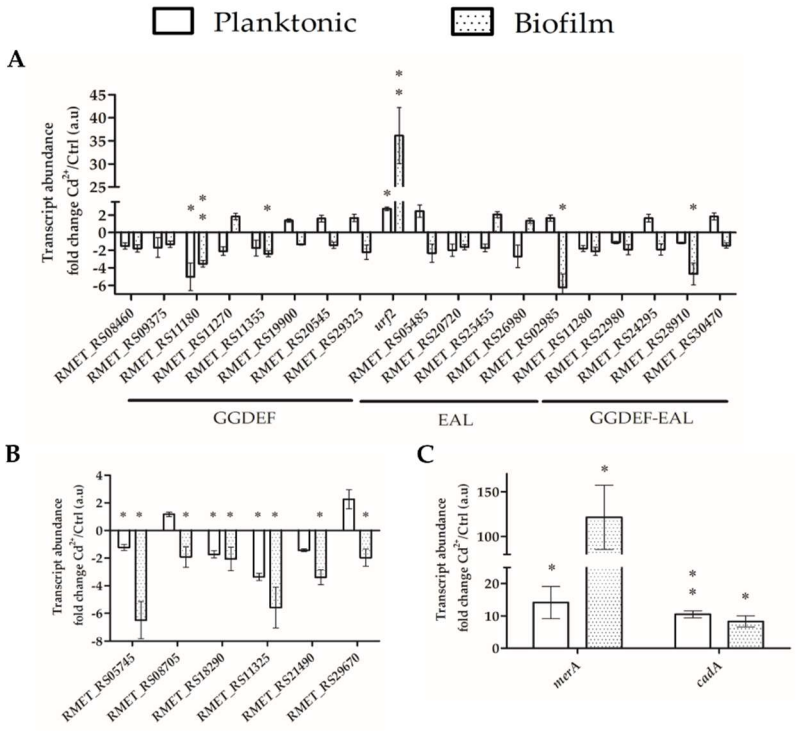

Figure 4. Effect of cadmium on c-di-GMP metabolism and heavy metal resistance transcripts in planktonic and biofilm *C. metallidurans* CH34 cells. The transcription was measured after exposure to cadmium (2 mM) during 45 min. **A,** Effect of cadmium on the expression of genes involved in c-di-GMP metabolism. **B,** Effect of cadmium on the expression of genes encoding c-di-GMP effectors **C,** Effect of cadmium on the expression of mercury and cadmium resistance genes. The means of values of three independent experiments, each analyzed in duplicate, and standard deviations are shown. Significant differences assessed by *t* test: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), ns: non-significant.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 26 July 2018 doi:10.20944/preprints201807.0520.v1

3.6 The urf2 gene encodes a functional PDE

The *urf2* gene is a component of the Tn*501 mer* operon. Although Tn*501* and its derivatives were described decades ago [59], the Urf2 function remains unknown. Here, we are able for the first time to suggest a function. A complementation assay was developed in the PDE null mutant strain *P. aeruginosa* PAO1 *ΔrocR* to evaluate the effects of the protein encoded by the *urf2* gene in biofilm formation and c-di-GMP levels. The complementation by the *urf2.2* gene restored biofilm formation (Figure 5A) and c-di-GMP levels (Figure 5B) at levels comparable to the wild-type PAO1 strain, indicating that *urf2.2* gene encodes a functional PDE. Based on this data we propose to rename this specific gene as *mrp2* gene for metal regulated phosphodiesterase and its copy on plasmid pMOL28 as *mrp1* gene.

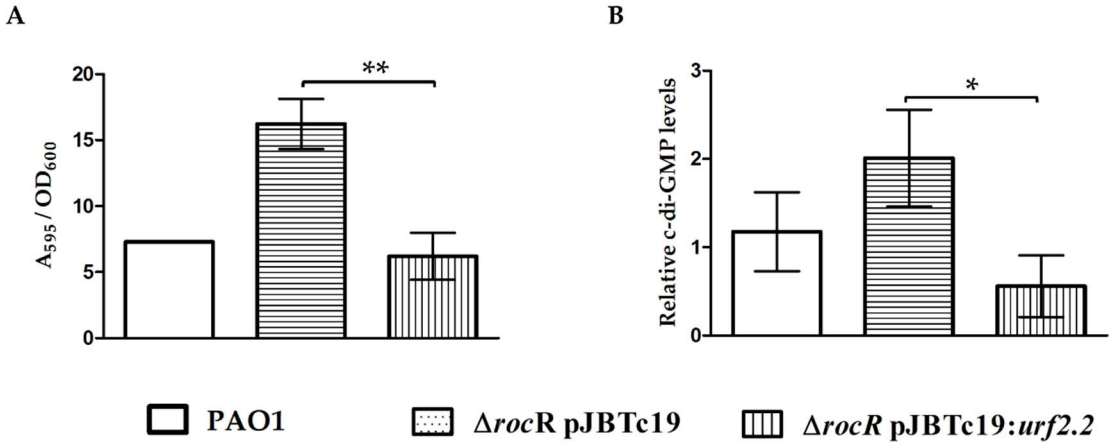

Figure 5. Complementation effects in *P. aeruginosa* PAO1 Δ*rocR* with pJBTc19:*urf*2.2. A, Biofilm formation at 6 h of incubation in 96 well plates. **B**: The c-di-GMP content in planktonic cells at 6 h of incubation in Erlenmeyer flasks. The means of three independent experiments and standard deviations are shown. Significant differences assessed by *t* test: $P < 0.05$ (*), $P < 0.01$ (**).

3.7 Effects of c-di-GMP metabolism disturbances in response to cadmium

The relevance of c-di-GMP decrease for biofilm formation and cadmium response in *C. metallidurans* CH34 was assessed through the overexpression of the *urf2.2* gene and the heterologous expression of the constitutive active DGC PleD* [26]. The overexpression of the *urf2.2* gene has no significant effects on biofilm formation (Figure 6A) and EPS content in macrocolonies (Figure 6B). However, after overexpression of the *urf2.2* gene a slight decrease in c-di-GMP levels in control planktonic cells were observed, which disappear after the exposure to cadmium (Figure 6C). Conversely, the heterologous expression of the PleD* DGC increased biofilm formation (Figure 6A), EPS content (Figure 6B) and c-di-GMP levels in control cells and in cells incubated with cadmium (Figure 6C).

To further assess the effects of these alterations in c-di-GMP metabolism in cadmium susceptibility, MBC and MBEC were determined in *C. metallidurans* transformed strains. The overexpression of the *urf2.2* gene has no effect on cadmium susceptibility. Interestingly, the heterologous expression of PleD* DGC resulted in significant detrimental effects in cadmium tolerance (Figures 7 and S10) in both bacterial lifestyles.

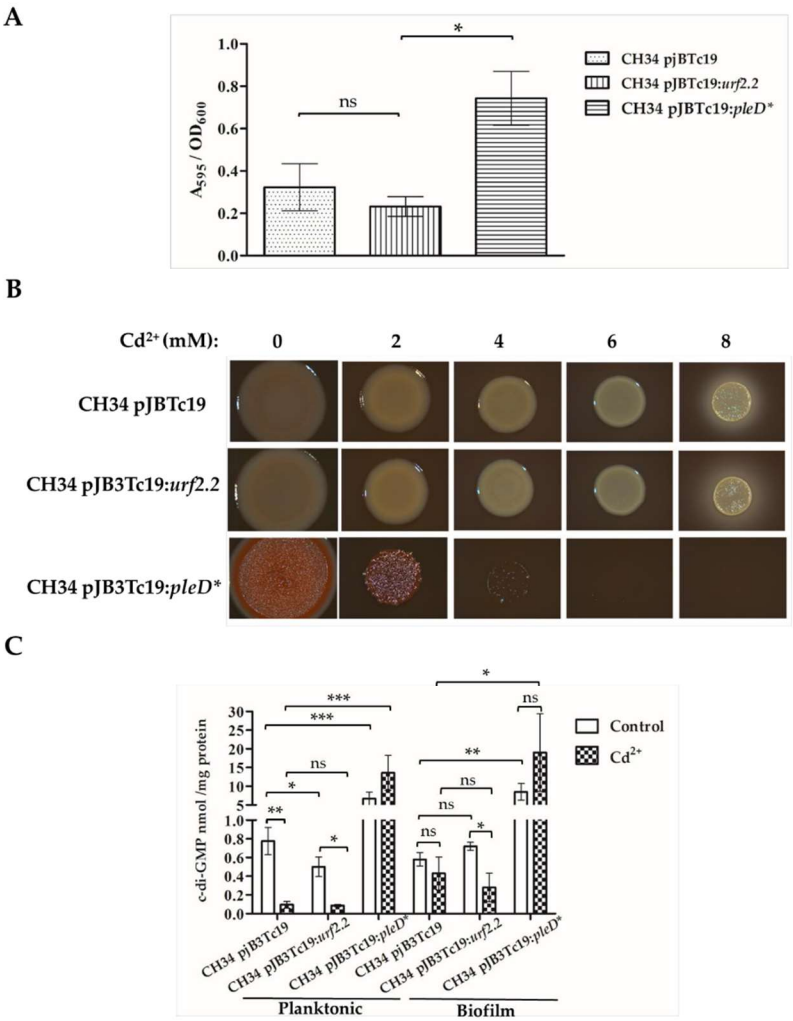

Figure 6. Effect of *urf2.2* overexpression and *pleD heterologous expression on *C. metallidurans* CH34 biofilm formation and c-di-GMP metabolism. A:** Biofilm formation. The means of three independent experiments and standard deviations are shown. Significant differences assessed by *t* test: $P < 0.05$ (*), ns: not significant. **B**: Macrocolonies morphotype in presence of cadmium. **C**: Response in c-di-GMP levels after cadmium exposure. The means of three independent experiments and standard deviations are shown. Significant differences assessed by *t* test: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), ns: non-significant.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 26 July 2018 doi:10.20944/preprints201807.0520.v1

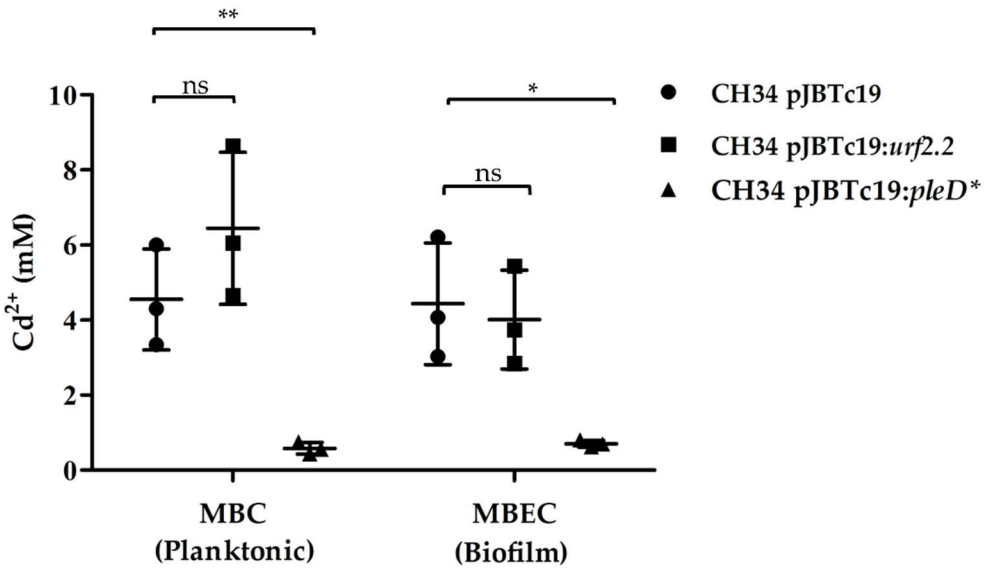

Figure 7. Effect of *urf*2.2 overexpression and *pleD heterologous expression on *C. metallidurans* CH34 tolerance to cadmium.** The means of three independent experiments and standard deviations are shown. Significant differences assessed by *t* test: $P < 0.05$ (*), $P < 0.01$ (**), ns: non-significant.

4. Discussion

This study shows that cadmium inhibits biofilm formation in the model heavy metal-resistant strain *C. metallidurans* CH34, which correlates with a drop in the c-di-GMP levels. The upregulation of the PDE encoded by the *urf*2 genes is probably involved in the drop in c-di-GMP concentration. We propose to rename it as *mrp* (metal regulated PDE) gene based on this study and the location of its two copies (*RMET_RS30310*/*mrp1* and *RMET_RS31035*/*mrp2*) in the two *mer* operons from the *C. metallidurans* pMOL28 and pMOL30 plasmids. The *mer* operons are part of transposons belonging to the *Tn*501/*Tn*21 family, the most widespread transposons families in terrestrial and marine environments [56,57]. Similar results on biofilms were reported by Agulló *et al.* (2017) describing that the toxic aromatic compound *p*-cymene reduced biofilm formation in the model aromatic-degrader *B. xenovorans* LB400. The reduced biofilm formation in LB400 cells grown on *p*-cymene correlated with a decrease of the DGC protein levels that probably reduced c-di-GMP levels [58].

In our study, of the 42 putative proteins involved in c-di-GMP metabolism coded in *C. metallidurans* CH34 genome, thirty six proteins with at least 50% conservation of amino acid determinants for the catalytic activity were predicted as active, whereas six were predicted to be non-active. The domain organization revealed a high proportion of stand-alone GGDEF (9) and EAL (6) domains, contrasting with the 0.03 % and 20 % of representation of these kind of architectures in Pfam database [59]. Strain CH34 is especially well adapted to harsh niches in which heavy metals are present [6], therefore, the

presence of unknown accessory domains in the c-di-GMP signaling pathway proteins should not been excluded.

Until this study, the role of the *urf2* gene from the mercury resistance operon was unknown [59]. This *urf2* gene was the first gene encoding a protein with an EAL domain to be reported in *Tn*501 [56] and *Tn*21 transposon families [57]. It has been previously described that *urf2.1* and *urf2.2* genes are upregulated by mercury, cadmium, lead and zinc [55,60]. These heavy metals belong to the group 12 in the periodic table, and clustered into the same transcriptomic profile, i.e. regulates the same set of genes, based on microarrays data [60]. Our study establishes for the first time the catalytic functionality of the *urf2* (*mrp)* gene product, due to its ability to functionally replace RocR in *P. aeruginosa* PAO1 Δ*rocR* in complementation assays. RocR is a PDE response regulator in the RocS1S2AR (or SadARS) two-component signaling system in *P. aeruginosa* [61]. This system controls bacterial biofilm formation by regulating the transcription of the *cup* fimbrial gene cluster by a poorly understood mechanism [61]. Crystallography studies revealed that RocR is a constitutively inhibited tetrameric PDE, which is activated after phosphorylation, a modification that exposes the catalytic EAL domains [62]. This mechanism can explain the absence of effects, either by overexpression or by transposon interruption of the *roc*R gene in *P. aeruginosa* biofilm formation in static conditions [61]. In contrast, the results obtained in our study showed a drop in c-di-GMP levels and a decreased biofilm formation in *P. aeruginosa* PAO1 Δ*rocR* complemented with the CH34 *mrp2 (urf2.2)* gene (Figure 5). This discrepancy may be explained by differences in experimental strategies. Cells from a static culture after 20 h incubation [61] are in a different physiological state compared to the adhered biomass after 6 h incubation under stirred conditions (this study). This condition generates shearing forces that promote biofilm compaction and viscosity loss in the EPS matrix [63], which result in a biofilm more resistant to hydrodynamic erosion [64]. Another important difference between both studies was the incubation time. After 6 h incubation cells are in an early and reversible biofilm formation stage, in which the RocR regulation of the adhesins CupB/C is more relevant than in a mature stage after 20 h incubation [61]. Thus, under the conditions used in our study, the *urf2.2* gene product could replace the RocR role in biofilm formation and c-di-GMP hydrolysis.

The absence of differences in cadmium susceptibilities (Figure 2) between both bacterial lifestyles and the significant decrease of c-di-GMP concentration in planktonic cells in response to cadmium (Figure 1C) suggest that the response exceeds planktonic lifestyle promotion. In this sense, some PDEs such as YjcC in *K. pneumoniae* [47], CdgR in *Salmonella typhimurium* [66] and YgfF in *E. coli* [67] play a role in oxidative stress survival. Since heavy metal toxicity may be related to oxidative stress, this decrease in c-di-GMP concentration could be expected. The heterologous expression of a constitutively active DGC (PleD*) increased the c-di-GMP levels and cadmium susceptibility in *C. metallidurans* CH34 (Figures 6 and 7). As the extracellular matrix production is an energetically expensive process [68], a constitutive activation of this machinery could deplete ATP cellular pools, depriving the cell to achieve efficient cadmium detoxification. Furthermore, a displacement of RNA polymerase II cores by a permanently active transcription factors in those operons could not be excluded, due to the wide diversity of sigma factors and transcriptional regulators which expression is regulated during exposure of *C. metallidurans* cells to heavy metals [60]. By overexpression of the *C. metallidurans* CH34 *mrp* gene, the role of the *mrp*

gene product in the c-di-GMP metabolism in response to cadmium was only observed in planktonic cells with a slight decrease in c-di-GMP that correlates with a tendency to decrease biofilm formation. It is possible that the overexpression of the *mrp* gene from the plasmid pJBTc19:*urf2.2* was not high enough to observe higher changes due to the high expression of the *mrp* genes from plasmids pMOL30 and pMOL28.

The *mrp* gene product domain architecture shows a C-terminal accessory domain DUF3030 (Pfam 11809), which is only present in a nitro reductase protein family. I-TASSER 3D prediction platform [69] identified five α-helix in this region beyond EAL domain towards the C-terminal region. Thus, the *mrp* gene product may be an exceptional case of domain organization in intracellular signaling proteins, combining cytoplasmic sensor domains in its N-terminal extreme with C-terminal output domains [70]. Although the unknown function of the DUF3030 domain, it may play a role in the c-di-GMP metabolism in *C. metallidurans* CH34. Therefore, it will be useful to generate a *C. metallidurans* CH34 double *mrp* null mutant Δ*urf2.1* Δ*urf2.2* strain to evaluate the contribution of these PDEs either for cadmium tolerance or for the c-di-GMP mediated response to cadmium. As other members of this family of proteins, the *mrp2* gene product is encoded in mobile genetic elements, indicating their biological significance during horizontal gene transfer [59]. Finally, an unexpected result was the strong increase (121 fold) by cadmium of the *merA* gene expression in *C. metallidurans* biofilm cells. A lower induction (14 fold) by cadmium of the *merA* gene expression was observed in planktonic cells. Previously, we have observed the upregulation by cadmium of the expression of the *merT* and *merP* genes in *C. metallidurans* planktonic cells [71]. The *mer* operon is also induced by cadmium in *Nitrosomonas europaea* [72]. All these results suggest a role of the *mer* operon in the protection towards cadmium. The flexible nature of the regulator MerR allows the interaction with other heavy metals such as cadmium, zinc, gold and silver, according to *in vitro* transcription studies [73], but with lower affinity than for mercury [74]. In contrast to the strong induction by cadmium of the expression of the *merA* gene (121 fold) in biofilm cells, a lower induction by cadmium of the *cadA* gene (~10 fold) was observed, indicating a differential behavior of both operons in the sessile biofilm lifestyle. Previously, differential mercury resistance mechanisms of *Desulfovibrio desulfuricans* planktonic cells and biofilms have been reported [75], which resembles the response to cadmium in strain CH34.

5. Conclusions

Cadmium inhibits biofilm formation in *C. metallidurans* CH34 through a decrease in c-di-GMP levels in both planktonic and biofilm modes of growth. The response to cadmium of C. *metallidurans* CH34, which was isolated from a heavy metal-polluted slurry, suggests that the inhibition by cadmium of the biofilm formation mediated by the decrease of c-di-GMP should be relevant for bacterial fitness under this condition, favoring the planktonic lifestyle. However, both lifestyles showed similar tolerance to cadmium, thus probably the adaptive advantage of planktonic cells is just to move towards less toxic environments. Transcription analysis suggests an active role of a previously unknown catalytic functional PDE encoded by the *urf2* genes of Tn*4378* (pMOL28) and Tn*4380* (pMOL30) *mer* operons from *C. metallidurans* CH34. Since this is the first report on the function of the *urf2* gene, the gene was renamed as *mrp2*, for "metal regulated *p*hosphodiesterase". In conclusion, this study indicates that the response to

cadmium in C. *metallidurans* CH34 involves a decrease in c-di-GMP content that inhibits the biofilm lifestyle.

Supplementary Materials: Table S1: Primers used in this work. Bioinformatic predictions of catalytic functionality of putative c-di-GMP metabolism protein was displayed in Table S2 (DGCs), Table S3 (PDE) and Table S4 (Hybrids). Table S5: Orthology analysis of putative DGC/PDEs encoded in *C. metallidurans* CH34 with *E. coli K12* and *P. aeruginosa PAO1* genomes. Figure S1: Experimental model used to expose planktonic and biofilm cells to cadmium.
Figure S2: Cadmium kill curves of *C. metallidurans* CH34 in planktonic and biofilm lifestyles. Figure S3: Multiple alignment of GGDEF domains encoded in *C. metallidurans* CH34 genome. Figure S4: Multiple alignment of EAL domains encoded in *C. metallidurans* CH34 genome. Figure S5: Multiple alignment of HD-GYP domains encoded in *C. metallidurans* CH34 genome. Figure S6: Genomic context of genes involved in c-di-GMP metabolism in *C. metallidurans* CH34. Figure S7: Multiple alignment of PilZ domains encoded in *C. metallidurans* CH34 genome. Figure S8: Global alignment between amino acidic sequences encoded by *bcam1349* (*B. cenocepacia*) and *RMET_RS18290* (*C. metallidurans* CH34) genes. Figure S9: Global alignment between aminoacidic sequences of c-di-GMP effector PelD encoded in genomes of *P. aeruginosa* PAO1 and *C. metallidurans* CH34. Figure S10: Cadmium kill curves of transformed strains of *C. metallidurans* CH34 in planktonic and biofilm lifestyles.

Acknowledgments: The authors thank Dr. Juan San Juan and Dr. Daniel Pérez Mendoza for the donations of pJBTc19 and pJBTc19:*pleD** vectors. To Nathalie Gugala for support with Calgary Biofilm Device. PA acknowledges MECESUP PhD fellowship (UCH 1107)). This work was supported by FONDECYT 1160702 (NG) and 1151174 (MS) (http://www.fondecyt.cl), Programa de Investigación Asociativa (PIA) Anillo ACT172128 GAMBIO (MS) and USM (MS) (http://www.usm.cl) grants.

Author Contributions: P.A., M.S., N.G. and R.T. conceived and designed the experiments; P.A. performed the experiments; P.A., M.S., N.G., R.T., S.F. and L.R. analyzed the data; M.S., N.G. and R.T. contributed reagents/materials/analysis tools; P.A., M.S., N.G., R.T. and S.F. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References:

1. Begg, S. L.; Eijkelkamp, B. A.; Luo, Z.; Couñago, R. M.; Morey, J. R.; Maher, M. J.; Ong, C. Y.; McEwan, A. G.; Kobe, B.; O'Mara, M. L.; Paton, J. C.; McDevitt, C. A. Dysregulation of transition metal ion homeostasis is the molecular basis for cadmium toxicity in *Streptococcus pneumoniae*. *Nat. Commun.* **2015**, 6:6418, doi:10.1038/ncomms7418.

2. Vallee, B. L.; Ulmer, D. D. Biochemical effects of mercury, cadmium, and lead. *Annu. Rev. Biochem.* **1972**, *41*, 91–128, doi:10.1146/annurev.bi.41.070172.000515.

3. Nies, D. H. Microbial heavy-metal resistance. *Appl. Microbiol. Biotechnol.* **1999**, *51*, 730–50, doi:10.1007/s002530051457.
4. World Health Organization Safety evaluation of certain food additivies and contaminants, Geneve 2011, cadmium (addendum).
5. Mosa, K. A.; Saadoun, I.; Kumar, K.; Helmy, M.; Dhankher, O. P. Potential biotechnological strategies for the cleanup of heavy metals and metalloids. *Front. Plant Sci.* **2016**, *7*, 1–14, doi:10.3389/fpls.2016.00303.
6. Rojas, L.A.; Yáñez, C.; González, M.; Lobos, S.; Smalla, K.; Seeger, M. Characterization of the metabolically heavy metal-resistant *Cupriavidus metallidurans* strain MSR33 generated for mercury remediation. *PLoS ONE* **2011**, *6*(3):e17555, doi: 10.1371/journal.pone.0017555.
7. Harrison, J. J.; Ceri, H.; Turner, R. J. Multimetal resistance and tolerance in microbial biofilms. *Nat. Rev. Microbiol.* **2007**, *5*, 928–938, doi:10.1038/nrmicro1774.
8. Harrison, J. J.; Ceri, H.; Stremick, C.; Turner, R. J. Differences in biofilm and planktonic cell mediated reduction of metalloid oxyanions. *FEMS Microbiol. Lett.* **2004**, *235*, 357–62, doi:10.1016/j.femsle.2004.05.005.
9. Chen, M.; Zhou, X.; Liu, X.; Zeng, R. J.; Zhang, F.; Ye, J.; Zhou, S. Facilitated extracellular electron transfer of *Geobacter sulfurreducens* biofilm with *in situ* formed gold nanoparticles. *Biosens. Bioelectron.* **2018**, doi:10.1016/j.bios.2018.02.030.
10. Espinoza-Tofalos, A.; Daghio, M.; González, M.; Pappacchini, M.; Franzetti A.; Seeger, M. Toluene degradation by *Cupriavidus metallidurans* CH34 in nitrate-reducing conditions and in Bioelectrochemical Systems. *FEMS Microbiol. Lett* **2018**, *16*, doi:10.1093/femsle/fny119/4996784.
11. Gugala, N.; Lemire, J. A.; Turner, R. J. The efficacy of different anti-microbial metals at preventing the formation of, and eradicating bacterial biofilms of pathogenic indicator strains. *J. Antibiot. (Tokyo).* **2017**, *70*, 775–780, doi:10.1038/ja.2017.10.
12. Harrison, J. J.; Ceri, H.; Stremick, C. A; Turner, R. J. Biofilm susceptibility to metal toxicity. *Environ. Microbiol.* **2004**, *6*, 1220–7, doi:10.1111/j.1462-2920.2004.00656.x.
13. Harrison, J. J.; Ceri, H.; Roper, N. J.; Badry, E. a; Sproule, K. M.; Turner, R. J. Persister cells mediate tolerance to metal oxyanions in *Escherichia coli*. *Microbiology* **2005**, *151*, 3181–95, doi:10.1099/mic.0.27794-0.
14. Jenal, U.; Reinders, A.; Lori, C. Cyclic di-GMP: Second messenger extraordinaire. *Nat. Rev. Microbiol.* **2017**, *15*, 271–284, doi:10.1038/nrmicro.2016.190.
15. Wassmann, P.; Chan, C.; Paul, R.; Beck, A.; Heerklotz, H.; Jenal, U.; Schirmer, T. Structure of BeF_3^- -modified response regulator PleD: implications for diguanylate cyclase activation, catalysis, and feedback inhibition. *Structure* **2007**, *15*, 915–27, doi:10.1016/j.str.2007.06.016.
16. Tchigvintsev, A.; Xu, X.; Singer, A.; Chang, C.; Brown, G.; Proudfoot, M.; Cui, H.; Flick, R.; Anderson, W. F.; Joachimiak, A.; Galperin, M. Y.; Savchenko, A.; Yakunin, A. F. Structural insight into the mechanism of c-di-GMP hydrolysis by EAL domain phosphodiesterases. *J. Mol. Biol.* **2010**, *402*, 524–538, doi:10.1016/j.jmb.2010.07.050.
17. Lovering, A. L.; Capeness, M. J.; Lambert, C.; Hobley, L.; Sockett, R. E. The structure of an unconventional HD-GYP protein from *Bdellovibrio* reveals the roles of conserved residues in this

class of Cyclic-di-GMP phosphodiesterases. *mBio* **2011**, *2*, 1–8, doi:10.1128/mBio.00163-11.

18. Jain, R.; Sliusarenko, O.; Kazmierczak, B. I. Interaction of the cyclic-di-GMP binding protein FimX and the Type 4 pilus assembly ATPase promotes pilus assembly. *PLoS Pathog.* **2017**, *13*, 1–23, doi:10.1371/journal.ppat.1006594.
19. Duerig, A.; Sören, A.; Folcher, M.; Nicollier, M.; Schwede, T.; Amiot, N.; Giese, B.; Jenal, U. Second messenger-mediated spatiotemporal control of protein degradation regulates bacterial cell cycle progression. *Gene Dev.* **2009**, *23*, 93–104, doi:10.1101/gad.502409.tion.
20. Habazettl, J.; Allan, M. G.; Jenal, U.; Grzesiek, S. Solution structure of the PilZ domain protein PA4608 complex with cyclic di-GMP identifies charge clustering as molecular readout. *J. Biol. Chem.* **2011**, *286*, 14304–14314, doi:10.1074/jbc.M110.209007.
21. Römling, U.; Galperin, M. Y.; Gomelsky, M. Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiol. Mol. Biol. Rev.* **2013**, *77*, 1–52, doi:10.1128/MMBR.00043-12.
22. Mergeay, M.; Nies, D.; Schlegel, H. G.; Gerits, J.; Charles, P.; Van Gijsegem, F. *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. *J. Bacteriol.* **1985**, *162*, 328–34.
23. Collard, J. M.; Corbisier, P.; Diels, L.; Dong, Q.; Jeanthon, C.; Mergeay, M.; Taghavi, S.; van der Leie, D.; Wilmotte, A.; Wuertz, S. Plasmids for heavy metal resistance in *Alcaligenes eutrophus* CH34: Mechanisms and applications. *FEMS Microbiol. Rev.* **1994**, *14*, 405–414, doi:10.1111/j.1574-6976.1994.tb00115.x.
24. Janssen, P. J.; van Houdt, R.; Moors, H.; Monsieurs, P.; Morin, N.; Michaux, A.; Benotmane, M. A.; Leys, N.; Vallaeys, T.; Lapidus, A.; Monchy, S.; Médigue, C.; Taghavi, S.; McCorkle, S.; Dunn, J.; van der Lelie, D.; Mergeay, M. The complete genome sequence of *Cupriavidus metallidurans* strain CH34, a master survivalist in harsh and anthropogenic environments. *PLoS One* **2010**, *5*, doi:10.1371/journal.pone.0010433.
25. Nies, D. H. The biological chemistry of the transition metal "transportome" of *Cupriavidus metallidurans*. *Metallomics* **2016**, *8*, 481–507, doi:10.1039/C5MT00320B.
26. Maes, S.; Props, R.; Fitts, J. P.; De Smet, R.; Vanhaecke, F.; Boon, N.; Hennebel, T. Biological recovery of platinum complexes from diluted aqueous streams by axenic cultures. *PLoS One* **2017**, *12*, 1–17, doi:10.1371/journal.pone.0169093.
27. Montero-Silva, F.; Durán, N.; Seeger, M. Synthesis of extracellular gold nanoparticles using *Cupriavidus metallidurans* CH34 cells. *IET Nanobiotechnology* **2018**, *12*, 40–46, doi:10.1049/iet-nbt.2017.0185.
28. Pérez-Mendoza, D.; Aragón, I. M.; Prada-Ramírez, H. A.; Romero-Jiménez, L.; Ramos, C.; Gallegos, M. -T.; Sanjuán, J. Responses to elevated c-di-GMP levels in mutualistic and pathogenic plant-interacting bacteria. *PLoS One* **2014**, *9*, doi:10.1371/journal.pone.0091645.
29. O'Toole, G.A.; Kolter, R. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol. Microbiol.* **1998**, *28*, 449–61.
30. Rao, F.; Yang, Y.; Qi, Y.; Liang, Z.-X. Catalytic mechanism of cyclic di-GMP-specific phosphodiesterase: A study of the EAL domain-containing RocR from *Pseudomonas aeruginos*a. *J.*

Bacteriol. **2008**, *190*, 3622–3631, doi:10.1128/JB.00165-08.

31. Romero, D.; Aguilar, C.; Losick, R.; Kolter, R. Amyloid fibers provide structural integrity to *Bacillus subtilis* biofilms. *Proc. Natl. Acad. Sci.* **2010**, *107*, 2230–2234, doi:10.1073/pnas.0910560107.
32. Harrison, J. J.; Stremick, C. A; Turner, R. J.; Allan, N. D.; Olson, M. E.; Ceri, H. Microtiter susceptibility testing of microbes growing on peg lids: a miniaturized biofilm model for high-throughput screening. *Nat. Protoc.* **2010**, *5*, 1236–54, doi:10.1038/nprot.2010.71.
33. Li, T. N., Chin, K. H., Liu, J. H., Wang, A. H. -J., Chou, S. H. XC1028 from X*anthomonas campestris* adopts a PilZ domain-like structure without a c-di-GMP switch. *Proteins* 2009, 75, *2*, 282-288, doi: 10.1002/prot.22330.
34. Fazli, M.; Almblad, H.; Rybtke, M. L.; Givskov, M.; Eberl, L.; Tolker-Nielsen, T. Regulation of biofilm formation in Pseudomonas and Burkholderia species. Environ. Microbiol. 2014, 16, 1961-81, doi: 10.1111/1462-2920.
35. Thompson, J. D.; Higgins, D. G.; Gibson, T. J. Clustal-W-Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **1994**, *22*, 4673–4680.
36. Katoh, K.; Misawa, K.; Kuma, K.; Miyata, T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* **2002**, *30*, 3059–3066, doi:10.1093/nar/gkf436.
37. Altschul, S. F.; Madden, T. L.; Schäffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D. J. Gapped BLAST and PSI-BLAST:a new generation of protein database search programs. *Nucleic Acids Res.* **1997**, *25*, 3389–3402, doi:10.1093/nar/25.17.3389.
38. Geer, L. Y.; Domrachev, M.; Lipman, D. J.; Bryant, S. H. CDART: protein homology by domain architecture. *Genome Res.* **2002**, *12*, 1619–1623, doi:10.1101/gr.278202.
39. Bernstein, J. A.; Khodursky, A. B.; Lin, P.-H.; Lin-Chao, S.; Cohen, S. N. Global analysis of mRNA decay and abundance in *Escherichia coli* at single-gene resolution using two-color fluorescent DNA microarrays. *Proc. Natl. Acad. Sci.* **2002**, *99*, 9697–9702, doi:10.1073/pnas.112318199.
40. Sung, K.; Khan, S. A.; Nawaz, M. S.; Khan, A. A. A simple and efficient Triton X-100 boiling and chloroform extraction method of RNA isolation from Gram-positive and Gram-negative bacteria. *FEMS Microbiol. Lett.* **2003**, *229*, 97–101, doi:10.1016/S0378-1097(03)00791-2.
41. Hellemans, J.; Mortier, G.; De Paepe, A.; Speleman, F.; Vandesompele, J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol.* **2007**, *8*, R19, doi:10.1186/gb-2007-8-2-r19.
42. Matamouros, S.; Hager, K. R.; Miller, S. I. HAMP domain rotation and tilting movements associated with signal transduction in the PhoQ sensor kinase. *mBio* **2015**, *6*(3):e00016-15 doi:10.1128/mBio.00616-15.
43. Upadhyay, A. A.; Fleetwood, A. D.; Adebali, O.; Finn, R. D.; Zhulin, I. B. Cache domains that are homologous to, but different from PAS domains comprise the largest superfamily of extracellular sensors in prokaryotes. *PLoS Comput. Biol.* **2016**, *12*(4):e1004862, doi:10.1371/journal.pcbi.1004862.

44. Biemans-Oldehinkel, E.; Mahmood, N. A. B. N.; Poolman, B. A sensor for intracellular ionic strength. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 10624–10629, doi:10.1073/pnas.0603871103.
45. Tajima, H., Imada, K., Sakuma, M., Hattori, F., Nara, T., Kamo, N., Homma, M., Kawagishi, I. Ligand specificity determined by differentially arranged common ligand-binding residues in bacterial amino acid chemoreceptors Tsr and Tar. *J. Biol. Chem.* **2011**, *49*, 42200-42210, 10.1074/jbc.M111.221887.
46. Heikaus, C. C., Pandit, J., Klevit, R. E. Cyclic nucleotide binding GAF domains from phosphodiesterases: structural and mechanistic insights. *Structure.* **2009**, 17 , 1551-1557, doi:10.1016/j.str.2009.07.019.
47. Huang, C.-J.; Wang, Z.-C.; Huang, H.-Y.; Huang H.-D.; Peng, H.-L. YjcC, a c-di-GMP phosphodiesterase protein, regulates the oxidative stress response and virulence of *Klebsiella pneumoniae* CG43. *PLoS One* **2013**, *8*, e66740, doi:10.1371/journal.pone.0066740.
48. Galperin, M. Y., Gaidenko, T. A., Mulkidjanian, A. Y., Nakano, M., Price, C. W. MHYT, a new integral membrane sensor domain. *FEMS Microbiol. Lett.* **2005**, *205*, 17-23, 10.1016/S0378-1097(01)00424-4.
49. Nikolskaya, A. N., Mulkidjanian, A. Y., Beech, I. B., Galperin, M. Y. MASE1 and MASE2: Two novel integral membrane sensory domains. *J. Mol. Microbiol. Biotech.* **2003**, *5*, 11-16, 10.1159/000068720.
50. Henry, J. T.; Crosson, S. Ligand-binding PAS domains in a genomic, cellular, and structural context. *Annu. Rev. Microbiol.* **2011**, *65*, 261–86, doi:10.1146/annurev-micro-121809-151631.
51. Sanchez-Torres, V., Hu, H., Wood, T. K. GGDEF proteins YeaI, YedQ, and YfiN reduce early biofilm formation and swimming motility in *Escherichia coli*. **2011**, 2, 651-658, 10.1007/s00253-010-3074-5
52. Xu, K., Li, S. Y., Wen, L., Kan, B.A, Yuwei, Xu, Y., Jin, J., Wang, Y., Bartlam, M. Structural and biochemical analysis of tyrosine phosphatase related to biofilm formation A (TpbA) from the opportunistic pathogen *Pseudomonas aeruginosa* PAO1. *PLoS One.* **2015**, 10, 1-16, 10.1371/journal.pone.0124330
53. Ryjenkov, D. A., Tarutina, M., Moskvin, O. V., Gomelsky, M. Cyclic diguanylate is a ubiquitous signaling molecule in bacteria: Insights into biochemistry of the GGDEF protein domain. *J Bacteriol*. **2005**, *5*, 1792-1798, 10.1128/JB.187.5.1792-1798.2005
54. Li, Y.; Heine, S.; Entian, M.; Sauer, K.; Frankenberg-Dinkel, N. NO-induced biofilm dispersion in *Pseudomonas aeruginosa* is mediated by an MHYT domain-coupled phosphodiesterase. *J. Bacteriol*. **2013**, 195, 3531–3542, doi:10.1128/JB.01156-12.
55. Monsieurs, P.; Moors, H.; Van Houdt, R.; Janssen, P. J.; Janssen, A.; Coninx, I.; Mergeay, M.; Leys, N. Heavy metal resistance in *Cupriavidus metallidurans* CH34 is governed by an intricate transcriptional network. *Biometals* **2011**, *24*, 1133–51, doi:10.1007/s10534-011-9473-y.
56. Brown, N. L.; Misra, T. K.; Winnie, J. N.; Schmidt, A.; Seiff, M.; Silver, S. The nucleotide sequence of the mercuric resistance operons of plasmid R100 and transposon Tn*501*: further evidence for *mer* genes which enhance the activity of the mercuric ion detoxification system. *MGG Mol. Gen. Genet.* **1986**, *202*, 143–151, doi:10.1007/BF00330531.

57. Liebert, C. A; Hall, R. M.; Summers, A. O. Transposon Tn*21*, flagship of the floating genome. *Microbiol. Mol. Biol. Rev.* **1999**, *63*, 507–522.
58. Agulló, L.; Romero-Silva, M.J.; Domenech, M.; Seeger, M. P-Cymene promotes its catabolism through the p-Cymene and the p-Cumate pathways, activates a stress response and reduces the biofilm formation in *Burkholderia xenovorans* LB400. *PLoS One* **2017**, *12* (1):e00179-17, doi:10.1371/journal.pone.0169544.
59. Mouali, Y. E; Kim, H.; Ahmad, I.; Brauner, A.; Liu, Y.; Skurnik, M.; Galperin, M. Y.; Römling, U. Stand-alone EAL domain proteins form a distinct subclass of EAL proteins involved in regulation of cell motility and biofilm formation in enterobacteria. *J. Bacteriol.* **2017**, *199*, 1–17, doi:10.1128/JB.00179-17.
60. Monchy, S.; Benotmane, M. a; Janssen, P.; Vallaeys, T.; Taghavi, S.; van der Lelie, D.; Mergeay, M. Plasmids pMOL28 and pMOL30 of *Cupriavidus metallidurans* are specialized in the maximal viable response to heavy metals. *J. Bacteriol.* **2007**, *189*, 7417–25, doi:10.1128/JB.00375-07.
61. Kulasekara, H. D.; Ventre, I.; Kulasekara, B. R.; Lazdunski, A.; Filloux, A.; Lory, S. A novel two-component system controls the expression of *Pseudomonas aeruginosa* fimbrial cup genes. *Mol. Microbiol.* **2005**, *55*, 368–380, doi:10.1111/j.1365-2958.2004.04402.x.
62. Chen, M. W.; Kotaka, M.; Vonrhein, C.; Bricogne, G.; Rao, F.; Chuah, M. L. C.; Svergun, D.; Schneider, G.; Liang, Z.-X.; Lescar, J. Structural insights into the regulatory mechanism of the response regulator RocR from *Pseudomonas aeruginosa* in cyclic di-GMP signaling. *J. Bacteriol.* **2012**, *194*, 4837–46, doi:10.1128/JB.00560-12.
63. Goeres, D. M.; Loetterle, L. R.; Hamilton, M. A.; Murga, R.; Kirby, D. W.; Donlan, R. M. Statistical assessment of a laboratory method for growing biofilms. *Microbiology* **2005**, *151*, 757–762, doi:10.1099/mic.0.27709-0.
64. Koechler, S.; Farasin, J.; Cleiss-Arnold, J.; Arsène-Ploetze, F. Toxic metal resistance in biofilms: diversity of microbial responses and their evolution. *Res. Microbiol.* **2015**, doi:10.1016/j.resmic.2015.03.008.
65. Merritt, J. H., Brothers, K. M., Kuchma, S. L., O'Toole, G.A. SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. **2007**, 189, 8154-8164, 10.1128/JB.00585-07.
66. Hisert, K. B.; MacCoss, M.; Shiloh, M. U.; Darwin, K. H.; Singh, S.; Jones, R.A; Ehrt, S.; Zhang, Z.; Gaffney, B. L.; Gandotra, S.; Holden, D. W.; Murray, D.; Nathan, C. A glutamate-alanine-leucine (EAL) domain protein of *Salmonella* controls bacterial survival in mice, antioxidant defence and killing of macrophages: role of cyclic diGMP. *Mol. Microbiol.* **2005**, *56*, 1234–45, doi:10.1111/j.1365-2958.2005.04632.x.
67. Lacey, M. M.; Partridge, J. D.; Green, J. *Escherichia coli* K-12 YfgF is an anaerobic cyclic di-GMP phosphodiesterase with roles in cell surface remodelling and the oxidative stress response. *Microbiology* **2010**, *156*, 2873–86, doi:10.1099/mic.0.037887-0.
68. Karunakaran, E., Mukherjee, J. Ramalingam, B., Biggs, C.A. "Biofilmology": a multidisciplinary review of the study of microbial biofilms. *App. Microbiol. Biotech.* **2011**, *90,* 1869-81, 10.1007/s00253-011-3293-4.

69. Roy, A.; Kucukural, A.; Zhang, Y. I-TASSER: A unified platform for automated protein structure and function prediction. *Nat. Protoc.* **2010**, *5*, 725–738, doi:10.1038/nprot.2010.5.
70. Galperin, M. Y. Bacterial signal transduction network in a genomic perspective. *Environ. Microbiol.* **2004**, *6*, 552–567, doi:10.1111/j.1462-2920.2004.00633.x.
71. Rojas, L. Studies of bacterial bioremediation in soil microcosms contaminated with mercury (II) species, cadmium and copper, Universidad Técnica Federico Santa María, Valparaíso, Chile. 2011.
72. Park, S.; Ely, R. L. Candidate stress genes of *Nitrosomonas europaea* for monitoring inhibition of nitrification by heavy metals. *Appl. Environ. Microbiol.* **2008**, *74*, 5475–5482, doi:10.1128/AEM.00500-08.
73. Ralston, D. M.; O'Halloran, T. V. Ultrasensitivity and heavy-metal selectivity of the allosterically modulated MerR transcription complex. *Proc. Natl. Acad. Sci. U. S. A.* **1990**, *87*, 3846–3850, doi:10.1073/pnas.87.10.3846.
74. Wang, D.; Huang, S.; Liu, P.; Liu, X.; He, Y.; Chen, W.; Hu, Q.; Wei, T.; Gan, J.; Ma, J.; Chen, H. Structural Analysis of the Hg(II)-Regulatory Protein Tn*501* MerR from *Pseudomonas aeruginosa*. *Sci. Rep.* **2016**, *6*, 1–9, doi:10.1038/srep33391.
75. Lin, T. Y.; Kampalath, R. A.; Lin, C. C.; Zhang, M.; Chavarria, K.; Lacson, J.; Jay, J. A. Investigation of mercury methylation pathways in biofilm versus planktonic cultures of *Desulfovibrio desulfuricans*. *Environ. Sci. Technol.* **2013**, *47*, 5695–5702, doi:10.1021/es400079

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 26 July 2018 doi:10.20944/preprints201807.0520.v1

Table S1. Primers used in this study

ORF	Orientation	5´- 3' Sequence	Amplicon (pb)
RMET_RS02985	Fwd	AGCGCGGTTTCGGTGATTT	127
	Rev	TGGTGTCGATCAACCTGTCT	
RMET_RS05485	Fwd	GTGCATTGCTTGGATGGCTT	145
	Rev	ACGCCTACGCATTCGTTGT	
RMET_RS05490	Fwd	GCTTGGGTTCGCAACTCCTT	124
	Rev	ACATGCTGGTGCATTGTGCT	
RMET_RS08460	Fwd	GGAATCATATGCCCAACCGCTAAG	120
	Rev	ACGTATCCGTCAAACCGCAACA	
RMET_RS08465	Fwd	GCCAAGAGTACCGATTGCGT	133
	Rev	ATGTGGATGCGCTGCACTTT	
RMET_RS09375	Fwd	GGAAACAGCGGCCTGATCAT	135
	Rev	AATTGCCAGCGTGAAGCAGA	
RMET_RS11180	Fwd	TGGCCATCGCCAATCATGTT	125
	Rev	AGAAAGGCCCGTGATCTGGT	
RMET_RS11270	Fwd	CGCGTTTGGACAGCGAATG	113
	Rev	CGTGCTGAAGCTGGTTTTGC	
RMET_RS11280	Fwd	TGCCGTCGCGTGGAAAGAAT	121
	Rev	TGTCGAATGCGGCAAGCAGA	
RMET_RS11355	Fwd	CCATCGCCGGACATCGTTT	139
	Rev	GCCTCGCTTGGCTTCATACA	
RMET_RS16170	Fwd	TCTGGCTGCATTCGCTGTTT	133
	Rev	CGGATTTCGCCACGGTAGTT	
RMET_RS19900	Fwd	GCCGACGTCACGATCGATTA	139
	Rev	CGTTTGGGCCAGATTGCTTG	
RMET_RS20545	Fwd	AAGCTTCCGGATTCGATCGAGTTG	148
	Rev	TTAGCCGCTGCAGTTCGAGATT	
RMET_RS20720	Fwd	GCTGGTCAGATCACGCAACT	127
	Rev	TGGCCAAGCCGCTGAATTT	
RMET_RS22980	Fwd	GTCGATGGCGGTCAACCTTT	115
	Rev	TCGAGCGTCAATCGTCTTGG	
RMET_RS28910	Fwd	GTGCATGGCCAGATGGTTCA	149
	Rev	CGAAAGAACGGCGAGCTGTA	
RMET_RS29325	Fwd	ACGCCTTCGAAAGCCAGTT	123
	Rev	TGGCATGGCCGTGTATGTC	
RMET_RS29670	Fwd	TCCGCCAGGTTAAGCAAACA	127
	Rev	AAGCGAATCGTGCCCTTTGT	
RMET_RS30470	Fwd	TCGCGCCTTTATCCATGACA	132
	Rev	ACCGACACTTGCCAAACGTA	
urf2	Fwd	CACGCTCAAGATCGACCAAT	133
	Rev	AAGACTCGCCGATGTTTCCA	
rpoZ	Fwd	CGCGTATTACCGTCGAAGA	123

 doi:10.20944/preprints201807.0520.v1

	Rev	TGTCCTTTGCCTCGACCTT	
gyrB	Fwd	AGAAAACGAGGTCGCCAAGA	132
	Rev	GAAACGAGCTTGTCCTTGGT	
urf2.2	Fwd	AATAATAAGCTTATGAGCGCTTTCCGG	1050
	Rev	AATAATGAATTCATGCCGCCGCCGGCA	

Table S2. Prediction of the diguanylate cyclase functionality of GGDEF domain proteins encoded in *C. metallidurans* CH34 genome.

GGDEF domain DGCs					
Locus	**Replicon**	**Active site (7 aa)**	**Ip[a] site (3 aa)**	**Is[b] site (1 o 2 aa)**	**Prediction**
RMET_RS04365	CHR1	6	0	0	Active
RMET_RS05490	CHR1	7	3	0	Active
RMET_RS08460	CHR1	6	1	0	Active
RMET_RS09375	CHR1	7	3	2	Active
RMET_RS10905	CHR1	7	3	0	Active
RMET_RS11180	CHR1	6	2	0	Active
RMET_RS11270	CHR1	6	0	0	Active
RMET_RS11355	CHR1	7	0	0	Active
RMET_RS16170	CHR1	6	3	0	Active
RMET_RS17815	CHR1	7	1	2	Active
RMET_RS19450	CHR2	6	0	0	Active
RMET_RS19900	CHR2	6	1	2	Active
RMET_RS20545	CHR2	7	3	2	Active
RMET_RS21415	CHR2	7	3	0	Active
RMET_RS25835	CHR2	7	0	1	Active
RMET_RS28515	CHR2	7	0	0	Active
RMET_RS28900	CHR2	3	0	0	Non Active
RMET_RS29325	CHR2	7	0	0	Active

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 26 July 2018 doi:10.20944/preprints201807.0520.v1

Table S3. Prediction of the phosphodiesterase functionality of EAL domain proteins encoded in *C. metallidurans* CH34 genome.

EAL domain PDEs			
Locus	**Replicon**	**Active site (10 aa)**	**Prediction**
RMET_RS05485	CHR1	10	Active
RMET_RS08465	CHR1	9	Active
RMET_RS19345	CHR2	6	Non active
RMET_RS20720	CHR2	10	Active
RMET_RS21885	CHR2	9	Active
RMET_RS24820	CHR2	10	Active
RMET_RS25455	CHR2	9	Active
RMET_RS26980	CHR2	9	Active
RMET_RS30310	pMOL30	10	Active
RMET_RS31035	pMOL28	10	Active

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 26 July 2018 doi:10.20944/preprints201807.0520.v1

Table S4. Bioinformatic prediction of the catalytic functionality of EAL & GGDEF domain proteins encoded in *C. metallidurans* CH34 genome.

Hybrid proteins							
		GGDEF				EAL	
Locus	**Replicon**	**Active site (7 aa)**	**Ip[a] site (3 aa)**	**Is[b] site (1 o 2 aa)**	**Prediction**	**Active site (12 aa)**	**Prediction**
RMET_RS02985	CHR1	7	2	0	Active	12	Active
RMET_RS11280	CHR2	4	2	0	Active	12	Active
RMET_RS19120	CHR2	3	1	0	Non active	12	Active
RMET_RS20580	CHR2	5	0	0	Active	11	Active
RMET_RS24490	CHR2	2	0	0	Non active	6	Non active
RMET_RS22980	CHR2	7	0	0	Active	12	Active
RMET_RS24295	CHR2	7	0	0	Active	10	Active
RMET_RS28625	CHR2	7	1	0	Active	12	Active
RMET_RS28910	CHR2	6	1	2	Active	12	Active
RMET_RS29320	CHR2	6	0	0	Active	12	Active
RMET_RS29670	pMOL30	1	1	0	Non active	4	Non active
RMET_RS30470	pMOL30	7	2	0	Active	12	Active

a: Primary inhibition site, b: Secondary inhibition site

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 26 July 2018 doi:10.20944/preprints201807.0520.v1

Table S5. Orthology analysis of putative DGC/PDEs encoded in *C. metallidurans* CH34 and in *E. coli* K12 and *P. aeruginosa* PAO1 genomes.
Parenthesis represents the length of each aminoacidic sequence. Genes in bold were selected for transcriptomic analysis.

***C. metallidurans* CH34 gene products**		**Related gene products**				
Locus	**Domain architecture (aa)**	**Ref. Sequence**	**Organism**	**Function**	**% Id**	**Domain architecture (aa)**
GGDEF ORF						
RMET_RS04365	**PAS-GGDEF(492)**	**MorA**	***P. aeruginosa* PAO1**	**DGC-PDE**	**73**	**BaeS-PAS-GGDEF-EAL (1415)**
RMET_RS05490	GGDEF (653)	NP_251560.1	*P. aeruginosa* PAO1	Hyp	33	GGDEF (525)
RMET_RS08460	**Tar_Tsr-PAS-GGDEF (546)**	**NP_249266.1**	***P. aeruginosa* PAO1**	**Hyp**	**44**	**PBP-PAS-PAS-PAS-GGDEF-EAL (1245)**
RMET_RS09375	**GGDEF (586)**	**YedQ**	***E. coli* K12**	**DGC**	**27**	**GGDEF (558)**
RMET_RS10905	GGDEF (416)	AID76695.1	*P. aeruginosa* PAO1	Hyp	43	DNAPollII- SMCN-GGDEF (671)
RMET_RS11180	**CACHE-HAMP-PAS-GGDEF (654)**	**EHS35231.1**	***P. aeruginosa* PAO1**	**Hyp**	**30**	**CHASE-HAMP-PAS-GGDEF (638)**
RMET_RS11270	**HAMP-GGDEF (426)**	**TpbB**	***P. aeruginosa* PAO1**	**DGC**	**55**	**HAMP-GGDEF (435)**
RMET_RS11355	**GGDEF (237)**	**AID76049.1**	***E. coli* K12**	**DGC**	**42**	**REC-REC-GGDEF (542)**
RMET_RS16170	HAMP-GGDEF (599)	NP_251560.1	*P. aeruginosa* PAO1	Hyp	45	GGDEF (525)
RMET_RS17815	CACHE-GGDEF (587)	YedQ	*E. coli* K12	DGC	27	GGDEF (558)
RMET_RS19450	GGDEF (392)	NP_250418.1	*P. aeruginosa* PAO1	Hyp	37	MHYT- GGDEF (685)
RMET_RS19900	**GAF-GGDEF (338)**	**YeaP**	***E. coli* K12**	**DGC**	**41**	**GAF-GGDEF (341)**
RMET_RS20545	**REC-GGDEF (335)**	**EHS43875.1**	***P. aeruginosa* PAO1**	**Hyp**	**72**	**REC-GGDEF (347)**
RMET_RS21415	GGDEF (409)	AID74892.1	*P. aeruginosa* PAO1	DGC	44	REC-GGDEF (347)
RMET_RS25835	GGDEF (400)	WP_077873855.1	*E. coli* K12	Hyp	35	GGDEF (252)
RMET_RS28515	HisK-PAS-GGDEF (530)	NP_252391.1	*P. aeruginosa* PAO1	Hyp	35	REC-REC-GGDEF (347)
RMET_RS29325	**HAMP-PAS-GGDEF (662)**	**NP_249266.1**	***P. aeruginosa* PAO1**	**Hyp**	**30**	**PBP-PAS-PAS-PAS-GGDEF (1245)**
EAL ORF						
RMET_RS05485	**CSS-EAL (530)**	**YJcC**	***E. coli* K12**	**PDE**	**33**	**CSS-EAL (516)**
RMET_RS08465	**EAL (385)**	**MucR**	***P. aeruginosa* PAO1**	**PDE**	**33**	**MHYT-GGDEF-EAL (685)**
RMET_RS20720	**REC-EAL (404)**	**RocR**	***P. aeruginosa* PAO1**	**PDE**	**30**	**REC-EAL (392)**

Continuing from Table S3Table S3..

RMET_RS21885	EAL (353)	AID75563.1	*P. aeruginosa* PAO1	PDE	33	GGDEF-EAL (687)
RMET_RS24820	EAL (254)	AID74466.1	*P. aeruginosa* PAO1	Hyp	42	EAL-CBS-GGDEF (582)
RMET_RS25455	**EAL (298)**	**MorA**	***P. aeruginosa* PAO1**	**PDE**	**36**	**HisK-PAS-GGDEF- EAL (1415)**
RMET_RS26980	**CSS-EAL (529)**	**YJcC**	***E. coli* K12**	**PDE**	**34**	**CSS-EAL (516)**
RMET_RS30310	**EAL-DUF3030 (333)**	**MorA**	***P. aeruginosa* PAO1**	**PDE**	**45**	**HisK-PAS-GGDEF- EAL (1415)**
RMET_RS31035	**EAL-DUF3030 (329)**	**MorA**	***P. aeruginosa* PAO1**	**PDE**	**45**	**HisK-PAS-GGDEF- EAL (1415)**
Hybrid ORF						
RMET_RS02985	**MASE1-PAS-GGDEF-EAL (1121)**	**NP_249872.1**	***P. aeruginosa* PAO1**	**Hyp**	**47**	**PAS-PAS-GGDEF- EAL (1120)**
RMET_RS11280	**PAS-PAS-GAF-GGDEF-EAL (850)**	**DosP**	***E. coli* K12**	**PDE**	**32**	**PAS-PAS-GGDEF - EAL (799)**
RMET_RS19120	GGDEF-EAL (660)	AID74522.1	*P. aeruginosa* PAO1	Hyp	33	MHYT-GGDEF-EAL (726)
RMET_RS20580	GGDEF-EAL (441)	AID75563.1	*P. aeruginosa* PAO1	Hyp	39	GGDEF-EAL (687)
RMET_RS22980	**MHYT-GGDEF-EAL (709)**	**MucR**	***P. aeruginosa* PAO1**	**PDE**	**65**	**MHYT-GGDEF-EAL (685)**
RMET_RS24295	EAL-CBS-GGDEF (676)	WP_079279815.1	*P. aeruginosa* PAO1	Hyp	34	EAL-CBS-GGDEF (601)
RMET_RS28625	PAS-GGDEF-EAL (589)	MucR	*P. aeruginosa* PAO1	PDE	42	MHYT-GGDEF-EAL (685)
RMET_RS28910	CBS-CBS-GGDEF-EAL (839)	NP_249266.1	*P. aeruginosa* PAO1	PDE	48	PBP - PAS - GGDEF-EAL (1245)
RMET_RS29320	GGDEF-EAL (581)	AID74522.1	*P. aeruginosa* PAO1	Hyp	41	MHYT-GGDEF-EAL (726)
RMET_RS30470	**CBS-GGDEF-EAL (705)**	**AID71820.1**	***P. aeruginosa* PAO1**	**Hyp**	**40**	**PBP-PAS-PAS-PAS-GGDEF-EAL (1245)**

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 26 July 2018 doi:10.20944/preprints201807.0520.v1

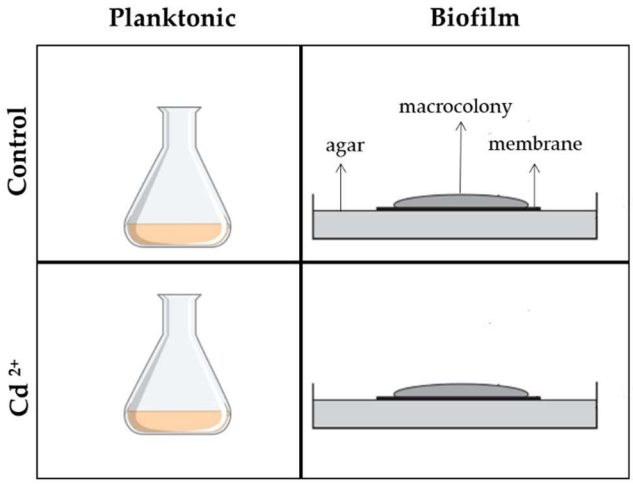

Figure S1. Experimental model used for the exposure to cadmium of planktonic and biofilm cells. Exposure of planktonic cells to cadmium (2 mM) during 45 min. Exposure of biofilm cells to cadmium (2 mM) by transferring the macrocolony grown on polycarbonate membranes from a control to a 2 mM cadmium-containing medium.

 doi:10.20944/preprints201807.0520.v1

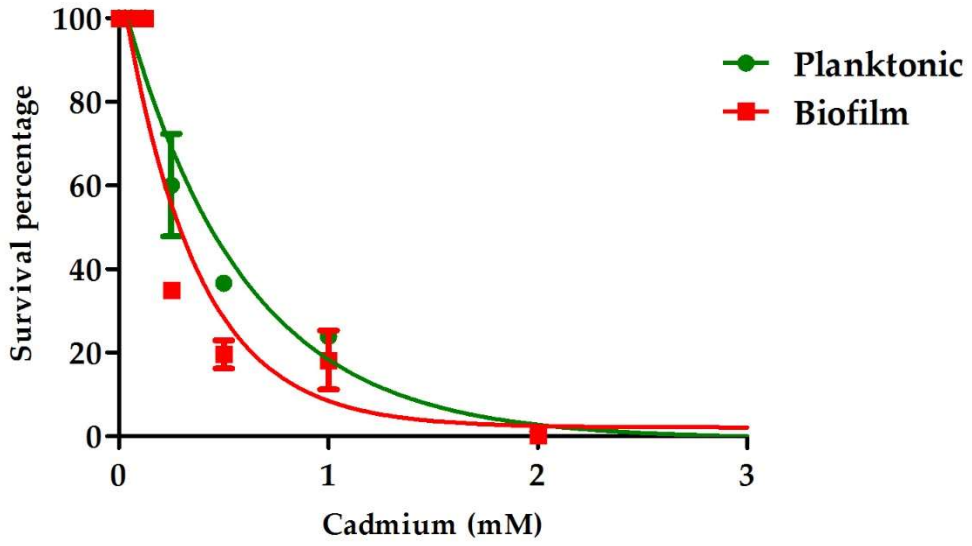

Figure S2: Cadmium kill curves of *C. metallidurans* CH34 in planktonic and biofilm lifestyles. Dots corresponds to relative viability means ± standard deviation obtained from two biological replicates, each one by three technical replicates. Continuous lines represent non-linear regressions calculated for each strain to get susceptibility parameters (MBC & MBEC) trough interpolation.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 26 July 2018 doi:10.20944/preprints201807.0520.v1

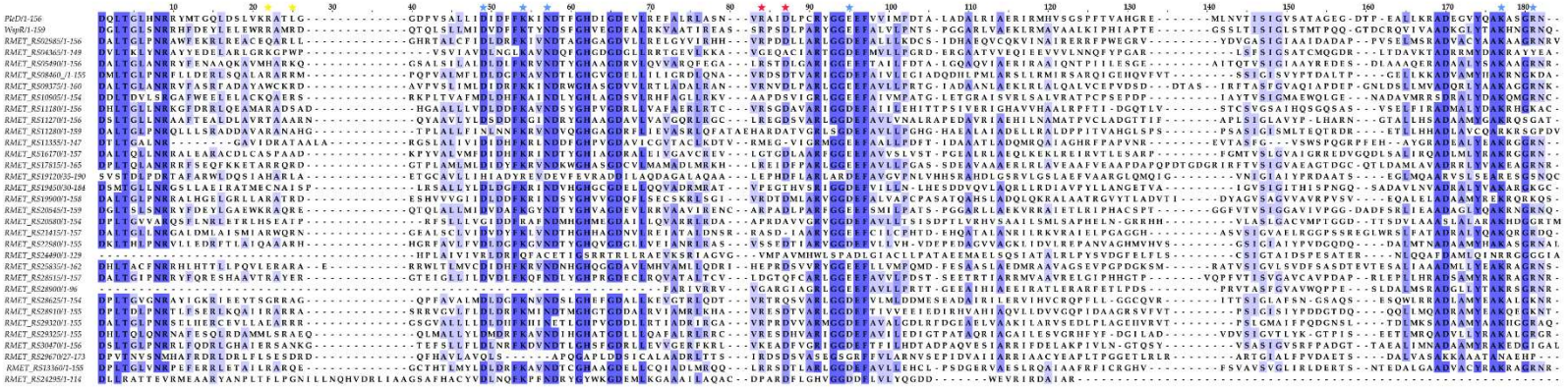

Figure S3. Multiple alignment of GGDEF domains encoded in *C. metallidurans* CH34 genome. As reference GGDEF domains of the functional DGCs PleD (*Caulobacter crescentus*) and WspR (*P. aeruginosa*). Symbols corresponds to determinant residues in catalytic activity. Blue: Active site, Red: Primary inhibition site, Yellow: Secondary inhibition site. Alignments performed trough ClustalW method.

 doi:10.20944/preprints201807.0520.v1

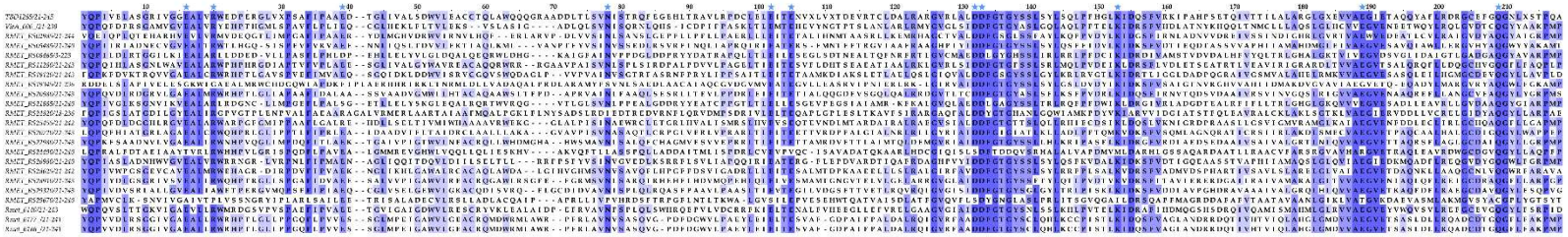

Figure S4. Multiple alignment of EAL domains encoded in *C. metallidurans* CH34 genome. As reference EAL domains of the functional PDEs TBD_1265 (*Thiobacillus denitrificans*) and VieA (*Vibrio cholerae*). Blue Symbols corresponds to determinant residues in catalytic activity. Alignments performed trough ClustalW method.

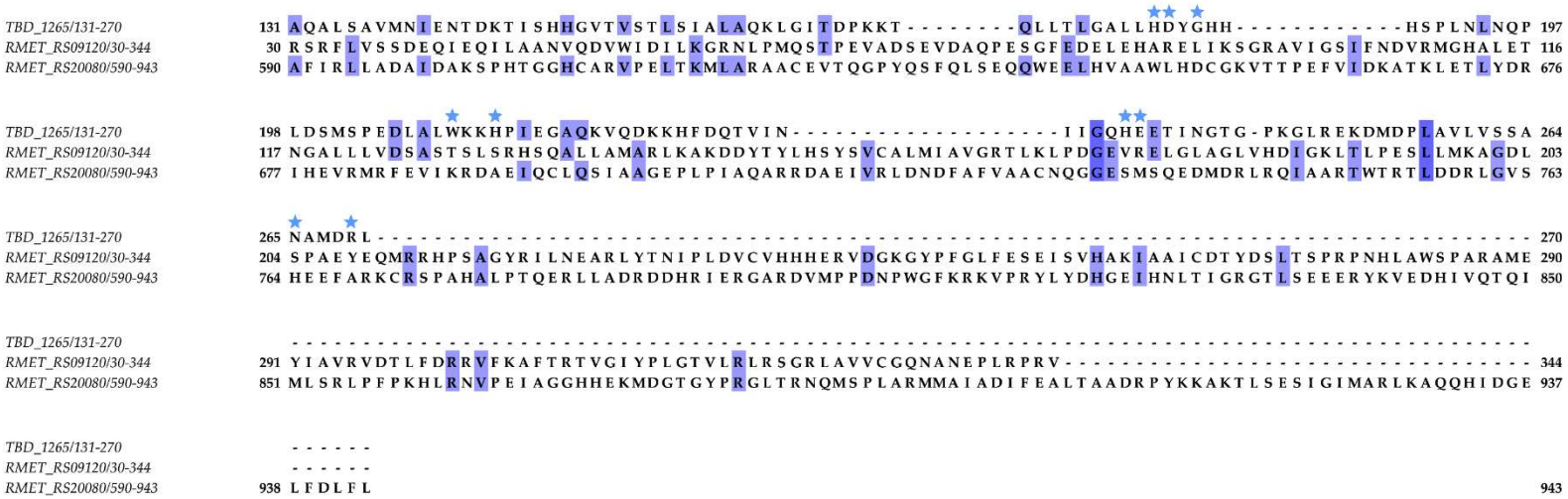

Figure S5. Multiple alignment of HD-GYP domains encoded in *C. metallidurans* CH34 genome. As reference HD-GYP domains of the functional PDE Bd1817 (*Bdellovibrio bacteriovorus*). Blue symbols corresponds to determinant residues in catalytic activity. Alignments performed trough ClustalW method.

 doi:10.20944/preprints201807.0520.v1

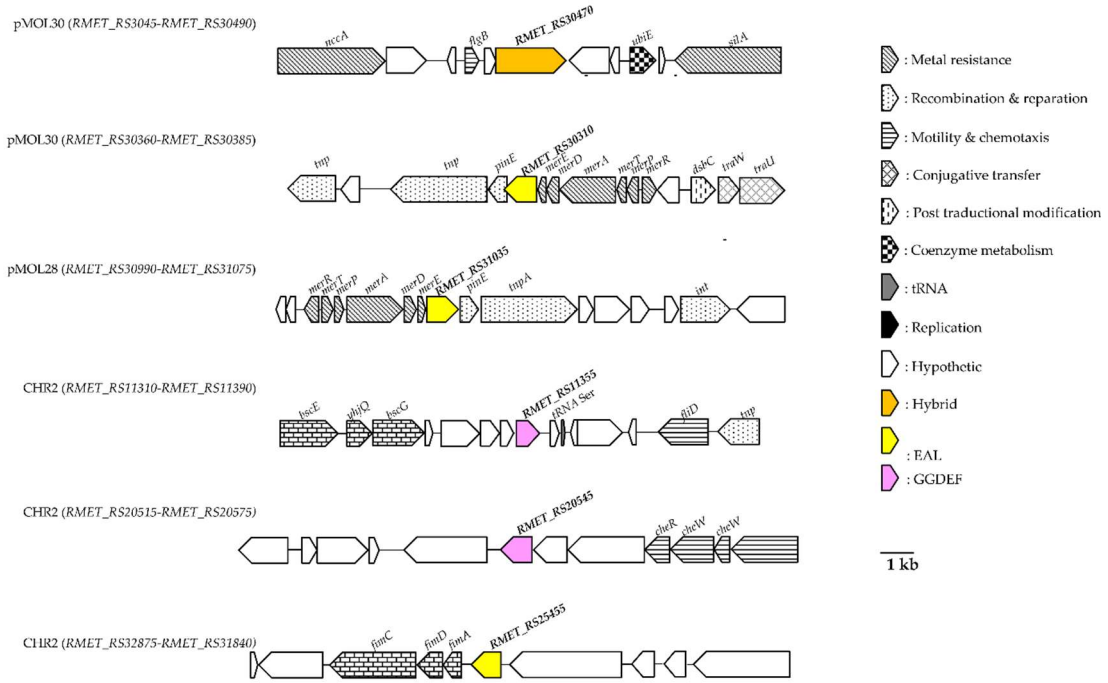

Figure S6. Genomic context of genes involved in c-di-GMP metabolism in *C. metallidurans* CH34. Sizes of the genes and the intergenic regions are on scale. *nccA:* nickel & cobalt resistance, *flgB:* flagellar basal-body rod, *ubiE:* methyltransferase, *silA:* silver efflux, *repA*: replication protein, *tnp:* transposase, *pinE: resolvase, merR:* transcriptional regulator, *merP:* periplasmic mercuric-ion binding, *merT:* mercuric transport, *merA:* mercuric reductase, *merD:* mercuric resistance transcriptional repressor, *merE:* mercuric transport, *urf2*: unknown related function, *bcsG:* cellulose biosynthesis, y*hjQ:* cellulose synthase, *bscE:* cellulose production, *fliD:* flagellar hook *cheR:* methylase/chemotaxis, *cheW:* kinase/chemotaxis, *fimA:* major type 1 subunit fimbrin , *fimD:* outer membrane usher protein, *fimC:* fimbrial chaperone. Both *urf2* ORFs are located between the *merE* gene and the transposon recombinase (PinE) coding gene of the *mer* operon Tn*4380* and Tn*4378*, which are located in different heavy metal resistance plasmids. The *urf2* ORF in pMOL30 overlaps with the Tn*4380* resolvase ORF by 94 nucleotides.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 26 July 2018 doi:10.20944/preprints201807.0520.v1

Figure S7. **Multiple alignment of PilZ domains encoded in *C. metallidurans* CH34 genome**. As references, were used the PilZ of PA4608 of *P. aeruginosa*, as active form, and the inactive form present in XC1028 of *X. campestris*. Symbols corresponds that comprises the "c-di-GMP swicht" binding motif. Alignments performed trough MAFFT method

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 26 July 2018 doi:10.20944/preprints201807.0520.v1

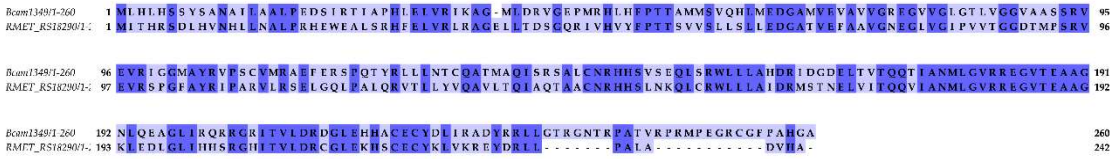

Figure S8. Global aligment between aminoacidic sequences encoded by *bcam1349* (*B. cenocepacia*) and *RMET_RS18290* (*C. metallidurans* CH34) genes. Sequences with 47.1% identity and 63.2 % similarity.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 26 July 2018 doi:10.20944/preprints201807.0520.v1

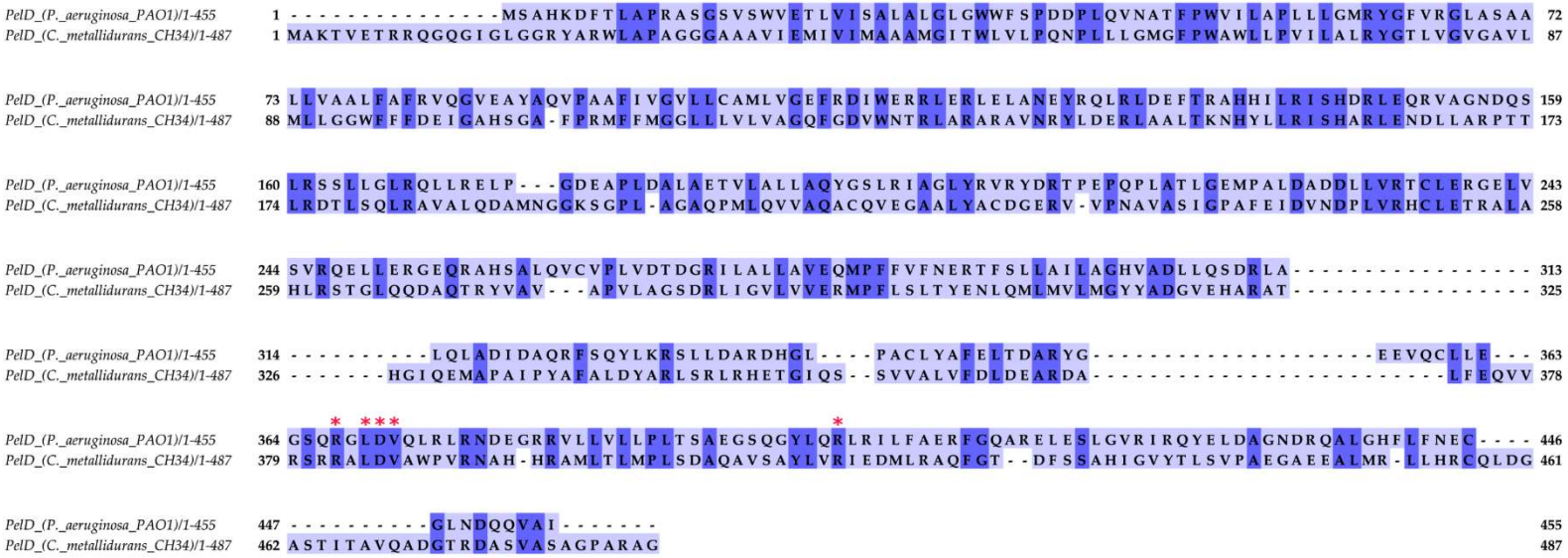

Figura S9. Global aligment between aminoacidic sequences of c-di-GMP efector PelD encoded in genomes of *P. aeruginosa* PAO1 and *C. metallidurans* CH34. Determinant residues in c-di-GMP binding are highlighted in asterik.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 26 July 2018 doi:10.20944/preprints201807.0520.v1

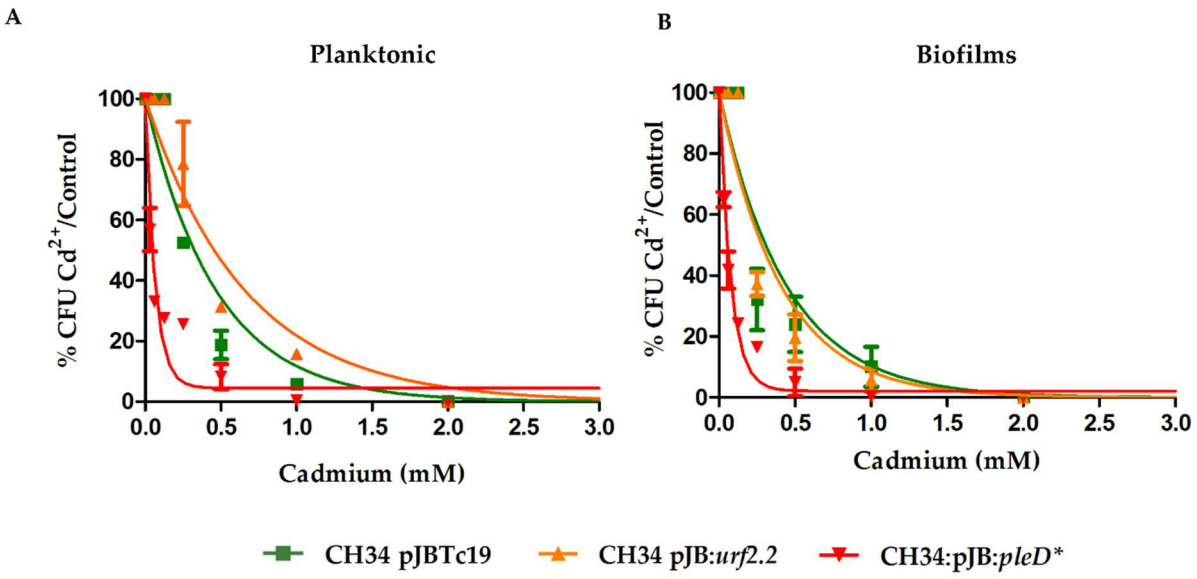

Figure S10. Cadmium kill curves of transformed strains of *C. metallidurans* CH34 in planktonic and biofilm lifestyles. Dots corresponds to relative viability means ± standard deviation obtained from two biological replicates, each one by three technical replicates. Continuous lines represent non-linear regressions calculated for each strain to get susceptibility parameters (MBC & MBEC) trough interpolation.