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A novel bacterial L-arginine sensor controlling c-di-GMP levels in *Pseudomonas aeruginosa*

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Abstract

Nutrients such as amino acids play key roles in shaping the metabolism of microorganisms in natural environments and in host–pathogen interactions. Beyond taking part to cellular metabolism and to protein synthesis, amino acids are also signaling molecules able to influence group behavior in microorganisms, such as biofilm formation. This lifestyle switch involves complex metabolic reprogramming controlled by local variation of the second messenger 3′, 5′-cyclic diguanylic acid (c-di-GMP). The intracellular levels of this dinucleotide are finely tuned by the opposite activity of dedicated diguanylate cyclases (GGDEF signature) and phosphodiesterases (EAL and HD-GYP signatures), which are usually allosterically controlled by a plethora of environmental and metabolic clues. Among the genes putatively involved in controlling c-di-GMP levels in *P. aeruginosa*, we found that the multidomain transmembrane protein PA0575, bearing the tandem signature GGDEF-EAL, is an L-arginine sensor able to hydrolyse c-di-GMP. Here, we investigate the basis of arginine recognition by integrating bioinformatics, molecular biophysics and microbiology. Although the role of nutrients such as L-arginine in controlling the cellular fate in *P. aeruginosa* (including biofilm, pathogenicity and virulence) is already well established, we identified the first L-arginine sensor able to link environment sensing, c-di-GMP signaling and biofilm formation in this bacterium.

KEYWORDS

arginine, C-di-GMP, hybrid, protein, phosphodiesterase, venus fly trap

1 | INTRODUCTION

The 3′,5′-cyclic diguanylic acid (c-di-GMP) is an ubiquitous dinucleotide in bacteria, controlling complex processes such as the transition from single-cell motile state to biofilm and cellular differentiation.[1] The intracellular levels of c-di-GMP are modulated by the opposite activities of diguanylate cyclases (DGCs), containing GGDEF domains, and phosphodiesterases (PDEs), containing either EAL or HD-GYP domains, responsible for the synthesis and degradation of c-di-GMP, respectively.[1] In general, high levels of c-di-GMP are associated to biofilm formation, while a decrease in its intracellular levels pairs with biofilm dispersal.[1] However, it has been demonstrated that the complex and redundant network of DGCs/PDEs enzymes impacts on several other processes that are not necessarily related to biofilm, by finely regulating c-di-GMP levels.[2–4] To further complicate the scenario, genetic mutants in putative DGCs and/or PDEs showed unexpected phenotypes in terms of biofilm formation/dispersal.[5,6]

A recent analysis on the regulation of the c-di-GMP network in *Pseudomonas fluorescens* Pf01 clearly showed that multifaceted regulatory strategies can take place to achieve the final phenotype, including combinations of ligand-mediated signals and/or transcriptional regulation.[5] Therefore, it appears that multiple signaling pathways could act together to obtain a unique c-di-GMP-dependent phenotype, and that different pathways could lead to the same output. In this regard, the activity of DGCs and PDEs is often allosterically regulated by environmental/metabolic signals acting on upstream sensory domains.[1] Indeed, several environmental and metabolic signals characterized so far dramatically change the overall structure of the target protein, affecting cooperativity in catalysis, protein oligomerization or protein-protein interaction.[7–9] However, regardless of the molecular

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mechanisms, the common trait of c-di-GMP network is that nutrients are among the most important driving signals leading to c-di-GMP-mediated metabolic reprogramming. In particular, it has been shown that sensing of metabolites relevant to central metabolism, such as amino acids, links the reshaping of such metabolism with the modifications of c-di-GMP-levels.[10–12] To date, few data are available on such strategies of nutrients sensing, although the re-profiling of central metabolism represents a novel perspective to tackle the biofilm issue.[11,13]

Among periplasmic domains known to be involved in nutrients sensing, the Venus flytrap (VFT) of the periplasmic binding proteins (PBPs) is the most representative.[14] Nutrients recognized by PBPs include mono- and oligosaccharides, amino acids, oligopeptides, oxyanions, cations, and vitamins. In general, PBPs trap nutrients for their internalization into bacteria by ABC transporters; they are commonly in the same operon of the permease counterpart.[15] Nevertheless, the PBP-fold and specificity is conserved also when fused to diverse sensory/transduction systems, not necessarily linked to the import event (ie, the so-called "sensor-PBPs"), conserved both in prokaryotes and in eukaryotes.[14,16–18]

Regardless of the function, ligand binding triggers closure of the VFT by bringing its two domains together, like a Venus flytrap of the carnivorous plant *Dionaea muscipula*, from which its name derives. This closure allows the PBP-ligand complex to be competent for the interaction with the permease or, if fused to a membrane protein, to initiate the intracellular signal transduction downstream the polypeptide.

To deepen the knowledge on the control of c-di-GMP levels in response to nutrients related to central metabolism, in this work we focused our analysis on PA0575, a so far poorly characterized protein from *P. aeruginosa*. PA0575 is a multidomain hybrid protein containing a VFT domain, four Per-Arnt-Sim (PAS) domains and a GGDEF-EAL effector superdomain. Initial biological studies on the PA0575 hortologue gene in *P. aeruginosa* PA14 (named PA14_07500, 99% sequence identity) could not assign an enzymatic activity,[19] while in a following study a PA14 strain carrying PA14_07500 deletion showed severely reduced swarming and swimming motility.[20] More recently, the same PA14_07500 gene has been shown to display slightly different surface behaviors, while controlling matrix production and colony morphogenesis in response to the cellular redox state and pyocyanin, and accordingly named RmcA (Redox regulator of c-di-GMP).[21] It is likely that redox-sensing is mediated by one of the PAS domains in the cytoplasmic side of the protein, given that these domains function as input modules in proteins that sense oxygen, redox potential, light, and some other stimuli.[22] The diversity of phenotypes reported strongly suggest that the function of this protein is deeply affected by the environmental signals, and suggests that rather small variations in cultural conditions may result in different phenotypes.

Using a multidisciplinary approach, including bioinformatics, protein engineering and microbiology, we describe here a novel mechanism of L-arginine sensing related to the VFT domain of PA0575, and relevant to c-di-GMP metabolism. L-arginine plays a key role in *P. aeruginosa* pathogenesis and antibiotic resistance.[23–25] Noteworthy, in *P. aeruginosa* L-Arginine is at the crossroads of multiple anabolic and catabolic/energy pathways,[11] particularly under anaerobic conditions, thus widening the metabolic versatility of this bacterium. L-arginine metabolism is emerging as a crucial factor in complex biological processes involving metabolic-reprogramming, such as those related to bacterial biofilm formation and virulence, immune response and cancer.[26,27] Interestingly, recent data suggest that L-arginine is perceived and catabolized by bacteria during infection to escape the host defense strategies by (i) changing the pH (due to the ammonium production) and (ii) contrasting the iNOS-mediated nitric oxide production, (by depleting the host of the iNOS substrate, ie, L-Arginine).[27]

Therefore, the identification of a novel L-Arginine sensor in *P. aeruginosa*, as presented in this article, represents a contribution to the characterization of the mechanisms controlling host-pathogen interaction.

2 | MATERIALS AND METHODS

For experiments reported in the Supporting Information materials, the procedures are reported in the corresponding figure legends.

2.1 | PA0575 domains modeling

The program Phyre[228] was used to detect template folds for the N-terminal periplasmic domain (PP domain; 20-274) and the best hits (Accuracy = 100%) found were chosen for model construction, and aligned to detect structurally conserved regions (SCRs) using PyMod 2.0.[29,30] A multiple sequence alignment between PA0575 and templates was obtained using the program Muscle.[31] The alignment was then used for the secondary structure prediction (PhD server)[32] and for detection of evolutionary conserved residues.[33] An initial alignment between the structural templates and the PA0575 sequence constructed based solely on sequence similarity. The alignment was then manually optimized to match several characteristics, including the observed and predicted secondary elements, the hydrophobic regions in the three-dimensional structures, the structurally and functionally conserved residues and the SCRs. Care was taken to insert gaps preferably in regions which correspond to loops in the crystal structures. Pymod 2.0 was used for the manipulation of alignments and to merge the predicted models in a multidomain model. Protein monomeric models were constructed using the Modeler package.[34] One hundred different models were built and evaluated using several criteria: the model displaying the lowest objective function was taken as the representative one, and analyzed with Dope Score and Verify_3d[35] to monitor its stereochemical quality.

2.2 | Protein expression and purification

PA0575 gene was amplified by PCR from genomic PAO1 DNA and subcloned (NdeI/BamHI) as N-terminal His-tag construct in Pet28b. From this template, the construct encompassing residues 21-255 (hereinafter VFT) was subcloned by PCR (NdeI/XhoI) into the same vector.

Site-directed mutants were obtained using the Quikchange Lightning kit (Agilent Technologies, Santa Clara, CA) according to the

manufacturer's instructions; PCR and mutagenesis were confirmed by DNA sequencing.

VFT and full-length were overexpressed in the *E. coli* BL21 (DE3) strain. Bacterial cultures were grown at 37°C in Luria-Bertani (LB) liquid medium supplemented with 30 μg mL^{-1} Kanamycin (Sigma). When $OD_{600} \sim 0.8$, the temperature was reduced to 20°C and protein expression was induced by adding 0.5 mM IPTG (isopropyl β-D-thiogalactoside; Sigma). Cells were harvested by centrifugation after 20 h and stored at −20°C. Bacterial pellets were suspended in lysis buffer containing 250 mM NaCl, 50 mM Tris-HCl pH 8, 1 mM PMSF and 1 complete protease inhibitor (Roche) (+2% v/v Tween20 in the full-length sample) and lysed by sonication on ice. After centrifugation, the proteins were purified by affinity chromatography using a HisTrap column (GE Healthcare) loaded with Ni^{2+} and equilibrated with Buffer A (20 mM Tris-HCl pH 8.5, 150 mM NaCl, for VFT; 20 mM Tris-HCl pH 7.5, 250 mM NaCl, +2% v/v Tween20, for full-length). Elution was carried out increasing the imidazole concentration, with the full-length protein eluting at 300 mM imidazole and VFT at 200 mM imidazole. Fractions containing VFT pure protein or full-length PA0575 (see Supporting Information materials for details) were pooled, imidazole was removed with PD-10 desalting columns (GE Healthcare) and concentrated with 10 kDa cut-off Ultracel Amicon concentrators. The VFT sample was than loaded on a FPLC column (Superdex 75 16/600, GE Healthcare) and eluted with buffer A using an FPLC apparatus (AKTA system). Full-length protein purity was assessed to be ∼50%; activity assay was performed with freshly purified fractions. Purified VFT protein was flash frozen in liquid nitrogen and stored at −20°C. Protein content was evaluated with BCA assay (Sigma-Aldrich) and for VFT also spectroscopically.

2.3 | Isothermal titration calorimetry (ITC) assays

Isothermal titration calorimetry (ITC) experiments were carried out using an iTC200 microcalorimeter (MicroCal). Optimal conditions for VFT titrations with different amino acids were: 1.2 μL aliquots of amino acid solution (0.6 mM, pH 8.5, in the same buffer of protein solution) injected into a 32 μM VFT solution (20 mM Tris-Hcl, pH 8.5, 150 mM NaCl); time interval between injections was 180 seconds. ITC data were analyzed by integrating the heat exchange for each addition and normalized for the amount of injected protein; the heat exchange due to the dilution of the amino acid solution (0.2 mM) into the ITC buffer was subtracted, as normalized value, to the titration data. The heat of binding (*H*), the stoichiometry (*n*), and the dissociation constant (K_D) were then calculated from plots of the heat evolved per mole of ligand injected versus the molar ratio of ligand to protein using the Origin software provided by the vendor (single binding site equation). Data are the mean of at least three experiments ±SD.

2.4 | Kinetic assays

Possible PDE and/or DGC activities were evaluated by reverse-phase high-performance liquid chromatography (RP-HPLC), as previously described.[36] Briefly, protein solution (2 μM) was incubated at 25°C for 10 min in 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 2.5 mM $MnCl_2$, 0,5% v/v Tween20 and then 100 μM GTP or 30 μM c-di-GMP was added to evaluate DGC or PDE activity, respectively. About 150 μL of reactions were stopped at selected times by adding 150 μL of 50 mM EDTA pH 6.0 and boiled for 10 min. Reactions were centrifuged for 10 min at 13 000 rpm and the protein precipitate was removed with 0.2 μm filters (Bilk GHP Acrodisc 13 mm). The reactions products were separated using a 150 x 4.6 mm reverse phase column (Prevail C8, Grace Davison Discovery Science, particle size of 5 μm) with 100 mM phosphate buffer pH 5.8/ methanol (98/2, v/v, 1 mL min^{-1}) as mobile phase and set the UV detector at 252 nm.

2.5 | Bacterial strains, growth conditions and chemicals

E. coli and *P. aeruginosa* strains used in this study are listed in Supporting Information Table S1. Bacterial cultures were grown at 37°C in Luria-Bertani broth,[37] LB supplemented with 1.5% (w/v) agar, or M9 Minimal Medium[37] supplemented with 20 mM L-arginine, L-lysine, L-glutamic acid or L-leucine as carbon sources. When required, the following chemicals were added to the growth media: ampicillin (Ap, 100 μg mL^{-1}), chloramphenicol (Cm, 30 μg mL^{-1}), tetracycline (Tc, 10 μg mL^{-1}), nalidixic acid (Nal, 15 μg mL^{-1}), for *E. coli*; carbenicillin (Cb, 300 μg mL^{-1}), chloramphenicol (Cm, 375 μg mL^{-1}), tetracycline (Tc, 200 μg mL^{-1}), sucrose 10% (w/v), for *P. aeruginosa*. Chemicals, including synthetic pyocyanin, were purchased from Sigma–Aldrich.

2.6 | Construction of *P. aeruginosa* ΔPA0575 and pPA0575 plasmid

The *P. aeruginosa* PAO1 ΔPA0575 chromosomal deletion mutant was constructed by allelic exchange using the suicide vector pDM4-PA0575 (Supporting Information Table S1) as previously described[38,39] pDM4-PA0575 was made as follows; using PAO1 template DNA, upstream (UP) and downstream (DW) fragments of the PA0575 genes were amplified with primer pairs FW-PA0575-UP (5'-TATTCTAGAGCTTGGCGAGCGCATG-3′) and RV-PA0575-UP (5'-TATGAATTCCTGCATGAGGCGGTCTCG-3′), or FW-PA0575-DW (5'-TATGAATTCGAGCAGATCCAGGGCTTC-3′) and RV-PA0575-DW (5'-TATGTCGACGCCTGTTCAGGGTGACTGA-3′), respectively.

The resulting PA0575-UP and PA0575-DW PCR products were cloned in pBluescript-II KS+ with XbaI-EcoRI and EcoRI-SalI restriction enzymes, respectively, resulting in the plasmids pBS-PA0575-UP and pBS-PA0575-DW (Supporting Information Table S1). The PA0575-UP and PA0575-DW fragments were then excised with the corresponding restriction enzymes and both cloned in the suicide vector pDM4,[38] resulting in the pDM4-PA0575 plasmid. Allelic exchange in *P. aeruginosa* PAO1 following conjugal mating with the *E. coli* S17.1 λ*pir* pDM4-PA0575 donor strain and sucrose counter-selection was performed as described by.[39] Among sucrose-resistant clones, the PAO1 ΔPA0575 mutant was selected by PCR and verified by sequence analysis.

The complementation plasmid pPA0575 is based on the pUCP18 vector[40] (Supporting Information Table S1). Briefly, the PA0575 gene was amplified from PAO1 chromosomal DNA with primers FW-PA0575 (5'-TATGGATCCGCCAGATCGAAGC-3′) and RV-PA0575,

and the resulting PCR product was cloned in pUCP18 *via* BamHI-XbaI restriction.

2.7 | Phenotypic assays

Measurement of P*cdrA*::lux activity was performed as previously described in.[41,42] Briefly the mini-CTX-*lux* empty vector[43] (Supporting Information Table S1) and its derivative mini-CTX-P*cdrA*::*lux*[41] (Supporting Information Table S1) were conjugated in PAO1 wild type and ΔPA0575 strains by standard methods.[37] The resulting strains, containing or not pUCP18 or pPA0575, were grown for 16 h at 37°C with shaking in M9 supplemented with 20 mM L-arginine, L-lysine, L-glutamic acid or L-leucine as carbon sources, and 300 μg mL^{-1} Cb for strains containing pUCP18 or pPA0575 plasmids. These pre-cultures were diluted to an A_{600} of 0.05 into M9 supplemented with 20 mM of the same carbon source as in the pre-culture, and 200 μL of the resulting bacterial suspensions were dispensed in black, flat-clear-bottom 96-well microtiter plates. Microtiter plates were incubated at 37°C with shaking at 120 rpm. Cell density (A_{600}) and bioluminescence (relative light units, RLU) were simultaneously measured after 9 h incubation in a Spark 10 M multilabel plate reader (Tecan). P*cdrA*::*lux* activity was calculated as RLU normalized to A_{600}.

For biofilm formation assays, PAO1 wild type and ΔPA0575 strains containing or not the pUCP18 empty vector or the pPA0575 plasmids were grown for 16 h at 37°C with shaking in M9 minimal medium supplemented with 20 mM L-arginine, L-lysine, L-glutamic acid or L-leucine as carbon sources, and 300 μg mL^{-1} Cb for strains containing pUCP18 or pPA0575 plasmids. These precultures were diluted to an A_{600} of 0.05 into M9 supplemented with 20 mM of the same carbon source as in the preculture, and 200 μL of the resulting bacterial suspensions were dispensed in polystyrene 96-well microtiter plates. Microtiter plates were incubated at 37°C in static condition for 16 h before measuring cell density (A_{600}) and biofilm formation by standard crystal violet staining (A_{595}).[44] Biofilm adhesion units were calculated as A_{595} normalized to A_{600}.

Swarming and swimming motility assays were performed as previously described,[45] in the presence or the absence of 20 mM L-arginine.

3 | RESULTS AND DISCUSSION

3.1 | In silico analysis of the VFT domain of PA0575

To identify the most promising GGDEF/EAL candidates involved in nutrient sensing, we looked for the presence of VFT-periplasmic domains among the 38 genes from *P. aeruginosa* genome harboring GGDEF and/or EAL domains.[19] We found that the PA0575 gene contains a VFT domain at the periplasmic N-terminal region (from residue 23, till residue 274), likely involved in signal recognition and transduction. To get insights into the structure-function relationships of this domain and to identify the signal controlling PA0575 activity, we built a homology model of the VFT domain, using the known crystal structures of the two most similar homologous proteins, that is, an arginine-binding protein (AncQR) from *Escherichia coli* (PDB: 4ZV1, arginine-bound complex; sequence identity: 29%) and the periplasmic VFT2 domain of *Bortedella pertussis* (PDB: 3MPK; sequence identity: 28%) (Figure 1 A and Supporting Information Figure S1 for energy graph).

The VFT domain of PA0575 presents the typical dual-lobe α/β subdomain architecture shown by AncQR and VFT2, as expected for this-fold (Figure 1 B). The two mobile sub-domains are connected by a two-stranded β-sheet (residues 120-124 and 218-221), acting as a hinge and allowing the opening/closure conformational transition upon ligand binding.[46]

In the AncQR VFT domain, an L-arginine molecule is bound at the interface between the two subdomains[47]: L-arginine binds Glu17, with ion-pair and ion–dipole interactions between its guanidinium group and the side-chain and main-chain of Glu17, respectively. Moreover, the guanidinium group of the bound L-arginine is π-stacked between the aromatic rings of Phe20 and Phe58. In PA0575, the corresponding residues are represented by Asp44, Trp47, and Trp86. Residues interacting with the α-amino and α-carboxylate moieties of L-arginine are also well conserved (Arg83 and Asp167 in AncQR; Arg110 and Asp192 in PA0575) (Figure 1 C). Similar binding geometries were found also in other arginine-binding proteins (eg, PDB: 1LAF, 2Q2A, and 2Y7I;[47]), supporting the hypothesis that PA0575 could also sense the presence of L-arginine *via* its VFT domain.

It should be mentioned that another structure of a VFT domain bound to L-arginine has been recently published, belonging to the periplasmic portion of CdgH protein from *V. cholera*, which is connected to a GGDEF domain responsible for the rugosity of this bacterium.[48] In spite of sharing the presence of coupled GGDEF and VFT modules, a sequence comparison of the CdgH and PA0575 VFT domains returned a very low sequence identity (≈13.6%), suggesting a very remote homology. This is also evident by comparing the diverse orientation and binding mode of L-arginine into the active site of the two domains, and the identity of the active site residues involved in L-arginine binding (Figure 1 D), which are instead almost identical to AncQR from *Escherichia coli* (Figure 1 C).

Moreover, an analysis of the sequences homologous to PA0575 in PFAM[49] highlighted the presence of the same domains architecture of PA0575 only in the *Pseudomonas* genus, thus suggesting that in this genus L-arginine sensing required the evolution of dedicated means to fine tune intracellular metabolism.

3.2 | The N-terminal domain of PA0575 binds to L-arginine

To validate the in silico predictions, the periplasmic portion of PA0575 (residues 21-255 of VFT) was expressed and purified to homogeneity; the domain is monomeric in solution, both in the presence and in the absence of 1 mM L-arginine in the buffer (Supporting Information Figure S2A). Excess L-arginine increases the thermal stability of the protein (Supporting Information Figure S2B), thus suggesting that an interaction between the VFT and this amino acid occurs. To quantitatively determine the thermodynamics of the interaction, we performed a titration by Isothermal Titration Calorimetry (ITC). ITC data clearly show that the VFT domain binds L-arginine with a K_D = 11.9 ±0.9 μM and a stoichiometry of 0.8 ±0.1 per monomer (Figure 2 and Supporting Information Figure S3). The thermodynamic

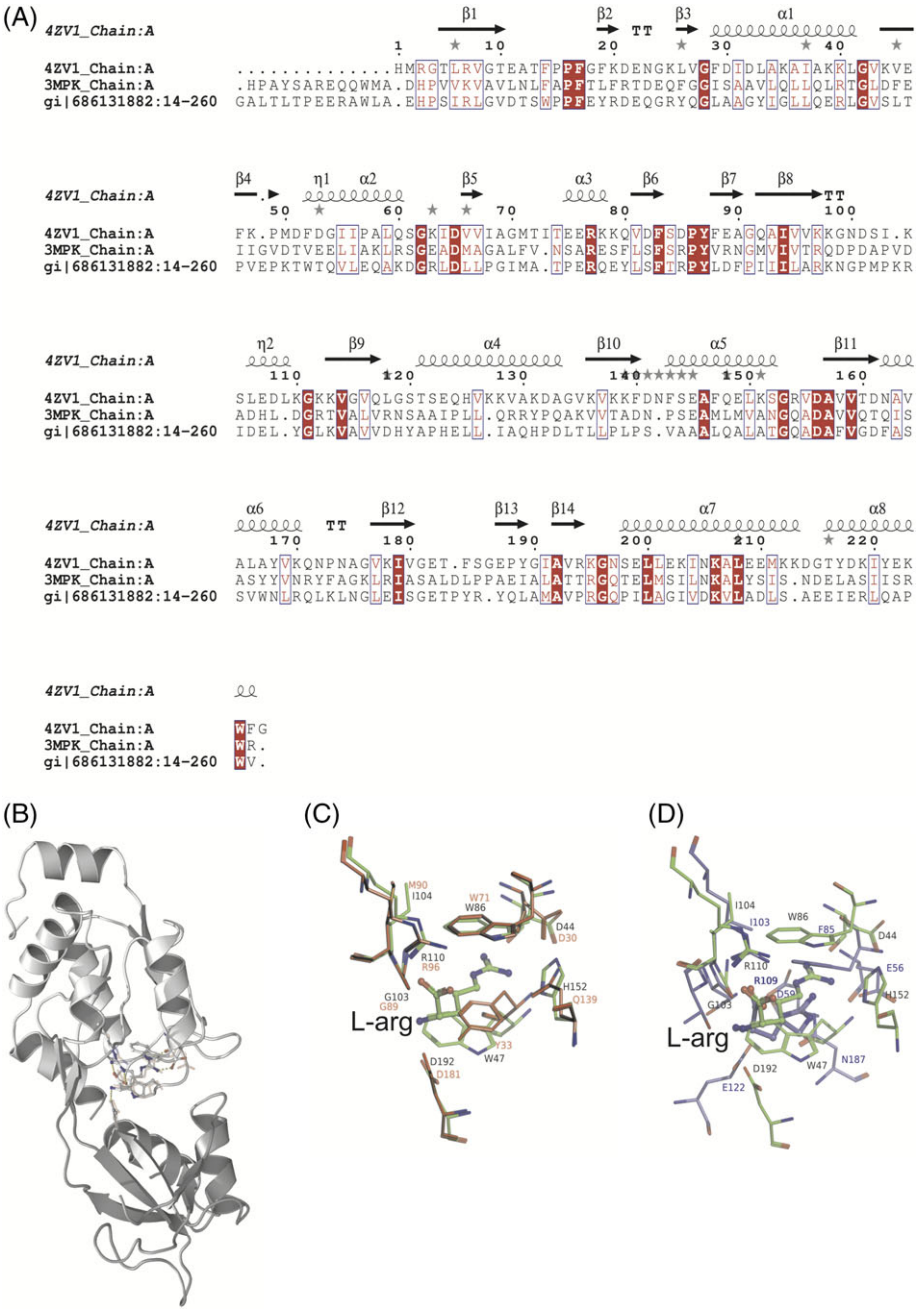

FIGURE 1 Homology model of the periplasmic N-terminal Venus flytrap (VFT) domain of PA0575 and comparison with other L-arginine binding VFT domains. (A) Sequence alignment of the VFT domain of PA0575 and the two structural templates used to build the homology model, that is, AncQR (4ZV1 in the figure), an ancestral arginine-binding protein and the periplasmic VFT2 domain of *Bortedella pertussis* (3MPK in the figure). (B) Cartoon representation of the entire domain. Polar interactions between L-arginine and the protein moiety are shown in yellow. The corresponding positions of AncQR from *Escherichia coli* (PDB: 4ZV1) are represented as transparent pink sticks. (C) Structural superposition between the VFT model of PA0575 (green sticks) and AncQR (pink sticks). Residues are labeled according to sequence numbering. Nitrogen and oxygen atoms are colored blue and red, respectively. (D) Structural superposition between the VFT model of PA0575 (green sticks) and the periplasmic portion of CdgH protein from *V. Cholera* (PDB: 5GZS; cyan sticks)

signature indicates that the binding is both entropy and enthalpy driven, thus suggesting that hydrogen bonding and hydrophobic interactions represent the driving force of the affinity (Figure 2, upper panel, inset). The measured affinity is comparable to that of the structural template AncQR (K_{D_VFT} = 11.9 vs K_{D_AncQR} = 5.7 μM), but in AncQR the binding is enthalpy driven, and the unfavorable entropic factor indicates that a conformational rearrangement occurs upon binding of L-arginine. The diversity of thermodynamic signatures, including the considerable range of ΔH and $T\Delta S$, observed for similar binding to homologous VFTs has been already acknowledged by previous studies.[47] More in general, members of the polar amino acid-binding protein (AABP) family may display different thermodynamic profiles, though sharing conserved structural determinants for the recognition of amino acids.[50]

Interestingly, binding is highly specific for L-arginine, given that no other amino acids such as lysine, leucine or glutamic acid were

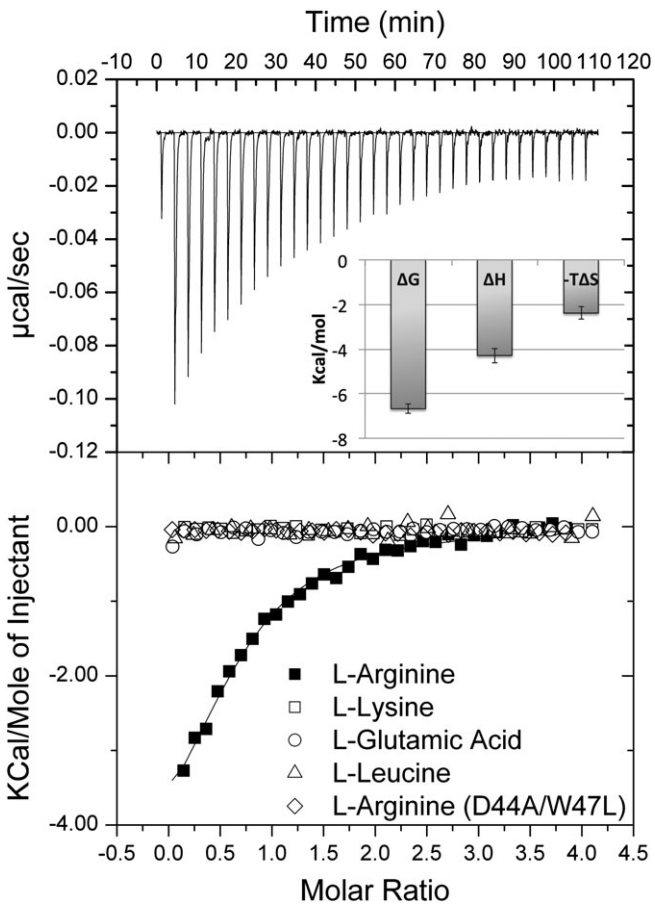

FIGURE 2 Binding of L-arginine to purified VFT. Binding of L-arginine to VFT was assayed by isothermal titration calorimetry (ITC), by titrating a VFT solution (32 μM) with different amounts of L-arginine (600 μM in the ligand syringe) at 25°C in 20 mM Tris pH 8.5, 150 mM NaCl. The upper panel shows the heat exchange in time; in the lower panel the normalized enthalpy exchange (black squares, reported as kcal/Mol of injectant) has been reported as a function of the molar ratio between the injectant and the macromolecule. Data were fitted with the single binding site equation with the origin software, as provided by the vendor (continuous line), yielding the following parameters: $N = 0.8 \pm 0.1$; $K_D = 11.9 \pm 0.9$ μM; $\Delta H = -4.3 \pm 0.3$ kcal mol^{-1}; $\Delta S = 8.0 \pm 0.9$ cal mol^{-1} deg^{-1}; the corresponding thermodynamic signature plot has been also reported (upper panel, inset). Titration was repeated with the D44A/W47 L double mutant of VFT, under the same experimental conditions (empty diamonds, in the lower panel); the mutations do not alter the overall secondary structure, as verified by CD spectrum (Supporting Information igure S3C). To verify the specificity of binding, wild-type VFT was titrated with other amino acids such as L-lysine, L-glutamic acid and L-leucine (empty squares, circles and triangles, respectively), under the same experimental conditions

able to bind to VFT (Figure 2). To further validate the structural determinants of L-arginine binding, the two residues predicted to be crucial, that is, Asp44 and Trp47, were mutated into Ala and Leu, respectively. The double mutation completely abolishes the ability of VFT to bind L-arginine (Figure 2), thus confirming the predicted binding mode, which is deeply different from that observed in the periplasmic VFTs from the *Vibrio cholerae* CdgH protein.[48] Nevertheless, regardless of the different ligand-recognition mechanism, arginine sensing, independent of its internalization could represent a common, yet poorly explored trait of bacteria connected to the control of c-di-GMP levels.[48,51]

3.3 | PA0575 modulates c-di-GMP levels in response to L-arginine

To probe the possible effect of L-arginine on PA0575 activity in vivo, a *P. aeruginosa* PAO1 mutant strain deleted in the PA0575 gene was generated by allelic exchange[38] and the intracellular levels of c-di-GMP produced by the wild type PAO1 and its isogenic ΔPA0575 mutant were compared by monitoring the activity of the c-di-GMP-reporter cassette P*cdrA*::*lux* in these strains. Because the P*cdrA* promoter is induced by high c-di-GMP levels, light emission in *P. aeruginosa* strains carrying the P*cdrA*::*lux* cassette is proportional to the intracellular concentration of c-di-GMP.[41,42] Briefly, PAO1 wild type and ΔPA0575 strains, both carrying the P*cdrA*::*lux* cassette, were grown in M9 minimal medium supplemented (alternatively) with 20 mM arginine, lysine, glutamic acid, or leucine as sole carbon sources, and NH_4Cl as saturating nitrogen source. Light emission and cell density were recorded in the resulting cultures after 9 h incubation at 37°C. As shown in Figure 3A, P*cdrA* activity was >50% higher in the ΔPA0575 strain relative to PA01 wild type when L-arginine was used as carbon source, and this phenotype could be complemented by constitutive expression of PA0575 via the pUCP18-derived plasmid pPA0575. Conversely, no significant difference was observed in P*cdrA* activity between the wild type and ΔPA0575 strains when M9 was supplemented with lysine, glutamic acid, or leucine as sole carbon sources (Figure 3 A). No difference in light emission from the same strains containing the mini-CTX-*lux* empty vector was observed, irrespective of the amino acid used as carbon source, supporting the specificity of PA0575 effect on the P*cdrA* promoter (Supporting Information Figure S4A). These data strongly indicate that PA0575 acts as a c-di-GMP PDE in PAO1, as previously shown for its homologous protein RmcA in PA14,[21] and that the ability of PA0575 to decrease c-di-GMP levels in PAO1 is L-arginine dependent.

Previous results indicated that the secreted virulence factor pyocyanin could be involved in the cytoplasmic regulation of RmcA activity in PA14.[21] To investigate this issue in PAO1 and to identify possible synergistic/competitive interactions between L-arginine and pyocyanin in controlling PA0575 activity, P*cdrA*::*lux* activity was monitored in PAO1 wild type and ΔPA0575 grown in M9 minimal medium supplemented alternatively with 20 mM arginine or glutamic acid, in the absence or in the presence of different concentrations of exogenously added pyocyanin. Our data indicate that pyocyanin does not affect P*cdrA* activity in the tested strains up to a concentration of 25 μM (ie, ~4 folds higher than the maximal concentration of pyocyanin produced by PAO1[52]), irrespective of the presence of arginine or glutamic acid in the medium (data not shown).

A decrease in intracellular c-di-GMP levels often negatively affects biofilm formation[1] and other surface/motility behaviors; hence, we compared the effect exerted by arginine and the other amino acids on biofilm formation in wild type PAO1 and ΔPA0575. When L-arginine was used as sole carbon source, biofilm adhesion units were ~12% higher in PAO1 ΔPA0575 relative to PAO1 wild type, while decreased of ~35% in PAO1 ΔPA0575-carrying plasmid

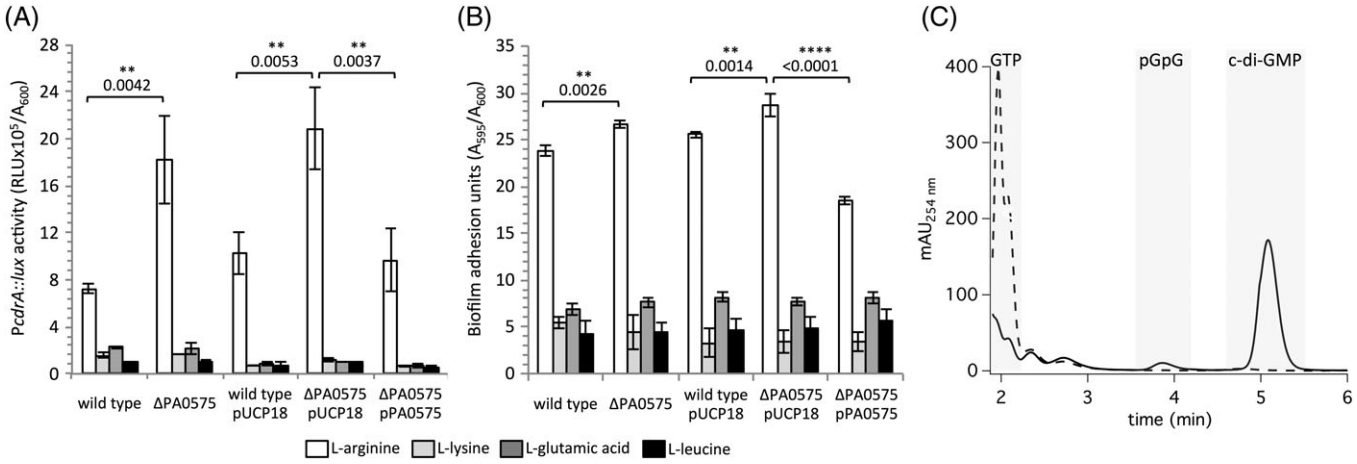

FIGURE 3 PA0575 decreases c-di-GMP levels. Effect of PA0575 gene deletion (ΔPA0575 in the figure) on c-di-GMP levels in PAO1. P*cdrA::lux* activity (A) and biofilm adhesion units (B) measured in the indicated strains grown in M9 minimal medium supplemented with L-arginine (white bars), L-lysine (light-gray bars), L-glutamic acid (dark-gray bars), or L-leucine (black bars) as sole carbon sources. Means of three independent cultures are reported with SD. Indicated *P*-values were calculated by one-way ANOVA with Tukey's multiple comparison test. Data were compared with the wild type strain and in the corresponding strain carrying the pUCP18 plasmid. The latter conditions represent the reference for the complemented strain, ΔPA0575 pPA0575 in the figure. (C) Pilot enzymatic assays on partially purified full-length PA0575. The nucleotide content of the reaction mixture, containing 2 μM of protein sample, excess GTP (100 μM; dashed line), or c-di-GMP (50 μM, continuous line), was separated by HPLC (on a C8 RP column) to assess the DGC or the PDE activity, respectively. While the PDE reaction yields the expected product (ie, pGpG), no c-di-GMP accumulates starting from GTP, under these experimental conditions

pPA0575 relative to the same strain carrying the empty vector pUCP18 (Figure 3 B). The same trend is not observed when lysine, glutamic acid, or leucine was used as carbon sources (Figure 3B). Increased biofilm biomass in the PA0575 mutant was not due to altered cell-density of the corresponding planktonic culture (Supporting Information Figure S4B). These data are in accordance with the hypothesis that PA0575 decreases c-di-GMP levels in response to L-arginine.

The PA0575 mutation did not affect PAO1 swarming and swimming motility, irrespective of the presence of L-arginine as carbon source (Supporting Information Figure S4 panels C and D). It should be mentioned that discrepancies on the role of RmcA on swarming motility in PA14 were previously reported, with our data on this pleiotropic phenotype being consistent with results published by Okegbe et al.,[21] but not with data obtained by Ha et al.[20]

As also highlighted in a recent review[2] it appears that the sensory activity of PA0575/RmcA in PAO1 and PA14 is significantly affected by the environmental conditions and that the phenotypes controlled by this protein could be strain-dependent. Moreover, it is not possible to exclude that the PA0575-dependent effector(s) could tune other aspects of cell metabolism, which are known to be modulated by L-arginine, than surface behaviour.[11]

Our in silico analysis proposes that PA0575 binds to L-arginine and our in vivo data indicate that this protein responds to L-arginine by lowering c-di-GMP levels (and consequently decreasing biofilm formation) thus suggesting a PDE activity. Accordingly, we observed PDE activity (but not a DGC) in vitro with the partially purified PA0575 protein sample (Figure 3 C and Supporting Information Figure S5), which is compatible with the aforementioned in vivo data (ie, PA0575-dependent c-di-GMP decrease).

In agreement with other studies carried out on *V. cholera*,[48], our results underline that nutrient-dependent metabolism adaptation is mediated, at least in part, by the second messenger c-di-GMP, whose role is clearly wider than previously expected.

4 | CONCLUSIONS

L-arginine metabolism is emerging as a crucial factor in complex processes involving metabolic-reprogramming, such as those related to biofilm formation, immune response and cancer.[26,27] L-arginine is at the crossroads of complex metabolic networks that enable *P. aeruginosa* to utilize this amino acid as a source of carbon, energy, and nitrogen. It can be metabolized by several enzymes, such as arginine decarboxylase (ADC), dehydrogenase (ADH), succinyl-transferase (AST, aerobic conditions) and deiminase (ADI, which produces ATP under low oxygen tension and is inducible by exogenous L-arginine).[53] Arginine fermentation (ie, the ADI pathway) allows *P. aeruginosa* to survive under anaerobic conditions when denitrification (ie, nitrates respiration) is not induced[54] and is associated to chronic infections, biofilm/virulence,[23,24] and antibiotic resistance.[25] Interestingly, also the ADC pathway modulates the host inflammatory response.[55]

Under aerobic conditions, the presence of L-arginine (even together with ammonium) in the growth medium induces the maximal expression of the Arginine-specific ABC transporters via the ArgR transcription factor, mainly to sustain the AST pathway.[53,56] It is known that in the *P. aeruginosa* PA14 strain, L-arginine negatively affects swarming motility, increases biofilm formation and, to a lower extent, c-di-GMP levels as compared to glucose-supplemented medium; moreover, metabolic fluxes involving arginine catabolism are enhanced.[57,58]

Given the plethora of pathways dependent on L-arginine, it is not surprising that *P. aeruginosa* has evolved a protein system such as the multidomain protein PA0575 to directly connect this metabolite to

the fine tuning of c-di-GMP and biofilm formation. Structural and mechanistic details on PA0575 will contribute to depict the metabolic events occurring in this bacterium in response to nutrients such as L-arginine, to possibly target in the future the environment-mediated metabolic reprogramming leading to biofilm formation.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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