A bipartite periplasmic receptor–diguanylate cyclase pair (XAC2383–XAC2382) in the bacterium *Xanthomonas citri*

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**ABSTRACT**

The second messenger cyclic diguanylate monophosphate (c-di-GMP) is a central regulator of bacterial lifestyle, controlling several behaviors, including the switch between sessile and motile states. C-di-GMP levels are controlled by the interplay between diguanylate cyclases (DGCs) and phosphodiesterases (PDEs), which synthesize and hydrolyze this second messenger, respectively. These enzymes often contain additional domains that regulate activity via binding of small molecules, covalent modification or protein–protein interactions. A major challenge remains to understand how DGC activity is regulated by these additional domains or interaction partners in specific signaling pathways. Here, we identified a pair of co-transcribed genes (*xac2382* and *xac2383*) in the phytopathogenic, Gram-negative bacterium *Xanthomonas citri* subsp. *citri*, whose mutations resulted in opposing motility phenotypes. We show that the periplasmic cache domain of XAC2382, a membrane-associated DGC, interacts with XAC2383, a periplasmic binding protein, and provide evidence that this interaction regulates XAC2382 DGC activity. Moreover, we solved the crystal structure of XAC2383 with different ligands, indicating a preference for negatively charged phosphate-containing compounds. We propose that XAC2383 acts as a periplasmic sensor that, upon binding its ligand, inhibits the DGC activity of XAC2382. Of note, we also found that this previously uncharacterized signal transduction system is present in several other bacterial phyla, including Gram-positive bacteria. Phylogenetic analysis of homologs of the XAC2382–XAC2383 pair supports several independent origins that created new combinations of XAC2382 homologs with a conserved periplasmic cache domain with different cytoplasmic output module architectures.

Cyclic diguanylate monophosphate (c-di-GMP) is a nucleotide second messenger responsible for the control of a variety of bacterial features, most of them involved in lifestyle transitions (1). Several reports have demonstrated the role of c-di-GMP in exopolysaccharide production, biofilm formation, chemotaxis and different modes of motility (swimming, twitching and swarming), virulence, antibiotic resistance, cell morphology and cell cycle regulation (2-6). Generally, high intracellular c-di-GMP levels correlate with a sessile state and biofilm formation whereas low levels correlate with increased motility.

Intracellular c-di-GMP levels are controlled by diguanylate cyclases (DGCs), which are GGDEF domain-containing proteins, and by phosphodiesterases (PDEs) harboring EAL or the less common HD-GYP domains.
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DGCs join two GTP molecules to form one molecule of c-di-GMP and two molecules of pyrophosphate. These enzymes act as dimers since each GGDEF domain can bind only one GTP at its active site (9) and the two domains must come together in the correct orientation to form a competent active site that leads to the generation of the cyclic product. Furthermore, many EAL domains that catalyze the hydrolysis of c-di-GMP to pGpG may depend on dimerization to be active (10-12). The requirement of a dimerization step and the frequent presence of accessory domains, most often located at the N-terminal of DGCs and PDEs, suggest that these enzymes are under tight regulation in order to precisely control when, where, and how much c-di-GMP is to be produced or degraded. In fact, over half of these proteins contain partner domains such as PAS (13), GAF (14) and HAMP (15) which could function as sensors or signal transmitters to the catalytic part of the molecule. Furthermore, approximately half of the many thousands of these enzymes annotated in the protein databases are also integral membrane proteins, often with a sensor domain located within the bacterial periplasm and the DGC or PDE domain located in the cytoplasm (16).

Despite the broad existence of inner membrane-spanning DGCs and PDEs in bacterial genomes, only a few studies have so far delved into their mechanisms of activation. In Pseudomonas aeruginosa, the YfiBNR operon, required for cystic fibrosis persistence, codes for a transmembrane DGC which has its activity finely controlled by the two other proteins coded by the operon, one localized in the periplasm and the other localized in the outer membrane (17). LapD from Pseudomonas fluorescens contains a dual GGDEF-EAL domain and is the best-understood member of this class of proteins. Instead of being an active DGC or PDE, LapD senses intracellular c-di-GMP fluctuations through the EAL domain and transmits this information to its periplasmic domain, which in turn recruits a protease (LapG) that cleaves a crucial adhesin that promotes biofilm formation in response to phosphate starvation (18). In this case, information regarding intracellular c-di-GMP concentration is transmitted from the bacterial cytoplasm to the periplasm, and is thus labeled an “inside-out” mechanism of signal transduction, opposite to that which occurs in the YifBNR system.

Xanthomonas is a bacterial genus comprising pathogens that infect dozens of plant species, many of which are of significant economic interest, such as orange, rice, sugarcane and crucifers. In order to cope with constant environmental changes as they colonize different host tissues, the genomes of these bacteria code for dozens of signaling proteins, among which are transmembrane DGCs and PDEs. Xanthomonas citri subsp. citri (Xac), the causal agent of citrus canker, has 18 GGDEF, 3 EAL, 3 HD-GYP and 11 GGDEF-EAL domain-containing proteins, evidence of a complex c-di-GMP signaling network in this organism (19). Of these proteins, fifteen are predicted to contain membrane-spanning helices and periplasmic domains (19). One of these is XAC2382, which has an uncharacterized N-terminal periplasmic Cache domain followed by a cytoplasmic portion that contains HAMP, GGDEF and EAL domains. As we show here, the xac2382 gene is co-transcribed with its neighboring gene xac2383. Interestingly, homologs of the xac2382-xac2383 pair can be found in a wide variety of bacterial phyla, though, in some cases the cytosolic GGDEF-EAL module has been replaced with another signaling output module (i.e. Histidine kinase or sigma54 activator domains). We therefore embarked to study XAC2382 and XAC2383, their interactions and their in vivo functions. We present genetic and biochemical evidence that supports the hypothesis that XAC2383 acts as a periplasmic sensor which regulates the DGC activity of XAC2382 via protein-protein interactions. We also determined the crystal structure of XAC2383 in complex chloride ion, phosphate, pyrophosphate and adenosine triphosphate. These results suggest that the XAC2383-XAC2382 system responds to the extracellular levels of anionic phosphate-derived ligands.
RESULTS

The xac2382 and xac2383 genes are co-transcribed and code for an integral membrane Cache_HAMP_GGDEF_EAL protein and a periplasmic protein

The xac2382 gene codes for a 76.8 kDa protein containing a periplasmic Cache domain, delimited by two transmembrane helices (residues 10-32 and 195-217), followed by three cytoplasmic domains: HAMP (named for its occurrence in Histidine kinases, Adenyl cyclases, Methyl-accepting proteins and Phosphatases, residues 198-268), GGDEF (diguanylate cyclase, residues 277-438) and EAL (c-di-GMP phosphodiesterase, residues 457-692) (Fig 1A, 1B and S1 Fig). Cache domains are the largest superfamily of extracellular sensor domains in bacteria, often encountered as an N-terminal module of multi-domain transmembrane proteins (20,21). The HAMP domain contains a canonical heptad repeat signature (residues 252-266; S1 Fig) known to mediate the formation of coiled-coil structures (22) and hence may be important for XAC2382 dimerization (23). The GGDEF domain possesses all of the nine conserved residues that are considered important for catalytic activity based on previous mutagenesis studies on DGC enzymes (9,24). The XAC2382 EAL domain is also predicted to be catalytically active since it presents conserved residues at positions predicted to be involved in metal coordination, c-di-GMP binding and general base catalysis (25,26). Furthermore, the so-called loop 6 motif (DFG(T/A)GYSS) recognized to be important for the activity of this class of PDEs has only two substitutions (Y to F and S to G at positions 6 and 8 respectively). S2 Fig present alignments of the XAC2382 GGDEF and EAL domains with well-characterized homologs and highlights the details of the features described above.

The xac2383 gene (Fig 1A and 1B) was originally annotated as a phosphonate binding protein (PhnD domain) (27) that belongs to the superfamily of periplasmic binding proteins (PBPs) that bind to different solutes, including amino acids, vitamins, sugars and ions (28-31). It has been shown that some PhnD homologs bind phosphates instead of phosphonates, which illustrates how the ligand specificity for these proteins is often not clear from the primary structure (32,33). We note that the stop codon of xac2383 overlaps with the start codon of xac2382 and a fragment extending from the end of the xac2383 gene to the beginning of the xac2382 gene can be amplified from a preparation of Xac cDNA (Fig 1C). These observations immediately bring up the possibility that these two proteins could work together in a common pathway. Another gene (xac2384) is found upstream of xac2383 (the two genes do not overlap and are separated by 54 nucleotides) and is annotated as a methylase belonging to the SpoU family (Fig 1A). Transcription sites have been mapped in the genome of the closely related species Xanthomonas campestris pv campestris strain B100 (34). In that species, the homologous locus has an almost identical genetic structure and nucleotide sequence and initiation start sites were mapped to position 2223411, which is 25 bp upstream of the start site of xcc-b100_1902, the xac2384 homolog, and to position 2224252, which is 21 bp upstream of the start site of xcc-b100_1903, the xac2383 homolog (34) (S3 Fig). No transcription start site was observed in the vicinity of start codon of xcc-b100_1904, the xac2382 homolog (34). Therefore, the evidence so far is consistent with independent transcriptional control of xac2384 and xac2383/xac2382. Since xac2384 homologs are not present in this cluster in other bacterial species beyond the Xanthomonadaceae family, it was not an object of study in the present investigation.

XAC2383 interacts with the XAC2382 periplasmic Cache domain

XAC2383 has a signal peptide for periplasmic localization and XAC2382 has a predicted Cache periplasmic domain flanked by transmembrane helices (S1 Fig). Since their genes are co-transcribed, we asked if XAC2383 might be interacting with the XAC2382 periplasmic Cache domain (XAC2382Cache). To test this hypothesis we co-expressed in E. coli XAC2383 lacking its signal peptide (XAC238331-309, 31.3 kDa) and residues 37-192 of XAC2382 (XAC2382Cache_37-192, 16.9 kDa), the latter carrying an N-terminal His-tag which was used to purify the complex by affinity
chromatography using a Ni^{2+}-His-trap column (S4 Fig). The two proteins are co-eluted from a size exclusion column (GE Superdex 200 10/300 GL) as a single peak with an average mass of 126±8 kDa calculated by multi-angle light scattering (Fig 1D). Additionally, size exclusion chromatography analyses of isolated XAC2382_{Cache,37-192} and XAC2383_{31-309} shows that the proteins elute with apparent masses close to their theoretical monomer masses (Table 1). These results indicate that oligomerization is induced upon interaction between XAC2383_{31-309} and XAC2382_{Cache,37-192}. Since XAC2382 is predicted to be an active DGC, we make the assumption that XAC2382_{Cache,37-192} is a dimer or multiple of dimers in the complex. For a XAC2382_{Cache,37-192}:XAC2383_{31-309} complex with 2:2 stoichiometry, we would expect a mass of 96.4 kDa for a (2:2)$_1$ complex and a mass of 192.8 kDa for a (2:2)$_2$ complex. For a XAC2382_{Cache,37-192}:XAC2383_{31-309} complex with 2:1 stoichiometry, we calculate theoretical masses of 65.1 kDa for the (2:1)$_1$ complex and a mass of 130.3 kDa for a (2:1)$_2$ complex. The experimentally observed apparent mass lies between the calculated theoretical masses for the (2:2)$_1$ and (2:2)$_2$ complexes and very close to the theoretical mass of the (2:1)$_2$ complex. It is important to point out that the peak in which the complex elutes is asymmetric, which may be indicative of an equilibrium of oligomeric states (Fig 1D). We therefore cannot at the moment precisely determine the stoichiometry of the XAC2382_{Cache,37-192}:XAC2383_{31-309} complex. The physiologically relevant stoichiometry may be influenced by the transmembrane and heptad repeat motifs in the XAC2382 HAMP domain (both absent in the soluble XAC2382_{Cache} construct used in the interaction studies).

The crystal structure of XAC2383 reveals a periplasmic binding protein topology and a positively charged surface lining the central ligand-binding groove

We solved the crystal structure of XAC2383$_{31-309}$ at 1.9 Å resolution using single-wavelength anomalous dispersion (S1, S2, S3 Tables and see details in Materials and Methods; structure deposited with PDB ID: 5UB3). The crystal belongs to the P2$_1$2$_1$2$_1$ space group with two protein molecules in the asymmetric unit. The molecules interface one another through an area of 798.8 Å$^2$, calculated by PISA (35). This area is not predicted to be sufficient to sustain a dimer in solution, which is consistent with the monomeric structure deduced from analytical gel filtration of XAC2383$_{31-309}$ (Table 1).

The protein has two topologically similar lobes (lobes 1 and 2) separated by a central groove (Fig 2A and B). Each lobe is composed of a core of a mixed 6-stranded beta sheet and five alpha-helices. Two antiparallel beta strand extensions link the two lobes and an additional helix from the C-terminus of the protein lies between the lobes (Fig 2B). This topology (sometimes called Venus flytrap) corresponds to that of the periplasmic binding proteins (PBPs) family that often act as components of ABC transporters (36) but have also been observed as parts of integral membrane signaling proteins, as a result of gene fusion (37). In all cases in which these proteins have been structurally characterized, the cleft at the interface between the two domains is involved in the binding of a variety of solutes (38). Furthermore, PBPs have been observed to undergo structural changes upon ligand binding, bringing the two lobes into closer proximity (39-41). A structural search using the Dali Server (42) identified the phosphonate binding protein PhnD from Escherichia coli, as the database entry with the highest degree of structural similarity in spite of a low sequence identity (21%). Two PhnD structures are available, one with the 2-aminoethylphosphonate (2AEP) ligand bound (PDBID: 3P7I) and one with no ligand (apo; PDBID: 3S4U) (41). While the XAC2383 structure is more similar to the 2AEP-bound form of PhnD (RMSD for backbone atoms of 3.4 Å) than to apo-PhnD (RMSD = 5.6 Å), comparison of the structures reveals that XAC2383’s central cleft between the two lobes of the protein is more open than that of 2AEP-bound PhnD but less open than that of apo-PhnD (Fig 2C). When this aperture is measured using the distances between the α carbons of residues at homologous positions in both structures, the XAC2383 aperture is 0 to 7 Å more open than in 2AEP-PhnD but is 3 to 13 Å less open than in apo-PhnD.
Inspection of the electron density in the vicinity of the putative XAC2383 ligand-binding cleft identified a spherically symmetric density that could not be satisfactorily modeled as water (Fig 3A). We modeled a chloride ion to fit this density since (i) the crystallization conditions contained $>300 \text{ mM NaCl}$; (ii) NH groups are frequently observed to coordinate chloride ions (43) and the ligand is coordinated by two main chain NH groups from residues T153 and S154; and (iii) the coordination distances are all greater than 3 Å ($3.05, 3.18, 3.26, 3.59$; Fig 3A), consistent with chloride ion coordination (43).

In addition to the main-chain NH groups of T153 and S154, the chloride ion is coordinated by the S154 side-chain hydroxyl group and a water molecule that bridges to the S152 side-chain hydroxyl (Fig 3A). This Ser-Thr-Ser (STS) motif also participates in the binding of the $-\text{PO}_3^{2-}$ group of the 2AEP ligand bound to \textit{E. coli} PhnD (Fig 3B). Furthermore, this STS triad is not absolutely conserved in the annotated phosphonate binding family in the Pfam-database (20) which suggests that the phosphonate-binding family contains members that bind other types of ligands as well.

While XAC2383 and PhnD from both \textit{E. coli} and \textit{P. aeruginosa} all have an STS motif, XAC2383 differs from the latter two proteins at positions in which these make contacts with the amino moiety of the 2AEP ligand. In the case of \textit{E. coli} PhnD (PDBID: 3P71), negatively charged residues E177 and D205 interact with the positively charged amino group (41) (Fig 3B). For \textit{P. aeruginosa} PhnD (PDBID: 3N5L), the corresponding residues participate in the coordination of an unidentified ligand whose electron density is also consistent with 2AEP (not shown). In the case of XAC2383, the corresponding residues are hydrophobic (V224 and A241, respectively; Fig 3C). These observations suggest that the phosphonate binding protein family has a well-conserved primary STS motif responsible for $-\text{PO}_3^{2-}$ binding plus a more evolutionarily diverse secondary site that may distinguish between different R-groups.

\textit{XAC2383 binds to compounds harboring phosphate moieties}

The surface representation of XAC2383 shows that the central groove has a high density of positively charged residues that could favor the binding of anionic ligands (Fig 4A). We therefore performed soaking experiments to test if XAC2383 crystals could bind different phosphonates or phosphates with anionic R-groups. Crystals soaked overnight with the relatively small phosphonoacetic acid (negatively charged R-group) or 2-aminoethyl-phosphonate (positively charged R-group) failed to provide evidence for ligand binding, except for the previously observed chloride ion. But when we turned our attention to phosphate derivatives, we were able to observe unambiguous electron densities for the following bound ligands: inorganic phosphate (PDB ID: 5UB4), pyrophosphate (PDB ID: 5UB6) and the triphosphate nucleotide ATP (PDB ID: 5UB7) (Fig 4B, 4C, 4D, and S1, S2 and S3 Tables). In all cases, the added phosphates at 2 mM concentration were able to compete with and displace the bound Cl$^-$ ion (present in the mother liquor at a concentration of $>300 \text{ mM}$). The electron density maps show that the terminal phosphate moiety interacts with the $\text{ST}_{154}$ motif (Fig 4B, 4C and 4D). Besides the $\text{ST}_{154}$ motif, residues T95 and K193 were also frequently found making contacts with the terminal phosphate of all the ligands. Subtle differences were observed in the position of side chains of contacting residues in the two molecules of the asymmetric unit. For example, in the phosphate bound structure, K193 from chain A interacts with an oxygen atom from phosphate molecule 1 at a distance of 3.1 Å while K193 from molecule B is too far to contact oxygen from phosphate molecule 2 (5.0 Å). In the pyrophosphate-bound structure, the same residues interact with the P2 phosphate group, as described above, while the P1 phosphate interacts with K193 and with the R240 side chain, which was modeled in a double conformation (Fig 4C). Furthermore, the water occupancy at the binding site varies in both the number of water molecules and in the diversity of coordination types among the structures.

In the ATP-bound structure, the beta and gamma phosphates occupy the site occupied by pyrophosphate in the pyrophosphate-bound structure described above. R240 interacts with
both the alpha and beta phosphate groups and an additional basic side chain, R116, interacts with the alpha phosphate. We observe clear electron density for the whole ATP molecule bound to chain B of the asymmetric unit (Fig 4D). Here, the ribose 2'OH group makes contacts with the H46 side-chain and the adenine 6-amino group hydrogen-bonds with the Q148 side-chain. In chain A of the asymmetric unit, we observe density only for the triphosphate moiety of the bound ligand, indicative of flexibility of the ribose and adenine base. Omit maps for all the ligands bound to the two chains in the asymmetric unit are shown in S5 Fig. We also collected data for XAC2383 crystals soaking with GTP and could observe density for the triphosphate groups but no for the ribose ring and nitrogenous base. Interestingly, we did not observe any ligand electron density in crystals soaked with nucleotide monophosphates, nucleotide diphosphates, hexaphosphate or phytate.

An atomic sphere representation of XAC2383 whose coloring scheme is based on the degree of sequence conservation derived from an alignment of 44 XAC2383 homologs was generated (S6 Fig) (44). This figure shows that the ligand binding site in the cleft region between the two lobes is highly conserved. Also, lobe 1 contains a greater portion of conserved residues outside of the ligand binding site than does lobe 2. This suggests that lobe 1 might constitute an important site for interaction with other proteins; for example with the XAC2382 Cache domain.

In conclusion, the results of the limited number of soaking assays that we performed seem to indicate that XAC2383 preferentially binds compounds harboring phosphate moieties, and not phosphonates, but further studies must be done to more specifically define ligand-binding preferences in solution. Soaking assays can sometimes conceal the true affinities for potential ligands because crystal packing forces restrict dynamics and may lock the receptor in a suboptimal configuration. In any case, the XAC2383 binding groove is lined by basic residues which strongly suggests that it binds phosphates or phosphonates with a negatively charged R-group.

**XAC2382 and XAC2383 both regulate bacterial motility**

Diguanylate cyclases and c-di-GMP phosphodiesterases regulate bacterial lifestyle, often through the control of motility (4,45). We therefore produced a Δxac2382 mutant and evaluated its sliding motility on a semisolid agar surface. Sliding motility is impeded by the presence of type IV pili (46,47). After four days of growth at 30 °C, the knockout strain’s motility was clearly greater than that of the wild-type strain (Fig 5A and S7 Fig), a phenotype compatible with a decrease in c-di-GMP levels (48,49). This suggests that under the experimental conditions used, the DGC activity of the GGDEF domain may predominate over the PDE activity of the EAL domain. Complementation of the mutant strain with a pBRA-derived plasmid carrying the full-length xac2382 gene under the control of the araBAD promoter restored the motility to levels observed for the wild type strain, even under low-level leaky expression under non-inducing conditions (Fig 5A). Furthermore, the Δxac2382 knock-out strain complemented with the full-length construct presented a tendency to aggregate when cultivated in liquid medium (S8 Fig). This phenotype is also characteristic of high c-di-GMP levels (49,50).

We then sought to evaluate the role of the protein coded by the upstream open reading frame, xac2383, in this phenotype. Contrary to what was observed for the Δxac2382 strain, the Δxac2383 strain displayed decreased surface sliding motility (Fig 5B). When Δxac2383 was transformed with a plasmid expressing the full-length xac2383 construct, sliding motility was restored to levels even greater than that observed for the wild-type strain (Fig 5B). The opposite effects on motility observed for the Δxac2382 and Δxac2383 strains suggest that both proteins may act in the same signaling pathway. The observation that the Δxac2383 motility phenotype is similar to that observed in cells over-expressing catalytically active XAC2382 or its fragments raised the hypothesis that XAC2383 interacts with the periplasmic Cache domain of XAC2382 and that this interaction results in an inhibition of XAC2382 DGC activity. Below we present data that are consistent with this hypothesis.
XAC2382 is an active DGC in vivo and requires functional GGDEF and HAMP domains for this activity

Different expression constructs of XAC2382 in the pBRA vector were introduced into the Δxac2382 knockout strain to address the importance of each domain on protein function (Fig 5A and S7 Fig). We observed that a construct expressing only the HAMP-GGDEF domains (residues 198-446) was sufficient to revert the knockout strain’s sliding motility to wild-type levels under the experimental conditions tested. Reversion of the phenotype was also observed for the cytosolic fragment containing HAMP-GGDEF-EAL (residues 198-705) domains. The cellular aggregation phenotype was also observed for the Δxac2382 knock-out strain complemented with the HAMP-GGDEF and HAMP-GGDEF-EAL constructs (S8 Fig). However, cells expressing constructs for only the GGDEF domain, only the EAL domain or the GGDEF-EAL dual domain did not abolish the high motility phenotype (Fig 5A) nor did they result in cellular aggregation (S8 Fig).

The above results suggest that the high motility phenotype observed in the knockout strain may be due to the absence of DGC activity of the XAC2382 GGDEF domain. To more clearly test the ability of the GGDEF domain to produce c-di-GMP, we transformed E. coli BL21(DE3) cells with the pBRA-XAC2382 HAMP-GGDEF and HAMP-GGDEF-EAL constructs and grew them under inducing conditions (1% arabinose). Liquid chromatography coupled to mass spectrometry analysis of the extracts from the cell lysates showed that these strains present 30 and 22 times greater c-di-GMP levels, respectively, than E. coli cells carrying the empty pBRA vector (S9 Fig).

In order to test whether DGC activity is required for XAC2382-dependent reversion of the high motility phenotype, we abolished activity by mutating the conserved GGDEF motif to AAAEF (24). Fig 5A shows that this mutant protein was not able to revert the knockout strain’s high sliding motility phenotype. Furthermore, complementation with this mutant did not result in cellular aggregation, different to that observed for complementation using the wild-type protein (S8 Fig). These results suggest that XAC2382’s effects on Xac motility is dependent on its diguanylate cyclase activity. For this activity to manifest itself, the adjacent HAMP domain is expected to be required to facilitate dimerization and the proper relative orientation of the two GGDEF domains (51,52). HAMP domains contain heptad repeats (abcdefg) where positions a and d are occupied by hydrophobic residues that are required for coiled-coil formation (S1 Fig). The heptad repeats in the XAC2382 HAMP domain correspond to residues 252-266 (S1 Fig). The influence of the coiled-coil structure important for HAMP domain-mediated dimerization was therefore evaluated. A mutation at position a of the second repeat was produced (F259E), replacing a hydrophobic residue with a negatively charged residue. Fig 5A shows that this mutant is unable to revert the knockout strain’s phenotype, and S8 Fig shows that complementation with the F259E mutant also did not result in cellular aggregation, different to that observed for complementation using the wild-type protein (S8 Fig). These results indicate that a functional HAMP domain is necessary for diguanylate cyclase activity, probably by facilitating dimerization and proper orientation of the GGDEF domains. Recombinant XAC2382 fragments corresponding to the HAMP-GGDEF (residues 198-446), HAMP-GGDEF-EAL (residues 198-705) and GGDEF (residues 269-446) domains were expressed in E. coli and purified in soluble forms. None of these fragments had detectable in vitro DGC activity (see Materials and Methods). We speculate that this may be due to a necessity for the protein to be properly inserted into the bacterial membrane, via the second transmembrane helix at the N-terminus of the HAMP domain (S1 Fig), for activity.

The 152STS154 motif is required for XAC2383-dependent sliding motility

The Δxac2383 strain has reduced sliding motility while the Δxac2383 strain containing a plasmid expressing wild-type xac2383 presents sliding motility in excess of that observed for the wild-type strain (Fig 5B). To test whether this effect requires ligand binding to XAC2383, we
mutated the conserved 152STS154 motif to 152AAA154. Complementing the knockout strain with this mutant produced a strain whose motility is very similar to that of the Δxac2383 strain and much less than that observed for the mutant complemented with wild-type XAC2383 (Fig 5B and S7B Fig). This result suggests that ligand binding to XAC2383 is required for its proper function.

**Homologs of the XAC2382_cache-XAC2383 pair are found in many bacterial species**

The protein-protein interaction between the XAC2382 Cache domain and XAC2383 stimulated us to explore the evolutionary relationships between this pair of periplasmatic proteins. Homology searches were performed in order to evaluate the conservation of the xac2382-xac2383 pair in other organisms. Firstly, four iterations using PSI-blast (53) identified 4229 annotated microbial genes coding for proteins with Cache domains similar to that of XAC2382 (residues 32-194; inclusion threshold of 10^{-4}). Of these genes, 871 (approximately 20%) were found in the same orientation as, and in almost all cases downstream to, a gene coding for a XAC2383 homolog (from a total of 14522 XAC2383 homologs identified by two PSI-blast iterations using an inclusion threshold of 10^{-5}). In Gammaproteobacteria, homologs of operons coding a XAC2382_cache-XAC2383 pair seem to be well distributed in families of the order Xanthomonadales (175 gene pairs) but are also present in individuals of the Alteromonadales (39 pairs) and Vibrionales (309 pairs) orders, including the medically important species Vibrio cholerae and Vibrio parahaemolyticus. The presence of the pair was also observed in some alphaproteobacteria (25 pairs), betaproteobacteria (47 pairs), and deltaproteobacteria (102 pairs) species as well as in some Gram-positive bacteria from the Firmicutes phylum (78 pairs), for example in Bacillus.

Interestingly, the 871 XAC2382 homologs in the above XAC2382_cache-XAC2383 homolog pairs exhibit a variety of domain architectures, many of which (67%) are significantly different from the Cache-HAMP-GGDEF-EAL architecture of XAC2382. Fig 6 shows a sample of the architectures observed and reveals that they can be classified into two main groups, based on their cytoplasmic “output” modules. One group of architectures always presents a cytoplasmic GGDEF or GGDEF-EAL module that is separated from the Cache domain by intervening HAMP, PAS and/or GAF domains (Fig 6A). In the other major group of architectures, the XAC2382_cache homolog has a cytoplasmic portion containing a histidine kinase or histidine kinase and response regulator module, again, with intervening HAMP and/or PAS domains (Fig 6B). A relatively small fraction of XAC2382_cache homologs have output modules that do not fall into one of the above two groups, including, for example, sigma-54 activation with Helix-Turn-Helix (HTH), HD phosphodiesterase and guanylate cyclase domains (Fig 6C). This analysis shows that conserved periplasmatic input modules made up of a XAC2382_cache-XAC2383 pair can be coupled to different cytoplasmatic output modules, in some cases controlling second messenger levels while in other cases regulating protein phosphorylation, or even gene expression.

Fig 7 shows the phylogeny of XAC2382_cache homologs found in association with XAC2383-like genes. Two main groups of XAC2382 cytoplasmic output module architectures are associated with mixed branches of the phylogenetic tree. Interestingly, essentially all members of the Xanthomonadaceae family have a single *bona fide* ortholog of the full-length XAC2382, *i.e.* a single gene that belongs to the same monophyletic group characterized by the fusion of a periplasmic XAC2382_cache domain to a GGDEF cytoplasmic output domain. In contrast, lineages such as the Vibrionaceae and Betaproteobacteria possess a more numerous and diverse set of XAC2382_cache homologs in their genomes, including genes that code for fusions of XAC2382_cache to GGDEF or histidine kinases domains and, most notably, some instances where a single polypeptide chain contains both types of output domains (S1 File).

**DISCUSSION**

The co-transcribed *xac2383* and *xac2382* genes in the phytopathogen
**Periplasmic regulation of a diguanylate cyclase**

*Xanthomonas citri* subsp. citri code for a bipartite periplasmic sensor made up of XAC2383 and the XAC2382 Cache domain that controls a cytoplasmic output module made up of GGDEF and EAL domains. The bipartite XAC2383-XAC2382_{Cache} module is quite widespread among bacteria (from gammaproteobacteria to firmicutes). In contrast, the output modules encountered in the XAC2383-XAC2382_{Cache} phylogeny do not always possess GGDEF and EAL domains and are therefore not always predicted to participate in the control of c-di-GMP levels. In many cases they instead carry histidine kinases or sigma 54 activators, indicative of participation in pathways involving protein phosphorylation cascades and/or the control of gene transcription.

In the XAC2383-XAC2382 system, the communication conduit between the sensor and output modules is the HAMP domain that contains an N-terminal transmembrane helix and a C-terminal heptad repeat that may be required for dimerization and DGC activity. This may explain why *in vitro* DGC and phosphodiesterase activities could not be detected using purified soluble recombinant XAC2383 fragments in the absence of the bacterial membrane.

Our results, summarized below, support a scheme, depicted in Fig 8, in which XAC2383 regulates c-di-GMP production by the XAC2382 GGDEF domain. Knocking out the xac2382 gene or over-expressing xac2383 results in increased motility, indicative of reduced c-di-GMP levels. When XAC2383 is not interacting with XAC2382, a state simulated by the Δxac2383 knockout strain or by the over-expression of the xac2382 gene due to its presence on a multi-copy plasmid, sliding motility is decreased, consistent with higher levels of c-di-GMP. Mutations that abolish XAC2382 DGC activity or destroy the XAC2383 ligand binding site fail to revert the phenotypes of the Δxac2382 or Δxac2383 knockout strains. These observations are consistent with the hypothesis that in the absence of XAC2383, XAC2382 is able to adopt a catalytically active configuration. Of course, under physiological conditions, in which xac2383 and xac2382 are co-expressed, the nature of the XAC2383-XAC2382_{Cache} interaction is probably regulated by ligand binding to XAC2383.

One expected feature of the active XAC2382 configuration is that it exhibits C2 symmetry, at least transiently, during the cyclization reaction (54). We do not have any information regarding the orientation of the two proteins in the XAC2382-XAC2383 complex except to note that induced asymmetry in PAS-like domains (which include Cache complex) upon binding by a periplasmic protein has been proposed by other groups for the LapD-LapG (55) and LuxP-LuxQ systems (56). In both these examples, the periplasmic domains derived from the membrane bound component (LapD or LuxQ) interact with its partner in a non-symmetrical manner. Therefore, if XAC2383 binding to XAC2382, or ligand binding to the XAC2383-XAC2382 complex, can stabilize an asymmetric configuration in the XAC2382 dimer, this could inhibit the latter from adopting a catalytically competent conformation (Fig 8).

Other sensory pathways which more closely resemble the XAC2382-XAC2383 pair in having one membrane-bound GGDEF-EAL component with a periplasmic domain and a second periplasmic binding protein component have been characterized, but the stoichiometry and organization of the complexes were not determined. The *Pseudomonas aeruginosa* YfIBNR tripartite signaling system (17,57) consists of YfIR, a periplasmic protein that interacts with YfIN, a transmembrane DGC that regulates exopolysacharide production. An additional protein, YfIB is located at the outer membrane and binds to YfIR, thereby preventing its interaction with the diguanylate cyclase. In the *Vibrio parahaemolyticus* scrABC system, which regulates bacterial capsule production, the phosphodiesterase activity of ScrC (a GGDEF-EAL protein) is regulated by the periplasmic binding protein, ScrB, which in turn acts as a receptor for the quorum sensing molecule produced by ScrA (58). Finally, in the *Agrobacterium tumefaciens* DcpA-PruR system, it was proposed that PruR (a pterin binding protein) activates the phosphodiesterase activity of DcpA (a membrane-bound GGDEF-EAL protein) by interacting with its EAL domain in the cytoplasm (59). We analyzed the PruR primary structure and found an N-terminal
signal peptide for periplasmic localization (S10 Fig). We therefore propose that PruR-DcpA might function in a manner similar to XAC2382-XAC2383, in which PruR is in fact a periplasmic binding protein that interacts with the N-terminal periplasmic domain of the transmembrane GGDEF-EAL protein DcpA.

The physiologically relevant ligand of the XAC2383-XAC2382 signal transduction system is still unknown. The positively charged nature of the ligand binding site, in lieu of the results obtained by soaking XAC2383 crystals with a limited number of different ligands, are both consistent with a preference for phosphate or phosphonate compounds with an additional negatively charged R group. The fact that we were able to observe electron density for one complete ATP molecule bound to one XAC2383 subunit, as well as the triphosphate moiety of another ATP bound to the second subunit in the asymmetric unit, is particularly intriguing. Extracellular ATP can be derived from a number of sources, including the lysis of eukaryotic hosts or other bacterial species killed by toxins released by macromolecular secretion systems (60-62). Mammalian cells can also actively release ATP (63). Furthermore, various bacteria, including *Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter junii*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Staphylococcus aureus*, *Enterococcus gallinarum*, *Enterococcus mundii*, and *Enterococcus faecalis* have been shown to secrete ATP (64,65). Mammalian cells can also actively release ATP (63). Furthermore, various bacteria, including *Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter junii*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Staphylococcus aureus*, *Enterococcus gallinarum*, *Enterococcus mundii*, and *Enterococcus faecalis* have been shown to secrete ATP (64,65). Finally, extracellular ATP, dATP and CTP have been shown to inhibit *P. aeruginosa* twitching motility while no effect was observed for AMP, ADP, GTP, UTP, cdi-GMP, cAMP and the non-hydrolyzable ATP homolog AMP-PNP (66). The lack of an effect for AMP-PNP led to the suggestion that the twitching motility response involves hydrolysis of ATP as opposed to direct sensing of the ATP molecule by a signal transduction system (66). An alternative interpretation is that the AMP-PNP molecule is not recognized by the ATP receptor due to loss of specific interactions mediated by the H-bond-accepting oxygen atom linking the beta and gamma phosphates when substituted by the H-bond donating NH group. Future studies will have to test these possibilities, determine whether and in what manner *X. citri* responds to extracellular ATP (and other nucleotide triphosphates) and whether the XAC2383-XAC2382 system is involved in this response.

The phylogeny of XAC2382_Cache homologous domains found in association with XAC2383-like genes (highlighted in Fig 7) supports several independent origins that created new combinations with different cytoplasmic output module architectures. Such events might be the product of gene fusion and domain loss that create new domain combinations from neighboring genes. This hypothesis is supported by the observation that, in some species, the xac2383-xac2382 homologs have a third downstream gene that codes for another output protein (represented by colored branches in Fig 7). In many instances, these downstream genes code for proteins with effector/output domains (ie GGDEF, histidine kinase, sigma-54 activator) that occur as fusions in nearby sibling lineages. This suggests that XAC2382_Cache homologs are recruited to new domain architectures in combination with a XAC2383-like gene, i.e. as a stable pair that remains evolutionary and functionally linked.

**Experimental procedures**

**Bacterial strains, culture conditions, and media**

*Escherichia coli* DH5α was used for DNA cloning. S4 Table shows all strains used in this study. Xac cells were grown at 30°C in 2×TY medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl). Motility assays were performed in SB-agar medium (5 g/L sucrose, 5 g/L yeast extract, 5 g/L peptone, 1 g/L glutamic acid, pH 7.0, 0.5% agar). Antibiotics were used at the following final concentrations: kanamycin 100 µg/mL, ampicillin 100 µg/mL. Selenomethionine derivatized recombinant protein was produced by growing *E. coli* BL21(DE3) cells in minimal medium (6 g/L Na2HPO4, 3 g/L KH2HPO4, 0.5 g/L NaCl, 2 mM MgSO4, 0.4% glucose, 100 µM CaCl2, 1 g/L NH4Cl, pH 7.35) as described below.

**Site-directed mutagenesis**

A two step PCR was performed to achieve produce site-directed mutants as previously described (46). Briefly, four primers
were designed, two flanking the ends of the gene and two internal complementary primers containing the desired mutation (S5 Table). Firstly, two fragments were generated, one from the beginning of the gene to the desired mutation and a second from the desired mutation to the end of the gene. The PCR products were then combined and another PCR reaction was performed using the primers that flank the ends of the gene. The resulting PCR product was cloned into pET-28a and pBRA for expression in E. coli or Xac cells respectively. The mutations were confirmed by sequencing.

Recombinant XAC2383 purification and crystallization

The xac2383 gene coding for the protein lacking the signal peptide was cloned (residues 31-309) into the pET3a vector and used to transform BL21 DE3 cells and colonies were selected with ampicillin. Cells were grown in 2xTY medium to an optical density of 0.8 at 600 nm. Cells were induced with 400 µM IPTG for four hours at 37°C and harvested by centrifugation. Cells were resuspended in 50 mM Tris pH 8.0, 25% sucrose, 1% glycerol, 0.03% Triton X-100, 0.03% Tween 20 and purified using a 55 mL SP-Sepharose (GE Healthcare) cationic exchange column. The column was previously equilibrated with 50 mM Tris pH 8.0 and bound proteins were eluted using a 0-1 M NaCl gradient in the same buffer. The protein spontaneously crystallized three hours after elution in the chromatography buffer containing approximately 0.3 M NaCl in the same buffer. The crystals were collected with a pipette and no further purification step was required. For selenomethionine (Se-Met) derivatized protein production, cells were grown in 5 mL of 2xTY medium overnight at 37°C, washed and transferred to 500 mL of minimal medium. After the cells reached an optical density of 0.8, the following amino acids were added: 60 mg of selenomethionine, 50 mg each of valine, isoleucine, lysine, threonine and phenylalanine. Recombinant gene expression was then induced by the addition of 400 µM of IPTG and allowed to grow for 6 hours at 37°C.

Diguanylate cyclase activity

XAC2382 constructs, HAMP-GGDEF (residues 198-446), HAMP-GGDEF-EAL (residues 198-705) and GGDEF (residues 269-446), at a concentration of 20 µM, were incubated at 30°C in a solution containing 20 mM Tris pH 8.0, 100 mM NaCl, 2 mM MgCl2 and 1 mM GTP in a final volume of 100 µL. After 16 hours the reaction was applied in a Resource Q column (GE Healthcare) and the nucleotides were eluted using a gradient 0-125 mM NaCl in 10 mM solution of HCl. The production of c-di-GMP was evaluated by comparison with c-di-GMP standard purchased from Biolog (Bremen, Germany).

X ray data collection and structure determination

XAC2383 Se-Met crystals were used to collect a single wavelength anomalous dispersion dataset at the MX2 beamline at the Laboratório Nacional de Luz Síncrotron (LNLS) in Campinas, Brazil using a wavelength of 0.979 Å at -180°C under a nitrogen gas flux (S1 Table). Data were processed using the HKL2000 suite and phases were solved using the Autosol software from the Phenix package (67-69). The initial model was further used for molecular replacement using Phaser (70) to obtain a higher resolution model (1.9 Å) from a native crystal, also collected at the MX2 beamline. The model was improved by cycles of automatic refinement using REFMAC5 and Phenix Refine and manual real-space refinement using Coot (71,72). For soaking experiments, crystals were transferred from the purification tube (where they were initially grown) to Eppendorf tubes containing the same solution plus 2 mM ligands and incubated at 4°C overnight prior to data collection. Data were collected at the Institute of Chemistry of the University of São Paulo using a rotatory copper anode MicroMax 007 (Rigaku) X ray source at a wavelength of 1.542 Å with crystals maintained at -180°C under a nitrogen gas flux. The crystals were rotated by 180° and images images were collected at 1° intervals. Phases were solved by molecular replacement using Phaser (70). All models were validated using MolProbity (73).

Sliding motility assay
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*Xanthomonas citri* cells were grown overnight with agitation in liquid 2xTY medium at 30°C. Cells were washed with water and optical density was adjusted to 0.3 at 600 nm. Plastic petri plates were prepared with 30 mL of SB medium containing 0.5% agar and 3 μL of the cell suspensions were pipetted onto the center of the plates. After drying the drops, the plates were sealed and incubated at 30°C for four days on a flat surface (46). Spectinomycin was used at 100 μg/ml and no arabinose was added.

**RT-PCR**

Xac cells were collected from plates after 2 days of growth at 30°C. Cells were washed with water and RNA was isolated using Trizol reagent (Invitrogen). DNase I (Thermo Scientific) was added to avoid genomic DNA contamination. The reverse transcription reaction was performed using 1 μg of RNA and RevertAid H Minus First-Strand cDNA Synthesis Kit (Fermentas) with random hexamer primers, accordingly to the manufacturer’s instructions. The cDNA was then used as a template to amplify three regions of the *xac2382-xac2383* gene locus: i) the first 186 bp of the *xac2382* gene (oligos RT_xac2382F RT_xac2382R); ii) the final 193 bp of the *xac2383* gene (oligos RT_xac2383F RT_xac2383R); and iii) 396 bp overlapping the *xac2382-xac2383* junction (oligos RT_xac2383F and RT_xac2382R). The oligos sequences are shown in S5 Table. A control reaction in which reverse transcriptase was omitted was also performed to confirm the absence of genomic DNA in the sample.

**Production of Xac gene knockout strains**

In-frame deletions of targeted genes were performed using a two-step allelic exchange procedure as previously described (46). Briefly, forward and reverse primers were designed to amplify 1000 bp flanking regions upstream (oligos F1 and R1) and downstream (oligos F2 and R2) of the target genes (S5 Table). These upstream and downstream 1000 bp fragments were ligated to produce a deleted version of the gene. This approximately 2000 bp fragment was cloned into the pNPTS138 suicide vector and Xac cells were transformed by electroporation. Transformants were selected sucrose sensitivity and kanamycin resistance. Cells were then grown without selection for three days and then plated and selected for sucrose resistance and kanamycin sensitivity. Deleted alleles and wild-type alleles were identified by PCR.

**XAC2382-XAC2383 interaction**

The pET-28a-derived construct for the expression of the XAC2382 Cache domain (residues 37-192) with an N-terminal His-tag and the pET3a-derived construct for the expression of XAC2383 (residues 31-309) were used to simultaneously transform BL21(DE3) cells. Transformants were selected for kanamycin and ampicillin resistance and recombinant protein production was induced with 400 μM IPTG at an OD_{600nm} of 0.8 for 4 hours at 37°C with agitation. The cells were harvested by centrifugation and resuspended in 50 mM Tris pH 8.0, 150 mM NaCl, 10 mM imidazol at 1 mL/min. Bound proteins were eluted with a 10-500 mM imidazol gradient (10 column volumes). Eluted fractions were analyzed by SDS-PAGE and those containing both proteins were pooled and applied to a Superdex 200 gel filtration column (GE 10/300) with a flow rate of 0.5 mL/min coupled to a multi-angle light scattering system (minIDAWN TREOS) and to a refractive index detector (Optilab rEX). A refractive index increment dn/dc = 0.185 ml/g was assumed for the mass calculation.

**C-di-GMP quantification in E. coli cells**

C-di-GMP extraction was performed as described (74). Briefly, *E. coli* BL21(DE3) cells carrying pBRA vector constructs expressing XAC2382_HG (residues 198-446) and XAC2382_HGE (residues 198-705) were grown in 2XTY media overnight. An aliquot of each overnight culture was inoculated in 10 ml of
fresh 2XTY media and incubated at 37 °C. After the bacterial culture reach an OD between 0.6 – 0.8, arabinose was added to a final concentration of 1% (w/v) and the culture was incubated for 2 hours at 37 °C at 200 rpm. One ml of the culture was transferred to a 1.5 ml eppendorf tube and centrifuged at 9300 x g for 2 minutes at 4 °C. The cellular pellet was washed twice with ice-cold PBS (pH 7.2) and resuspended in 100 µl of ice-cold PBS and incubated at 100 °C for 5 minutes. Ice-cold ethanol to a final concentration of 65% and vortexed for 15 seconds. The sample was centrifuged at 9300 x g for 2 minutes and the supernatant was transferred to a new microcentrifuge tube. This extraction procedure was repeated two more times and all the supernatants were pooled together. The insoluble fraction was retained for subsequent determination of protein content by BCA assay. The pooled ethanol-soluble fraction was vacuum dried after which the pellet was resuspended in 100 µl of distilled water. 40 µl of these samples were subjected to HPLC-ESI-MS analysis using a C-18 column (Jupiter 5u, Phenomenex, 250 mm x 4.6 mm) coupled to a C-18 precolumn (Phenomenex, 4 mm x 3 mm) equilibrated with 2 % of buffer B (100 % methanol) and 98 % of buffer A (25 mM ammonium formate, pH 7) at 0.6 mL/min. The mixture was eluted using 2 % of buffer B for 7 minutes followed by a 2%-80% B gradient up to 17 min, followed 80 %B up to 25 min. Under these conditions, c-di-GMP elutes at approximately 15 min. The separated components from this c-di-GMP peak were analyzed with an Amazon Speed ETD (Bruker Daltonics) mass spectrometer equipped with an ESI interface. The operating parameters were set as follows: drying gas flow rate, 12 L/min; temperature, 300 °C; nebulizer, 70 psi; HV, 4500 V. The samples were analyzed in positive mode and mass spectra data recorded across the m/z range of 50-1000. Peak areas of the MS spectrum corresponding to the reference mass for cdiGMP (691 +/-0.2) were recorded and corrected with reference to the total protein content of the particular sample. Three biological replicas were performed, each in triplicate.

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CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article.
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Table 1. Analytical size exclusion chromatography analysis of XAC2382\textsubscript{Cache\_37-192} and XAC2383\textsubscript{31-309} and SEC-MALS analysis of the XAC2382\textsubscript{Cache\_37-192}+XAC2383\textsubscript{31-309} complex.

| Protein Sample | Theoretical mass (kDa) | Mass (kDa) | Oligomeric state |
|----------------|------------------------|------------|------------------|
| XAC2382\textsubscript{Cache\_37-192} | 16.9 | 13.0 * | Monomer |
| XAC2383\textsubscript{31-309} | 31.3 | 26.9 * | Monomer |
| XAC2382\textsubscript{Cache\_37-192} + XAC2383\textsubscript{31-309} | 126 ± 8 ** | (2:1)\textsubscript{n} or (2:2)\textsubscript{n} |

* mass estimated by size exclusion chromatography (SEC)
** mass estimated by SEC-MALS
Figure legends

Figure 1. The xac2382 and xac2383 genes are co-transcribed in Xanthomonas citri subsp. citri and the proteins interact in vitro. (A) Diagram of the locus containing the xac2383, xac2382 and xac2384 genes (shown as thick arrows). Black lines represent fragments (1, 2 and 3) amplified from the Xac cDNA preparation in panel C. (B) Domain architecture of XAC2382 and XAC2383. Black boxes show transmembrane regions of XAC2382 and the signal peptide of XAC2383. The lines above XAC2382 domain architecture indicate the constructs realized using pBRA vector. Asterisks (*) indicate the sites of introducing a single amino acid modification in the heptad repeat of the HAMP domain (F259E) and the modification of the GGDEF motif to AAAEF. (C) Lanes 1, 4 and 7: PCR reactions using oligonucleotides that amplify a 186 bp fragment (fragment 1 in part a) corresponding to the beginning of the xac2382 gene. Lanes 2, 5 and 8: PCR reactions using oligonucleotides that amplify a 193 bp fragment (fragment 2) corresponding to the end of the xac2383 gene. Lanes 3, 6 and 9: PCR reactions using oligonucleotides that amplify a 396 bp fragment (fragment 3) that overlaps genes xac2382 and xac2383. Lanes 1, 2 and 3: PCR amplification using Xac cDNA produced from total messenger RNA as template. Lanes 4, 5 and 6: Negative control. PCR amplification using a mock cDNA sample (prepared by omitting reverse transcriptase) as template. Lanes 7, 8 and 9: Positive control. PCR amplification using genomic DNA as template. (D) Size exclusion chromatography coupled to Multi-Angle Light Scattering (SEC-MALS) analysis of the XAC2382-Cache_37-192-XAC2383_31-309 complex using a Superdex 200 10/300 column. Black line indicates the relative concentration of the proteins and the grey line indicates the mass determined by the scattering analysis. (E) SDS-PAGE of the purified complex.

Figure 2. Crystal structure of XAC2383. (A) XAC2383 topology and secondary structure. β strands are shown in red and α helices in green. (B) Cartoon representation of XAC2383 crystal structure showing the two structurally similar lobes (PDB ID: 5UB3). (C) Comparison of the aperture between the lobes in between XAC2383 (PDB ID: 5UB3), PhnD bound to 2-aminoethylphosphonate (2AEP) ligand (PDB ID: 3P7I) and apo-PhnD (PDB ID: 3S4U). Lobe 1: yellow, lobe 2: orange. The aperture between lobes in XAC2383 smaller than that observed for apo-PhnD but larger than that observed for the PhnD-2AEP complex. The chloride ion bound to XAC2383 is shown as a green sphere. The 2AEP ligand bound to PhnD is completely buried and therefore not visible.

Figure 3. The XAC2383_152STS154 motif in the ligand binding site. (A) 2Fo-Fc map contoured at 1.0 σ of the conserved 152STS154 motif and a coordinated chloride ion. (PDB ID: 5UB3). (B) E. coli PhnD in complex with 2-aminoethylphosphonate (PDB ID: 3P7I). The numbers in parts (A) and (B) indicate distances in Å. (C) Alignment of PhnD from E. coli and P. aeruginosa and XAC2383 highlighting the residues in part B responsible for 2-aminoethyl-phosphonate binding by PhnD. Black contour boxes show the residues responsible for the binding to the -PO3²- moiety. Grey contour boxes represent the residues responsible for the 2-aminoethyl R-group. The N-terminal signal peptides of the three sequences are highlighted.

Figure 4. Ligand binding to the central groove of XAC2383. (A) XAC2383 surface representation with the chloride ion (green) at the binding site (PDB ID: 5UB3). (B)-(D): Binding site with different ligands showing 2Fo-Fc map contoured at 1.0 σ. (B) Phosphate (PDB ID: 5UB4). (C) Pyrophosphate (PDB ID: 5UB6). (D) ATP (PDB ID: 5UB7). The 2Fo-Fc map for side chains in D was omitted for clarity. The numbers in parts (B), (C) and (D) indicate distances in Å.

Figure 5. XAC2382 and XAC2383 are involved in Xanthomonas citri subsp. citri motility and have opposing phenotypes. (A) Sliding motility assay for the Xac wild type strain containing the empty pBRA vector, the Δxac2382 containing the empty pBRA vector and the Δxac2382 strain containing the pBRA vector expressing different XAC2382 fragments shown in Fig 1B. (B) Xac wild
type strain containing the empty pBRA vector, the Δxac2383 containing the empty pBRA vector and the Δxac2383 strain containing the pBRA vector expressing XAC2383 wild type and XAC2383 with the 152STS154 motif mutated to AAA. Pictures taken after four days growth at 30° C on SB medium 0.5% agar plates.

**Figure 6.** Common domain architectures found for XAC2382 homologs whose genes are predicted to be co-transcribed with xac2383 homologs. (A) GGDEF domain containing proteins. (B) Histidine kinases. (C) Sigma 54 with a helix-turn-helix (HTH) motif domain, HD domain from phosphodiesterases and guanylate cyclase.

**Figure 7.** Phylogeny of XAC2382\textsubscript{Cache} homologous domains found in association with XAC2383-like genes. The phylogeny tree was built by FastTree (Price et al. 2010), using default options, from a multiple sequence alignment generated by MUSCLE (75). Only the conserved columns of the alignment, as identified by TrimAL (76) using the heuristic similarity statistics, were used. Species names are colored after the domain effector type, as described in the legend in the bottom left corner. Branch color (when not black) refers to the domain architecture of an occasionally encountered third gene, located downstream to the effector GGDEF or histidine kinase XAC2382 homolog and unrelated to the XAC2383 homologs. Such genes also encode signal transduction proteins and contain effector domains indicated by the colors in the legend). We note some instances of highly supported sibling branches that correspond to proteins with different effector domains but whose Cache domains are more closely to each other than to any other homologs with a similar architecture, thus pointing to novel and independent origins for these architectures.

**Figure 8.** Model of the XAC2382-XAC2383 signal-transduction pathway. The periplasmic binding protein XAC2383 has two topologically similar (non-equivalent) lobes and can exist in two conformations whose equilibrium is determined by the binding of a specific ligand (L, unknown). It is not known if XAC2383 binding to the XAC2382 Cache domain is affected by ligand binding, nor do we know the precise stoichiometry of the XAC2382-XAC2383 complex (here shown as 2:1 for simplicity). In the absence of the ligand (left), the XAC2383 interaction with the XAC2382 Cache domain permits XAC2382 to adopt a symmetric, catalytically active conformation. In the presence of ligand (right), the XAC2383-XAC2382 interaction changes, breaking the symmetry of the XAC2382 dimer, thereby inhibiting the latter’s DGC activity.
Figure 1

A

Xac2382

Xac2383

Xac2384

1

185 bp

2

193 bp

3

396 bp

B

XAC2382

198

HG

446

705

198

HGE

705

269

G

446

705

269

GE

E

705

Cache domain

HAMP domain

GGDEF domain

EAL domain

705

C

cDNA

Mock

DNA

1

2

3

4

5

6

7

8

9

10

D

Relative Concentration (Normalized DRI)

Volume (mL)

200000

180000

160000

140000

120000

100000

80000

60000

40000

20000

0

10

12

14

16

18

20

XAC2383

XAC2382_Cache1

66 kDa

45 kDa

35 kDa

25 kDa

18.4 kDa

14.4 kDa

E
Figure 2

A

B

C

Periplasmic regulation of a diguanylate cyclase
Figure 3

Periplasmic regulation of a diguanylate cyclase
Figure 5

Periplasmic regulation of a diguanylate cyclase
Figure 6
Figure 7
Figure 8
A bipartite periplasmic receptor–diguanylate cyclase pair (XAC2383–XAC2382) in the bacterium Xanthomonas citri

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