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Progress in understanding of the molecular basis underlying functional diversification of cyclic di-nucleotide turnover proteins

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Running title: Diversity of cyclic di-GMP metabolizing proteins

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ABSTRACT

Cyclic di-GMP was the first cyclic di-nucleotide second messenger described, presaging the discovery of additional cyclic di-nucleotide messengers in bacteria and eukaryotes. The GGDEF diguanylate cyclase (DGC) and EAL and HD-GYP phosphodiesterase (PDE) domains conduct the turnover of cyclic di-GMP. These three unrelated domains belong to superfamilies that exhibit significant variations in function, to include both enzymatically active and inactive members with a subset involved in synthesis and degradation of other cyclic di-nucleotides. Here we summarize current knowledge of sequence and structural varitions that underpin the functional diversification of cyclic di-GMP turnover proteins. Moreover, we highlight that superfamily diversification is not restricted to cyclic di-GMP signaling domains, as particular DHH/DHHA1 domain and HD domain proteins have been shown to act as cyclic di-AMP phosphodiesterases. We conclude with a consideration of the current limitations that such diversity of action places on bioinformatic prediction of the roles of GGDEF, EAL and HD-GYP domain proteins.

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INTRODUCTION

The di-nucleotide cyclic di-GMP is the most abundant second messenger in Bacteria. It promotes the environmental life style switch between sessility and motility as well as the host-related life style switch between acute and chronic/benign infection. A hallmark of the cyclic di-GMP signaling network is an apparent redundancy of cyclic di-GMP turnover proteins encoded in one genome. However many of these proteins have distinct N-terminal sensing and signaling domains, suggesting that their activities in cyclic di-GMP turnover respond post-translationally to various (and different) intra- and extra-cellular signals. In gross terms, the number of cyclic di-GMP turnover proteins is linearly correlated with genome size within the different bacterial phyla with *Thermotogae* having one of the highest cyclic di-GMP related "IQs", density of enzymes per Mbp, with some species harboring over 100 cyclic di-GMP turnover proteins (http://www.ncbi.nlm.nih.gov/Complete_Genomes/c-di-GMP.html). As in other domain superfamilies, extensive sequence diversity exists. Here, we review the knowledge on the translation of sequence diversity of cyclic di-GMP turnover proteins into functional diversity. We conclude by discussing whether and how a unified nomenclature for cyclic di-GMP turnover proteins can be established.

FUNCTIONAL DIVERSIFICATION OF THE GGDEF DOMAIN

The approx. 180 amino acid long GGDEF domain catalyzes synthesis of cyclic di-GMP from two molecules of GTP with the release of pyrophosphate (Fig. 1; (1, 2)). So far, the GGDEF domain is the only identified protein domain to carry out this specific condensation reaction. Even before functional characterization, the GGDEF domain was recognized to be a structural homologue of the adenylate cyclase domain, both belonging to the RRM (ferredoxin) fold palm domain family, which includes other enzymes forming 3'-5' phosphodiester bonds such as reverse transcriptases, class A and B DNA polymerases and RNA dependent RNA polymerases (3, 4). In approximately 40% of

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proteins, the GGDEF domain is not only coupled to an N-terminal signaling domain, but also a C-terminal EAL domain. Stand-alone GGDEF domains are rare and have not been characterized extensively (5). The GGDEF domain frequently possesses suboptimal catalytic activity and requires dimerization for the condensation reaction to occur at the active half-sites of the two monomers. Dimerization can be further promoted by allosteric activation of the N-terminal sensor domain (6). Various mechanisms of activation are emerging reflecting the diversity of cytoplasmic, transmembrane and periplasmic signaling domains as well as linker and signal transducing domains, which are potentially associated with sequence diversification of the turnover domain (1, 6-10). Notably, the DgcZ (YdeH) DGC is an active dimer with Zn^{2+} ion binding to inhibit the catalytic activity (10). GGDEF domains can be differentiated into three major classes: enzymatically functional domains; enzymatically functional domains, linked to an EAL domain; and enzymatically non-functional domains ((Fig. 2); (11)). This classification is based on the homology of the entire domain in combination with the conservation of the extended signature motif (Fig. 2; (12, 13)).

Key residues in catalysis and allosteric regulation

The GGDEF domain consists of the defining GG(D/E)EF sequence motif that includes the D/E catalytic base and other residues intimately involved in substrate binding and coordination of one of the two divalent cations (14). The position of the substrate GTP in the crystal structure(s) of GGDEF domain proteins indicates the presence of the glycines provides space for the ribosyl sugar and phosphates thus explaining conservation of these residues (Fig. 3; (14, 15)). In PleD, the most well investigated di-guanylate cyclase for which a crystal structure is available, the guanine base is bound in a pocket with N335 and D344 as key contact residues curtailed by apolar side chains of L294, F331 and L247. D/E is the catalytic base, while K332 stabilizes the transition state. All those residues are well conserved in catalytically competent diguanylate cyclases (for further information see Fig. 2).

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Non-functional GGDEF domains are usually characterized by a degenerate GGDEF motif as any mutation within the GGDEF motif of catalytically active GGDEF domain proteins usually abolishes the catalytic activity, although there are exceptions. For example, the GGDEF domain protein of *Staphylococcus aureus* and *Staphylococcus epidermidis* with a well-conserved GGDEF motif has been experimentally proven to be non-functional (16). The structural basis of non-functionality of *Staphylococcal* GGDEF domains still remains an enigma. As to alteration in the signature motif, it is fairly common that GGDEF domains contain a degenerate GG(D/E)EF motif with the first G not conserved. Recent experimentally characterized proteins with a G>A or G>S substitution still exhibit significant functionality demonstrating unexpected flexibility in the GGDEF containing active site hairpin (Fig. 2; (17-19)).

Besides the gross classification into catalytically active and non-active GGDEF domains, the inhibitory site (I-site), designated by the central signature motif RxxD is another functional feature which characterizes the activity profile (14, 20). The I-site, which is formed at an intra- or intermolecular interface bridged by a cyclic di-GMP dimer, variably extends beyond the central conserved RxxD cyclic di-GMP binding motif and mediates allosteric non-competitive product inhibition, feedback control of cyclic di-GMP synthesis (20, 21). The RxxD motif is absent in a proportion of GGDEF domains; alternative mechanisms to control cyclic di-GMP synthesis have been described for some of these proteins (6, 22, 23). A second recently discovered function of the I-site is the participation in protein-protein interaction with cyclic di-GMP receptor, which ensures a stringent specificity of cyclic di-GMP signaling even in the presence of cyclic di-GMP production (21). In divergent GGDEF domain proteins (see below), a retained I-site in catalytically non-functional GGDEF domains converts these domains into cyclic di-GMP receptors (24-27). It should be noted that the enzymatic activity of the GGDEF domain can also be positively regulated by cooperative binding of the GTP substrate (19).

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Some GGDEF domains have diverged to be enzymatically nonfunctional. These nonfunctional GGDEF domains can act as sensor domains that bind the substrate GTP, thereby allosterically regulating the enzymatic activity of a C-terminal EAL phosphodiesterase (28). In this way the degenerate GGDEF motif is involved in allosteric control (20, 29). A surprisingly high catalytic plasticity has been demonstrated as a highly degenerate GGDEF domain has been shown to display ATPase activity, albeit at suboptimal levels (30).

Alternative cyclic dinucleotides synthesized by GGDEF domain proteins

A hallmark of binding of nucleotide and sugar derivatives to proteins is the low stringency of the specificity of the binding site. Accordingly, alteration of few amino acids can alter substrate specificity of nucleotides and sugars. Although it is the common perception that cyclic di-GMP synthases can be readily identified in bacterial genomes as being members of the GGDEF domain superfamily, GGDEF domain proteins that predominantly synthesize cyclic GMP-AMP, but also cyclic di-GMP and cyclic di-AMP have recently been identified (31). The relative specificity of cyclic GMP-AMP synthase activity as opposed to stringently using GTP as substrate on this specific protein scaffold is determined by the amino acid serine, which has replaced aspartate 344 (designation according to PleD sequence), a key contact residue in the base-binding pocket. As the exchange of aspartate for serine in an established di-guanylate cyclase did not lead to the conversion into a cyclic GMP-AMP synthase, additional features of the protein scaffold must also contribute to substrate specificity.

Specificity in regulatory action

In general, GGDEF domains encoded by a single genome are functional paralogues, which have a low amino acid sequence identity/similarity below 40%, while orthologues with identical domain structure and high sequence identity can be found even in distantly related species (32). One of the

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hallmarks of cyclic di-GMP signaling is a relative or absolute specificity of a phenotypic output of an individual chromosomally encoded GGDEF domain protein. This specificity is partly explained by the close proximity of signal production/degradation with receptor and/or effector proteins mediated through protein-protein interactions, a first example being the involvement of the I-site of a GGDEF domain in interaction with an EAL domain cyclic di-GMP receptor (21, 33). Interactions between the EAL domain protein YciR and diguanylate cyclase YdaM control a key step in *E. coli* biofilm formation through modulation of localised cyclic di-GMP levels (34). Functionality is also provided, however, by specific protein-protein interactions that are independent of the catalytic activity (19, 35). In this case, the xxDxDx motif, which is highly conserved in GGDEF domains, is required for the interaction with the HD-GYP domain. HD-GYP::GGDEF complex formation serves to control motility through recruitment of a PilZ domain protein and interaction with the pilus biogenesis machinery (35, 36). Overall, these data indicate that GGDEF domain proteins possess several protein interaction interfaces, which participate in the formation of supramolecular complexes.

FUNCTIONAL DIVERSIFICATION OF THE EAL DOMAIN

The EAL domain was the first identified cyclic di-GMP specific phosphodiesterase, and remains the most well characterized (Fig. 4, 5; Fig. S1; (2, 37, 38)). The product of EAL phosphodiesterase activity is the di-nucleotide pGpG, while hydrolysis of pGpG into GMP is considered to be too slow to be physiologically relevant. EAL phosphodiesterases require a divalent cation for enzymatic activity, which in most cases is Mg^{2+} or Mn^{2+} ion, while Ca^{2+} and Zn^{2+} efficiently inhibit the enzymatic activity (39, 40). Catalytically active EAL domains usually have a high substrate affinity in the physiological nanomolar range and cyclic di-GMP binding can increase the dimerization affinity (41). Although monomers can be catalytically active, dimerization substantially enhances protein stability and catalytic activity (37).

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Key residues for activity

Systematic alanine substitutions of conserved signature amino acids have given insights into the catalytic mechanism, even before a crystal structure had become available (42, 43). That work showed that the EAL motif is part of a larger conserved signature motif that is required for catalytic activity including amino acids required for binding of divalent cations, the substrate and catalysis. In addition, a flexible loop ("loop 6") extensively characterized in (β/α) barrel proteins mediates dimerization and controls substrate and cation binding, thus being required for catalytic activity (42, 44). The findings from this mutagenesis study enabled the differentiation of EAL domains in three classes: catalytically active, potentially catalytically active and catalytically inactive EAL domains (32, 42), thus facilitating the prediction of the function of further EAL domains. Based on the functional characterization of additional EAL domains, a further sub-classification can be made (see Fig. 4).

The crystal structures of several EAL domain-containing proteins revealed that these proteins possess a protein fold variant of the (β/α) TIM-barrel structure arranged as eight alternating alpha helices and beta-strands (Fig. 5; (44). This arrangement of secondary structures is found in over 50 diverse protein superfamilies (45). The functionality of this highly conserved arrangement of secondary structures is highly flexible as these protein families bind different substrates and catalyze different reactions. In case of the light-inducible phosphodiesterase Blrp1 of *Klebsiella pneumoniae*, interdomain interaction between the sensor domain and a non-conserved connector in the EAL domain of only four amino acids in length controls the catalytic activity in response to light (Fig. 5A; (44)).

Classification of divergent domain members

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188 As with the GGDEF domain, the EAL domain superfamily contains diverged members. Most EAL
189 domains are class I EAL domains, which possess a N-terminal signaling domain and features
190 substantial, but still suboptimal catalytic activity in the non-activated state, requiring the correct
191 positioning of conserved loop 6 (42, 44). Class II EAL domains potentially possess catalytic activity
192 with deviations of some amino acids from the conserved signature motifs; they are most poorly
193 characterized. Of note, catalytically active EAL-only domain proteins comprise a specific subgroup
194 within the class II family. Class III EAL domains can already be recognized by bioinformatic analysis
195 to be catalytically inactive, since class III domains possess deviations from the conserved signature
196 motifs of active enzymes in several determinative positions. Nevertheless some class III domains
197 can still bind cyclic di-GMP, thus serving as cyclic di-GMP receptors (class IIIa), whereas others are
198 unable to bind the di-nucleotide (Fig. 5B; (class IIIb)).
199 Cyclic di-GMP binding and non-binding EAL domains cannot be distinguished with certainty (Fig.
200 4). However, in both cases, several conserved signature amino acids are missing and loop 6 is not
201 conserved. Binding of cyclic di-GMP to a receptor EAL domain allosterically controls subsequent
202 events. In the conserved Lap system with the GGDEF-EAL receptor LapD, interactive inside-
203 out/outside-in signals mediated by the HAMP domain couple cytoplasmic cyclic di-GMP binding to
204 reinforcement of periplasmic protein-protein interactions controlling e.g. periplasmic proteolysis of
205 cell surface proteins (46, 47). Interestingly, homologous GGDEF-EAL receptors have variations in
206 their cyclic di-GMP binding sites and bind cyclic di-GMP in different conformations, which reflects
207 the structural polymorphism of this second messenger (48, 49) as well as binding site flexibility
208 (Fig. 5C; (50)). Such polymorphisms make it still challenging to predict cyclic di-GMP binding
209 residues by bioinformatics.
210 Catalytically inactive, non-cyclic di-GMP binding EAL proteins function solely through protein-
211 protein interactions. Several well-investigated class IIIb proteins of *Escherichia coli* and *Salmonella*
212 *typhimurium*, YdiV and *Salmonella* specific STM1697, bind to the major flagella regulator FlhDC

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213 with apparently similar, but highly distinct interfaces (51-53). Furthermore, the class IIIb protein
214 YdiV, interacts in complex with FlhDC, with the ClpXP protease guiding FlhDC for degradation (54)
215 and regulates other physiological traits besides motility (55).
216
217 *Regulation of dual-function diguanylate cyclase-phosphodiesterases*
218 Of particular complexity is the regulation of the activity of GGDEF-EAL domain proteins in case
219 where the two domains are both catalytically functional (56). Notably, the three DGCs and three
220 PDEs of *Komatagaeibacter xylinus* that affect cellulose production, the first biological function
221 recognized to be affected by cyclic di-GMP signaling, are GGDEF-EAL domain proteins, and both
222 domains are predicted to be functional by bioinformatics analysis (39). Differential regulation of
223 the catalytic activity of these domains can include allosteric regulation by ligand binding, signal
224 perception or protein-protein interactions which favors one catalytic activity over the other (7, 57-
225 60), but could also include a combination of regulatory mechanisms such as proteolytic cleavage in
226 combination with signal perception (61). This points to a multifactorial regulation of catalytic
227 activity *in vivo*. However, catalytically active domains can even predominantly affect certain aspects
228 of physiology through protein-protein interactions. For example, the GGDEF-EAL
229 phosphodiesterase YciR of *E. coli* affects expression of *csgD*, a major biofilm regulator, through
230 interaction with a DGC and a transcriptional regulator, which inhibits biofilm formation (34).
231
232 *A phosphodiesterase involved in pGpG degradation*
233 The observations that the EAL domain hydrolyses cyclic di-GMP into 5'-pGpG (Fig. 1) have raised
234 the question of the possible cellular role and fate of this di-nucleotide product (62). As an inhibitor
235 of the enzymatic activity of particular EAL domain proteins, this molecule could potentially impinge
236 on cyclic di-GMP levels and signaling. Furthermore, it has been suggested that this nano-RNA is a
237 signaling molecule in its own right, involved in the initiation of transcription by RNA polymerase

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(63). Two classes of enzymes are implicated in 5'-pGpG degradation: a subgroup of HD-GYP domain phosphodiesterases that can hydrolyse both cyclic di-GMP and 5'-pGpG (see below) and the oligoribonuclease Orn, recently identified as the primary degradative enzyme for 5'-pGpG in *Pseudomonas aeruginosa* (64, 65). Homologues of Orn are widely distributed in bacteria, although Cohen and colleagues (65) identified over 200 species that lack an Orn homolog, but have EAL and HD-GYP domain proteins, as well as over 100 species that lack both an Orn homolog and EAL domains, but have HD-GYP domain proteins. Thus in some bacteria, HD-GYP domain proteins may influence cyclic di-GMP levels both directly, by hydrolysis of the nucleotide, and indirectly by preventing product inhibition of the activity of EAL domain enzymes.

FUNCTIONAL DIVERSIFICATION OF THE HD-GYP DOMAIN

There are fewer studies of HD-GYP domain proteins compared to those with the GGDEF and EAL domains. Although well-studied model organisms harbor mostly EAL domain phosphodiesterases, the HD-GYP domain is one third as abundant throughout the phylogenetic tree (https://www.ncbi.nlm.nih.gov/Complete_Genomes/c-di-GMP.html). The prototype of an HD-GYP domain protein is the response regulator RpfG from *Xanthomonas campestris* (36, 66). This protein is part of a two component system that affects expression of multiple virulence functions in this plant pathogen (67, 68). *In vitro*, RpfG converts cyclic di-GMP to GMP via the intermediate 5'-pGpG dependent on Mn^{2+} (66, 69). Alanine substitution within the signature HD dyad leads to loss of both enzyme activity and regulatory action (66). In contrast, although alanine substitutions in the signature GYP motif have little or no effect on enzyme activity, they do counteract interaction of RpfG with particular GGDEF domain proteins to modulate a specific subset of RpfG mediated phenotypes (35, 66, 70).

Diversity in metal binding

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263 The crystal structure of the enzymatically-active HD-GYP phosphodiesterase *Pm*GH from
264 *Persephonella marina* EX-H1 unexpectedly showed a trinuclear Fe center with iron in two redox
265 states as Fe(II) and central Fe(III) buried at the bottom of the cavity forming the c-di-GMP binding
266 site (Fig. 6; (71)). In general, the HD domain superfamily of enzymes has been shown to catalyze
267 phosphomonoesterase and phosphodiesterase reactions depending on their catalytic metal center
268 being mono- or binuclear, respectively. Variations in the metallic center of the HD-GYP domain
269 were seen in the structure of the unconventional, catalytically inactive Bd1817 from *Bdellovibrio*
270 *bacteriovorans* (72) and PA4781, a two component regulatory protein from *Pseudomonas*
271 *aeruginosa* (73) which harbor bi-nuclear metal centers, although of a distinct nature.
272 A phylogenetic comparison of HD-GYP domains showed a distinct separation into two evolutionary
273 groups independent of the type of associated regulatory and/or sensory domain (71) with seven
274 out of the eight *Pm*GH metal ligand residues shared (Fig. 7; (71)). The variable ligand which
275 corresponds to E185 in *Pm*GH is embedded in the signature motif E/D-T-G for the *Pm*GH subfamily.
276 E185 has been predicted to be determinative for a three-metal center valency (71, 74). Conversely,
277 the other subfamily primarily presents a tyrosine or phenylalanine (Y/F) and lacks a unique
278 signature.. The separation of HD-GYP proteins into these two subfamilies is not entirely clear-cut
279 though (Fig. 7); (73), (75)). For example, RpfG from *X. campestris*, despite phylogenetically
280 clustering within the E/D-T-G subgroup, aligns a glycine in place of the E/D residue, as well as
281 variation in a H-site metal ligand (Fig. 7). Thus RpfG is more likely to possess a binuclear metal ion
282 center.
283 Recent work has provided evidence that the differences in the occupancy of the metal site and the
284 redox status affect catalysis (74). The activity of VCA0681 requires Fe(II) at the bimetallic center,
285 and derivatives with Fe(III) are inactive suggesting that the activity of this protein is redox-
286 regulated (76). Also isolated TM0186 from *Thermotoga maritima* with two Fe(III) atoms is inactive;
287 reduction to Fe(II) enables the enzyme to generate 5'-pGpG but not GMP. Additional

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supplementation with either Mn(II) or Fe(II) leads to production of GMP. The phylogenetic clustering of TM0186 within the E/D-T-G subgroup of HD-GYP domain proteins suggests that it has a tri-metallic center. Furthermore, a variant protein with an alanine substitution of the glutamate generates only 5'-pGpG as a product. The findings point to the association of a tri-metallic center with the ability to generate GMP from 5'-pGpG. Also, the action of HD-GYP domains in converting 5'-pGpG to GMP suggests regulation by the intracellular availability of metals and metal site occupancy. Finally, catalytically inactive SO2541 HnoD from *Shewanella oneidensis* and PA2572 from *P. aeruginosa* are variant at the HD dyad (SE and YN respectively) and have only 1 conserved residue involved in metal chelation (77, 78); as a result, these proteins may exert their effect through protein interactions involving the GYP motif (77, 78).

Diversity in substrate binding and catalysis

Determination of the structure of *Pm*GH in complex with the substrate cyclic di-GMP and final reaction product GMP has revealed the mode of binding and shed light on the possible catalytic mechanism (71, 79). Adequate space is available for the substrate to bind and both hydrolysable phosphates to interact with the metal center to sequentially hydrolyze cyclic di-GMP to GMP. Cyclic di-GMP is bound in a *cis* conformation (71), in contrast to the more extended conformation observed when cyclic di-GMP is bound to EAL domain proteins (80) or predicted in binding to the HD-GYP domain protein PA4108 (81).

The structural analysis of *Pm*GH-cyclic di-GMP complex shows that the bound cyclic dinucleotide interacts with the central (M-site) Fe(III) and is involved in diverse hydrogen bonds and hydrophobic interactions (Fig. 6). As in RpfG, in *Pm*GH alanine substitutions of six residues involved in metal binding in addition to the HD dyad (H221, D222) (see Fig. 7) essentially abolish or markedly reduce the phosphodiesterase activity. Alanine mutation of other conserved residues near the metal center (D183, D308, and K225) have a similar impact on activity (71). Alanine

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substitutions of residues implicated in cyclic di-GMP recognition do not, however, result in a substantial decrease in catalytic activity (71). The proposed enzymatic mechanism is that M-site Fe(III) directly interacts with a non-bridging oxygen of one of the scissile phosphate diesters of cyclic di-GMP to provide a strong Lewis acid catalyst, whereas a metal-activated bridging hydroxide ion of the M-H Fe pair is the likely nucleophile for the hydrolysis of the scissile bond (71). The occurrence of a hydroxide ion bridging ligand is consistent with the metal-ligand bond lengths (72, 82). The structure does not reveal how the O3' leaving group is protonated, however.

The structure of PA4781 reveals potential steric hindrance of cyclic di-GMP binding by a glutamate at position 314 (73). Accordingly, the purified enzyme has a relatively low affinity for cyclic di-GMP (K_M ~120 μM) compared to 5'-pGpG (K_M ~27 μM). In other enzymatically active HD-GYP domain proteins, position 314 is occupied by an alanine (see Fig. 7), and an E314A variant of PA4781 shows substantially enhanced affinity for cyclic di-GMP (81). Detailed kinetic analyses indicate that PA4781 has a low enzymatic activity but hydrolyses 5'-pGpG more effectively than cyclic di-GMP (81). Although similar kinetic experiments on other HD-GYP domain proteins have not been reported, the available evidence suggests that differences in the relative activity against 5'-pGpG compared to cyclic di-GMP do occur (83) (76) (84) (77) (66, 69, 71).

Structural insights into the multifunctional roles of HD-GYP domains

A sequence-based analysis identified the GYP signature motif of HD-GYP proteins as part of a larger widely conserved motif HHExxDGxGYP (66). The *Pm*GH structure suggests an extension of this consensus motif to HHExxDGxGYPxxxxxxxI, to include a conserved isoleucine residue (I294 in *Pm*GH) that stabilizes the structure of the loop by hydrophobic interactions with G284 from the GYP motif (71). The structural conservation of the 'GYP loop' (Fig. 6) (73) between *Pm*GH and PA4781 suggests that it is integral to the functions(s) of HD-GYP domain proteins. The GYP motif is

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337 critical for protein-protein interactions of RpfG with specific GGDEF domain proteins in *X.*
338 *campestris*, but is not necessary for the phosphodiesterase activity (66).
339 The available evidence suggests that the HD-GYP domain of RpfG can also interact with proteins of
340 other classes, including the transcriptional regulator NtrC (36, 85). Furthermore the enzymatically
341 inactive HD-GYP domain response regulator HnoD can inhibit the activity of the EAL domain
342 response regulator HnoB to regulate cyclic di-GMP levels in *Shewanella oneidensis* (77). The
343 mechanistic basis of this inhibition is not known. Different HD-GYP domain proteins within the
344 same organism may interact with different partners *in vivo*, although this remains to be tested
345 experimentally.
346 The structure of the *Pm*GH HD-GYP complex with cyclic di-GMP reveals that Y285 of the GYP motif
347 is placed inside the substrate-binding pocket, where it H-bonds to cyclic di-GMP (Fig. 6). This
348 presents a conundrum for the action of RpfG. If GGDEF domains interact directly with Y285, they
349 need to intercalate with the inner side of the HD-GYP nucleotide-binding pocket. This would
350 prevent cyclic di-GMP binding and phosphodiesterase activity, although such effects have not been
351 observed *in vitro* (35). An intriguing alternative is that RpfG involvement in protein-protein
352 complexes is determined not only by cyclic di-GMP binding but also by conformational alterations
353 associated with cyclic di-GMP degradation, which would be 'reported' via the GYP loop. In this way,
354 RpfG would act as a trigger enzyme for protein complex formation and regulation similar as
355 suggested for the EAL domain protein YciR of *Escherichia coli* (34). However mutation of the HD
356 dyad of the HD-GYP domain of RpfG does not significantly affect its *in vivo* interaction with GGDEF
357 domain proteins, as revealed by FRET analysis (35). Only further work can reveal whether
358 particular regulatory actions of HD-GYP domain proteins occur independently of their ability to
359 bind or hydrolyse cyclic di-GMP or 5'-pGpG.
360
361 *Further substrates for HD-GYP domain proteins*

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In addition to cyclic di-GMP, bacteria have been shown to utilise cyclic di-AMP and most recently the dinucleotide 3'3'-cyclic GMP-AMP as intracellular signal molecules. The latter molecule was discovered in *Vibrio cholerae* as a regulator of chemotaxis and of factors contributing to colonisation of the intestine (86). A screen of potential phosphodiesterases for 3'3'-cyclic GMP-AMP from *V. cholerae* identified three HD-GYP domain proteins, VCA0210, VCA0681 and VCA0931 which were capable of hydrolysis of the cyclic dinucleotide into 5'-pApG, with VCA0681 having an additional 5' nucleotidase activity to generate 5'-ApG (87). The nucleotidase and phosphodiesterase activities were associated with the HD and HD-GYP domains respectively that are present in tandem (87). All three proteins hydrolyse 3'3'-cyclic GMP-AMP specifically, with no activity against other cyclic GMP-AMP forms with different phosphodiester linkages, to include the mammalian innate immunity regulator 2'3'-cGMP-AMP. Variant VCA0681 proteins with alanine substitutions in the signature HD dyad and GYP motif have no detectable activity (87)(Gao et al., 2015), in contrast to the role of the GYP motif in P*m*GH and RpfG (Bellini et al., 2014; Ryan et al, 2010).

FUNCTIONAL DIVERSIFICATION OF CYCLIC DI-AMP PHOSPHODIESTERASES

The functional diversification also extends to other cyclic di-nucleotide signaling networks. As the currently most prominent example, DHH/DHHA1 proteins usually function as phosphatase or phosphodiesterases for hydrolyzing a wide variety of substrates that range from pyrophosphate to ssDNA. The substrate specificity of DHH/DHHA1 enzymes is usually governed by the DHHA1 domain rather than the DHH domain. A bioinformatics search of potential phosphodiesterases for cyclic di-AMP, a universally essential cyclic di-nucleotide second messenger in Gram-positive bacteria (88, 89), led to the discovery of a DHH domain protein (YybT or GdpP) from *B. subtilis* as a cyclic di-AMP phosphodiesterase (30). GdpP is a metal ion-dependent phosphodiesterase that breaks down cyclic di-AMP into 5'-pApA at physiologically relevant substrate concentrations (μM). In accordance with its specificity towards cyclic di-AMP, the DHHA1 domain of GdpP does not share significant sequence

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387 homology with the DHHA1 domains of other DHH/DHHA1 proteins. Importantly, a number of Arg
388 residues critical for the binding of polyphosphate, RNA, or ssDNA in other DHHA1 domain proteins
389 (e.g. RecJ and YtqI) are not conserved in YybT. Another DHH/DHHA1 protein (Pde2) that lacks the
390 PAS and GGDEF domain of GdpP and degrades cyclic di-AMP into AMP was discovered in *S.*
391 *pneumoniae* (90). Pde2 is an ortholog of *B. subtilis* YtqI (also named NrnA) that was claimed to be
392 responsible for degrading nanoRNA (RNA oligonucleotides of ≤5 nucleotides) and dephosphorylating
393 pAp to AMP (91, 92)
394 In addition to the DHH-DHHA1 proteins, a subfamily of HD domains possesses cyclic di-AMP
395 phosphodiesterase activity. The first example is the *Listeria monocytogenes* protein PgpH (93).
396 Biochemical and structural studies revealed binding of cyclic di-AMP with high affinity (K_d = 0.3–0.4
397 μM) and hydrolysis to 5'-pApA in the presence of divalent metal ions such as Mn^{2+} and Fe^{2+}.
398 The discovery of the DHH/DHHA1 and HD-domain based phosphodiesterases for degrading
399 cyclic di-AMP mirrors the converging evolution of the EAL and HD-GYP domains involved in cyclic
400 di-GMP degradation. Although the structural basis for the recognition of cyclic di-AMP by the PDEs
401 remains to be fully defined, the crystal structure of the stand-alone DHH/DHHA1 protein Rv2837c
402 in complex with the hydrolytic intermediate 5-pApA suggests that a set of residues from both DHH
403 and DHHA1 domains contribute to the binding of cyclic di-AMP (94). Even assuming that only two
404 families of cyclic di-AMP phosphodiesterases are found in nature, identification of the members of
405 the two families by bioinformatic should still proceed with caution and experimental validation is
406 necessary.
407
408 **CONCLUDING REMARKS**
409 As outlined above, diversity in the function of GGDEF, EAL and HD-GYP domains is evident in terms
410 of enzymatic activity, the ability to synthesize or degrade alternate di-nucleotides as well as in
411 interactions with other proteins. This functional diversity certainly extends to other cyclic di-

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412 nucleotide turnover proteins such as the DHH/DHHA1 enzymes. Further biochemical and
413 structural work is required to gain knowledge of the molecular bases for the substrate specificity or
414 preference. Work on stringent cyclic mononucleotide synthases shows that quite limited variations
415 give rise to different specificities; cyclic GMP synthases can be experimentally changed to cyclic
416 AMP synthases and vice versa by just two or three amino acid exchanges (95, 96). On the other
417 hand, relaxed enzymes can produce several different cyclic nucleotides (97). In addition, a three
418 amino acid replacement in the human cyclic di-nucleotide synthase cGAS changes the
419 phosphodiester linkage specificity so that 3'3' cyclic GMP-AMP rather than the non-canonical 2'3'
420 cyclic GMP-AMP is synthesized (98). The three new residues incorporated were the determinative
421 amino acids in DncV, a bacterial homolog of cyclic GMP-AMP synthase (98). Indeed ancient cGAS is
422 a 3'3' cyclic GMP-AMP synthase (99). As outlined above, distinct GGDEF domain proteins that have
423 been shown to produce cyclic GMP-AMP (31) and some HD-GYP domain phosphodiesterases can
424 have cyclic GMP-AMP hydrolytic activity (87). Similar changes in substrate specificity to those
425 within the GGDEF and HD-GYP domain protein families could also occur within the EAL domain. In
426 addition, novel enzymes with cyclic di-nucleotide turnover activity might be recognized. Recently,
427 CpdB which is characterized by a diffusion determined speed of 3'-AMP hydrolytic activity, was also
428 shown to hydrolize cyclic di-AMP with a reasonable turnover rate (100). With the current stage of
429 knowledge, it thus appears difficult to assign substrate specificity and product outcome with
430 certainty by bioinformatics. Thus, current species-specific nomenclatures might limit the
431 comparison to distantly related species, which frequently harbor orthologous proteins, while
432 functional paralogues of di-nucleotide turnover proteins dominate within a species. The elucidation
433 of the structures of cyclic di-GMP turnover domains in complex with other cyclic di-GMP turnover
434 domains and other interacting proteins will also be necessary to provide a deeper understanding of
435 the regulatory action of the diversity of these families of signaling proteins and to fully explore their
436 true functions. This is certainly the case for those proteins that may be multifunctional and which

437 may regulate different functions through protein-protein interactions and modulation of cyclic di-

438 GMP levels.

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453 **Figure legends**

454

455 **FIG 1** Enzymes involved in the turnover of second messengers cyclic di-GMP (B) and cyclic di-AMP
456 (B). GGDEF domain proteins are cyclic di-GMP synthases (2), but a few members can preferentially
457 synthesize cyclic GMP-AMP (31). Cyclic di-GMP is degraded by EAL domain or HD-GYP domain
458 phosphodiesterases into 5'-pGpG and GMP, respectively (66). 5'-pGpG is further hydrolyzed to GMP
459 by the oligoribonuclease Orn. Cyclic di-AMP is synthesized by the DAC domain and hydrolysis has
460 been demonstrated by HD and DHH/DHHA1 domain proteins. Major phenotypes affected upon
461 cyclic di-nucleotide synthesis in many bacteria are indicated.

462

463 **FIG 2** Classification of GGDEF domains according to protein structure and conservation of signature
464 motifs. Amino acids on a grey background interact with the substrate in the diguanylate cyclase
465 PleD. K_{332} stabilizing the transition state is on a cyan background. The RxxD I-site core motif is in
466 blue. Unconventional amino acids still conferring enzymatic activity are on a blue background.
467 Amino acids conferring cyclic GMP-AMP specificity are on a green background. Amino acids
468 involved in the interaction with the HD-GYP domain are underlined. Conserved amino acids in
469 color. GGDEF domain protein names are in black and GGDEF-EAL proteins in green. Unconventional
470 GGDEF domain names in violet and cGMP-AMP synthesizing proteins in orange. Protein designation
471 in Supplemental material. Modified after (32).

472

473 **FIG 3** Ribbon diagram of the GGDEF domain of PleD binding the substrate analog GTPαS (PDB code:
474 2v0n). A. Amino acids interacting with the substrate analog αS-GTP (including Lys_{442} and Arg_{446}
475 interacing with the phosphate group and the Mg^{2+} ions (Asp_{327} and Glu_{370}) (1, 6, 15) are indicated. Mg^{2+}
476 ions in green. B. Amino acid motifs providing additional functionality to GGDEF domains. $R_{359}xxD_{362}$ is

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477 the core motif of the I-site. The $xxD_{327}xD_{329}$ motif was demonstrated in protein-protein interactions in
478 GGDEF domain proteins others than PleD.
479
480 **FIG 4** Classification of EAL domains according to protein structure and conservation of signature
481 motifs. The catalytic base glutamate is shown in red. In green, amino acids involved in Mg^{2+} binding;
482 in blue, amino acids involved in substrate binding. Alternative amino acids involved in cyclic di-
483 nucleotide binding are underlined. The glutamate stabilizing loop 6 is shown in orange. Loop 6
484 amino acids are on a grey background. Names of EAL proteins in black, EAL only proteins in red
485 and GGDEF-EAL proteins in green. Protein designation in Supplemental material. Modified after
486 (32).
487
488 **FIG 5** Substrate binding by EAL domains. A. Ribbon diagram structure of the EAL domains of BlrP1,
489 a fully functional class I PDE activated by light (44) and YahA (41) binding to the substrate cyclic di-
490 GMP. In the middle, enlarged view of the cyclic di-GMP binding site of BlrP1. Cations are shown in
491 violet and pink. Cyclic di-GMP is shown as sticks with carbon atoms colored yellow. B. Comparison
492 of electrostatic surface representation of class III EAL domains FimX of *P. aeruginosa* and YdiV of *E.*
493 *coli*. While the cyclic di-GMP binding site of class IIIa FimX is conserved (model is shown with cyclic
494 di-GMP bound), the cyclic di-GMP binding pocket is not conserved in class IIIb member YdiV. The
495 electrostatic surface potential shows highly electronegative (red) and electropositive patches
496 (blue) of the two proteins. C. Ribbon diagram structure of three Class IIIa cyclic di-GMP binding EAL
497 domains (EAL_{FimX_PSEAE} (Q9HUK6 of *P. aeruginosa*), EAL_{XcFimX} (A0A0H2X6E4 of *Xanthomonas*
498 *campestris* pv. *campestris*) and $EAL_{LapD_Psfluor}$ (Q3KK31 of *Pseudomonas fluorescens* Pf0-1). Note the
499 different conformations and binding modes of cyclic di-GMP, which is displayed as sticks with
500 carbon atoms in yellow, oxygen in red, phosphate in orange and nitrogen in blue.
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FIG 6 Substrate binding by the HD-GYP domain of PmGH. A: Surface representation of the PmGH HD-GYP domain monomer subunit showing the binding cavity for cyclic di-GMP, which is represented in stick mode and colored by atom type. B: Superposition of the structures of PmGH bound to cyclic di-GMP and GMP. Both nucleotides are shown in stick mode. Bonding interactions are represented by dashed lines. The central metal iron has been labeled as the middle site (M) and the two flanking metal sites as H and G, to reflect their proximity to the HD and GYP motifs, respectively. Residues that interact with cyclic di-GMP include Y285 of the GYP motif. Red spheres represent solvent and SIN-1 a succinate molecule (71).

FIG 7 Primary sequence alignment of HD-GYP domains from proteins that have been characterized structurally and/or enzymatically reveals the diversity within the domain. Protein designation in supplemental material. The top line indicates the helices in the structure of *Pm*GH with an annotation of the inter-helix loops. Metal ligands are given in red, proposed catalytic residues are given in green. The GYP motif is highlighted in cyan and the substrate binding ligands in magenta. The region of the sequences with consensus motifs E(D)TG/YTY are highlighted in yellow. (Note that these are not fully conserved). The blue triangle points to the E residue in PA4781 that may act in steric hindrance of cyclic di-GMP binding.

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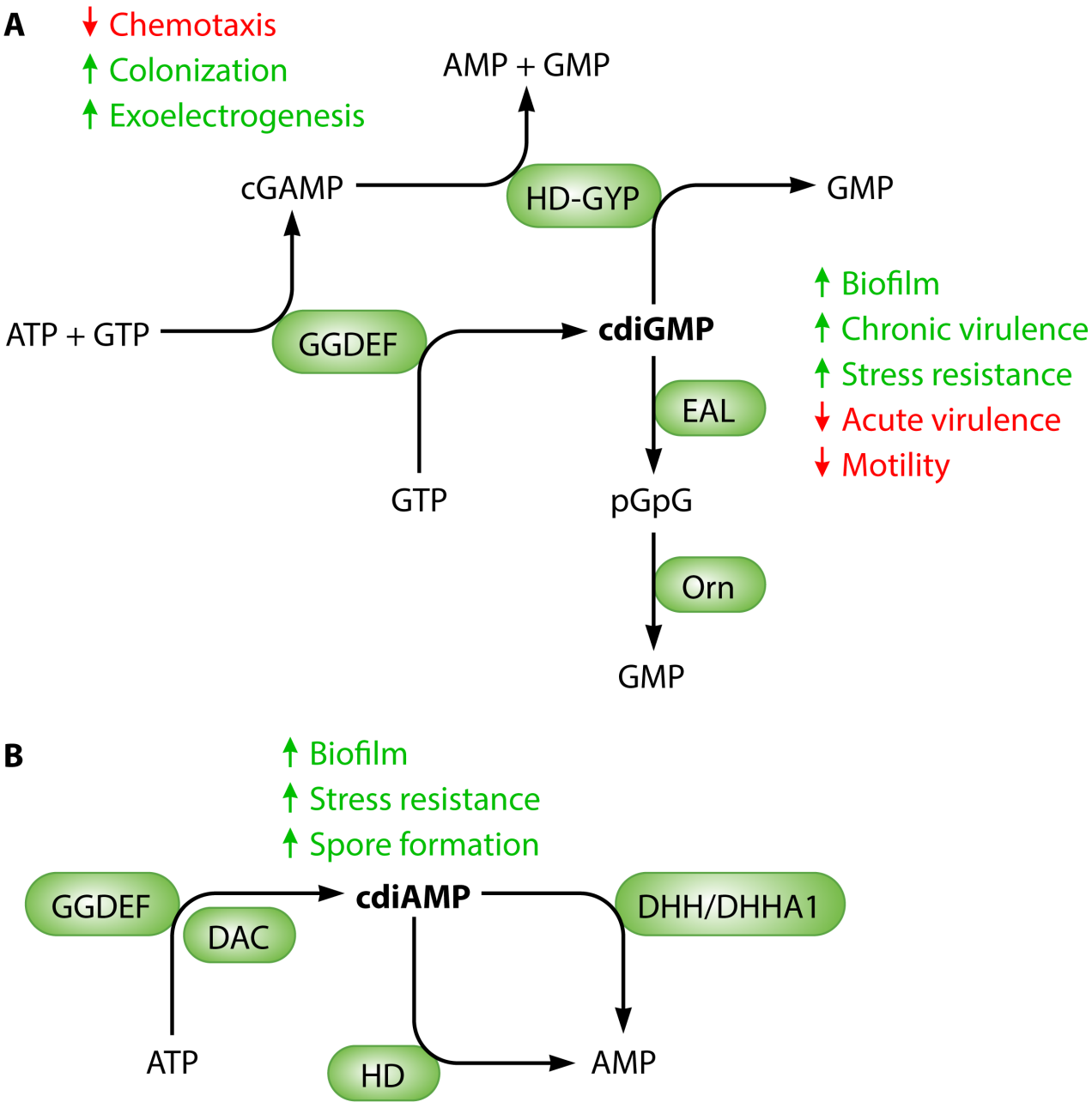
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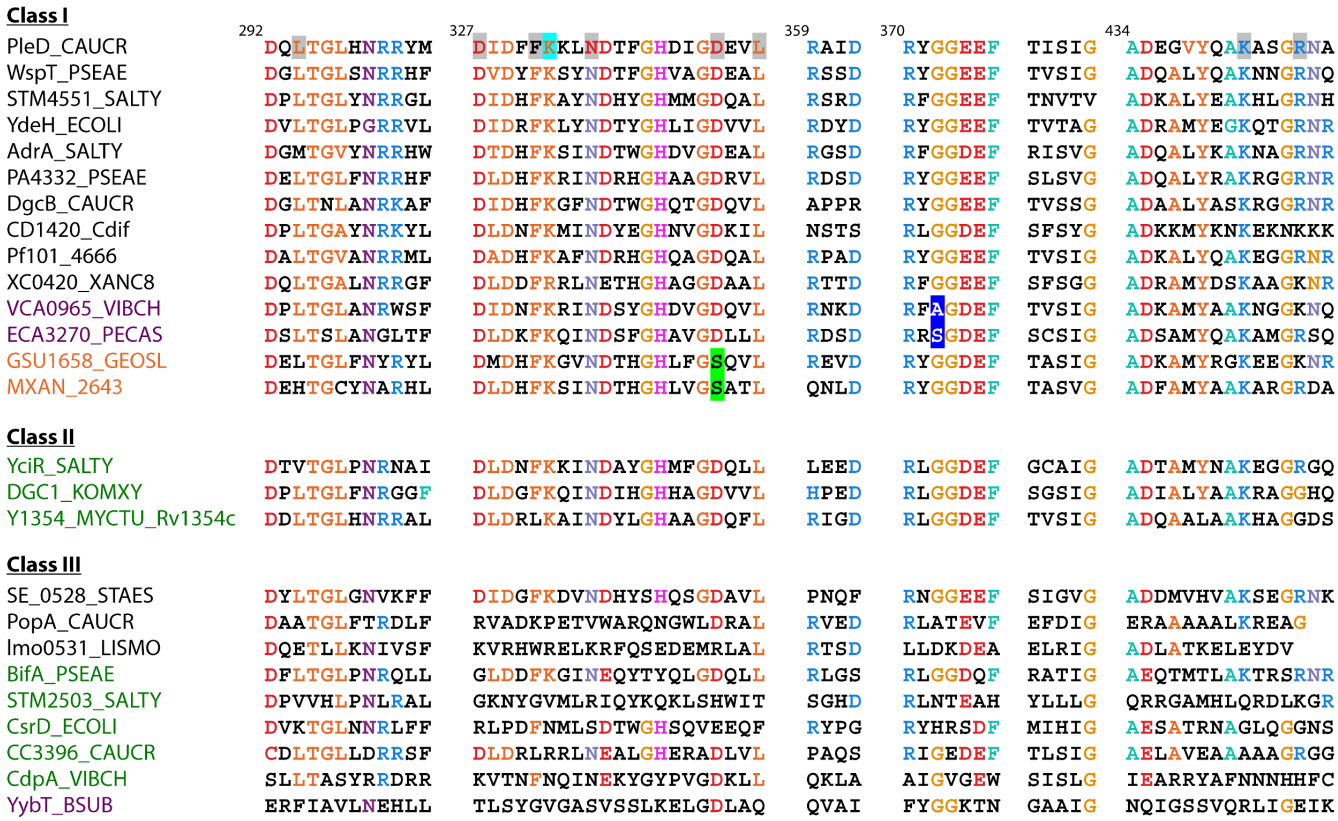
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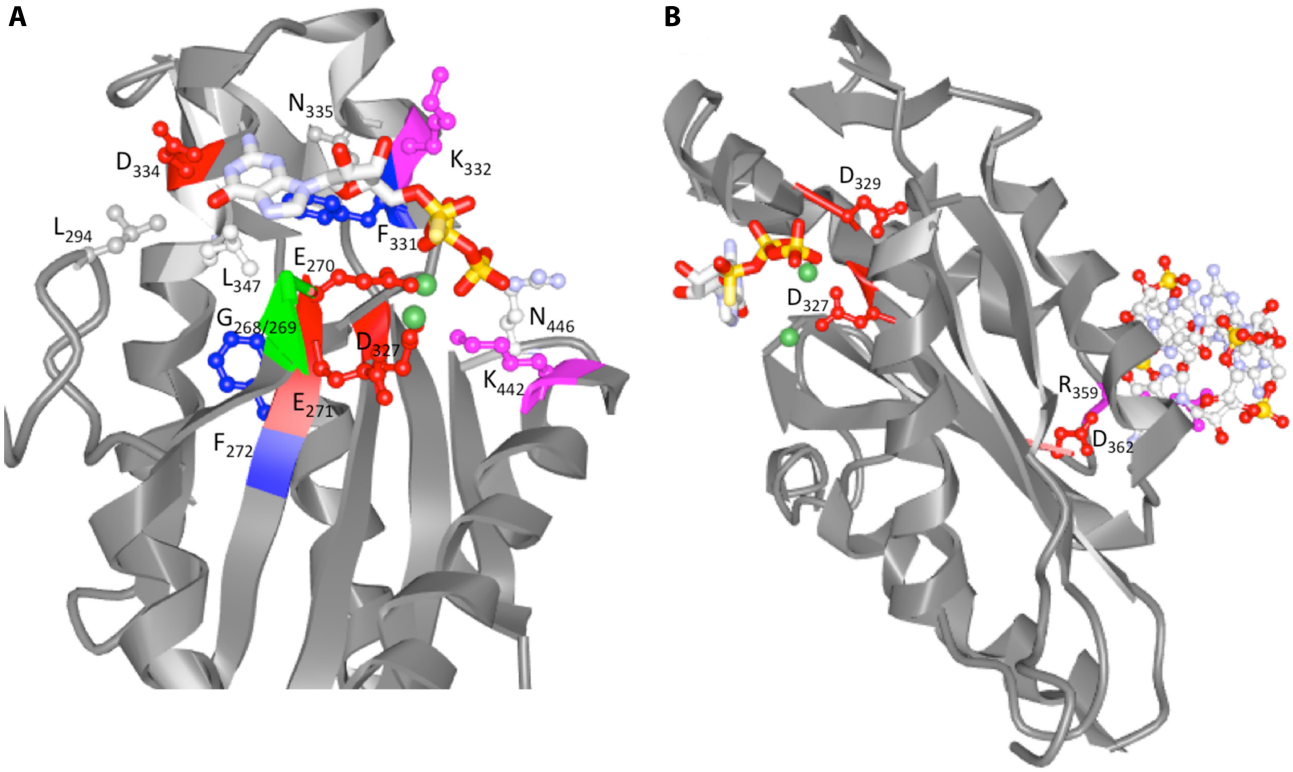


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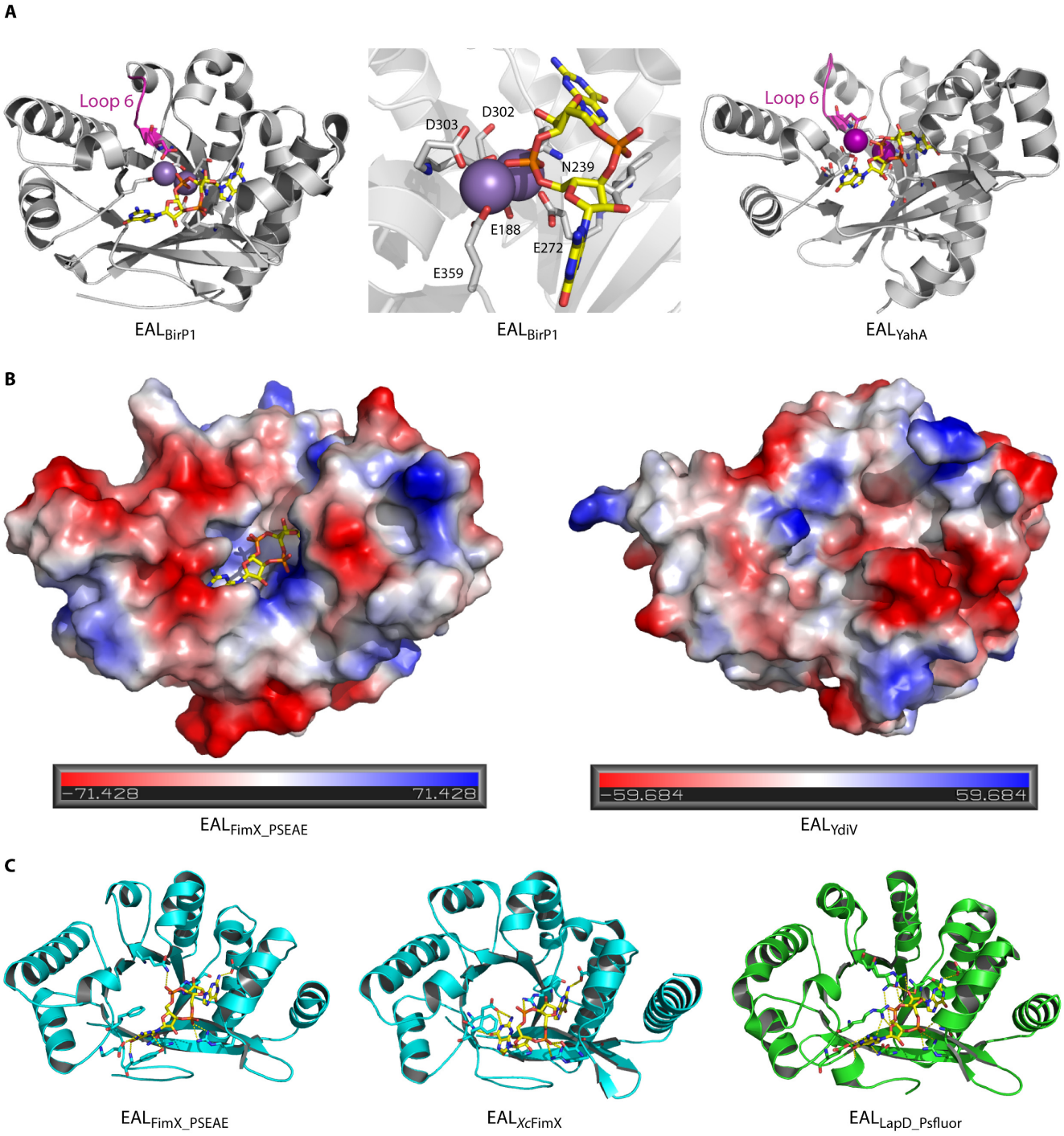
	160	175	190	197	232	265	282	316	321	349	372
Class I											
RocR_Paer	YQP	EVLAR	PS-HF	ME	FN	EITE	LVRLRIMGCGLAMDDFGAGYSSLDR	KLD	FV	LVVEGVES	QGYL
VieA_Vchol	YQP	EALVR	PSIEQ	HE	VN	EMTE	LARLRMYGVGLSIDDFGTGYASLGQ	KID	FV	CVVEGVEN	QGYY
Tbd1265	YQP	EALVR	PAAED	IV	VN	EITE	LDALRARGVRLALDDFGTGYSSLSY	KID	FV	VVAEGIET	QGNL
Blrp1_Kpneu	LQA	EALIR	PVEMF	AE	IN	EVTE	LKALRVAGMKLAIDDFGAGYSGLSL	KVD	EV	VVAEGVET	QGFL
Y1354_MYCTU_Rv1354c	YQP	EALVR	PG-CF	AE	IN	EITE	LARLKEVGVHIAIDDFGTGYSAISL	KID	FV	VVAEGVET	QGFL
DosP_Ecoli	YQP	EALAR	PS-RF	AE	VN	EITE	IQILRDMGVGLSVDDFGTGFSGLSR	KID	FV	VVAEGVET	QGYF
MorA_Paer	YQP	EALLR	PS-EF	LE	VN	ELTE	LSGLKRLGLAIAVDDFGTGYSSLNY	KID	FV	VIAEGVES	QGYL
Atu3495_Agfab	YQP	EALLR	PS-VF	AE	VN	EITE	LSRLRIMGCGLAMDDFGTGYSSLAN	KID	FL	TIAEGIET	QGYL
PDEA3_Koxyl	YQP	EALSR	PS-RF	AE	VN	EITE	LQSIRNIGCGLSMDDFGTGYSSLSR	KID	FI	VVTEGVET	QGYL
Ip10329LePne	YQP	EALIR	PL-DF	AE	CN	EITE	LKKLKSLGVLIILDDFGTGNSSLNL	KID	FI	IIAEGVET	QGFL
YciR_Salty	YQP	EALVR	PL-EF	AE	VN	ELTE	LQQFSQLGAQIHLDDFGTGYSSLSQ	KLD	FV	VIAEGVEN	QGFL
cc3396	FQP	EALAR	PD-EF	IE	VN	EVTE	LKTLRDAGAGLALDDFGTGFSSLSY	KID	FV	VVAEGVEN	QGFG
BifA_Paer	YQP	EALLR	PD-HF	AE	VN	EVTE	LLSLRRAGALIAIDDFGTGYSSLSY	KID	FV	VIAEGVET	QGYL
HmsP_Ypest	LQP	EALLR	PS-GF	AE	VN	EITE	LRELQGLGLLIALDDFGIGYSSLRY	KLD	FV	VMAEGVET	QGFL
Class II											
Class IIa											
YhjH	YQP	ELLTV	PDRYF	AV	VN	ELVE	FASMCEFGPL-WLDDFGTGMANFSA	KVA	FV	VIVEGVET	QGYFL
YE2225_Yente	CEP	ELLSR	YTERF	LN	IN	EIME	LSKLSKRYS-LWLDDFGSGNAHLAA	KID	FY	VVVEGVET	QGYL
Class IIb											
YkuI_Bsub	YQA	EVLGR	GP-FF	PE	IF	EITE	LAYYRTYGIKIAVDNIGKESSNLDR	KID	AL	LLYEDIEA	QGYY
DGC2_Komyxl	YQP	EALLR	AE-DF	FT	IN	EVTE	LRELARAGFRIALDNFGKGITVLNH	KID	MV	VTVEGVEN	QGFF
Class III											
Class IIIa											
LapD_Psfluor	FQP	KVLSR	PAGRF	LE	LN	EIGE	TRRLRELGFSLSLQRFGGRFSMIGN	KID	YI	LIAERVET	QGQL
FimX_Paer	FQP	EVLLR	PA-EF	KE	VH	QISE	LTQGLATLHCQAAISQFGGSLNPFN	KID	FV	PIVPFVES	QGYYL
PXO_00403	YQP	QAFLR	PN-AF	AE	VR	QTPE	LSAVSAMGCKVGLEQFGSGLDFSQL	KLD	IT	TMAEFVAD	QGDF
Lpg1057_Lpneu	YQP	EVLLR	PD-EF	AE	IN	ELEI	LSTLRNMGVKLAEDNFGSGYSFLSH	KLK	LI	VVVGGVET	QGYY
Class IIIb											
YcgF_Ecoli	FHP	EAIVQ	VGQRK	EI	IN	EFTE	IKSLKAAGISVAIDHFGAGFAGLLL	KIS	LI	VSAMGVAT	QGDL
YE1324	CEP	ELLSR	DVENF	LD	IN	EIME	LRDLAER-YPLWLDDLGSGGSTLNA	KID	FF	VIVVGVEN	QGYL
STM1697	AEP	EITTH	P--EF	WD	FC	ELQV	IHALHKQPNPLWLGDLGVGNATAAP	KLD	FF	IVVGGQEN	QGTL
STM1344	FLP	EIIAT	P-TEL	RL	WL	ELAI	TLANLAMHFPLMLANFGAGEASTKA	MLD	FI	LMIAGIET	QGGL
ToxR_Paer	FAH	KLSLR	LSAFL	AE	FI	ELCL	LARLRDSGVRIALHPQRIDTDARQC	GLD	LL	MLCLNVEN	HGRH
CsrD_Ecoli	YQK	ELMCR	SA-EY	VL	IQ	ELAE	LRLVNALGVRVAVNQAGLTLVSTSW	KLH	LV	VYATGVRS	QGDF

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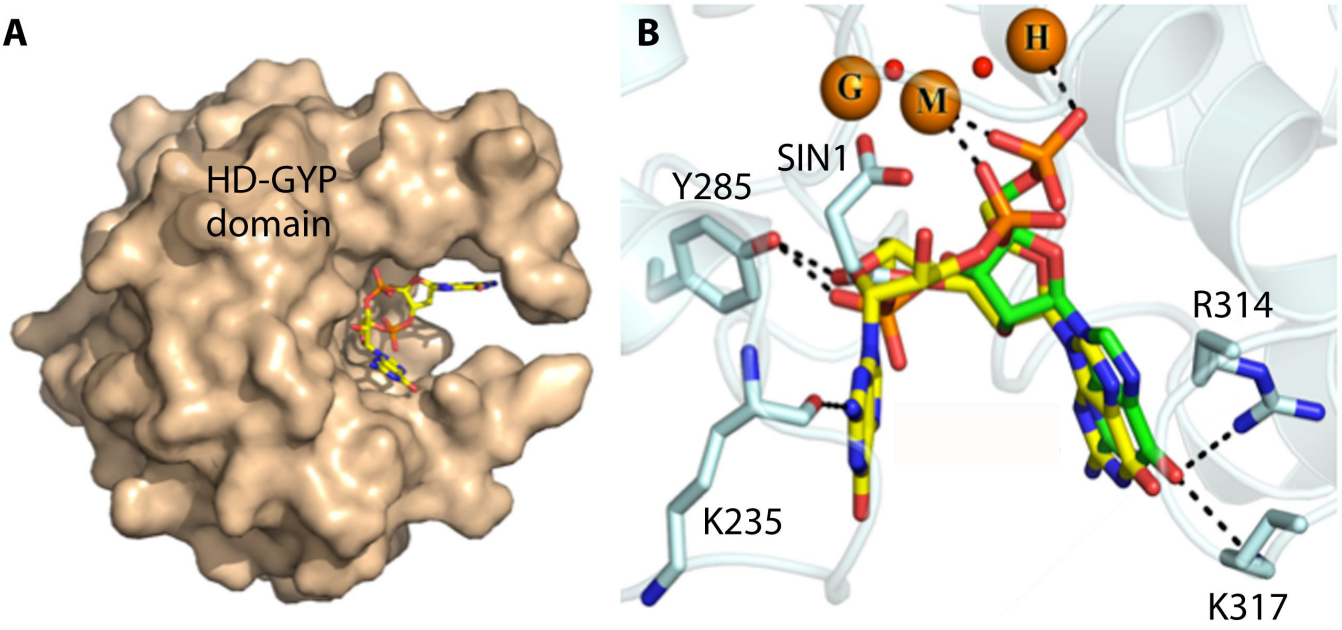
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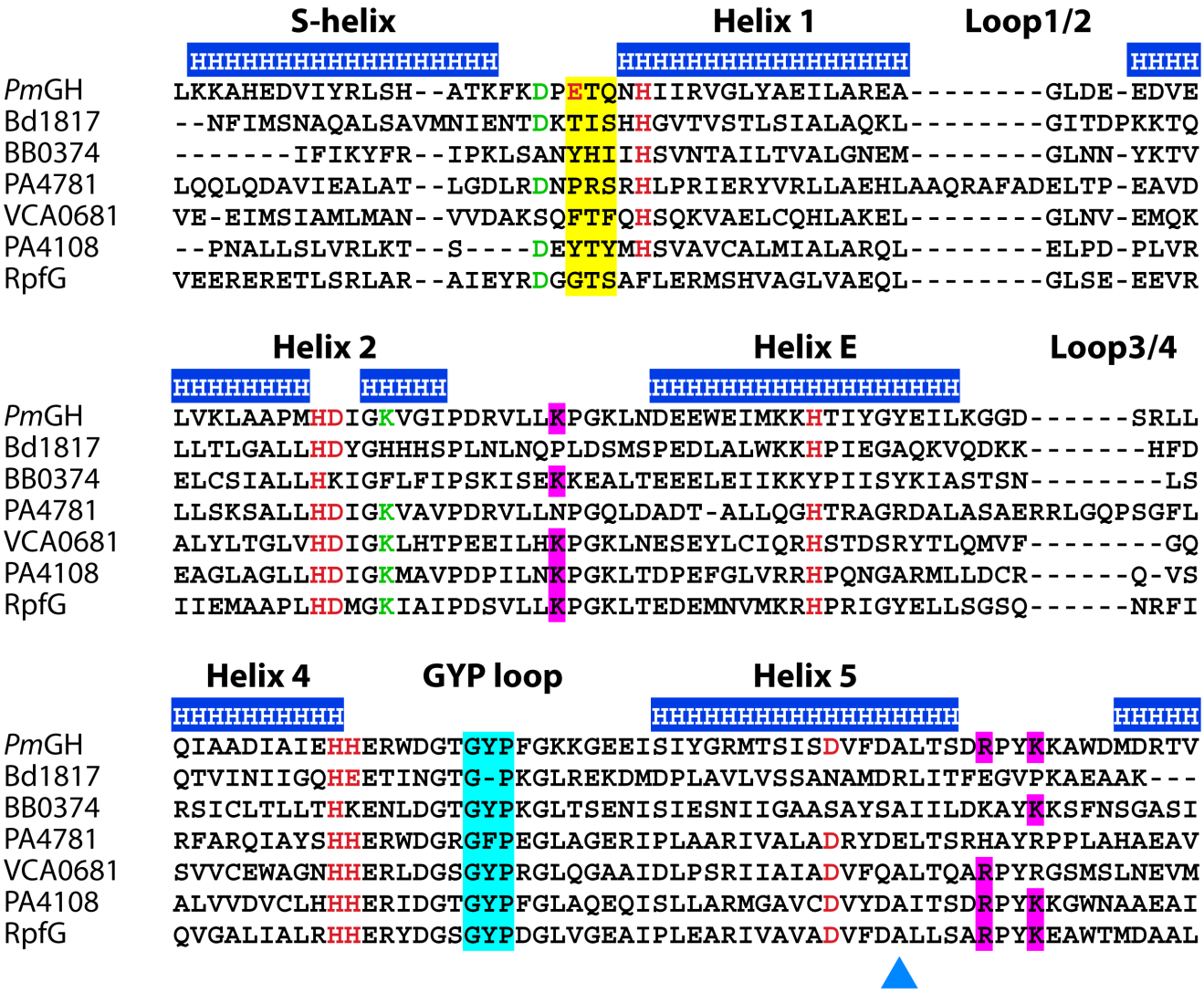
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