

## COMMENTARIES

# Rationalizing the Evolution of EAL Domain-Based Cyclic di-GMP-Specific Phosphodiesterases<sup>∇</sup>

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Degradation of the bacterial second messenger cyclic (5'→3')-diguanosine monophosphate (c-di-GMP) is performed by c-di-GMP-specific phosphodiesterases (PDEs). EAL domain-based c-di-GMP-specific PDEs catalyze the hydrolysis of the 3' cyclic phosphate bond of c-di-GMP, resulting in the linear dimeric GMP molecule 5'pGpG as an end product (Fig. 1) (20, 21, 23). In this issue of the *Journal of Bacteriology*, Zhao-Xun Liang's research group analyzed the impact of conserved loop 6 on the c-di-GMP-dependent catalytic activity of EAL domains (16). Together with a recent publication of the same group dissecting the function of 14 conserved polar residues in EAL domain signature motifs (Fig. 2) (17), this work has started to rationalize the impact of the sequence diversity in EAL domain proteins through systematic structure-function analysis.

**Requirement for tight regulation of the c-di-GMP signal.** The activity of GGDEF domain-based diguanylate cyclases (DGCs) results in the synthesis of the second messenger molecule c-di-GMP, which, as a key signal, triggers the transition between sessility and motility and regulates other common phenotypes, such as virulence in many bacteria (5, 19, 29). If c-di-GMP synthesis were left unchecked, the c-di-GMP concentration would increase rapidly throughout the bacterial cell. To allow dynamic temporal and spatial control, one mean to restrict the magnitude and duration of the c-di-GMP signal is the action of c-di-GMP-specific PDEs (24). Thereby, c-di-GMP degradation is conferred by the unrelated EAL and HD-GYP domains (22, 23).

**Discovery of catalytically active and inactive EAL domains.** EAL domains, named after the characteristic EAL motif, comprise a large superfamily of proteins, restricted to bacteria. Early studies have already indicated the existence of at least two classes of EAL domains, one class of EAL domains with apparent c-di-GMP-specific PDE activity and a second class that seemed to lack this catalytic activity. In vitro c-di-GMP-specific PDE activity was demonstrated for the *Escherichia coli*-derived EAL domain proteins YahA and DOS (23). Other examples of EAL proteins with c-di-GMP PDE output included YhjH from *Salmonella enterica* serovar Typhimurium and VieA from *Vibrio cholerae* (25, 31). On the other hand, three diguanylate cyclases producing c-di-GMP dedicated to the activation of cellulose biosynthesis in the fruit-degrading

bacterium *Gluconacetobacter xylinus* were GGDEF-EAL domain proteins (28). Similarly, GGDEF-EAL domain proteins stimulated c-di-GMP-dependent biofilm formation in *S. Typhimurium* (9). On the basis of these findings, distinct signature motifs indicative for the presence of enzymatically active EAL domains were suggested (23). Due to the complexity of the consensus sequence, however, the impact and function of most of these residues remained an enigma.

**Amino acids required for the PDE activity of EAL domains.** Using the c-di-GMP phosphodiesterase RocR from *Pseudomonas aeruginosa* as a model, Rao et al. systematically mutagenized 14 highly conserved polar residues that were all integral components of the EAL domain signature motifs and determined their function in catalysis (17). Exchange of seven of these residues led to a >10<sup>5</sup>-fold decrease in the turnover number  $k_{cat}$ , a measurement of the maximal reaction velocity, indicating that these residues were absolutely required for catalysis (Fig. 2). Consistent with this finding was the positioning of these amino acids in the structural model of RocR (17). Seven amino acids were directly or indirectly involved in the positioning of the Mg<sup>2+</sup> ion required for catalysis. The glutamate at position 352 was identified as the general base catalyst, which accepts the proton from a water molecule. The created hydroxid ion subsequently performs the nucleophilic attack which leads to the breakage of the phosphate-sugar bond.

**Role of loop 6 in EAL domain activity.** In addition, the extended conserved motif DDFG(T/A)GYSS was suggested to be essential for enzymatic activity (17, 23). The structural model of RocR revealed that the amino acids of the DFG(T/A)GYSS motif form loop 6, which connects elements of secondary structure, a  $\beta$ -sheet and an  $\alpha$ -helix. In support for the functional importance of the loop, alanine replacement of the glutamate at position 268, which stabilized the loop according to the structural model of RocR, led to a complete loss of the enzymatic activity (17).

Loops, nonregular secondary structures in proteins, play important roles in protein function, stability, and folding (6). Between the members of the same protein family, sequence differences leading to functional differences frequently correspond to loop structures, and therefore, loops often determine the functional specificity of a given protein framework (8). Observing the association between the sequence degeneration of loop 6 and the mutation of essential catalytic residues prompted Rao et al. to investigate the role of loop 6 in the functionality of the EAL domain proteins (16). Indeed, alanine replacement of loop amino acids (Fig. 2) led to a dramatic

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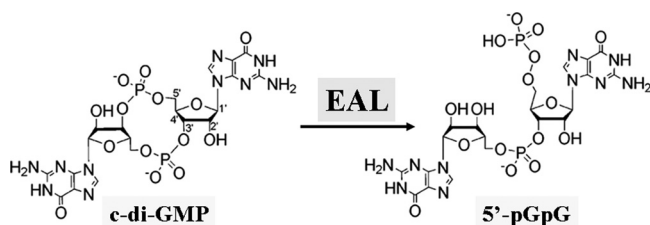


FIG. 1. Hydrolysis of c-di-GMP to linear guanylic acid 5'-pGpG is catalyzed by the enzymatic activity of phosphodiesterase A, which is conferred by EAL domains.

change in the oligomerization status of the RocR protein and caused the EAL domain protein PA2567 to form inclusion bodies. A substrate inhibition kinetic was observed in some mutants, indicating altered binding of the c-di-GMP substrate.

**Loop 6 is involved in signal transduction.** In the majority of cases, EAL domains are part of multidomain proteins connected with N-terminal signaling and sensing domains. A significant fraction of EAL domain proteins are response regulators intimately integrated into two-component systems, signaling systems that transmit information by phosphotransfer. RocR is a response regulator and part of a complex two-component system in *P. aeruginosa* (10, 11). Conventionally, phosphorylation of a conserved aspartic acid residue (at position 56) located in the N-terminal receiver domain activates

the effector domain of the response regulator in a manner that is still incompletely understood.

Replacement of the conserved aspartate by asparagine in the receiver domain of RocR altered the effector output in that a 16-fold-lower  $K_m$  (corresponding to higher substrate affinity), but also a lowered  $k_{cat}$ , a measure of reaction velocity, was observed. This change in kinetic parameters corresponded with enhanced solvent accessibility of the loop 6 region and the putative dimerization interface. Although the replacement of the conserved aspartate by asparagine in the receiver domain conventionally locks response regulators in an inactive state (14), these results intriguingly showed that specific transmission of an alteration in the receiver domain controls the catalytic activity of the EAL effector domain. Importantly, these results indicated that loop 6 plays a role in signal transduction.

**Restoration of loop 6 recovers the catalytic activity of an EAL domain.** The recently solved structure of the EAL domain protein YkuI in complex with c-di-GMP (12) provides additional experimental evidence for the impact of loop 6 on functionality. Although YkuI contains the glutamate serving as the general base catalyst (Fig. 2), it does not show c-di-GMP hydrolyzing activity. Loop 6 has degenerated in YkuI, and the positioning of key residues is not favorable for the successful performance of catalytic activity.

YkuI harbors class 2 EAL domains (Fig. 2, as discussed below) (16) with conserved catalytic residues, but degenerated loop 6. Intriguingly, Rao et al. were able to demonstrate re-

#### Class 1 EAL domain

RocR	161	QP	175	<u>EV</u> VLAR	233	<u>N</u>	265	<u>E</u> ITE	295	<u>DD</u> FGAGYSS	316	<u>K</u> LDRTF	352	<u>E</u> GVE	372	QG
PDEA1		QP		EALSR		N		EITE		DDFGTYSS		KIDRSF		EGVE		QG
DOS		QP		EALAR		N		EITE		DDFGTFSG		KIDKSF		EGVE		QG
VieA		QP		EALVR		N		EMTE		DDFGTYAS		KIDRSF		EGVE		QG
PdeA		QP		EALAR		N		EVTE		DDFGTFSS		KIDRTF		EGVE		QG
YciR		QP		EALVR		N		ELTE		DDFGTYSS		KLDQAF		EGVE		QG
BlrP1		QA		EALIR		N		EVTE		DDFGAGYSG		KVDAEL		ETLE		QG

#### Class 2 EAL domain

									<u>DD</u> FGTYTV							
DGC2		QP		EALLR		N		EVTE		DNFGKITV		KIDQSM		EGVE		QG
YkuI		QA		EVLGR		F		EITE		DNIGKESSN		KIDLQA		EDIE		QG
YahA		QP		EVLVR		L		ELTE		DDFGTYAT		KIDKSF		EGVE		QG
YhjH		ER		ELLTV		N		ELVE		DDFGTGMAN		KVAREL		EGVE		QG

#### Class 3 EAL domain

YcgF		SP		DPLSR		N		EFTE		DHFGAGFAG		KISQEL		MGVA		QG
YdiV		LP		EIIAT		N		AINE		ANFGAGEAS		MLDKNF		AGID		QG
LapD		QP		YKVLS		N		EIGE		QRFGGRFSM		KIDGSY		ERVE		QG
CsrD		KP		EIRMC		Q		ELAE		NQAGLTLVS		KLHPGL		TGVR		QG

FIG. 2. Alignment of signature motifs of functionally characterized class 1, 2, and 3 EAL domains. The proteins with class 1 EAL domains displayed showed c-di-GMP-specific PDE activity in vitro (1, 3, 17, 23, 30, 33). YhjH and YahA, which have class 2 EAL domains, display c-di-GMP-specific PDE activity (15, 23, 26), while this activity is lacking in YkuI and DGC2 (12, 28). Proteins with class 3 EAL domains lack c-di-GMP-specific PDE activity (13, 26, 27, 32). LapD, however, binds c-di-GMP (13). For further explanations, see text. RocR was used as a model to investigate the role of conserved residues on catalytic activity (17). Amino acids involved in  $Mg^{2+}$  binding are shown in green (light green, direct binding; dark green, indirectly involved); amino acids involved in substrate binding are shown in blue. The glutamate serving as the general base catalyst is shown in red, and the glutamate stabilizing loop 6 is shown in pink. The exchange of underlined amino acids by alanine led to  $>10^5$ -fold reduction in catalytic activity in RocR (17). Amino acids on gray background were mutagenized to study the role of loop 6 in RocR (16). Exchange of three amino acids (dark red and underlined in the sequence above the original loop 6 sequence) in loop 6 of the EAL domain of DGC2 restored the c-di-GMP-specific PDE activity (17). Underlined amino acids in LapD were demonstrated to participate in c-di-GMP binding (13).

verse evolution of a catalytically nonfunctional class 2 EAL domain back to a catalytically functional protein. The diguanylate cyclase 2 (DGC2) of *G. xylinus* is a GGDEF-EAL domain protein (28). As in YkuI, the EAL domain of DGC2 contains the Mg<sup>2+</sup>-coordinating residues and the general base catalyst; however, PDE activity was undetectable in full-length DGC2 and the stand-alone EAL domain. Through mutagenesis of three amino acids in the degenerated loop 6 (Fig. 2), however, Rao et al. could recover the catalytic activity of the stand-alone EAL domain of DGC2. In summary, loop 6 plays a determinative role in oligomerization of subunits, c-di-GMP binding, and catalysis.

**A classification scheme for EAL domains.** On the basis of their results, Rao et al. categorized the EAL domains in the database in three classes and correlated sequence conservation with the proposed enzymatic activity (16, 17). EAL domains belonging to class 1 possess conserved catalytic residues and a conserved loop 6. Class 1 EALs function as PDEs (4, 17, 23, 24, 30, 33) (see Fig. 2 for examples). Class 2 EAL domains contain conserved catalytic residues and a degenerated loop 6 (present, for example, in YkuI and DGC2 [Fig. 2]). These EAL domains are most likely catalytically inactive, but the potential to be activated by terminal signaling domains cannot be excluded. Class 3 EAL domains lack one or more of the catalytic residues, have a degenerated loop 6, and are predicted to be catalytically inactive. The recently characterized proteins CsrD and YcgF from *E. coli*, STM1344 (YdiV) from *S. Typhimurium*, and LapD from *Pseudomonas fluorescens* harbor EAL domains which belong to this class (13, 26, 27, 32).

**A request for an EAL domain structure supporting suboptimal catalytic activity.** How general is the impact of loop 6 for EAL domain functionality? Does a dependence on the sequence context exist? Lack of catalytic activity of BlrP1, a PDE with an N-terminal BLUF domain from *Klebsiella pneumoniae* (distinct from the BLUF-EAL domain protein YcgF) upon mutagenesis of loop 6 residues supports a general role for loop 6 (1). On the other hand, the stand-alone EAL domain protein YhjH, which is an effective PDE (15, 25, 26), contains a degenerated loop 6. In addition, YhjH lacks several amino acids demonstrated to be involved in substrate binding in RocR (Fig. 2).

The catalytic activity of EAL domains can be readily detected without activation (23, 30). Assuming efficient multi-layer control of second messenger signaling, however, most likely many EAL domain proteins are designed for suboptimal function and require a signal input that triggers a physiologically relevant activity (4). Further experimentation is required to characterize the catalytic activities of EAL domains in detail and to define amino acids required for high catalytic activity of EAL domains.

**Analysis of substrate binding specificity of EAL domains.** The studies of Rao et al. (16, 17) also identified sequence and structural requirements for substrate binding. These results provide the basis to rationalize substrate binding of other EAL domains. YhjH with an degenerated loop 6 and missing several amino acids contributing to c-di-GMP binding in RocR still binds and processes c-di-GMP (Fig. 2) (15, 25). As YhjH is a highly effective PDE, it can be speculated that alternative amino acids lead to a more efficient binding and turnover of c-di-GMP. In contrast, YdiV (STM1344), which shows high

overall similarity to YhjH, does not even bind c-di-GMP (Fig. 2) (26). The behavior of the protein is consistent with a degenerated loop 6 and the lack of most amino acids required for c-di-GMP binding and catalysis, including the base catalyst. Failure to bind c-di-GMP was also reported for the BLUF-EAL domain protein YcgF from *E. coli* (32). To date, LapD from *P. fluorescens* is the only EAL domain protein found to bind c-di-GMP but to lack catalytic activity. At least four amino acids are required for c-di-GMP binding, among them the glutamate serving as the general base catalyst in functionally active PDEs (13) (Fig. 2).

It is interesting to note that the EAL domain protein YkuI originates from *Bacillus subtilis*, an organism that is thought to produce not only the second messenger c-di-GMP but also the putative second messenger c-di-AMP (18, 34). While c-di-AMP-specific PDEs await discovery, it can only be speculated whether a subgroup of EAL domains also bind and turn over c-di-AMP. Promiscuous substrate specificity is observed in certain PDE subclasses in eukaryotes (2).

**Conclusions.** Functional characterization of a protein sequence is one of the most challenging problems in biology. The systematic structure-function studies initiated by Zhao-Xun Liang's research group (16, 17) laid the groundwork for more in-depth analysis. Of particular interest is the evolution of highly similar proteins. For example, the GGDEF-EAL protein CsrD from *E. coli* does not display PDE activity (27). Currently, PDE activity cannot be excluded for PigX, the CsrD ortholog in *Serratia marcescens* (7), while LapD binds c-di-GMP (13). Combined with in vivo studies to analyze the physiological output of sequence changes, these studies will continue to unravel the secrets of c-di-GMP signaling.

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