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Systems-level c-di-GMP Signaling in Shewanella during Adaptation to Fluctuating Environments

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Introduction

Cyclic diguanylate (c-di-GMP) is a ubiquitous bacterial second messenger that has been previously shown to control diverse cellular processes such as the transition between the motile and sessile lifestyle, as well as virulence. Intracellular c-di-GMP levels are controlled by the activities of diguanylate cyclases (DGCs), characterized by the amino acid sequence motif GGDEF, and c-di-GMP-specific phosphodiesterases (PDE), characterized by the amino acid sequence motifs EAL and HD-GYP, respectively. Interestingly, the number of these domain proteins encoded by bacteria is highly variable, with some species having none and some having as many as 100 of these proteins. Although significant progress has been made in understanding how c-di-GMP levels affect bacterial motility and virulence, many questions regarding c-di-GMP-related signaling remain unanswered. For example, what are the targets of c-di-GMP signaling? Given the large number of DGCs and PDEs in some bacterial species, how are their activities coordinated to achieve the appropriate levels of c-di-GMP? Finally, what are the processes by which environmental signals influence c-di-GMP metabolism?

The metal-reducing bacterium, *Shewanella oneidensis*, plays a fundamental role in the geochemical cycling of minerals, and is a potential agent for bioremediation of environmental contaminants. *Previously, we showed that h*igh levels of c-di-GMP lead to formation of greater *S. oneidensis* biofilm biomass, while lower levels of c-di-GMP lead to reduced biofilm formation and dissolution. These intracellular levels of c-di-GMP are controlled by DGCs and c-di-GMP-specific phosphodiesterases. The *S. oneidensis* genome has a relatively large number of genes that encode for proteins containing the GGDEF and EAL domains (54 and 30 genes, respectively). Of these genes, 10 of have both GGDEF and EAL domains, as well as a PAS sensor domain (Table 1). The PAS domains have been previously shown to be involved in monitoring changes in light, oxygen, redox potential, and other small ligands. Given the ability of *S. oneidensis* to utilize a wide range of compounds as terminal electron acceptors for anaerobic respiration and growth, we decided to focus on how these PAS-GGDEF-EAL proteins may affect *S. oneidensis* metabolism, biofilm formation, and motility by utilizing a wide range of genetic and biochemical methods.

Results

Genetic analyses

We have constructed in-frame deletions of 7 of the 10 PAS-GGDEF-EAL proteins in *Shewanella oneidensis* MR-1. Because we are interested in c-di-GMP signaling in response to environmental conditions, we are first focusing on genes that demonstrate differential expression between aerobic and anaerobic growth as determined by microarray analyses conducted by A. Beliaev as part of the Shewanella Federadtion. In addition to differential gene expression, we also chose to focus on the genes located in close proximity to genes involved in energy storage, translation, and respiration.

We are currently in the process of characterizing the deletion mutants under a wide range of growth conditions. These include aerobic growth and survival in stationary phase, as well as anaerobic growth with ferric citrate, fumarate, nitrate, thiosulfate, TMAO, and DMSO as terminal electron acceptors. The anaerobic growth tests are of particular interest because of the possible link between c-di-GMP signaling and respiration suggested by published microarray data. We also plan to compare the motility and biofilm formation of the knockout mutants with those of the wild-type strain.

Some preliminary data is shown in Table 1. Mutants carrying in-frame deletions in the genes SO0341 and SO0427 demonstrate increased doubling times during anaerobic growth with fumarate as the terminal electron acceptor. In addition to the PAS-GGDEF-EAL proteins, we are also examining the growth phenotype of a gene (SO4734) that encodes the PAS-EAL domains but no GGDEF domain. This gene was selected because it is constitutively expressed under aerobic and anaerobic conditions, and is located near the 16S, 23S, and 5S rRNA genes, which suggests a possible role for SO4734 in regulating overall gene expression in *S. oneidensis*.

In addition to characterizing growth, we will also examine the biofilm formation of the deletion mutants. In particular, we are interested in the potential role that SO3084 plays in *S. oneidensis* biofilm formation. SO3084 was identified by us in a transposon mutagenesis screen for gene disruptions resulting in a transition from “rigid” to “creamy” colony morphology. Previously, we studied biofilm attachment and detachment in response to intracellular c-di-GMP levels, which were altered through expression of DGC and PDE genes. During these studies, we isolated rigid variants that arose from biofilm cultures grown under high salt conditions or when an *ΔmxdB* biofilm was grown for an extended period of time. (The *mxdB* gene is part of the *mxd* operon, which is important for biofilm development.) These rigid variants gave rise to colonies that were matte in appearance and “rigid” when prodded with a toothpick. In biofilms, these rigid variant cells were less motile, and this motility did not change in response to altered intracellular c-di-GMP levels. A transposon mutagenesis screen yielded 33 defects in the “rigid” morphology. Of these 33 mutants, 28 of them had a “creamy” or wild-type morphology. Five of these 28 mutants had insertions at SO3084. We are currently in the process of constructing an in-frame deletion of SO3084 in the wild-type and rigid variant strains. Because we have a potential phenotype for SO3084 that can be scored, we hope to better understand how each domain (PAS-GGDEF-EAL) is involved in SO3084’s overall

activity.

Biochemical analyses

In addition to the genetic analyses of the mutants, we are also interested in comparing the activities of the various PAS-GGDEF-EAL proteins to address the following questions:

1) Do these proteins have DGC and/or PDE activity?

2) How does each domain affect the activity of the others?

3) Is there a ligand involved in the activity? If so, which domain directly binds it and what activity is affected by it?

We are purifying C-terminally His_6-tagged PAS-GGDEF-EAL proteins from *E. coli and S. oneidensis* and testing for DGC and PDE activity *in vitro*. DGC and PDE activity are tested using α-^{32}P-labeled-GTP or ^{32}P–c-di-GMP as substrates, separating the reaction products via thin-layered chromatography (TLC), and visualizing them by phosphorimagery. Products are identified based on Rf values. In addition to TLC, LCMS and HPLC analyses are performed to confirm the reactions as well as to determine the kinetic parameters of the proteins. Currently, we have preliminary activity results for SO0427, SO0437, SO1500, SO4734, and possibly SO4552, which are summarized in Table 1. Thus far, almost all the purified constructs from *E. coli* demonstrate PDE activity. SO0427 demonstrates PDE as well as possible DGC activity. No directly observable cofactor or ligand (i.e., heme, flavin, chromophore) has been isolated with the proteins. However, this may be due to the fact that these proteins were purified from *E. coli* and not from *S. oneidensis*. Purifications from *S. oneidensis* are currently in progress.

In addition to characterizing the wild-type PAS-GGDEF-EAL proteins, we are also generating mutants in each of the domains via site-directed mutagenesis to further probe overall protein activity. Once phenotypes for the deletion mutant strains have been identified, we will investigate potential ligands that may directly affect protein activity.

Conclusions

Proteins containing PAS, GGDEF and EAL amino acid sequence motifs may play an important role in regulating c-di-GMP signaling in response to environmental conditions. A genetic and biochemical analysis into the roles of these proteins is underway. PDE activity was observed for several PAS-GGDEF-EAL proteins. One of these proteins, SO0427, also demonstrates possible DGC activity *in vitro*. Currently, we are studying the growth, motility and biofilm formation characteristics of deletion mutants, as well as the activity of the purified proteins. It is our hope that understanding the role of PAS-GGDEF-EAL proteins may help elucidate the complex c-di-GMP signaling system.

Candidate gene	Genetic neighborhood	Conditions when upregulated	Aerobic phenotype of knockout	Anaerobic phenotype of knockout	Predicted activity	Observed activity
SO0141	Molybdopterin synthase, ferritin	Aerobic growth			DGC, PDE	
SO0341	Methyl citrate synthase		Biofilm biomass 20-60% WT	Higher doubling time (125% WT)		
SO0427	Pyruvate dehydrogenase complex	Anaerobic growth	Biofilm biomass 20-70% WT	Higher doubling time (177% WT)	DGC, PDE	Possible DGC, PDE
SO0437	Aconitase, heme-synthesis gene				None (GGDEF, EAL predicted inactive)	PDE
SO1500	Glycogen synthesis genes				DGC, PDE	PDE
SO2216 (not under investigation)						
SO2498 (not under investigation)						
SO3084	M16 family peptidase	Anaerobic growth on $NaNO_3$	Rigid variant reverts to creamy (disruption of gene)		DGC (EAL predicted inactive)	
SO3389 (not under investigation)	ATP-dependent RNA helicase			Higher doubling time (124% WT)		
SO4552					DGC (EAL predicted inactive)	possible PDE
SO4734	Ribosomal 16S, 23S and 5S		Higher doubling time (122% WT), biofilm biomass 120% WT	Higher doubling time (138% WT)		PDE

Figure 1. Characterization of PAS-GGDEF-EAL proteins of S. oneidensis. Data is preliminary.

Publications resulting from this research:

1. Saville RM, Rakshe S, Haagensen JAJ, Shukla S, Spormann AM. 2011. Energy-dependent Stability of *Shewanella oneidensis* MR-1 Biofilms. J. Bacteriol. 193(13):3257-64.

2. Chao L. , Rakshe S., Leff M., and Alfred M. Spormann. 2013. A cyclic di-GMP-specific phosphodiesterase, SarP, that regulates sulfate assimilation in *Shewanella oneidensis* MR-1. submitted

References

1. Galperin, M. Y., A. N. Nikolskaya, and E. V. Koonin. 2001. Novel domains of the prokaryotic two-component signal transduction systems. *FEMS Microbiol Lett* **203**: 11-21.

2. Beliaev, A. S., D. M. Klingeman, J. A. Klappenbach, L. Wu, M. F. Romine, J. M Tiedje, K. H. Nealson, J. K. Fredrickson, and J. Zhou. 2005. Global transcriptome analysis of *Shewanella oneidensis* MR-1 exposed to different terminal electron acceptors. *J Bacteriol* **187**: 7138-45.

3. Tamayo, R., A. Tischler, and A. Camilli. 2005. The EAL domain protein VieA is a cyclic diguanylate phosphodiesterase. *J Biol Chem* **280**: 33324-33330.

4. Thormann, K. M., R. M. Saville, S. Shukla, D. A. Pelletier, and A. M. Spormann. 2004. Initial phases of biofilm formation in *Shewanella oneidensis* MR-1. *J. Bacteriol* **186**: 8096-104.

5. Thormann, K. M., S. Duttler, R. M. Saville, M. Hyodo, S. Shukla,Y. Hayakawa, and A. M. Spormann. 2006. Control of biofilm formation and cellular detachment from *Shewanella oniedensis* MR-1 biofilms by c-di-GMP. *J Bacteriol* **188**: 2681-91.

6. Taylor, B. L., and Igor B. Zhulin. 1999. PAS domains: Internal sensors of oxygen, redox potential, and light. *Microbiol and Mol Bio Reviews* **63**: 479-506.