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Sustained sensing as an emerging principle in second messenger signaling systems

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Abstract

Bacteria utilize a diverse set of nucleotide second messengers to regulate cellular responses by binding macromolecular receptors (RNAs and proteins). Recent studies on cyclic di-GMP (c-di-GMP) have shown that this signaling molecule binds multiple receptors to regulate different steps in the same biological process. We propose this property of the same molecule regulating multiple steps in the same process is biologically meaningful and have termed this phenomenon “sustained sensing”. Here, we discuss the recent findings that support the concept of sustained sensing of c-di-GMP levels and provide additional examples that support the utilization of sustained sensing by other second messengers. Sustained sensing may be widespread in bacteria and provides an additional level of complexity in prokaryotic signal transduction networks.

Introduction

Bacteria utilize signal transduction systems to enact suitable responses to changing environments in order to optimize growth and survival. This is typically a three step process in which the signal is detected, cellular response is triggered, and then the response is terminated to return the system to its initial state. Some of the most widely utilized signal transduction mechanisms in bacteria are the second messenger signaling systems (reviewed in [1]). These signaling systems are found in the majority of bacterial and archaeal species that have completely sequenced genomes (Figure 1). Recently, c-di-GMP, cyclic di-AMP (c-di-AMP) and cyclic-AMP-GMP (cAGMP) have been characterized as new second messengers in bacteria that regulate transcription initiation, post-initiation regulation of the

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mRNA, and allosteric regulation of translated proteins (Figure 2). Here, we discuss the recent observations that c-di-GMP second messenger signaling may regulate the same phenotype at multiple regulatory steps. We introduce the term “sustained sensing” to describe this phenomenon. We describe several examples of sustained sensing, the mechanism and utility of sustained sensing, and discuss whether sustained sensing could be a general principle of secondary signaling molecules. If sustained sensing proves to be conserved, it represents another layer of complexity in second messenger signaling.

Discovery of c-di-GMP, its receptors and their implications for second messenger signaling systems

The cyclic dinucleotide second messenger c-di-GMP was first described in 1987 by the Benziman lab as an allosteric activator of cellulose synthase in *Acetobacter xylinum* (since renamed *Komagataeibacter xylinus*) [2]. Since then, c-di-GMP has been shown to be a widely utilized signaling molecule in diverse bacterial species, generally regulating major lifestyle transitions such as the switch from motile, free-swimming planktonic cells to sessile, biofilm-dwelling forms (see [3] for a comprehensive review of c-di-GMP signaling). C-di-GMP regulates these processes by binding to receptors that include transcription factors [4–8], riboswitches [9,10], and other proteins such as those containing a PilZ domain [11,12] and reviewed in [3,13,14]. For many years, the number of c-di-GMP receptors identified in any given genome was far below the number of c-di-GMP synthetases and hydrolases encoded in the same genome. However, just in the past several years, a variety of new c-di-GMP receptors have been characterized [15–17] that partly resolved that conundrum. Furthermore, recent studies show an emerging pattern in which c-di-GMP appears to regulate the same cellular processes at multiple levels. We have proposed to refer to this phenomenon as “sustained sensing” [15].

Two examples of sustained sensing that have been described for c-di-GMP are regulation of the synthesis of the Pel polysaccharide in *Pseudomonas aeruginosa* and the mannose-sensitive hemagglutinin (MSHA) pilus in *Vibrio cholerae* (Table 1). In the first example, c-di-GMP regulates *P. aeruginosa* Pel polysaccharide by interacting with two protein receptors. The first receptor is FleQ, a transcription factor that enhances transcription of the *pel* operon [4,17]. The second receptor is PelD, which allows Pel polysaccharide synthesis upon binding to c-di-GMP [18]. In the second example, c-di-GMP regulates the MSHA pilus in *V. cholerae* at also at two levels, the transcriptional level and the level of secretion. Elevated levels of c-di-GMP increase transcription of the *msh* operon that encodes proteins responsible for the assembly and function of the MSHA pilus [19]. The second c-di-GMP-dependent regulatory step is its binding to secretory ATPase MshE in order to allow export of the MshA pilus to the cell surface [15,20].

In addition to regulating transcription factors and protein complexes, c-di-GMP regulates RNA through direct binding to at least two types of riboswitches [9,10]. In many cases, these riboswitches are upstream of operons known to be regulated by c-di-GMP, such as those encoding flagella, which are negatively regulated by c-di-GMP binding, and type IV pili, which are positively regulated by c-di-GMP binding in *C. difficile* [10,21]. Interestingly,

the PilB1 (CD3512) protein encoded in the operon downstream of the c-di-GMP-responsive riboswitch in *C. difficile* is an MshE homolog (36.3 % identity) that also contains the R9 and Q32 residues that are important for c-di-GMP binding in MshE [15]. If PilB1 indeed binds c-di-GMP in a manner similar to MshE, type IV pili biogenesis in *C. difficile* would serve as another example of c-di-GMP sustained sensing. In contrast to *C. difficile*, *Clostridium perfringens* has a similar *pil* operon with an apparent c-di-GMP binding MshE-like ATPase (CPF_2570) that, however, is not preceded by a c-di-GMP riboswitch. Therefore, in *C. perfringens*, c-di-GMP appears to regulate formation of type IV pili solely at the level of secretion, i.e. not to involve sustained sensing in regulation of this system. This comparison suggests that recombination of these regulatory elements may aid organisms to adapt their regulatory systems for their unique niches.

Mechanism of sustained sensing

Sustained sensing occurs when two c-di-GMP receptors are involved in regulating the same process. If the receptors for mRNA regulation and post-translational allosteric regulation have different relative affinities, it could result in different regulatory mechanisms playing the key role under certain conditions. There are three theoretically possible scenarios: 1. the affinity (the inverse of dissociation constant (K_d)) of receptor 1 is lower than receptor 2 ($K_{d1} > K_{d2}$), 2. the affinity of receptor 1 is higher than receptor 2 ($K_{d1} < K_{d2}$) and 3. the affinity for both receptors are the same ($K_{d1} = K_{d2}$) (Figure 3). The different combinations of relative affinities of the two regulated steps permit additional possibilities of c-di-GMP regulation beyond a simple on and off switch.

In scenario 1 for positive regulation, at concentrations of c-di-GMP substantially below K_{d1} and K_{d2} , the regulated process would not be activated. At concentrations between K_{d1} and K_{d2} , two outputs are possible when a cell begins without any c-di-GMP. If the transcriptional and/or translational regulation is tightly regulated by c-di-GMP, these concentrations of c-di-GMP would have little regulatory effect as receptor 2 is not yet present in the cell. However, if transcriptional and/or translational regulation is leaky, then these c-di-GMP concentrations will activate any receptor 2 that is made from the leaky expression leading to an intermediate phenotypic output. A different outcome is possible when the cell starts with higher concentrations of c-di-GMP and drops to a concentration between K_{d1} and K_{d2} . In this case, c-di-GMP will continue to activate previously synthesized molecular machinery to sustain the phenotypic output. The last possibility for scenario 1 is when c-di-GMP concentrations exceed K_{d2} . At these concentrations, both receptors will be activated resulting in maximal transcription/translation and phenotypic output. In scenario 1, the bacterium can not only gauge the c-di-GMP concentration, but also increases or decreases of the current c-di-GMP concentration relative to the previous concentration will have regulatory consequences.

In the second scenario, receptor 1 has a lower K_d than receptor 2 for c-di-GMP. For concentrations of c-di-GMP below K_{d1} and K_{d2} , the regulated process should not be activated in a manner similar to scenario 1. Increasing c-di-GMP to concentrations between K_{d1} and K_{d2} activates transcription/translation to allow expression of the proteins that compose the molecular machinery, but there is not enough c-di-GMP present to fully drive the allosteric activation of the protein receptor. As a consequence, the phenotypic output

remains unchanged. Once c-di-GMP exceeds K_{d2} , the bacterium is already primed to fully engage in producing the phenotype. This allows a rapid burst of phenotypic output when c-di-GMP exceeds K_{d2} . For scenario 2, a decrease of c-di-GMP concentration from above K_{d2} to between K_{d1} and K_{d2} inactivates the molecular machinery while more proteins are produced. This allows the bacterium to be primed to quickly return to expressing the phenotype upon returning to environmental conditions that trigger high levels of c-di-GMP. In scenario 2, the directionality of the change in the concentration of the signaling molecule has little impact in the phenotypic outcome, i.e. when the concentration of c-di-GMP is between K_{d1} and K_{d2} , the previous c-di-GMP concentration, whether high or low, does not alter the biological output.

In the third scenario, if the time required for transcription/translation and complex assembly is substantial, then the c-di-GMP concentration can be reassessed over time to ensure that it remains elevated. In cases where the c-di-GMP levels drop, the bacterium will be nonetheless primed with assembled protein complexes that allow for a faster response to subsequent elevated levels of c-di-GMP. Otherwise, there is no particular benefit of having two regulatory levels over just a single regulatory event. For processes in which the c-di-GMP negatively regulates both receptors, the outcomes described will be in reverse. Together, the three categories of sustained signaling based on the relative affinities of the two receptors allows for many additional types of biological regulation.

In the known example of sustained sensing for Pel polysaccharide biosynthesis in *P. aeruginosa*, the K_d of FleQ and PelD for c-di-GMP is $\sim 7 \mu\text{M}$ [22] and ~ 0.5 to $2 \mu\text{M}$ [18,23,24], respectively. The two K_d s indicate that c-di-GMP regulation of Pel polysaccharide operates as category 1 of sustained sensing. The *pel* operon is expressed at a baseline level in the absence of c-di-GMP induction, suggesting that the PelD protein is present in the cell under low c-di-GMP concentrations. At concentrations below the K_d of PelD for c-di-GMP, there should be little production of the Pel polysaccharide and baseline levels of *pel* transcription. At c-di-GMP concentrations between the K_d for PelD and FleQ, the cell will produce Pel polysaccharide, but *pel* transcripts will remain at baseline levels. When c-di-GMP concentrations exceed the K_d for FleQ, then the bacteria will produce more PelA-G proteins and induce them to synthesize copious amounts of Pel polysaccharide. When c-di-GMP levels drop below what is required to activate FleQ, the bacteria will continue to produce Pel through activation of PelD. Despite not generating new proteins, the bacteria can nonetheless continue to make Pel polysaccharide and sustain their participation in the biofilm. This level of Pel polysaccharide will eventually drop to the baseline level through protein turnover.

Advantages of sustained sensing

The regulatory advantages of sustained sensing can be considered by the phenotypes that are regulated. C-di-GMP regulates numerous processes that involve lifestyle decisions with large metabolic costs such as the formation of multicellular biofilms or the development of the motility apparatus. The commitment to a sessile lifestyle demands a high cost in cellular resources and the opportunity cost of forgoing the planktonic lifestyle. While the biofilm lifestyle provides advantages under some conditions, it is well known that bacteria also have

a process that allows departure from the biofilm [25–27]. Therefore bacteria need a way to determine their commitment level to a specific lifestyle. For motility, flagella biosynthesis and rotation requires a commitment of cellular resources [28,29]. Sustained sensing as presented in scenario 1 provides the bacteria with a mechanism to continually gauge their enthusiasm for a particular lifestyle. By repeatedly measuring the level of the same signaling molecule, the bacteria can move their level of enthusiasm for a behavioral change from nonexistent to medium to high as conditions increase in favorability. When the favorability is diminished, the bacteria can reduce their enthusiasm. In this case, they can continue to participate in the process with residual production through activation of already assembled protein complexes, but also be ready for a lifestyle change if and when the existing protein complexes are degraded. Within the heterogeneous microenvironments of the biofilm, each individual can continually assess their enthusiasm for participation in the biofilm. Thus, sustained sensing also allows bacterial cells to behave heterogeneously in the population. This property is different from bistable switches [30,31] through positive feedback loops in that the behavior of bacterial cells is gradated as determined by the affinity of each of the receptors.

In addition to gauging enthusiasm, sustained sensing also provides the bacterial cells a mechanism to prepare themselves for a lifestyle change as described in scenario 2. In cases where the bacteria can sense a need for an impending rapid response, the bacteria can use low concentrations of secondary signaling molecules to produce proteins that remain inactive due to insufficient concentrations of the signaling molecule. These cells will be prepared for subsequent increase in the signaling molecule, which is indicative of changes in the conditions that require protein function. Since these cells already have the proteins synthesized, their response time will be greatly reduced by bypassing the need for *de novo* transcription and translation. Sustained sensing of the same signaling molecule over time allows bacteria to gauge enthusiasm and to prepare for future conditions that require rapid response.

To test sustained sensing, one option is to constitutively express the c-di-GMP regulated promoters. However, this will only allow testing one part of the sustained sensing mechanism. Ideally, constitutive alleles of both receptors that mimic the c-di-GMP bound form would need to be generated. Competition experiments between the wild type parental strain and the isogenic mutant with the constitutive allele that mimics c-di-GMP binding will reveal whether there are advantages to sustained sensing. Preferably, these constitutive alleles can be generated for both receptors. Currently, alleles that alter c-di-GMP signaling lack the ability to bind c-di-GMP and therefore act as constitutively unbound forms of the receptor. These alleles have taught us the importance of c-di-GMP binding to various regulated pathways. However, they are not suitable for experiments designed to test the concept of sustained sensing.

Implications of sustained sensing for identification of additional c-di-GMP receptors

If the concept of sustained sensing holds true, it could enable prediction of additional c-di-GMP receptors. Processes that are known to be regulated by c-di-GMP at the transcriptional and/or translational level may also be regulated by some c-di-GMP binding proteins at the post-translational level. Conversely, processes that are known to be allosterically governed by c-di-GMP receptor proteins may also have transcriptional/translational c-di-GMP regulation. Examples of genes that encode biosynthetic operons that are regulated by c-di-GMP-binding transcription factors include the *psl* operon in *P. aeruginosa* via FleQ [4], the *vps* operon in *V. cholerae* via VpsT and VpsR [5,19,32], the *xag* operon in *X. campestris* via Clp [7,33], *Bcam1330-Bcam1341* operon in *Burkholderia cenocepacia* via Bcam1349 [8] and *E. coli* curli [34–37]. Examples of processes that are regulated allosterically by c-di-GMP binding include cellulose synthesis via binding to BcsA [2,12,38,39] and BcsE in *S. Typhimurium* [40], alginate production via binding to Alg44 in *P. aeruginosa* [41,42] and cell adhesion via binding to both GcbC and LapD in *P. fluorescens* [43–45]. While not all of these systems necessarily have additional c-di-GMP dependent regulatory steps (see for example the difference for type IV pili regulation between *C. difficile* and *C. perfringens*), if additional c-di-GMP regulation for any of the above systems is identified, sustained sensing will emerge as larger theme in c-di-GMP signaling. Thus, sustained sensing in the regulation of these phenotypes would be beneficial to the bacterium as it would allow repeated sampling to the environment prior to committing to a major change in behavior.

Is sustained sensing a general feature of secondary signaling nucleotides?

In addition to c-di-GMP, other second messengers may utilize sustained sensing. C-di-AMP was first described in 2008 in *Bacillus subtilis* as a regulator of DNA checkpoint control [46]. Since that initial report, c-di-AMP has also been shown to act as a bacterial second messenger that regulates such processes as reporting DNA integrity [47], cell envelope stress [48,49] and potassium homeostasis [50] (see review [51]). Due to its recent discovery, few binding partners for c-di-AMP have been reported. Two unbiased binding studies in *Staphylococcus aureus* [50] and *Listeria monocytogenes* [52], as well as targeted studies have revealed several c-di-AMP binding proteins: the transcription factor DarR in *Mycobacterium smegmatis* [53], pyruvate carboxylase PycA and proteins of unknown function CbpA (Lmo0553) and CbpB (Lmo1009) in *L. monocytogenes* [52], a PII-like protein PstA in *S. aureus* [52] and *L. monocytogenes* [52] (called DarA in *B. subtilis* [54]), a cation/proton antiporter CpaA, sensor histidine kinase KdpD, and the potassium transporter KtrA in *S. aureus* [55,56]. In addition to binding proteins, a c-di-AMP responsive riboswitch (*ydaO*) [57] has also been identified upstream of operons containing KtrA homologs (COG0569), the *kdp* operon that contains the KdpD homolog, and a PstA homolog. The presence of the c-di-AMP-sensing riboswitch upstream of genes encoding putative c-di-AMP binding proteins suggests that sustained sensing may also be a property of c-di-AMP signaling.

Even more recently, a cAGMP has been shown to regulate *V. cholerae* virulence [58] and Geobacter electron transfer [59,60]. Furthermore, a cAGMP riboswitch has been identified [59–61]. Whether cAGMP is also subject to sustained sensing will become clear after cAGMP receptors and regulated phenotypes are better characterized.

Conclusions

Here we present the concept of sustained sensing as a property of second messenger signaling in bacteria. This feature allows bacteria to utilize the same signaling molecule to control multiple steps in the same regulated process, allowing them to gauge their enthusiasm for rapidly respond to environmental cues using previously synthesized proteins. Sustained sensing also allows an individual bacterium within a biofilm to sense changing microenvironment resulting from the spatial heterogeneity within the biofilm and thereby allows individual cells to decide whether to continue to participate in the biofilm community or depart from it. Sustained sensing provides another potential level of regulatory complexity in bacterial signaling.

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Highlights

- Sustained sensing is proposed as a regulatory mechanism used by bacterial cyclic dinucleotides
- Sustained sensing uses the same signaling molecule to regulate multiple receptors at distinct steps
- Sustained sensing allows bacteria to make decisions over time that require commitment of cellular resources
- This mechanism adds another layer of complexity in second messenger signaling regulation

Figure 1.

A. Co-occurrence of two-component systems, c-di-GMP-mediated (yellow) and/or c-di-AMP-mediated (red) regulation in 628 bacterial genera, covered by the recent version of the COG database [62]. There are 90 genera with HisK only (green), 183 with HisK and c-di-GMP (yellow), 195 with HisK and c-di-AMP (red), and 129 with all three signaling systems (orange). There are 10 genera that have only c-di-AMP (pink) and 21 genera that lack all three signaling systems (gray). **B.** Co-occurrence of two-component systems and c-di-AMP-mediated regulation in 83 archaeal genera, covered by the recent version of the COG database. There are 6 genera with HisK only (green), 38 with both HisK and c-di-AMP (orange), 4 with c-di-AMP only (red). There are 35 genera that lack HisK and c-di-AMP signaling systems (gray). Archaea do not encode genes with COGs related to c-di-GMP signaling.

Figure 2.

Schematic of the processes regulated by c-di-GMP signaling. Signals can activate diguanylate cyclase to synthesize c-di-GMP, which then acts on transcription, transcript termination and/or translation, and post-translation protein activity.

Scenario	Affinities	[c-di-GMP]	Increasing c-di-GMP production			Decreasing c-di-GMP concentration		
			Receptor 1	Receptor 2	Phenotype	Receptor 1	Receptor 2	Phenotype
1	$K_{d1} > K_{d2}$	High ($>K_{d1}$)	ON	ON	++	ON	ON	++
		Middle (Between K_{d1} and K_{d2})	OFF	No protein	-	OFF	ON	++
		Low ($<K_{d2}$)	OFF	OFF	-	OFF	OFF	-
2	$K_{d1} < K_{d2}$	High ($>K_{d2}$)	ON	ON	++	ON	ON	++
		Middle (Between K_{d1} and K_{d2})	ON	OFF	-	ON	OFF	-
		Low ($<K_{d1}$)	OFF	OFF	-	OFF	OFF	-
3	$K_{d1} = K_{d2}$	High ($>K_{d1}$ and K_{d2})	ON	ON	+	ON	ON	+
		Low ($<K_{d1}$ and K_{d2})	OFF	OFF	-	OFF	OFF	-

Figure 3.

Schematic of possible combinations of affinities for receptor 1 and receptor 2 to c-di-GMP. Shown are the possible combinations of relative dissociation constants of receptor 1 (K_{d1}) and receptor 2 (K_{d2}), the levels of c-di-GMP (black triangle), and the resulting phenotypic output for positive regulation by c-di-GMP.

Table 1

Examples of c-di-GMP-mediated sustained sensing

Regulated system & organism	Receptor 1, K_d^a	Receptor 2, K_d^a	Reference
Pel polysaccharide, <i>Pseudomonas aeruginosa</i>	FleQ, 7 μ M	PelD, 0.5–1.9 μ M	[22] [18] [23] [24]
MshA pili, <i>Vibrio cholerae</i>	?	MshE, 1.9 μ M	[19] [15]
Biofilm dispersal <i>Pseudomonas aeruginosa</i>	FleQ, 7 μ M	? (BdlA cleavage) ^b	[22] [63] [64]
Type IV pili <i>Clostridium difficile</i>	type II c-di-GMP riboswitch	PilB1	[21] [15]
Surface adhesin CD2831 <i>Clostridium difficile</i>	type II c-di-GMP riboswitch	type I c-di-GMP riboswitch	[65]

^a Affinity values (dissociation constants, K_d) are listed where known^b BdlA does not seem to bind c-di-GMP by itself but its cleavage is regulated by c-di-GMP [64]