Nucleotide second messengers in bacterial decision making
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Since the initial discovery of bacterial nucleotide second messengers (NSMs), we have made huge progress towards understanding these complex signalling networks. Many NSM networks contain dozens of metabolic enzymes and binding targets, whose activity is tightly controlled at every regulatory level. They function as global regulators and in specific signalling circuits, controlling multiple aspects of bacterial behaviour and development. Despite these advances there is much still to discover, with current research focussing on the molecular mechanisms of signalling circuits, the role of the environment in controlling NSM pathways and attempts to understand signalling at the whole cell/community level. Here we examine recent developments in the NSM signalling field and discuss their implications for understanding this important driver of microbial behaviour.

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Introduction
Models for bacterial nucleotide second messenger (NSM) signalling have evolved substantially since the discovery of cAMP catabolite repression in the late 1960s [1]. The first pathways discovered, for example, cAMP signalling [1] and the stringent response [2], typically contained few discrete NSM metabolic proteins. However, the advent of whole genome sequencing revealed that bacterial NSM signalling is substantially more complex than previously thought. This has led to the discovery of more NSMs, such as cyclic-di-GMP (cdG, [3]), cyclic-di-AMP (cdA, [4]) and recently cyclic-GAMP [5], which have been shown to control a wide variety of bacterial behaviours. Furthermore, many NSM pathways have been shown to contain far more metabolic enzymes than were initially suspected, with dozens of cdG cyclases and phosphodiesterases identified in many bacterial species [6]. Finally, the complexity and variability of NSM binding domains have become more apparent, with NSMs shown to bind specifically to diverse protein and RNA folds, defying straightforward bioinformatic prediction [7].

To explain this remarkable complexity, sophisticated models for NSM networks have been developed that incorporate several key findings. NSM signalling proteins function under tight temporal and spatial control, with NSM synthases and degradative enzymes controlled at multiple regulatory levels in response to the cell cycle and the surrounding environment [7,8]. Discrete signalling circuits, where specific NSM enzymes control a small number of downstream proteins have been identified, often accompanied by direct protein–protein interaction between signalling enzymes and NSM effectors [7,8]. Related to this, unconventional enzyme functions have been discovered that explain certain specific NSM responses, for example, trigger enzymes, whose enzyme activity alters their interaction with an effector protein [9]. Supported by bioinformatic and computational analyses, comprehensive models have been built for several NSM networks, particularly cdG signalling in Caulobacter crescentus [10] and Escherichia coli [11,12].

NSM-binding effector proteins
Building on their initial characterisation as controllers of the stringent response (tp)ppGpp, [2]), the motile-sessile transition (cdG, [13]) and potassium/glutamate homeostasis (cdA, [14]) NSM signalling molecules have been implicated in the control of a diverse range of bacterial processes. In many cases these are quite different to the initial NSM regulon, for example, decreased cdA levels are implicated in eDNA release during Staphylococcus aureus biofilm formation [15], while cdG controls Sinorhizobium meliloti peptidoglycan biosynthesis [16]. The Streptomyces life cycle is also controlled by σ-factor binding to cdG [17]. Given the inherently leaky nature of second messenger signal transduction, a major factor in shaping this expanded NSM regulon has been the development of reliable biochemical methods to identify and characterise NSM binding proteins [18,19]. For example, Perez-Mendoza et al. used surface plasmon resonance to show that S. meliloti β-glucan synthesis is controlled by cdG binding to the BgsA synthase subunit [20]. The absence of an obvious NSM binding fold suggests that BgsA contains a novel binding motif.
The cdG-binding transcriptional regulator BrlR mediates the correlation between biofilm formation and antibiotic tolerance in *Pseudomonas aeruginosa*. Intriguingly, BrlR was also recently shown to bind to a second intracellular regulatory molecule: pyocyanin, a phenazine [21*]. This finding, alongside other recent work [22] identify mechanisms for regulatory crosstalk between cdG and phenazine signalling, more closely integrating processes such as redox adaptation and antibiotic tolerance with biofilm formation and motility control. This dual regulatory mechanism is reminiscent of the master regulator FleQ, which responds to both cdG and ATP levels and integrates biofilm formation with flagella-driven motility [23]. This raises the prospect of finding other multiple-input regulators that integrate input signals from discrete signalling pathways.

Alongside the identification of new NSM binding proteins, recent biochemical and structural biology research has led to significant advances in our mechanistic understanding of NSM regulatory pathways. For example, Anderson et al. showed how (p)ppGpp inhibits the purine salvage enzyme HPRT by binding a conserved motif with its substrate phosphoribosyl pyrophosphate (PRPP), and used their findings to propose a more general mechanism for the evolution of ligand binding sites [24]. Wood et al. [25] conducted an analysis of Era, a GTPase involved in *S. aureus* ribosomal assembly and showed it interacted with the DEAD-box helicase CshA and the (p)ppGpp synthase RelSaur. Interaction with the latter was shown to boost Era GTPase activity, impacting its function as a hub protein for enzymes involved in ribosomal assembly and rRNA processing/degredation [25].

Two new studies from the Sondermann lab examined previously identified NSM regulatory systems; the LapD cdG-binding adhesin regulator and the Orn di-ribonucleotidease, which degrades linearised cyclic di-nucleotides [26,27]. In both cases, these studies refined our understanding of these proteins and uncovered new insights into the molecular mechanisms of NSM signalling. Orn was shown to possess a unique specificity for di-ribonucleotides in contrast to other cellular ribonucleotidases [26]. Meanwhile, LapD binding was shown to strongly enhance the DGC activity of its cognate cyclase, GebC [27], suggesting a mechanism for the establishment of signal specificity, a longstanding problem in the field of NSM regulation.

**Advances in our understanding of NSM input signals**

While our understanding of the diversity and mechanisms underlying NSM-effector binding has rapidly advanced, comparatively few of the direct sensory inputs controlling NSM metabolism have been identified. The low number of unambiguously identified NSM input proteins stems from the pleiotropic, leaky nature of NSM signalling, making it difficult to interpret overexpression or deletion studies with confidence. Those direct sensors that have been identified often have predictable input domains, for example, for phosphorylation [28] or light-sensing [29], or have been identified following detailed structural analysis, for example, the DgcZ zinc-binding diguanylate cyclase [30], or the redox/L-tryptophan/vitamin B6-regulated YbBNR system [31,32]. Nonetheless, in recent years several new input signals have been attributed to specific NSM proteins, including a novel class of phosphodiestersases (PDEs) identified in *E. coli* [33]. These PDEs contain an N-terminal periplasmic CSS domain with two highly conserved cysteine residues, and are controlled by a cysteine disulphide REDOX switch. The presence of a cystine cross bridge inhibits the cytoplasmic EAL (PDE) domain, while disulphide bond reduction is associated with increased PDE activity [33].

The transmembrane GGDEF-EAL hybrid protein RmcA from *P. aeruginosa* is another important NSM signalling protein whose inputs have recently been discovered [22,34]. RmcA controls colony morphology in response to changing redox conditions, which are transmitted through phenazine binding to one of four cytosolic PAS domains leading to PDE stimulation under oxidising conditions. Intriguingly, RmcA also has been implicated in direct sensing of the amino-acid L-arginine [34] that binds to its periplasmic Venus Flytrap domain and again stimulates PDE activity. This dual-input mechanism is reminiscent of the BrlR pyocyanin/cdG binding regulator [21*], which also contributes to phenazine-linked regulation. Going forward, it will be interesting to determine how many more NSM signalling pathways are controlled by proteins with multiple signal inputs, both at the NSM-binding and metabolic levels.

**Molecular characterisation of NSM signalling pathways**

The identification and characterisation of individual NSM proteins has been accompanied by detailed analysis of several well-known NSM-controlled phenotypes. Recent studies have progressed beyond simple community-level phenotyping to define the underlying molecular mechanisms of NSM control. An excellent example of this is initial surface attachment in *P. aeruginosa* and *C. crescentus*. Recent work implicates the flagellar motor proteins MotA and MotB (MotAB) in initial surface detection by both species [35,36]. In *C. crescentus*, the initial chemotaxis input comes from the Cle family of chemotaxis proteins, where cdG-binding promotes interaction with the MotAB complex [35]. This interaction in *C. crescentus* re-orientates the cell [13] and increases the tactile response initiated by surface proximity [13,36]. A similar feedback system that triggers the initial cascade of surface attachment is also likely to exist in other bacteria, with recent work showing that MotAB are essential for the switch from flagellar tethering to surface attachment in *P. aeruginosa* [37].
Once activated, the flagellar motor complex triggers an increase in cellular cdG levels through the upregulation of DGCs [13,36,38,39**]. *C. crescentus* has a specialised flagellar motor-coupled DGC, DgcB, which is specifically upregulated during surface sensing. Meanwhile, several DGCs are implicated in the case of *P. aeruginosa* surface attachment [13,36,39**]. The increase in cellular cdG leads to an upregulation of type IV pili (T4P) and adherence of the cell to the surface (illustrated in Figure 1) [13,39**]. In *P. aeruginosa*, the synthesised cdG specifically binds to FimW, causing it to localise to the cell poles and induce multiple T4P to adhere to the surface [39**]. However, in *C. crescentus* the high levels of cdG bind to the protein HfS, which is essential for the biogenesis of the holdfast, the retraction of T4P and consequently to irreversible surface adherence [36]. The commitment to surface attachment in *P. aeruginosa* has been shown to lead to asymmetric division driven by different cdG levels in mother and daughter cells, ultimately leading to a mixed population of surface attached and motile cells reminiscent of the lifecycle of *C. crescentus* [39**].

**Complex microbial communities and NSM signalling networks**

Much of our knowledge of NSM signalling has been developed for pathways isolated from the wider bacterial signalling network, and for model organisms growing in defined laboratory conditions. Refining our current models to incorporate crosstalk with other NSM networks, and using these models to explain bacterial responses to complex, dynamic environments remain major challenges in molecular microbiology. It is easy (and often practical) to assume that bacterial gene expression is reasonably uniform throughout a clonal bacterial colony. However, work over the last decade has shown that this is generally not the case. Spatial and temporal diversity within colonies and biofilms has been shown for species including *P. aeruginosa* and *E. coli* [12,40], suggesting in turn that NSM signalling is a major driver of stratification and diversification within bacterial communities.

Biofilm matrix heterogeneity in *E. coli* is generated by a cdG-dependent network characterised by nested positive and negative feedback loops [12]. The transcription factor CsgD is a key regulator in this complex signalling cascade, controlling localised expression of key cdG enzymes [41]. The cascade begins when two DGC/PDE pairs; PdeH/DgcE and DgcM/PdeR activate the transcription factor MlrA, which in turn drives expression of CsgD. Another input to biofilm architecture comes from the PDE PdeC [33], whose activity is linked to oxygen levels that decrease along a steep vertical gradient within the biofilm. Microaerobic conditions deep within biofilms promote the free-thiol form of the PdeC periplasmic domain, activating the enzyme and decreasing intracellular cdG levels, and suppressing EPS production [33].

*Pseudomonas* spp. have particularly complex cdG-signalling networks, with dozens of metabolic enzymes and binding targets. Recently, several groups have used high-throughput analysis methods to define the functions and environmental responses of different *Pseudomonas* spp. cdG networks [42*,43,44]. Dahlstrom et al. [42*] investigated the role of a large number of *Pseudomonas fluorescens* cdG-signalling proteins under multiple nutrient conditions. This work uncovered a large scale multimodal network...
of PDEs and DGCs that were able to respond to a variety of signals, giving rise to a complex, context-dependent regulatory network based on a combination of ligand-binding, protein–protein interaction, and transcriptional regulation to fine-tune cdG outputs and tightly control biofilm formation [42]. Nie et al. recently conducted a similar study in Pseudomonas putida. By screening the phenotypes associated with deletion/overexpression mutants in 42 predicted cdG-metabolic genes, the authors assigned preliminary roles of each gene in colony morphology, biofilm formation and swimming motility [43]. These papers show that the roles of individual PDEs and DGCs play in bacterial behaviour are dynamic, with individual proteins responding to a diverse set of external stimuli and producing specific phenotypic responses. These studies go some way towards answering the question: ‘why do bacteria encode so many NSM metabolic enzymes?’.

Finally, Little et al. investigated the functions of a large group of P. fluorescens PDEs and DGCs during plant colonisation. They showed that cdG-gene upregulation was not uniform during root colonisation, nor did patterns of gene regulation group exclusively by enzyme family [44]. These data suggest that different groups of NSM enzymes are required at different stages of the root colonisation process, likely due to differing environmental stimuli (illustrated in Figure 2). This study was one of a series of recently published papers examining different aspects of cdG-signalling during bacterial plant colonisation [44–48]. While there is still a great deal of work needed to fully understand how NSM signalling functions during complex host–microbe interactions, the past few years have seen encouraging steps in this direction.

**Figure 2**

A predicted model for signalling during rhizosphere colonisation, (adapted from Ref. [44]). In the early stages of root colonisation, the balance of DGC (pentagons) and PDE (circle) transcription suggests an increase in cdG favouring root attachment and siderophore production (red). As colonisation progresses, transcriptional levels of the tested genes suggest a balance between motile, surface-attached and siderophore producing bacteria. During the later stages of colonisation, upregulation of the PDE PFLU6074 leads to a shift towards cdG degradation and a more motile lifestyle. The size of each shape denotes the level of transcription with larger shapes corresponding to higher transcript abundance.

**Novel NSM molecules: more than microbial signals?**

One of the most exciting recent discoveries in NSM signalling has uncovered the true diversity of NSM signalling found in nature [49]. Whiteley et al. combined bioinformatic analyses with a systematic biochemical screen to discover a large family of cGAS/DncV-like nucleotidyltransferases (CD-NTases) that synthesise a diverse range of NSM molecules.
of cyclic-NSMs, including asymmetric molecules and ones based on pyrimidine nucleotides. CD-NTase enzymes were also discovered that produced cyclic trinucleotides, further expanding the NSM signalling space [49**]. While the biological function and importance of individual novel NSM molecules is still unclear, the implications of this finding for our understanding of the diversity and complexity of NSM signalling cannot be understated.

Since their discovery, research into NSM molecules has focussed almost exclusively on their roles as microbial signalling molecules. However, a recent study by Ahmad et al. [50**] has uncovered a new role for the NSM molecule (p)ppApp synthase held by P. aeruginosa (p)ppApp synthesis is catalysed by a (p)ppGpp synthase homolog: TaS1, which is injected into neighbouring cells through the type VI secretion system effector [50**]. This novel mechanism of bacterial warfare acts by depleting the target cells pool of ATP and inhibiting ATP synthesis [50**]. Furthermore, TaS1 mediated (p)ppApp synthesis was shown to decrease essential pathway metabolites, dysregulate the proton motive force and interact with known (p)ppGpp binding proteins to disrupt their function.

Conclusions

The last few years have seen remarkable advances in our understanding of NSM signalling in bacteria. New binding proteins and signal inputs have been characterised. Molecular and structural analyses have established new principles of signalling, crosstalk and network formation. Research into whole-cell, multicellular and host–microbe signalling networks have begun to uncover how NSM signalling operates in increasingly complex environments. Finally, our understanding of the nature of NSM function in bacteria has expanded, both in terms of the known NSM repertoire, and the discovery of new, non-signalling functions for these molecules. On this basis, the future for NSM signalling research promises to be equally exciting.

Conflict of interest statement

Nothing declared.

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The authors describe both the discovery and novel function of (ppp)App in interbacterial warfare. This insight is an exciting development in the field of NSMs, moving away from classical signalling pathways into bacterial competition.