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Control of bacterial second messenger signaling and motility by heme-based direct oxygen-sensing proteins

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Bacteria sense and respond to their environment, allowing them to maximize their survival and growth under changing conditions, such as oxygen levels. Direct oxygen-sensing proteins allow bacteria to rapidly sense concentration changes and adapt by regulating signaling pathways and/or cellular machinery. Recent work has identified roles for direct oxygen-sensing proteins in controlling second messenger levels and motility machinery, as well as effects on biofilm formation, virulence, and motility. In this review, we discuss recent progress in understanding O_2-dependent regulation of cyclic di-GMP signaling and motility and highlight the emerging importance in controlling bacterial physiology and behavior.

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Introduction

Bacteria have complex systems to sense and respond to environmental oxygen (O_2) concentrations. The balance between bacterial proliferation and stress adaptation through the formation of multicellular aggregates known as biofilms requires the adaptation of metabolism, growth, and the expression of stress responses in localized environments [1]. The oxygen gradient formed within a biofilm matrix affects physiological differentiation. Modulations to O_2 concentration impact virulence-related phenotypes such as motility and biofilm formation. For example, in the opportunistic pathogen *Pseudomonas aeruginosa*, O_2 concentrations impact downstream gene expression in systems ranging from antibiotic resistance to colony morphology [2–5].

To balance hypoxia, normoxia, and hyperoxia, prokaryotes have developed complex pathways to signal oxygen saturation. Bacteria generally reply on protein cofactors such as hemes, which directly bind O_2, or [FeS] clusters and flavins, which alter the oxidative state of the cofactor, to regulate O_2-dependent gene expression. While redox sensors are impacted by O_2 concentration, organisms that can use alternative electron acceptors can mediate their redox potential under hypoxia [5].

In addition to the systems controlling metabolism, O_2 sensing is also used to directly modulate bacterial signaling pathways and phenotypes, such as biofilm formation and motility [6,7], and direct O_2-sensing protein activity is tuned specifically to O_2 saturation. These proteins are often linked to transcription regulators or signaling cascades, which alter bacterial phenotypes. For example, the direct oxygen-sensing membrane receptor FixL, isolated from *Rhizobia*, regulates the expression of nitrogen fixation genes through an oxygen-binding heme domain [8]. Understanding the mechanism of O_2 sensing has been an area of significant research, however, many questions remain on the mechanisms of O_2-dependent signal transduction in bacteria [8–10].

Numerous heme-containing metalloenzymes have been studied as diatomic gas sensors [11]. These enzymes can be categorized by their domain architecture into globin-coupled sensors (GCS), heme-binding Per-Arnt-Sim (PAS) domain proteins, heme-binding GAF domain proteins (GAF domains are named for their occurence in c**G**MP-specific phosphodiesterases, **A**denylyl cyclases and **F**hlA), CooA proteins, and heme–NO/O_2 (H-NOX) proteins [12–14]. Some characterized heme proteins, such as FixL [10], *Af*GcKH from the bacterium *Anaeromyxobacter* sp. Fw 109-5 [15–17], and DosT/DevS from *Mycobacterium tuberculosis* [18–20], are part of two-component signal transduction systems. These multidomain sensory proteins consist of a sensing domain and a histidine kinase, which autophosphorylates upon ligand binding and phosphorylates the response regulatory protein, which regulates downstream gene expression. The mechanisms of O_2-dependent kinase regulation and functional roles of many of these two-component systems, including *Af*GcHK and FixL, have been investigated and recently have been reviewed in detail [10,14,17].

In this brief review, we highlight recent advances in our understanding of microbial O_2-dependent signaling

outside of classic two-component signal transduction systems and their physiological implications in secondary messenger formation and motility. We have focused on two distinct systems, heme-PAS domain containing methyl-accepting chemotaxis proteins (MCP) and sensor globin-containing proteins that regulate the bacterial second messenger bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP), to highlight the diversity of direct O_2-sensing mechanism in bacteria. Emerging work in nonheme iron [21,22] and small RNA (sRNA)-based [23] O_2 sensors underscores the need for further investigation into the breadth of direct O_2-sensing proteins and their physiological effects.

Oxygen-dependent nucleotide second messenger signaling

Bacterial nucleotide secondary messengers are used across the domains of life to link sensory inputs to regulatory responses [24–27]. In prokaryotes, the metabolic enzymes, effectors, and targets involved in the function of secondary messengers, such as bis-(3,5)-cyclic diguanosine monophosphate and cyclic adenosine monophosphate, have been identified in numerous species. The diversity of regulatory responses in bacteria, including growth, metabolic homeostasis, stress responses, cellular differentiation, and phage resistance, suggests the broad importance of understanding the molecular drivers of nucleotide secondary messenger activity [28,29].

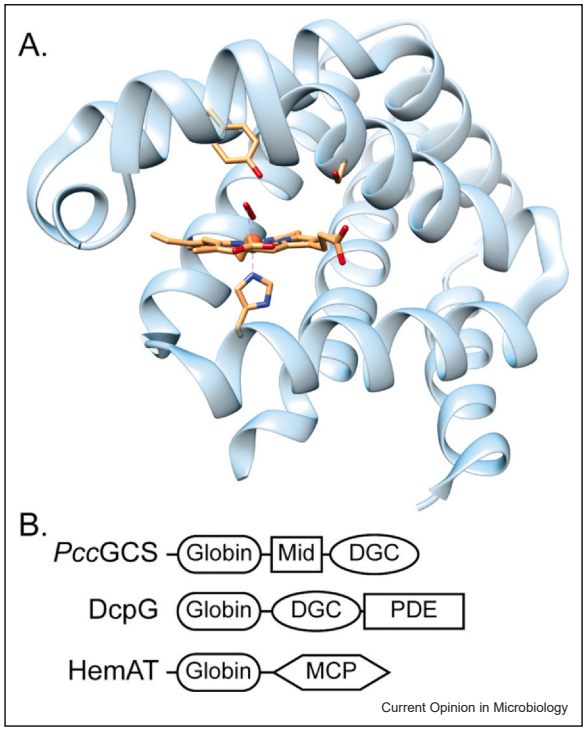
GCS proteins are a class of heme-containing O_2 sensors found in bacteria, archaea, and lower eukaryotes. The GCS proteins characterized to date are multidomain proteins, with an N-terminal-sensing globin domain linked to a C-terminal catalytic domain. Output domains that have been characterized include MCP, kinases, diguanylate cyclases (DGCs), phosphodiesterases (PDEs), and adenylate cyclases [24]. GCS proteins from several species, including the *E. coli* (*Ec*DosC) [28,29], *Bordetella pertussis* (*Bpe*GReg) [30,31], *Shewanella putrefaciens* (DosD) [32], and *Pectobacterium carotovorum* subspecies *carotovorum* (*Pcc*GCS of *Pc*DgcO) [30], contain DGC output domains and have been demonstrated to exhibit O_2-dependent c-di-GMP production *in vitro*. Studies on *Ec*DosC, *Bpe*GReg, and *Pcc*GCS have demonstrated that the proteins exhibit a range of O_2 affinities, suggesting that each GCS is tuned to increase c-di-GMP production at a different environmental O_2 saturation based on the requirements of the bacterial species.

In addition to O_2-sensing GCSs, DcpG, a bifunctional DGC/PDE GCS from *Paenibacillus dendritiformis*, was recently characterized as dual O_2/nitric oxide (NO) sensor [33,34] and has expanded our understanding of bacterial gas sensing. Ligation of O_2 to DcpG heme iron decreases DGC activity, as compared with Fe(II) unligated state, which NO binding increases DGC activity. In contrast, O_2 binding increases PDE activity, relative to Fe(II), while NO binding causes little effect. The *in vitro* data suggest that under high O_2 saturation, DcpG will function primarily as a PDE, while NO binding under anaerobic conditions will result in c-di-GMP production. Quantification of *P. dendritiformis* biofilm formation under aerobic, anaerobic, and anaerobic + NO conditions yielded results consistent with the *in vitro* studies [33] and suggests a role for DcpG in responding to both O_2 levels and NO produced within the environment, as well as highlights the potential for bifunctional c-di-GMP metabolic enzymes to respond to multiple signals.

Structures of sensor globins and mutagenesis studies have highlighted key features involved in O_2 binding, affinity, and signaling transduction to regulate DGC activity (Figure 1) [15,35–37]. Within the heme pocket, typically a distal tyrosine and serine/threonine hydrogen bonds with the bound O_2 and stabilizes the Fe(II)–O_2 form. *Ec*DosC, which has an alanine in the homologous position of the distal serine/threonine residue, and Ser-to-Ala variants of *Bpe*GReg and *Pcc*GCS exhibit markedly weaker O_2 affinity, underscoring the role of the hydrophilic residues in stabilizing ligand binding [29,38]. Within *Ec*DosC, a distal pocket leukine is involved in stabilizing the bound O_2 and, within DcpG, a π-stacking heme edge residue, typically tryptophan, histidine, or tyrosine, modulates O_2 binding without concomitant heme auto-oxidation [34]. A structure of *Bpe*GReg globin domain in the Fe(II)–O_2 ('on') and Fe(III) ('off') states identified changes in heme distortion, which can be propagated through the heme edge residue, and lead to conformational changes in the protein and changes in c-di-GMP metabolic domain activity [35].

While experiments to determine the cellular effects of O_2-dependent GCS signaling have been limited, the results hint at important, and often-overlooked roles, in second messenger signaling. In each of the species mentioned above, the Δ*GCS* strain exhibited decreased biofilm formation, relative to wild-type (WT) strain [30–32,40]. More in-depth studies in *P. carotovorum* have demonstrated that *Pcc*GCS regulates O_2-dependent motility and virulence within a potato host [39]. Regulation of cellular functions by *Pcc*GCS has been demonstrated to occur through both global changes in transcript levels and local interactions with downstream proteins, allowing for multiple levels of c-di-GMP-dependent regulation of cellular behavior [41]. These findings suggest that DGC-containing GCS proteins, and likely other direct O_2-sensing c-di-GMP metabolic proteins, have important roles in c-di-GMP signaling within bacteria and highlight the need for comparisons of WT/sensor deletion strains in aerobic and anaerobic environments.

Figure 1



GCS proteins. **(a)** Crystal structure of BpeGReg globin domain with distal tyrosine and serine and proximal hisitidine shown [36]. **(b)** Domain architectures of representative GCS protein.

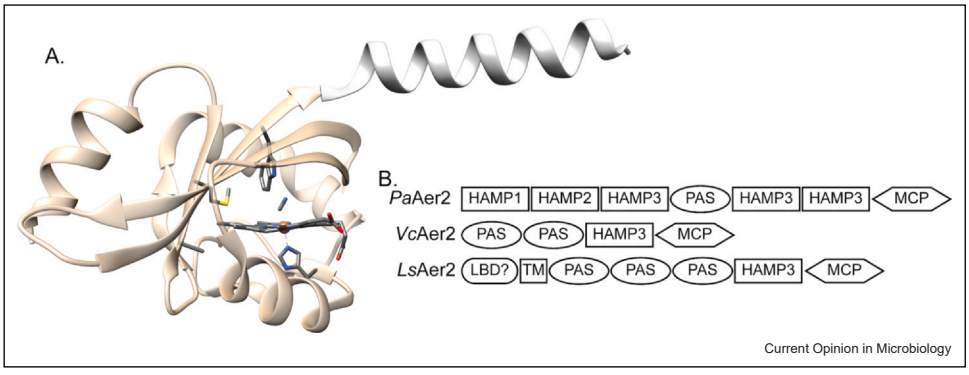
Oxygen-sensing methyl-accepting chemotaxis proteins

Bacteria have evolved several mechanisms of movement to colonize a breadth of environments and acquire resources [42]. Whether through swimming in aqueous media or moving over solid surfaces, motile bacteria can sense spatial gradients of chemicals, pH, temperature, or redox signals through complex chemotaxis signaling pathways. Chemosensory arrays detect changes in the environment through a range of mechanisms, including direct ligand binding to periplasmic receptors, indirect sensing mediated by periplasmic binding proteins, and by coupling chemotactic responses to metabolism [43]. Bacteria utilize the chemotaxis machinery to move toward O_2 concentrations and redox environments optimum for growth and proliferation [44]. Aerotaxis (O_2 sensing) has been studied most prominently in *E. coli*, which uses Aer and Tsr proteins to indirectly sense oxygen. Unlike other chemotaxis receptors, Aer senses redox changes inside the cell using a flavin adenine dinucleotide (FAD)-containing PAS domain facing the cytoplasm. Tsr, a serine chemoreceptor, senses a change in proton motor force [45].

To date, chemotaxis machinery involved in directly sensing O_2 concentrations has been found to often use either a sensing globin domain (HemAT-*Bs* and HemAT-*Hs*) or a PAS domain (Aer2). Recent work in the analogous Aer2 receptors from *Pseudomonas aeruginosa* [46–51], *Leptospira interrogans* [52], *Vibrio cholerae* [53,54], and *Vibrio vulnificus* [55] underlines the diversity of O_2-sensing mechanisms involved in taxis. Aer2 proteins are heme-based soluble gas-sensing receptors that contain PAS folds and poly-HAMP (histidine kinase–adenylyl cyclase–MCP–phosphatase) domains. Unlike *E. coli* Aer receptors [56], which are redox-based sensors, Aer2 receptors are soluble, membrane-associated proteins that directly bind O_2 using a heme-bound PAS domain(s) (Figure 2) and changes in PAS domain N-terminal cap upon O_2 binding yield signaling to the HAMP domain [49].

When expressed in *E. coli*, each of the Aer2 proteins can mediate O_2-dependent motility, with nature of the response (attractant versus repellant) dependent on the

Figure 2



Aer2 protein architectures. **(a)** Structure of *Pa*Aer2 PAS (tan) and HAMP3 linker (gray) domains with key residues (distal tryptophan and methionine, proximal histidine) shown [49]. **(b)** Domain architectures of Aer2 proteins from various species.

Aer2 homolog. In addition, *P. aeruginosa* Aer2 has been demonstrated to associate with flagellum-mediated chemotaxis proteins CheA2 and CheW2 and has been implicated in stress response and virulence [50] The role of Aer2 in *V. cholerae* has also been investigated and was demonstrated to be the MCP responsible for O_2-dependent swarming motility. *V. cholerae* Aer2 only responds to O_2 levels, as anaerobic assays in the presence of alternative electron acceptors did not yield any differences between WT and Δ*aer2* strains, demonstrating its physiological role as a direct O_2 sensor [54]. Furthermore, Aer2 modulates expression of *V. cholerae* virulence factors TcpA and TcpP, with virulence factor production increased under microaerobic and anaerobic conditions. Surprisingly, the Δ*aer2* strain outcompeted WT *V. cholerae* in a mouse model of infection, suggestion that Aer2 signaling may have more complex roles during bacterial infection of a host.

Additional species, including *Bacillus subtilis* and *Halobacterium salinarum*, utilize globin-coupled sensors with methyl-accepting chemotaxis domains to directly control O_2-dependent motility [57–59]. Based on *in vitro* spectroscopic studies, O_2 binding to the sensing globin domain results in conformational changes that are propagated through the protein and result in altered motility, [60–63] as has been observed for other GCS family members (discussed above). Similar to the Aer2 family of MCPs, HemAT proteins can control chemotaxis toward or away from high O_2 levels, depending on the bacterium from which the HemAT originates. Within *B. subtilis*, HemAT-*Bs* modulates an aerophilic response, while *H. salinarum* HemAT-*Hs* controls an aerophobic response [58,59]. These studies suggest that subtle differences in either the sensing globin domain or intraprotein signaling pathway modulate bacteria chemotactic responses to O_2 and highlight a need for further studies into both the O_2-sensing/signaling mechanism and physiological effects in a wider range of bacterial species.

Conclusions

Recent work investigating the roles of direct O_2-sensing proteins has identified roles in controlling intricate signaling pathways in bacteria modulating motility, biofilm formation, and virulence. While the GCS and Aer2 protein families have been under investigation for their roles in c-di-GMP signaling and motility, respectively, further work is necessary to understand their signaling mechanism(s) and the physiological roles in a wider range of bacterial species.

The recent discoveries of additional O_2-sensing domains suggest that many pathways controlled by O_2 levels remain uninvestigated and could have significant implications for our understanding of bacterial signaling and physiology under changing conditions. Nonheme iron proteins such as DcrH-Hr found in *Desulfovibrio vulgaris*, which contains a hemerythrin-like domain, sense O_2 via autoxidation of the iron center [22,64,65]. A distinct family of bacterial and archaeal oxygen-sensing di-iron proteins, ODPs, has emerged as a novel class of O_2 and iron sensors. In the human pathogen *Treponema denticola*, reversible binding of O_2 to the ODP $Fe(II)_2$ center leads to the formation of the *cis* μ-1,2 peroxo species, which destabilizes phosphorylated CheA, a histidine kinase that serves as a primary regulator of bacterial chemotaxis [21]. ODPs fall within the metallo-β-lactamase superfamily and serve as the regulatory link between sensory input and chemoreceptors without transmembrane regions and periplasmic ligand-binding domains.

sRNA has also been linked to O_2-dependent virulence. In enterohemorrhagic *Escherichia coli* O157:H7 (EHEC), the sRNA DicF is expressed in hypoxic conditions and modulates Shiga toxin and host colonization-related gene expression [23]. While the precise mechanism sensing is still unknown, O_2-dependent sRNA-mediated transcriptional regulation highlights the diversity of mechanisms employed by bacteria to sense and respond to O_2. Given the widespread occurrence of putative O_2-sensing systems in bacterial genomes, elucidating the mechanisms of sensing and signaling by all classes of O_2 sensors will help to explain how bacteria adapt to changing O_2 levels in the environment, as well as potentially identify novel methods to control O_2-dependent bacterial phenotypes.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Emily Weinert reports financial support and travel were provided by National Institutes of Health. Emily Weinert reports financial support and travel were provided by National Science Foundation.

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