Towards Understanding the Molecular Basis of Nitric Oxide-Regulated Group Behaviors in Pathogenic Bacteria

Dominique E. Williams Elizabeth M. Boon

Department of Chemistry and Institute of Chemical Biology and Drug Design, Stony Brook University, Stony Brook, NY, USA

Keywords

Nitric oxide signaling · Cyclic di-GMP · Biofilm · Nitric oxide sensor

Abstract

Pathogenic bacteria have many strategies for causing disease in humans. One such strategy is the ability to live both as single-celled motile organisms or as part of a community of bacteria called a biofilm. Biofilms are frequently adhered to biotic or abiotic surfaces and are extremely antibiotic resistant. Upon biofilm dispersal, bacteria become more antibiotic susceptible but are also able to readily infect another host. Various studies have shown that low, nontoxic levels of nitric oxide (NO) may induce biofilm dispersal in many bacterial species. While the molecular details of this phenotype remain largely unknown, in several species, NO has been implicated in biofilm-to-planktonic cell transitions via ligation to 1 of 2 characterized NO sensors, NosP or H-NOX. Based on the data available to date, it appears that NO binding to H-NOX or NosP triggers a downstream response based on changes in cellular cyclic di-GMP concentrations and/or the modulation of quorum sensing. In order to develop applications for control of biofilm infections, the identification and

characterization of biofilm dispersal mechanisms is vital. This review focuses on the efforts made to understand NO-mediated control of H-NOX and NosP pathways in the 3 pathogenic bacteria *Legionella pneumophila*, *Vibrio cholerae*, and *Pseudomonas aeruginosa*.

© 2018 The Author(s)
Published by S. Karger AG, Basel

Introduction

Bacteria are adaptable microorganisms that can cause infectious disease in humans. Not only can pathogenic bacteria evade host defenses, they can evolve resistance to those defenses as well as antibiotic treatments. Due to the increased prevalence of antibiotic-resistant strains, as well as the emergence and reemergence of infectious diseases, pathogenic bacteria pose an imminent threat to public health and welfare [1]. Therefore, it is imperative to identify and characterize the complex mechanisms used by microorganisms to cause infection, especially those used to develop persistent and chronic infections.

There is a diverse set of bacterial pathogens, but they use common strategies to invade, survive, and proliferate

inside a human host [1]. A particularly important strategy for pathogenicity and antibiotic resistance is biofilm formation [2, 3]. Biofilms are surface-associated, multi-cellular communities of bacteria that are often associated with disease, such as lung infections in cystic fibrosis (CF) patients and nosocomial infections [3]. It has been estimated that 80% of acute and chronic infections are biofilm related. Furthermore, biofilms are 100- to 1,000-fold harder to kill with antibiotics in comparison to free-swimming planktonic cells [4]. Thus, inducing dispersal from biofilm could be a key approach to making bacteria more susceptible to antibiotics in addition to biofilm eradication [3].

The biological signaling molecule nitric oxide (NO) has been shown to induce biofilm dispersal in many bacterial species, including the human pathogen *Pseudomonas aeruginosa* [5, 6]. Not only is NO involved in the regulation of biofilm formation, but it is also a mediator of quorum sensing [7, 8], a bacterial cell-to-cell communication mechanism that is interconnected with biofilm formation [9]. An understanding of the molecular basis of NO-assisted biofilm dispersal, as well as other NO-triggered group behaviors, could be vital to the development of more effective approaches to preventing and treating antibiotic-resistant biofilm infections. Henceforth, this review focuses on NO signal transduction pathways that are associated with the cooperative group behaviors biofilm formation and quorum sensing in the facultative human pathogens *Legionella pneumophila*, *Vibrio cholerae*, and *P. aeruginosa*.

The Two Faces of NO: Intracellular Signaling Molecule and Bactericidal Agent

Originally identified as a poison and pollutant, NO, a diatomic, cell-permeable, free-radical gas, is now also recognized as a ubiquitous signaling molecule in both eukaryotic and bacterial organisms [10]. At higher concentrations ($>1\ \mu\text{M}$), NO has cytotoxic properties, but at lower concentrations ($<1\ \mu\text{M}$), NO mediates downstream signal transduction pathways to control many physiological processes [11].

As an intracellular cytotoxic agent, NO converts to reactive nitrogen species in the presence of oxygen or superoxide radicals to induce damage of biological macromolecules, such as lipids, DNA, and proteins [11]. Owing to the power of nitrogen radical production from NO, humans use it as a key component of the immune system: dendritic cells, neutrophils, and macrophages produce

NO by inducible NO synthases (iNOS) to defend against harmful cells, including bacteria, viruses, and cancerous cells [11–13]. The cytotoxic properties of NO have also been harnessed for practical applications; for example, the addition of nitrite (NO_2^-), which dismutates to NO at low pH, to meat packaging prevents bacterial spoilage [13, 14].

Because of the antimicrobial properties of NO, bacteria have evolved strategies to protect themselves from high NO levels and nitrosative stress. For example, in *P. aeruginosa*, the NO-responsive transcription regulator FhpR activates the gene of flavohemoglobin (fhp), which detoxifies NO by converting it to nitrate under aerobic conditions [15]. This and other NO-responsive regulators involved in NO detoxification, such as respiratory and flavorubredoxin NO reductases, have been reviewed previously [16]. Of particular relevance for the bacteria of interest in this review, nitrosative stress resistance in *V. cholerae* O1 and O139 [17] and biofilm development in *P. aeruginosa* [6] are well characterized as NO stress responses. For more information about the stress response pathways triggered by NO in bacteria, please refer to reviews by Cole [18] and Bowman et al. [19].

Paradoxically, in some cases, high concentrations of NO may serve a protective role in bacteria. For example, NO may protect bacteria from antibiotic-induced oxidative stress by either chemical modification of antibiotics or induction of aerobic respiration [20, 21]. This strategy has been shown in a few bacterial species, primarily gram-positive pathogens, that have the ability to endogenously produce NO by bacterial NO synthases (bNOS) [21]. Furthermore, because biofilm formation is a protective response in bacteria, the high concentrations of NO produced during mammalian inflammatory responses have been shown to facilitate biofilm adhesion on human tissue as well as intravenous medical devices [22].

Intracellular NO signaling, on the other hand, occurs at sub-micromolar concentrations. In mammals, NO endogenously produced from the oxidation of arginine by endothelial or neuronal NO synthase (eNOS, nNOS) binds to the heme cofactor of soluble guanylyl cyclase (sGC), which induces production of the secondary messenger cyclic 3',5'-guanosine monophosphate (cGMP), which in turn goes on to control vasodilation, myocardial function, neurotransmission, and platelet aggregation [23]. In bacteria, NO has been implicated in signaling processes to regulate physiological processes, including virulence, biofilm regulation, gene expression, and cell morphology [24]. For example, low levels of NO (10^{-12} to $10^{-9}\ \mu\text{M}$) from NO donors have been shown to induce

biofilm dispersal in various bacterial species, including the pathogens *P. aeruginosa* [6], *L. pneumophila*, and *V. cholerae* [25].

Insight into how and when pathogens encounter NO during the natural course of infection has both clinical and physiological relevance. Bacteria may encounter NO produced from endogenous or exogenous sources. A schematic overview of some of these mechanisms is shown in Figure 1. Briefly, as mentioned above, a few bacterial species encode a protein similar to mammalian NOS, called bNOS, which produces NO through the oxidation of arginine [21]. Many bacteria, including *P. aeruginosa*, produce NO as an intermediate in anaerobic respiration known as denitrification [24, 26]. Exogenous sources of NO may include host production from NOS or the breakdown of environmental nitrate (NO_3^-) or nitrite (NO_2^-) [27]. For example, bacteria in the gut may experience NO produced from iNOS expression in human epithelial cells of the small intestine during a host immune response [28]. Bacteria may also experience exogenous NO produced from nitrite by nonenzymatic dismutation in acidic environments [27], including the airway surface liquid in CF lung disease (relevant to *P. aeruginosa* infections) [29] or the stomach (relevant to *V. cholerae* infections) [27]. A common source of nitrite is from dietary nitrates which are reduced by nitrate reductases of anaerobes located in the oral cavity [27] or gut [30].

Clinical Relevance of NO as an Antibiofilm Strategy

Biofilm formation is a complex and highly regulated process, which is often an adaptation response to environmental cues, such as changes in temperature, pH, nutrient limits, and iron or oxygen levels [31]. There are 3 defined stages in a biofilm life cycle: initial attachment, maturation, and dispersal. Initial attachment of planktonic cells to a surface is facilitated by flagella-mediated motility and pili-mediated adhesion [31]. After attachment, cells in the biofilm upregulate extracellular polysaccharide synthesis and continue to grow [31]. The last step, dispersal, involves release of cells from the biofilm where they are free to colonize another surface or host [31].

Biofilm production is a protective growth mode for bacteria and a major player in bacterial pathogenicity and infectious diseases. In a human host, biofilm-derived infections are often chronic infections and are the main contributor to nosocomial infections, such as respiratory pneumonia, blood stream and gastrointestinal infections,

Fig. 1. Pathways for NO production from endogenous and exogenous sources. Denitrification involves 4 reduction steps: nitrate (NO_3^-) to nitrite (NO_2^-) by nitrate reductase (NAR), NO_2^- to NO by nitrite reductase (NIR), NO to nitrous oxide (N_2O) by nitric oxide reductase (NOR), and N_2O to nitrogen (N_2) by nitrous oxide reductase (N_2OR). Dietary sources of NO_3^- may be reduced to NO_2^- by NAR and then dismutate at low pH to NO. NO may also be produced from the oxidation of arginine by bacterial or mammalian nitric oxide synthases (NOS).

endocarditis, and infections resulting from contaminated medical devices (e.g., intravenous catheters and heart valves) [2, 31, 32]. Along with the protective barrier provided by extracellular polysaccharide, bacterial cells display increased resistance towards antibiotics and host defenses in part due to the upregulation of efflux pumps. Current biofilm removal and control strategies (e.g., chlorine disinfection and antibiotics) are insufficient for the eradication of biofilm-derived contaminants and infections [4].

A key approach to preventing biofilm-related diseases and infections may be applications targeting the bacterial NO signal transduction pathways that control biofilm dispersal and related group behaviors. Thus, the identification of NO regulatory pathways involved in the biofilm-to-planktonic cell transition will lead to strategies to eradicate and prevent clinically relevant bacterial infections and diseases. The remainder of this section will focus on what has been established about the clinical relevance of NO in fighting biofilm infections caused by our pathogens of focus.

L. pneumophila

The waterborne, gram-negative facultative anaerobe and opportunistic pathogen *L. pneumophila* can cause Legionnaires' disease, a severe form of pneumonia and Pontiac fever in humans [33]. In the environment, *L. pneumophila* lives in many forms: its planktonic state, inside an amoeba host [34], or as part of a multi-species biofilm, such as those that are frequently adhered to water systems, such as cooling towers and ventilation or air conditioning systems [33]. *L. pneumophila* infects humans through the inhalation of contaminated water droplets. Once in a human host, *L. pneumophila* is able to inhabit and replicate in alveolar macrophages in lung tissue, causing disease.

Current methods, such as long-term chlorine treatment of water systems, are effective in the removal of planktonic cells but are ineffective against biofilm-derived *L. pneumophila* [33]. Interestingly, Barraud et al. [25] have demonstrated that low, nontoxic levels of an NO donor called sodium nitroprusside (SNP) can induce biofilm dispersal and markedly enhance the antimicrobial efficacy of antibiotic applications against many bacterial biofilms, including multi-species biofilms isolated from water systems. In particular, pretreatment with approximately 500 nM SNP (~500 pM NO) for 18 h increased chlorine efficacy by 20-fold and resulted in ~85% removal of multi-species biofilms from a recycled water distribution system [25].

V. cholerae

Similar to *L. pneumophila*, *V. cholerae*, the causative organism of cholera, is a waterborne, gram-negative facultative anaerobe and opportunistic pathogen. *V. cholerae* causes infection through contaminated water or food. Once ingested, *V. cholerae* colonizes the surface of the small intestine and releases virulence factors (toxin co-regulated pilus and cholera toxin) that cause acute diarrhea in humans [28]. Transmission of *V. cholerae* can also occur from host to host [28]. Effective transmission of *V. cholerae*, either from host to host or environment to host, depends on the protection imbued by biofilm formation. In order for *V. cholerae* to enter the intestine, it has to avoid acid shock in the stomach and avoid bile and other host defenses inside the gut; furthermore, ingestion of a biofilm delivers a higher concentration of the bacterium and increases the likelihood of the production of virulence factors once inside the intestine [28].

Although quite ineffective against biofilms, the use of antibiotics in combination with NO to fight *V. cholerae* infections is proving to be a convincing strategy. Barraud

et al. [25] showed that *V. cholerae* biofilms were only reduced by 21% in vitro when treated with 14 μ M tetracycline, whereas a nontoxic concentration of NO (500 μ M SNP = ~500 pM NO) reduced *V. cholerae* biofilms by 73%. Combining the two was shown to be even more effective in biofilm reduction: exposure to 14 μ M tetracycline after pretreating *V. cholerae* biofilms with 500 nM SNP provided a 90% reduction in biofilms compared to the untreated controls [25].

P. aeruginosa

Like *L. pneumophila* and *V. cholerae*, *P. aeruginosa* is a gram-negative facultative anaerobe and an opportunistic pathogen. *P. aeruginosa* is a leading cause of respiratory infections in CF patients and is thought to be responsible for a majority of nosocomial infections [35]. *P. aeruginosa* is capable of both planktonic and biofilm modes, which are frequently linked to oxygen availability. When oxygen levels are low, *P. aeruginosa* switches from aerobic to anaerobic respiratory growth to obtain energy from nitrate respiration, also known as the denitrification pathway (Fig. 1) [36, 37]. Denitrification involves the sequential 8-electron reduction of NO_3^- to nitrogen gas by 4 reductases [37]. Aside from low oxygen concentrations, denitrification can also be activated by NO [38]. The anaerobic and aerobic metabolic pathways are both dependent on nitrite reductase activity in *P. aeruginosa* [26]. Under aerobic conditions, NO favors swarming motility derived from an upregulation of the production of flagella and type IV pili and the biosynthesis of biosurfactant rhamnolipid [6]. Under anaerobic metabolism, accumulation of NO, an intermediate of denitrification, contributes to biofilm formation due to the repression of nitrite reductase activity [39]. This ability to switch between metabolic pathways and to form biofilms under anaerobic conditions greatly contributes to *P. aeruginosa* survival and persistence during host infection.

The pathogenesis of *P. aeruginosa* in the CF airway illustrates how the pathogen thrives in the adverse environment of the CF lung, adapting and responding to environmental changes through regulation of biofilm formation. There are two general forms of *P. aeruginosa* found in the CF lung: a nonmucoid-type strain found in the aerobic airway lumen and a mucoid-type strain found within the hypoxic mucus lining of the CF airways [37]. Although both form biofilm, mucoid *P. aeruginosa* grows more robust biofilms and is more resistant to antibiotics than the nonmucoid strain, due to an increase in alginate biosynthesis and extracellular DNA [37]. Likewise, in vitro biofilms grown anaerobically are more robust and

more resistant to antibiotics than those grown under aerobic conditions; thus, it has been concluded that denitrification markedly aids in *P. aeruginosa* pathogenesis and physiology [37].

As with *L. pneumophila* and *V. cholerae*, antibiotic-assisted death of planktonic cells upon NO-induced biofilm dispersal almost completely eradicates *P. aeruginosa* biofilms in vitro [6]. This approach may be especially important for treatment of *P. aeruginosa* biofilm-based infections, especially considering that treatment with antibiotics alone has been shown to elicit an enhancement of biofilm in vitro [40]. Indeed, a proof-of-concept study suggests that this strategy is very effective against chronic *P. aeruginosa* infections in CF patients [41]. Clinical isolates from CF sputum were treated with NO and 5 $\mu\text{g mL}^{-1}$ tobramycin, which resulted in almost complete eradication, while tobramycin alone induced 243% more biofilm growth. NO treatment alone resulted in a reduction in biofilm biomass along with an enhancement of planktonic cell count [41]. The investigators went on to apply the same treatment to 6 CF patients, which resulted in a marked reduction in detectable levels of *P. aeruginosa* biofilms in their lungs after 5–7 days of treatment with 10 ppm of inhaled NO gas for 8 h per day and treatment with antibiotics [41]. Unfortunately, however, detectable levels of *P. aeruginosa* were restored in the patients 10–13 days after treatment [41]. Nonetheless, this approach could be optimized or be more effective in treating early or chronic CF lung infections.

NO Sensors that Affect Group Behaviors: H-NOX and NO Sensor Protein

As discussed above, there is reason to believe that NO, at low concentrations, could be an effective treatment for some biofilm-based infections due to its ability to trigger biofilm reduction [5, 6, 25]. NO is also involved in other aspects of bacterial group behaviors, particularly quorum sensing [42]. The remainder of this article will focus on what is currently understood about the biochemistry of the NO sensors and NO-responsive signaling pathways that have been implicated in responding to NO and regulating biofilm and quorum sensing.

The best understood NO signaling pathways are mediated by hemoproteins. In eukaryotes, the NO receptor sGC, mentioned briefly above, features a NO-responsive heme-binding domain fused to a catalytic guanylate cyclase domain that responds to picomolar amounts of NO to catalyze the production of cGMP from GTP [23]. The

Fig. 2. Common signaling pathways involving the NO sensors H-NOX and NosP in facultative anaerobes. **a** Cyclic di-GMP signaling involving the NO sensor regulating an associated cyclic di-GMP processing enzyme that contains a diguanylate cyclase (DGC) domain and a phosphodiesterase (PDE) domain. **b** Two-component signaling involving a sensor histidine kinase (HK) that phosphorylates a cognate response regulator (RR). **c** Two-component signaling involving a hybrid HK that initiates phosphorelay between a histidine-containing phosphotransfer protein (Hpt) and a RR.

cyclic nucleotide cGMP is a secondary messenger molecule that, in mammals, mediates downstream signal transduction pathways by activating protein kinases, phosphodiesterases (PDEs), and ion-gated channels [43]. In sGC, the heme domain is a member of the H-NOX (heme-nitric oxide and oxygen binding domain) family of NO sensors, members of which are also encoded in bacterial genomes [44].

In facultative anaerobes, H-NOX proteins are typically found as stand-alone hemoproteins that have been shown to regulate c-di-GMP (bis-[3'-5']-cyclic dimeric guanosine monophosphate) metabolizing enzymes or histidine kinases (HKs) *in trans* [8]. We have collectively named these H-NOX partner proteins H-NOX-associated c-di-GMP processing enzymes (HaCE; Fig. 2a) or H-

Table 1. Summary of NO signaling pathways involving H-NOX and NosP

Pathways (see Fig. 2)	Sensor/HK	Hpt	RR	NO effect		
				HK or HaCE	c-di-GMP levels	biofilm
<i>Vibrio cholerae</i>						
2C	NosP/VpsS (VC1444/VC1445)	LuxU (VC1022)	LucO (VC1021)	↓ ^a	ND	↓ ^b
2B	H-NOX/HahK (VC0720/VC0719)	–	PDE (VC1086)	↓ ^c	↑ ^d	↑ ^d
<i>Legionella pneumophila</i>						
2B	NosP/NahK (lpg0279/lpg0278)	–	DGC-PDE (lpg0277)	↑ ^e	↑ ^f	↓ ^f
2A	H-NOX/HaCE (lpg1056/lpg1057)	–	–	↓ ^g	↓ ^g	↓ ^g
<i>Pseudomonas aeruginosa</i>						
2C	NosP/NahK (Pa1975/Pa1976)	HptB (PA3345)	ND	↓ ^h	ND	↓ ^g

ND, not determined. ^a Heckler et al., submitted. ^b Shikuma et al. [55], 2009. ^c Plate et al. [56], 2012. ^d Waters et al. [54], 2008. ^e Fischer et al., unpubl. data. ^f Pécastaings et al. [34], 2016. ^g Carlson et al. [47], 2010. ^h Hossain and Boon [46], 2017.

NOX-associated HKs (HahK; Fig. 2b, c), respectively. Upon NO binding to the Fe(II) ion of the heme cofactor, a signal is transmitted from the H-NOX domain to the effector domain, which regulates HahK autophosphorylation or HaCE activity. In some *Vibrio*, H-NOX-associated HKs have been shown to be involved in quorum sensing circuits, and thus may be known as HqsKs (H-NOX-associated quorum sensing HKs).

H-NOX was the first dedicated NO sensor discovered in bacteria [45]; it is conserved in ~250 independent bacterial species, but it is not encoded in all NO-responsive bacteria [44]. Thus, a recent pursuit for the identification of an alternative bacterial NO sensor led to the discovery of a new NO sensor named NO sensor protein (NosP) [7, 46]. It was discovered that, like H-NOX, NosP, also a hemoprotein, responds to low levels of NO and is commonly found co-cistronic with signaling proteins, including HKs and c-di-GMP metabolizing enzymes (Fig. 2c) [7]. Compared to H-NOX, NosP is more widely conserved, found in greater than 800 independent bacterial species, but, unlike H-NOX, NosP is found almost exclusively in bacteria [46].

Well-characterized H-NOX pathways [8, 23, 42, 44, 47, 48], along with the recent progress in the characterization of NosP pathways [7, 46], have directed our understanding of the molecular basis of NO signaling in bacteria, particularly in gram-negative facultative anaerobes. These sensors have been shown to regulate bacterial com-

munal behaviors in response to NO, usually involving c-di-GMP signaling, two-component signaling, or quorum sensing. To be consistent with H-NOX nomenclature, we have collectively named NosP-associated HKs NahKs and NosP-associated quorum sensing kinases NqsKs, but in some cases these kinases are also known by other names, which will be noted below.

Cyclic di-GMP Signaling Involving Catalytic PDEs and Cyclases

One commonality of bacterial pathogenicity is biofilm formation, which is strongly correlated with intracellular c-di-GMP levels [49]. The bacterial 2nd messenger c-di-GMP regulates various bacterial physiological processes and contributes to several aspects of bacterial pathogenesis, including virulence, pathogen transmission, and chronic infections [32]. C-di-GMP signaling promotes motility-to-sessility transition in bacteria by targeting downstream pathways responsible for the biosynthesis of flagella, pili, and extracellular DNA and polysaccharides, components that are necessary for surface-associated biofilms [49]. Typically, high intracellular levels of c-di-GMP favor biofilm formation, whereas planktonic motile cells are favored at low c-di-GMP levels. Intracellular levels are controlled by c-di-GMP metabolizing enzymes: PDEs lower c-di-GMP levels by the hydrolytic cleavage of the phosphate ester bond of c-di-GMP to yield its linear

form pGpG (5'-phosphoguanylyl-[3'-5']-guanosine), and, on the other hand, diguanylate cyclases (DGCs) enhance c-di-GMP levels by catalyzing the condensation of two GTP molecules to yield c-di-GMP.

In response to NO, H-NOX or NosP may control c-di-GMP metabolism by the regulation of an associated DGC and/or PDE enzyme directly (Fig. 2a) or indirectly through regulation of a kinase that, in turn, regulates a DGC or PDE [7, 8]. For instance, in H-NOX pathways, NO may “switch on” PDE activity [48] or “switch off” cyclase activity [47, 48], which results in decreased intracellular c-di-GMP concentrations upon NO binding to the prosthetic heme of the NO sensor [47, 48]. Altogether, NO-induced biofilm dispersal [5, 6, 25] may be a result of reduced intracellular levels of c-di-GMP via H-NOX or NosP, regulating its cognate c-di-GMP metabolizing enzyme *in trans* in response to NO.

Two-Component Signaling Involving Sensor HKs

NO-dependent modulation of HKs can be generally classified as two-component sensory pathways. Two-component signaling pathways involving H-NOX or NosP proteins that regulate their respective HKs (HahK or NahK) are commonly found in gram-negative facultative aerobes [7, 8, 44]. These systems are typically composed of the NO sensor, the HK, and one or more response regulator proteins. In short, the NO sensor regulates the autokinase activity of its associated HK, and the signal phosphate is then transferred from the HK to a response regulator protein via a histidine-to-aspartate phosphotransfer reaction (Fig. 2b). Another commonality, as shown in Figure 2c, is the involvement of an additional accessory protein, a histidine-containing phosphotransfer protein (Hpt), that works in conjunction with a hybrid HK (a HK that contains an internal receiver domain), and, thus, instead of one, two histidine-to-aspartate phosphorelay steps occur. Here, after HK autophosphorylation, the phosphoryl group is shuttled within the HK (via histidine-to-aspartate phosphorelay) and then transferred to the histidine residue of the Hpt, which ultimately transfers the phosphoryl group to the receiver domain of a cognate response regulator (Fig. 2c). Ultimately, the activated response regulator controls the output of the NO-dependent signaling pathways. Frequently, this response regulator is an effector protein that has PDE activity or is a transcription factor [44].

Quorum Sensing

Quorum sensing is cell-to-cell communication used by bacteria to regulate group behaviors in a cell density-

dependent manner [50]. Quorum sensing circuits control the expression of genes involved in host colonization [51], biofilm formation and dispersal [51], and the expression of virulence factors in microbes [50], among other group behaviors. In this regulatory system, bacteria cells communicate with other cells by synthesizing, releasing, and responding to chemical signaling molecules called autoinducers. In some cases, NO signaling pathways involving H-NOX or NosP may be directly or indirectly involved in quorum sensing (discussed in the forthcoming section).

The remainder of this review will discuss common mechanisms seen in H-NOX and NosP regulatory systems that mediate biofilm formation, c-di-GMP signaling, and quorum sensing in 3 gram-negative facultative pathogens. A summary of all mechanisms reviewed in this paper is found in Table 1. For more in-depth reviews on the molecular basis of NO signaling pathways, please refer to recent reviews by Nisbett and Boon [8], Williams et al. [52], and Hossain et al. [7].

NO Signaling in *V. cholerae*

In *V. cholerae*, as well as other bacteria, responding to environmental cues to switch between motile and sessile states contributes to persistence and survival inside a human host. For example, for propagation, *V. cholerae* must in turn transverse the human gut, colonize the small intestine, and then detach from epithelial cells to prepare for transmission to another host [53]. There are two NO-associated signaling pathways that have been characterized in *V. cholerae*, one involving NosP and the other involving a H-NOX protein, and both modulate biofilm formation [7]. Along with regulating biofilm formation, the H-NOX pathway is indirectly associated with density-dependent quorum sensing, and NosP is directly involved in *V. cholerae*'s quorum sensing pathway. Thus, these two NO-associated pathways both contribute to the interconnectivity between biofilm formation and quorum sensing in *V. cholerae*. Along with briefly describing these two signaling pathways and their role in biofilm formation in *V. cholerae*, this section will give a glimpse into how NO-associated signaling may work synergistically with density-dependent quorum sensing.

*Connection between Quorum Sensing and Biofilm Formation in *V. cholerae**

In *V. cholerae*, quorum sensing plays a vital role in host colonization and disease transmission by coordinating

motile-to-sessile transitions in bacteria by responding to cell density population. In this bacterium, cells communicate using two autoinducers named CA-1 ([S]-3-hydroxytridecan-4-one) and AI-2 ([2S,4S]-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate) [54], and information relayed from the autoinducers is processed by two-component signaling. In more detail, quorum sensing in *V. cholerae* involves several HKs that initialize the phosphotransfer from a Hpt named LuxU (VC1022) to a response regulator named LuxO (VC1021), which ultimately leads to the repression of virulence factors and the expression of genes responsible for polysaccharide biosynthesis at low autoinducer levels [50, 55]. Specifically, for *V. cholerae*, in the low cell density state, autoinducer receptors function as kinases to transfer phosphate to the response regulator LuxO [50]. Phosphorylated LuxO ultimately leads to the expression of genes required for biofilm formation and virulence factor production. Conversely, at high cell density, the receptors switch to phosphatases, leading to dephosphorylated LuxO which contributes to the repression of biofilm formation and virulence factor production [50]. This output is a result of HapR, a quorum sensing transcription factor, activation by the dephosphorylation of LuxO leading to the activation or repression of genes responsible for the degradation or biosynthesis of cyclic di-GMP and polysaccharides, respectively [54]. Overall, in *V. cholerae*, motility is favored at high cell density, and biofilm formation is favored at low cell density, which is regulated by the dephosphorylation and phosphorylation of LuxO via quorum signaling.

NO/NosP-Regulated Two-Component Signaling Pathway Involving Quorum Sensing and Repressed Biofilm Formation

In the same quorum sensing pathway described above, there is a HK that is not dependent on cell density but on a NosP-mediated NO-signaling pathway that follows the system in Figure 2c. Accordingly, *V. cholerae* contains a NosP gene (VC1444, previously referred to as VpsV) that is co-cistronic with a hybrid HK and, corresponding to its involvement in quorum sensing, named VpsS (VC1445). We have shown that upon NO binding, NosP inhibits VpsS ATP-dependent autophosphorylation [Heckler et al., submitted]. Similar to high autoinducer concentration in density-dependent quorum sensing, biofilm dispersal may result from kinase inhibition. This result is consistent with a study showing enhanced biofilm formation when VpsS is overexpressed in wild-type *V. cholerae* [55]. Thus, NO may be able to mediate biofilm dispersal using a NosP/VpsS pathway that is directly involved in quorum sensing.

NO/H-NOX-Regulated Two-Component Signaling Pathway Involving c-di-GMP Signaling and Enhanced Biofilm Formation

V. cholerae encodes a H-NOX/HK system (Fig. 2b) consisting of a H-NOX (VCA0720) and a cognate HahK (VCA0719). The HahK regulates two response regulators: one is an active PDE (VC1086) containing a conserved EAL amino acid motif and the other is a degenerate PDE (VC1087) containing a conserved HD-GYP amino acid motif [44]. The EAL response regulator VC1086 is activated by HahK via phosphotransfer [44] and favors biofilm dispersal; a phenotype displayed by VC1086 overexpression in a quorum sensing-deficient mutant $\Delta luxO$ due to a reduction in intracellular c-di-GMP levels and repression of biofilm formation in *V. cholerae* [54]. VC1086 is activated by the quorum sensing transcription factor HapR, which also represses other genes responsible for the biosynthesis of polysaccharide and c-di-GMP, at low cell density [54]. Thus, although this H-NOX/HahK system involving VC1086 is independent from the quorum sensing pathway, this NO-responsive pathway indirectly contributes to *V. cholerae*'s motility response at low cell density.

However, in the presence of NO, this H-NOX/HahK pathway favors biofilm formation due to suppression of the activity of HahK which subsequently decreases phosphorelay-dependent activation of VC1086 [56]. This effect, NO-bound H-NOX inhibition of HahK, was also shown in other gram-negative facultative anaerobes, including *L. pneumophila* [47], *Shewanella oneidensis* [56], *Vibrio fischeri*, *Vibrio harveyi*, *Vibrio parahaemolyticus*, and *Pseudoaeromonas atlantica* [8]. Also, corresponding to the NO-mediated repression of VC1086 activity, but shown in a H-NOX/HK pathway containing an analogous response regulator, in the presence of NO, H-NOX contributes to increasing intracellular c-di-GMP levels by the inhibition of HahK, which, in turn, increases biofilm formation in *S. oneidensis* [56]. Although nontoxic levels of NO donor induce biofilm dispersal in *V. cholerae* [25], this particular NO signaling pathway may favor NO-induced biofilm formation. Hence, it was proposed that this specific H-NOX/HahK signaling pathway may play a role in biofilm formation during *V. cholerae* colonization, with the assistance of NO, in the acidic environment of the gut [56].

NO Signaling in *L. pneumophila*

Thus far, two NO-associated signaling pathways have been characterized in *L. pneumophila*, one involving a H-NOX/HaCE system (Fig. 2a) and the other involving a

NosP/NahK system (Fig. 2b). Although the mechanisms are different, both pathways regulate c-di-GMP signaling and mediate biofilm dispersal via NO binding to the respective sensor. Thus, these two NO signal transduction pathways studied in the waterborne pathogen *L. pneumophila* and described below could be linked to NO-induced dispersal of mixed-species biofilms from water systems treated with NO donors [25].

NO/NosP-Regulated Two-Component Signaling Pathway Involving c-di-GMP Signaling and Repressed Biofilm Formation

L. pneumophila encodes a NosP/NahK (Fig. 2b) system comprised of a NosP (lpg0279) and a cognate NahK (lpg0278), which regulates a response regulator containing a dual-functioning DGC/PDE enzyme (lpg0277). A homologue of lpg0277, lpl0329 from the Lens strain of *L. pneumophila*, was shown to be involved in the repression of biofilm formation [34] and c-di-GMP metabolism [57]. In addition, activated response regulator, by NahK-assisted autophosphorylation, led to the inhibition of cyclase activity ([57], [Fischer et al., unpubl. data]) and was coupled with an enhancement in PDE activity [Fischer et al., unpubl. data]. In the presence of NosP, in both the unligated and NO-bound states, kinase activity of NahK is markedly inhibited [Fischer et al., unpubl. data]. However, unlike the other two-component signaling pathways discussed, NO-bound NosP presented slightly more kinase activity than the kinase activity in the presence of unligated NosP [Fischer et al., unpubl. data]. Thus, in this case, NO-bound NosP enhances NahK activity [Fischer et al., unpubl. data], which may contribute to NO-induced biofilm dispersal via the upregulation of the PDE domain of the cognate response regulator in this NosP/NahK pathway in *L. pneumophila*. Lastly, it is important to note that, although deletion of the homologous response regulator in the Lens strain led to a hyper-biofilm phenotype [34], decreased intracellular c-di-GMP levels were also observed [34]. Generally, c-di-GMP levels are directly proportional to biofilm growth; this anomaly underscores the complexity of c-di-GMP-regulated intracellular signaling pathways and the difficulty involved in understanding these pathways.

*NO/H-NOX-Regulated c-di-GMP Metabolism and Repression of Biofilm Formation in *L. pneumophila**

L. pneumophila encodes a H-NOX/HaCE system (Fig. 2a) composed of a H-NOX gene (lpg1056) adjacent to a HaCE protein (lpg1057) containing an active digua-

nylate cyclase and an inactive PDE domain. The HaCE catalyzes the synthesis of c-di-GMP from two GTP molecules via the cyclase domain, which contains the conserved amino acid motif GGDEF. This cyclase dominant HaCE contributes to biofilm formation shown by the hyper-biofilm phenotype caused by the overexpression of HaCE in *L. pneumophila* [47]. In the presence of NO-bound H-NOX, cyclase activity of HaCE is inhibited, which subsequently lowers c-di-GMP levels [47]. A similar response was shown for another H-NOX/HaCE system in the marine bacterium *Shewanella woodyi*, in which, in the presence of NO-bound H-NOX, cyclase activity was inhibited and the active PDE domain activity was upregulated [48]. Notwithstanding the upregulation of the PDE domain in HaCE, decreased c-di-GMP levels ultimately led to biofilm dispersal in *S. woodyi* [48]. Likewise, in *L. pneumophila*, the H-NOX/HaCE system response to NO was decreased c-di-GMP levels by inhibition of cyclase activity of HaCE. Altogether, like *S. woodyi*, this NO signaling pathway involving H-NOX/HaCE may favor NO-induced biofilm dispersal in *L. pneumophila*.

NO Signaling in *P. aeruginosa*

Unlike *L. pneumophila* and *V. cholerae*, but similar to many other bacteria, *P. aeruginosa* does not encode a H-NOX gene, yet responds to NO. A recent study suggests that an alternative NO sensor named NosP plays a role in NO-associated biofilm dispersion in *P. aeruginosa* [46].

NO/NosP-Regulated Two-Component Signaling Pathway and Repression of Biofilm Formation

Accordingly, in *P. aeruginosa*, a NosP gene (Pa1975) is co-cistronic with a hybrid HK (PA1976). The hybrid NosP-associated HK (NahK) is composed of a histidine-containing sensor domain, as well as, at the C-terminus, an aspartate-containing receiver domain. After ATP-dependent phosphorelay within HahK, the phosphate is transferred from the HahK's receiver domain to a histidine phosphotransfer protein called HptB (Pa3345) (Fig. 2c). Similar to H-NOX/HahK systems, in the presence of NO-bound NosP, NahK activity is inhibited, which subsequently decreases phosphorelay from NahK to HptB [46]. Though the response regulators downstream of HptB have not been identified, NO-induced biofilm dispersal in *P. aeruginosa* was linked to NosP/NahK-assisted phosphotransfer to HptB due to a NahK-disrupted mutant inability to disperse in the presence of NO [46].

Finally, it must be mentioned that in addition to NosP, the chemotaxis transducer BdlA (biofilm dispersion locus A) and the MHYT-coupled PDE NbdA (NO-induced biofilm dispersion locus A) have both been implicated in biofilm dispersal upon NO detection, each through decreasing cyclic di-GMP concentrations [58, 59]. It is not yet clear if or how these proteins may be functionally linked to NosP.

Conclusions

Biofilm-forming pathogens have negative implications in medical and industrial settings. This bacterial physiological response, biofilm formation, is a cellular adaption and survival strategy that prepares the cell for host infection and contributes to antibiotic resistance. Biofilm dispersal facilitates the transmission of pathogens, although planktonic cells are more susceptible to antibiotics. NO, an essential signaling molecule sensed by bacteria, is involved in regulatory pathways that appear to

primarily aid in bacterial cell release from biofilms (Table 1). Furthermore, studies have shown that combined NO and antimicrobial treatment is an excellent strategy to treat biofilm-derived infections in a host [41] or, as seen with NO-enhanced chlorine disinfection of *L. pneumophila* biofilms, can be used as a biofilm control and removal strategy in the environment and industrial setting [6, 25]. Thus, further analysis of the NO-based biofilm dispersal by NosP and H-NOX regulatory systems may lead to improvement of current approaches or lead to new strategies to alleviate or prevent harmful biofilms.

Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors declare no conflicts of interest.

References

- 1 Wilson JW, Schurr MJ, LeBlanc CL, Ramamurthy R, Buchanan KL, Nickerson CA. Mechanisms of bacterial pathogenicity. *Postgrad Med J*. 2002 Apr;78(918):216–24.
- 2 Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science*. 1999 May;284(5418):1318–22.
- 3 Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. *Lancet*. 2001 Jul;358(9276):135–8.
- 4 Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A. The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J Clin Microbiol*. 1999 Jun;37(6):1771–6.
- 5 Arora DP, Hossain S, Xu Y, Boon EM. Nitric oxide regulation of bacterial biofilms. *Biochemistry*. 2015 Jun;54(24):3717–28.
- 6 Barraud N, Hassett DJ, Hwang SH, Rice SA, Kjelleberg S, Webb JS. Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. *J Bacteriol*. 2006 Nov;188(21):7344–53.
- 7 Hossain S, Nisbett LM, Boon EM. Discovery of Two Bacterial Nitric Oxide-Responsive Proteins and Their Roles in Bacterial Biofilm Regulation. *Acc Chem Res*. 2017 Jul;50(7):1633–9.
- 8 Nisbett LM, Boon EM. Nitric Oxide Regulation of H-NOX Signaling Pathways in Bacteria. *Biochemistry*. 2016 Sep;55(35):4873–84.
- 9 Li YH, Tian X. Quorum sensing and bacterial social interactions in biofilms. *Sensors (Basel)*. 2012;12(3):2519–38.
- 10 Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev*. 1991 Jun;43(2):109–42.
- 11 Wink DA, Mitchell JB. Chemical biology of nitric oxide: insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Radic Biol Med*. 1998 Sep;25(4-5):434–56.
- 12 Fang FC. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat Rev Microbiol*. 2004 Oct;2(10):820–32.
- 13 MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. *Annu Rev Immunol*. 1997;15(1):323–50.
- 14 Tarr HL. Bacteriostatic action of nitrates. *Nature*. 1941;147(3727):417–8.
- 15 Arai H, Hayashi M, Kuroi A, Ishii M, Igarashi Y. Transcriptional regulation of the flavohemoglobin gene for aerobic nitric oxide detoxification by the second nitric oxide-responsive regulator of *Pseudomonas aeruginosa*. *J Bacteriol*. 2005 Jun;187(12):3960–8.
- 16 Spiro S. Regulators of bacterial responses to nitric oxide. *FEMS Microbiol Rev*. 2007 Mar;31(2):193–211.
- 17 Patra SK, Bag PK, Ghosh S. Nitrosative Stress Response in *Vibrio cholerae*: Role of S-Nitrosoglutathione Reductase. *Appl Biochem Biotechnol*. 2017 Jul;182(3):871–84.
- 18 Cole JA. Anaerobic bacterial response to nitrosative stress. *Adv Microb Physiol*. 2018;72:193–237.
- 19 Bowman LA, McLean S, Poole RK, Fukuto JM. The diversity of microbial responses to nitric oxide and agents of nitrosative stress close cousins but not identical twins. *Adv Microb Physiol*. 2011;59:135–219.
- 20 Chaudhari SS, Kim M, Lei S, Razvi F, Alqarzaee AA, Hutfless EH, et al. Nitrite Derived from Endogenous Bacterial Nitric Oxide Synthase Activity Promotes Aerobic Respiration. *MBio*. 2017 Aug;8(4):8.
- 21 Gusarov I, Shatalin K, Starodubtseva M, Nudler E. Endogenous nitric oxide protects bacteria against a wide spectrum of antibiotics. *Science*. 2009 Sep;325(5946):1380–4.
- 22 Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol*. 2004 Feb;2(2):95–108.
- 23 Cary SP, Winger JA, Derbyshire ER, Marletta MA. Nitric oxide signaling: no longer simply on or off. *Trends Biochem Sci*. 2006 Apr;31(4):231–9.
- 24 Toyofuku M, Yoon SS. Nitric oxide, an old molecule with noble functions in *Pseudomonas aeruginosa* biology. *Adv Microb Physiol*. 2018;72:117–145.

- 25 Barraud N, Storey MV, Moore ZP, Webb JS, Rice SA, Kjelleberg S. Nitric oxide-mediated dispersal in single- and multi-species biofilms of clinically and industrially relevant microorganisms. *Microb Biotechnol*. 2009 May; 2(3):370–8.
- 26 Cutruzzola F, Frankenberg-Dinkel N. Origin and impact of nitric oxide in *Pseudomonas aeruginosa* biofilms. *J Bacteriol*. 2016 Jan; 198(1):55–65.
- 27 Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov*. 2008 Feb;7(2):156–67.
- 28 Conner JG, Teschler JK, Jones CJ, Yildiz FH. Staying alive: vibrio cholerae's cycle of environmental survival, transmission, and dissemination. *Microbiol Spectr*. 2016 Apr;4(2):1–32.
- 29 Yoon SS, Karabulut AC, Lipscomb JD, Hennigan RF, Lyman SV, Groce SL, et al. Two-pronged survival strategy for the major cystic fibrosis pathogen, *Pseudomonas aeruginosa*, lacking the capacity to degrade nitric oxide during anaerobic respiration. *EMBO J*. 2007 Aug;26(15):3662–72.
- 30 Sobko T, Reinders CI, Jansson E, Norin E, Midtvedt T, Lundberg JO. Gastrointestinal bacteria generate nitric oxide from nitrate and nitrite. *Nitric Oxide*. 2005 Dec;13(4):272–8.
- 31 O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. *Annu Rev Microbiol*. 2000;54(1):49–79.
- 32 Römmling U, Balsalobre C. Biofilm infections, their resilience to therapy and innovative treatment strategies. *J Intern Med*. 2012 Dec; 272(6):541–61.
- 33 Abu Khweek A, Amer AO. Factors mediating environmental biofilm formation by *Legionella pneumophila*. *Front Cell Infect Microbiol*. 2018 Feb;8:38.
- 34 Pécastaings S, Allombert J, Lajoie B, Doublet P, Roques C, Vianney A. New insights into *Legionella pneumophila* biofilm regulation by c-di-GMP signaling. *Biofouling*. 2016 Sep; 32(8):935–48.
- 35 Pierce GE. *Pseudomonas aeruginosa*, *Candida albicans*, and device-related nosocomial infections: implications, trends, and potential approaches for control. *J Ind Microbiol Biotechnol*. 2005 Jul;32(7):309–18.
- 36 Arai H. Regulation and function of versatile aerobic and anaerobic respiratory metabolism in *Pseudomonas aeruginosa*. *Front Microbiol*. 2011 May;2:103.
- 37 Yoon SS, Hennigan RF, Hilliard GM, Ochsen UA, Parvatiyar K, Kamani MC, et al. *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. *Dev Cell*. 2002 Oct;3(4):593–603.
- 38 Castiglione N, Rinaldo S, Giardina G, Cutruzzola F. The transcription factor DNR from *Pseudomonas aeruginosa* specifically requires nitric oxide and haem for the activation of a target promoter in *Escherichia coli*. *Microbiology*. 2009 Sep;155(Pt 9):2838–44.
- 39 Yoon MY, Lee KM, Park Y, Yoon SS. Contribution of cell elongation to the biofilm formation of *Pseudomonas aeruginosa* during anaerobic respiration. *PLoS One*. 2011 Jan; 6(1):e16105.
- 40 Hoffman LR, D'Argenio DA, MacCoss MJ, Zhang Z, Jones RA, Miller SI. Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature*. 2005 Aug;436(7054):1171–5.
- 41 Howlin RP, Cathie K, Hall-Stoodley L, Cornelius V, Duignan C, Allan RN, et al. Low-Dose Nitric Oxide as Targeted Anti-biofilm Adjunctive Therapy to Treat Chronic *Pseudomonas aeruginosa* Infection in Cystic Fibrosis. *Mol Ther*. 2017 Sep;25(9):2104–16.
- 42 Henares BM, Higgins KE, Boon EM. Discovery of a nitric oxide responsive quorum sensing circuit in *Vibrio harveyi*. *ACS Chem Biol*. 2012 Aug;7(8):1331–6.
- 43 Derbyshire ER, Marletta MA. Structure and regulation of soluble guanylate cyclase. *Annu Rev Biochem*. 2012;81(1):533–59.
- 44 Plate L, Marletta MA. Nitric oxide-sensing H-NOX proteins govern bacterial communal behavior. *Trends Biochem Sci*. 2013 Nov; 38(11):566–75.
- 45 Iyer LM, Anantharaman V, Aravind L. Ancient conserved domains shared by animal soluble guanylyl cyclases and bacterial signaling proteins. *BMC Genomics*. 2003 Feb;4(1):5.
- 46 Hossain S, Boon EM. Discovery of a Novel Nitric Oxide Binding Protein and Nitric-Oxide-Responsive Signaling Pathway in *Pseudomonas aeruginosa*. *ACS Infect Dis*. 2017 Jun; 3(6):454–61.
- 47 Carlson HK, Vance RE, Marletta MA. H-NOX regulation of c-di-GMP metabolism and biofilm formation in *Legionella pneumophila*. *Mol Microbiol*. 2010 Aug;77(4):930–42.
- 48 Liu N, Xu Y, Hossain S, Huang N, Coursolle D, Gralnick JA, et al. Nitric oxide regulation of cyclic di-GMP synthesis and hydrolysis in *Shewanella woodyi*. *Biochemistry*. 2012 Mar; 51(10):2087–99.
- 49 Römmling U, Galperin MY, Gomelsky M. Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiol Mol Biol Rev*. 2013 Mar;77(1):1–52.
- 50 Rutherford ST, Bassler BL. Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harb Perspect Med*. 2012 Nov;2(11). pii: a012427.
- 51 Zhu J, Mekalanos JJ. Quorum sensing-dependent biofilms enhance colonization in *Vibrio cholerae*. *Dev Cell*. 2003 Oct;5(4):647–56.
- 52 Williams DE, Nisbett LM, Bacon B, Boon E. Bacterial heme-based sensors of nitric oxide. *Antioxid Redox Signal*. 2018 Dec;29(18):1872–1887.
- 53 Nelson EJ, Harris JB, Morris JG Jr, Calderwood SB, Camilli A. Cholera transmission: the host, pathogen and bacteriophage dynamic. *Nat Rev Microbiol*. 2009 Oct;7(10):693–702.
- 54 Waters CM, Lu W, Rabinowitz JD, Bassler BL. Quorum sensing controls biofilm formation in *Vibrio cholerae* through modulation of cyclic di-GMP levels and repression of vpsT. *J Bacteriol*. 2008 Apr;190(7):2527–36.
- 55 Shikuma NJ, Fong JC, Odell LS, Perchuk BS, Laub MT, Yildiz FH. Overexpression of VpsS, a hybrid sensor kinase, enhances biofilm formation in *Vibrio cholerae*. *J Bacteriol*. 2009 Aug;191(16):5147–58.
- 56 Plate L, Marletta MA. Nitric oxide modulates bacterial biofilm formation through a multi-component cyclic-di-GMP signaling network. *Mol Cell*. 2012 May 25;46(4):449–60.
- 57 Levet-Paulo M, Lazzaroni J-C, Gilbert C, Atlan D, Doublet P, Vianney A. The atypical two-component sensor kinase Lpl0330 from *Legionella pneumophila* controls the bifunctional diguanylate cyclase-phosphodiesterase Lpl0329 to modulate bis-(3'-5')-cyclic dimeric GMP synthesis. *J Biol Chem*. 2011 Sep; 286(36):31136–31144.
- 58 Barraud N, Schleheck D, Klebensberger J, Webb JS, Hassett DJ, Rice SA, et al. Nitric oxide signaling in *Pseudomonas aeruginosa* biofilms mediates phosphodiesterase activity, decreased cyclic di-GMP levels, and enhanced dispersal. *J Bacteriol*. 2009 Dec;191(23):7333–42.
- 59 Li Y, Heine S, Entian M, Sauer K, Frankenberg-Dinkel N. NO-induced biofilm dispersion in *Pseudomonas aeruginosa* is mediated by an MHYT domain-coupled phosphodiesterase. *J Bacteriol*. 2013 Aug;195(16):3531–42.