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Nitric Oxide Regulation of Bacterial Biofilms

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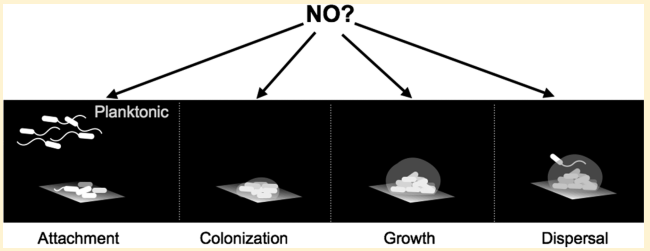
ABSTRACT: Biofilms are surface-associated, multicellular communities of bacteria. Once established, they are extremely difficult to eradicate by antimicrobial treatment. It has been demonstrated in many species that biofilm formation may be regulated by the diatomic signaling molecule nitric oxide (NO). Although this is still a relatively new area of research, we review here the literature reporting an effect of NO on bacterial biofilm formation, emphasizing dose-dependent responses to NO concentrations when possible. Where it has been investigated, the underlying NO sensors or signaling pathways are also discussed. Most of the examples of NO-mediated biofilm regulation have been documented with exogenously applied NO, but we also survey possible natural sources of NO in biofilm regulation, including endogenously generated NO. Finally, because of the apparent broad-spectrum, antibiofilm effects of NO, NO-releasing materials and prodrugs have also been explored in this minireview.



NITRIC OXIDE AS A SIGNALING MOLECULE

Nitric oxide (NO) is a diatomic, uncharged gas molecule with one unpaired electron. At high concentrations, NO is a poisonous gas. At low concentrations, however, NO has come to be known as a vital signaling molecule in eukaryotic biology,[1−5] for which it was selected in 1992 as *Science Magazine*'s Molecule of the Year.[6] In mammals, NO is produced from L-arginine by nitric oxide synthases (NOSs).[7−9] As a highly diffusible gas molecule, NO is ideal for intracellular signaling and is able to act in adjacent cells where it binds its specific protein receptor, an enzyme called soluble guanylyl cyclase (sGC). sGC binds NO at the ferrous iron center of a protoporphyrin IX heme cofactor; upon ligation of NO, the enzymatic conversion of guanosine triphosphate (GTP) to second-messenger cyclic guanosine 3′,5′-monophosphate (cGMP) is increased several hundred-fold above basal levels.[10] cGMP has an array of downstream targets, and its increased level of production results in the regulation of calcium channels and protein phosphorylation cascades to ultimately regulate essential physiological processes such as blood flow, platelet aggregation, myocardial function, tissue formation, and angiogenesis, to name a few.[1,2,4,7,11] As a testament to the importance of NO signaling in human health, impaired NO signaling can play a role in a wide variety of ailments, including cardiovascular disease, diabetes, erectile dysfunction, and cancer.

Because of the clinical implications of NO signaling, most of the research on the biological function of NO has focused on its role in eukaryotes. In the past several decades, however, bacterial detection of NO has emerged as a widespread phenomenon and fascinating area of study. NO is toxic to bacteria at high concentrations (approximately micromolar). This is, in fact, one line of defense that NO-producing eukaryotes have against bacterial infection.[12,13] Bacteria may also encounter relatively high concentrations of NO during denitrification, a process by which some bacteria can respire on nitrate or nitrite under oxygen-limiting conditions. Many NO-responsive bacterial proteins involved in the elimination of NO through denitrification and detoxification pathways have been characterized, including FNR-like transcription factors,[14] the NO-responsive transcriptional activator NorR,[15] and the nitrite-sensitive repressor NsrR.[16] These and other NO-responsive sensors and regulatory proteins have been reviewed previously and are not the subject of this review.[17−24]

Our laboratory has instead been fascinated by an increasing number of reports in which bacteria apparently respond to relatively low, nontoxic, concentrations of NO (approximately nanomolar to micromolar) to elicit physiological responses other than those involved in denitrification and detoxification, i.e., other than processes primarily aimed at the elimination of NO from the cell.[25−31] In this review, we focus on this new, or perhaps overlapping, paradigm for the role of NO in bacteria. It is likely that these two broadly outlined roles of NO overlap and provide a continuum of bacterial responses. For example, in *Escherichia coli*, the transcription factors NorR and NsrR have both been implicated in NO removal;[23] however, NorR exhibits nanomolar sensitivity to NO,[15] and NsrR has been linked to bacterial motility in addition to its role in NO elimination.[32]

This duality of NO signaling in bacteria is analogous to mammalian responses to NO. Endothelial cells produce NO at

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low concentrations, and it is used for intercellular signaling upon ligation to sGC, as previously indicated.[10] Macrophages, however, produce high concentrations of NO to kill tumor cells and invading pathogens, but this NO also results in damage to mammalian cells. Mammalian cells are therefore also able to respond to NO present at concentrations higher than that needed to activate sGC.[12,13,33,34]

Concurrent with reports that, like mammals, bacteria may also respond to low concentrations of NO, homologues of both NOS[35] and the heme domain of sGC[36] have been discovered in bacteria, reinforcing the possibility of sensitive and selective NO signal transduction circuits in bacteria, and helping to catalyze intensive research efforts focused on a more complete understanding of the role of NO in bacteria. Although our understanding of the role of NO in bacterial signal transduction is in its infancy, from the available data, a common observation is that, aside from detection of NO for the purpose of its elimination, bacteria use NO-mediated signal transduction for regulating biofilm formation.[37,38] Interestingly, even within the biofilm-related NO-dependent phenotypes that have been observed, there appear to be NO concentration dependencies, which we will explore below. To be clear, NO is not the only known regulator of biofilms. There is also no evidence that it is a master regulator of biofilms. Furthermore, low concentrations of NO may also regulate additional bacterial processes other than biofilm. On the basis of the current evidence, however, there does appear to be a strong link between NO signaling and biofilm formation; this review will focus on those studies.

BACTERIAL BIOFILMS

Most bacteria have a strong predisposition for growth in a sessile multicellular community called a biofilm.[39−43] Biofilms are matrix-encapsulated microbial colonies that adhere to all kinds of surfaces, including soil, medical implants, tissue, metals, and plastics; biofilms form on just about any surface in a moist environment. Biofilms can be formed by a single bacterial species, but more often than not, they consist of many bacterial species as well as other microbes such as fungi, algae, and protozoa. These slimy films are formed when bacteria attach themselves to a surface and secrete a hydrated polymeric substance, often called the exopolysaccharide (EPS) matrix.[44] This matrix serves many purposes: it cements the colony to the surface, attracts new members, helps to capture and concentrate nutrients, and offers protection from the surrounding environment.

Biofilms constitute a widespread and extremely persistent form of bacterial growth.[45−47] The biofilm community is able to conduct diverse functions, some harmful to and some beneficial to humans. These functions are distinct from those conducted by planktonic cells. In the environment, biofilms promote remediation of contaminated groundwater and soil; however, they also cause persistent biofouling of ship hulls, underwater sensors, and oil and water transport and storage containers. Of clinical interest, biofilms in the human body, including those lining surfaces in the mouth and the gastrointestinal tract, can both promote and harm human health. For example, biofilms coating the intestinal lining contribute to the production of vitamins and digestion of food,[48] but they can also harbor pathogens and contribute to inflammatory diseases such as irritable bowel syndrome.[49] Other examples of clinically relevant biofilms include dental plaque, responsible for tooth decay and gum disease, and bacterial biofilms that form on medical equipment, which are a major cause of secondary infections contracted in medical facilities. Disease-causing biofilms are particularly problematic because host immune systems are, typically, unable to respond to biofilm infections. In addition, current antibiotic solutions are largely ineffective against these compact and well-protected structures; cells in a biofilm are 10−1000-fold more protected from antibiotics than planktonic cells.[50,51]

Although biofilms are the most abundant form of bacterial growth, they are currently poorly understood. Significant effort is being spent to improve our understanding of the fundamental processes involved in the biofilm life cycle and to develop new antibiofilm agents for practical and therapeutic use. Biofilm regulation is likely multifactorial and complex, and understanding discrete contributing factors is expected to have a significant impact.[52] NO has emerged as an important factor in regulating biofilm formation (Figure 1). The molecular details of these NO-regulated signal transduction pathways are also not well understood, but current evidence indicates that NO likely mediates changes in the biofilm through two-component signaling, cyclic-di-GMP signaling (cyclic diguanylate; 3′,5′-cyclic diguanylic acid; c-di-GMP), and/or quorum sensing. These are briefly described below.

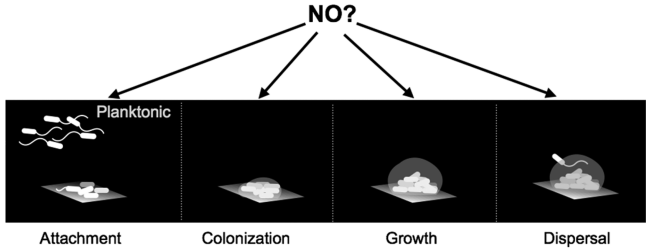

Figure 1. During biofilm development, free-swimming planktonic cells settle on a surface and within minutes lose their flagella and begin to excrete an extracellular polysaccharide matrix. Over hours to days, this structure grows as bacteria divide and more microbes join the colony. The final stage in biofilm development is dispersal. Recent evidence suggests that NO is involved in the regulation of bacterial biofilms.

Two-Component Signal Transduction. Two-component signal transduction is an important mechanism for stimulus response in bacteria.[53,54] In its simplest form, a two-component signaling pathway consists of a sensor histidine kinase and a response regulator. The variable sensor domain of the histidine kinase is responsible for detecting a stimulus, which results in a change in the autophosphorylation state of the histidine kinase.[55] The signal is relayed downstream by means of the transfer of phosphoryl from the histidine in the kinase domain to an aspartic acid in the receiver domain of the response regulator. The activated response regulator then elicits a response to the initial stimulus. However, many two-component systems deviate from this canonical system.[55,56] For example, in three-component systems, an accessory protein that detects a signal and regulates kinase autophosphorylation *in trans* replaces the kinase sensor domain. In hybrid signaling, the histidine kinase has a receiver domain; thus, a His-to-Asp phosphotransfer takes place within the hybrid kinase, and then a histidine-containing phosphotransfer protein is required to relay the signaling phosphate from the histidine kinase to the appropriate response regulator in two additional phosphotransfer steps.[57]

Quorum Sensing. Biofilm formation is one of many behaviors bacteria coordinate as a group. Key to understanding

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Table 1. Effect of Nitric Oxide on Bacterial Biofilms

species	NO source	NO donor concentration	approximate NO concentration	ref	effect on biofilm	growth mode
			Gram-Negative			
Pseudomonas aeruginosa	SNP	25 nM to 2.5 mM	0.025−2500 nM[a]	25	decreases	aerobic
P. aeruginosa	SNP	>25 mM	>25000 nM[a]	25	enhances	aerobic
Vibrio cholerae	SNP	25−500 nM	0.025−0.5 nM[a]	31	decreases	aerobic
Serratia marcescens	SNP	25−500 nM	0.025−0.5 nM[a]	31	decreases	aerobic
E. coli	SNP	500 nM	0.5 nM[a]	31	decreases	aerobic
E. coli	DETA NONOate	100 μM	∼100−300 nM	32	decreases	aerobic
Fusobacterium nucleatum	SNP	1−10 μM	1−10 nM[a]	31	decreases	aerobic
Acinetobacter baumannii	gaseous NO	200 ppm	∼7000000 nM[b]	135	decreases	aerobic
Nitrosomonas europaea	gaseous NO	>30 ppm	>1000000 nM[b]	30	decreases	aerobic
N. europaea	gaseous NO	<5 ppm	<170000 nM[b]	30	enhances	aerobic
Shewanella woodyii	DETA NONOate	200 μM	∼50 nM[b]	28	decreases	aerobic
Vibrio harveyi	DPTA NONOate	50 μM	40−80 nM[b]	110	enhances	aerobic
V. harveyi	DPTA NONOate	100 μM	100−300 nM[b]	110	decreases	aerobic
Neisseria gonorrheae	SNP	500 nM	0.5 nM[a]	86	decreases	aerobic
Ne. gonorrheae	SNP	20 μM	20 nM[a]	87	enhances	anaerobic
Shewanella oneidensis	DETA NONOate	200 μM	400−2700 nM[b]	29	enhances	anaerobic
S. oneidensis	DPTA NONOate	50 μM	∼100 nM[b]	*c*	decreases	aerobic
			Gram-Positive			
Bacillus licheniformis	SNP	100−500 nM	0.1−0.5 nM[a]	31	decreases	aerobic
Staphylococcus epidermidis	SNP	10 μM	10 nM[a]	31	decreases	aerobic
Staphylococcus aureus	gaseous NO	200 ppm	∼7000000 nM[b]	135	decreases	aerobic
St. aureus	DETA NONOate	1−1000 μM	>125000 nM	88	decreases	aerobic
St. aureus	DETA NONOate	1−1000 μM	∼900−2000 nM	88	enhances	aerobic
			Multispecies Biofilm			
from water system	SNP	100−500 nM	0.1−0.5 nM[a]	31	decreases	aerobic

[a]The concentration of NO released by SNP is calculated according to the measurements reported in ref 31. [b]Concentration of NO directly measured. [c]Unpublished data from the Boon laboratory.

the group behavior of bacteria is understanding the ability of bacteria to communicate with each other, as well as their environment, by exchanging chemical signals. Quorum sensing (QS), as the phenomenon is termed, involves the production and detection of small molecules known as autoinducers. Detection of autoinducers in the environment allows cells to gauge cell density. Arrival at a critical population size or "quorum" triggers a signaling cascade that ultimately regulates gene expression.[58−60] In many organisms, this signaling cascade involves His-to-Asp phosphotransfer similar to what is described above. QS regulates changes in global gene expression of entire bacterial communities affecting behaviors such as virulence, biofilm formation, bioluminescence, and antibiotic resistance, all activities that are best served by large population sizes.

Cyclic-di-GMP Signaling. It has become apparent that a wide variety of bacteria use c-di-GMP to regulate biofilm formation.[61−64] Indeed, many of the histidine kinase pathways mentioned above ultimately regulate c-di-GMP concentrations. Generally speaking, as the intracellular concentration of c-di-GMP increases, a higher percentage of bacteria enter biofilm modes; as the intracellular concentration of c-di-GMP decreases, bacteria become motile or more virulent. Although this is a relatively new area of inquiry, the emerging theme is that the total concentration of intracellular c-di-GMP is tightly regulated by a variety of enzymes that both synthesize and degrade c-di-GMP. c-di-GMP is synthesized by proteins with diguanylate cyclase activity, which can be identified by a conserved GGDEF (or GGEEF) amino acid sequence.[65] Proteins with phosphodiesterase activity degrade c-di-GMP. These are identified by a conserved EAL or HD-GYP amino acid motif.[66,67] The details of c-di-GMP regulation in bacteria are under investigation, and the current evidence indicates that c-di-GMP synthase and phosphodiesterase activities are controlled by signal transduction. GGDEF-, EAL-, and HD-GYP-containing domains are invariably found to be associated with sensing domains specific for a certain stimulus. Most of the downstream targets of c-di-GMP discovered to date include proteins involved in production and maintenance of the exopolysachharide (EPS) matrix and proteins involved in motility. These mechanisms have recently been reviewed in great detail.[64]

NITRIC OXIDE REGULATION OF BIOFILM FORMATION

A summary of documented NO-mediated biofilm regulation responses is listed in Table 1. As noted above, in several species, distinct concentration-dependent biofilm-related responses to NO have been documented. The better understood examples of NO-dependent biofilm phenotypes are described in more detail below. In several cases, it is difficult to definitively separate NO detoxification and denitrification responses from NO signaling responses. For example, an increase in the level of biofilm formation may be attributed to a defensive bacterial growth mode. Likewise, it is also possible that at high NO concentrations, an observed reduction in biofilm biomass is due to cell death. Nonetheless, in many bacteria, NO is observed to cause biofilm dispersal at low NO concentrations, which is not consistent with a defense mechanism.

NO Regulation of a Biofilm in *Nitrosomonas europaea*. One of the first documented observations of the effect of NO on biofilm formation was documented in *N. europaea*, a

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bacillus-shaped, Gram-negative, obligate chemolithoauxotroph. Because *N. europaea* oxidize ammonia for energy production, they are commonly found in sewage and untreated water. In *N. europaea*, it has been shown that NO levels above 30 ppm (~1 mM) result in biofilm formation and below 5 ppm (<200 μM) in biofilm dispersal, consistent with a separate, non-stress-related response to NO at lower concentrations.[30] The bacteria were grown in a chemostat laboratory reactor so that the gas mixtures in the chamber could be precisely defined. NO was applied to the bacteria as the pure gas, and its concentration was monitored with a nitric oxide analyzer (Eco Physics, Dürnten, Switzerland). The oxygen concentration in these experiments was maintained at 2.6 mg/L. Proteomics experiments revealed 11 proteins that are differentially expressed at the two NO concentrations. Notably, the flagellar and flagellar assembly proteins were among those modulated by NO and expressed at higher levels in planktonic cells, which is consistent with the observed change in growth mode. Although the underlying biochemical pathway(s) responsible for this NO-induced phenotype remains unknown, it is an NO-specific phenomenon; ammonium ion, nitrite, O_2, temperature, and pH have no significant effect on the growth mode or proteome of *N. europaea*.

NO Regulation of a Biofilm in *Pseudomonas aeruginosa*. The most extensively studied example of NO-dependent biofilm regulation is in *P. aeruginosa*. *P. aeruginosa* is a principal pathogen in cystic fibrosis and many hospital-acquired infections, and its pathogenicity is linked to biofilm formation.[45] In an early study of the effect of NO on biofilm formation, it was shown that *P. aeruginosa* remain in the biofilm state until exposed to 25 nM to 2.5 mM sodium nitroprusside (SNP), which corresponds to approximately 0.025−2500 nM NO, at which point they revert to a free-swimming, planktonic lifestyle.[25] When the bacteria are exposed to NO concentrations greater than ~25 μM, however, biofilm formation was enhanced relative to biofilm formation in the absence of NO. Thus, in *P. aeruginosa*, nanomolar NO is seen to cause biofilm dispersal. In addition, it was demonstrated that *P. aeruginosa* lacking the *rhl* quorum sensing circuit die because of overproduction of NO;[68] quorum sensing is a critical mechanism involved in the regulation of biofilm formation in *P. aeruginosa*,[69] as well as many other bacteria.[58,60,70−72]

It should be noted that SNP is used as a NO donor in many of the studies reviewed here (Table 1). Effective concentrations of NO are estimated to be 1000 times lower than the concentration of NO donor used.[31] For example, 25 μM SNP is expected to produce ~25 nM NO in solution.[25] While it certainly releases NO, SNP can also release cyanide and has been linked to nitrosative stress independent of NO production.[22] Therefore, studies in which SNP has been used as an NO donor require some caution before one can conclude that NO causes the observed effect.

It has been suggested that the response of *P. aeruginosa* to NO is linked to its denitrification abilities. For example, biofilms of a *P. aeruginosa* mutant lacking nitrite reductase (Δ*nirS*) do not disperse; NirS generates NO from nitrite under anaerobic conditions. However, a NO reductase mutant (Δ*norCB*) exhibits greatly enhanced dispersal.[25] Furthermore, the *P. aeruginosa* transcription factors ANR and DNR help regulate a network of genes needed to respond to low oxygen tension and the presence of nitrate, nitrite, and other N-oxides.[73,74] Interestingly, DNR, a member of the FNR family, has been demonstrated to be a heme protein capable of binding NO.[75] Its affinity for NO appears to be in the micromolar range, however, and thus, DNR is likely not responsible for the observation that low nanomolar concentrations of NO can disperse *P. aeruginosa* biofilms.

Little is known about the detailed molecular mechanism of *P. aeruginosa* biofilm dispersal, but it appears that this response to NO is a metabolic response and not a toxic response. Exposure to NO is associated with the regulation of intracellular levels of c-di-GMP through the sophisticated interaction of many diguanylate cyclases and phosphodiesterases.[76] However, of the proteins known to play a role in NO-mediated biofilm dispersal in *P. aeruginosa*, it is unclear if any of them serve as a primary, sensitive NO sensor. A chemotaxis transducer BdlA (biofilm dispersal locus A) has been implicated in c-di-GMP degradation and biofilm dispersal upon detection of NO through a currently unknown mechanism.[76,77] The PASa domain of BdlA can bind heme and may detect NO.[78] However, BdlA appears to also respond to many environmental cues in addition to NO, including succinate, Ag^+, Hg^{2+}, and As^{3+}. The c-di-GMP synthase GcbA has also been implicated in NO-induced biofilm dispersal because it contributes to the activation of BdlA, but it does not appear to bind NO itself.[79] The c-di-GMP phosphodiesterases DipA[80] and NbdA[81] have been linked to decreasing cyclic-di-GMP concentrations upon exposure to NO. However, the mechanism of action for both is NO-induced upregulation of *dipA* and *nbdA* expression, suggesting they each act downstream of initial NO sensing. Nonetheless, a bioinformatics study has suggested that conserved Met and His residues in NbdA could coordinate with copper ions and potentially sense NO.[82]

Other Examples of Clinically Relevant NO-Regulated Biofilm Formation. NO-mediated regulation of biofilm formation was first observed in *N. europaea*[30] and *P. aeruginosa*,[25] but NO is now generally considered to regulate biofilm formation in a wide variety of bacteria (Table 1).[31,38] A few additional representative examples of NO-mediated biofilm formation are discussed below.

For example, *Ne. gonorrheae*, the causative agent for gonorrhea, has been shown to form biofilms during cervical infection. As a human pathogen, *Ne. gonorrheae* has evolved the ability to resist NO-mediated killing (from host inducible nitric oxide synthase) and reduce intracellular NO levels using NorB, a respiratory nitric oxide reductase.[83] Furthermore, *Ne. gonorrheae* can grow under anaerobic conditions in the presence of nitrite, and NorB is also responsible for the reduction of NO produced by the respiratory nitrite reductase AniA.[84] Thus, *Ne. gonorrheae* encodes NO binding proteins such as NsrR[85] that respond to NO for denitrification or detoxification purposes. In terms of NO-responsive biofilm phenotypes, one study found both *norB* and *aniA* insertion mutants of *Ne. gonorrheae* strain 1291 to be defective in biofilm formation, which led the authors to conclude that biofilm formation is linked to NO concentration.[86] Interestingly, in the same study, it was demonstrated that application of a very low concentration of NO, 500 nM SNP (~500 pM NO), at the beginning of biofilm growth (under aerobic conditions where NorB is not expected to be expressed) resulted in a significant reduction in biomass. In a follow-up study, however, it was demonstrated that *Ne. gonorrheae* biofilms are tolerant to high concentrations of NO (up to 20 μM SNP, ~20 nM) if grown under anaerobic conditions where *norB* is expressed, but nitrite has been removed.[87] This may be due to the fact that *Ne. gonorrheae* can use NO as a substrate for anaerobic growth when nitrite is

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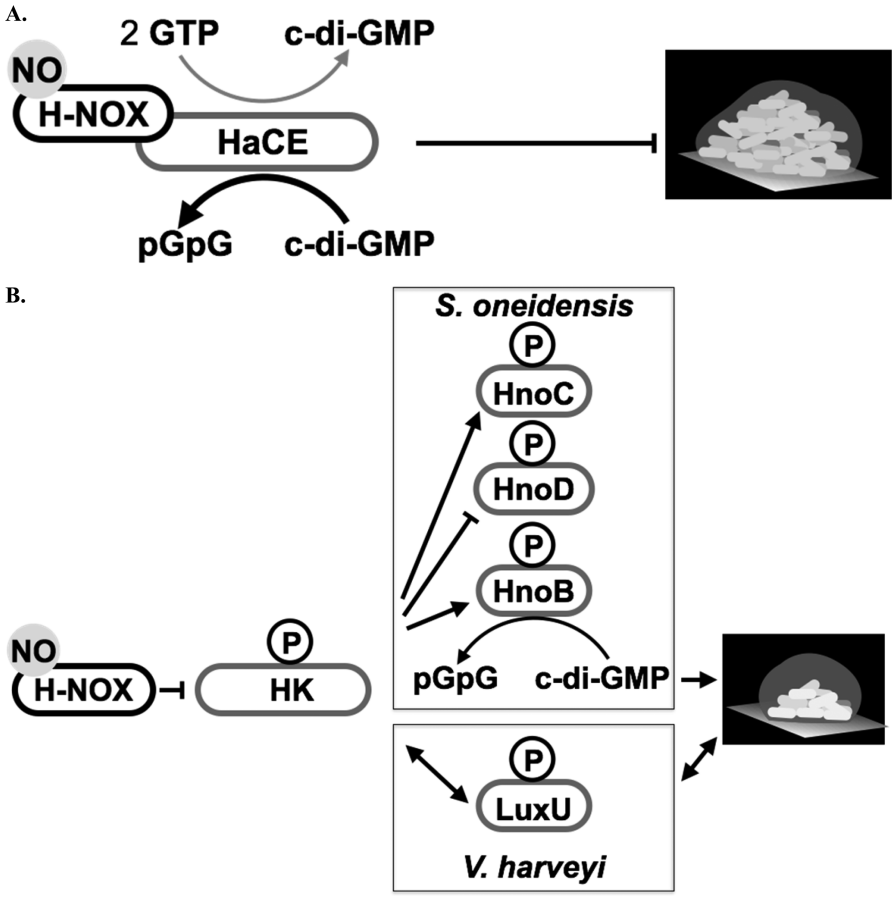

Figure 2. NO regulates biofilm formation through ligation to H-NOX. (A) NO-bound H-NOX directly regulates both the c-di-GMP synthesis and phosphodiesterase activities of a bifunctional HaCE in *Shewanella woodyi*. A decreased c-di-GMP concentration leads to biofilm repression in the presence of NO. (B) NO-bound H-NOX directly regulates histidine kinase autophosphorylation, which ultimately regulates downstream biofilm formation through c-di-GMP signaling in *Shewanella oneidensis* and quorum sensing in *Vibrio harveyi*.

limited. Taken together, the authors of these studies suggest that when NO is present at sublethal concentrations, either under aerobic conditions or when *norB* is expressed in the presence of nitrite, biofilm formation is inhibited, implying that *Ne. gonorrheae* is capable of sensing and responding to low concentrations of NO. No pico- or nanomolar NO sensors have been described for *Ne. gonorrheae* to date, however.

In *Staphylococcus aureus*, a causative agent for rhinosinusitis, a recent study found that ∼10-fold less biofilm biomass accumulates in the presence of relatively high NO concentrations (∼125−1000 μM as estimated from the concentration of the NO donor)[88] than in the absence of NO. In this study, the NO donor used was a NONOate. NONOates are *N*-diazeniumdiolate compounds that are stable as solids but release NO in solution at neutral or acidic pH; as such, they are commonly used as NO-donating reagents.[89] In contrast, when *S. aureus* biofilms were exposed to lower levels of NO (0.9−2 μM), biofilm biomass increased. Interestingly, in a healthy individual, a normal concentration of sinonasal NO is considered to be between 6 and 25 ppm (∼200 and 830 μM),[90] while the NO levels in patients with rhinosinusitis are usually much lower, between 0.021 and 0.07 ppm (∼0.7 and 2.3 μM).[91] The mechanism for this dual nature of NO-dependent biofilm regulation is not yet understood, but these results suggests that *S. aureus* can sense NO in a concentration-dependent manner and, further, that NO may be physiologically important for regulating the human microbiome.

In addition to the examples discussed above, NO regulation of biofilm formation has been documented in *Legionella pneumophila* and several species of *Shewanella* and *Vibrio*. In these organisms, however, unlike those described above, significantly more is currently understood about the mechanism of NO sensing and signal transduction. Here, the primary NO sensor has been demonstrated to be a member of the H-NOX family. These sensors will be described below.

A PRIMARY NO SENSOR IN BACTERIA: THE H-NOX FAMILY

A family of bacterial heme proteins with as much as 40% primary sequence identity to the heme domain from mammalian sGC has been identified on the basis of genomic analysis.[36] We and others have confirmed this discovery through biochemical characterization of predicted bacterial family members.[27−29,92−102] They all have ligand binding properties similar to those of sGC, confirming their membership in a family of sensitive and selective NO-sensing proteins. H-NOX family members are able to discriminate between NO and molecular oxygen as ligands; the molecular basis of ligand discrimination has been debated.[94,103,104] Nonetheless, this ability to discriminate between ligands is essential to the role of H-NOX role as a NO sensor.

H-NOX domains have now been predicted in the genomes of more than 300 sequenced species from all subgroups of bacteria. Generally, H-NOX genes are encoded in putative

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operons with signaling proteins such as two-component histidine kinases, GGDEF diguanylate cyclases, and/or EAL and HD-GYP c-di-GMP phosphodiesterases. Histidine kinases found in NO/H-NOX signal transduction pathways are collectively termed HaHKs (H-NOX-associated histidine kinase), and c-di-GMP synthases/phosphodiesterases in NO/H-NOX signaling pathways are collectively termed HaCEs (H-NOX-associated cyclic-di-GMP processing enzymes). Although the function of H-NOX proteins is not yet fully understood, from currently available data, it appears they are bacterial NO sensors that broadly regulate biofilm formation and other bacterial community behaviors such as quorum sensing and symbiosis. H-NOX proteins have been recently reviewed;[105] thus, here we only revisit NO/H-NOX signaling in the context of NO regulation of biofilm formation (Figure 2).

NO/H-NOX Regulation of Biofilm Formation in *L. pneumophila*. The first published observation of biofilm regulation by an NO/H-NOX signaling pathway was in *L. pneumophila*.[97] *L. pneumophila* encodes two H-NOX proteins, one cocistronic with a HaHK and one cocistronic with a HaCE. The HaCE in *L. pneumophila* has both GGDEF and EAL domains but was found to have only c-di-GMP synthase and not phosphodiesterase activity. NO-bound H-NOX was shown to inhibit this cyclase activity, thereby inhibiting the formation of c-di-GMP. The authors showed that deletion of the HaCE-adjacent H-NOX gene resulted in a *L. pneumophila* strain with a hyperbiofilm phenotype. This phenotype was reversed by deletion of the associated HaCE, suggesting that this pathway, and therefore NO, plays an essential role in biofilm regulation of *L. pneumophila*.

NO/H-NOX Regulation of Biofilm Formation in *S. woodyi*. In contrast to the HaCE from *L. pneumophila*, the HaCE from *S. woodyi* has been characterized as an enzyme with dual activity,[106] capable of acting as both diguanylate cyclase and phosphodiesterase. Using purified proteins, we have shown that *S. woodyi* H-NOX and HaCE are binding partners.[28,107] Bound to H-NOX in the absence of NO, HaCE behaves primarily as a c-di-GMP synthase, but bound to NO-H-NOX, HaCE acts primarily as a phosphodiesterase.[28] Consistent with these results, we found that 50 nM NO, delivered from a NONOate, causes a decrease in both the level of biofilm formation and *in vivo* c-di-GMP concentration, a phenotype that is absent in the *hnoX* deletion strain, confirming that NO affects biofilm formation in *S. woodyi* via H-NOX/c-di-GMP signaling.

NO/H-NOX Regulation of Biofilm Formation in *S. oneidensis*. *S. oneidensis* encodes one H-NOX gene cocistronic with a HaHK. With purified proteins, it was shown that the autophosphorylation activity of HaHK is regulated by the ligation state of H-NOX.[101] We identified and biochemically characterized the first response regulator cognate to an H-NOX-associated histidine kinase (HaRR; H-NOX-associated response regulator) from *Pseudoalteromonas atlantica*, an organism with an H-NOX/HaHK operon[92] homologous to that in *S. oneidensis*. This response regulator was identified by use of bioinformatic tools and is annotated as an HD-GYP c-di-GMP phosphodiesterase. A multiple-sequence alignment revealed degeneracy, however, and we found that this HD-GYP HaRR lacks phosphodiesterase activity. It does, however, exhibit autophosphatase activity,[92] presumably as a means of autoregulation, as seen with other response regulators.[108] It is possible that this HD-GYP HaRR indirectly manipulates intracellular c-di-GMP levels via a binding event. Subsequently, the homologous HD-GYP HaRR, as well as two additional HaRRs, an EAL-type c-di-GMP phosphodiesterase, and a helix−turn−helix transcription factor were identified and characterized in *S. oneidensis*.[29] Further, it was documented that c-di-GMP hydrolysis by the EAL HaRR was inhibited by NO-bound H-NOX, not only directly by inhibiting HaHK phosphorylation of this EAL response regulator but also indirectly by the unphosphorylated HD-GYP HaRR. The authors reported further that ∼200 μM DETA NONOate (the authors report production of ∼400 to ∼2700 nM NO under these conditions) caused an increase in *S. oneidensis* biofilm biomass, hypothesizing that biofilm formation is a defense mechanism against environmental NO.[29] It should be noted, however, that this biofilm growth was conducted anaerobically, which is not ideal for *S. oneidensis* biofilm formation; in fact, an anaerobic environment has been reported to cause rapid dispersal of *S. oneidensis* biofilms.[109] When *S. oneidensis* is grown aerobically in the presence of lower NO concentrations (∼100 nM from NONOate), our laboratory found that NO actually inhibits its biofilm formation (unpublished results). This discrepancy could be due to aerobic versus anaerobic conditions or the difference in NO concentration. These findings in *S. oneidensis* highlight the complexity of multi-component signaling networks in bacteria, suggesting stringent regulation of biofilm formation.

NO/H-NOX Regulation of Quorum Sensing and Biofilm Formation in *V. harveyi*. *V. harveyi* is an extremely well-studied model organism for QS because cultures become bioluminescent via luciferase expression in response to a high cell density (*V. harveyi* QS is reviewed in ref 60). *V. harveyi* has at least three QS circuits that together regulate the phosphorylation state of a phosphotransfer protein called LuxU, which regulates synthesis of the QS master transcriptional regulator LuxR, ultimately regulating bioluminescence and other QS responses. Unexpectedly, several years ago, we demonstrated that 50 nM NO from NONOate caused a significant increase in *V. harveyi* bioluminescence,[27] suggesting NO may affect QS.

Interestingly, several species of *Vibrio* bacteria have a predicted *hnoX* gene in the same putative operon as a gene that encodes a soluble histidine kinase that is highly identical to the kinase domain of one of the *V. harveyi* quorum-sensing kinases, LuxQ. These specific H-NOX-associated histidine kinases are termed HqsK (H-NOX-associated quorum-sensing kinase). We were able to demonstrate that in *V. harveyi*, H-NOX participates in the LuxU/LuxR QS pathway by regulating the activity of HqsK,[27] which like the other QS kinases is able to exchange phosphate with LuxU. We further showed that NO-bound H-NOX regulates HqsK autophosphorylation and phospho-exchange with LuxU, providing a mechanistic explanation for our observation that NO affects QS. In a subsequent study, we found that NO regulates biofilm formation and flagellar synthesis via H-NOX/HqsK in a dose-dependent manner.[110]

NO/H-NOX Regulation of Symbiosis in *Vibrio fischeri*. Another organism with an H-NOX/HqsK signaling pathway is *V. fischeri*, a bioluminescent marine bacterium that forms a specific symbiotic relationship with *Euprymna scolopes*, the Hawaiian bobtail squid. Squid-generated NO is thought to play a role in establishing this symbiosis.[26,111] NO is normally detectable in the ducts, appendages, and mucus lining of the squid, but upon colonization by *V. fischeri*, NO production is weakened. Furthermore, when the squid is treated with NO

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Biochemistry 2015, 54, 3717−3728

scavengers during incubation with *V. fischeri*, aggregates of *V. fischeri* are significantly larger (50−200 μm in diameter) than their normal size in the presence of NO (<20 μm in diameter), suggesting a role for NO in the regulation of bacterial clumping, which is likely related to biofilm formation.

The role of H-NOX as an NO sensor involved in colonization has been investigated. Surprisingly, an *hnoX* deletion strain of *V. fischeri* is able to outcompete the wild-type strain for colonization of the squid light organ.[102] It is currently hypothesized that NO/H-NOX is involved in the tight regulation of iron acquisition in *V. fischeri*. The light organ is an iron-deficient environment, so bacteria tend to scavenge the nutrient upon colonization. High concentrations of iron can be toxic to growth, so NO/H-NOX may be involved in the tight regulation of iron uptake. This explains why an *hnoX* deletion would display an initial growth advantage upon colonization because of the absence of this strict regulation of iron uptake. In support of this hypothesis, the *hnoX* deletion strain loses fitness after a longer time, presumably because of the absence of iron uptake regulation.

SYNTHESIS OF NITRIC OXIDE IN BACTERIA

One of the major outstanding questions about the role of NO in bacteria is the source of the NO signal. The *V. fischeri*/*Eu. scolopes* symbiosis suggests that, at least in some cases, NO may serve as an interkingdom signal, generated by eukaryotic NOS enzymes. Another possible source is NO produced by nitrite reductases, which are used by facultative anaerobic bacteria in the presence of nitrate/nitrite under anaerobic conditions. Interestingly, nitrate/nitrite-reducing bacteria have been suggested to be an important mammalian symbiont with roles in the nitrogen cycle.[112,113] Another intriguing possibility is bacterial homologues of the oxygenase domain of mammalian NOS (bNOS), which are briefly reviewed here. A more extensive review of bNOS can be found in ref 35.

Bacterial homologues of mammalian NOS enzymes were recently discovered,[114] and thus far, 403 species of bacteria, mostly Gram-positive, are known to encode a bNOS gene. Unlike mammalian NOSs, which are composed of an oxygenase domain and a reductase domain connected by a calmodulin binding domain, bNOS genes encode an oxygenase domain only. Although bNOS enzymes can convert L-arginine to L-citrulline and NO if an appropriate reductase domain is provided *in trans*, it is currently not clear if bNOS necessarily produces NO *in vivo*, although many studies are consistent with NO production. For example, in *Streptomyces turgidiscabis*, a plant pathogen responsible for potato scab disease, bNOS is thought to be involved in the nitration of tryptophan,[115] rather than explicit NO production. In other organisms, bNOS is believed to generate NO as a final product.

The function of synthesized NO is not fully understood, but it appears to be cytoprotective. In *Bacillus subtilis* and *Bacillus anthracis*, many studies have suggested that bNOS is involved in the protection of bacteria against oxidative damage, presumably by NO-mediated suppression of Fenton chemistry and/or upregulation of catalase activity.[116−118] Furthermore, deletion of the *nos* gene from the extremophile *Deinococcus radiodurans* severely compromises this organism's ability to survive exposure to radiation.[119]

Interestingly, there is also evidence of a possible interkingdom signaling role for NO in studies of bNOS function. *Caenorhabditis elegans* is a nonparasitic, transparent nematode that lives in temperate soil environments. *C. elegans* subsists on *Bacillus* sp. as their primary food and is a rare eukaryote that does not encode a NOS gene. *C. elegans* that are fed wild-type *Bacillus* or *E. coli* transgenically expressing bNOS live longer than *C. elegans* fed either wild-type *E. coli* (do not encode bNOS) or *bnos*-deficient *Bacillus*.[120]

Finally, and especially pertinent to this review, investigators have explored biofilm regulation as a function of bNOS. Schreiber et al. demonstrated that NO from bNOS does not appear to affect biofilm development or bacterial motility in *B. subtilis* 3610 but found ∼10-fold less biofilm in a Δ*bnos* mutant than in the wild type, suggesting NO may inhibit biofilm dispersal.[121] In *Lactobacillus plantarum*, it was found that even nanomolar concentrations of exogenous NO from SNP were toxic and therefore reduced the level of biofilm formation. Endogenously produced NO from bNOS, however, was found to be critical for biofilm formation.[122] Furthermore, when heterologously expressed in *Pseudomonas putida*, bNOS from *B. subtilis* was shown to produce NO endogenously, enhance bacterial motility, and decrease the level of biofilm formation.[123]

NITRIC OXIDE FOR ANTIBIOFILM APPLICATIONS

Finally, despite lacking a fundamental understanding of NO signaling in biofilm regulation, because NO has been shown to be efficacious against both Gram-negative and Gram-positive bacteria, investigators have proceeded to explore applications of NO as an antibiofilm therapeutic agent.[124] It has been hypothesized that bacteria will develop minimal resistance to NO.[125] One of the challenges, however, is to deliver NO gas constantly and steadily, and for its ultimate application, within a human body. To that end, NO-releasing materials are being explored as antibiofilm agents in the treatment of pathogens.

As previously mentioned, *N*-diazeniumdiolates (NONOates) are commonly used as NO donors (Figure 3). Because of

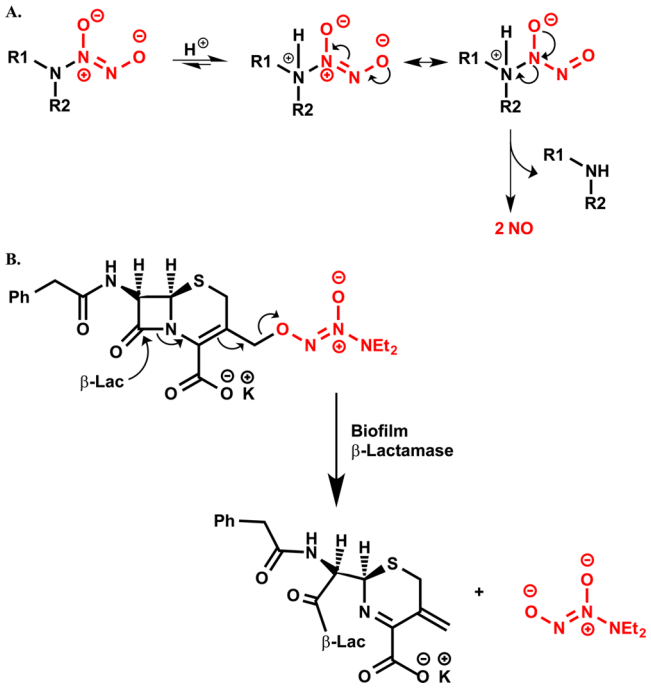

Figure 3. (A) Nitric oxide release by *N*-diazeniumdiolate. (B) *β*-Lactamase-catalyzed cephalosporin 3′-diazeniumdiolate decomposition. One mole of parent compound releases 1 mol of NONOate.

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instability at room temperature and in neutral or acidic solution, they have been incorporated into macromolecule vehicles to deliver NO to bacteria for antibacterial and antibiofilm applications. The Schoenfisch group has constructed effective antibiofilm materials by modifying silica nanoparticles, amphiphilic poly(amidoamine) dendrimers, and chitosan oligosaccharides with NONOates.[126−128] The total amount of released NO ranges from nanomolar to low micromolar per milligram of material, and half-lives vary from minutes to hours.[126−129] It has been reported that at a 1−10 mg/mL dosage, surviving cells are suppressed to <0.001%. Recently, the Teplitski laboratory tested several NONOate variants, as well as molsidomine, for biofilm dispersal.[130] Results suggested that at 10 pM, molsidomine and a NONOate induced an ∼50% reduction in *Salmonella enterica* biofilm biomass.

The Boyer group has tested the effect of NONOate-functionalized gold nanoparticles and star polymers on *P. aeruginosa* biofilm formation. They incorporated NONOate into a polymer/gold hybrid nanoparticle called AuNP@P(OEGMA)-*b*-P(VBHA) and tested its properties. At 10 μg/mL, the particles release NO sufficient to suppress biofilm mass by 83%.[131] They also generated a NONOate-functionalized star polymer. At pH 7.0, 400 μg/mL NONOate-modified star polymer continuously releases an amount of NO (356 nM/h) comparable to that of 6 μg/mL SNP (408 nM/h) over 70 h, resulting in a solution NO concentration that slowly builds from 20 to 60 μM over the 70 h time course. With a dose as low as 100 μg/mL, they observed a 90% reduction in biofilm biomass, which amounts to dispersal of *P. aeruginosa* better than that observed using SNP.[132]

Although NO-releasing macromolecules are adequate for broad-spectrum bactericidal purposes, it has been hypothesized that a biofilm-specific NO prodrug may be more efficient and cause less NO exposure to host tissues. Thus, Barraud et al. synthesized cephalosporin 3′-diazeniumdiolate as a NO-releasing prodrug,[133] designed so that NO would be released by biofilm-specific β-lactamases (Figure 3). They found that this compound displays a dose-dependent (they tested 2−200 μM prodrug) effect on *P. aeruginosa* biofilm dispersal.

Alternates to *N*-diazeniumdiolates-based NO-releasing compounds are also being investigated for antibiofilm purposes. For example, the stable free radical nitroxides ($RNO^{\bullet}$) have been shown to mimic the biofilm dispersal effect of NO on *P. aeruginosa*.[134] Sulemankhil et al. have tested the bactericidal effect of enzyme-catalyzed NO-releasing dressings[135] on the bacterial pathogens *Acinetobacter baumannii*, MRSA, and *P. aeruginosa*. They found that a dressing releasing >200 ppm of NO (∼7 mM NO) induced a 1000-fold reduction in bacterial viability after 3 h.

Furthermore, anticipating the use of NONOates in humans, Engelsman et al. have synthesized carbon-based NO-generating compounds,[136] because nitrogen-based NONOates may form carcinogenic nitrosamines during their decomposition *in vivo*.[137] When a surgical mesh was coated with these NONOate alternative compounds and exposed to multiple pathogens *in vitro*, pathogen survival rates dropped significantly. No evidence of biofilm dispersal *in vivo* was found with these compounds, however.[136]

Finally, it is hypothesized that more antibacterial potency may be achieved by combining a NO-releasing drug with a traditional antibiotic.[133] For example, in studies in which the QS inhibitor Fimbrolide was functionalized with an NO-releasing group and applied to *P. aeruginosa*, both less virulence factor production and less biofilm aggregation were observed than in studies with Fimbrolide alone.[138]

■ PERSPECTIVES AND CONCLUSIONS

As first reported for *N. europaea* and *P. aeruginosa*, NO-mediated regulation of biofilm formation is now generally considered to regulate biofilm formation in a wide variety of bacteria. In this review, we have presented a summary of documented NO-mediated biofilm regulation responses. In most species, low concentrations of NO cause biofilm dispersal, although there have been reports of biofilm enhancement observed as a function of NO (Table 1). In general, however, when enhancement is observed, distinct responses are documented when conditions are altered. For example, in *N. europaea* and *V. harveyi*, enhancement is observed at one concentration and dispersal at another. For *Ne. gonorrheae* and *S. oneidensis*, NO-mediated enhancement of biofilm formation is reported under anaerobic conditions and dispersal under aerobic conditions. Because antibiofilm applications of bacterial NO signaling are currently being developed on the basis of the assumption that NO always causes biofilm dispersal, understanding the condition- and concentration-dependent conditions of NO signaling in bacteria is especially timely and important.

Furthermore, on the basis of studies with Gram-positive bacteria, there is the suggestion that the source of NO, endogenous (bNOS) versu exogenous (NO donor), may be very important in determining how bacteria respond. This is especially interesting in light of the fact that the majority of studies that have reported an effect of NO on biofilm formation have been performed with exogenous NO donors (SNP or NONOate). Therefore, this observation may also be an important part of understanding the effect of NO in Gram-negative bacteria where, generally, there is no endogenous bNOS and exogenous NO has been shown to cause biofilm dispersal. Additionally, on the basis of the limited data available, it seems to take much higher concentrations of gaseous NO to cause biofilm phenotypes than in experiments in which NO is generated from an NO donor. This could be because aqueous NO concentrations are generally measured in a manner independent of the concentration of NO donor applied, while in most experiments, the aqueous concentration of NO was not determined when gaseous NO was used as the NO source. In the future, it will be interesting to learn if the source of NO is truly important and whether NO produced endogenously from nitrite reduction, in bacteria that do not encode bNOS, also causes a response different from that of NO applied exogenously from NO donors.

Finally, in general, the molecular details of NO sensing and signaling pathways in bacteria are poorly understood at present. The H-NOX domain has been demonstrated to be a primary NO sensor in several environmental and facultative pathogenic bacteria that regulates biofilm formation in response to NO ligation. Many bacteria, including *P. aeruginosa* and *N. europaea*, do not encode H-NOX but have documented nanomolar NO-mediated biofilm phenotypes; therefore, it is likely that an additional biofilm-regulating NO sensor is yet to be characterized. Indeed, the condition- and concentration-dependent aspects of NO signaling in bacteria may indicate the existence of multiple NO signaling pathways that regulate biofilm formation, aside from the better established NO signaling pathways for detoxification and denitrification.

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NO-mediated biofilm formation is an accelerating area of bacteriology that promises important contributions to both understanding the fundamentals of bacterial biofilms and applications for combating the public health threats of biofilms.

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Notes

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ABBREVIATIONS

NO, nitric oxide; H-NOX, heme-nitric oxide/oxygen binding protein; HaCE, H-NOX-associated cyclic-di-GMP processing enzyme; HaHK, H-NOX-associated histidine kinase; HaRR, H-NOX-associated response regulator; HqsK, H-NOX-associated quorum-sensing kinase; c-di-GMP or cyclic-di-GMP, bis(3′−5′)-cyclic dimeric guanosine monophosphate; GGDEF, conserved amino acids in the catalytic sites of diguanylate cyclases (the domain containing these conserved residues is often termed a GGDEF domain); EAL, conserved amino acids in the catalytic site of some phosphodiesterases (the domain containing these conserved residues is often termed an EAL domain); HD-GYP, conserved amino acids in the catalytic site of some phosphodiesterases (the domain containing these conserved residues is often termed an HD-GYP domain); QS, quorum sensing; NOS, nitric oxide synthase; sGC, soluble guanylyl cyclase (a eukaryotic H-NOX domain-containing protein); SNP, sodium nitroprusside; NONOate, *N*-diazeniumdiolate.

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