

# Origin and Impact of Nitric Oxide in *Pseudomonas aeruginosa* Biofilms

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The formation of the organized bacterial community called biofilm is a crucial event in bacterial physiology. Given that biofilms are often refractory to antibiotics and disinfectants to which planktonic bacteria are susceptible, their formation is also an industrially and medically relevant issue. *Pseudomonas aeruginosa*, a well-known human pathogen causing acute and chronic infections, is considered a model organism to study biofilms. A large number of environmental cues control biofilm dynamics in bacterial cells. In particular, the dispersal of individual cells from the biofilm requires metabolic and morphological reprogramming in which the second messenger bis-(3'-5')-cyclic dimeric GMP (c-di-GMP) plays a central role. The diatomic gas nitric oxide (NO), a well-known signaling molecule in both prokaryotes and eukaryotes, is able to induce the dispersal of *P. aeruginosa* and other bacterial biofilms by lowering c-di-GMP levels. In this review, we summarize the current knowledge on the molecular mechanisms connecting NO sensing to the activation of c-di-GMP-specific phosphodiesterases in *P. aeruginosa*, ultimately leading to c-di-GMP decrease and biofilm dispersal.

## PSEUDOMONAS AERUGINOSA: A MODEL SYSTEM FOR BIOFILM RESEARCH

The ubiquitous Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic pathogen responsible for both acute and chronic infections. *P. aeruginosa* chronic lung infections are the major cause of death in cystic fibrosis (CF) patients, a genetic disease affecting 1/2,500 newborns in Europe (1). *P. aeruginosa* is frequently resistant to conventional antibiotic therapy and to host antimicrobial effector mechanisms and forms biofilms in many infection sites, including CF lung chronic infections (2). Therefore, this bacterium has become a model system for biofilm research.

The life span of a *P. aeruginosa* biofilm can be divided into several stages, starting with the reversible interaction of a planktonic cell with a surface (3). The cells subsequently attach to the surface and mature into three-dimensional mushroomlike structures, also known as microcolonies (4). This process is accompanied by the production of an extracellular hydrated matrix, also known as extracellular polymeric substance (EPS), composed of exopolysaccharides, extracellular DNA (eDNA), proteins, amyloid fibers, and bacteriophages, in which the bacteria are embedded (5, 6). *P. aeruginosa* produces three secreted polysaccharides, Pel, Psl, and alginate, each of which provides different physiological properties to the matrix (7). eDNA is also considered an important component of the bacterial matrix, particularly during early stages of biofilm formation (3). Overall, the extracellular matrix facilitates the formation of three-dimensional structures that give the bacteria increased access to nutrients and advantages of multicellular living and protects the bacteria from outside predators and toxic chemicals, including antibiotics.

Many adaptive traits may arise during *P. aeruginosa* infections, which may favor biofilm formation: these novel traits include nonmotility, small-colony variant formation, deficiency in the population density-sensing system known as quorum sensing (QS), changes in the chemical structure of the membrane, and increased mutation rates (8). Bacteria within a biofilm are signif-

icantly more tolerant of antibiotic treatment than their planktonic counterparts (9, 10).

During dispersal, motile cells from microcolonies leave the biofilm to colonize new locations (4, 11). Dispersed cells are often considered identical to planktonic ones; however, recent reports support earlier findings that dispersed cells represent a specific intermediate state between biofilm and planktonic lifestyles in *P. aeruginosa* (4, 12). This proposal is mainly based on the observation that the physiology and virulence of dispersed cells are highly different from those of biofilm and planktonic ones (12, 13). The dispersal phenotype can be achieved and maintained by either the addition of a dispersing agent or the overexpression of a phosphodiesterase (PDE) (see below) (12).

## BIOFILM DISPERSAL

While passive detachment of bacterial cells is triggered by external physical forces and can occur during all stages of biofilm development, active dispersal is initiated by the bacteria themselves and can be specifically triggered by certain environmental cues in various bacterial species. These include the availability of nutrients (i.e., carbon and iron) (11, 14–18) and oxygen (19), changes in temperature (20, 21), and low levels of exogenous and endogenous nitric oxide (NO) (22–25). In addition, native dispersal has also been described which involves the self-synthesized signaling molecule *cis*-2-decenoic acid (26). Active dispersal is specific and tightly regulated and involves modulation of the intracellular concentration of the second messenger bis-(3'-5')-cyclic dimeric

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GMP (*c*-di-GMP), which also plays a role in biofilm formation (5, 10).

### ROLE OF *c*-DI-GMP IN BIOFILM FORMATION AND DISPERSAL

In recent years, it became clear that the second messenger *c*-di-GMP is a key player in the regulation of biofilm formation and dispersal (27). While high levels of *c*-di-GMP promote the sessile lifestyle and the formation of biofilms, low levels of *c*-di-GMP lead to biofilm dispersal and favor the planktonic lifestyle. In response to environmental cues, the intracellular level of this second messenger is modulated by enzymes that build (diguanylate cyclases [DGCs]) or break (phosphodiesterases [PDEs]) *c*-di-GMP. These enzymes possess characteristic domains with conserved amino acid motifs: GGDEF domains in DGCs and EAL or HD-GYP domains in PDEs (27). GGDEF and EAL/HD-GYP domains are often part of multidomain proteins that harbor additional signal input or transduction domains (i.e., PAS [Per Arnt Sim], GAF [found in *c*GMP-specific phosphodiesterases, adenylyl cyclases, and FhlA], and REC [signal receiver] domains) (27). This organization implies that the upstream signal input domains may regulate the activity of the catalytic domains, and indeed, several reports support this hypothesis (28, 29). The GGDEF and EAL domains are not only linked to signal input domains but are often found fused in a single polypeptide chain. These proteins are either bifunctional or one of the domains is catalytically inactive and adopts a new regulatory function (30, 31). Interestingly, one single organism usually harbors more than one each of putative DGCs and PDEs, indicating a certain amount of redundancy; it is also evident that specific DGCs and PDEs may control distinct phenotypes. DGC and PDE activities are able not only to control the global intracellular level of *c*-di-GMP but also to regulate localized pools of the second messenger, for example, during cell division (32, 33); the presence of target receptors (like PilZ domains) thus leads to the specific cellular responses (34).

Given that NO is able to induce dispersal of biofilms formed by several bacterial species, including *P. aeruginosa*, *Escherichia coli*, *Vibrio cholerae*, *Staphylococcus epidermidis*, *Bacillus licheniformis*, *Serratia marcescens*, *Legionella pneumophila*, *Nitrosomonas europaea*, and *Neisseria gonorrhoeae* (23, 35–38), a wealth of studies has been conducted in the last 5 years addressing the effectiveness of NO-based antibiofilm strategies. Several classes of NO-releasing compounds active in biofilm modulation have been found, whose properties were very recently reviewed by Barraud et al. (39).

Within this minireview, we focus mainly on the effect of NO on *P. aeruginosa* biofilm dispersal, the origin and perception of NO, and the subsequent effect of NO on *c*-di-GMP levels, trying to summarize what is known on the molecular mechanisms underlying this complex biological process.

### PROPERTIES AND SOURCES OF NO

Nitric oxide is a colorless diatomic gas with the chemical formula NO. Due to an unpaired electron, NO is a free radical with a half-life of only a few seconds, causing lipid peroxidation, nitration, and S-nitrosylation of proteins (40). NO readily interacts with transition metals found in metalloproteins (41). This topic has been intensely studied, for instance, for the interaction of NO with the heme-iron of hemoglobin (42). In addition, NO is a signaling molecule in vertebrates, controlling a plethora of biological

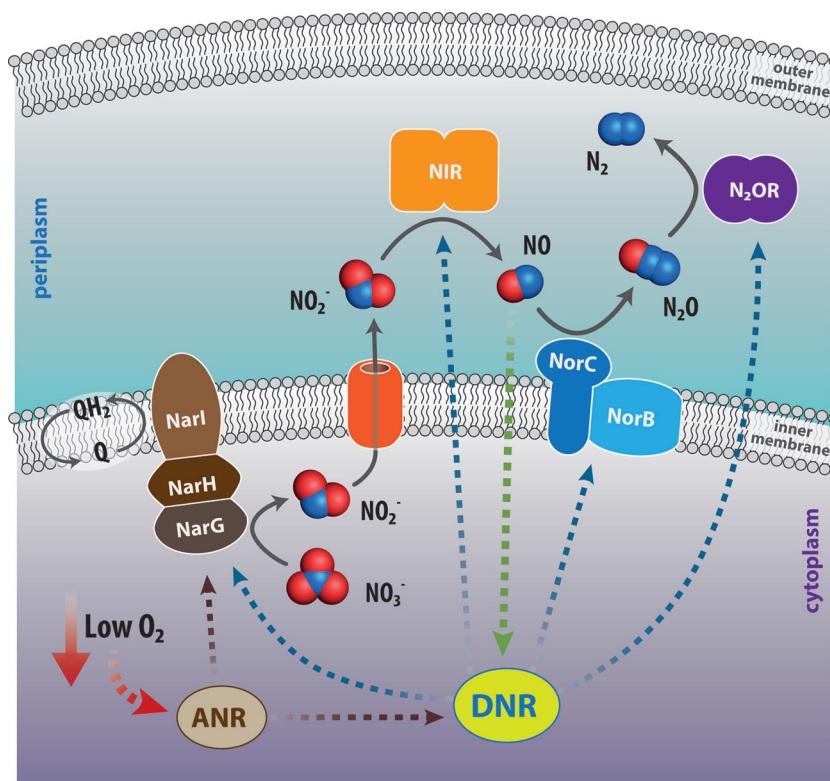
processes, such as muscle relaxation leading to vasodilation and increased blood flow. In the latter example, the receptor for NO is also a heme-containing protein, i.e., soluble guanylate cyclase, which upon NO binding is activated to produce cyclic GMP (*c*GMP) (43).

In bacterial systems, including *P. aeruginosa*, NO can be produced directly from the microorganism itself, as an intermediate or by-product of specific metabolic pathways, such as denitrification, a form of anaerobic respiration. NO may also come from outside the bacterial cell, produced by eukaryotic host cells that attack pathogens with NO (44, 45). To characterize the response of biofilms formed by several bacteria to exogenous NO, different NO-releasing compounds have been employed, mostly under aerobic conditions. These compounds release variable amounts of NO, ranging from low nanomolar to micromolar concentrations, with different half-lives, mainly depending on their kinetics of NO release. In *P. aeruginosa*, biofilm dispersal has been shown to occur in the presence of exogenous compounds spontaneously releasing NO, such as sodium nitroprusside (SNP), 6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-hexanamine (MAHMA NONOate) or aminoxyl free radicals (nitroxides) (reference 39 and references therein). SNP as an NO donor has been shown to deliver an effective NO concentration about 1,000-fold lower than the concentration of the donor over several minutes (up to 30 min) (22), whereas the half-life of MAHMA NONOate is much shorter (1 to 2 min) (39). More recently, prodrugs releasing NO after an activation step in the bacterium, such as diethylamine (DEA) NONOate-cephalosporin prodrug (DEACP), were also shown to promote dispersal (46, 47).

### ENDOGENOUS SOURCES OF NO IN *P. AERUGINOSA* AND THEIR EFFECTS ON BIOFILM

*P. aeruginosa* is a facultative anaerobe, able to respire under anaerobic conditions in the presence of the alternative electron acceptors nitrate and nitrite, employing denitrification. In this metabolic pathway, nitrate is reduced to NO and then to N<sub>2</sub> in four reaction steps, each catalyzed by a specific reductase (Fig. 1) whose expression is tightly controlled, mainly by the arginine nitrate regulation (ANR) and dissimilative nitrate respiration regulator (DNR) transcription factors (48–53). Two putative nitrate reductases are present in *P. aeruginosa*, i.e., the inner membrane-bound nitrate reductase NarGHI (Fig. 1), encoded within the *narK1K2GHJI* operon, and the periplasmic nitrate reductase NapAB, encoded within the *napEFDABC* operon. NarGHI has been shown to be the predominantly expressed nitrate reductase under anaerobic conditions in the presence of nitrate (54). Nitrite reductase, the bacterial enzyme that reduces nitrite to NO during denitrification, may contain either copper or heme. The corresponding *nirK* and *nirS* genes are found in both Gram-negative and Gram-positive bacteria, with *nirS* being the most frequent one. Denitrification has been implicated in the virulence of several bacterial species, including members of the *Brucella*, *Pseudomonas*, and *Neisseria* genera (55–57).

In *P. aeruginosa*, the enzyme responsible for nitrite reduction to NO (Fig. 1) is cytochrome *cd*<sub>1</sub> nitrite reductase (hereinafter NIR), a homodimer containing one *c*-heme and one *d*<sub>1</sub>-heme group in each subunit (58), belonging to the NirS family of nitrite reductases (59). The *d*<sub>1</sub>-heme is a partially saturated macrocycle, unique to this enzyme and synthesized by a specialized pathway present only in denitrifiers (strongly induced in *P. aeruginosa*



**FIG 1** Scheme of the denitrification pathway in *Pseudomonas aeruginosa*. In denitrification, a form of anaerobic respiration, nitrate ( $\text{NO}_3^-$ ) is reduced to molecular nitrogen ( $\text{N}_2$ ) in four reductive steps, each catalyzed by a specific reductase, namely, nitrate reductase (NAR), nitrite reductase (NIR), NO reductase (NOR), and nitrous oxide reductase ( $\text{N}_2\text{OR}$ ). The expression of these enzymes is mainly controlled by two transcriptional regulators that sense low-oxygen conditions (ANR, an iron-sulfur protein) and NO (DNR, an heme-containing protein), respectively. Q, coenzyme Q or ubiquinone (2,3-dimethoxy-5-methyl-6-multiprenyl-1,4-benzoquinone);  $\text{QH}_2$ , reduced form of ubiquinone, i.e., ubiquinol.

upon nitrite treatment): this specialized cofactor is required for the efficient release of NO (60).

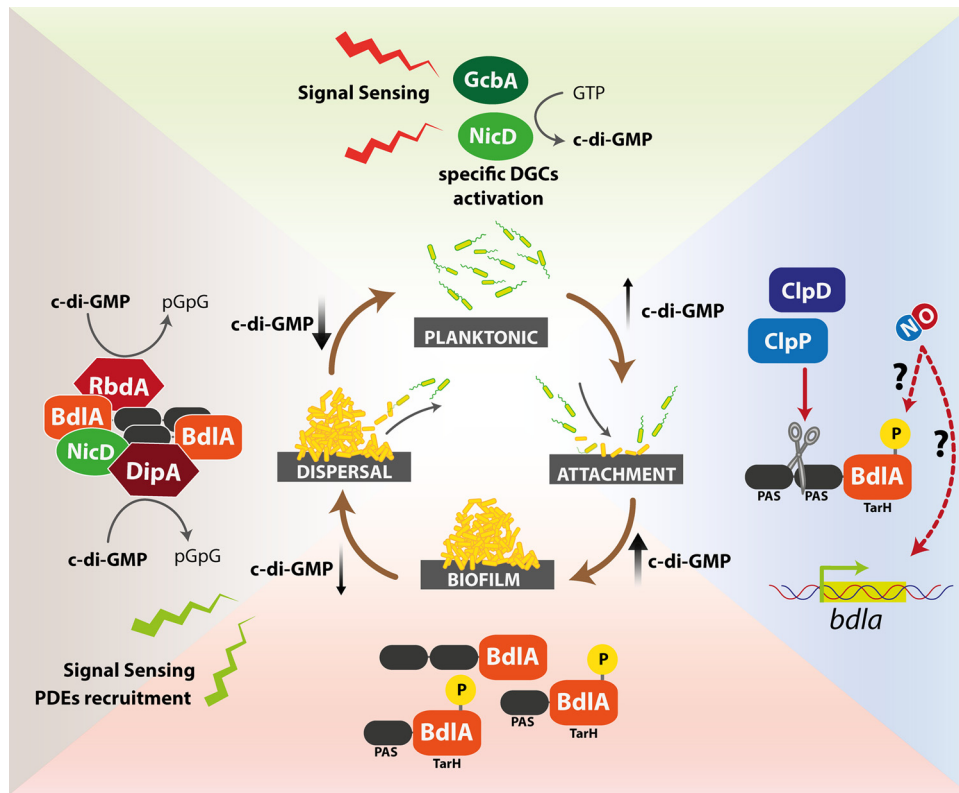
The last two enzymes in the denitrification chain are the membrane-bound NO reductase (NorCB) (61) and the periplasmic copper-containing  $\text{N}_2\text{O}$  reductase (62), reducing nitrous oxide ( $\text{N}_2\text{O}$ ) to molecular nitrogen (Fig. 1).

Several studies suggest the involvement of the NO produced by denitrification in biofilm dynamics and virulence in *P. aeruginosa*; these studies have been carried out under different environmental conditions, including oxygen availability. Under aerobic conditions, nitrate sensing and metabolism control swarming motility, a type of motility which requires the production of flagella, type IV pili, and the biosurfactant rhamnolipid (encoded by the *rhlAB* operon) and affect biofilm architecture, showing a central role for NIR-derived NO in these events (57, 63). In addition, biofilm dispersal under aerobic conditions requires NIR activity and NO production (22, 38, 57): a *P. aeruginosa* strain lacking NIR does not disperse in the presence of SNP, whereas a NO reductase mutant exhibits a hyperdispersal phenotype (38). Under these experimental conditions, NO affects the expression or activity of proteins related to motility (such as those encoded by *pilA* and *rhlAB*) (22, 63).

The overall picture is somewhat different under strict anaerobic conditions, when NO accumulation may ultimately favor biofilm formation (64). As shown by Yoon and coworkers and confirmed in a more recent study (64, 65), *P. aeruginosa* PAO1 cells

grown anaerobically are more elongated than that grown aerobically and easily form highly cohesive clumps, yielding a robust biofilm. Cell elongation is dependent on the presence of NIR and is repressed in the presence of an NO antagonist. Under low-oxygen tension, the nonelongated NIR-deficient mutant failed to form biofilm, while the wild-type PAO1 was highly elongated and formed robust biofilm.

Very recently, NIR was also shown to control flagellum production and swimming motility under anaerobic conditions, independently from its ability to produce NO, by serving as a scaffold to form a ternary complex with the chaperone DnaK and the flagellar protein FliC in the periplasm (66). In the same study, the *nirS* mutant showed a partial restoration of swimming ability under aerobic conditions, whereas swarming mobility was previously shown to be affected under aerobiosis (63). An intriguing possibility suggested by the latter study, which will require further investigation, is that NIR promotes motility in different ways, depending on oxygen availability, either by producing NO to increase rhamnolipid synthesis or activate other signaling pathways, or by directly controlling flagellum formation by protein-protein interactions. As underlined by Borrero-de Acuna et al. (66), to fully understand these phenomena, the assembly of flagella under aerobic and anaerobic conditions will have to be analyzed in further detail. Since it is known that NO transcriptionally activates *nirS* expression, the production of a catalytically inactive version



**FIG 2** Nitric oxide activation of the BdlA signaling cascade. The BdlA protein is a key player in the modulation of biofilm formation and dispersal. It may be activated by nutrient and/or other signals (NO); the complex events occurring during and after BdlA activation are summarized in this figure (clockwise). Signal sensing initially leads to a transient increase in c-di-GMP, possibly triggered by specific DGCs, such as NicD or GcbA. Activation of BdlA by site-specific proteolysis by ClpD/ClpP at the level of the PAS domains or by phosphorylation leads to a further increase in c-di-GMP levels and biofilm formation. Other signaling events may trigger the recruitment of c-di-GMP-specific PDEs (RbdA and DipA), ultimately leading to a decrease in c-di-GMP levels and biofilm dispersal.

of NIR may also be a route for motility control by exogenously added NO donors.

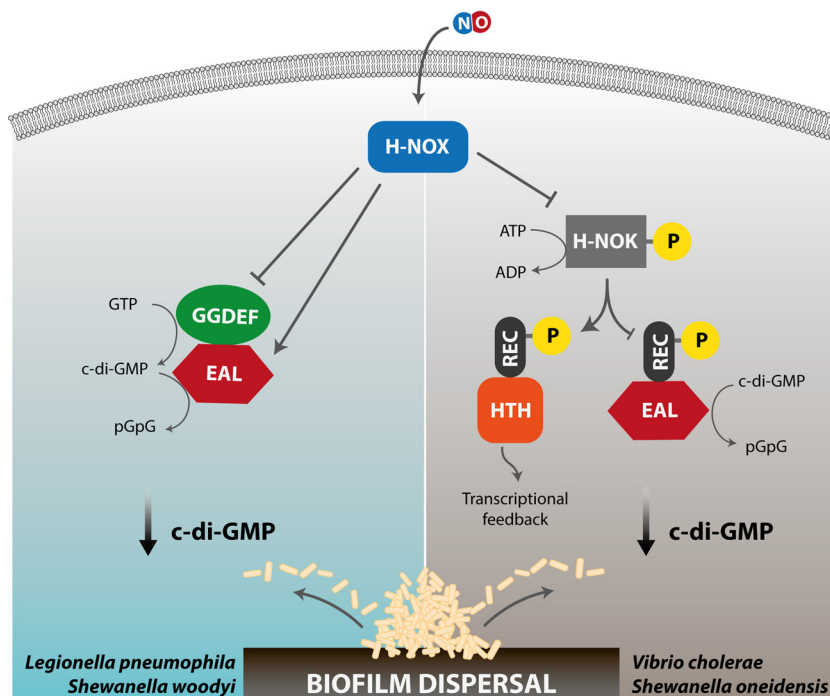
Another interesting link between denitrification and biofilm modulation in *P. aeruginosa* involves the cell-to-cell communication signaling systems collectively known as quorum sensing (QS) systems that coordinate gene expression in response to population density. *P. aeruginosa* possesses at least three different QS systems: two *N*-acyl-L-homoserine lactone (AHL) signals, the LasR-LasI (*las*) and RhIR-RhII (*rhl*) systems (67), and a third one, 2-heptyl-3-hydroxy-4-quinolone, referred to as the *Pseudomonas* quinolone signal (PQS) (68). LasI directs the synthesis of the AHL signal *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), and RhII directs the synthesis of another AHL signal, *N*-butyryl-L-homoserine lactone (C4-HSL). The transcription regulatory proteins LasR and RhIR are specifically activated by 3-oxo-C12-HSL and C4-HSL, respectively.

Regulation of denitrification by QS may thus be important in both aerobically and anaerobically grown biofilms. The denitrification pathway is regulated by both the AHL and PQS systems via different mechanisms, including transcriptional regulation and iron chelation (38, 69–72). C4-HSL represses denitrification via its cognate regulator RhIR (69); on the other hand, the addition of PQS to the growth medium specifically promotes NO accumulation (70), thus suggesting a possible mechanism of endogenous regulation of biofilm dispersal (38). Anr, the oxygen-responsive

transcription factor controlling the expression of denitrification genes, also controls QS in biofilms under low-oxygen conditions (1%) (73).

It is noteworthy that bacterial species devoid of *nirK* and *nirS* are also able to reduce nitrite to NO anaerobically; this capability likely involves products of *fir* and *hmp* genes, as shown for *Salmonella* (74). Dispersal of *Salmonella* biofilms by NO donors was shown to depend on the presence of the *recA-hydN* genomic region, suggesting its involvement in NO sensing (75).

An alternative group of bacterial enzymes capable of producing NO are the bacterial nitric oxide synthases (bNOS), only found in Gram-positive bacteria. These enzymes catalyze the oxidation of the amino acid L-arginine in the presence of oxygen ( $O_2$ ) as an essential substrate (76). Mammalian NOS are composed of both oxygenase and reductase domains, whereas bNOS from *Bacillus* and *Staphylococcus* contain only an oxygenase domain. In Gram-positive bacteria, bNOS-produced NO modulates several aspects of bacterial physiology, including protection from oxidative stress and antimicrobials (77, 78). Interestingly, although NOS-derived NO appears to inhibit biofilm dispersal of *Bacillus subtilis* 3610 (79), heterologous expression of *B. subtilis* bNOS in *Pseudomonas putida* increases motility and decreases biofilm formation (80), suggesting that the same signal (NO) may exert different effects depending on the cellular background.



**FIG 3** Nitric oxide-induced signaling events mediated by the heme-based sensor domain H-NOX. The binding of NO to the heme moiety of H-NOX may result in biofilm dispersal following different signaling cascades in different bacterial species harboring this protein domain. Left, in *Legionella pneumophila* or *Shewanella woodyi*, the NO derivative of the H-NOX protein interacts with the GGDEF-EAL (HaCE) protein, thus lowering c-di-GMP levels by stimulation of the PDE activity of the HaCE EAL domain. Right, in *Vibrio cholerae* or *Shewanella oneidensis*, interaction of the NO-bound H-NOX domain with a coupled histidine kinase (H-NOK) controls the phosphorylation activity of the kinase. Specific phosphorylation events lead to a decrease in c-di-GMP levels, either by stimulating the hydrolysis of c-di-GMP to pGpG by a cognate PDE (REC-EAL) via the fused REC domain or by controlling the transcriptional response through a dedicated transcription regulator (REC-HTH).

### DISPERSAL OF *P. AERUGINOSA* BIOFILMS INDUCED BY EXOGENOUS NO SOURCES

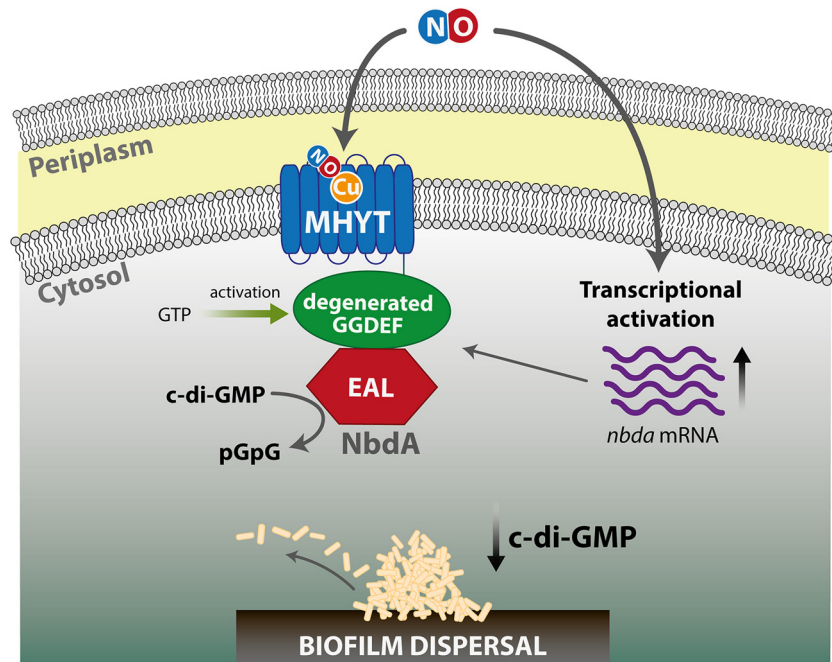
The molecular events leading to biofilm dispersal in *P. aeruginosa* are still far from being completely understood. One of the first proteins identified to play a role is the chemotaxis transducer protein BdlA (biofilm dispersion locus A), now considered a key player in this process. BdlA was identified during a search for a chemotaxis transducer protein involved in sensing different environmental cues that might trigger biofilm dispersal (81). It is a multidomain protein of ~47 kDa consisting of two adjacent Per Arnt Sim (PAS)-domains, putatively involved in binding a heme cofactor, followed by a C-terminal TarH domain (82), related to ligand binding domains of methyl-accepting chemotaxis receptors. Although BdlA lacks domains directly responsible for modulating c-di-GMP levels, strains devoid of *bdla* are impaired in dispersal of *P. aeruginosa* biofilms in response to several environmental cues, including NO (22, 81). Functional BdlA requires an unusual nonprocessive ClpP/ClpD-dependent proteolytic cleavage, which is stimulated by elevated c-di-GMP levels and BdlA phosphorylation (83) (Fig. 2).

In *P. aeruginosa*, the response to nutrient (glutamate)-induced dispersal was recently proposed to be governed by a signal transduction mechanism involving a multiprotein complex composed of BdlA together with the DGC NicD and the PDE DipA, ultimately leading to the modulation of the cellular c-di-GMP pool (Fig. 2) (82, 83). Upon signal (i.e., glutamate) sensing, the DGC NicD, a seven-transmembrane receptor, becomes dephosphoryl-

ated, leading to enhanced DGC activity and resulting in a transient increase in c-di-GMP levels (84). NicD contributes to BdlA activation in two ways, by elevating c-di-GMP levels and via phosphorylation. Once activated, BdlA stimulates the PDE DipA and recruits a second PDE (RbdA), which ultimately leads to decreased c-di-GMP levels and biofilm dispersal (82, 83). RbdA itself contains sensory PAS and PAC domains, in addition to GGDEF and EAL domains. The PAS domain of RbdA appears to be involved in sensing low-oxygen stress. The EAL domain was shown to be catalytically active as a PDE, while the GGDEF domain controls PDE activation through GTP binding (19).

Very recently, an additional player in the BdlA signal transduction system has been identified, i.e., the catalytically active DGC GcbA (85). GcbA was shown to contribute to the regulation of BdlA cleavage shortly after initial cellular attachment to surfaces. It was shown that the levels of both proteins are inversely regulated, depending on the mode of growth (motile versus sessile), through the cellular c-di-GMP level. Therefore, both GcbA and BdlA translate different cues received by sensor proteins into the molecular cascade of events ultimately leading to dispersal (Fig. 2).

Although the involvement of BdlA and its cognate DGCs and PDEs in controlling c-di-GMP levels and biofilm dispersal is becoming increasingly clear, much less is known on the nature of other *P. aeruginosa* proteins which may directly sense NO and activate downstream response pathways. The features of possible candidates are summarized in the next two paragraphs.



**FIG 4** Scheme of NbdA activation. Possible mechanism of NO-induced biofilm dispersal via the MHYT domain-containing NbdA protein. This protein is composed of three domains, a membrane-spanning MHYT domain, a degenerated GGDEF domain, and a PDE-EAL domain. Binding of NO is supposed to occur via copper ions (Cu) located in the MHYT domain. The signal is then transmitted via the GGDEF domain to the EAL domain, triggering its PDE activity and, thus, c-di-GMP hydrolysis to pGpG. GTP may increase the PDE activity of the EAL domain by binding to the degenerated GGDEF. In parallel, NO may also activate the transcription of NbdA by a yet-unknown mechanism.

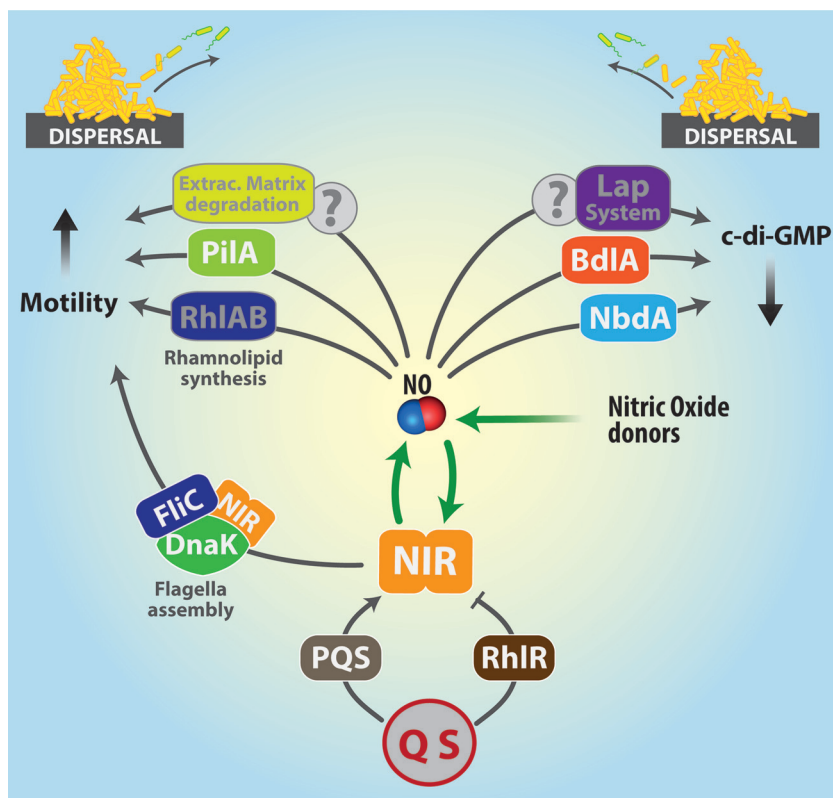
## NO SENSOR PROTEINS

Almost a decade ago, the exposure of bacteria to NO was linked to changes in intracellular levels of c-di-GMP (38). In the meantime, heme-based sensor domains (H-NOX [heme-nitric oxide/oxygen]), involved in detecting NO, have been identified and linked to c-di-GMP signaling in a number of species. The H-NOX domain is a member of a large family of hemoproteins, including mammalian soluble guanylate cyclase, which sense diatomic gases (86, 87). In many bacteria, H-NOX domains act as NO sensors that regulate biofilm formation by modulating the intracellular level of c-di-GMP (88); these domains are often found adjacent to GGDEF and EAL domains, yielding a simplified one-component signaling which may either increase or decrease the c-di-GMP levels. In *Legionella pneumophila*, NO binding to the Hnox1 protein (composed of a fused H-NOX–GGDEF–EAL polypeptide) directly inhibits DGC activity and, thus, biofilm formation (Fig. 3) (35). The two-component system present in *Shewanella woodyi* comprises the SwH-NOX (H-NOX of *S. woodyi*) and SwGGDEF-EAL (HaCE [H-NOX-associated c-di-GMP-processing enzyme]) proteins: NO binds to SwH-NOX and regulates the partner protein by simultaneously downregulating its cyclase activity and upregulating the phosphodiesterase activity (89, 90). In general, NO binding events in bacterial H-NOX domains seem to cause conformational changes involving the N-terminal helices, which mediate interaction with partner enzymes, such as HaCEs (see above), or control the activity of a dedicated histidine kinase (H-NOX-associated histidine kinase [HnoK]), as seen in *Shewanella oneidensis* (91), ultimately resulting in changes of c-di-GMP within the cell (88) (Fig. 3).

Although this signaling system is quite well understood, several

bacteria, including *P. aeruginosa*, lack H-NOX proteins but are still able to respond to NO (38). Interestingly, in *P. aeruginosa*, the response to NO has been linked to increased PDE activity (22, 24). Thus far, only one PDE (i.e., NbdA) specifically involved in the NO-specific reduction of c-di-GMP has been identified (24). While two additional PDEs (i.e., DipA and RbdA) have been identified to be involved in the dispersal response to various environmental cues (including NO) and are shown to be part of the BdlA signal transduction system (see previous paragraph), it is still not completely understood how NbdA is interwoven in this signal transduction network. This is especially intriguing since the overexpression of the *E. coli* PDE YhjH in *P. aeruginosa* leads to biofilm dispersal, as does the addition of a dispersal-inducing agent (12). Similar behavior has been observed for other bacteria, including *Shewanella oneidensis*, where overexpression of *yhjH* also led to rapid cellular detachment from biofilms (92).

NbdA possesses a so-called MHYT-domain (93), a transmembrane domain composed of seven predicted membrane spanning helices, proposed to possess a putative sensory function for diatomic gases like oxygen, carbon monoxide, or NO through protein-bound copper ions (93) (Fig. 4). In addition to the MHYT domain, NbdA harbors cytoplasmic GGDEF and EAL domains. Biochemical studies revealed that NbdA has PDE activity, stimulated by GTP bound to the degenerated GGDEF domain. Lack of NbdA resulted in biofilms impaired in the dispersal response to NO but not glutamate and could be linked to changes in c-di-GMP levels (24). In NO-dispersed biofilms, *nbdA* mRNA levels are increased, suggesting that an (additional) event involving transcriptional regulation is taking place (Fig. 4) (24). However, whether one of the NO-responsive transcriptional regulators is



**FIG 5** Summary of the possible effects of NO leading to biofilm dispersal in *P. aeruginosa*. In *P. aeruginosa*, NO can be produced by the endogenous enzyme nitrite reductase (NIR) or come from external sources (NO donors). Since NO is known to stimulate NIR synthesis, the two sources (endogenous and exogenous) may also cooperate, as shown in the figure. Biofilm dispersal is triggered by events that increase motility by stimulating synthesis of pili (PiIA) or rhamnolipids (RhlAB). Flagellum assembly can also be stimulated by the formation of a ternary complex of NIR (in the active and inactive form), FliC (the major flagellar subunit), and DnaK (a chaperone). NO-induced biofilm dispersal also involves the decrease of c-di-GMP intracellular levels. Two major signaling pathways are known to lower c-di-GMP concentrations, namely, those involving the chemotaxis receptor BdlA and the membrane protein NbdA. Another possible effector of c-di-GMP decrease is the Lap system, whose mechanism of action is yet to be fully elucidated. Many details of these processes will require further investigation, to clarify how NO may affect remodeling of the extracellular matrix to facilitate the escape of individual motile cells and to identify which NO-sensitive transcriptional regulators are involved in regulating gene expression during dispersal.

involved in this process and whether the MHYT-domain itself has a NO-sensory function has to be determined. In this context, it is also worth mentioning that *P. aeruginosa* possesses a second c-di-GMP-modulating enzyme with a domain organization similar to that of NbdA. MucR was shown to be involved in regulating alginate synthesis through its DGC activity (94). However, weak PDE activity could also be demonstrated *in vitro* (24). The lack of MucR resulted in biofilms that did not disperse either in response to NO or to glutamate.

In addition to the c-di-GMP-modulating enzymes, a preliminary report suggests that the periplasmic protease LapG may be involved in the dispersal response to NO, as *P. aeruginosa* biofilms devoid of LapG did not show a dispersal response upon NO addition (39). LapG is a membrane-bound protease that, in a c-di-GMP-dependent fashion via an inside-out signaling mechanism through the membrane receptor LapD, cleaves the surface adhesion LapA in *Pseudomonas putida* to mediate dispersal (95, 96). Since a LapA homolog is absent in *P. aeruginosa*, it is yet to be determined how the Lap system is integrated in the dispersal response in this pathogenic *Pseudomonas* species.

#### NO RESPONSIVE TRANSCRIPTIONAL REGULATORS

As summarized above, the addition of NO often also leads to biofilm dispersal by modulating the transcriptional profile of the bac-

terium. NO is an established signaling molecule in bacteria, interacting with many bacterial regulatory components, such as OxyR, SoxR, NsrR, NorR, FhpR, DNR, and other regulators of the fumarate nitrate reductase transcriptional regulator (FNR) family (97, 98). These regulators display iron-containing cofactors (iron-sulfur clusters, mononuclear iron, heme) or reactive cysteine thiols that react with NO, thereby changing their affinity to the target DNA. Many molecular studies have elucidated the role of these regulators in NO metabolism and NO detoxification. On the other hand, although it has been shown that NO sensing during biofilm dispersal requires transcriptional activation of selected members of signaling or metabolic pathways, ultimately leading to c-di-GMP decrease, there is little evidence on which regulators are involved in this process.

In *P. aeruginosa*, during biofilm dispersal, low doses of NO were shown to modulate the expression of a subset of genes, including *nirS* and other components of the denitrification chain and genes involved in attachment (12, 22). The expression of the denitrification genes is controlled by the activity of the DNR transcription factor (52), which is also highly expressed under biofilm dispersal conditions (12) and may play a role in this process. DNR is a heme-based gas sensor of the cAMP receptor protein (CRP)-FNR superfamily, which positively responds to NO (48, 49, 51).

The affinity of the DNR-NO complex for its cognate DNA was recently determined to be in the nanomolar range (50).

A recent study has correlated the appearance after biofilm dispersal of a superinfective (SI) version of the *Pseudomonas aeruginosa* filamentous phage Pf4 with oxidative and nitrosative stress response mediated by OxyR, a LysR-type transcriptional regulator (99). Although studies reporting S-nitrosylation of regulatory proteins are not available for *P. aeruginosa*, it is known that OxyR is a master regulator of S-nitrosylation under anaerobic conditions in *E. coli* (100). In *P. aeruginosa*, the OxyR regulon includes genes involved in iron homeostasis and the production of cytochromes (*cyoA* and *snr1*) (101), all found to be significantly altered in dispersed cells (12), suggesting that an OxyR-dependent mechanism could also be envisaged.

## CONCLUSIONS AND OPEN QUESTIONS

Dispersed cells are physiologically different from planktonic cells, as previously suggested (4, 13) and remarked in a recent study showing that freshly dispersed cells (induced by either NO or overexpression of a PDE) are highly virulent and sensitive to iron stress (12). Gene expression patterns are different between planktonic and dispersed cells, but the SNP- (NO donor) and PDE-induced dispersal patterns overlap. As summarized in this mini-review, although the dispersed cells show a defined physiological profile, distinct from those of the planktonic and biofilm cells, all available evidences suggest that the bacterium may employ different routes to reach this dispersed state after sensing external or internal stimuli, including NO. A summary of the possible effects of NO as a signaling molecule leading to biofilm dispersal in *P. aeruginosa* is presented in Fig. 5.

NO can be either endogenously produced as a metabolic intermediate of denitrification or come from exogenous NO donors (such as SNP). The NO molecule can increase motility directly, by acting on flagella, pili, and/or rhamnolipid production, or indirectly stimulate NIR expression to produce more NO or to facilitate flagellum assembly (Fig. 5). NIR expression or activity is also controlled by QS (RhlR and PQS), which thereby modulate NO levels in response to population clues. The response to NO in terms of biofilm formation/dispersal is, however, different under aerobic and anaerobic conditions: anaerobiosis favors the accumulation of NO, ultimately leading to biofilm formation as a stress defense mechanism. Thus, investigations of the effects of endogenously generated NO and/or those obtained with NO donors (such as SNP), should take oxygen availability into account more carefully, and such studies should be done under anaerobic or microaerobic conditions in both nonbiofilm (planktonic and dispersed) and biofilm cells.

A common theme in the dispersal of *P. aeruginosa* biofilms is the observed decrease of c-di-GMP intracellular levels: the major signaling pathways are those involving BdlA (an intracellular effector) and NbdA (a membrane-bound effector) (Fig. 5). However, many other molecular mechanisms of NO sensing and the downstream signaling events are still to be fully clarified. This includes not only the events leading from perception of various environmental signals to decreased cellular c-di-GMP levels but also the events following thereafter. How is the c-di-GMP concentration transduced into physiological responses, such as secretion of extracellular enzymes that degrade the matrix to release individual motile cells?

The other open questions to be answered concern the type and

role of transcriptional regulators involved and the mechanism of NO sensing by individual receptors (metals, prosthetic groups, and active thiols). To shed more light on the role of single transcriptional regulators in biofilm dispersal, it would be interesting to compare the behavior of genetic mutants of some of the proteins mentioned above (DNR and OxyR) with respect to dispersal. Moreover, it would be highly desirable to enlarge our knowledge regarding dispersal by investigating, in *P. aeruginosa* (as well as other denitrifying bacteria), the influence of quorum sensing. Last but not least, the effects of the growth medium should also be considered, using, instead of a rich undefined medium, other media, possibly more closely resembling the growth conditions encountered by the pathogen during colonization of host tissues, which were recently shown to greatly influence gene expression patterns (102).

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