JOURNAL OF BACTERIOLOGY, Dec. 2009, p. 7333–7342 Vol. 191, No. 23
0021-9193/09/$12.00 doi:10.1128/JB.00975-09

Nitric Oxide Signaling in *Pseudomonas aeruginosa* Biofilms Mediates Phosphodiesterase Activity, Decreased Cyclic Di-GMP Levels, and Enhanced Dispersal▽†

Nicolas Barraud,[1] David Schleheck,[1]‡ Janosch Klebensberger,[1] Jeremy S. Webb,[1]§ Daniel J. Hassett,[2] Scott A. Rice,[1] and Staffan Kjelleberg[1]*

School of Biotechnology and Biomolecular Sciences and Centre for Marine Bio-Innovation, University of New South Wales, Sydney, New South Wales 2052, Australia,[1] and Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267[2]

Received 24 July 2009/Accepted 22 September 2009

Bacteria in biofilms often undergo active dispersal events and revert to a free-swimming, planktonic state to complete the biofilm life cycle. The signaling molecule nitric oxide (NO) was previously found to trigger biofilm dispersal in the opportunistic pathogen *Pseudomonas aeruginosa* at low, nontoxic concentrations (N. Barraud, D. J. Hassett, S. H. Hwang, S. A. Rice, S. Kjelleberg, and J. S. Webb, J. Bacteriol. 188:7344–7353, 2006). NO was further shown to increase cell motility and susceptibility to antimicrobials. Recently, numerous studies revealed that increased degradation of the secondary messenger cyclic di-GMP (c-di-GMP) by specific phosphodiesterases (PDEs) triggers a planktonic mode of growth in eubacteria. In this study, the potential link between NO and c-di-GMP signaling was investigated by performing (i) PDE inhibitor studies, (ii) enzymatic assays to measure PDE activity, and (iii) direct quantification of intracellular c-di-GMP levels. The results suggest a role for c-di-GMP signaling in triggering the biofilm dispersal event induced by NO, as dispersal requires PDE activity and addition of NO stimulates PDE and induces the concomitant decrease in intracellular c-di-GMP levels in *P. aeruginosa*. Furthermore, gene expression studies indicated global responses to low, nontoxic levels of NO in *P. aeruginosa* biofilms, including upregulation of genes involved in motility and energy metabolism and downregulation of adhesins and virulence factors. Finally, site-directed mutagenesis of candidate genes and physiological characterization of the corresponding mutant strains uncovered that the chemotaxis transducer BdlA is involved in the biofilm dispersal response induced by NO.

Bacterial biofilms are highly dynamic communities which display a range of differentiated phenotypes during the course of development. By exchange of cell-cell signals, subpopulations of cells can coordinate their activity and undertake particular metabolic tasks or defense strategies (56). At times, the bacterial community releases single cells that escape from the biofilm and revert to a free-swimming, planktonic mode of growth, leaving behind hollow voids in the biofilm architecture (5, 37, 57). This process, referred to as dispersal, completes the biofilm life cycle and is thought to be important for successful colonization of new surfaces. Although the mechanisms underlying these events remain to be fully elucidated, previous studies of various species, including the opportunistic pathogen *Pseudomonas aeruginosa*, have revealed that dispersal events correlate with the induction of a specific phenotype that involves cellular motility (37, 42).

In *P. aeruginosa*, biofilm dispersal can be triggered by environmental factors, including nutrient (42, 45) and iron (4, 36) availability, and has recently been linked to the intracellular second messenger cyclic di-GMP (c-di-GMP) (45, 47). Numerous studies revealed that decreased c-di-GMP levels are related to a motile mode of growth and to cell dispersal in eubacteria. In this second messenger system, diguanylate cyclases (DGCs) and specific phosphodiesterases (PDEs) are responsible for the biosynthesis and the degradation of c-di-GMP, respectively. DGCs and PDEs contribute to a genetic network that responds to a broad range of environmental cues and/or cell-cell signals and modulate intracellular levels of c-di-GMP, which has been shown to regulate various cellular functions, including biofilm formation, virulence, and dispersal, in many bacterial species (47, 51–53). Recently, we identified the gas nitric oxide (NO) as an important factor in the regulation of dispersal in *P. aeruginosa* biofilms (5). Exogenous addition of nontoxic concentrations of NO, typically in the low nanomolar range, was found to stimulate motility and biofilm dispersal in *P. aeruginosa*. A role for anaerobic metabolism and NO in biofilm dispersal and survival was further supported by other studies of *P. aeruginosa* (54, 61), *Staphylococcus aureus* (44), and various single and multispecies biofilms (6).

NO is a water-soluble, hydrophobic free radical that can freely diffuse in biological systems. At high concentrations (micromolar to millimolar range), NO and downstream reactive nitrogen species (e.g., peroxynitrite [$ONOO^-$]) can have se-

* Corresponding author. Mailing address: School of Biotechnology and Biomolecular Sciences and Centre for Marine Bio-Innovation, Biological Sciences Building, University of New South Wales, Sydney, NSW 2052, Australia. Phone: 61 (2) 9385 2102. Fax: 61 (2) 9385 1779. E-mail: s.kjelleberg@unsw.edu.au.

† Supplemental material for this article may be found at http://jb.asm.org/.

‡ Present address: Department of Biology, University of Konstanz, D-78457 Konstanz, Germany.

§ Present address: School of Biological Sciences, University of Southampton, Southampton SO16 7PX, United Kingdom.

▽ Published ahead of print on 2 October 2009.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or characteristic(s)[a]	Source or reference
Strains		
P. aeruginosa		
PAO1	Wild type	19
Δ*nirS* strain	PAO1; Δ*nirS* Gm^r	61
KO[*bdlA*]	PAO1; *bdlA*::pKO[*bdlA*]	This study
KO[*fimX*]	PAO1; *fimX*::pKO[*fimX*]	This study
KO[*morA*]	PAO1; *morA*::pKO[*morA*]	This study
KO[*rocS1*]	PAO1; *rocS1*::pKO[*rocS1*]	This study
KO[PA0575]	PAO1; PA0575::IS*phoA*/hah in nucleotide 1900; Tet^r	21
KO[PA1181]	PAO1; PA1181::IS*lacZ*/hah in nucleotide 1757; Tet^r	21
KO[PA2072]	PAO1; PA2072::IS*lacZ*/hah in nucleotide 279; Tet^r	21
NSGFP	PAO1; *nirS*::*gfp* transcriptional reporter fusion	5
E. coli		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*Z*ΔM15::Tn*10* (Tet^r)]	Stratagene
S17-1 λ*pir*	*thi pro hsdR hsdM*$^+$ *recA* RP4-2-Tc::Mu-Km::Tn*7* λ*pir*	48
Plasmids		
pKnockout-G	Suicide vector used for insertional knockout construction; Gm^r	59
pKO[*bdlA*]	pKnockout-G harboring an internal fragment (750 bp) of *bdlA*	This study
pKO[*fimX*]	pKnockout-G harboring an internal fragment (1,484 bp) of *fimX*	This study
pKO[*morA*]	pKnockout-G harboring an internal fragment (1,201 bp) of *morA*	This study
pKO[*rocS1*]	pKnockout-G harboring an internal fragment (668 bp) of *rocS1*	This study

[a] Gm^r, gentamicin resistance; Tet^r, tetracycline resistance.

vere deleterious effects on cells. In recent years, it has become apparent that multiple regulatory systems in bacteria can mediate responses to NO exposure and activate detoxification mechanisms, thereby preventing damage from nitrosative stress (38, 49). Nevertheless, bacteria do not fully eliminate this radical, and lower concentrations of NO (nanomolar range) are commonly present in healthy bacteria (62, 63). For example, in denitrifying bacteria, NO is endogenously produced as an obligate intermediate of the anaerobic respiratory pathway, mainly through the activity of the nitrite reductase (Nir). To limit toxicity, its concentration is maintained at low levels within the bacterial cells by tight transcriptional control of *nirS* and the *norCB* genes, encoding the nitrite reductase precursor and two subunits of the nitric oxide reductase, respectively. In *P. aeruginosa*, NO itself may exert feedback control by inducing or repressing expression of the entire denitrification pathway, including *nirS* and *norCB* genes, via direct interactions with the regulators DNR (induction [2, 15]) and ANR (repression [62]). However, very little is known about the cellular and genetic responses to such low, physiological concentrations of NO. In biological systems, NO can react with high affinity to a broad variety of molecules, giving it a very short life span, in the order of seconds. Autooxidation with molecular oxygen (O_2) is the most abundant reaction, the most rapid of which occurs with superoxide (O_2^-) (11). Molecular targets of NO encompass heme/nonheme iron cofactors, iron-sulfur clusters, and other redox metal sites, all forming metal-nitrosyl complexes as well as cysteine thiols (S nitrosylation), amines, and others (50). Reactions with iron moieties appear predominant, and interestingly, in several bacterial species, NO can derepress iron acquisition mechanisms through inactivation of the ferric uptake regulator (Fur) (12, 34). The amount of NO required for inactivation of Fur was found to be in the micromolar range (12, 34), and thus, it remains unclear whether endogenous NO could exert such regulatory effect.

Sensory receptors encoded by conserved PAS (*P*er-*A*rnt-*S*im) or H-NOX (*h*eme-*n*itric oxide/*ox*ygen binding) domains that can potentially bind and respond to NO are commonly present in both eukaryotes and prokaryotes (9, 13, 20). Interestingly, genome sequence analyses revealed that *P. aeruginosa* contains multiple genes with such redox sensor domains associated with conserved GGDEF and EAL motifs with known or putative DGC and PDE activities, respectively (30). This strongly suggests a potential link between NO-sensing and modulation of c-di-GMP levels in *P. aeruginosa*.

The goal of this study was to test this hypothesis by performing PDE inhibitor studies, PDE enzyme activity measurements, and quantification of c-di-GMP levels in cells by liquid chromatography-tandem mass spectrometry (LC–MS-MS) analysis in the presence or absence of NO. Results from this study demonstrated that NO-mediated dispersal requires the chemotaxis transducer BdlA (*b*iofilm *d*ispersion *l*ocus) and involves increased PDE activity, resulting in an overall decrease in intracellular c-di-GMP levels in *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains, culture media, and chemicals. The *P. aeruginosa* strains used in this study are listed in Table 1. Overnight cultures were routinely grown in Luria-Bertani (LB) medium with 10 g liter^{-1} NaCl with shaking at 37°C and supplemented with antibiotics where appropriate. Biofilms were grown in modified M9 minimal medium (57) containing 48 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl, 2 mM $MgSO_4$, 100 μM $CaCl_2$ (pH 7.0), and glucose at 5 mM for continuous-culture-flow-reactor experiments and 20 mM for batch culture petri dish experiments. Fresh solutions of the NO donor sodium nitroprusside (SNP) (Sigma) or the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) (Sigma) were made daily in culture medium and protected from light. Although the exact amount and location of NO liberated in

vivo within biofilms from SNP have not yet been elucidated, a 1,000-fold linear relationship between SNP concentrations and NO concentrations was measured in vitro ($R^2 \geq 0.95$) by using a NO analyzer (Apollo 4000 with ISO-NOP electrode; World Precision Instruments, Sarasota, FL). NO concentrations (100 nM to 1 μM) were determined for a range of SNP solutions (100 μM to 1 mM) in M9 medium. In these experiments, NO levels reached steady state after 10 to 15 min and were maintained over several hours. The sensitivity limit for the electrode system was approximately 20 nM NO. c-di-GMP (Biolog, San Diego, CA) and GTP (Sigma) stock solutions of 1 mM and 200 mM, respectively, were made in Milli-Q H_2O and stored at −20°C. bis(*p*-nitrophenyl) phosphate (bis-pNPP) (Sigma) was directly dissolved in buffer prior to the enzymatic assay.

Biofilm experiments. (i) Flow reactor (continuous culture) biofilm experiments. *P. aeruginosa* biofilms were cultivated on the interior surfaces of tubing of a once-through continuous-flow-reactor system at room temperature with a flow rate of 10 ml h^{-1}. Earlier studies showed that *P. aeruginosa* biofilms grown on the inner walls of silicone tubing exhibited morphological similarities with biofilms grown on glass substrata in flow cells (17). The reactor was composed of Tygoprene XL-60 silicone tubing (Saint-Gobain Performance Plastics) (internal diameter, 3.2 mm; wall thickness, 1.6 mm; length, 200 mm; internal volume, 1.6 ml). Biofilms were cultivated for 5 days in M9 medium in triplicate. Then, biofilm dispersal was induced by adding SNP at a final concentration of 5 μM to the growth medium. This concentration of SNP was determined to generate steady-state NO concentrations of 5 nM in M9 medium in a static glass vial by using the Apollo 4000 NO analyzer (data not shown). Because the type of tubing had an impact on biofilm biomass, we selected Tygoprene tubing and 5 μM SNP for our experiments, as these conditions allowed sufficient biofilm biomass remaining after induction of dispersal to be harvested for subsequent extractions and analysis. The total number of viable bacteria in the effluent runoff of biofilms was measured by performing serial dilutions and CFU counts on LB agar at 37°C. Data are presented as changes in effluent runoff CFU numbers for SNP-treated biofilms in comparison to the level for untreated biofilms.

(ii) Batch culture biofilm experiments. Biofilms of *P. aeruginosa* wild-type and mutant strains were grown in batch cultures in petri dishes (90 mm in diameter) containing glass microscope slides, as previously described (5). Biofilms were first allowed to develop for 24 h in M9 medium in the absence of NO. After 24 h, planktonic bacteria were removed and replaced with fresh M9 medium with or without the NO donor SNP at a final concentration of 500 nM to induce biofilm dispersal (5) and with or without 250 μM or 1 mM GTP. Inhibition of PDE activity upon exogenous addition of GTP was validated by using a PDE enzymatic assay as described below. The system was then incubated for an additional 24 h with constant incandescent lighting. Slides were rinsed gently in sterile phosphate-buffered saline (PBS). The biofilm on the slides was then stained with 250 μl of SYTO 9 (3 μl ml^{-1}) (Molecular Probes, Eugene, OR) for 20 min in a humidified chamber. Fifteen selected fields of view per slide were imaged in the *x-y* plane by using an epifluorescence microscope (Leica model DMR) at regular intervals and across the entire slide. Image analysis (ImageJ software; NIH) was performed to determine the percentage of the glass surface covered with biofilm. Experiments were performed in triplicate, and a statistical comparison of the percentages of surface covered by biofilms was performed using one-way analysis of variance and Tukey's multiple comparison tests.

In vitro PDE activity assay. The effect of NO on PDE activity in *P. aeruginosa* was assessed in vitro by using the PDE-specific synthetic substrate bis-pNPP, as previously described (8, 29). Three milliliters of late-log-phase *P. aeruginosa* cells grown in LB medium and adjusted to an optical density at 600 nm (OD_{600}) of 2.0 were harvested by centrifugation at 6,000 × *g* at 4°C and resuspended in buffer containing 50 mM NaCl, 50 mM Tris base (pH 8.1), 1 mM $MnCl_2$, and bis-pNPP at a final concentration of 5 mM. Cells were either lysed with CelLytic Express (Sigma) or kept intact. Then, SNP was added to the mixtures in 50-ml Falcon tubes (BD), and the tubes were incubated vertically in the dark at 37°C with shaking at 60 rpm in triplicate. In these experiments, degradation of bis-pNPP and the release of *p*-nitrophenol was linear and quantified with a 200-μl aliquot by measuring OD_{405} with a spectrophotometer (Wallac Victor^2; PerkinElmer, Waltham, MA) after 2 h of incubation, at a time point when the highest differences in *p*-nitrophenol production could be observed as identified in preliminary experiments (not shown). The controls consisted of medium without cells with or without SNP or CelLytic reagent in medium without cells.

Detection and quantification of c-di-GMP in *P. aeruginosa*. For c-di-GMP extraction from planktonic bacteria, overnight cultures were diluted to an OD_{600} of 0.2 in fresh LB or M9 medium and grown with shaking at 37°C to an OD_{600} of 0.4. SNP was applied to 5 ml aliquots of the culture, and the solutions were incubated for 2 h. After treatment, heat inactivation of cells and ethanol extraction of c-di-GMP were performed as previously described (47, 51). Specifically, cell pellets were heated at 100°C for 3 min, cooled on ice, resuspended in 1 ml of ice-cold 65% ethanol, and homogenized by passing five times through a 20-gauge needle. Then, samples were incubated 20 min on ice, followed by centrifugation for 10 min at 4°C, and the supernatants retained. The extraction was repeated, and the supernatants were combined. The remaining cell debris was used for subsequent protein quantification by using a Lowry-based total protein assay (25). Nucleotide extracts were frozen at −80°C and lyophilized. Samples were then dissolved in 500 μl of Milli-Q H_2O, and chloroform (1 ml) was added to the suspensions, vortexed, and separated by centrifugation for collection of 400 μl. For c-di-GMP extraction from biofilm bacteria, the biofilm on the inner walls of the tubing reactor were washed by a gentle bath of 3 ml ice-cold PBS and the tubing containing the biofilm was immersed in boiling water for 5 min. After cooling, biofilm cells were resuspended in 3 ml ice-cold 65% ethanol and harvested by compressing the exterior of the tubing by hand. Then, the extraction procedure was continued as described above. Nucleotides were extracted in at least three independent experiments.

For detection of c-di-GMP in cell extracts, reversed-phase high-pressure LC on a Nucleosil C_{18} 100-5 column (3 by 125 mm; particle size, 5 μm) (Macherey-Nagel, Düren, Germany) coupled with the use of an electrospray ionization triple-quadrupole mass spectrometer in positive-ion mode was performed using the TSQ Quantum Access Max system (Thermo Scientific, Waltham, MA). The mobile phases were 50 mM ammonium acetate in water (pH 4.0) (phase A) and 100% acetonitrile (phase B). The gradient program was initiated with 100% phase A at a flow rate of 500 μl min^{-1}, and after 0.5 min, the portion of phase A was decreased linearly to 10% in 4 min and then restored to 100% in 1 min; then, the portion of phase A was again decreased to 10% in 1 min and restored to 100% in 0.5 min to eliminate carryover between injections. The temperature was set at 30°C, and the cycle time was 10 min. Authentic c-di-GMP (Biolog) was used to identify c-di-GMP in cell extracts on the basis of (i) identical elutions (i.e., cochromatography) at 3.6 min, (ii) identical masses of the protonated molecular ion ($[M+H]^+$) ($m/z = 691$), and (iii) identical MS-MS fragmentation patterns of isolated precursor ions for major components with m/z values of 152 and 540 (loss of guanine) (47).

For quantification of c-di-GMP in cell extracts, since no isotopically labeled c-di-GMP was available, a standard curve, whereby defined concentrations of authentic c-di-GMP were spiked to pooled cell extracts and analyzed against unspiked, pooled cell extracts, was established. The areas of the major fragments of c-di-GMP ($m/z = 152$ and 540) were used to estimate the amount of c-di-GMP in a sample, referred to in mg of cellular protein. One-way analysis of variance and Tukey's multiple comparison tests were used for statistical analysis of these experiments.

RNA isolation from *P. aeruginosa* biofilms. To determine gene expression patterns associated with NO-mediated biofilm dispersal in *P. aeruginosa*, the relative abundances of mRNA were compared for biofilm cells grown in continuous-flow reactors, with and without exposure to NO in two settings. First, *P. aeruginosa* biofilms were grown for 5 days in the absence of SNP, before a mature biofilm was exposed to 5 μM SNP to initiate dispersal and another biofilm was left untreated as a control (SNP versus untreated control experiment). After 1 h, RNA was extracted from both biofilm populations. Three independent replicate experiments were performed. Second, wild type and Δ*nirS* mutant (unable to produce endogenous NO) biofilms were grown for 5 days, and RNA was extracted from both biofilm populations (wild type versus Δ*nirS* mutant experiment). This experiment was performed in duplicate. For RNA extraction, the biofilm remaining on the walls of the tubing was washed by a gentle bath of 3 ml ice-cold PBS to remove planktonic bacteria. Biofilm cells were immediately resuspended in 3 ml ice-cold RNA*later* RNA stabilization reagent (Qiagen, Hilden, Germany) and harvested from the interior surface of the tubing. Total RNA was extracted by using RNeasy RNA isolation kits (Qiagen) in accordance with the manufacturer's instructions and precipitated with 0.1 volume of 3 M sodium acetate, pH 5.2, and 3 volumes of ethanol. The RNA concentration and purity were determined by OD_{260} and OD_{280} measurements ($OD_{260} = 1.0 = 40$ μg RNA ml^{-1}, and $2.0 < OD_{260}/OD_{280} < 2.1$, respectively). Biotinylated cDNA samples were synthesized and hybridized to PaeG1a oligonucleotide arrays by using Affymetrix GeneChip microarrays (Affymetrix, Santa Clara, CA) in accordance with the manufacturer's manual at the Ramaciotti Centre for Gene Function Analysis, The University of New South Wales.

Analysis of microarray results. Gene name, number, and annotation are from the Pseudomonas Genome Database (60). ArrayAssist version 5.0 (Stratagene, La Jolla, CA) was used for hybridization data processing, filtering, and statistical analysis. The probe array images were inspected for any image artifact. Polyadenosine RNA control intensities were used to monitor the labeling process. The overall fluorescence intensity of each microarray was normalized using the probe logarithmic intensity error estimate method to remove intensity-dependent effects of the arrays. Then, statistical analysis was done by unpaired *t* testing,

and *P* values were obtained from calculated *t* statistics by using asymptotic analysis under the assumptions of normal distributions of expression values and equal variances. Genes were considered to be differentially expressed if the differences in change over three independent experiments (SNP versus untreated control) or two independent experiments (wild type versus Δ*nirS* mutant) had a *P* value of ≤0.05 and the absolute value of the average was equal to or greater than the standard deviation (≥1.3-fold) for the whole-microarray experiments (53).

Real-time PCR. To validate changes in gene expression observed in the microarray experiments, the mRNA levels of six selected genes and one housekeeping gene (*proC*) (43) from biofilm cells treated with or without 5 μM SNP by using the same experimental conditions as those for microarray analysis were examined by real-time quantitative PCR (qPCR) in triplicate. cDNA strands were synthesized by a SuperScript III reverse transcriptase reaction mixture (Invitrogen, Carlsbad, CA), and qPCR was carried out with a Rotor-Gene RG-3000A qPCR detection system (Corbett Research, New South Wales, Australia) by using Platinum SYBR green qPCR SuperMix-UDG (Invitrogen) with 100 ng of cDNA and a 500 nM concentration of each primer. Controls consisted of samples to which no cDNA template had been added and to which unmodified mRNA was added. Similar levels of mRNA for the housekeeping gene *proC* were found in treated and untreated biofilm cells (change, 1.0-fold). Primers for each selected gene were designed using PrimerSelect software (DNASTAR, Madison, WI) (see Table S1 in the supplemental material). Following PCR, relative expression ratios were calculated using $2^{\Delta CT}$, where ΔCT represents the difference in cycling times for the two samples being compared. Unpaired *t* testing was used for statistical analysis.

***nirS* transcription reporter strain (NSGFP) assay.** To confirm upregulation of the *nirS* gene upon exposure to low doses of NO, a *P. aeruginosa* transcriptional fusion reporter strain, NSGFP, which expresses green fluorescent protein (GFP) when the anaerobic gene *nirS* is expressed (5), was used. Overnight cultures of NSGFP were diluted to an OD_{600} of 0.2 in fresh LB medium and grown with shaking at 37°C to an OD_{600} of 0.4. SNP treatments were added to 10-ml aliquots of the cultures in 50-ml Falcon tubes (BD) in triplicate, and the bacteria were incubated in the dark for a further 2 h. After exposure, cells were washed once in ice-cold PBS and resuspended in 1 ml ice-cold PBS. Two-hundred-microliter aliquots were transferred to a microtiter plate for fluorescence measurements (excitation, 485 nm; emission, 535 nm) (Wallac Victor^2).

Pyoverdine release assay. To assess the effect of low doses of NO on the production of the virulence factor pyoverdine in *P. aeruginosa*, overnight cultures of wild-type bacteria were washed once with PBS and diluted 50 times in 5 ml King's medium B (26) in 50 ml Falcon tubes (BD, Franklin Lakes, NJ) in the presence or absence of the NO donor SNP in triplicate. SNP was added to the tubes to give final concentrations of 500 nM or 5 μM or was not added, and the cells were incubated in the dark at 37°C at 100 rpm for 4 h to allow for sufficient release of pyoverdine in untreated control cultures. The relative concentrations of pyoverdine siderophore in the supernatants were quantified from 200-μl aliquots by using a microtiter plate fluorometer (excitation, 355 nm; emission, 460 nm) (Wallac Victor^2).

Construction of *P. aeruginosa* insertional mutants. To construct insertional mutants of the genes *bdlA*, *fimX*, *morA*, and *rocS1*, internal fragments of these genes were amplified by PCR, using *P. aeruginosa* PAO1 genomic DNA as a template, with appropriate primer pairs (see Table S1 in the supplemental material). The purified PCR products of *bdlA*, *fimX*, *morA*, and *rocS1* were digested with SalI, SpeI-PstI, BamHI-HincII, and BamHI-HincII, respectively, and the correct-sized fragments were gel purified and cloned into the respective restriction sites of the suicide vector pKnockout-G (59) before transformation into *Escherichia coli* XL1-Blue (Stratagene). The resulting suicide vectors pKO[*bdlA*], pKO[*fimX*], pKO[*morA*], and pKO[*rocS1*] were transferred in strain PAO1 by biparental mating, with *E. coli* strain S17-1 as a donor, as described previously (27). Correct chromosomal insertion of the vectors was confirmed by PCR with appropriate primer pairs (see Table S1 in the supplemental material).

RESULTS

PDE activity in *P. aeruginosa* biofilms during NO-mediated dispersal. Previously, NO was found to induce a transition from the biofilm to the planktonic mode of growth and to increase swimming and swarming motilities in *P. aeruginosa* (5). Exposure to the NO donor SNP also induces dispersal events in biofilms cultivated under continuous-flow laboratory conditions. After 1 h of treatment with 5 μM SNP, biofilms exhibited a 2.4-fold increase in CFU released in their effluent runoff compared to untreated biofilms (Fig. 1A). In this assay, SNP was added at 5 μM rather than 500 nM to compensate for the higher O_2 levels in the biofilm medium from diffusion through the silicone tubing than in the batch culture biofilm assay, which has previously demonstrated the NO-induced dispersal event (5). Because the effects of NO on *P. aeruginosa* physiology closely resemble those regulated by the intracellular secondary messenger c-di-GMP (47), and because genes putatively involved in the turnover of c-di-GMP are often found to be associated with NO sensors (9, 20), the potential role of this signaling dinucleotide in the response to low doses of NO was investigated.

To assess the potential involvement of PDE activity during NO-mediated dispersal in *P. aeruginosa* biofilms, GTP, the precursor of c-di-GMP, which is known to inhibit some PDE activity and the degradation of c-di-GMP (40), was used. First, the inhibition of intracellular PDE activity by GTP in *P. aeruginosa* was confirmed when intact cells grown in suspension cultures were incubated with the PDE-specific substrate bis-pNPP, as previously described (8, 29). Addition of 250 μM and 1 mM GTP to intact cells of the *P. aeruginosa* wild type in buffer with bis-pNPP resulted in dose-dependent inhibition of PDE activity as determined by *p*-nitrophenol production (Fig. 1B). Second, with the use of the previously described batch culture biofilm dispersal assay (5), which focuses on NO induction effects during the initiation of dispersal, preestablished *P. aeruginosa* biofilms were exposed to 500 nM SNP to trigger dispersal in the presence or absence of various concentrations of GTP. SNP treatment alone induced a 68% reduction in biofilm surface coverage compared to the level for untreated control biofilms, indicative of dispersal events (Fig. 1C). NO-mediated dispersal was significantly abrogated in the presence of 250 μM and 1 mM GTP in a dose-dependent manner (Fig. 1C) ($P < 0.001$). Treatment with 500 nM SNP in the presence of 250 μM GTP and 1 mM GTP resulted in only 43% and 10% reduction in biofilm surface coverage, respectively, compared to the level for untreated biofilms. The addition of GTP alone was not found to induce any increase in surface coverage when assessed for biofilms without NO addition (Fig. 1C). Thus, the results revealed that NO induction of biofilm dispersal was significantly impaired in the presence of GTP and linked to altered PDE activity within the cells.

To determine whether NO stimulates the activity of PDEs present in *P. aeruginosa*, the overall PDE activity in cell extracts of *P. aeruginosa* grown in suspension was determined by using bis-pNPP as a substrate in the presence or absence of SNP. Addition of SNP at final concentrations of 500 nM, 5 μM, and 50 μM increased the overall PDE activities by 20%, 45%, and 31%, respectively, compared to the level for untreated cell extracts (Fig. 1D). In contrast, higher concentrations of SNP, 5 mM and 50 mM (releasing 5 μM and 50 μM NO, respectively), caused inhibitions of PDE activity of 66% and 75%, respectively, in cell extracts. Moreover, addition of the NO scavenger PTIO (100 μM), which was previously found to be effective at preventing exogenous NO from inducing biofilm dispersal (5), decreased the apparent PDE stimulation in cell extracts after SNP addition. Interestingly, PTIO treatment also decreased the overall PDE activity in cell extracts not treated with SNP (Fig. 1D). This observation suggests that low, basal levels of

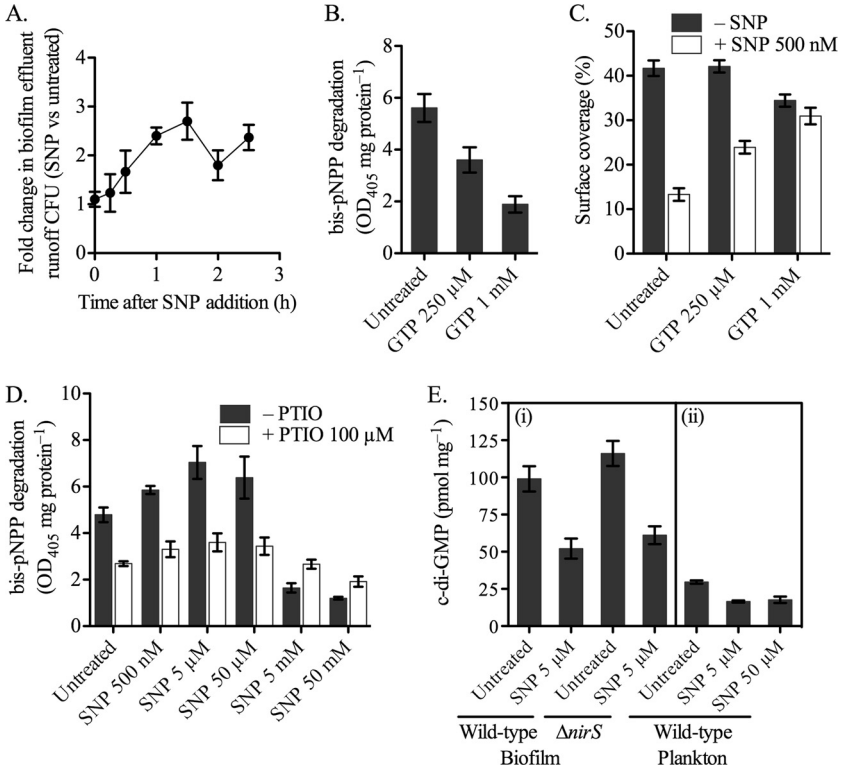

FIG. 1. NO-mediated dispersal in *P. aeruginosa* involves PDE activity and decreased levels of intracellular c-di-GMP. (A) Induction of *P. aeruginosa* biofilm dispersal by the addition of the NO donor SNP in continuous cultures. Five-day-old biofilms were exposed to 5 μM SNP, and the induction of dispersal was measured by comparing CFU counts released in the effluent runoff with those for the untreated control biofilms. (B) Addition of GTP inhibits the degradation of the PDE-specific substrate bis-pNPP by intact *P. aeruginosa* cells in a dose-dependent manner. PDE activity was quantified as *p*-nitrophenol production at OD_{405}. (C) Inhibition of NO-mediated dispersal in *P. aeruginosa* by GTP. Preestablished *P. aeruginosa* biofilms were treated with (white bars) or without (dark bars) SNP at 500 nM as previously described (5) in the absence or presence of 250 μM or 1 mM GTP; then, the biofilms on the slides were quantified (percent surface coverage) using digital image analysis. (D) Low doses of NO stimulate PDE activity in cell extracts of *P. aeruginosa*. Cell extracts of *P. aeruginosa* were incubated with 5 mM bis-pNPP in the presence of various concentrations of SNP and in the presence (white bars) or absence (dark bars) of 100 μM of the NO scavenger PTIO. The release of *p*-nitrophenol was quantified at OD_{405} after 2 h. (E) Effect of low concentrations of NO on c-di-GMP levels in *P. aeruginosa*. (i) Five-day-old *P. aeruginosa* wild-type and Δ*nirS* mutant biofilms were treated with 5 μM SNP for 1 h to initiate dispersal or were left untreated before nucleotides were extracted from biofilm cells as described in Materials and Methods. (ii) *P. aeruginosa* in suspension cultures was exposed to various concentrations of SNP for 2 h, and nucleotides were extracted. c-di-GMP was detected in the nucleotide samples, quantified by LC–MS-MS, analysis and referred to in mg of cellular protein. Data are mean values, and error bars indicate standard errors of the means ($n = 3$).

NO may have been present in the cell extracts and contributed to the basal PDE activity, even without exogenous addition of NO via SNP. Addition of PTIO was also tested with lysed cell solutions in the presence of *p*-nitrophenyl phosphate, a substrate specific for phosphomonoesterase. No significant difference from solutions without PTIO could be detected, suggesting that PTIO itself does not have any unspecific inhibitory effect on phosphomonoesterase activity (not shown). Finally, similar stimulatory effects on PDE activity by low doses of NO were also observed by using intact *P. aeruginosa* cells or cells from 5-day-old biofilms grown in continuous-flow reactors (data not shown).

Low doses of NO reduce c-di-GMP levels in *P. aeruginosa* cells. The stimulatory effect of NO on PDE activity described above implied an increased degradation of c-di-GMP in *P. aeruginosa* during NO-mediated dispersal. In order to test this, a method for quantification of intracellular c-di-GMP by use of LC–MS-MS analysis was adapted (see Materials and Methods). With this method, the average c-di-GMP concentration was determined to be 101 pmol per mg cellular protein (pmol mg^{-1}) in cells of *P. aeruginosa* taken from 5-day-old biofilms grown in continuous-flow cultures (Fig. 1E). In contrast, in planktonically grown cells of *P. aeruginosa*, the concentration of c-di-GMP was 30 pmol mg^{-1}, representing a 3.4-fold-decreased c-di-GMP content in planktonic cells compared to the level for biofilm-grown cells. We applied this method to quantify c-di-GMP in biofilm and planktonic bacteria that were exposed to low levels of the NO donor SNP. Mature biofilms of the parent strain and a Δ*nirS* mutant, which is unable to produce endogenous NO, grown for 5 days in the absence of SNP were exposed to 5 μM SNP to induce dispersal (Fig. 1A). After 1 h of treatment, at a time point when dispersal had been initiated but sufficient amounts of biofilm could still be harvested, c-di-GMP was extracted from the biofilm cells. Treatment with SNP resulted in 47% (54 pmol mg^{-1}) and 45% (63 pmol mg^{-1}) ($P < 0.01$) decreases of intracellular c-di-GMP levels in cells of the parent strain and the Δ*nirS* mutant, respectively, compared to the level for untreated controls (Fig. 1E). Further, cells from planktonic cultures of *P. aeruginosa* exposed to 5 μM and 50 μM SNP for 2 h showed 44% (17 pmol

TABLE 2. Comparison of microarray and qPCR analyses of six selected genes in *P. aeruginosa* biofilm cells exposed to NO donor SNP (5 μM) and in untreated biofilm cells

Gene	Fold change (*P*) (*n* = 3)	
	qPCR	Microarray
Upregulated		
nirS	12.51 (<0.001)	2.23 (0.008)
pilA	3.42 (<0.001)	2.31 (0.008)
Downregulated		
pelC	−1.93 (0.035)	−1.29 (0.038)
cupB2	−1.72 (0.038)	−1.92 (0.013)
fhp	−1.79 (0.029)	−1.44 (0.027)
pvdE	−2.01 (<0.001)	−1.58 (0.042)
proC (unchanged; housekeeping gene)	1.01 (0.688)	−1.08 (0.787)

mg^{-1}) and 40% (18 pmol mg^{-1}) decreases in c-di-GMP levels, respectively, compared to the level for untreated planktonic bacteria (Fig. 1E) ($P < 0.01$). Overall, these results strongly suggest that low levels of NO stimulate PDE activity in *P. aeruginosa*, leading to a decrease of intracellular c-di-GMP levels, which results in enhanced dispersal of biofilm bacteria.

Changes in gene expression profiles upon exposure to low concentrations of NO in *P. aeruginosa* biofilms. To further investigate the regulatory mechanisms involved in NO-mediated dispersal in *P. aeruginosa*, we examined changes in gene expression in biofilm cells during induction of dispersal by NO. For each microarray analysis, we focused on genes with expression levels that were consistently affected across replicate experiments, with a *P* value of ≤0.05 (unpaired *t* test), and examined those that were the most up- or downregulated, with an absolute level of change higher than the standard deviation for the whole-microarray experiments. The observed changes in expression were supported by real-time qPCR analysis for six genes from 5-day-old biofilms after exposure to 5 μM SNP, which confirmed the transcriptomic pattern of the corresponding microarray analysis (Table 2).

The results indicated that the expression of genes involved in anaerobic metabolism, motility, attachment, cell envelope biogenesis, adaptation, protection virulence, and c-di-GMP signaling as well as bacteriophage genes were differentially expressed (Table 2; see also Table S2 in the supplemental material). The *pilA* gene, involved in twitching and swarming motilities (28), was found to be upregulated in the presence of NO, which correlates with increased motility behavior as previously observed for *P. aeruginosa* after NO exposure (5). In contrast, expression of adhesion genes, such as *cupB* and *cupC*, was reduced (Table 2; see also Table S2 in the supplemental material). The *cupB* and *cupC* genes, which are involved in interconnection of cells and biofilm formation, are known to be regulated by the Roc two-component system in which RocS1 is a membrane sensor with a PAS domain and one of the corresponding response regulators has an EAL domain (31). Further, genes involved in the production of virulence factors, such as *pvd*, essential for pyoverdine biosynthesis, and genes involved in defense mechanisms, such as *fhp*, implied in resistance to nitrosative stress (14), were found to be downregulated in biofilms exposed to 5 μM SNP versus untreated biofilms (Table 2). To confirm these NO-induced changes in gene expression, production of pyoverdine was directly assessed in the presence or absence of NO in *P. aeruginosa* grown in King's B medium (26). Addition of 500 nM and 5 μM SNP showed dose-dependent responses, resulting in 2.6- and 8.8-fold decreases in pyoverdine production, respectively, compared to the level for untreated cells (Fig. 2A).

Genes involved in energy metabolism, in particular the denitrification pathway, including *nirS* and *norC*, were found to be upregulated in biofilms treated with SNP (Table 2; see also Table S2 in the supplemental material). To further investigate this transcriptional activation, we used a *P. aeruginosa* reporter strain, NSGFP (5), which expresses GFP under the control of the *nirS* promoter, to monitor *nirS* expression levels in response to low doses of NO. SNP addition to NSGFP cells at final concentrations of 5 μM, 50 μM, and 250 μM induced 1.6-, 2.1-, and 3.1-fold increases in *nirS* expression, respectively, compared to the level for untreated cells (Fig. 2B), verifying the microarray data described above.

DNA microarray results comparing the wild type versus Δ*nirS* also indicated that NO altered the expression of multiple genes suggested to be involved in the turnover or regulation of the secondary messenger c-di-GMP (see Table S2 in the supplemental material), including *morA* (downregulated) and *bdlA* (upregulated). MorA has been linked to the expression of adhesive surface structures essential for biofilm formation,

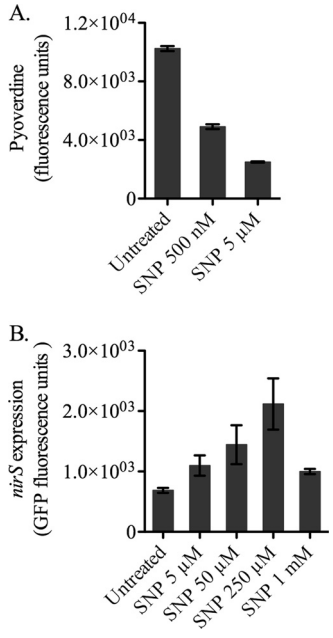

FIG. 2. Low doses of NO induce physiological changes in *P. aeruginosa*. (A) Pyoverdine production of *P. aeruginosa* cells was quantified by fluorescence measurement (excitation, 355 nm; emission, 460 nm) after growth in King's B medium in the presence or absence of low doses of SNP. (B) Expression of the denitrification gene *nirS*. A *P. aeruginosa* reporter strain, NSGFP, which expresses GFP under the control of the *nirS* promoter, was used to monitor the relative expression levels of the denitrification gene *nirS* in planktonic cells exposed to various concentrations of SNP. Data are mean values, and error bars indicate standard errors of the means ($n = 3$).

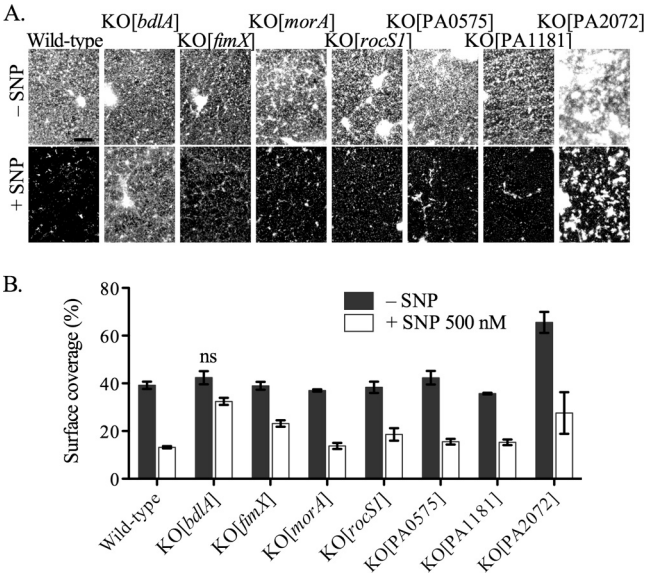

FIG. 3. NO-mediated dispersal in *P. aeruginosa* knockout mutants of selected genes. The mutants were assessed in a batch culture biofilm dispersal assay in the presence or absence of 500 nM SNP; then, the biofilms on the slides were stained with SYTO 9 to allow analysis using fluorescence microscopy and were quantified (percent surface coverage) using digital image analysis. (A) The images show microscopic pictures of the biofilms on glass slides treated without (−) or with (+) 500 nM SNP. Bar, 50 μm. (B) The bars show levels of biofilm surface coverage when treated with 500 nM SNP (white bars) or untreated controls (dark bars). Data are mean values, and error bars indicate standard errors of the means (n ≥ 3). ns, not significant.

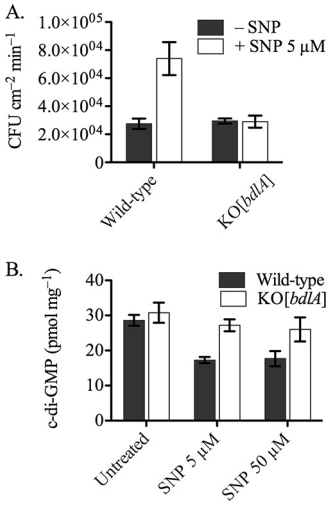

FIG. 4. (A) Biofilms of the BdlA mutant strain, KO[*bdlA*], do not disperse when exposed to low doses of NO in continuous-flow cultures. Five-day-old biofilms of the *P. aeruginosa* wild-type and KO[*bdlA*] mutant strains were exposed to 5 μM SNP for 1 h. Subsequently, the dispersal of cells was determined by CFU counts in the biofilm effluent runoff and compared to the level for an untreated control. (B) Effect of low concentrations of NO on c-di-GMP levels in the *P. aeruginosa* wild-type and KO[*bdlA*] strains. *P. aeruginosa* wild-type (dark bars) and KO[*bdlA*] mutant (white bars) cells in suspension cultures were exposed to 5 μM and 50 μM SNP and controls for 2 h, and c-di-GMP was extracted and quantified by using LC–MS-MS. Data are mean values, and error bars indicate standard errors of the means ($n = 3$).

thus suggesting that MorA may exhibit DGC activity (33), while BdlA is known to regulate c-di-GMP levels in *P. aeruginosa* (35).

NO-mediated dispersal in *P. aeruginosa* involves the chemotaxis regulator BdlA. The results presented thus far strongly suggest that low levels of NO trigger dispersal events in biofilm cells via c-di-GMP signaling by altering gene expression patterns that favor a planktonic lifestyle, e.g., increased motility and decreased surface attachment, and by changes in the expression of genes which are involved in c-di-GMP signaling. On the basis of these results, we selected genes which might be involved in the NO-induced dispersal by applying the following criteria: (i) the gene was found to be differentially expressed in the microarray data or is involved in the regulation of a gene which was identified in the microarray, (ii) the gene product is known or predicted to be involved in the turnover of c-di-GMP (EAL/GGDEF domains [31]) or known to influence intracellular c-di-GMP levels, (iii) the gene product is predicted to harbor a PAS domain for potential NO sensing (13, 16), and (iv) the gene product is known to regulate or alter the expression of surface structures involved in biofilm development or motility. Finally, genes which met at least three of these criteria were selected for further analysis using a site-directed mutagenesis approach with *P. aeruginosa*. This screening process identified seven genes, namely, *bdlA* (i to iii) (35), *fimX* (ii to iv) (24), *morA* (i to iv) (33), *rocS1* (i to iv) (31), PA0575 (i to iii) (30), PA1181 (i to iv) (30), and PA2072 (i to iii) (30). Insertional knockout mutants of these genes were then tested for their ability to disperse from batch culture biofilms in response to low doses of NO. Preestablished biofilms grown on glass slides were exposed to 500 nM SNP to induce biofilm dispersal as previously reported (5). Addition of NO to all strains resulted in 40 to 66% reductions (statistically significant [$P < 0.05$]) in biofilm surface coverage in comparison to the level for the untreated control, except for the BdlA mutant strain KO[*bdlA*], which showed only a 23% reduction and was not significantly different from the untreated control (Fig. 3).

To confirm the involvement of BdlA in the NO-mediated dispersal, biofilms were allowed to grow under continuous-flow conditions for 5 days without SNP, and then 5 μM SNP was added to the growth medium. After 1 h of exposure and initiation of biofilm dispersal (Fig. 1A), dispersing bacteria in the biofilm effluent runoff were enumerated by CFU counts on agar plates and compared to the corresponding untreated control. In the untreated controls, no significant difference was observed for the biofilm effluent runoff of strain KO[*bdlA*] in comparison to the *P. aeruginosa* wild-type strain (3.0 and 2.8 CFU cm^{-2} min^{-1}, respectively). Exposure to 5 μM SNP increased the number of cells that detached from the wild-type biofilm by 2.7-fold ($P < 0.05$), whereas the number of cells detached from the KO[*bdlA*] strain biofilm remained unchanged (Fig. 4A).

To further investigate the role of BdlA in the response to NO, levels of c-di-GMP in planktonically grown cells of strain KO[*bdlA*] were determined and compared to the level for the parent strain in the presence or absence of SNP. When strain KO[*bdlA*] was cultured in the presence or absence of NO treatment, no significant difference in intracellular concentration of c-di-GMP (31, 27, and 26 pmol mg^{-1} with 0, 5, and 50

μM SNP, respectively) (Fig. 4B) was observed in comparison to the level for the parent strain, which showed significant reduction of c-di-GMP levels upon exposure to NO (30, 17, and 18 pmol mg^{-1} with 0, 5, and 50 nm SNP, respectively) (Fig. 4B).

DISCUSSION

NO was previously found to induce a transition from a biofilm to a planktonic lifestyle in *P. aeruginosa*. In this study, we show that inhibition of PDE activity impaired NO-mediated biofilm dispersal and that low levels of NO stimulated PDE activity in *P. aeruginosa* and induced an overall decrease in intracellular c-di-GMP levels. In addition, microarray analysis revealed that several responses are affected upon addition of low, physiological doses of NO, mostly corresponding to c-di-GMP-regulated phenotypes and a transition from a biofilm to a free-living mode of growth. Hence, the data suggest that NO signaling is part of a global regulatory network that controls the switch between biofilm and planktonic phenotypes and involves the secondary messenger c-di-GMP. Further, analysis of several mutant strains showed that NO-mediated dispersal in *P. aeruginosa* requires the chemotaxis transducer BdlA.

NO operates via the c-di-GMP signaling network. Comparison of c-di-GMP levels in wild-type *P. aeruginosa* biofilm and planktonic cells demonstrated lower levels of c-di-GMP present in cell extracts of planktonic bacteria than for biofilm-grown cells. This is in agreement with previous reports, which demonstrated that reduced c-di-GMP is associated with dispersal or a planktonic phenotype in various bacterial species, including *P. aeruginosa* (30, 41, 47). Furthermore, our results show that exogenous NO addition reduced the overall intracellular c-di-GMP content in both biofilm and planktonic cells. A recent study demonstrated that cells of *P. aeruginosa* exist mostly in small aggregates during growth in suspension cultures and that c-di-GMP levels are significantly higher in these cell cultures than in those consisting of freely suspended cells (45). Therefore, the decrease in c-di-GMP levels in planktonically grown cells after exposure to SNP observed here is in agreement with the current hypothesis of c-di-GMP-regulated phenotypes, as it indicates a dispersal event from mostly aggregated cells toward freely suspended cells in the culture.

DGC and PDE enzymes that regulate the turnover of c-di-GMP signals are predicted to respond to environmental stimuli on the basis of their association with putative sensory receptors or of results from in vitro experiments. In this study, differences in intracellular levels of c-di-GMP in response to exogenous stimuli in wild-type bacteria were observed. Our data suggest that c-di-GMP signaling involves continuous changes in the intracellular content of this secondary messenger and does not imply an on/off mechanism. These observations correlate with the hypothesis that several, most likely localized, c-di-GMP pools are present in the cell and are representative of several, parallel c-di-GMP signal transduction cascades for various environmental signals (23, 58). Thus, changes of individual environmental signals might only induce alterations of discrete c-di-GMP pools, which can result in a partial decrease of the total cellular c-di-GMP levels. The changes in the expression levels of several genes encoding potential PDEs and/or DGCs observed in this study support the involvement of multiple pathways in the response to NO. All of those genes identified in this study encode proteins harboring both a GGDEF and an EAL domain, respectively, with the exception of the putative DGC gene PA0290 and the known PDE gene *arr* (18). However, the function of these composite proteins is not entirely understood, as some of the domains might have regulatory rather than catalytic properties (22).

Signal transduction mechanisms involving c-di-GMP were previously suggested to imply multilayer control at the transcriptional, translational, and posttranslational levels (22). For example, it was found that c-di-GMP binds to PelD to regulate production of the PEL polysaccharide (32). Moreover, the PDE stimulation by NO in cell extracts observed in this study suggests a posttranslational activation of PDE activity rather than a transcriptional control by NO. Such posttranscriptional regulatory effects could translate the relatively small changes in transcriptomic response into significant phenotypic effects, such as increased pyoverdine production, motility, and biofilm dispersal, which were observed here.

Finally, the chemotaxis transducer BdlA, which harbors two PAS domains and can indirectly modulate c-di-GMP levels (35), was found to be required for the dispersal response to low doses of NO in *P. aeruginosa* biofilms. Disruption of the corresponding single-gene operon encoding BdlA caused the loss of the dispersal phenotype in response to NO exposure in strain KO[*bdlA*]. We further demonstrated that BdlA is able to influence c-di-GMP levels, as cells of strain KO[*bdlA*] did not respond with decreased c-di-GMP concentrations upon exposure to NO in comparison to the level for the parent strain. These results strongly support our hypothesis that NO-induced dispersal is regulated via a c-di-GMP-dependent regulatory network. Since BdlA is not predicted to have any PDE activity itself, the effect of BdlA on c-di-GMP levels is most likely based on a regulatory mechanism, although the target is currently unknown. Further studies will be carried out to establish whether BdlA can directly bind NO signals at concentrations found to induce dispersal.

NO-induced dispersal is not a toxic response and is linked to increased metabolism. The nontoxic effect of low concentrations of NO in our experiments included the observed reduction in transcription of the flavohemoprotein-encoding gene, *fhp*, involved in aerobic NO scavenging (Table 2). In contrast, expression of this gene was previously reported by Firoved et al. (14) to be highly upregulated as a result of nitrosative stress, i.e., in the presence of damaging levels of NO generated from 5 mM *S*-nitroso-L-glutathione (GSNO). Here, we exposed bacteria to 5 μM SNP, which generates low, physiological concentrations of NO, kinetically lower on a molar basis by at least 1,000-fold. Moreover, NO-mediated dispersal was unchanged in a *P. aeruginosa* knockout mutant of *fhpR* (data not shown), encoding a NO-responsive regulator (FhpR, ortholog to NorR) that activates transcription of *fhp* under nitrosative stress (1). Thus, we suggest that FhpR is not involved in the dispersal response induced by NO in *P. aeruginosa* biofilms but is primarily responsible for inducing *fhp* in the presence of damaging concentrations of NO.

Many genes involved in the anaerobic respiratory pathway were positively affected in biofilms that were exposed to exogenous NO. The induction of *nirS* and *norC* transcription by NO was shown previously and found to be controlled by the NO-

responsive activator DNR and the regulatory protein NirQ in *P. aeruginosa* (2). In *Pseudomonas stutzeri*, similar activations were observed in the presence of 5 to 50 nM NO but not in the presence of 500 nM NO under anoxic conditions (55). Bacteria in biofilms usually exhibit reduced metabolic activity, and by enhancing anaerobic metabolism, NO may favor a transition to a more active state allowing dispersal from the biofilm. Interestingly, recent studies have shown that the addition of nitrate (NO_3^-), nitrite (NO_2^-), or arginine to *P. aeruginosa* biofilms resulted in an increase in the sensitivity of the biofilm cells toward antibiotics (10) similar to that observed with NO (5). A NO_3^- sensor-response regulator system was also found to be involved in biofilm formation and motility in *P. aeruginosa* (54). Therefore, it is possible that NO may mediate these effects in *P. aeruginosa* biofilms.

NO as a conserved evolutionary pathway mediating dispersal. In this study, we provide evidence for a direct link between NO and c-di-GMP in *P. aeruginosa*. This regulatory pathway may be conserved across microbial species. Indeed, NO, which is ubiquitous in nature, was found to induce dispersal in biofilms of several gram-positive and gram-negative species and *Candida albicans* as well as in mixed-species biofilms (6). Further, GGDEF and EAL domains are widely distributed among bacteria and are often associated with redox sensors capable of sensing NO (3, 39). These observations support the hypothesis that NO is an ancient and highly conserved regulator of dispersal and life histories (7).

The molecular machinery underlying NO-induced dispersal in bacterial biofilms shares striking similarities with the well-characterized NO/cGMP signaling pathway in eukaryotes, where NO sensing domains are commonly found associated with guanylate cyclases and/or PDEs (46). In animals, the NO/cGMP regulatory system is involved in sophisticated multicellular processes, including vasodilation and neurotransmission. Thus, multiple components of NO signaling mechanisms in biofilms appear to be represented in higher multicellular organisms, suggesting an intriguing relationship between biofilm dispersal signaling and the evolution of eukaryotic regulatory pathways.

ACKNOWLEDGMENTS

We thank A. L. Vivant for help with the BdlA mutant biofilm experiments and our colleagues at the University of New South Wales (UNSW) for their support. The microarray experiments were performed at the Clive and Vera Ramaciotti Centre for Gene Function Analysis (UNSW). Mass spectrometric results were obtained at the Bioanalytical Mass Spectrometry Facilities within the Analytical Centre (UNSW). Subsidized access to this facility is gratefully acknowledged.

This work was partially supported by grants from the Australian Research Council, the Centre for Marine Bio-Innovation, and the Environmental Biotechnology Cooperative Research Centre. D.S. was supported by UNSW Vice-Chancellor's Postdoctoral Fellowship. D.J.H. was supported by the National Institutes of Health (GM-69845), the Cystic Fibrosis Foundation, and Cure Finders, Inc. (Sevierville, TN).

REFERENCES

1. **Arai, H., M. Hayashi, A. Kuroi, M. Ishii, and Y. Igarashi.** 2005. Transcriptional regulation of the flavohemoglobin gene for aerobic nitric oxide detoxification by the second nitric oxide-responsive regulator of *Pseudomonas aeruginosa*. J. Bacteriol. **187:**3960–3968.
2. **Arai, H., T. Kodama, and Y. Igarashi.** 1999. Effect of nitrogen oxides on expression of the *nir* and *nor* genes for denitrification in *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **170:**19–24.
3. **Aravind, L., V. Anantharaman, and L. M. Iyer.** 2003. Evolutionary connections between bacterial and eukaryotic signaling systems: a genomic perspective. Curr. Opin. Microbiol. **6:**490–497.
4. **Banin, E., K. M. Brady, and E. P. Greenberg.** 2006. Chelator-induced dispersal and killing of *Pseudomonas aeruginosa* cells in a biofilm. Appl. Environ. Microbiol. **72:**2064–2069.
5. **Barraud, N., D. J. Hassett, S. H. Hwang, S. A. Rice, S. Kjelleberg, and J. S. Webb.** 2006. Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. J. Bacteriol. **188:**7344–7353.
6. **Barraud, N., M. V. Storey, Z. P. Moore, J. S. Webb, S. A. Rice, and S. Kjelleberg.** 2009. Nitric oxide-mediated dispersal in single- and multi-species biofilms of clinically and industrially relevant microorganisms. Microb. Biotechnol. **2:**370–378.
7. **Bishop, C. D., and B. P. Brandhorst.** 2003. On nitric oxide signaling, metamorphosis, and the evolution of biphasic life cycles. Evol. Dev. **5:**542–550.
8. **Bobrov, A. G., O. Kirillina, and R. D. Perry.** 2005. The phosphodiesterase activity of the HmsP EAL domain is required for negative regulation of biofilm formation in *Yersinia pestis*. FEMS Microbiol. Lett. **247:**123–130.
9. **Boon, E. M., and M. A. Marletta.** 2005. Ligand specificity of H-NOX domains: from sGC to bacterial NO sensors. J. Inorg. Biochem. **99:**892–902.
10. **Borriello, G., L. Richards, G. D. Ehrlich, and P. S. Stewart.** 2006. Arginine or nitrate enhances antibiotic susceptibility of *Pseudomonas aeruginosa* in biofilms. Antimicrob. Agents Chemother. **50:**382–384.
11. **Buerk, D. G.** 2001. Can we model nitric oxide biotransport? A survey of mathematical models for a simple diatomic molecule with surprisingly complex biological activities. Annu. Rev. Biomed. Eng. **3:**109–143.
12. **D'Autréaux, B., D. Touati, B. Bersch, J. M. Latour, and I. Michaud-Soret.** 2002. Direct inhibition by nitric oxide of the transcriptional ferric uptake regulation protein via nitrosylation of the iron. Proc. Natl. Acad. Sci. USA **99:**16619–16624.
13. **Delgado-Nixon, V. M., G. Gonzalez, and M. A. Gilles-Gonzalez.** 2000. Dos, a heme-binding PAS protein from *Escherichia coli*, is a direct oxygen sensor. Biochemistry **39:**2685–2691.
14. **Firoved, A. M., S. R. Wood, W. Ornatowski, V. Deretic, and G. S. Timmins.** 2004. Microarray analysis and functional characterization of the nitrosative stress response in nonmucoid and mucoid *Pseudomonas aeruginosa*. J. Bacteriol. **186:**4046–4050.
15. **Giardina, G., S. Rinaldo, K. A. Johnson, A. Di Matteo, M. Brunori, and F. Cutruzzola.** 2008. NO sensing in *Pseudomonas aeruginosa*: structure of the transcriptional regulator DNR. J. Mol. Biol. **378:**1002–1015.
16. **Gilles-Gonzalez, M. A., and G. Gonzalez.** 2005. Heme-based sensors: defining characteristics, recent developments, and regulatory hypotheses. J. Inorg. Biochem. **99:**1–22.
17. **Hentzer, M., L. Eberl, and M. Givskov.** 2005. Transcriptome analysis of *Pseudomonas aeruginosa* biofilm development: anaerobic respiration and iron limitation. Biofilms **2:**37–61.
18. **Hoffman, L. R., D. A. D'Argenio, M. J. MacCoss, Z. Zhang, R. A. Jones, and S. I. Miller.** 2005. Aminoglycoside antibiotics induce bacterial biofilm formation. Nature **436:**1171–1175.
19. **Holloway, B. W.** 1955. Genetic recombination in *Pseudomonas aeruginosa*. J. Gen. Microbiol. **13:**572–581.
20. **Iyer, L. M., V. Anantharaman, and L. Aravind.** 2003. Ancient conserved domains shared by animal soluble guanylyl cyclases and bacterial signaling proteins. BMC Genomics **4:**5–12.
21. **Jacobs, M. A., A. Alwood, I. Thaipisuttikul, D. Spencer, E. Haugen, S. Ernst, O. Will, R. Kaul, C. Raymond, R. Levy, L. Chun-Rong, D. Guenthner, D. Bovee, M. V. Olson, and C. Manoil.** 2003. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **100:**14339–14344.
22. **Jenal, U., and J. Malone.** 2006. Mechanisms of cyclic-di-GMP signaling in bacteria. Annu. Rev. Genet. **40:**385–407.
23. **Kader, A., R. Simm, U. Gerstel, M. Morr, and U. Romling.** 2006. Hierarchical involvement of various GGDEF domain proteins in rdar morphotype development of *Salmonella enterica* serovar Typhimurium. Mol. Microbiol. **60:**602–616.
24. **Kazmierczak, B. I., M. B. Lebron, and T. S. Murray.** 2006. Analysis of FimX, a phosphodiesterase that governs twitching motility in *Pseudomonas aeruginosa*. Mol. Microbiol. **60:**1026–1043.
25. **Kennedy, S. I., and C. A. Fewson.** 1968. Enzymes of the mandelate pathway in Bacterium N.C.I.B. 8250. Biochem. J. **107:**497–506.
26. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. **44:**301–307.
27. **Klebensberger, J., K. Lautenschlager, D. Bressler, J. Wingender, and B. Philipp.** 2007. Detergent-induced cell aggregation in subpopulations of *Pseudomonas aeruginosa* as a preadaptive survival strategy. Environ. Microbiol. **9:**2247–2259.
28. **Köhler, T., L. K. Curty, F. Barja, C. van Delden, and J. C. Pechère.** 2000. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. J. Bacteriol. **182:**5990–5996.
29. **Kuchma, S. L., K. M. Brothers, J. H. Merritt, N. T. Liberati, F. M. Ausubel, and A. G. O'Toole.** 2007. BifA, a cyclic-di-GMP phosphodiesterase, inversely

regulates biofilm formation and swarming motility by *Pseudomonas aeruginosa* PA14. J. Bacteriol. **189:**8165–8178.
30. **Kulasekara, H., V. Lee, A. Brencic, N. Liberati, J. Urbach, S. Miyata, D. G. Lee, A. N. Neely, M. Hyodo, Y. Hayakawa, F. M. Ausubel, and S. Lory.** 2006. Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3′–5′)-cyclic-GMP in virulence. Proc. Natl. Acad. Sci. USA **103:**2839–2844.
31. **Kulasekara, H. D., I. Ventre, B. R. Kulasekara, A. Lazdunski, A. Filloux, and S. Lory.** 2005. A novel two-component system controls the expression of *Pseudomonas aeruginosa* fimbrial *cup* genes. Mol. Microbiol. **55:**368–380.
32. **Lee, V. T., J. M. Matewish, J. L. Kessler, M. Hyodo, Y. Hayakawa, and S. Lory.** 2007. A cyclic-di-GMP receptor required for bacterial exopolysaccharide production. Mol. Microbiol. **65:**1474–1484.
33. **Meissner, A., V. Wild, R. Simm, M. Rohde, C. Erck, F. Bredenbruch, M. Morr, U. Romling, and S. Haussler.** 2007. *Pseudomonas aeruginosa cupA*-encoded fimbriae expression is regulated by a GGDEF and EAL domain-dependent modulation of the intracellular level of cyclic diguanylate. Environ. Microbiol. **9:**2475–2485.
34. **Moore, C. M., M. M. Nakano, T. Wang, R. W. Ye, and J. D. Helmann.** 2004. Response of *Bacillus subtilis* to nitric oxide and the nitrosating agent sodium nitroprusside. J. Bacteriol. **186:**4655–4664.
35. **Morgan, R., S. Kohn, S. H. Hwang, D. J. Hassett, and K. Sauer.** 2006. BdlA, a chemotaxis regulator essential for biofilm dispersion in *Pseudomonas aeruginosa*. J. Bacteriol. **188:**7335–7343.
36. **Musk, D. J., D. A. Banko, and P. J. Hergenrother.** 2005. Iron salts perturb biofilm formation and disrupt existing biofilms of *Pseudomonas aeruginosa*. Chem. Biol. **12:**789–796.
37. **Purevdorj-Gage, B., W. J. Costerton, and P. Stoodley.** 2005. Phenotypic differentiation and seeding dispersal in non-mucoid and mucoid *Pseudomonas aeruginosa* biofilms. Microbiology **151:**1569–1576.
38. **Rodionov, D. A., I. L. Dubchak, A. P. Arkin, E. J. Alm, and M. S. Gelfand.** 2005. Dissimilatory metabolism of nitrogen oxides in bacteria: comparative reconstruction of transcriptional networks. PLoS Comput. Biol. **1:**415–431.
39. **Römling, U., M. Gomelsky, and M. Y. Galperin.** 2005. C-di-GMP: the dawning of a novel bacterial signalling system. Mol. Microbiol. **57:**629–639.
40. **Ross, P., R. Mayer, H. Weinhouse, D. Amikam, Y. Huggirat, M. Benziman, E. de Vroom, A. Fidder, P. de Paus, L. A. Sliedregt, et al.** 1990. The cyclic diguanylic acid regulatory system of cellulose synthesis in *Acetobacter xylinum*. Chemical synthesis and biological activity of cyclic nucleotide dimer, trimer, and phosphothioate derivatives. J. Biol. Chem. **265:**18933–18943.
41. **Ryan, R. P., Y. Fouhy, J. F. Lucey, L. C. Crossman, S. Spiro, Y. W. He, L. H. Zhang, S. Heeb, M. Camara, P. Williams, and J. M. Dow.** 2006. Cell-cell signaling in *Xanthomonas campestris* involves an HD-GYP domain protein that functions in cyclic-di-GMP turnover. Proc. Natl. Acad. Sci. USA **103:**6712–6717.
42. **Sauer, K., M. C. Cullen, A. H. Rickard, L. A. Zeef, D. G. Davies, and P. Gilbert.** 2004. Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. J. Bacteriol. **186:**7312–7326.
43. **Savli, H., A. Karadenizli, F. Kolayli, S. Gundes, U. Ozbek, and H. Vahaboglu.** 2003. Expression stability of six housekeeping genes: a proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR. J. Med. Microbiol. **52:**403–408.
44. **Schlag, S., C. Nerz, T. A. Birkenstock, F. Altenberend, and F. Gotz.** 2007. Inhibition of staphylococcal biofilm formation by nitrite. J. Bacteriol. **189:**7911–7919.
45. **Schleheck, D., N. Barraud, J. Klebensberger, J. S. Webb, D. McDougald, S. A. Rice, and S. Kjelleberg.** 2009. *Pseudomonas aeruginosa* PAO1 preferentially grows as aggregates in liquid batch cultures and disperses upon starvation. PLoS ONE **4:**e5513.
46. **Schlossmann, J., R. Feil, and F. Hofmann.** 2003. Signaling through NO and cGMP-dependent protein kinases. Ann. Med. **35:**21–27.
47. **Simm, R., M. Morr, A. Kader, M. Nimtz, and U. Römling.** 2004. GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. Mol. Microbiol. **53:**1123–1134.
48. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. Nat. Biotechnol. **1:**784–791.
49. **Spiro, S.** 2007. Regulators of bacterial responses to nitric oxide. FEMS Microbiol. Rev. **31:**193–211.
50. **Stamler, J. S., D. J. Singel, and J. Loscalzo.** 1992. Biochemistry of nitric oxide and its redox-activated forms. Science **258:**1898–1902.
51. **Thormann, K. M., S. Duttler, R. M. Saville, M. Hyodo, S. Shukla, Y. Hayakawa, and A. M. Spormann.** 2006. Control of formation and cellular detachment from *Shewanella oneidensis* MR-1 biofilms by cyclic di-GMP. J. Bacteriol. **188:**2681–2691.
52. **Tischler, A. D., and A. Camilli.** 2004. Cyclic diguanylate (c-di-GMP) regulates *Vibrio cholerae* biofilm formation. Mol. Microbiol. **53:**857–869.
53. **Ueda, A., and T. K. Wood.** 2009. Connecting quorum sensing, c-di-GMP, Pel polysaccharide, and biofilm formation in *Pseudomonas aeruginosa* through tyrosine phosphatase TpbA (PA3885). PLoS Pathog. **5**:e1000483.
54. **Van Alst, N. E., K. F. Picardo, B. H. Iglewski, and C. G. Haidaris.** 2007. Nitrate sensing and metabolism modulate motility, biofilm formation, and virulence in *Pseudomonas aeruginosa*. Infect. Immun. **75:**3780–3790.
55. **Vollack, K. U., and W. G. Zumft.** 2001. Nitric oxide signaling and transcriptional control of denitrification genes in *Pseudomonas stutzeri*. J. Bacteriol. **183:**2516–2526.
56. **Webb, J. S., M. Givskov, and S. Kjelleberg.** 2003. Bacterial biofilms: prokaryotic adventures in multicellularity. Curr. Opin. Microbiol. **6:**578–585.
57. **Webb, J. S., L. S. Thompson, S. James, T. Charlton, T. Tolker-Nielsen, B. Koch, M. Givskov, and S. Kjelleberg.** 2003. Cell death in *Pseudomonas aeruginosa* biofilm development. J. Bacteriol. **185:**4585–4592.
58. **Weber, H., C. Pesavento, A. Possling, G. Tischendorf, and R. Hengge.** 2006. Cyclic-di-GMP-mediated signalling within the sigma network of *Escherichia coli*. Mol. Microbiol. **62:**1014–1034.
59. **Windgassen, M., A. Urban, and K. E. Jaeger.** 2000. Rapid gene inactivation in *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **193:**201–205.
60. **Winsor, G. L., T. Van Rossum, R. Lo, B. Khaira, M. D. Whiteside, R. E. Hancock, and F. S. Brinkman.** 2009. Pseudomonas Genome Database: facilitating user-friendly, comprehensive comparisons of microbial genomes. Nucleic Acids Res. **37:**D483–D488.
61. **Yoon, S. S., R. F. Hennigan, G. M. Hilliard, U. A. Ochsner, K. Parvatiyar, M. C. Kamani, H. L. Allen, T. R. DeKievit, P. R. Gardner, U. Schwab, J. J. Rowe, B. H. Iglewski, T. R. McDermott, R. P. Mason, D. J. Wozniak, R. E. Hancock, M. R. Parsek, T. L. Noah, R. C. Boucher, and D. J. Hassett.** 2002. *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. Dev. Cell **3:**593–603.
62. **Yoon, S. S., A. C. Karabulut, J. D. Lipscomb, R. F. Hennigan, S. V. Lymar, S. L. Groce, A. B. Herr, M. L. Howell, P. J. Kiley, M. J. Schurr, B. Gaston, K. H. Choi, H. P. Schweizer, and D. J. Hassett.** 2007. Two-pronged survival strategy for the major cystic fibrosis pathogen, Pseudomonas aeruginosa, lacking the capacity to degrade nitric oxide during anaerobic respiration. EMBO J. **26:**3662–3672.
63. **Zumft, W. G.** 2002. Nitric oxide signaling and NO dependent transcriptional control in bacterial denitrification by members of the FNR-CRP regulator family. J. Mol. Microbiol. Biotechnol. **4:**277–286.