Effect of Nitroxides on Swarming Motility and Biofilm Formation, Multicellular Behaviors in Pseudomonas aeruginosa

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The ability of nitric oxide (NO) to induce biofilm dispersion has been well established. Here, we investigated the effect of nitroxides (sterically hindered nitric oxide analogues) on biofilm formation and swarming motility in Pseudomonas aeruginosa. A transposon mutant unable to produce nitric oxide endogenously (nirS) was deficient in swarming motility relative to the wild type and the complemented strain. Moreover, expression of the nirS gene was upregulated by 9.65-fold in wild-type swarming cells compared to planktonic cells. Wild-type swarming levels were substantially restored upon the exogenous addition of nitroxide containing compounds, a finding consistent with the hypothesis that NO is necessary for swarming motility. Here, we showed that nitroxides not only mimicked the dispersal activity of NO but also prevented biofilms from forming in flow cell chambers. In addition, a nirS transposon mutant was deficient in biofilm formation relative to the wild type and the complemented strain, thus implicating NO in the formation of biofilms. Intriguingly, despite its stand-alone action in inhibiting biofilm formation and promoting dispersal, a nitroxide partially restored the ability of a nirS mutant to form biofilms.

Bacteria are able to develop multicellular growth states, such as swarming motility and biofilms (1–3). Swarming motility is a distinct growth state representing a complex multicellular adaptation to semisolid (viscous) surfaces. It is widespread among flagellated bacteria, including Pseudomonas aeruginosa and Salmonella, Vibrio, Yersinia, Serratia, and Proteus spp. (4). During swarming, cells move in a highly coordinated manner (e.g., giving a dendritic colonial appearance in P. aeruginosa PA14 [4–6]) that is dependent on hundreds of genes, including many regulators, and exhibits the differential expression of many genes (4, 6), leading to increased antibiotic resistance and altered metabolism and increased virulence factor expression (2, 4, 6).

Biofilms are structured, surface-associated microbial communities that are highly resistant to antibiotics (3, 5, 7). Biofilms are formed as a result of a complex developmental life cycle that often includes the coordinated dispersal of biofilm cells from the mature biofilm and differentiation into planktonic cells, presumably to enable new biofilm colonies to form elsewhere (1, 3, 8).

Here, we focused on the bacterial pathogen P. aeruginosa since it is a model for the study of swarming motility and biofilms (5). P. aeruginosa is a Gram-negative opportunistic bacterium that is the leading cause of nosocomial and chronic lung infections in cystic fibrosis (CF) patients (5). CF is the most common inherited lethal disorder of Caucasian populations. P. aeruginosa biofilms have been found in the infected lungs of individuals with CF and are thought to contribute to their persistent phenotype (3, 5, 9). Both biofilm formation leading to microcolonies in the CF lung and swarming motility in the context of the viscous mucoid environment of the CF lung are thought to be important in influencing colonization and persistence.

Biofilm dispersal can be induced by a variety of small molecules, including nitric oxide (NO) (10, 11). NO is a signaling molecule involved in many biological processes (12). Interestingly, NO produced by bacterial nitric oxide synthases has been shown to increase the resistance of bacteria to a broad spectrum of antibiotics through the chemical medication of toxic compounds and reduction of antibiotic-induced oxidative stress (13, 14). Previous reports identified and described the role of NO in the regulation of dispersal in P. aeruginosa biofilms (10, 11, 15). NO has also been shown to be related to anaerobic metabolism in the context of biofilm survival and dispersal (15, 16). The dispersal of biofilms to free swimming planktonic bacteria occurs at low nontoxic concentrations of NO in a variety of Gram-positive and Gram-negative bacterial species (8, 10). Furthermore, several studies have reported a link between NO and turnover of the second messenger c-di-GMP, indicating that a decrease in the levels of c-di-GMP results in a switch to dispersal and the planktonic mode of existence (17, 18). In P. aeruginosa, NO is synthesized by the enzyme nitrite reductase encoded by the gene nirS (Fig. 1) (10). To limit toxicity, NO is then reduced to nitrous oxide by a nitric oxide reductase (norBC) (Fig. 1) (10).

Nitroxides (or aminoxyls) are long-lived free radical species consisting of a substituted nitrogen atom bound to a univalent oxygen atom (19). Their stability arises because of the high delocalization energy of the unpaired electron over both the nitrogen and the oxygen atoms, making them resistant to dimerization. They are also resistant to degradation through disproportionation when the carbon atoms adjacent to the nitroxide center are substituted with bis (tetr-alkyl) groups (20). Since both nitric oxide and nitroxide structures possess an unpaired electron that is delocalized over the nitrogen-oxygen bond, nitroxides represent a sterically hindered version of nitric oxide. These stable free-radical species are commonly used as potent antioxidants in biological systems (21, 22). Like NO, these compounds can reduce levels of...
oxidative stress caused by reactive oxygen species (ROS). Many of the biological effects of nitroxides can be explained by their nitric oxide-mimetic properties, with both compounds exerting superoxide dismutase mimic properties. Nitroxides and nitric oxide are both efficient scavengers of protein-derived radicals (23).

**MATERIALS AND METHODS**

**Strains and media.** The strains utilized included the wild-type (wt) bacterium *P. aeruginosa* PA14 and two mutants from the transposon-MAR2xT7 Harvard library (24), namely, *nirS* and *norB* mutant strains. To complement the *nirS* mutation, we cloned the gene as follows. First, the *nirS* gene was amplified from strain PA14 by using primers 0519F (CCA CAAGCCGCAAGCAAGCAG) and 0519R (TGGATGCGATGGCAAGGCC), and the 2-kb amplified DNA fragment containing the *nirS* gene was subsequently cloned into a pUC18T-mini-Tn7/Tet plasmid (pUC18T-mini-Tn7/Tet-nirS+). The insertion of the plasmid carrying gene *nirS* in a single attTn7 site within the chromosome of *P. aeruginosa* was performed according to the protocol described by Choi and Schweizer (25). Briefly, the plasmid pUC18T-mini-Tn7/Tet-nirS+ was coelectroporated with the plasmid helper pTNS2 in *nirS* mutant electrocompetent cells. Positives clones containing the complementation were selected on lysogeny broth (LB) plus 50 μg of tetracycline/ml. The insertions were confirmed by PCR using the primers PTn (GTCTGAACGCTCCTAGG) and PTn (CTGGCAGTGTCGACGCT), and subsequently cloned into a pUC18T-mini-Tn7/Tet-nirS+.

**Nitroxides.** Carboxy-TEMPO (4-carboxy-2,2,6,6-tetramethylpiperidine-1-oxyl; Fig. 2) was obtained commercially (catalog no. 382000, Sigma-Aldrich). CTMIO (5-carboxy-1,1,3,3-tetramethyloxisolin-2-ylxoyl; Fig. 2) and DCTEIO (5,6-dicarboxy-1,1,3,3-tetramethyloxisolin-2-ylxoyl; Fig. 2) were prepared according to previously described methods (28).

**Growth curves.** PA14 wild-type (wt) and *nirS* mutant strains were grown overnight in BM2 swarming medium (62 mM potassium phosphate buffer [pH 7], 2 mM MgSO4, 10 μM FeSO4, 0.4% [wt/vol] glucose, 0.1% [wt/vol] Casamino Acids). Cultures were diluted to obtain equal absorbance at 620 nm every 20 min cultures at 37°C under shaking conditions was monitored with a TECAN Cary 300 Bio spectrophotometer. Then, 1/100 f of these cultures were added to 195 ml of 10-fold growth medium and pumped through the flow cells as described above for 48 h in the absence of nitroxides. After this time, biofilms were exposed to 20 μM of the nitroxide compounds for 24 h to assess their ability to disperse preformed biofilms. For this, nitroxides were added to the medium and pumped through the flow cells as described above. Staining and visualization of the resulting biofilms was performed using the Live/Dead BacLight bacterial viability kit and a confocal laser scanning microscope (Olympus, Fluoview FV1000), and three-dimensional reconstructions were generated using the Imaris software package (Bitplane AG).

**Biofilm dispersal flow cell assays.** Biofilms were grown for 72 h, in the absence or presence of 20 μM concentrations of the nitroxide compounds, at 37°C in flow chambers with channel dimensions of 1 by 4 by 20 μm. Silicone tubing (VWR, 0.062 in. [inner diameter] by 0.125 in. [outer diameter] by 3 ft) was autoclaved, and the system was assembled and sterilized by pumping a 0.5% hypochlorite solution through the system at 6 rpm for 30 min using a Watson Marlow 205S multichannel peristaltic pump. The system was then rinsed at 6 rpm with sterile water and medium for 30 min each. Flow chambers were inoculated by injecting 400 μl of an overnight culture diluted to an OD600 of 0.05. After inoculation, chambers were left without flow for 2 h, after which medium was pumped through the system at a constant rate of 0.75 rpm (3.6 ml/h). Biofilm cells were stained using the Live/Dead BacLight bacterial viability kit (Molecular Probes, Eugene, OR) prior to microscopy experiments. A 1:5 ratio of SYTO-9 (green fluorescence, live cells) to propidium iodide (PI; red fluorescence, dead cells) was used. Microscopy was performed using a confocal laser scanning microscope (Olympus, Fluoview FV1000), and three-dimensional reconstructions were generated using the Imaris software package (Bitplane AG).

**Biofilm assays.** Briefly, the nitroxides were dissolved in water, and MIC assays were performed by using a confocal laser scanning microscopy-broth microdilution method (29, 30) in sterile 96-well polypropylene microtiter plates (catalog no. 3790; Costar). Nitroxides were added to the plate at the desired concentrations, and the bacteria were inoculated at a final concentration of 5 × 10^3 CFU/ml per well. The plates were incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of nitroxide at which no growth was observed.

**RESULTS AND DISCUSSION**

Effect of a *nirS* mutation on swarming motility; genetic and chemical complementation. To examine the role of endogenous NO in motility and virulence of *P. aeruginosa* PA14, we tested the ability of a strain PA14 *nirS* transposon mutant (24) to undergo swarming motility. The *nirS* mutant was considerably less able to swarm...
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Demonstrating a 76% decrease in surface coverage (Fig. 3B). In contrast, a norB mutant, deficient in the ability to reduce NO to nitrous oxide, showed no defect in swarming. Genetic complementation of the nirS mutant with the nirS gene fully restored swarming (Fig. 3B), thus confirming that nirS was responsible for the observed deficiency. Importantly, planktonic growth of the nirS mutant was indistinguishable from that of the wild-type strain (Fig. 3A), thus minimizing the possibility that the deficiency in swarming may have been due to a growth deficiency.

In an attempt to chemically complement the deficiencies in the switch to multicellularly observed in the nirS mutant, we investigated the use of water-soluble nitroxides. First, we determined the direct antimicrobial activity of the compounds carboxy-TEMPO, CTMIO, and DCTEIO since NO is known to exhibit direct antimicrobial activity at high concentrations (31). An MIC of >160 μM was obtained for all three compounds using the broth microdilution method. Growth curve experiments further revealed that sublethal levels of nitroxide derivatives (20 μM) did not affect planktonic growth of mutants nirS and norB mutants (data not shown).

The effects of sublethal levels of the nitroxides on swarming by the PA14 wild-type strain and its nirS mutant were then tested. All experiments were performed at least three times. The nitroxides by themselves did not affect the swarming motility of the wild type, but the exogenous addition of 20 μM of each of the nitroxides complemented the swarming phenotype to various extents (Fig. 3C). The more hydrophobic derivatives DCTEIO (90.6%) and CTMIO (89.1%) were more efficient at restoring the swarming ability of a nirS mutant to nearly wild-type levels compared to the more hydrophilic compound carboxy-TEMPO (62.1%). Moreover, the complementation exerted by exogenous treatment with the nitroxides appeared to be concentration-dependent. Indeed, while 0.2 μM of the compounds enhanced dendrite formation, this effect was increased when 2 μM nitroxide concentrations were used (data not shown). Higher concentrations of nitroxide analogs (20 μM) complemented the swarming phenotype (Fig. 3C).

Therefore, the nitroxides appeared to complement the swarming deficiency that was apparently caused by a lack of endogenous NO, implying that these compounds maintained some properties of endogenous NO.

The expression of genes nirS, norB, and norC under swarming conditions, as opposed to planktonic growth, was tested by RT-qPCR. Consistent with its role in swarming motility, the nirS gene was (9.65 ± 0.16)-fold more highly expressed in wild-type strain PA14 swarming cells compared to the same strain grown under planktonic conditions. Conversely, norB and norC were not found to be differentially expressed in these experiments. Neither nirS nor norB mutants (PA14 transposon mutants) (24) were altered in growth (compared to the PA14wt strain) in LB or BM2 medium with or without Casamino Acids (data not shown), suggesting that endogenous NO synthesis is not essential for normal planktonic growth. From these observations, we concluded that endogenously produced NO was required for normal swarming motility but not for planktonic growth.

Effect of a nirS mutation on biofilm formation. The nirS mutant was unable to form biofilms when grown in flow cell chambers, in contrast to the wild-type PA14, the genetically complemented strain and the norB mutant (Fig. 4A). Indeed, PA14 wt biofilms were 28.6 μm thick, whereas the unstructured monolayer of cells formed by the nirS mutant was only 6.7 μm in thickness. On the other hand, biofilms formed by the norB mutant showed similar thickness to that of the wild type (22.7 μm). Unlike the nirS mutant, the nirS genetically complemented strain formed structured biofilms in the flow cell assay (thickness, 16.7 μm).
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results recently reported (34) describing the ability of NO to stimulate biofilm formation by controlling c-di-GMP levels are in agreement with the biofilm deficiency of the nirS mutant presented here.

Since mutant strain nirS was deficient in biofilm formation, we hypothesized that the nitroxides might be able to complement the deficient phenotype. We grew the nirS mutant in flow cell chambers in the presence of the nitroxides and observed increased cell aggregation (biofilm thickness, 9.3 μm) after 3 days with carboxy-TEMPO addition (Fig. 4A) and structured cell aggregates when adding DCTEIO (Fig. 4A). However, no major biofilm structures comparable to the ones formed by wild-type PA14 were identified in these samples. In conclusion, the nitroxide derivatives were able to partially complement the biofilm-deficient phenotype of the nirS strain. We argue that the phenotype is only partially rescued due to the absence of NO in the nirS mutant strain. TEMPO and DCTEIO, when added, presumably acted as a surrogate for NO in the cell but did not fully complement NO absence.

To investigate the basis for this limited chemical complementation, cf. the case for swarming motility, we examined the effect of the nitroxide analogs on P. aeruginosa biofilms. When the wild-type strain PA14 was grown in flow cells for 3 days in the presence of 20 μM concentrations of nitroxides, biofilms were unable to develop (Fig. 4B), a finding consistent with the conclusion that these compounds were able to inhibit the initial stages of biofilm formation. In addition, treatment with the analogs triggered the dispersal of 2-day-old preformed PA14 biofilms (Fig. 4B). In fact, nitroxide-induced dispersion led to biofilm dissolution with only a thin monolayer of cells (thickness, 4 μm) attached to the flow cell chamber. From these results we infer that the addition of nitroxides to an otherwise NO-producing strain (PA14 wt) had the same impact as excess levels of NO, which increase the biofilm dispersal processes.

Nitroxide-induced biofilm dispersal did not involve cell death since there was no observable increase in dead cells (revealed by a lack of staining with red propidium iodide). Both the inhibition of initial biofilm formation and the dispersal of mature biofilms may be explained by the presumptive biofilm dispersal properties of the nitroxide derivatives. In other words, newly differentiated biofilm cells may be readily dispersed by the compounds both at the beginning of biofilm development and during the final dispersal events. The well-established ability of NO to disperse biofilms was therefore conserved in the different analogs.

The gene encoding for enzyme nitrite reductase nirS was essential for swarming and biofilm formation, as shown by phenotypic characterization assays. Indeed, upregulation of nirS was observed in swarming but not in planktonic cells. However, biosynthesis of endogenous NO did not appear to contribute to planktonic growth since the nirS mutant displayed growth dynamics identical to the wild-type strain. Since endogenous NO is known to increase resistance to antibiotics, we propose that the production of this molecule may be in part responsible for the antibiotic-resistant phenotypes observed in swarming and biofilm cells. Overall, the inability of nirS transposon mutant to swarm or form biofilms appeared to be due to its lack of endogenous NO biosynthesis since synthetic NO analogs were able to at least partially complement both phenotypes. Moreover, treatment of PA14 biofilms with nitroxides led to the inhibition of biofilm development and triggered biofilm dispersion, possibly due to excess NO levels. We therefore hypothesize that the concentration of intracellular NO (and/or NO-like molecules) is critical to either trigger or repress biofilm formation. These studies have revealed new features of
multicellular behavior in *Pseudomonas aeruginosa* and underline the potential of nitroxides as agents that can inhibit biofilm formation and trigger biofilm dispersal.

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