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

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Antimicrob. Agents Chemother. **2013, 57(10):4877. DOI: 10.1128/AAC.01381-13.**
Published Ahead of Print 22 July 2013.

AAC Journals.ASM.org

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 disubstituted 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 (*tert*-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

Received 28 June 2013 **Accepted** 15 July 2013
Published ahead of print 22 July 2013
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doi:10.1128/AAC.01381-13

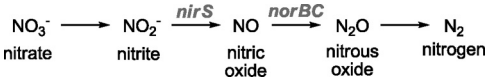

FIG 1 Nitric oxide metabolism in *P. aeruginosa*.

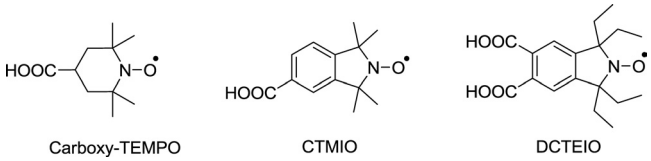

FIG 2 Structures of the nitroxide compounds.

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 mimetic properties. Nitroxides and nitric oxide are both efficient scavengers of protein-derived radicals (23).

MATERIALS AND METHODS

Strains and media. The stains utilized included the wild-type (wt) bacterium *P. aeruginosa* PA14 and two mutants from the transposon-MAR2xT7 Harvard library (24), namely, *nirS* and *norS* 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 CAAGCGCAAAGCAACG) and 0519R (TCGATGGCATGGCAGGCC), and the 2-kb amplified DNA fragment containing the *nirS* gene was subsequently cloned into a pUC18T-mini-Tn*7*T-Tet plasmid (pUC18T-mini-Tn*7*T-Tet-*nirS*+). The insertion of the transposon carrying gene *nirS* in a single attTn*7* site within the chromosome of *P. aeruginosa* was performed according to the protocol described by Choi and Schweizer (25). Briefly, the plasmid pUC18T-mini-Tn*7*T-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*7*-up and PglmS-down as previously described (25). Strains were maintained and routinely grown on LB medium and plates.

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-tetramethylisoindolin-2-yloxyl; Fig. 2) (26, 27) and DCTEIO (5,6-dicarboxy-1,1,3,3-tetraethylisoindolin-2-yloxyl; 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 $MgSO_4$, 10 μM $FeSO_4$, 0.4% [wt/vol] glucose, 0.1% [wt/vol] Casamino Acids). Cultures were diluted to obtain equal optical densities. Portions (5 μl) of these cultures were added to 195 μl of fresh swarming medium in 96-well microtiter plates. The growth of these cultures at 37°C under shaking conditions was monitored with a TECAN Spectrofluor Plus by determining the absorbance at 620 nm every 20 min for 28 h.

Swarming motility assays. Swarming assays were performed as previously described (4, 29) on BM2 swarming agar plates (62 mM potassium phosphate buffer [pH 7], 2 mM $MgSO_4$, 10 μM $FeSO_4$, 0.4% [wt/vol] glucose, 0.1% [wt/vol] Casamino Acids, 0.5% [wt/vol] Difco agar) in the absence or presence of different concentrations of the nitroxide derivatives. Then, 1-μl aliquots of mid-log-phase (i.e., an optical density at 600 nm [OD_{600}] of 0.4 to 0.6) cultures grown in BM2 minimal medium [62 mM potassium phosphate buffer (pH 7), 7 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 10 μM $FeSO_4$, 0.4% (wt/vol) glucose supplemented with 0.1% (wt/vol) Casamino Acids] were inoculated onto petri dish plates. Each experiment was carried out at least three times. All resulting dendritic colonies were analyzed by measuring the surface coverage on agar plates after 20 h of incubation at 37°C by using ImageJ software and represented as a percentage of the wild type.

RT-qPCR assays. Reverse transcription-quantitative PCR (RT-qPCR) was performed to examine the expression of genes *nirS*, *norB*, and *norC* under swarming conditions as opposed to planktonic growth. Actively swarming cells from the edges of swarming colonies were harvested. For planktonic growth assays, mid-logarithmic-phase cells (OD_{600} = 0.4) grown in liquid cultures were isolated prior to RNA extraction. For RNA extraction, RNeasy mini-columns (Qiagen, Mississauga, Ontario, Canada) were used, and total RNA was isolated from swarming cells and mid-log-phase *P. aeruginosa* PA14 cultures. RNA samples were treated with DNase I to remove contaminating genomic DNA. A total of 750 ng of total RNA was added to a reaction mixture containing each deoxynucleoside triphosphate at concentration of 0.5 μM, 500 U of SuperaseIN (Ambion, Austin, TX)/ml, and 10 μM dithiothreitol in 1× reaction buffer and reverse transcribed for 1 h at 37°C and for 2 h at 42°C with 10,000 U of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA)/ml. The resulting cDNA was used as a template for PCR. The number of cycles used to amplify each gene of interest was chosen to ensure that the PCR was not saturated. All reactions were normalized to the *rpsL* gene encoding the 30S ribosomal protein S12.

Biofilm 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 40 mm. Silicone tubing (VWR, 0.062 in. [inner diameter] by 0.125 in. [outer diameter] by a 0.032-in. wall) 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 OD_{600} 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 by using the Imaris software package (Bitplane AG).

Biofilm dispersal flow cell assays. *P. aeruginosa* biofilms were grown as indicated above for 48 h in the absence of nitroxides. After this time, biofilms were exposed to 20 μM 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 (Fluoview FV1000) as previously described.

MIC 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 5×10^5 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 on multicellular bacterial behaviors, 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

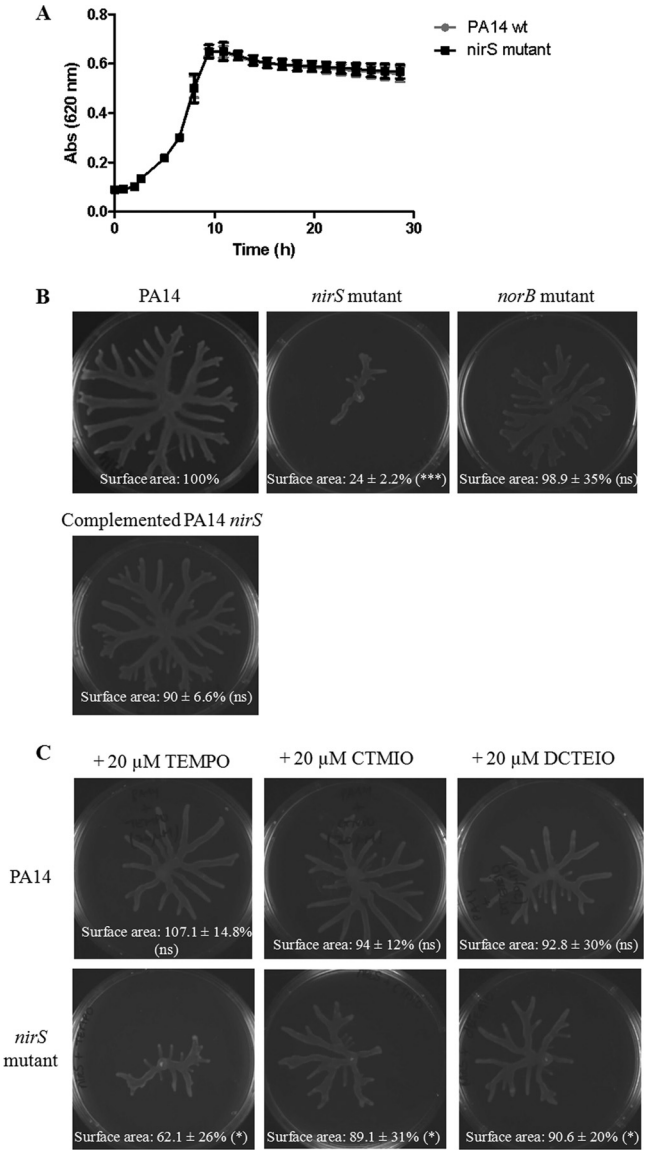

FIG 3 Involvement of NO in swarming motility. (A) A *nirS* mutant was not growth deficient compared to its parent strain PA14. (B) A *nirS* mutant, but not the complemented strain or a *norB* mutant, was deficient in swarming motility. (C) Chemical complementation of the *nirS* mutant swarming deficiency by subinhibitory levels (20 μM, the minimum concentration of nitroxides that almost fully complemented the swarming phenotype) of nitroxides. Representative images are shown of at least three independent swarming experiments performed per condition. The average percentage (%) surface area values are relative to wild-type swarming in all cases. Student *t* tests were performed to assess significance levels (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). In panel B, Student *t* test analyses were used to compare the surface area values of swarming colonies of mutants *nirS* and *norB* and the complemented PA14 *nirS* strain to PA14 wild type. In the upper row of panel C, the statistical significance of PA14 wild-type swarming in the presence of nitroxides was compared to the swarming of PA14 wild type alone. Significant *P* values shown in the lower row of panel C represent swarming of mutant *nirS* strains treated with nitroxide compounds relative to untreated *nirS* strains.

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 multicellularity 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 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 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),

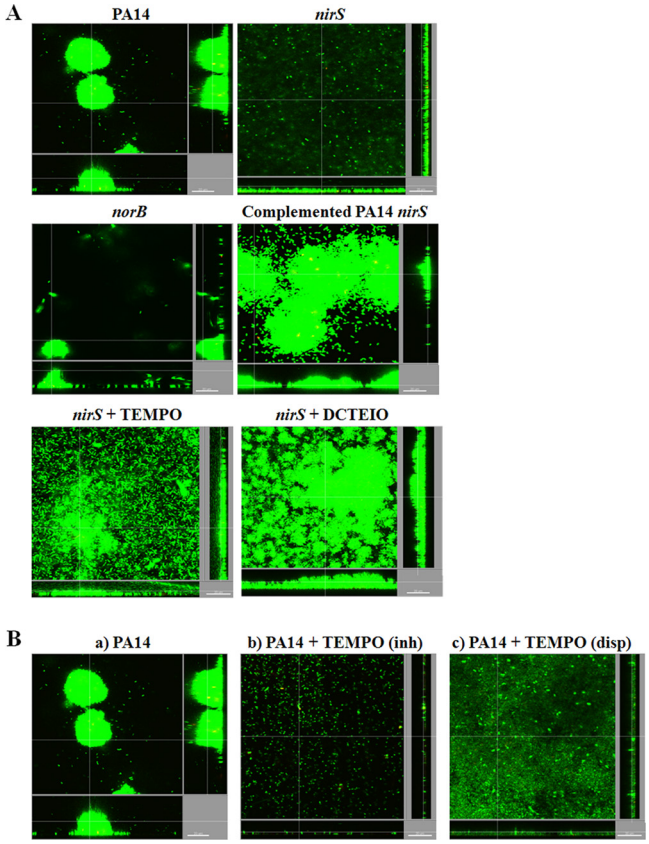

FIG 4 Effect of *nirS* on biofilm development and the biofilm inhibitory activity of nitroxide compounds. (A) Biofilms were cultivated in minimal medium for 3 days at 37°C in flow cell chambers and stained with SYTO-9 (green; live cells) and propidium iodide (PI) (red; dead cells). No increase in dead cells was observed in *nirS* and *norB* mutants compared to PA14 wild-type biofilms. Exogenous addition of 20 μM concentrations of the nitroxide derivatives carboxy-TEMPO and DCTEIO partially complemented *nirS* mutant biofilm formation in flow cells. (B) Low levels of nitroxide compounds (20 μM) prevented initial biofilm formation in flow cells (b) and triggered biofilm dispersal (c). Biofilms were visualized using SYTO-9 to stain live cells green and propidium iodide (PI) to stain dead cells red. All samples were then examined using confocal laser scanning microscopy. The scale bar represents 20 μm in length, and each panel shows the *xy*, *yz*, and *xz* dimensions.

suggesting that the biofilm deficiency of the *nirS* mutant was due to the mutation in the gene *nirS*. All thickness values were calculated based on the 20-μm scale present in all biofilm images. This indicates that NO might have another role in biofilm formation other than the known role in biofilm dispersal (10), since an inability to make NO strongly inhibited biofilm formation. It is possible that these different effects would be related to the concentration of NO in the cell and the differentiation stage of the cells. The inability of the *nirS* mutant to form biofilms and the role of NO in biofilm dispersion may at first seem contradictory. However, the processes involved in the initial and dispersal stages of biofilm development are distinct. For example, the type IV pilus-mediated twitching motility allows early microcolony formation (1, 3, 32) and is therefore essential for the eventual development of fully mature biofilms. However, overstimulation of twitching motility leads to detachment of cells from biofilms (33). Furthermore, 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 only 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.

ACKNOWLEDGMENTS

We thank Florian Le Bras for technical support and expertise.

This study was supported by grants from the Canadian Institutes for Health Research, Cystic Fibrosis Canada (to R.E.W.H.) and the ARC Centre of Excellence for Free Radical Chemistry and Biotechnology (CE 0561607). C.D.L.F.-N. received a scholarship from the Fundación "la Caixa" and Fundación Canadá (Spain). R.E.W.H. holds a Canada Research Chair.

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