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Nitric oxide-mediated dispersal in single- and multi-species biofilms of clinically and industrially relevant microorganisms

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Summary

Strategies to induce biofilm dispersal are of interest due to their potential to prevent biofilm formation and biofilm-related infections. Nitric oxide (NO), an important messenger molecule in biological systems, was previously identified as a signal for dispersal in biofilms of the model organism *Pseudomonas aeruginosa*. In the present study, the use of NO as an anti-biofilm agent more broadly was assessed. Various NO donors, at concentrations estimated to generate NO levels in the picomolar and low nanomolar range, were tested on single-species biofilms of relevant microorganisms and on multi-species biofilms from water distribution and treatment systems. Nitric oxide-induced dispersal was observed in all biofilms assessed, and the average reduction of total biofilm surface was 63%. Moreover, biofilms exposed to low doses of NO were more susceptible to antimicrobial treatments than untreated biofilms. For example, the efficacy of conventional chlorine treatments at removing multi-species biofilms from water systems was increased by 20-fold in biofilms treated with NO compared with untreated biofilms. These data suggest that combined treatments with NO may allow for novel and improved strategies to control biofilms and have widespread applications in many environmental, industrial and clinical settings.

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Introduction

In nature, bacteria predominantly live in surface-associated matrix-encased communities called biofilms, which can cause significant damage in many industrial and clinical settings (Costerton *et al*., 1995). For example, in the water industry biofilms can block filtration membranes or induce fouling and corrosion in distribution systems (Coetser and Cloete, 2005; Pang *et al*., 2005). In the clinical context, it is estimated that 80% of acute and chronic infections are biofilm-related (Hall-Stoodley *et al*., 2004). Pathogens such as *Pseudomonas aeruginosa*, *Serratia marcescens* or *Vibrio cholerae* in single- or multi-species microbial communities can form biofilms on living tissues in humans, causing for example infections of the respiratory, gastrointestinal and urinary tracts, or periodontal diseases. Biofilms also readily form on biomedical devices such as prostheses and catheters (Khardori and Yassien, 1995).

Bacteria in biofilms are generally highly tolerant to biocides, antibiotics and natural host defences, often becoming up to 10 000 times more resistant compared with their free-swimming counterparts (Buckingham-Meyer *et al*., 2007). Antimicrobials, such as antibiotics or chlorine-based treatments, have traditionally been designed to inhibit planktonic bacteria. These treatments are inappropriate for biofilm control, as their use may be toxic to the environment, require unacceptably high cost and energy inputs or lead to fatal outcomes in clinical settings. To address the need for novel and improved measures against biofilms, a clear strategy is to study the biofilm life cycle and identify key trigger points that regulate biofilm development. Thus, several such switches that mediate surface attachment mechanisms (Valle *et al*., 2006), cell–cell signalling and biofilm maintenance (Hentzer *et al*., 2002) have been the target for biofilm control strategies in recent years. In addition, the last stage of biofilm development that describes the coordinated dispersal of biofilm cells presents several advantages with respect to biofilm control. Induction of biofilm dispersal could potentially use the microorganisms' own energy to remove established biofilms, revert cells to a planktonic phenotype and restore their vulnerability to antimicrobials.

In a recent study, the biologically ubiquitous gas molecule, nitric oxide (NO), was identified as an important

factor mediating biofilm dispersal in the model organism *P. aeruginosa* (Barraud *et al*., 2006). Low, non-toxic concentrations of NO were shown to induce a transition from the sessile, resistant biofilm mode of growth to the motile planktonic phenotype in *P. aeruginosa*. Furthermore, the addition of various antimicrobial compounds was found to almost completely remove remaining *P. aeruginosa* biofilms that were exposed to NO, suggesting a general effect of NO on *P. aeruginosa* physiology (Barraud *et al*., 2006). The involvement of NO in regulating biofilm formation and dispersal in *P. aeruginosa* was also supported by other studies (Darling and Evans, 2003; Van Alst *et al*., 2007). Nitric oxide-mediated dispersal in *P. aeruginosa* biofilms appears to involve cyclic di-GMP (N. Barraud and S. Kjelleberg, unpublished), a conserved secondary messenger, which level is regulated by diguanylate cyclases and phosphodiesterases (Ryan *et al*., 2006). Genes encoding for diguanylate cyclases and phosphodiesterases are widely distributed among bacteria, and are often associated with redox sensors, such as PAS (Per-Arnt-Sim) domains capable of sensing NO (Delgado-Nixon *et al*., 2000; Römling *et al*., 2005). This suggests that NO-mediated dispersal is not restricted to *P. aeruginosa* but may occur among various bacterial species.

Several lines of evidence support the hypothesis that NO-mediated dispersal may be conserved across species. First, a recent study showed that staphylococcal biofilms were inhibited upon exposure to nitrite (NO_2^-), a process that is thought to involve NO (Schlag *et al*., 2007). Second, several aspects of NO biology appear to be conserved across microorganisms, such as NO production apparatuses, either via reduction of NO_2^- during denitrification (Zumft, 1993) or via oxidation of arginine by NO synthases (Adak *et al*., 2002; Gusarov *et al*., 2008), as well as NO responsive networks (Rodionov *et al*., 2005). Third, NO has been suggested to be an ancient and highly conserved regulator of dispersal and life histories across eukaryotic organisms (Bishop and Brandhorst, 2003). For example, dissolution and dispersal of aggregated mycelial cells of fungi, *Neurospora crassa* (Ninnemann and Maier, 1996) and *Candida tropicalis* (Wilken and Huchzermeyer, 1999), and the amoeba *Dictyostelium discoideum* (Tao *et al*., 1997) were shown to rely on NO signalling. Taken together, these observations strongly suggest that NO may mediate biofilm dispersal across a broad range of microorganisms.

The objective of the present study was to examine the effects of NO on various biofilms of clinical and industrial significance. Single-species biofilms of Gram-negative and Gram-positive bacterial species and one yeast, as well as multi-species biofilms from water distribution and treatment systems were exposed to low doses of NO, typically in the picomolar and low nanomolar range. Low concentrations of various NO donors were found to induce dispersal of biofilm cells and reduce biofilm formation across all of the species tested. Moreover, multi-species biofilms in water systems were observed to show decreased surface coverage and heterotrophic counts in the presence of nanomolar concentrations of the NO donors sodium nitroprusside (SNP) and disodium 1-[2-(carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (PROLI). Pretreatment with NO significantly increased the efficacy of chlorine disinfection in removing biofilms established from chlorinated water distribution and treatment systems.

Results

Nitric oxide induces dispersal of single-species biofilms of Gram-negative and Gram-positive bacteria and yeasts

The effect of NO as a broad biofilm-dispersing agent was assessed in a range of biofilm-forming microorganisms of industrial and/or clinical significance (Table 1). Delivery of exogenous NO to biofilms was achieved by using NO-releasing compounds, called NO donors. Nitric oxide donors establish steady-state levels of NO, thus mimicking endogenous NO production. Although the exact amount and location of NO liberated *in vivo* within biofilms from NO donors have not yet been established, approximately a 1000-fold linear relationship between NO donor concentrations and NO steady-state levels was

Table 1. Microbial strains used in this study.

Strain	Site of biofilm formation or infection	Source or reference
Gram-negative		
Serratia marcescens MG1	Respiratory and urinary tracts	Givskov *et al.* (1988)
Vibrio cholerae 92A1552	Gastrointestinal tract and wounds	Yildiz and Schoolnik (1998)
Escherichia coli BW20767	Gastrointestinal and urinary tracts	Metcalf *et al.* (1996)
Fusobacterium nucleatum	Oral cavity	UNSW culture collection
Gram-positive		
Bacillus licheniformis	Food processing surfaces	UNSW culture collection
Staphylococcus epidermidis	Catheters and medical prostheses	UNSW culture collection
Yeast		
Candida albicans	Oral cavity, skin	UNSW culture collection

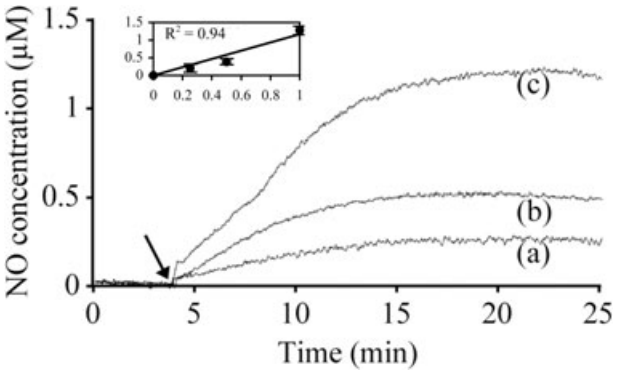

Fig. 1. Nitric oxide release profiles from the NO donor SNP. After the NO baseline signal was stabilized for at least 30 min in the PBS solution, SNP was added (arrow) at final concentrations of (a) 250 μM, (b) 500 μM and (c) 1 mM and the amount of NO released was quantified by using the NO electrode. The inset shows the linear relationship between SNP concentration (mM, *x*-axis) and NO increase (μM, *y*-axis); error bars indicate standard deviation ($n = 3$).

measured *in vitro* ($R^2 = 0.94$) by using solutions of the NO donor SNP in phosphate-buffered saline (PBS) in the range 250 μM–1 mM (Fig. 1). Similar release profiles were observed with the nitrosothiols S-nitroso-N-acetylpenicillamine (SNAP) and S-nitroso-L-glutathione (GSNO) (data not shown). Thus, effective concentrations of NO delivered to the cells are estimated to be 1000 times lower than the concentration of NO donor used herein.

Biofilms of the opportunistic pathogen *S. marcescens* dispersed in response to SNP at concentrations between 25 nM and 500 nM, with a 60% reduction in biofilm coverage at a concentration of 500 nM SNP (Table 2). S-nitroso-N-acetylpenicillamine (100 nM) was also effective in dispersing *S. marcescens* biofilms. The SNP, SNAP and GSNO were effective at inducing dispersal of *V. cholerae* biofilms, where 500 nM SNP induced a 73% reduction in surface coverage and 1 μM SNAP and 1 μM GSNO led to a 29% and 34% reduction respectively (Table 2, $P < 0.01$). *Escherichia coli* biofilms were also dispersed by exposure to low levels of NO as demonstrated by using SNP (Table 2). In these experiments, addition of NO resulted in an increased number of bacteria in suspension as revealed by colony-forming units (cfu) counts (data not shown). To confirm the involvement of NO in the observed effects on biofilms, the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) was used. With added PTIO, the SNP effect was reduced by 93% in *S. marcescens* biofilms, 72% in *V. cholerae* biofilms and 65% in *E. coli* biofilms.

The effect of NO exposure was also investigated on a key organism for biofilms of oral consortia, the anaerobic bacterium *Fusobacterium nucleatum*. The presence of SNP was found to inhibit biofilm formation by *F. nucleatum* (Table 2). The most effective concentrations of SNP were 1 μM and 10 μM, which achieved 48% and 55% reduction in biofilm surface coverage respectively ($P < 0.01$). These concentrations appear to be one order of magnitude higher than those observed with other organisms. This may reflect that, compared with other bacteria, *F. nucleatum* may use a distinct mechanism such as a different NO-sensor domain to activate biofilm dispersal in response to NO signalling.

A strong effect of SNP on the stability of Gram-positive *Bacillus licheniformis* biofilms was observed, with 500 nM SNP inducing greater than 90% reduction in surface coverage of the biofilms (Table 2, $P < 0.001$). *Staphylococcus epidermidis* was found to have reduced biofilm formation

Table 2. Effects of low concentrations of NO donors on various biofilm-forming microorganisms.

Biofilm-forming microorganism	Optimum NO donor[a] concentrations		Maximum percentage removal (SE)[b]
Gram-negative			
Serratia marcescens	SNP	25–500 nM	60.0% (±4.1%)
	SNAP	100 nM	37.8% (±10.5%)
Vibrio cholerae	SNP	25–500 nM	72.5% (±1.9%)
	SNAP	1 μM	28.6% (±3.6%)
	GSNO	1 μM	33.6% (±7.4%)
Escherichia coli	SNP	500 nM	38.1% (±8.7%)
Fusobacterium nucleatum	SNP	1–10 μM	55.6% (±5.6%)
Gram-positive			
Bacillus licheniformis	SNP	100–500 nM	93.2% (±2.0%)
Staphylococcus epidermidis	SNP	10 μM	58.6% (±2.8%)
Yeast			
Candida albicans	SNP	25–100 nM	61.4% (±6.7%)

a. NO donors used: SNP, sodium nitroprusside; SNAP, S-nitroso-N-acetylpenicillamine; and GSNO, S-nitroso-L-glutathione.
b. Percentage removal indicates the percentage of total biofilm surface that was removed after exposure to NO relative to control biofilms that were not exposed to NO. SE, standard error ($n \geq 3$).
Biofilm culture conditions and analysis methodology for each strain are detailed in *Experimental procedures*.

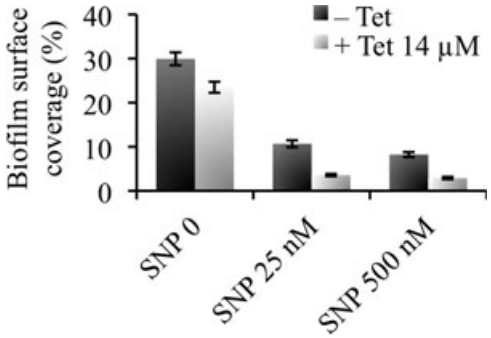

Fig. 2. Nitric oxide effect on *V. cholerae* biofilm antimicrobial sensitivity. Pre-established *V. cholerae* biofilms were treated for 24 h in the presence or absence of the NO donor SNP, and/or the antibiotic tetracycline (Tet) at 14 μM. Biofilm cells remaining on the slides were stained with SYTO 9 to allow analysis using fluorescence microscopy and quantification (per cent surface coverage) using digital image analysis. Data are mean values and error bars indicate standard error ($n = 3$).

in the presence of low concentrations of SNP. A 59% reduction of surface coverage was achieved in the presence of 10 μM SNP.

Nitric oxide-mediated biofilm dispersal was also tested on the fungus *Candida albicans*. When pre-established *C. albicans* biofilms were exposed to low concentrations of SNP, it was found that extremely low concentrations of SNP, i.e. 25 nM and 100 nM, were able to induce a reduction in biofilm formation by up to 61% (Table 2, $P < 0.01$).

Finally, combinatorial treatments of NO and an antibiotic were assessed on *V. cholerae* biofilms. Tetracycline is the usual antibiotic of choice to treat *V. cholerae* infections, but resistance to this drug is increasing (Dromigny *et al*., 2002). Although tetracycline (14 μM) alone, at a concentration below the minimum inhibitory concentration (approximated at 22 μM in this study) for *V. cholerae*, had very limited effect on biofilms of this organism resulting in only a 21% reduction in surface coverage (Fig. 2), SNP was able to enhance tetracycline activity against biofilm cells. Addition of tetracycline after 25 nM SNP and 500 nM SNP treatment induced 67% and 65% reduction of biofilm bacteria respectively, when compared with biofilms treated with tetracycline alone. Overall, combined exposure to 500 nM SNP and tetracycline resulted in 90% reduction in biofilm coverage compared with the untreated controls (Fig. 2).

Nitric oxide induces dispersal of multi-species biofilms in water systems and increases the efficacy of chlorine treatments

Recycled and potable water distribution. To study the effect of NO on multi-species biofilms formed in recycled water distribution systems, an annular reactor (AR) containing unplasticized polyvinyl chloride (uPVC) coupons was connected to a recycled water network for 3 months. After this time, biofilms were sampled and treated in the laboratory. The data demonstrate that SNP treatment was effective at removing multi-species biofilms as revealed with both microscopy and viability analyses by performing heterotrophic cfu counts of biofilm bacteria (Fig. 3). The most efficient concentration of SNP was 500 nM, which induced dispersal of 47 ± 3% of the biofilm compared with the untreated controls, as revealed by cfu measurements of biofilm bacteria (Fig. 3B). As seen on the microscopy images, control biofilms established from recycled water distribution system harbour mature microcolonies that contain both living and dead cells. After SNP treatments, the size of biofilm aggregates (microcolonies) on the surface was considerably reduced, indicative of dispersal events (Fig. 3A). In addition, biofilms treated with SNP displayed increased number of cells (cfu) in their effluent runoff (data not shown). Furthermore, the ratios of cfu counts to biofilm surface coverage were calculated for each treatment and normalized to the control experiments. The ratios did not vary significantly: 1.0, 0.9 and 0.9 for untreated, 100 nM SNP and 500 nM SNP respectively. These data indicate that SNP treatment effectively induced the removal of viable cells from the surface.

The total number of viable bacteria in biofilms decreased after exposure to SNP and it was observed that the relative proportions of different colony morphologies on plates did not change as a result of SNP treatment. Moreover, by using denaturing gradient gel electrophoresis no significant change in the biofilm microbial community could be detected after induction of dispersal with SNP (data not shown). This suggests that exposure to SNP was not selective for specific species within the mixed community; rather, SNP treatment appears to be broadly effective across the entire microbial community. Such an effect is in agreement with the observations of NO-mediated dispersal across a broad range of biofilm-forming organisms (Table 2).

Biofilms exposed to 100 nM and 500 nM SNP, respectively, also exhibited increased sensitivity to chlorine (HOCl) treatments. For example, 1 ppm HOCl, which is within the recommended range (1–2 ppm) for water distribution systems, was up to 20-fold more efficient at removing 500 nM SNP-treated biofilms compared with control biofilms as determined by cfu counts (Fig. 3B). Overall, combined NO and chlorine treatments resulted in 85–90% removal of recycled water biofilms compared with the untreated controls. Consistent data were obtained when coupons were analysed for total biofilm surface by quantification of surface coverage (not shown). Similar results were observed in an independent replicate experiment.

Sodium nitroprusside treatment was also assessed on multi-species biofilms established from a potable

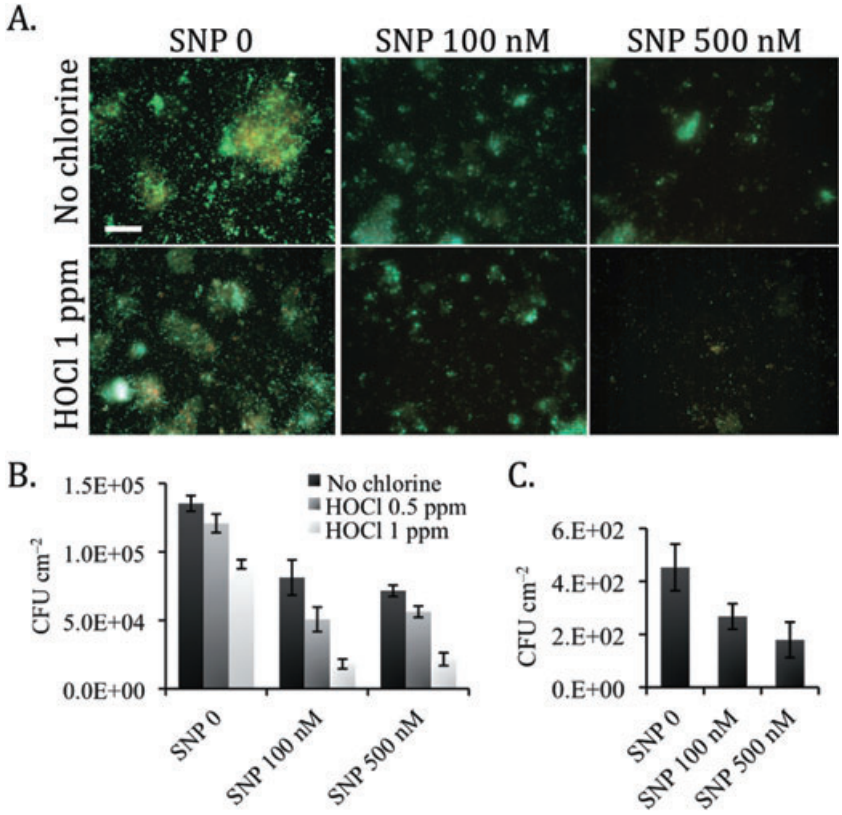

Fig. 3. Effect of NO on multi-species biofilms established from water distribution systems. Three-month-old biofilms from recycled and potable water distribution systems were exposed to 0 (control), 100 nM and 500 nM SNP for 18 h and then (recycled water biofilms) treated for 10 min with free chlorine (HOCl) at 0.5 ppm and 1 ppm and no chlorine controls. (A) The images show microscopic pictures of recycled water biofilms after 1 ppm HOCl exposure (lower panels) or no chlorine controls (upper panels) and stained with the LIVE/DEAD reagents. Live cells appear green, dead cells appear red. Bar, 50 μm. Viability analyses of the biofilms were assessed by heterotrophic colony-forming units (cfu) measurements of (B) recycled water biofilms and (C) potable water biofilms. Data are mean values and error bars indicate standard error ($n = 3$).

water distribution system. Control potable water biofilms generally contained 2-log lower number of cells as seen by heterotrophic cfu measurements (typically 4.5×10^2 cfu cm^{-2}) compared with control biofilms established in recycled water (typically 1.4×10^5 cfu cm^{-2}). Multi-species biofilms established in potable water distribution system were also reduced in total cfu counts upon exposure to nanomolar concentrations of SNP. The most efficient concentration of SNP was 500 nM, which induced dispersal of 60 ± 15% of the biofilm compared with the untreated controls (Fig. 3C).

Reverse osmosis filtration membrane. Biofilms established on a reverse osmosis (RO) filtration membrane connected in line with a full-scale water filtration system for 3 months were exposed to 100 nM SNP for 1 h followed by disinfection with chlorine for 10 min. Sodium nitroprusside itself induced a 30% reduction in biofilm viability compared with untreated controls, as revealed by cfu measurements (Fig. 4A). Moreover, the efficacy of chlorine disinfection was increased by twofold when assessed on biofilms that were pretreated with SNP compared with untreated biofilms. Overall, combined exposure to SNP and chlorine induced a 94% reduction in biofilms (Fig. 4A).

Reverse osmosis membrane biofilms were also assessed for simultaneous exposure to NO and chlorine. For these experiments, the NO donor PROLI was used. 1-[2-(carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate shows an unusual, fast release of NO in water (Fig. 4B) making it a preferred candidate for treatment of filtration membranes, systems which allow for a short exposure time of the compound only. Moreover, PROLI was previously shown to have very low potential toxicity, as tested against L929 Mouse Fibroblasts (Hetrick *et al.*, 2008), which appears suitable for use in potable water systems. After 2 h exposure, 20 nM and 500 nM PROLI induced, respectively, 41% and 32% reduction in total biofilm surface as revealed by cfu counts (Fig. 4A). Further, treatment with PROLI dramatically increased the efficacy of chlorine at removing biofilm cells. Thus, 20 nM PROLI increased 19-fold the efficacy of 10 ppm chlorine, and the

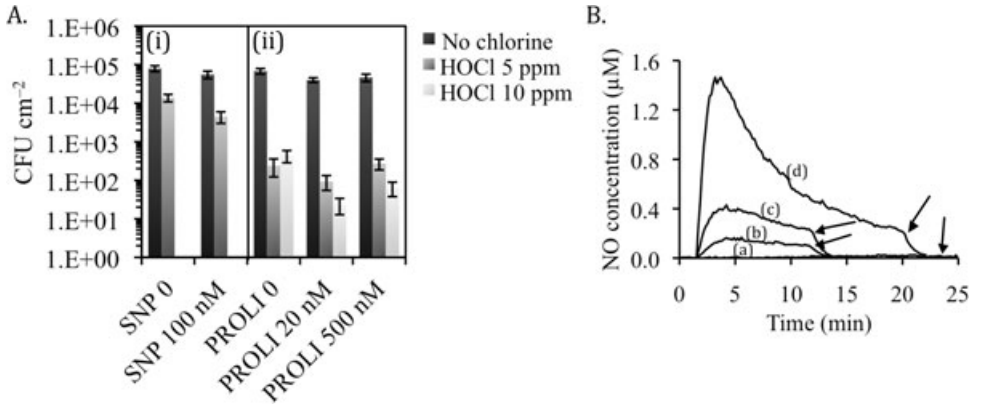

Fig. 4. Multi-species biofilms on a RO filtration membrane exposed to SNP or the fast NO donor PROLI in combination with chlorine.
A. Reverse osmosis membrane coupons harbouring multi-species biofilms were treated: (i) in the presence or absence of 100 nM SNP and subsequently exposed to 5 ppm HOCl for 10 min; or (ii) simultaneously in the presence or absence of 20 nM or 500 nM PROLI and/or free chlorine at 5 ppm or 10 ppm for 2 h. Biofilms were analysed by performing cfu counts. Data are mean values and error bars indicate standard error ($n \geq 3$).
B. Nitric oxide release profiles from PROLI in water. (a) Control, (b) 625 nM, (c) 1.25 μM and (d) 2.5 μM PROLI. Arrows indicate addition of NO scavenger PTIO (100 μM) to the solutions.

combination of PROLI and chlorine resulted in an overall 3-log reduction in the number of biofilm cells (Fig. 4A). PROLI 500 nM was also able to increase the efficacy of chlorine by up to sevenfold.

Discussion

The results presented in this study demonstrate that exposure to low levels of NO donors induces dispersal of diverse single-species biofilms of Gram-positive and Gram-negative bacteria and yeast, as well as multi-species biofilms from water systems. These observations suggest that NO-mediated biofilm dispersal is widespread among biofilm-forming microorganisms. Moreover, NO donors were also observed to increase the sensitivity of *V. cholerae* and multi-species biofilms to antimicrobial treatments. This suggests that low doses of NO can have profound effects on the physiology of bacteria in biofilms and function widely as a signal mediating the transition to a planktonic-like mode of growth, thus rendering cells more vulnerable to antimicrobials. In *P. aeruginosa*, transcriptomic analyses revealed that exposure to low levels of NO induces global responses in biofilm bacteria, including upregulation of genes involved in motility and energy metabolism and downregulation of adhesins and defence mechanisms (N. Barraud and S. Kjelleberg, unpublished).

Nitric oxide-based strategies to induce biofilm dispersal involve extremely low concentrations of NO, in the picomolar to low nanomolar range that should be safe to humans and to the environment. Indeed NO gas and NO donors are currently used clinically. For example, GSNO, which is endogenously produced in mammals, was suggested for its potential use as a therapeutic treatment of respiratory diseases with doses up to 30 μmol administered with a nebulizer (Snyder *et al*., 2002; Que *et al*., 2005). Further, systemic administration of SNP solutions, of up to 650 μM SNP, was approved by the Food and Drug Administration for the treatment of hypertension in humans. At the higher concentrations required to achieve these clinical effects, NO may be toxic and can lead to pathologies such as neurologic excitotoxicity or hypotension. In contrast, our data show that NO donors are active at extremely low levels against biofilms, for example at 500 nM for SNP, a 3-log difference compared with the concentrations used for treatment of hypertension. Hence, NO treatments should not induce any systemic toxic effect at the levels used here. In natural environments, basal NO levels appear to be below the detection limit of most measurement systems, therefore likely not to exceed 10–100 pM (Zafiriou *et al*., 1980), and thus are unable to induce dispersal of biofilms without exogenous addition of NO donors. From an environmental perspective, NO in aqueous solutions is quickly oxidized mostly to nitrite (NO_2^-) (Ignarro *et al*., 1993), for which the maximum contaminant level in drinking water recommended by the Environmental Protection Agency is 1 ppm (22 μM). Again, these concentrations are several orders of magnitude above the concentrations of NO that are effective for inducing biofilm dispersal.

The findings presented in this study identify a novel and unprecedented measure to control biofilms. Nitric oxide donors were active against all biofilms tested and the data suggest that NO can be effective across multi-species microbial communities and is not selective for any particular strain. Combinatorial treatments of low levels of NO and chlorine, which appear acceptable for use in water environments, achieved almost complete eradication of the biofilms, up to 99.97% removal. This reflects

considerable progress in biofilm control practices and would allow control and removal of biofilms in water systems on a continuous basis without any disruption of industrial processes. In addition, the efficacy of NO and tetracycline, as observed in this study, again at levels that appear suitable for treatment of infections in humans, provides a great opportunity for the eradication of recalcitrant infections of *V. cholerae* and possibly other pathogens. This strongly suggests that NO donor compounds warrant thorough investigation as biofilm-dispersing agents useful in the treatment of drug-resistant biofilm infections and control of environmental biofilms.

Furthermore, the use of compounds to modulate NO activity may also be valuable for enhanced control of beneficial microbial communities in biotechnology processes; for example, for the rejuvenation of biofilms to improve productivity and stability in bioremediation or biotransformation systems (Schachter, 2003).

The development of NO-based biofilm control biotechnology may be facilitated by information available from previous research on the use of NO for therapeutic treatments. A broad range of NO donors, which directly or indirectly release NO, or agents that increase NO bioactivity have been developed (Wang *et al*., 2002; Keefer, 2003). Several methods can be used to achieve controlled delivery of NO to biofilm cells in various application areas, including chemical carriers for dosing of NO donors directly in the liquid phase, or delivery at the target site, such as toothpastes for the eradication of dental biofilms. Moreover, NO donors may also be embedded in polymer coatings that can be applied onto surfaces (Reynolds *et al*., 2004; Frost *et al*., 2005).

Overall, combined treatments of low doses of NO with standard antimicrobials offer great potential for the control of biofilms in environmental, industrial and clinical settings, with clear benefits such as reduced ecological impact and reduced treatment costs. Finally, because NO appears to induce the transition from a biofilm to a free swimming phenotype, and thus reduces the antimicrobial tolerance of bacteria on surfaces, via a signalling mechanism rather than toxic effect, NO-based biofilm control strategies would not be expected to select for resistant strains as seen with antibiotics.

Experimental procedures

Single-species biofilms of various bacteria and the yeast C. albicans

Strains and culture conditions. The strains of bacteria and a yeast that were used in this study are listed in Table 1. Overnight cultures were grown at 30°C in Luria Bertani (LB) for *B. licheniformis*, *E. coli*, *S. marcescens* (formerly *Serratia liquefaciens*) and *V. cholerae*, in Tryptic Soy Broth (TSB) (BD Diagnostics) for *S. epidermidis*, and anaerobically in Schaedler Broth (BD Diagnostics) for *F. nucleatum*. The fungus *C. albicans* was grown at 30°C in Yeast Peptone Dextrose Broth (YPD) (Sigma).

Nitric oxide donors and amperometric NO measurements. Four NO donors were used in this study: SNP (Sigma), GSNO (MP Biomedicals), SNAP (Sigma), and disodium PROLI (Alexis Biochemicals). The NO scavenger PTIO (Sigma) was also used. Experiments were carried out where donors were freshly diluted in the respective biofilm medium, and used at final concentrations ranging from 20 nM to 10 μM as indicated. These concentrations were within the range that was previously observed to be effective at inducing biofilm dispersal in *P. aeruginosa* (Barraud *et al*., 2006). Amperometric measurements of NO release from NO donors were carried out by using a NO analyser (Apollo 4000, World Precision Instruments) with ISO-NOP electrode calibrated using SNAP and copper sulfate according to the manufacturer's instructions.

Biofilm experiments. Biofilms of *S. marcescens*, *V. cholerae*, *E. coli*, *B. licheniformis* and *S. epidermidis* were cultivated for 24 h on either glass slides (*V. cholerae*, *B. licheniformis* and *S. epidermidis*) or uPVC slides (*S. marcescens* and *E. coli*), immersed in biofilm medium in sterile petri dishes with gentle shaking, in triplicate. The biofilm media used were 1/5 strength LB for *S. marcescens*, *E. coli* and *B. licheniformis* biofilms, 2M medium as previously described (Paludan-Muller *et al*., 1996) for *V. cholerae* biofilms, and 1/5 strength TSB for *S. epidermidis* biofilms. The conditions used here were identified as those that were optimal for biofilm growth in the absence of any treatment for each organism. After 24 h, the supernatant was replaced with fresh medium containing the indicated concentrations of SNP, SNAP or GSNO (in addition to controls without NO donor), and 200 μM PTIO for the NO-scavenging experiments, and the biofilms were incubated for a further 24 h. For combined NO and antibiotic assays in *V. cholerae* biofilms, after the initial 24 h of biofilm development, the NO donor was added in combination with 14 μM tetracycline. The tetracycline concentration used was below the minimum inhibitory concentration for *V. cholerae* that was previously determined to be 22 μM by monitoring the OD_{600} over 24 h in a microtitre plate assay with *V. cholerae* inoculum in 2M medium and twofold serial dilutions of tetracycline (data not shown). For biofilm analysis, the slides were rinsed and the biofilms were stained with SYTO 9 (3 μl ml^{-1}) (Molecular Probes). Using epifluorescence microscopy (Leica model DMR), 15 selected fields of view per slide were imaged in the XY plane, at regular intervals and across the entire slide. Image analysis (ImageJ, NIH) was performed and total biofilm surface was determined as total surface coverage. The results in Table 2 are presented as the percentage of total biofilm surface reduction in cultures treated with NO relative to the total biofilm surface in control cultures that were not exposed to an NO donor. In Fig. 2, the results of combined NO and tetracycline treatments on *V. cholerae* biofilms are shown for each treatment condition as the percentage of biofilm surface coverage compared with the total substratum surface.

Fusobacterium nucleatum was selected as a model oral biofilm-forming organism. Briefly, cells were grown

anaerobically in Schaedler Broth to an OD_{600} of 0.1, at which time SNP was added. Bacteria were allowed to attach for 4 h on a sterile glass slide. Then the slides were rinsed and stained with 1% crystal violet (CV). Attached cells were enumerated microscopically by digital image capture and subsequent image analysis, and the total biofilm surface was determined as total surface coverage. Results are presented as the percentage of total biofilm surface reduction induced by NO treatments compared with the total biofilm surface in control cultures.

Candida albicans biofilms were grown for 24 h in 24-well polystyrene plates in 1/10 strength YPD with shaking at 100 r.p.m. After 24 h, the supernatant was replaced with fresh medium containing SNP and the cells were incubated for a further 24 h. The wells were then rinsed, stained with 1% CV, and washed again thoroughly, before the CV absorbed into the biofilm was dissolved in absolute ethanol and the total biofilm surface was quantified by measurement of OD_{540} (Wallac-Victor^2).

Experiments were carried out in triplicate and a statistical comparison of the percentage of surface covered by biofilms was performed using analysis of variance and Tukey's multiple comparison tests.

Multi-species biofilms established from water distribution systems

Model potable and recycled water system biofilms were grown in two continuous flow ARs (BioSurfaces Technologies) connected directly to a potable and recycled water distribution system. Sterile stainless steel and uPVC coupons were placed on the exposed face of the inner-rotating cylinder of the ARs, which received chlorinated potable and recycled water respectively, at a rate of 30 l h^{-1} making the hydraulic retention time 2.2 min. Biofilms were allowed to grow on coupon surfaces for a period of 90 days. Stainless steel coupons (potable) and uPVC coupons (recycled) were transferred to sterile bioreactors in the laboratory for NO exposure. The bioreactors consisted of 1 l polypropylene beakers with bottom inlet, top outlet and magnetic stirring, covered with aluminium foil and containing modified polypropylene microscope slide racks (Kartell) that fit the coupons. Coupons were placed in three separate bioreactors where they were exposed for 18 h to a continuous flow (50 ml h^{-1}) of 1/4 strength Ringers solution (Oxoid), pH 7.4, containing 0, 100 nM or 500 nM SNP respectively. For recycled water biofilm assays, coupons were then carefully transferred into sterile 25 ml glass vials containing 20 ml of chlorine treatments in Ringers solution that were freshly made from sodium hypochlorite (Sigma) and calibrated for free chlorine (HOCl) content by N,N-diethyl-p-phenylenediamine analysis using a Pocket Colorimeter II (HACH). After 10 min of gentle shaking, the reactions were stopped by adding 100 μM sodium thiosulfate.

The coupons were processed either for microscopy analysis, in duplicate, or for viability assay by performing heterotrophic plate counts, in triplicate. For microscopy analysis, biofilms were stained using the LIVE/DEAD *Bac*Light Kit (Molecular Probes), visualized by using fluorescent microscopy and the biofilm surface coverage was quantified by digital image analysis as described above. For viability analysis, the coupons were placed in stomacher bags containing 25 ml Ringers solution. Biofilm cells were firstly removed from the coupon surfaces by agitation of the coupon by hand, followed by sonication at 400 W for 60 s (Branson 2210) and then stomaching for 60 s (Seward Stomacher 80) to remove and homogenize the remaining biofilm. The homogenate was then serially diluted and plated on oligotrophic R2A medium agar (Oxoid), using a pour-plate technique. The plates were incubated at 25°C for 7 days. This protocol has previously been optimized for the removal of potable water biofilms for heterotrophic cfu measurements (data not shown). Analysis of variance tests at a significance level of 95% were used to compare the impact of the various combinations of low doses of NO, and chlorine disinfectant on biofilm growth.

Multi-species biofilms established on RO filtration membrane

A small-scale residential RO system (Crystal Clear Purification Systems) equipped with a RO cartridge model CSM RE1518-50 (Sae-Han) was connected to the Ravensthorpe (Western Australia) water treatment plant for 3 months. The pressure of the inlet was approximately 60 psi (400 kPa) at a flow rate of 100 ml min^{-1}. After this period, coupons were extracted under sterile conditions from the biofouled membranes, transferred to 3 ml Ringers solution containing NO and/or chlorine treatments in 12 well plates (Sarstedt) and incubated at 25°C with gentle shaking. For SNP experiments, coupons were exposed to 0 (controls) or 100 nM SNP for 1 h. Then coupons were rinsed in Ringers solution and treated for 10 min with 5 ppm HOCl and no chlorine controls. For PROLI experiments coupons were simultaneously exposed to the NO donor PROLI (20 nM, 500 nM and untreated controls) and HOCl (5 ppm, 10 ppm and no chlorine controls) for 2 h. After treatments, biofilms were analysed by performing heterotrophic cfu counts. Biofilm cells were removed from the membrane coupons by using a sterile swab, serially diluted, plated onto R2A agar plates and the plates were incubated at 25°C for 7 days before enumeration of cfu.

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