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Abstract

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Nitric oxide: a key mediator of biofilm dispersal with applications in infectious diseases

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Abstract

Studies of the biofilm life cycle can identify novel targets and strategies for improving biofilm control measures. Of particular interest are dispersal events, where a subpopulation of cells is released from the biofilm community to search out and colonize new surfaces. Recently, the simple gas and ubiquitous biological signaling molecule nitric oxide (NO) was identified as a key mediator of biofilm dispersal conserved across microbial species. Here, we review the role and mechanisms of NO mediating dispersal in bacterial biofilms, and its potential for novel therapeutics. In contrast to previous attempts using high dose NO aimed at killing pathogens, the use of low, non-toxic NO signals (picomolar to nanomolar range) to disperse biofilms represents an innovative and highly favourable approach to improve infectious disease treatments. Further, several NO-based technologies have been developed that offer a versatile range of solutions to control biofilms, including: (i) NO-generating compounds with short or long half-lives and safe or inert residues, (ii) novel compounds for the targeted delivery of NO to infectious biofilms during systemic treatments, and (iii) novel NO-releasing materials and surface coatings for the prevention and dispersal of biofilms. Overall the use of low levels of NO exploiting its signaling properties to induce dispersal represents an unprecedented and promising strategy for the control of biofilms in clinical and industrial contexts.

Increased antimicrobial tolerance in biofilms is the basis for chronic infections and failed antibiotic therapies

Antibiotic and antimicrobial strategies have traditionally been evaluated using suspension cultures of homogenous planktonic bacteria. However, in nature most bacteria live predominantly in heterogeneous multicellular biofilm communities encapsulated in a self-produced matrix of extracellular polymeric substances (EPS), while the free-swimming planktonic cells appear to be associated with a dispersal phase necessary to colonise new habitats. The biofilm lifestyle confers bacteria with greatly increased resistance compared to their planktonic counterparts, showing up to 10,000 fold higher tolerance towards immune defences, biocides and antibiotics [1-3], rendering biofilm infections extremely difficult to eradicate. Thus the formation of a biofilm often leads to persistent and chronic infections, which greatly increase morbidity and mortality. Bacterial biofilms are estimated to be the cause of 80% of all clinical infections (e.g. reviewed in [4]). The mechanisms of biofilm tolerance are still not fully understood but appear to involve a combination of physical effects together with specific and non-specific genetic determinants. Firstly, the biofilm EPS matrix provides a protective barrier by both reducing the penetration of antibiotics [5] and accumulating extracellular defence compounds such as β-lactamase enzymes [6], as well as guarding biofilm bacteria from macrophage engulfment and killing [7]. Secondly, within the biofilm, bacteria exhibit a high level of tolerance, either: (i) via expression of biofilm-specific traits such as periplasmic antibiotic-binding polysaccharides [8]; (ii) due to upregulation of enzymes to protect against endogenous oxidative stress [9]; or (iii) as a result of genetic modifications. The latter may occur in a non-specific manner due to increased frequency of mutations in biofilms that can lead to new resistance traits, e.g. constitutive expression of efflux pumps [10], or via

horizontal gene transfer when an invading pathogen acquires resistance genes from a commensal community [11]. Further, rapid adaptive induction of antibiotic resistance genes in cells at the biofilm peripheries can also serve to protect the rest of the biofilm community [12]. Thirdly, tolerance in biofilms is enhanced by the presence of a high number of persister cells. Nutrient gradients established within biofilm structures lead to starvation responses inducing a number of cells to switch to a transient antibiotic-tolerant persister phenotype, which can survive antibiotic treatments and rapidly resume growth once the treatment is stopped [13]. Antibiotic treatments are not only inefficient at controlling biofilms but exposure to sub-inhibitory concentrations of many antibiotics can also result in increased biofilm formation (reviewed in [14]), a process that is likely to have clinical relevance in numerous infectious diseases. Accordingly there is an urgent need to develop novel therapeutics and strategies to control biofilms and overcome biofilm resistance.

Inducing the natural biofilm dispersal response to control biofilm-related infections

One promising approach towards novel biofilm control measures is to study and target endogenous mechanisms that regulate the biofilm life cycle (Fig. 1A). Biofilm formation is a multi-stage process that involves the coordinated differentiation of cells. Following initial attachment, mediated by bacterial motility and cell surface appendages, such as pili and fimbriae that interact with abiotic and biotic surface materials, bacteria produce abundant EPS comprised of polysaccharides, proteins, extracellular nucleic acids, lipids and ions such as Ca^{2+}, which irreversibly commit the cells to the surface. During maturation, biofilms establish complex 3D structures comprised of highly differentiated bacteria, rendering the biofilm environment and the

bacterial communities highly heterogeneous, including steep nutrient and oxygen gradients. The final stage of biofilm development involves the coordinated release of differentiated, motile, chemotactic cells known as dispersal cells (reviewed in [15]). These specialized cells can colonize new surfaces and restart the biofilm life cycle. In several bacteria, biofilm dispersal correlates with the programmed death of a subpopulation of cells in mature microcolonies [16]. Surviving cells are then able to escape through break out points, leaving behind hollow structures in the biofilm. Dispersal events are generally thought to benefit the biofilm by releasing phenotypically diverse cells for the colonisation of new surfaces and by limiting overcrowding in a densely populated and genetically diversified mature biofilm [17].

Several molecular triggers have been identified that can induce the transition from a sessile, surface associated or suspended biofilm phenotype to a free-swimming dispersal phenotype, including: (i) environmental and physiological cues such as nutrient [18-20] or oxygen [21] availability, low concentrations of nitric oxide (NO) [22-24], iron levels [25, 26], and D-amino acids [27]; (ii) cell-cell communication signals such as quorum sensing (QS) acyl-homoserine lactone signals [28], autoinducing peptides [29] and diffusible fatty acids [30, 31]; and (iii) intracellular messengers such as cyclic di-GMP (c-di-GMP), which has emerged as a central element in the complex regulatory network controlling the switch between biofilm and planktonic bacteria [32], as well as cAMP, which was previously known to control the stringent response and was recently found to be implicated in biofilm formation and dispersal [33, 34]. Upon sensing a dispersal cue, bacteria can activate a range of cellular effectors that lead to dispersal, including the secretion of enzymes and surfactants that solubilise and degrade EPS components [35-37]. Finally,

induction of the dispersal response activates expression of motility mechanisms such as flagella and pili and proteins involved in chemotaxis [38, 39]. Dispersal is thus a highly regulated process that requires recruitment of the cellular machinery and energy resources to escape from the biofilm.

Manipulation of the endogenous biofilm development program, by inducing dispersal signals, has become a preferred strategy for developing novel control strategies in recent years. For instance, 2-aminoimidazole derivatives targeting QS have been designed and found to disperse established biofilms [40, 41]. Proof of concept studies showed that in vivo manipulation of c-di-GMP levels can effectively clear (by decreasing c-di-GMP) or prolong (by increasing c-di-GMP) *Pseudomonas aeruginosa* infections in murine models [42, 43]. Modification of the BdcA protein to enhance its c-di-GMP binding, thus reducing the intracellular c-di-GMP concentration, caused nearly complete removal of biofilms via dispersal in vitro [44]. Of particular interest is NO, a simple and versatile dispersal signal that is highly conserved across biofilm species. Much progress has been made in recent years to design efficient NO delivery strategies making it an outstanding candidate for novel therapeutic strategies. This review focuses on the discovery and use of NO for inducing biofilm dispersal.

Recent discovery of a key physiological signal for biofilm dispersal: nitric oxide (NO)

NO is produced endogenously during the biofilm life cycle to induce dispersal and trigger the transition to a planktonic lifestyle

NO is a ubiquitous gas and reactive lipophilic radical that can freely diffuse into cells. Its signaling role in regulating dispersal of bacterial biofilms was first discovered

while studying the biofilm life cycle of *P. aeruginosa*, where it was found to be produced at the same time and location as cell death and dispersal [22] (Fig. 1A). The use of a range of fluorescent dyes for detecting specific reactive oxygen and nitrogen species first indicated NO as the key mediator of cell death. This was confirmed by genetic studies demonstrating that dispersal events in biofilms are regulated by the endogenous production of NO [22]. A mutant strain unable to express nitrite reductase (NIR) required for production of NO did not show cell death and dispersal, whereas a mutant strain unable to scavenge NO (impaired in production of NO reductase, NOR) exhibited increased cell death and dispersal compared to the wild type [22]. Further, it was found that adding NO back to biofilms, by using donor compounds that spontaneously release NO in solution, showed that NO, at low, non-toxic concentrations in the picomolar to nanomolar range, triggered dispersal and the transition to the planktonic mode of growth. Importantly, exposure to low doses of NO restored the sensitivity of biofilm and dispersed bacteria towards several classes of antimicrobial agents, greatly increasing their efficacy (Fig. 2).

NO-mediated dispersal is conserved across species

Dispersal responses to NO have been observed in a range of monospecies biofilms. For instance addition of NO donors was shown to induce dispersal in biofilms of *P. aeruginosa, Escherichia coli*, *Vibrio cholerae*, *Bacillus licheniformis*, *Serratia marcescens, Fusobacterium nucleatum* [23], *Shewanella woodyi* [24], *Neisseria gonorrhoeae* [45] and a marine *Pseudoalteromonas* species [46], and in *Vibrio fischeri* addition of a NO scavenger prevented dispersal of aggregates [47]. Exposure to nitrite inhibited biofilm formation by *Staphylococcus aureus*, presumably through generating NO and inducing dispersal [48]. In *Bacillus subtilis* [49], changes in

endogenous production of NO resulted in loss of biofilm biomass. In *Legionella pneumophila*, a NO responsive sensor protein was found to mediate reduction in biofilm biomass [50]. Nitrifying biofilms also appear to be responsive to NO, for instance *Nitrosomonas europaea* biofilms were previously found to disperse in response to low NO levels [51]. In *Pseudomonas putida* studies showed that heterologous expression of a NO synthase (NOS) enzyme resulted in increased motility and biofilm dispersal [52]. Further, NO can also induce dispersal in multispecies biofilms. Addition of low dose (20-500 nM) NO donors caused dispersal of mixed species microbial biofilms formed in drinking water and recycled-water systems and on reverse osmosis water filtration membranes [23]. Suspended biofilm aggregates in expectorated sputum from chronically infected cystic fibrosis (CF) patients were dispersed by using NO donors [53]. Thus NO-mediated biofilm dispersal appears to be well conserved across bacterial species. Paradoxically, some studies have demonstrated the opposite effect where the addition of NO stimulated biofilm formation, for instance in *Shewanella oneidensis* [54] and the rhizobacterium *Azospirillum brasilense* [55]. It is possible that NO may not induce dispersal responses in some species, for instance in the context of host-microbe symbiotic or mutualistic relationships [56]. Intriguingly, disaggregation, dispersal and inhibition of attachment induced by NO have also been observed in several eukaryotic organisms, including fungi [23, 57], amoeba [58] and algal zoospores [59] (Table 1), which suggests that NO may be an ancient and highly conserved regulator of dispersal [60].

NO signaling involves the secondary messenger c-di-GMP

To elucidate the regulatory mechanisms involved in NO-mediated biofilm dispersal, transcriptomic analysis of the cellular response to low levels of NO were performed in

P. aeruginosa biofilms. These studies revealed 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 [39]. NO was found to decrease intracellular levels of c-di-GMP and stimulate phosphodiesterase (PDE) activity in cell-free extracts, the latter suggesting posttranslational regulation [39]. The ubiquitous messenger c-di-GMP functions as a central regulator of many important bacterial processes, including biofilm formation, virulence and dispersal (recently reviewed in [32, 61]). Intracellular levels of c-di-GMP are controlled through the opposing activities of diguanylate cyclases (DGC), for the synthesis of c-di-GMP, and PDEs, for its degradation. These are encoded by a conserved GGDEF domain and EAL or HDGYP domain containing genes, respectively. Many bacterial genomes encode multiple DGCs and PDEs often associated with other putative signaling domains, suggesting that their enzymatic activities may be responsive to different environmental cues. Downstream cellular targets of c-di-GMP include repression of EPS production, activation of EPS degrading enzymes and motility. A number of effectors of c-di-GMP signaling have been identified, such as transcription factors, PilZ domain and degenerate GGDEF and EAL domain-containing proteins, as well as mRNA riboswitches [61]. A link between NO and c-di-GMP has been established in several bacterial species in addition to *P. aeruginosa*, including *S. woodyi* [24], *L. pneumophila* [50], *Pseudoalteromonas atlantica* [62] and *E. coli* [63]. In *E. coli*, NO was found to induce motility and decrease surface attachment upon binding to the transcription repressor NsrR [64], although it is not clear if NsrR is linked to c-di-GMP or whether it operates via an independent pathway in *E. coli*.

In *P. aeruginosa* several c-di-GMP specific PDEs have been identified that appear to be involved in NO-mediated dispersal, including DipA, RbdA and NbdA [65, 66]. Further, NO signaling was shown to require the chemotaxis regulator BdlA [39], as well as the periplasmic protease LapG (dispersal in response to NO donors in in vitro batch and continuous flow *P. aeruginosa* biofilm assays was fully inhibited in the *lapG* knockout mutant strain compared to wild type, unpublished data), which in *P. putida* and *Pseudomonas fluorescens* was found to be repressed by a c-di-GMP receptor protein LapD and activated when intracellular c-di-GMP levels decreased [67, 68]. Although a receptor for NO associated with a c-di-GMP PDE remains to be identified in *P. aeruginosa*, such regulatory systems have been identified in *S. woodyi* [24]. *S. woodyi* encodes a heme nitric oxide/oxygen binding (HNOX) protein, which when complexed with NO binds to and activates a PDE enzyme resulting in dispersal (Fig. 1B). HNOX domains are conserved hemoproteins that are highly sensitive to NO, producing responses at femtomolar levels in *Clostridium botulinum* [69]. They are found in several Gram-negative and Gram-positive bacterial genomes and are often associated with a DGC or PDE [70]. However many bacterial strains known to disperse in response to NO, including *P. aeruginosa* and *E. coli*, do not have HNOX domain suggesting other systems can sense and transduce NO signals. Similarly, Gram-positive strains such as *S. aureus* do not possess any GGDEF, EAL or HDGYP domain, suggesting that in some organisms NO-mediated dispersal may operate via a signaling cascade independent of the secondary messenger c-di-GMP.

Dysregulation of NO production by the host immune system leads to chronic infection and disease

NO has been known to play an important role in the immune system and host defences against pathogenic bacteria for some time. Early studies showed that host tissues produce NO using nitric oxide synthase (NOS) enzymes from L-arginine after recognition of bacterial invasion [71-73]. Both constitutively expressed (cNOS) and inducible (iNOS) NOS are involved in immunity. In macrophage and epithelial cells, iNOS enzymes are activated by bacterial lipopolysaccharides (LPS) and inflammatory cytokines (e.g. IFN-γ, IL-1β, and TNF-α) [74].

In some cases, impairment of NOS function can lead to infectious disease. The cause of NOS malfunction may originate from the host or from the bacteria. For example, invading pathogens such as *E. coli* [75] or *Salmonella typhimurium* [76] can avoid host defences by secreting effectors that inhibit iNOS. *Helicobacter pylori* was found to secrete arginase to inhibit NO production in gastric mucosa [77]. In the oral cavity, NO production normally occurs during plaque deposition but in patients affected by smoking, where NO levels are reduced, higher bacterial counts are observed [78].

Cystic fibrosis (CF) lungs

NOS activity and generation of NO is required for clearance of infections in the respiratory tract [79]. In the lungs of patients suffering from CF, epithelial cells fail to produce NO in response to pathogen invasion and bacterial LPS sensing [80, 81]. Impaired NO production may be due to reduced NOS expression, including both iNOS [80, 82, 83] and possibly cNOS [84], as well as reduced availability of the NOS substrate L-arginine, possibly due to increased arginase activity in CF airways [85].

This inability to produce NO in response to pathogen invasion in CF patients appears to play a major role in the establishment of chronic infections, and compromised NOS activity has even been suggested as the primary reason for the poor antimicrobial defence of CF lungs [86]. In vitro studies using human airway epithelial cells from a CF patient showed reduced *P. aeruginosa* adhesion and infection in CF cells transfected with human iNOS cDNA compared to cells without iNOS [87]. Further, in these experiments while recombinant iNOS did not reduce internalisation of adhered bacteria, internalised cells were efficiently killed, suggesting that NO production mostly regulates adhesion as well as killing of cells that have infiltrated the epithelium.

NO and inflammation

NO plays an important role in regulating inflammatory responses. Dysregulation of its production in chronically infected host tissues can lead to immunopathology [88]. In healthy patients, NO can act as an autoregulatory feedback inhibitor serving to limit tissue damage after the onset of inflammation. At high levels, NO can inhibit iNOS expression in macrophages and terminate the inflammatory process [89]. The mechanisms underlying this regulation have recently been uncovered and found to involve *S*-nitrosylation of the inflammasome protein NLRP3 [90]. Thus it was suggested that impaired iNOS activity could potentially exacerbate autoimmune diseases including colitis, arthritis and multiple sclerosis [91]. In CF patients, lack of iNOS has also been linked to inflammation disorders [92]. Due to its role in inflammation and because it can be measured quickly and non-invasively in the respiratory airways, exhaled NO has become an important diagnostic marker for inflammatory airway conditions such as asthma and bronchitis [93, 94].

Adjunctive low dose NO combined with antibiotics: a promising new clinical strategy for biofilm control

Treatments to induce biofilm dispersal with NO and effectively inactivate dispersed bacteria with antibiotics

The ability of NO to induce the signaling cascade involving stimulation of PDE activity and decreased intracellular c-di-GMP leading to dispersal of biofilms offers great promise for developing novel and efficient therapeutics for controlling biofilm-related infections and for overcoming biofilm resistance. While exposure to low doses of NO alone appears to be non-toxic to bacteria, the released planktonic cells and cells still residing on surfaces both show increased susceptibility to a range of antibiotics and antimicrobials [23, 39, 95]. Thus NO-based anti-biofilm strategies probably benefit from combined treatments with standard antibiotic therapies to clear infections. Before considering NO as a standalone therapeutic, further studies are needed to determine whether exposure to low doses of NO in a host environment can facilitate recruitment of immune defences capable of clearing the dispersed cells.

Biofilm-related diseases are highly diverse as biofilms can form on both living tissues, e.g. lungs, nose (rhinosinusitis), urinary tract, ears (otitis media), heart (endocarditis), oral cavity (plaque, gingivitis) or wounds, as well as abiotic surfaces, e.g. dialysis catheters, prosthetic implants or contact lenses [4]. This variety of conditions makes it difficult to develop antibiofilm treatments that could be applied for treating multiple diseases. NO-based strategies to disperse biofilms will benefit from a broad range of delivery methods (reviewed below) that can be specifically adapted on a case-by-case basis.

At higher levels, NO may be effective at killing biofilms

At physiological concentrations, in the picomolar and nanomolar range, NO serves multiple signaling roles in both the host and pathogenic organisms. However, at higher concentrations NO can be converted to a number of more reactive derivatives, known collectively as reactive nitrogen species, which can have cytostatic and cytotoxic effects on pathogens as well as host cells [96]. These can cause damage to nucleic acids and proteins through nitrosylation (adding an NO group) or nitration (adding an NO_2 group) of amine, thiol and tyrosine residues, as well as metal centres [96]. At elevated concentrations, the high diffusivity and multiple modes of action of NO make it a broad-spectrum antimicrobial agent that could kill biofilms of Gram-negative and Gram-positive bacteria. In vitro experiments showed that exposure to 200 ppm NO gas (~8 µM NO) for up to 5 h could fully eradicate cultures of clinical isolates of *S. aureus*, methicillin-resistant *S. aureus* (MRSA), *E. coli*, Group B *Streptococcus*, *P. aeruginosa* and *Candida albicans* [97]. The potential of intermittent exposure to high levels of NO gas has been assessed in animal trials using rats. NO was delivered at 160 ppm (~7 µM NO) for 30 min every 4 h to rats with *P. aeruginosa* airway infection, and the results showed that the NO treatment was able to reduce the infection by more than 2 log [98]. Exposure to NO gas at 500 ppm (~20 µM NO) for 60 s every 24-48 h of external wounds colonised by *S. aureus* led to faster wound healing by 30%, compared to controls [99]. Wound dressings that release NO levels typically at 500 ppm appeared to be efficient at killing biofilms of nosocomial pathogens *Acinetobacter baumannii*, MRSA, and *P. aeruginosa* when assessed in in vitro experiments [100]. The use of toxic NO has also been investigated for treatment of urinary tract infections, where the addition of 10 mM ascorbate and

nitrite at 50 μM to 5 mM, which under these conditions generate equimolar NO, cleared *E. coli* infections in artificial urine and in an urinary tract model [101, 102]. Another study demonstrated the effectiveness of NO-charged catheters, typically releasing 2 to 60 μM NO, in preventing *E. coli* infections [103].

Several concerns have been raised when using NO as a bactericidal agent. Firstly, at high concentrations NO can be toxic to tissues and inhibit healing. Because NO can act as an immunosuppressant that limits inflammation, high levels could prematurely halt healing and reduce macrophage activity against infections. Further, during wound treatments excessive NO could be inhibitory to angiogenesis, decreasing endothelial cell and lymphocyte proliferation [104, 105]. Side effects of nitrosative stress in host tissues include the generation of carcinogenic N-nitrosamines [106]. In the lungs, high levels of NO can transfer to the blood and cause methaemoglomia [107]. Recently the safety of delivery and the physiologic effects of intermittent exposure to 160 ppm NO gas three times daily for 30 min for 5 days have been assessed in clinical trials. While a first cohort of healthy individuals appeared to tolerate the treatment well [108], in a second trial with CF patients, detrimental side effects were reported in several of the eight patients who received NO that included increased methaemoglomia, dry mouth, and one case of reduced lung function (forced expiratory volume in 1 s, FEV_1 max < 10%) [109]. Second, at elevated levels, NO may induce defence mechanisms in bacteria rendering them more tolerant to antibiotics. Thus in *P. aeruginosa*, while exposure to low concentrations of NO induced biofilm dispersal, treatment with higher concentrations in the micromolar to millimolar range resulted in increased biofilm formation, presumably as an adaptive response to protect against nitrosative stress [22]. In *B. subtilis*, 5 s exposure to 30 μM NO was found to enhance defence

against oxidative stress by depleting free cysteine and activating catalase [110]. In *Salmonella*, 750 μM NO donor spermine NONOate was found to block respiration, which induced an accumulation of NADH that protected against oxidative stress [111]. The same treatment was also shown to impair energy-dependent drug uptake after causing an arrest in respiration, which then led to increased resistance towards aminoglycoside antibiotics [112]. Finally, at high levels NO may directly react with antibiotic compounds leading to their inactivation [113]. Therefore the effectiveness of toxic levels of NO to kill biofilms may be strongly dependent on the bacterial species and infection conditions and may elicit undesirable secondary effects that could compromise clearance of the infection. In contrast, increased antibiotic resistance is not expected when using low, non-toxic concentrations of NO in the picomolar to nanomolar range to induce dispersal.

NO delivery methods

Due to its reactivity towards a wide range of molecules, including metalloproteins, heme and non-heme iron centres, thiols and amines, as well as oxygen and free radical species such as superoxide ($O_2^{-}\bullet$), NO has a half-life of only a few seconds in biological systems [114]. In order to be effective, NO needs to be available in the immediate vicinity of pathogenic biofilms. The method of delivery of NO to infectious biofilms is therefore crucial and can conceivably be achieved by several means.

Use of NO gas

If applicable, NO can be directly applied as a gas to infection sites exposed to air. NO gas has been used to treat skin infections, most notably leg ulcers [115]. Inhaled NO gas was approved as therapeutic agent by the US Food and Drug Administration (FDA) in 1999 and the European Medicine Evaluation Agency and European Commission in 2001. It has since been used as a pulmonary vasodilator in treating pulmonary hypertension, including in patients with chronic obstructive pulmonary disease (COPD) [116, 117]. However, the effect of inhaled NO on bacterial infections during these treatments was not investigated. Previous studies by two different research teams showed that exposure to 40 ppm NO in air [118] or 10 ppm NO in 100% oxygen [119] for 24 h reduced *P. aeruginosa* infiltration and helped clear lung infections in rats, decreasing bacterial load by 1.7 and 2 log, respectively. In the latter study, 10 ppm NO was also found to increase influx of inflammatory cells into the air space of infected rats [119]. Recently, the first clinical trial was conducted to evaluate the use of low dose inhaled NO gas combined with standard intravenous ceftazidime and tobramycin antibiotic therapy for the disruption of *P. aeruginosa* biofilms in 12 patients with CF. The results demonstrated that patients who received NO gas at 5-10 ppm (~200 nM NO) for 8 h daily during 7 days concomitant with standard ceftazidime and tobramycin treatments showed significant reductions, by 3.5 log in the number of *Pseudomonas* biofilm aggregates and marginal improvement in lung function (FEV_1 and forced vital capacity, FVC) compared to patients who received a placebo [120]. These data suggest that using NO as adjunctive therapy may be highly beneficial for the treatment of CF-related biofilm infections.

Stimulation of endogenous NO production

The delivery of NO to infectious biofilms should be achievable by stimulating endogenous production, either from the biofilm cells or from the surrounding infected tissues. In bacteria, NO is produced from NIR enzymes in denitrifying as well as non-denitrifying organisms [121], or from NOS enzymes using L-arginine as substrate [122]. Nitrate, nitrite and L-arginine have all been shown to enhance susceptibility of *P. aeruginosa* in biofilms to antibiotics by up to 2 log reduction in colony-forming units (CFU), presumably through an NO-mediated mechanism [123]. Exposure of *S. aureus* to nitrite, which was suggested to generate NO, prevented the formation of in vitro biofilms [48]. Addition of acidified nitrite, which can generate NO either spontaneously or from NIR activity, was found to effectively control *P. aeruginosa*, *S. aureus* and *Burkholderia cepacia* biofilms [124, 125], although in these cases high concentrations of nitrite (15 mM) were used and NO acted via a toxic, killing effect on biofilms. In preliminary clinical studies, treatments with nebulized L-arginine in CF patients infected with *P. aeruginosa* resulted in sustained improvement in lung function associated with significantly increased NOS activity within lung tissues, suggesting that the NO augmentation could potentially reduce the bacterial infection [126, 127]. However in these studies, the effect of increased L-arginine on *P. aeruginosa* growth was not investigated and will need to be confirmed in subsequent trials.

Use of NO donors

The most versatile option for the delivery of NO is to use NO-donor molecules that can liberate NO in vivo. Release of NO from donors can occur either spontaneously, upon activation by enzymatic activity or through activation under select chemical

conditions, e.g. pH. In general, the effective concentrations of NO delivered to the biofilms are estimated to be 100-1000 times lower than the concentration of NO donor used (Fig. 3, [23]). NO donors could potentially be administered in a variety of formulations, including tablets, ointments or nebulisers. Much progress has been made in developing usable NO donors and a large variety of compounds have been described in various reviews (e.g. [128, 129]). The metal nitrosyl sodium nitroprusside (SNP) and the organonitrate nitroglycerin are FDA-approved drugs that have been used for more than 50 years in the treatment of hypertension. SNP at 500 nM has been shown to effectively disperse various single species biofilms as well as multispecies biofilms, including those in CF sputum [22, 23, 53]. *S*-nitrosoglutathione (GSNO), a naturally occurring *S*-nitrosothiol, is also used clinically as a vasodilator, including in the lungs of CF patients [130, 131]. An important and highly versatile class of NO donors are the diazeniumdiolates (NONOates). Originally created as a laboratory curiosity, this class of compounds has evolved in the past 15 years into a vast range of compounds with wide ranging chemical properties and NO release profiles that are potentially useful in many short- or long-term healthcare applications [132] (Fig. 3). NONOate chemistry allows for storage of NO as part of an engineered molecule whose framework can be controlled to tune the level of NO storage, rate of NO release and molecule size. The compounds can be used to modify polymers and nanoparticles and can also be engineered to include prodrug moieties for targeted NO delivery (see sections below). Finally, dispersal of *P. aeruginosa* biofilms by long-lived aminoxyl free radicals (nitroxides), which are sterically hindered analogues of nitric oxide, has been demonstrated [133].

Targeted delivery of NO by using β-lactam prodrug antibiotics

Due to its short half-life in biological systems and its potential for non-selective reactivity towards many host targets, the use of donor compounds that spontaneously release NO in solution would often not be ideal for treating biofilm infections. The compounds could potentially have side effects and these typically polar chemicals would be difficult to deliver to biofilms. An innovative new class of NO-donor prodrugs was recently described that can liberate NO upon specific activation by bacterial enzymes [95, 134] (Fig. 4). The cephalosporin-3'-diazeniumdiolate compounds consist of a β-lactam analogue, cephalosporin, that provides a scaffold to prevent release of NO from an NONOate donor until activated by a substrate-tolerant, bacteria-specific enzyme β-lactamase. The modified cephalosporins were rationally designed to selectively release highly unstable NONOates ($t_{1/2}$ = 2.8 s-2 min for NO generation) following reaction with β-lactamase, and thus trigger biofilm dispersal. The lead compound, DEA NONOate-Cephalosporin Prodrug (DEACP) was synthesised and found to be highly stable in solution and release NO upon reaction with commercially available β-lactamase penicillinase as well as whole cell extracts from *P. aeruginosa* that produce β-lactamases. Interestingly, release of NO was also triggered by non-β-lactamase-producing *E. coli* extracts suggesting that the compounds can also be activated by transpeptidases, the target enzymes of β-lactam antibiotics. These compounds were effective at dispersing biofilms of several pathogenic species including mixed species biofilms from CF sputum, and when used in combination with tobramycin and ciprofloxacin greatly improved the outcome of the antibiotic therapy [53, 95]. In these experiments, the use of DEACP at 10 µM was found to be more effective than at 100 µM, increasing tobramycin and ciprofloxacin treatments by 1.8 and 1.5 log reduction in CFU, respectively [95]. The novel and

flexible synthetic chemistry route developed for DEACP was used to access five additional analogues carrying variations in both the acyl-amido side chain (R1) and O^2-alkyldiazeniumdiolate (R2) portions [134] (Fig. 5). The compounds showed activity similar to DEACP. Two compounds, DEACP and PyrroCP were tested for cytotoxicity in L929 murine fibroblast cells and showed no toxicity at 50 µM (DEACP) or 100 µM (PyrroCP, unpublished data), which suggests an excellent therapeutic window. The use of β-lactam-based prodrugs for the targeted delivery of NO to biofilms is immensely attractive as many cephalosporins have previously and continue to be used clinically [135]. The simple modification to incorporate a diazeniumdiolate NO donor may represent an effective method for treating biofilm-based chronic infections.

NO polymers and nanoparticles

Nanoparticle drug delivery has been widely studied as a means for increasing drug solubility and tissue specificity. The utility of nanoparticles arises from their various physicochemical properties (e.g., hydrophobicity, charge, size), which can be tuned by varying synthetic precursors and procedures. Silica- and gold-based nanoparticles have been developed that release low or high levels of NO [136-139] and show effectiveness against biofilms [140]. Nanoparticles also offer the advantage that they can be combined with other active molecules, such as antimicrobial agents, e.g. long chain quaternary ammonium salts [141]. NO-releasing polypropylenimine dendrimers have been developed which allow higher levels of NO release per ‘backbone’ molecule over traditional NO donors [142]. NO releasing polymers and nanoparticles could be used either as coatings to prevent biofilm formation on surfaces such as

catheters, prosthetic implants or contact lenses as well as industrial surfaces, or delivered in formulation either systemically or topically to treat biofilms on tissues.

NO-QS inhibitor dual-action hybrid compounds

Another important signaling pathway in biofilms is the quorum sensing (QS) system. QS regulates virulence, biofilm formation and dispersal in a range of organisms [143]. NO and QS-mediated regulation appear to share common molecular mechanisms, as exemplified in *P. aeruginosa* where QS deficient strains were found to accumulate more NO [144, 145]. This raises the intriguing possibility of interfering with multiple biofilm regulatory pathways using combinations of QS inhibitors and NO donors as a strategy towards therapeutics for controlling biofilms and bacterial virulence. A wide range of synthetic and natural product-based QS inhibitors have been identified in the last two decades, including the halogenated furanones isolated from the marine red algae *Delisea pulchra* which show highly potent QS inhibition and virulence attenuation activities [146]. Recently, novel dual-action furanone-NO donor hybrid compounds have been designed and synthesized. Two compounds were found to have both QS inhibition and NO releasing properties and were effective antibiofilm agents [147].

Surface modification for biofilm prevention and dispersal

The formation of biofilms on abiotic surfaces is a major clinical concern as biofilms on prosthetic implants, catheters or contact lenses, for example, can act as reservoirs for pathogenic bacteria leading to chronic and severe infections. NO releasing materials and coatings were originally developed to prevent platelet aggregation and improve biocompatibility of biological implants, such as vascular grafts (artificial

blood conduits) [148, 149]. Newer sol-gel NO releasing coatings based on NONOates have been developed that are compatible with artificial prosthetic implants and display antibacterial properties. The coatings were capable of inhibiting adhesion of biofilm bacteria, e.g. *P. aeruginosa*, *S. aureus*, and *S. epidermidis* [150, 151]. Polymer coatings that allow for modulation of NO release, controlled either by light, (e.g. *S*-nitrosothiols [152] or metal nitrosyls [153]) or by an electric pulse [154] have also been developed. Finally, coatings capable of catalytically generating NO via conversion of endogenous substrates such as *S*-nitrosothiols or nitrite are attractive for long-term applications since they are not limited by a finite reservoir of NO embedded in the surface [155, 156].

Future perspectives

The role of NO as a signaling molecule is vast. First discovered in the 1980s for its role in regulating vasodilation via the activation of soluble guanylate cyclase, NO has since emerged as a universal signal regulating a plethora of physiological functions in living organisms [157]. Its importance in human physiology was recognised with a number of awards and NO was named 'molecule of the year' by Science in 1992 [158]. The diffusivity of NO across cell membranes and its reactivity towards a range of target sites stand out as unique properties in signal transduction that allow rapid spreading and amplification of an initial cue and the coordination of a subset of adjacent cells. The signaling role of NO in regulating biofilm dispersal across microbial species offers an unprecedented opportunity to develop novel treatments to induce biofilm dispersal and improve treatments for chronic infections. Since the mechanisms linked to dispersal involve non-toxic activation of a signaling pathway there is reduced pressure for the evolution and spreading of variant bacteria. Thus,

resistance is not expected to arise from low-dose NO treatments. As the signaling pathways are further elucidated, novel markers both from infectious biofilms and host tissues will be identified that will facilitate the evaluation of novel biofilm-dispersing compounds in in vivo studies.

A range of NO donor compounds are already available that can be used as adjunctive therapies to improve antibiotic treatments. In addition, novel carriers, including nanoparticles and dual-action hybrid drugs, polymer coatings and prodrugs specifically designed to release NO to biofilm infection sites are being investigated. In the future, new compounds will be designed that exhibit multiple actions, including release of NO signals and/or other agents interfering with various effectors of the signaling cascades regulating dispersal and virulence, combined with potent antibiotic activity. Further, given the extreme simplicity of NO as an active ingredient, it may be possible to develop a wide range of targeted release chemistries for the precise delivery of NO signals to specific pathogenic bacteria, while leaving intact the commensal microbial community.

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Figures and Tables

Fig. (1). (A) The biofilm life cycle: oxygen (O_2) and nutrient gradients are present in the mature biofilm leading to production of NO signals that trigger cell death and dispersal. (B) NO signals activate phosphodiesterase (PDE) activity, which leads to decreased c-di-GMP levels and enhanced dispersal. In *Shewanella woodyi*, direct binding of NO to an H-NOX sensor stimulating PDE activity has been demonstrated [24].

Fig. (2). Add-back of NO to established biofilms using the NO donor sodium nitroprusside (SNP) triggers dispersal and increases susceptibility to various antimicrobial treatments: hydrogen peroxide (H_2O_2), tobramycin, sodium dodecyl sulphate (SDS) and ultraviolet light (UV). Partly reproduced from [22].

Fig. (3). (A) Representatives from the NONOate (diazeniumdiolate) class of NO donors. (B) Spontaneous NO release from 100 μM PROLI/NO, DEA/NO or SPER/NO in 0.1 M Tris buffer (pH 7.4). Measurements were obtained using an NO selective electrode. Arrow indicates addition of the NO scavenger 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide (PTIO).

Fig. (4). Novel prodrug strategy for biofilm-targeted NO delivery [95]. (A) Cephalosporins bearing O^2-alkyldiazeniumdiolates at the 3' position release NONOate anions following reaction with bacterial β-lactamases. (B) Electrode measurements of NO release from DEACP in the presence of penicillinase. Arrows from left to right indicate addition of: 100 μM DEACP, 0.05 U / ml penicillinase, 0.1

U / ml penicillinase and NO scavenger PTIO. (C) Schematic drawing of DEACP activation and NO-induced dispersal in biofilms. Red stars denote biofilm β-lactamase enzymes.

Fig. (5). Chemical structures of synthesised cephalosporin-3'-diazeniumdiolates. Half lives of the appended diazeniumdiolates in aqueous buffer at pH 7.4 are given. Compound numbers correspond to [134].

Table 1. List of microbial species dispersed by NO.

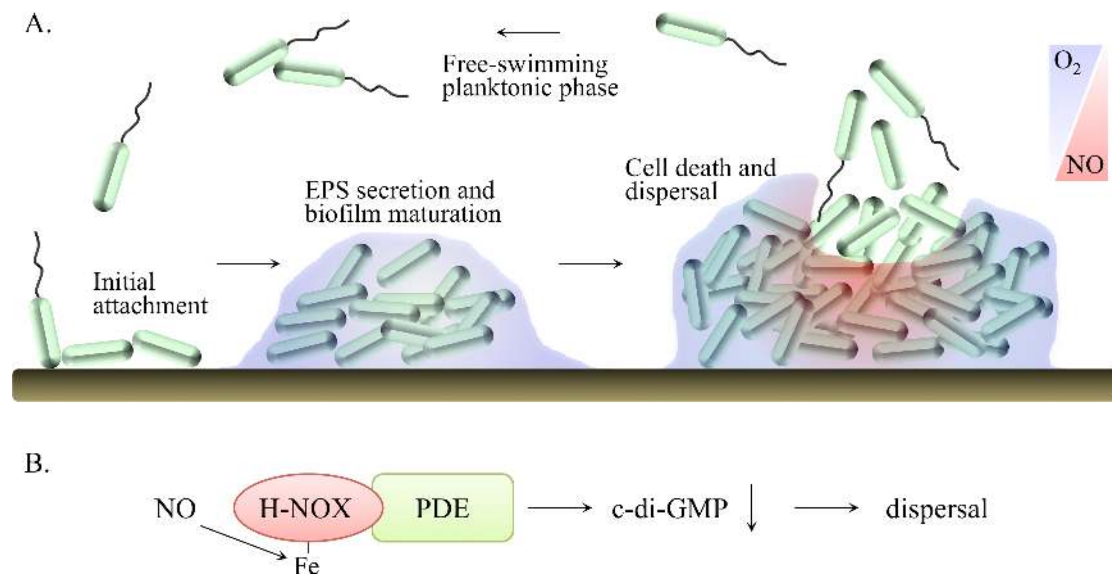
Microbial species	Description	Manipulation of NO levels	Ref
Single species biofilms			
Pseudomonas aeruginosa	Gram –ve opportunistic pathogen	NO donors SNP, nitroxides, MAHMA NONOate. Mutants in NIR and NOR	[22, 23, 66, 133, 159]
Escherichia coli	Gram –ve opportunistic pathogen	NO donor SNP	[23, 64]
Fusobacterium nucleatum	Gram –ve anaerobic oral pathogen	NO donor SNP	[23]
Serratia marcescens	Gram –ve opportunistic pathogen	NO donors SNP, SNAP	[23]
Vibrio cholerae	Gram –ve pathogen, agent of cholera	NO donors SNP, SNAP, GSNO	[23]
Bacillus licheniformis	Gram +ve soil bacterium	NO donor SNP	[23]
Shewanella woodyi	Gram –ve marine bacterium	NO donor DETA	[24]

		NONOate	
Neisseria gonorrhoeae	Gram –ve pathogen	NO donor SNP	[45]
Pseudoalteromonas sp. strain NCIMB 2021	Gram –ve marine bacterium	NO donor SNP	[46]
Vibrio fischeri	Gram –ve marine bacterium	NO scavenger PTIO	[47]
Staphylococcus aureus	Gram +ve pathogen	NO source sodium nitrite	[48]
Bacillus subtilis	Gram +ve opportunistic pathogen	Mutant in NOS	[49]
Legionella pneumophila	Gram –ve pathogen, agent of legionellosis	Mutant in H-NOX domain	[50]
Nitrosomonas europaea	Gram –ve autotroph nitrifier	Low NO gas	[51]
Pseudomonas putida	Gram –ve soil bacterium	Heterologous expression of NOS	[52]
Multispecies biofilms			
Mixed species biofilms	From water distribution systems and filtration membranes	NO donors SNP, PROLI NONOate	[23]
Mixed species biofilm aggregates	From cystic fibrosis sputum	NO donor SNP	[53]
Eukaryotes			
Candida albicans	Yeast; oral and genital infections	NO donor SNP	[23]
Candida tropicalis	Yeast; opportunistic pathogen	NO source L-arginine; NOS inhibitor L-NAME	[57]

Dictyostelium discoideum	Soil amoeba	NOS inhibitor L-NIO, NO gas, NO scavenger oxyhemoglobin	[58]
Ulva linza	Algal zoospores	NO donor SNAP, NO scavenger PTIO	[59]

NIR, nitrite reductase; NOR, NO reductase; SNP, sodium nitroprusside; SNAP, *S*-nitroso-*N*-acetylpenicillamine; GSNO, *S*-nitrosoglutathione; NOS, NO synthase; L-NAME, N^{ω}-nitro-L-arginine methyl ester; L-NIO, L-N^{5}-iminoethyl ornithine; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide.

Figures and Tables



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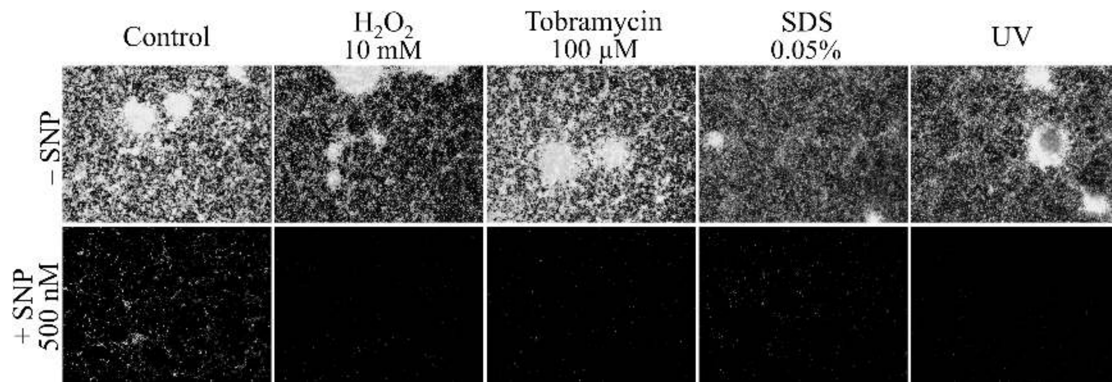

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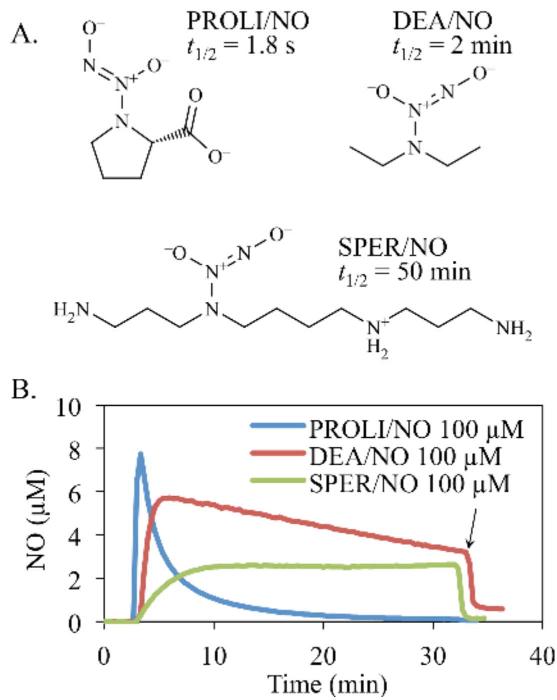

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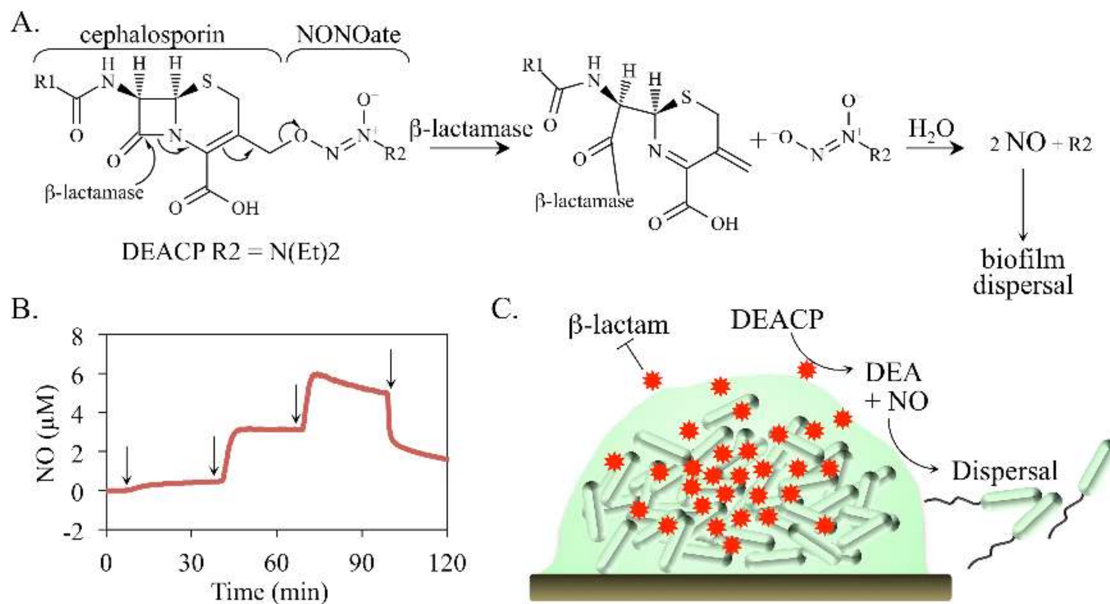

Fig. (4). Novel prodrug strategy for biofilm-targeted NO delivery [3]. (A) Cephalosporins bearing O^2-alkyldiazeniumdiolates at the 3' position release NONOate anions following reaction with bacterial β-lactamases. (B) Electrode measurements of NO release from DEACP in the presence of penicillinase. Arrows from left to right indicate addition of: 100 μM DEACP, 0.05 U / ml penicillinase, 0.1 U / ml penicillinase and NO scavenger PTIO. (C) Schematic drawing of DEACP activation and NO-induced dispersal in biofilms. Red stars denote biofilm β-lactamase molecules.

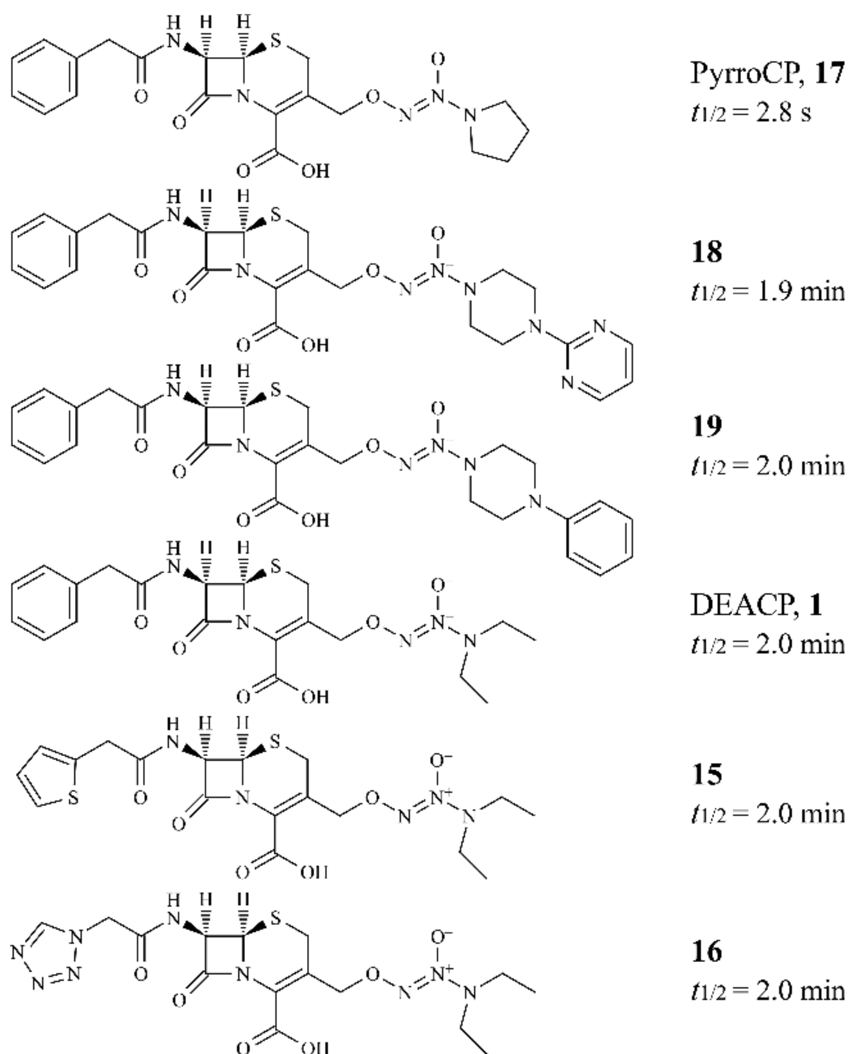

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Table 1. List microbial species dispersed by NO.

Microbial species	Description	Manipulation of NO levels	Ref
Pseudomonas aeruginosa	Gram –ve opportunistic pathogen	NO donors SNP, nitroxides, MAHMA NONOate. Mutants in NIR and NOR	[2, 5-8]
Escherichia coli	Gram –ve opportunistic pathogen	NO donor SNP	[5, 9]
Vibrio cholerae	Gram –ve pathogen, agent of cholera	NO donors SNP, SNAP, GSNO	[5]
Bacillus licheniformis	Gram +ve soil bacteria	NO donor SNP	[5]
Serratia marcescens	Gram –ve opportunistic pathogen	NO donors SNP, SNAP	[5]
Fusobacterium nucleatum	Gram –ve anaerobic oral pathogen	NO donor SNP	[5]
Candida albicans	Yeast, oral and genital infections	NO donor SNP	[5]
Shewanella woodyi	Gram –ve marine bacteria	NO donor DETA NONOate	[1]
Neisseria gonorrhoeae	Gram –ve pathogen	NO donor SNP	[10]
Pseudoalteromonas sp. strain NCIMB 2021	Gram –ve marine bacteria	NO donor SNP	[11]
Vibrio fischeri	Gram –ve marine bacteria	NO scavenger PTIO	[12]
Staphylococcus aureus	Gram +ve pathogen	NO source sodium nitrite	[13]
Bacillus subtilis	Gram +ve opportunistic pathogen	Mutant in NOS	[14]
Legionella pneumophila	Gram –ve pathogen, agent of legionellosis	Mutant in H-NOX domain	[15]
Nitrosomonas europaea	Gram –ve autotroph nitrifier	Low NO gas	[16]
Pseudomonas putida	Gram –ve soil bacteria	Heterologous expression of NOS	[17]
Multispecies biofilms from water distribution systems and filtration membranes		NO donors SNP, PROLI NONOate	[5]
CF sputum biofilm aggregates		NO donor SNP	[18]
Candida tropicalis	Yeast, opportunistic pathogen	NO source L-arginine; NOS inhibitor L-NAME	[19]
Dictyostelium discoideum	Soil amoeba	NOS inhibitor L-NIO, NO gas, NO scavenger oxyhemoglobin	[20]
Ulva linza	Algae zoospores	NO donor SNAP, NO scavenger PTIO	[21]

NIR, nitrite reductase; NOR, NO reductase; SNP, sodium nitroprusside; SNAP, S-nitroso-N-acetylpenicillamine; GSNO, S-nitrosoglutathione; NOS, NO synthase; L-NAME, N^{ω}-nitro-L-arginine methyl ester; L-NIO, L-N^5-iminoethyl ornithine; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide.

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