Low concentrations of nitric oxide modulate *Streptococcus pneumoniae* biofilm metabolism and antibiotic tolerance

Raymond N. Allan\(^{a,b}\), Samantha Morgan\(^a\), Sanjita Brito-Mutunayagama\(^a\), Paul Skippc\(^d\), Martin Feelisch\(^{e,f}\), Stephen M. Hayes\(^a\), William Hellier\(f\), Stuart C. Clarke\(^{a,g}\), Paul Stoodley\(^{c,g}\), Andrea Burgess\(^f\), Hasnaa Ismail-Koch\(f\), Rami J. Salib\(^{a,e,f}\), Jeremy S. Webb\(^{c,g}\), Saul N. Faust\(^{a,b,e,#}\) & Luanne Hall-Stoodley\(^{a,b,g}\)

Clinical and Experimental Sciences, Faculty of Medicine and Institute for Life Sciences, University of Southampton, Southampton, UK\(^a\); Southampton NIHR Wellcome Trust Clinical Research Facility, University Hospital Southampton NHS Foundation Trust, Southampton, UK\(^b\); Centre for Biological Sciences, University of Southampton, Southampton, UK\(^c\); Centre for Proteomic Research, Institute for Life Sciences, University of Southampton, Southampton, UK\(^d\); Southampton NIHR Respiratory Biomedical Research Unit, University Hospital Southampton NHS Foundation Trust, Southampton, UK\(^e\); Department of Microbial Infection and Immunity, Centre for Microbial Interface Biology, College of Medicine, The Ohio State University, Columbus, Ohio, USA\(^f\).

Running head: Nitric Oxide Treatment of *S. pneumoniae* Biofilms

#Address correspondence to Saul Faust, s.faust@soton.ac.uk
Abstract

*Streptococcus pneumoniae* is one of the key pathogens responsible for otitis media (OM), the most common infection in children and the largest cause of childhood antibiotic prescription. Novel therapeutic strategies that reduce the overall antibiotic consumption due to OM are required because although widespread pneumococcal conjugate immunization has controlled invasive pneumococcal disease, overall OM incidence has not decreased. Biofilm formation represents an important phenotype contributing to the antibiotic tolerance and persistence of *S. pneumoniae* in chronic or recurrent OM. We investigated the treatment of pneumococcal biofilms with nitric oxide (NO), an endogenous signaling molecule and therapeutic agent that has been demonstrated to trigger biofilm dispersal in other bacterial species. We hypothesised that addition of low concentrations of NO to pneumococcal biofilms would improve antibiotic efficacy and higher concentrations exert direct antibacterial effects. Unlike in many other bacterial species, low concentrations of NO, did not result in *S. pneumoniae* biofilm dispersal. Instead, treatment of both *in vitro* biofilms and *ex vivo* adenoid tissue samples (a reservoir for *S. pneumoniae* biofilms) with low concentrations of NO enhanced pneumococcal killing when combined with amoxicillin-clavulanic acid, an antibiotic commonly used to treat chronic OM. Quantitative proteomic analysis using iTRAQ (isobaric tag for relative and absolute quantitation) identified 13 proteins that were differentially expressed following low-concentration NO treatment, 85% of which function in metabolism or translation. Treatment with low-concentration NO therefore appears to modulate pneumococcal metabolism and may represent a novel therapeutic approach to reduce antibiotic tolerance in pneumococcal biofilms. [245]
**Introduction**

*Streptococcus pneumoniae* is a Gram-positive bacterium that asymptomatically colonizes the human nasopharynx. This opportunistic pathogen is responsible for invasive diseases such as pneumonia, bacteremia and meningitis, and localized mucosal infections such as otitis media and sinusitis. Globally, these infections represent a significant burden of disease, particularly in the very young and the elderly. The World Health Organization estimates that 1.6 million deaths occur annually due to pneumococcal infections, accounting for around 11% of the mortality in children under 5 (1). The majority of deaths occur in developing countries where invasive pneumococcal disease remains one of the most common fatal childhood illnesses.

Pneumococcus is a leading pathogen in otitis media (OM), the most common infection in young children and a principal reason for repeated physician visits. Upon colonization with *S. pneumoniae* and the establishment of carriage in children, bacteria may access the middle ear space by retrograde ascent from the nasopharynx due to the presence of fluid and/or disruption of mucociliary clearance. Recurrent or chronic otitis media causes much pain and morbidity at high economic cost to society (2). In spite of concerns about the selection of antibiotic resistant bacteria, OM continues to be the primary reason for antibiotic prescription in children (3–5). In addition, although pneumococcal conjugate vaccines (PCV) have reduced vaccine type invasive pneumococcal disease, PCVs have not led to a decrease in the incidence of otitis media due to pneumococci, most likely due to non-vaccine type replacement (1, 6, 7). Novel treatments for pneumococcal infection are therefore needed to address the problem of recurrent and/or chronic infections in children.
Infections occur following a breach of the mucosal epithelia subsequent to colonization and despite being a prerequisite for infection, little is known about how pneumococci colonize and persist in the nasopharynx. However, a growing body of literature suggests that bacterial biofilm development plays a prominent role in colonization and disease. *In situ* investigation of paediatric middle ear biopsies indicated that pneumococcal biofilms were present on the middle ear mucosal epithelium in children with chronic OM but not in children without chronic OM (8, 9). *S. pneumoniae* biofilms have also been identified *in situ* on adenoid mucosal epithelia from children undergoing adenoidectomy for the treatment of infective (chronic OM) or inflammatory (obstructive sleep apnoea - OSA) otolaryngological disease consistent with the adenoid serving as a reservoir of pathogens that may contribute to infection under circumstances that favor middle ear infection (10, 11). More recently, pneumococcal biofilms have been investigated with animal models and epithelial cell models (12–15).

Biofilms are highly adaptive surface-associated microbial aggregates that allow bacteria to survive the diverse stressful conditions encountered in the host such as nutrient limitation and host immune responses (16–19). The increased tolerance of biofilm bacteria to conventional antibiotic therapeutic concentrations compared with their planktonic counterparts also poses a significant problem in clinical settings, as does their propensity to acquire further antimicrobial resistance via horizontal gene transfer, underscoring the need for novel therapeutic strategies to limit the pneumococcal biofilm phenotype during disease (20, 21).

Nitric oxide (NO) is an important signaling molecule that is ubiquitous in both eukaryotes and prokaryotes, bridging the boundaries between host and pathogen.
human host, NO plays an important role in the innate immune response and is produced by
inducible nitric oxide synthases (iNOSs) in epithelial and phagocytic cells with its
production resulting in damage to bacterial cell membranes and DNA (22). Bacteria have
also been shown to possess NOSs that oxidise L-arginine to produce the low concentrations
of NO observed in several species (23, 24). Multiple regulatory systems have been
identified that mediate the diverse responses of bacteria to NO, including conferring
protection from oxidative stress and playing a role in toxin biosynthesis (23, 25). In
biofilms, however, low concentrations of exogenous NO have been shown to trigger a
dispersal response in several bacterial species including *Pseudomonas aeruginosa*,
*Escherichia coli* and *Staphylococcus epidermidis*, a response associated with increased
antibacterial efficacy when used as an adjuvant in conjunction with antibiotics (26–28).
Furthermore, NO-releasing nanoparticles and gaseous NO have been shown to exert potent
antimicrobial effects against *P. aeruginosa*, *Streptococcus pyogenes* and *Enterococcus
faecalis* (29–31).

Previous investigations into the role of NO in pneumococcal infection have given
conflicting results. In a murine pneumonia model NO was found to be associated with
increased bacterial loads and reduced survival during bacteremia in wild-type mice but not
in NOS2-deficient mice. In contrast during pneumonia following intranasal infection NO
decreased pneumococcal viability in the lung in both mouse strains via a direct
antibacterial effect (32).

Because several studies using biofilm models have shown that low concentrations of
NO trigger release of bacteria from the biofilm in various bacterial species we hypothesized
that adjunctive NO treatment would also improve the efficacy of antibiotic killing of
pneumococci in biofilms. We tested this hypothesis using *in vitro* and *ex vivo* pneumococcal biofilms. Biochemical studies and a high-throughput quantitative proteomic approach were utilized to interrogate possible mechanisms of action.

**Methods**

**Bacterial strains and growth conditions.** Clinical isolates of *Streptococcus pneumoniae* serotypes 14 (ST124), 19F and 23F (33), and the laboratory strain D39 (serotype 2) were selected to evaluate the *in vitro* antibacterial efficacy of NO and antibiotic adjunctive treatment. Strains were subcultured from frozen stocks onto Columbia blood agar (CBA) plates (Oxoid, U.K.) as described (33). Briefly, cultures were incubated at 37°C/5% CO₂ and colonies re-suspended in fresh Brain Heart Infusion (BHI) broth (Oxoid, U.K.) for experiments.

**Planktonic experiments.** Flat-bottomed 96-well culture plates (Fisher Scientific, U.K.) were inoculated with ~1.0 x 10⁷ bacteria per well (mid-exponential planktonic cultures) grown in BHI. All treatments were prepared in BHI. Sodium nitroprusside dihydrate (SNP) was added to wells for final concentrations ranging from 1 μM to 10 mM; diethylamine NONOate (DEA/NO; sodium salt), sodium nitrate (NaNO₃), sodium nitrite (NaNO₂) and potassium cyanide (KCN; all Sigma-Aldrich, U.K.) were added at a final concentration of 1 mM, and carboxy-PTIO potassium salt and L-methionine (both Sigma-Aldrich, U.K.) were added at final concentrations of 50 μM and 1 mM respectively. BHI was added in place of treatments for all untreated controls. BHI alone was used to measure background changes in absorbance. Cultures were incubated at 37°C/5% CO₂ and absorbance (OD₅₉₅)
measured every 30 min over 2 hours using an EZ Read 400 spectrophotometer (Biochrom) (n=3).

In vitro biofilm experiments. For biofilm formation mid-exponential planktonic cultures grown in BHI were used to inoculate individual wells of untreated polystyrene 6-well plates (1 x 10⁸ cells per well) (Corning Incorporated, Costar, U.S.A.), and supplemented with fresh BHI diluted 1:5 in distilled H₂O. Cultures were incubated at 37°C/5% CO₂ with replacement of warm, fresh diluted 1:5 BHI daily for 2 to 7 days.

Prior to treatment medium was removed and biofilms washed twice using diluted 1:5 BHI. NO donor (SNP and DEA/NO) treatments were prepared fresh in diluted 1:5 BHI and added to wells at final concentrations ranging from 100 nM to 1 mM. For adjuvant experiments amoxicillin + clavulanic acid (AMC) was added at a final concentration of 300/60 μg/ml. Biofilms were incubated at 37°C/5% CO₂ for 2 hours after which the NO donors or inhibitors were removed and the remaining biofilm rinsed twice with diluted 1:5 BHI. Biofilms were resuspended in Hank’s balanced salt solution (HBSS) as described (20).

Briefly, biofilms were scraped and vortexed, both resuspended biofilms and removed supernatants were diluted in HBSS, spot plated onto CBA plates and incubated at 37°C/5% CO₂. To assess total biofilm biomass 100 μl of the resuspended biofilms were diluted 10-fold in 1:5 BHI and turbidity measured by absorbance (OD595) using a Jenway 6300 spectrophotometer. All assays were performed on 48 h biofilms using 2 technical replicates of 2 biological replicates (n=4).
Confocal Laser Scanning Microscopy (CLSM). Mid-exponential planktonic cultures of strain ST124 (n=3) were grown in BHI and used to inoculate 35 mm untreated glass bottom CELLview cell culture dishes (Greiner Bio One, U.K.) and supplemented with fresh 1:5 BHI. Biofilms were grown under static conditions at 37°C/5% CO₂ for 48 h replacing medium daily with fresh 1:5 BHI. Biofilms were treated with 1 mM SNP in 1:5 BHI or 1:5 BHI (untreated control) at 37°C/5% CO₂ for 2 hours. Treatments were removed and the remaining biofilm rinsed twice with HBSS. Biofilms were stained with Live/Dead BacLight Bacterial Viability Kit (Life Technologies, U.S.A.) according to manufacturer instructions. Biofilms were examined immediately with an inverted Leica SP8 LSCM system using a 63x oil immersion lens and sequential scanning of 1 μm sections (Leica Microsystems, Milton Keynes, U.K.). To remove background eDNA staining, the Syto9 fluorescence intensity threshold was set to that of planktonic pneumococci. Images were analysed using Leica LCS Software.

Scanning Electron Microscopy (SEM). Serotype 14 (ST124) biofilms were grown for 48 h in 6-well plates containing ethanol-sterilized 13 mm glass cover slips (V.W.R., U.K.). Biofilms were treated with 1 mM SNP in 1:5 BHI or 1:5 BHI (untreated control) at 37°C/5% CO₂ for 2 hours then processed for SEM as described (33). Biofilms were imaged using an FEI Quanta 200 scanning electron microscope.

Protein extraction and iTRAQ (isobaric tag for relative and absolute quantitation) labelling. Comparative analyses of protein expression between biofilms treated with 100 μM SNP for 2 h at 37°C/5% CO₂ and untreated biofilms were performed on 3 technical
replicates of 3 biological replicates. Protein extraction and iTRAQ labelling were performed as described (33).

**Mass spectrometry, peak list generation, and database searching.** Mass spectrometry, peak list generation and database searching were performed as previously described (33).

Inclusion criteria for quantitative analysis were set at ≥3 peptide matches, ≥50 protein score, ≥5% sequence coverage (p < 0.05). Comparative protein data with >1.3 and <0.77 ratios were identified as having differential expression. For qualitative identification the inclusion criteria were 2 peptide matches, ≥50 protein score, and ≥5% sequence coverage.

**Ex vivo adenoid experiments.** Adenoids were obtained from paediatric patients <12 years of age undergoing adenoidectomy for the treatment of suspected inflammatory or infective Ear, Nose and Throat (ENT) disease (n=11). Adenoids were collected on ice in sterile HBSS + 5% fetal bovine serum (FBS; Sigma-Aldrich, U.K.), washed twice with HBSS to remove any unattached bacteria, then dissected into four equal-sized sections with similar luminal surface coverage. Tissue sections were normalized for weight and treated with 100 μM SNP alone, 300/60 μg/ml AMC alone or 100 μM SNP + 300/60 μg/ml AMC in HBSS/10% FBS for 2 h at 37°C/5% CO₂. Untreated control tissue was treated with HBSS/10% FBS alone. Tissue sections were washed twice with 10 ml HBSS, macerated in 1 ml HBSS through a 100 μm nylon cell strainer (Fisher Scientific, U.K.) and bacterial suspensions serially diluted and spot plated onto CBA plates.
Statistical analyses. Statistical analysis of in vitro planktonic and biofilm data was performed using one-way ANOVA and Tukey's multiple comparisons tests. Analysis of ex vivo adenoid data was performed using a Wilcoxon Signed Ranks test. Comparative data reported as p<0.05 were considered statistically different.

Results

Treatment with the NO donor SNP decreased viability of planktonic cells and the cell population remaining within in vitro biofilms. Since low-concentrations of NO have been shown to result in the release or dispersal of other bacterial species from biofilms, we first tested the hypothesis that low-concentrations of NO would have a similar effect on pneumococcal biofilms. Established 48 hour biofilms were treated for 2 hours with a range of NO concentrations generated from different concentrations of the NO-donor SNP (100 nM - 1 mM; Fig. 1). Measurement of biofilm biomass using turbidity, and viability using colony forming unit (CFU) enumeration, respectively, indicated that treatment with low concentrations of NO (100 nM to 100 μM SNP) did not have a similar effect on 48h pneumococcal biofilms. Treatment with 1 mM SNP however, resulted in a significant reduction in the biomass and a 3-log reduction in the number of viable cells remaining within the biofilm (p≤0.001). These results suggested that at 1mM SNP treatment may either be triggering the release of S. pneumoniae from the biofilm or had a direct bactericidal effect. To distinguish between these possibilities the number of viable cells present in the biofilm supernatant was measured following treatment with 1 mM SNP. Results indicated that there was a significant reduction in planktonic pneumococcal cells suspended in the supernatant as well as in the biofilm following NO treatment (Fig. 2,
p≤0.05). Since NO treatment of biofilms formed by other bacterial species typically results in increased numbers of bacterial CFUs in the supernatant (24) these data indicated that treatment with higher concentrations of SNP had a direct antibacterial killing effect. Furthermore, both SEM and CLSM imaging of biofilms treated with 1 mM SNP demonstrated no significant change in biofilm ultrastructure following treatment confirming the lack of dispersal (Fig. 3a-b, 3e-f). CLSM imaging did, however, demonstrate a reduction in the number of viable cells remaining within the biofilm following treatment, commensurate with the observed 3-log reduction in CFUs (Fig. 3c-d). To confirm that higher NO concentrations were toxic for pneumococcus, mid-exponential planktonic cultures were treated with the same range of concentrations of SNP. Cultures treated with SNP concentrations between 1 μM to 10 mM for 2 hours showed a significant reduction in growth with 500 μM to 5 mM SNP (p≤0.05), and complete cessation of growth with concentrations greater than 5 mM (Fig. 4).

The response of *S. pneumoniae* to treatment with SNP was mediated by NO. Having determined that both planktonic and biofilm pneumococci responded to SNP treatment, we next wished to confirm that the response was indeed mediated by NO and not due to other NO metabolites, intact SNP or SNP breakdown products other than NO. Treatment with 1 mM KCN, a control for the possible effect of cyanide anion liberation from the SNP molecule, caused no reduction in pneumococcal growth confirming that the reduction in viability compared with untreated bacteria was not due to cyanide toxicity (p=0.528; Fig. 5a). Moreover, treatment with 1 mM DEA/NO, an alternative NO-donor molecule that is chemically and mechanistically distinct from SNP, resulted in a significant decrease in
growth, similar in extent to treatment with 1 mM SNP (p=0.013, Fig. 5a), whilst treatment
with the NO-scavenger cPTIO abrogated the response to SNP (p=0.008, Fig. 5b). These
results indicated that the response to SNP treatment was NO-mediated. In contrast, treatment with 1 mM nitrate (p=0.321) or nitrite (p=0.078) failed to significantly reduce pneumococcal growth, indicating that the oxidative breakdown products of NO were not responsible for the observed reduction in viability (Fig. 5a). Since peroxynitrite (ONOO−) is an extremely toxic molecule that can be produced by reaction of NO with superoxide (O2−) to cause damage to DNA, proteins and lipids (24, 29), we further tested whether ONOO− toxicity might be involved in reducing pneumococcal viability during SNP treatment by using the ONOO− scavenger L-methionine. Indeed, the response to SNP was also reduced by the presence of L-methionine (p=0.005) suggesting that extracellular ONOO− formation secondary to reaction with O2− might be responsible for the bactericidal effects of higher concentrations of NO on pneumococcal cells (Fig. 5c).

**NO adjunctive treatment of *in vitro* pneumococcal biofilms enhanced antibiotic effectiveness.** Others have shown that NO combined with antibiotic treatment resulted in an additional reduction in the viability of biofilms in several types of bacteria (28). We therefore tested whether NO treatment of pneumococcal biofilms could further reduce bacterial viability when used as an adjunctive treatment in conjunction with a conventional antibiotic used to treat otitis media. Serotype 2 strain D39 biofilms and biofilms from 3 different clinical isolates representative of serotypes 14 (ST124), 19F and 23F and based on their high isolation frequency in OM (20) and high rates of antibiotic recalcitrance were...
used to assess NO adjunctive treatment (34, 35). Established biofilms were treated with 1 mM SNP and 300/60 μg/ml AMC for 2 h and the viability of the remaining pneumococcal biofilm bacteria assessed by CFU enumeration. Treatment of ST124, 19F and D39 biofilms with the NO donor alone resulted in a 2-log reduction in viable bacteria, whereas treatment of 23F biofilms resulted in a 3-log reduction (Fig. 6). AMC treatment alone resulted in a 3-log reduction in ST124 and 23F biofilm viability, and a 2-log reduction in 19F and D39 biofilm viability (Fig. 6). Combined NO/AMC treatment, however, resulted in a 3-log reduction in 19F and D39, a 5-log reduction in ST124, and complete killing of 23F when compared with untreated biofilms (Fig. 6), and a significant reduction in viable pneumococci in biofilms compared with antibiotic treatment alone (p < 0.05).

Combined antibiotic and NO treatment enhanced ex vivo killing of *S. pneumoniae* on adenoid tissue. Adenoids have been shown to act as a reservoir for *S. pneumoniae* and biofilm bacteria, and may provide a source for infection in some cases of chronic otitis media (10, 11, 36). Following research ethics committee approval and informed parental consent (NHS REC 09/H0501/74) we subsequently examined adenoid tissue *ex vivo* that was culture positive for *S. pneumoniae* from children <12 years of age undergoing adenoidectomy for the treatment of suspected inflammatory or infective ENT disease to evaluate the effect of NO on *S. pneumoniae* colonized mucosal epithelia (Fig. 7). Adenoid sections (n=11) were treated with 100 μM of SNP alone, 300/60 μg/ml AMC alone, or treated with both NO and AMC to determine if NO adjunctive therapy increased antibiotic efficacy (Fig. 7). Treatment with 100 μM of the NO-donor SNP alone did not significantly reduce colonized pneumococci determined by CFUs (p=0.722) and AMC treatment alone.
resulted in a 2-log reduction compared with the untreated adenoid (p=0.005). However, similar to results with *in vitro* biofilms, combined NO and AMC resulted in a significant reduction of pneumococci CFUs on *ex vivo* adenoid tissue by nearly 3 logs (p=0.005) compared with untreated adenoid tissue and further reduced the number of CFUs compared with AMC alone (p=0.04).

Treatment of *in vitro* *S. pneumoniae* biofilms with NO induced a change in translational and metabolic protein expression. Since low dose NO treatment of 48 h pneumococcal biofilms did not appear to be cytotoxic or induce dispersal we used a high-throughput gel-free proteomic approach to investigate whether NO treatment induced changes in protein expression to shed further light on the potential mechanisms involved. Previous data from our lab demonstrated that a total of 112 proteins were differentially expressed during biofilm development using iTRAQ (inclusion criteria ≥3 peptide matches; >5% sequence coverage and a 50+ protein score; p<0.05) (33). Of these, 13 proteins were differentially expressed in established pneumococcal biofilms treated with NO for 2 h, compared with biofilms treated with HBSS alone (Fig. 8). Eighty five percent (11/13) of these proteins were involved in *S. pneumoniae* translation or metabolism (Figure 9a). Five ribosomal proteins, all of which demonstrated significantly decreased expression in the biofilm phenotype, exhibited increased expression following NO treatment, suggesting a modulation of translational capacity similar to planktonic levels (33). Additionally, 6 metabolism-associated proteins were differentially expressed following NO treatment: 3-ketoacyl-(acyl-carrier-protein) reductase and PTS system fructose-specific II ABC components were upregulated upon NO treatment, whereas arginine deiminase (ArcA), a
PTS system mannose specific IID component, and 2 individual alcohol dehydrogenases exhibited decreased expression following NO treatment. The significant reduction in ArcA expression is of particular interest given its >4-fold increase in expression during biofilm growth (33). Two other proteins, a SPFH domain-containing protein and a hypothetical protein were also identified as having increased expression following NO treatment. These data were further supported by the qualitative identification of 12 proteins with differential expression following NO treatment including 7 associated with pneumococcal metabolism (Figure 9b).

Discussion

Consistent with other studies, S. pneumoniae biofilms were more tolerant to antibiotic treatment than planktonic pneumococci, including an antibiotic commonly used to treat otitis media (13, 20, 37). However, pneumococcal biofilm antibiotic tolerance was significantly diminished (by up to 2 logs) when accompanied by adjunctive treatment with a low concentration of NO. Additionally, we demonstrated biochemically that reduced viability of pneumococci in planktonic and biofilm growth conditions was mediated by NO, and the oxidative breakdown products of NO, nitrite and nitrate, did not mimic this effect. Although NO treatment reduced in vitro pneumococcal biofilm CFUs, our data suggest that the anti-pneumococcal effect was not due to a dispersal of bacteria. Rather, higher concentrations of NO demonstrated a direct antibacterial effect on pneumococcal growth. Treatment with 1 mM SNP resulted in a decrease in biofilm viability by up to 3 logs, and in the number of viable cells in the surrounding supernatant. These data were commensurate with CLSM and SEM imaging which demonstrated no obvious changes in biofilm...
ultrastructure, but did reveal a significant reduction in biofilm viability. A similar response was observed using the structurally distinct NO donor, DEA/NO. The reduced antibacterial response in the presence of the NO scavenger cPTIO, and the lack of response to nitrite and nitrate, indicated that the antimicrobial effects were indeed NO-mediated, and not associated with the formation of NO$_3^-$ and NO$_2^-$ which have also been shown to increase antibiotic efficacy in *P. aeruginosa* biofilms (38). The specificity of the NO-mediated response, along with the reduction in the planktonic growth rate observed with $\geq$500 μM of SNP, suggest a direct effect on growth and/or regulation of metabolism.

We also investigated the hypothesis that low concentrations of NO could enhance antibiotic efficacy in the treatment of pneumococcal biofilms. *In vitro* *S. pneumoniae* biofilms were more tolerant than planktonic pneumococci to AMC, an antibiotic commonly used to treat pneumococcal infections, commensurate with other studies (20, 21). Results indicated that the addition of 1 mM SNP significantly enhanced antibiotic efficacy by 1 to 2 logs in each of four strains tested, three of which represent serotypes (14, 19F and 23F) that are predominantly isolated from paediatric ENT patients and are associated with developing antibacterial recalcitrance (34, 35). Thus, when combined with NO, antibiotic tolerance within the biofilm was significantly diminished.

Pneumococcal interactions with epithelial cells have been shown to be important for colonization and biofilm formation (12, 13), and pneumococci are commonly present on adenoids from children with chronic OM or OSA (10, 11). We therefore used adenoid upper respiratory mucosal epithelial tissue colonized with pneumococci to further determine if a combination of NO and AMC might enhance pneumococcal killing using a lower concentration than for *in vitro* biofilms. Treatment of *ex vivo* adenoid tissue culture
positive for pneumococcus and rinsed to remove unattached bacteria, resulted in a significant reduction in pneumococcal CFUs on adenoid sections treated with antibiotic alone, but not NO-donor alone on treated tissue from the same adenoid sample. When combined however, NO/AMC treatment resulted in a significantly enhanced reduction in CFUs. These results suggest that low-concentrations of NO rendered biofilm pneumococci more susceptible to antibiotic killing. These results are also consistent with other data showing that NO reduced \textit{S. pneumoniae} viability \textit{in vivo} using iNOS knock-out mice (32).

The difference in susceptibility to \textit{S. pneumoniae} bacteremia and lung infection between wild-type and iNOS\textsuperscript{−/−} mice following intravenous infection versus intranasal infection may be due to the differential effects of inducible and constitutive NO production by endothelial and epithelial cells, suggesting that NO concentrations in the host are tissue dependent and regulated locally, and that NO in different mucosal sites is important in anti-pneumococcal host responses.

NO is constitutively synthesised in the respiratory epithelium and upregulated in response to infection or inflammation (39). Since \textit{S. pneumoniae} is highly adapted to the upper airway, a compartment characterised by higher constitutive NO concentrations compared with the lower airways as evidenced by exhaled breath analysis (40), it is likely that this bacterium has the ability to respond to NO. Moreover, since epithelial cells also produce iNOS, we speculate that exogenous NO may combine with endogenous NO levels to achieve the higher concentrations of NO sufficient to produce an enhanced anti-pneumococcal response observed \textit{in vitro}.

NO signaling has been shown to elicit different responses in bacterial biofilms, however its role in mediating dispersal from a biofilm by the reversal of a genetically
determined program inducing biofilm development via cyclic di-GMP (c-di-GMP) has garnered significant interest due to its potential as a treatment strategy for biofilm-associated infections (24). In the model biofilm bacterium *P. aeruginosa* the NO dispersal response results in increased motility and metabolic activity characteristic of the planktonic (colonizing) phenotype allowing propagation to new sites within an environmental niche (41). The single cell phenotype, as well as the increased metabolic and replicative capacity associated with the dispersed planktonic bacteria, are hypothesized to reduce biofilm antibiotic tolerance following NO treatment. Treatment of pneumococcal biofilms with low concentrations of the NO donor, SNP (100 nM to 100 μM), shown to disperse biofilms of other bacterial species resulted in no significant changes in biomass or viability at these concentrations. This is unsurprising since *S. pneumoniae* is a non-motile bacterium, which lacks proteins possessing the common EAL, GGDEF and HD-GYP domains that are involved in the turnover of the secondary messenger c-di-GMP known to mediate dispersal in other bacteria (27, 28).

Rather, proteomic analyses suggested that NO induced a shift to a planktonic-like profile in a subset of proteins, notably those involved in metabolism and translation. Proteomic analyses of *S. pneumoniae* remaining within biofilms following treatment with a low concentration (100 μM) of NO indicated that 13 of 112 quantitatively identified proteins were differentially expressed, indicating that NO was not directly cytotoxic at concentrations of 100 μM. The increased expression of five ribosomal proteins indicated up-regulation of translational capacity, which was previously shown to be substantially down-regulated in established pneumococcal biofilms (33). We previously hypothesized
that the decreased translation exhibited by the biofilm phenotype may contribute to antibiotic tolerance in the biofilm mode of growth in *S. pneumoniae* (33, 41, 42).

Six additional proteins differentially expressed after NO treatment play a role in pneumococcal metabolism. Arginine deiminase (ArcA) and two alcohol dehydrogenases (Adh) were notably decreased. In our previous study, expression of these proteins was markedly *increased* during biofilm formation, and may compensate for the dramatic reduction in glycolytic activity observed in *S. pneumoniae* biofilms by up-regulating arginine and pyruvate metabolism (33). Similar to the differential expression of translational proteins, the increased expression of metabolic proteins following NO treatment suggested that pneumococcus differentially modulates metabolism in planktonic and biofilm modes of growth. Qualitatively, seven other metabolic proteins exhibited differential expression following NO treatment, compared with untreated biofilms. However these proteins were below the threshold of >3 peptides required for inclusion in the quantitative iTRAQ dataset. Nonetheless, taken together these data suggest that while high concentrations of NO elicit a direct antibacterial effect, low dose NO may be involved in regulation of metabolism via a currently unknown signaling pathway.

The decreased expression of arginine deiminase following NO treatment is of particular interest. Regulation of *S. pneumoniae* arginine metabolism appears to be distinct from other bacteria and involves the regulators ArgR1, ArgR2 and AhrC (43, 44). ArgR1 is a transcriptional regulator of the arginine deminase system (ADS) consisting of arginine deiminase (*arcA*), ornithine carbamoyltransferase (*arcB*) and carbamate kinase (*arcC*) that mediate arginine acquisition and virulence in pneumococcus. Abrupt changes in arginine concentrations were recently shown to induce differential transcription of >450 genes in
Streptococcus gordonii, many of which were involved in adhesion and biofilm development (45). Furthermore, the difference in the effect of NO on two of the strains of pneumococcus used in our experiments is consistent with D39 and Serotype 14 having variable disruptions in arginine regulators ArgR1 and ArgR2 (43, 44). We speculate that NO may play a novel role in arginine metabolism and biofilm development in S. pneumoniae, and in regulating growth in pneumococcus. Our results suggest that low concentrations of NO modulate pneumococcal growth, possibly making dormant bacteria within the biofilm metabolically active and more susceptible to antibiotic killing. However, an alternative explanation is that the production of OONO⁻ may contribute to the antibacterial effects of NO on pneumococcus since the antibacterial effect was reduced in the presence of the peroxynitrite (OONO⁻) scavenger L-methionine. Elevated OONO⁻ levels have also been found to be associated with the dispersal response and cell death in P. aeruginosa (27). Peroxynitrite is a potent pro-oxidant and cytotoxic species produced by the interaction of superoxide (O₂⁻) and NO. Compromised pneumococcal superoxide dismutase (SOD) activity may lead to enhanced formation of O₂⁻ and subsequent reaction with host NO leading to the production of OONO⁻, a reaction that normally takes place in human macrophages (46). Peroxynitrite was a putative mediator of NO induced cytotoxic damage in pneumococcal infected microglial cell cultures in vitro and in vivo using pneumococcal mutants for pyruvate oxidase (spxB), and the arginine metabolism mutant carB in mice (22). Intriguingly, these authors made the novel observation that pneumococcus can release NO, suggesting that NO is an endogenous...
pneumococcal metabolite. Our previous proteomic analyses indicated pyruvate oxidase, which produces H2O2, was also markedly upregulated in pneumococcal biofilms (33).

A MerR-like transcriptional factor NmlRsp required for NO defense was identified in S. pneumoniae D39 using the NO donor S-nitrosoglutathione (GSNO) (47). There was no evidence of a MER-like transcriptional protein in our proteomic data, however the role of NmlRsp was subsequently noted to have broader functional roles including a role in H2O2 production and in arginine biosynthesis (48).

The NO donor SNP has been widely utilized for a number of clinical applications, primarily through its use as a vasodilator, however, prolonged treatment and/or high doses have been suggested to pose a risk of cyanide-mediated cytotoxicity (49, 50). The decomposition of SNP to cyanide has also been shown to be slow (<2.5% over 72 h) when protected from direct exposure to high intensity/natural light (51). For the purpose of our initial study SNP was used as a suitable NO-donor to explore the actions of NO on pneumococcal biofilms since relatively low doses were applied for a short period (26, 27).

Furthermore, treatment with equimolar concentrations of KCN had no effect on pneumococcal growth indicating that any observed responses to SNP were not the result of cyanide toxicity (52). However, future studies investigating NO-mediated anti-pneumococcal effects with alternative donors, such as Cephalosporin-3’-diazeniumdiolate NO-Donor Prodrugs, which have been specifically designed to release NO at sites of bacterial infection may offer better choices for clinical use (53).

The results of our study are consistent with other studies showing high concentrations of NO were toxic to bacteria including pneumococcus (32). However, to our knowledge our study is the first to show that: 1) planktonic and biofilm S. pneumoniae
responded differentially to low and high concentrations of NO; 2) the anti-pneumococcal response was not induced by nitrite or nitrate, but was NO specific; 3) unlike other bacteria, low concentrations of NO did not elicit a dispersal response by biofilm *S. pneumoniae*; 4) a low concentration of NO altered the protein expression profile of biofilm pneumococci; and 5) when accompanied by adjunctive treatment with NO, pneumococcal sensitivity to antibiotic treatment was enhanced *in vitro* and *ex vivo*. These results suggest that at lower concentrations, NO perturbs pneumococcal biofilm metabolism, but at higher concentrations NO is toxic to *S. pneumoniae*. Targeted adjunctive NO treatment may be a candidate novel therapy for reducing biofilm tolerance by pneumococcus.

**Funding Information**

Funding for this research was provided by Sparks Children’s Medical Research Charity (Grant 11STH01) and was supported by the NIHR Wellcome Trust Clinical Research Facility (RA, SNF and LH-S) and Southampton NIHR Respiratory Biomedical Research Unit (JSW, SCC, SNF and LH-S). The funders had no role in study design, data collection and interpretation.

**Acknowledgements**

We acknowledge the PRIDE team for the deposition of our mass spectrometry data to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD001182.
References


Chemotherapy 50:382–384.


Figure 1: SNP treatment of *in vitro* pneumococcal biofilms reduced biofilm viability and biomass. 48h *S. pneumoniae* serotype 14 *in vitro* biofilms were treated with SNP for 2 hours, and the biomass assessed by absorbance (OD600) and viability measured by CFU/cm². A significant reduction in total biomass and the number of viable cells remaining within the biofilm was observed following 1 mM SNP treatment. ***p≤0.001.

Figure 2: SNP treatment of *in vitro* *S. pneumoniae* biofilms reduced the viable cell population in the surrounding supernatant. The viability of 48h *S. pneumoniae* serotype 14 *in vitro* biofilm and supernatant populations was measured by CFU enumeration following treatment with 1 mM SNP. SNP treatment significantly reduced both the biofilm and supernatant populations. *p≤0.05.

Figure 3: *In vitro* *S. pneumoniae* biofilms treated with SNP demonstrated reduced viability and no evidence of dispersal. 48h *S. pneumoniae* serotype 14 *in vitro* biofilms were treated with 1 mM SNP for 2 hours then imaged using confocal microscopy and Live/Dead staining. 1 mM SNP-treated biofilms (b) demonstrated no obvious change in biomass when compared with untreated biofilms (a), however, a reduction in the number of Syto9-stained live bacteria in the 1 mM SNP-treated biofilms (d) was reduced in comparison with untreated biofilms (c), commensurate with CFU enumeration data. Scanning electron microscopy with Alcian Blue staining further demonstrated no obvious changes in biofilm ultrastructure between untreated (e) and 1 mM SNP-treated (f) biofilms (4,000x magnification; scale bar: 10 μm).
Figure 4: SNP treatment reduced the in vitro S. pneumoniae planktonic growth rate. S. pneumoniae serotype 14 exponential planktonic cultures were treated with SNP during exponential growth phase, and the growth rate was measured by the change in absorbance (OD595) over 2 hours, and compared with the untreated growth rate. A significant reduction in growth rate was observed using 500 μM SNP, and complete cessation of growth was observed with concentrations greater than 5 mM. *p≤0.05; ****p≤0.0001.

Figure 5: The response of S. pneumoniae to treatment with SNP was NO-mediated. a) S. pneumoniae serotype 14 exponential planktonic cultures were treated with the nitric oxide (NO) donors SNP, DEA/NO, nitrate and nitrite, and the CN− anion control potassium cyanide (KCN) over 2 hours. Significant decreases in the growth rate were observed upon treatment with two independent NO donors, SNP and DEA/NO, indicating that the response was NO-mediated. KCN treatment had no effect on growth rate confirming the response to SNP was not CN− mediated (p=0.528). Sodium nitrate (p=0.321) and sodium nitrite (p=0.078) treatments also had no effect on growth rate suggesting that nitrate and nitrite, respectively, were not utilised as sources of NO. Finally, the addition of b) the NO-scavenger carboxy-PTIO, and c) the peroxynitrite scavenger L-methionine reduced the response to SNP treatment suggesting the response may be mediated by either NO or peroxynitrite. *≤0.05; **≤0.01; ***≤0.001.

Figure 6: Adjunctive treatment of S. pneumoniae in vitro biofilms with SNP enhanced antibiotic efficacy. 48h S. pneumoniae serotype 14 (ST124), 19F, 23F and D39 in vitro biofilms were treated for 2 hours and the remaining viable cells measured by CFU
enumeration. When used separately both SNP and AMC treatment reduced the viable biofilm cell population, however, combined SNP and AMC treatment resulted in a further significant reduction in viability. *p≤0.05.

**Figure 7: Adjunctive treatment of *S. pneumoniae* biofilms on *ex vivo* adenoid tissue**

with SNP enhanced antibiotic efficacy. Adenoid tissue samples (n=11) were dissected into four equal sections (each with a similar proportion of luminal surface) treated for 2 hours, and the viability of *S. pneumoniae* was measured by CFU enumeration. SNP treatment alone had no significant effect on viable pneumococci (p=0.722), whereas AMC treatment alone resulted in a significant reduction (p=0.005). Combined SNP and AMC treatment however, resulted in enhanced antibiotic efficacy (p=0.041). *p≤0.05 (Wilcoxon Signed Ranks test).

**Figure 8: Treatment of *S. pneumoniae* in vitro biofilms with SNP resulted in the differential expression of a small subset of quantitatively identified proteins.** Comparative iTRAQ analyses of SNP treated (100 μM SNP/2 hours) and untreated *S. pneumoniae* serotype 14 7-day old *in vitro* biofilms quantitatively identified 112 proteins of which 13 were differentially expressed following treatment.

**Figure 9: Treatment of *S. pneumoniae* in vitro biofilms with SNP resulted in a change in metabolic and translation protein expression levels.** Comparative iTRAQ analyses of SNP treated (100 μM SNP/2 hours) and untreated *S. pneumoniae* serotype 14 *in vitro* biofilms a) quantitatively identified 13 differentially expressed proteins, and b)
qualitatively identified 12 differentially expressed proteins following treatment.

Quantitative inclusion criteria: ≥3 peptide matches, ≥50 protein score, ≥5% sequence coverage (p<0.05). Qualitative inclusion criteria: 2 peptide matches, ≥50 protein score, ≥5% sequence coverage (p<0.05). Comparative protein data with >1.3 and <0.77 ratios identified as having differential protein expression.
Viable Cells in Adenoid Tissue (CFU g⁻¹)

No Treatment

100 µM SNP

300/60 µg.ml Co-amoxiclav

SNP + Co-amoxiclav

*
Increased expression
Decreased expression
No change
### Table A

<table>
<thead>
<tr>
<th>Function</th>
<th>Gene</th>
<th>Protein</th>
<th>Accession No.</th>
<th>Expression Ratio (NO-treated/Untreated)</th>
<th>Peptide Matches</th>
<th>% Sequence Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Translation</td>
<td>rpsB</td>
<td>30S ribosomal protein S2</td>
<td>YP_001836898</td>
<td>↑ 1.38</td>
<td>4</td>
<td>37.5</td>
</tr>
<tr>
<td></td>
<td>rplQ</td>
<td>50S ribosomal protein L17</td>
<td>YP_001834961</td>
<td>↑ 1.46</td>
<td>5</td>
<td>33.6</td>
</tr>
<tr>
<td></td>
<td>rplM</td>
<td>50S ribosomal protein L13</td>
<td>YP_001835025</td>
<td>↑ 1.36</td>
<td>3</td>
<td>35.8</td>
</tr>
<tr>
<td></td>
<td>rplN</td>
<td>50S ribosomal protein L14</td>
<td>YP_001834945</td>
<td>↑ 1.87</td>
<td>4</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>rpsO</td>
<td>30S ribosomal protein S15</td>
<td>YP_001836318</td>
<td>↑ 1.32</td>
<td>3</td>
<td>29.2</td>
</tr>
<tr>
<td>Metabolism</td>
<td>arcA</td>
<td>arginine deiminase</td>
<td>YP_001836835</td>
<td>0.68</td>
<td>8</td>
<td>37.7</td>
</tr>
<tr>
<td></td>
<td>fabG</td>
<td>3-ketoacyl-(acyl-carrier-protein) reductase</td>
<td>YP_001835136</td>
<td>↑ 1.49</td>
<td>4</td>
<td>28.4</td>
</tr>
<tr>
<td></td>
<td>adhE</td>
<td>alcohol dehydrogenase, iron-containing</td>
<td>YP_001836708</td>
<td>↑ 0.73</td>
<td>6</td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td>adh</td>
<td>alcohol dehydrogenase, zinc-containing</td>
<td>YP_001836739</td>
<td>↑ 0.73</td>
<td>3</td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td>manN</td>
<td>PTS system, mannose-specific IIC component</td>
<td>YP_001835010</td>
<td>↑ 0.76</td>
<td>6</td>
<td>23.1</td>
</tr>
<tr>
<td></td>
<td>fruA</td>
<td>PTS system, fructose specific IIABC component</td>
<td>YP_001835543</td>
<td>↑ 1.32</td>
<td>4</td>
<td>13.8</td>
</tr>
<tr>
<td>Other</td>
<td>SPCG_2124</td>
<td>SPFH domain-containing protein</td>
<td>YP_001836841</td>
<td>↑ 1.80</td>
<td>3</td>
<td>18.1</td>
</tr>
<tr>
<td></td>
<td>SPCG_1532</td>
<td>hypothetical protein SPCG_1532</td>
<td>YP_001836249</td>
<td>↑ 1.31</td>
<td>5</td>
<td>26.8</td>
</tr>
</tbody>
</table>

### Table B

<table>
<thead>
<tr>
<th>Function</th>
<th>Gene</th>
<th>Protein</th>
<th>Accession No.</th>
<th>Expression Ratio (NO-treated/Untreated)</th>
<th>Peptide Matches</th>
<th>% Sequence Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Translation</td>
<td>rpsE</td>
<td>30S ribosomal protein S6</td>
<td>YP_001836244</td>
<td>↑ 1.32</td>
<td>2</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>glmS</td>
<td>D-fructose-6-phosphate amidotransferase</td>
<td>YP_001834993</td>
<td>↑ 0.77</td>
<td>2</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>tktA</td>
<td>transketolase</td>
<td>YP_001836712</td>
<td>↑ 0.75</td>
<td>2</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>acol</td>
<td>dihydroxyacetone dehydratase</td>
<td>YP_001835853</td>
<td>↑ 1.37</td>
<td>2</td>
<td>14.3</td>
</tr>
<tr>
<td>Metabolism</td>
<td>daph</td>
<td>2,3,4,5-tetrahydroxyphenylpyruvate-2-carboxylate N-succinyltransferase</td>
<td>YP_001836779</td>
<td>↑ 1.46</td>
<td>2</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>atpF</td>
<td>ATP synthase subunit B</td>
<td>YP_001836213</td>
<td>↑ 0.75</td>
<td>2</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>accD</td>
<td>acetyl-CoA carboxylase beta subunit</td>
<td>YP_001835141</td>
<td>↑ 0.76</td>
<td>2</td>
<td>14.6</td>
</tr>
<tr>
<td></td>
<td>metG</td>
<td>methionine-tRNA ligase</td>
<td>YP_001835454</td>
<td>↑ 1.64</td>
<td>2</td>
<td>7.1</td>
</tr>
<tr>
<td>Other</td>
<td>SPCG_1897</td>
<td>hypothetical protein SPCG_1897</td>
<td>YP_001836614</td>
<td>↑ 1.34</td>
<td>2</td>
<td>11.8</td>
</tr>
<tr>
<td></td>
<td>amiE</td>
<td>oligopeptide ABC transporter, ATP-binding protein</td>
<td>YP_001836579</td>
<td>↑ 1.64</td>
<td>2</td>
<td>10.1</td>
</tr>
<tr>
<td></td>
<td>gidA</td>
<td>glucose-inhibited division protein A</td>
<td>YP_001834840</td>
<td>↑ 0.66</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>SPCG_1659</td>
<td>Gfo/Idh/MocA family oxidoreductase</td>
<td>YP_001836376</td>
<td>↑ 0.67</td>
<td>2</td>
<td>7.6</td>
</tr>
</tbody>
</table>