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

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

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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.

Antimicrobial Agents and Chemotherapy

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. In the

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

Antimicrobial Agents and Chemotherapy

116 pneumococci in biofilms. We tested this hypothesis using *in vitro* and *ex vivo* pneumococcal
117 biofilms. Biochemical studies and a high-throughput quantitative proteomic approach were
118 utilized to interrogate possible mechanisms of action.
119
120 **Methods**
121 **Bacterial strains and growth conditions.** Clinical isolates of *Streptococcus pneumoniae*
122 serotypes 14 (ST124), 19F and 23F (33), and the laboratory strain D39 (serotype 2) were
123 selected to evaluate the *in vitro* antibacterial efficacy of NO and antibiotic adjunctive
124 treatment. Strains were subcultured from frozen stocks onto Columbia blood agar (CBA)
125 plates (Oxoid, U.K.) as described (33). Briefly, cultures were incubated at 37°C/5% CO_2 and
126 colonies re-suspended in fresh Brain Heart Infusion (BHI) broth (Oxoid, U.K.) for
127 experiments.
128
129 **Planktonic experiments.** Flat-bottomed 96-well culture plates (Fisher Scientific, U.K.)
130 were inoculated with ~1.0 x 10^7 bacteria per well (mid-exponential planktonic cultures)
131 grown in BHI. All treatments were prepared in BHI. Sodium nitroprusside dihydrate (SNP)
132 was added to wells for final concentrations ranging from 1 μM to 10 mM; diethylamine
133 NONOate (DEA/NO; sodium salt), sodium nitrate ($NaNO_3$), sodium nitrite ($NaNO_2$) and
134 potassium cyanide (KCN; all Sigma-Aldrich, U.K.) were added at a final concentration of 1
135 mM, and carboxy-PTIO potassium salt and L-methionine (both Sigma-Aldrich, U.K.) were
136 added at final concentrations of 50 μM and 1 mM respectively. BHI was added in place of
137 treatments for all untreated controls. BHI alone was used to measure background changes
138 in absorbance. Cultures were incubated at 37°C/5% CO_2 and absorbance (OD_{595})

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 10^8 cells per well) (Corning Incorporated, Costar, U.S.A.), and supplemented with fresh BHI diluted 1:5 in distilled H_2O. Cultures were incubated at 37°C/5% CO_2 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% CO_2 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% CO_2. To assess total biofilm biomass 100 µl of the resuspended biofilms were diluted 10-fold in 1:5 BHI and turbidity measured by absorbance (OD_{595}) 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% CO_2 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% CO_2 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% CO_2 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% CO_2 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% CO_2. 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 ($p\leq0.001$). 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

Antimicrobial Agents and Chemotherapy

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 (O_2^-) 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 O_2^- 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

Antimicrobial Agents and Chemotherapy

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 ≥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

366 positive for pneumococcus and rinsed to remove unattached bacteria, resulted in a
367 significant reduction in pneumococcal CFUs on adenoid sections treated with antibiotic
368 alone, but not NO-donor alone on treated tissue from the same adenoid sample. When
369 combined however, NO/AMC treatment resulted in a significantly enhanced reduction in
370 CFUs. These results suggest that low-concentrations of NO rendered biofilm pneumococci
371 more susceptible to antibiotic killing. These results are also consistent with other data
372 showing that NO reduced *S. pneumoniae* viability *in vivo* using iNOS knock-out mice (32).
373 The difference in susceptibility to *S. pneumoniae* bacteremia and lung infection between
374 wild-type and $iNOS^{-/-}$ mice following intravenous infection versus intranasal infection may
375 be due to the differential effects of inducible and constitutive NO production by endothelial
376 and epithelial cells, suggesting that NO concentrations in the host are tissue dependent and
377 regulated locally, and that NO in different mucosal sites is important in anti-pneumococcal
378 host responses.

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

387 NO signaling has been shown to elicit different responses in bacterial biofilms,
388 however its role in mediating dispersal from a biofilm by the reversal of a genetically

Antimicrobial Agents and Chemotherapy

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

403 Rather, proteomic analyses suggested that NO induced a shift to a planktonic-like
404 profile in a subset of proteins, notably those involved in metabolism and translation.
405 Proteomic analyses of *S. pneumoniae* remaining within biofilms following treatment with a
406 low concentration (100 μM) of NO indicated that 13 of 112 quantitatively identified
407 proteins were differentially expressed, indicating that NO was not directly cytotoxic at
408 concentrations of 100 μM. The increased expression of five ribosomal proteins indicated
409 up-regulation of translational capacity, which was previously shown to be substantially
410 down-regulated in established pneumococcal biofilms (33). We previously hypothesized

411 that the decreased translation exhibited by the biofilm phenotype may contribute to
412 antibiotic tolerance in the biofilm mode of growth in *S. pneumoniae* (33, 41, 42).
413 Six additional proteins differentially expressed after NO treatment play a role in
414 pneumococcal metabolism. Arginine deiminase (ArcA) and two alcohol dehydrogenases
415 (Adh) were notably decreased. In our previous study, expression of these proteins was
416 markedly *increased* during biofilm formation, and may compensate for the dramatic
417 reduction in glycolytic activity observed in *S. pneumoniae* biofilms by up-regulating
418 arginine and pyruvate metabolism (33). Similar to the differential expression of
419 translational proteins, the increased expression of metabolic proteins following NO
420 treatment suggested that pneumococcus differentially modulates metabolism in planktonic
421 and biofilm modes of growth. Qualitatively, seven other metabolic proteins exhibited
422 differential expression following NO treatment, compared with untreated biofilms.
423 However these proteins were below the threshold of >3 peptides required for inclusion in
424 the quantitative iTRAQ dataset. Nonetheless, taken together these data suggest that while
425 high concentrations of NO elicit a direct antibacterial effect, low dose NO may be involved
426 in regulation of metabolism via a currently unknown signaling pathway.
427 The decreased expression of arginine deiminase following NO treatment is of
428 particular interest. Regulation of *S. pneumoniae* arginine metabolism appears to be distinct
429 from other bacteria and involves the regulators ArgR1, ArgR2 and AhrC (43, 44). ArgR1 is a
430 transcriptional regulator of the arginine deminase system (ADS) consisting of arginine
431 deiminase (*arcA*), ornithine carbamoyltransferase (*arcB*) and carbamate kinase (*arcC*) that
432 mediate arginine acquisition and virulence in pneumococcus. Abrupt changes in arginine
433 concentrations were recently shown to induce differential transcription of >450 genes in

434 *Streptococcus gordonii*, many of which were involved in adhesion and biofilm development
435 (45). Furthermore, the difference in the effect of NO on two of the strains of pneumococcus
436 used in our experiments is consistent with D39 and Serotype 14 having variable
437 disruptions in arginine regulators ArgR1 and ArgR2 (43, 44).

438 We speculate that NO may play a novel role in arginine metabolism and biofilm
439 development in *S. pneumoniae,* and in regulating growth in pneumococcus. Our results
440 suggest that low concentrations of NO modulate pneumococcal growth, possibly making
441 dormant bacteria within the biofilm metabolically active and more susceptible to antibiotic
442 killing. However, an alternative explanation is that the production of $OONO^-$ may contribute
443 to the antibacterial effects of NO on pneumococcus since the antibacterial effect was
444 reduced in the presence of the peroxynitrite ($OONO^-$) scavenger L-methionine. Elevated
445 $OONO^-$ levels have also been found to be associated with the dispersal response and cell
446 death in *P. aeruginosa* (27). Peroxynitrite is a potent pro-oxidant and cytotoxic species
447 produced by the interaction of superoxide (O_2^-) and NO. Compromised pneumococcal
448 superoxide dismutase (SOD) activity may lead to enhanced formation of O_2^- and
449 subsequent reaction with host NO leading to the production of $OONO^-$, a reaction that
450 normally takes place in human macrophages (46). Peroxynitrite was a putative mediator of
451 NO induced cytotoxic damage in pneumococcal infected microglial cell cultures *in vitro* and
452 *in vivo* using pneumococcal mutants for pyruvate oxidase (*spxB*), and the arginine
453 metabolism mutant *carB* in mice (22). Intriguingly, these authors made the novel
454 observation that pneumococcus can release NO, suggesting that NO is an endogenous

pneumococcal metabolite. Our previous proteomic analyses indicated pyruvate oxidase, which produces H_2O_2, was also markedly upregulated in pneumococcal biofilms (33).

A MerR-like transcriptional factor *NmlR*$_{sp}$ required for NO defense was identified in *S. pnuemoniae* 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 $NmlR_{sp}$ was subsequently noted to have broader functional roles including a role in H_2O_2 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*

Antimicrobial Agents and Chemotherapy

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.

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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 (OD_{600}) and viability measured by CFU/ cm^2. A significant reduction in total biomass and the number of viable cells remaining within the biofilm was observed following 1 mM SNP treatment. ***$p \leq 0.001$.

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. *$p \leq 0.05$.

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).

685 **Figure 4: SNP treatment reduced the *in vitro S. pneumoniae* planktonic growth rate.** *S.*
686 *pneumoniae* serotype 14 *in vitro* planktonic cultures were treated with SNP during
687 exponential growth phase, and the growth rate was measured by the change in absorbance
688 (OD_{595}) over 2 hours, and compared with the untreated growth rate. A significant
689 reduction in growth rate was observed using 500 μM SNP, and complete cessation of
690 growth was observed with concentrations greater than 5 mM. *p≤0.05; ****p≤0.0001.
691
692 **Figure 5: The response of *S. pneumoniae* to treatment with SNP was NO-mediated. a)**
693 *S. pneumoniae* serotype 14 exponential planktonic cultures were treated with the nitric
694 oxide (NO) donors SNP, DEA/NO, nitrate and nitrite, and the CN^- anion control potassium
695 cyanide (KCN) over 2 hours. Significant decreases in the growth rate were observed upon
696 treatment with two independent NO donors, SNP and DEA/NO, indicating that the response
697 was NO-mediated. KCN treatment had no effect on growth rate confirming the response to
698 SNP was not CN^- mediated (p=0.528). Sodium nitrate (p=0.321) and sodium nitrite
699 (p=0.078) treatments also had no effect on growth rate suggesting that nitrate and nitrite,
700 respectively, were not utilised as sources of NO. Finally, the addition of **b)** the NO-
701 scavenger carboxy-PTIO, and **c)** the peroxynitrite scavenger L-methionine reduced the
702 response to SNP treatment suggesting the response may be mediated by either NO or
703 peroxynitrite. *≤0.05; **≤0.01; ***≤0.001.
704
705 **Figure 6: Adjunctive treatment of *S. pneumoniae in vitro* biofilms with SNP enhanced**
706 **antibiotic efficacy.** 48h *S. pneumoniae* serotype 14 (ST124), 19F, 23F and D39 *in vitro*
707 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.

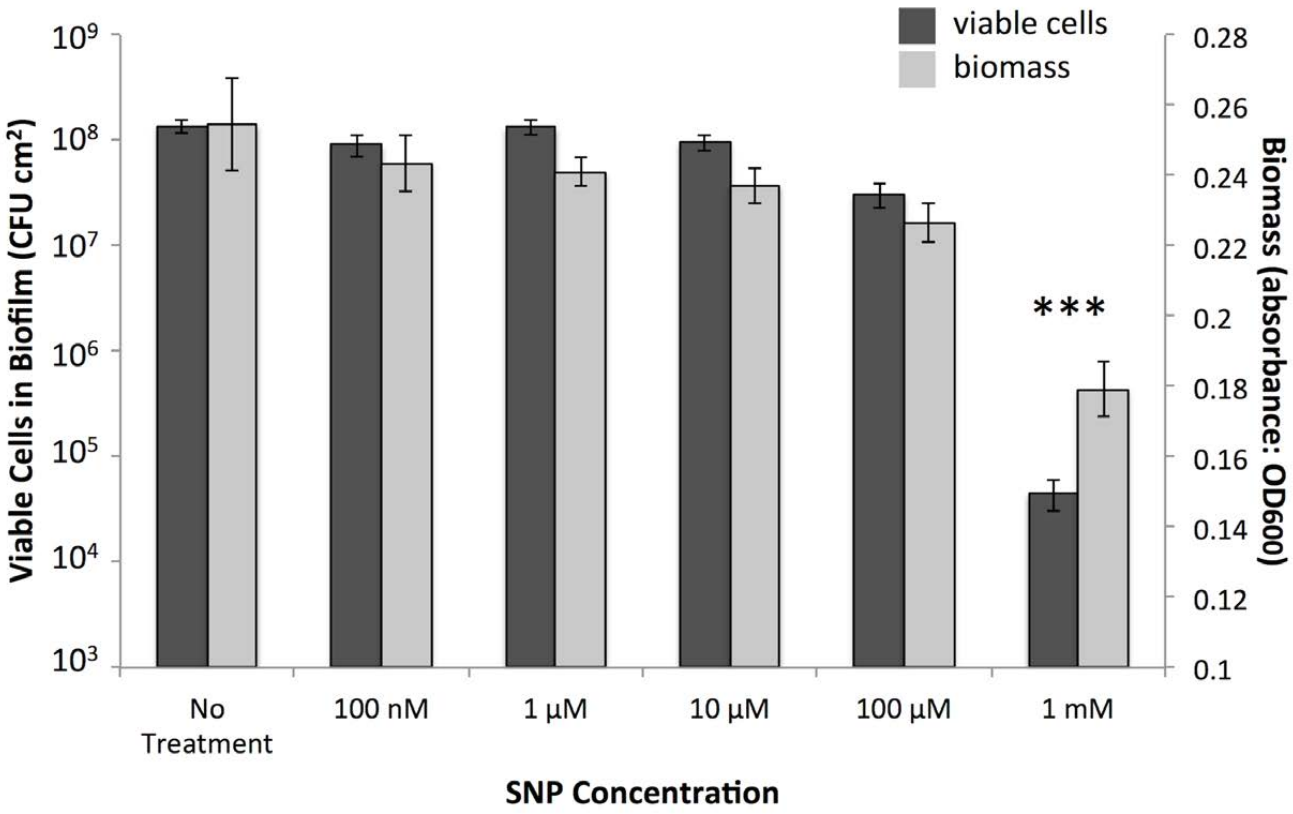
Figure 7: Adjunctive treatment of *S. pneumoniae* biofilms on *ex vivo* adenoid tissue with SNP enhanced antibiotic efficacy. Adenoid tissue samples (n=11) were dissected into four equal sections (each with a similar proportion of luminal surface) treated for 2 hours, and the viability of *S. pneumoniae* 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).

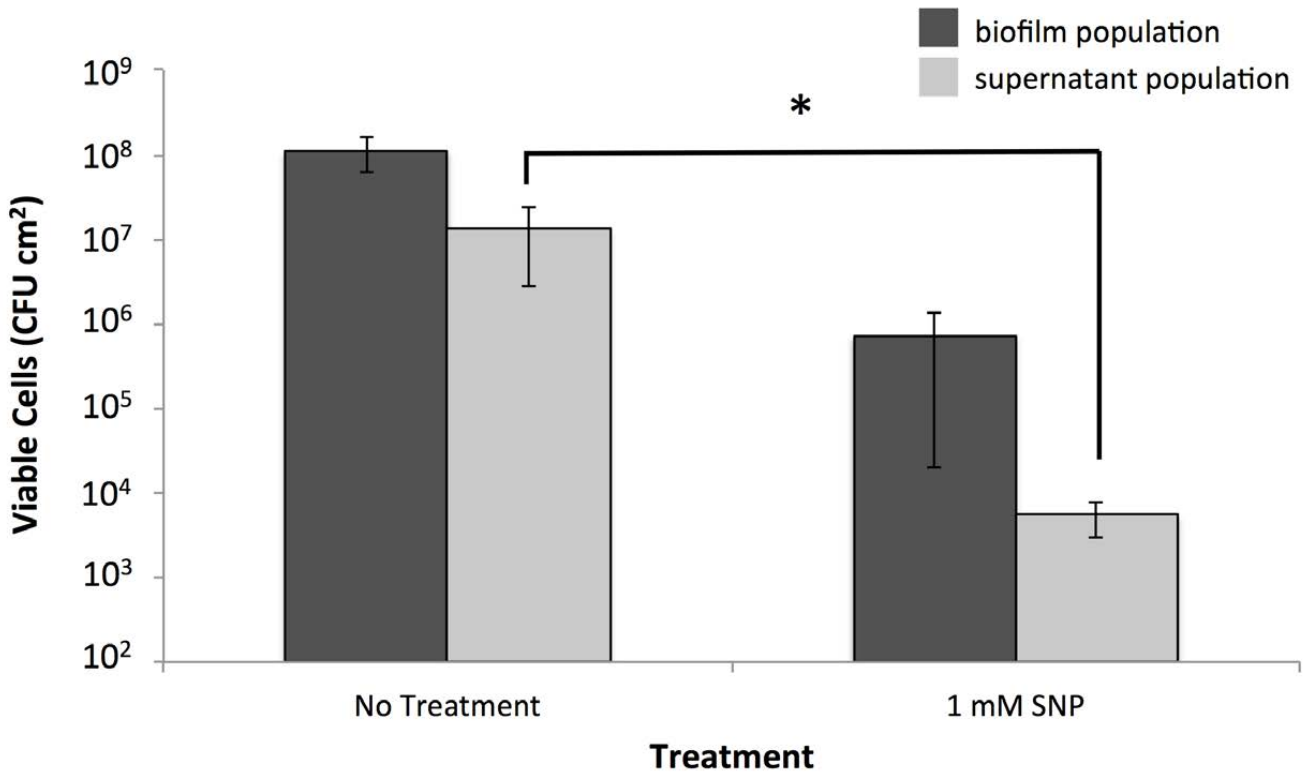
Figure 8: Treatment of S*. pneumoniae in vitro* biofilms with SNP resulted in the differential expression of a small subset of quantitatively identified proteins. Comparative iTRAQ analyses of SNP treated (100 ⍰M SNP/2 hours) and untreated *S. pneumoniae* serotype 14 7-day old *in vitro* biofilms quantitatively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

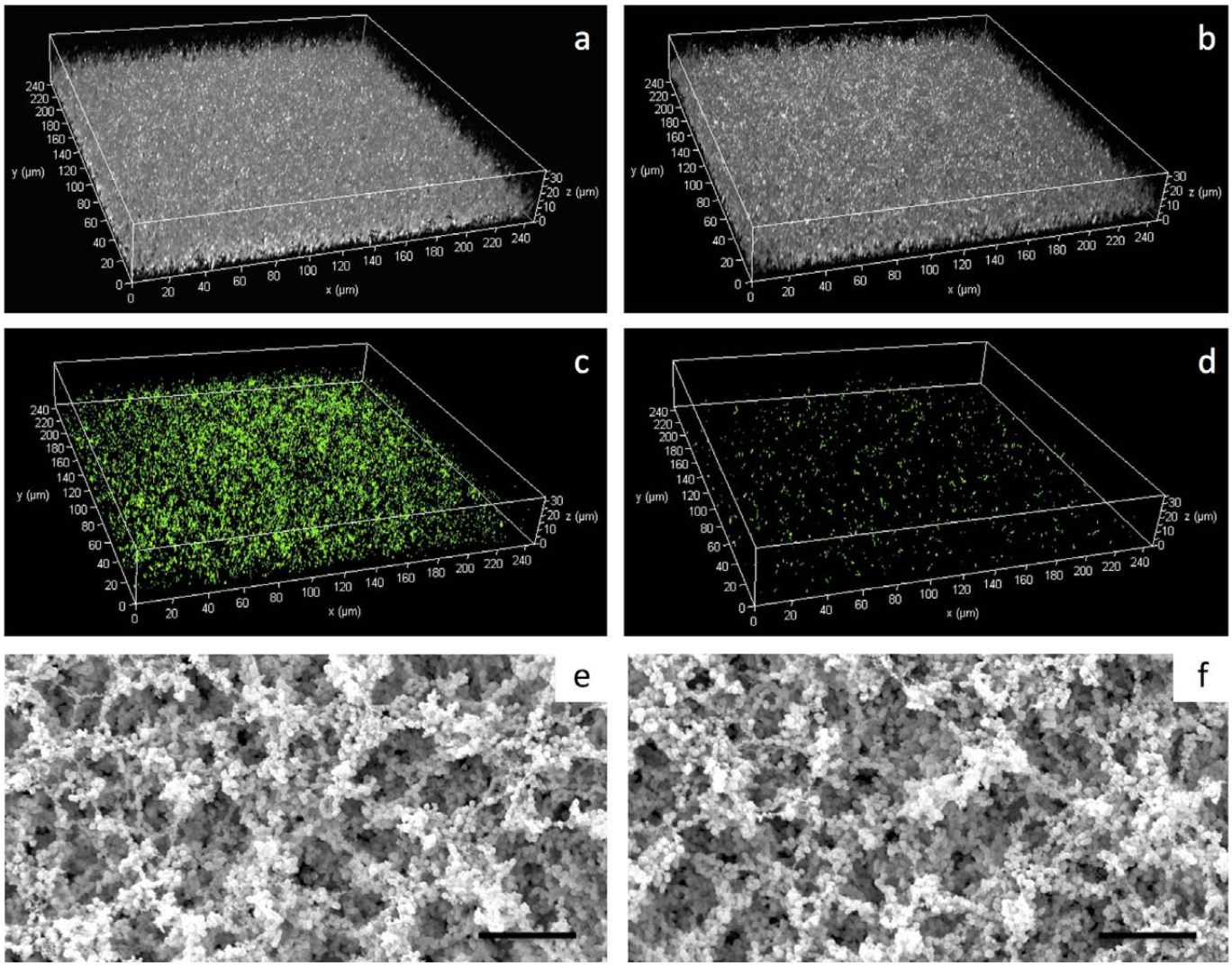
Figure 9: Treatment of *S. pneumoniae in vitro* biofilms with SNP resulted in a change in metabolic and translation protein expression levels. Comparative iTRAQ analyses of SNP treated (100 μM SNP/2 hours) and untreated *S. pneumoniae* serotype 14 *in vitro* biofilms **a)** quantitatively identified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

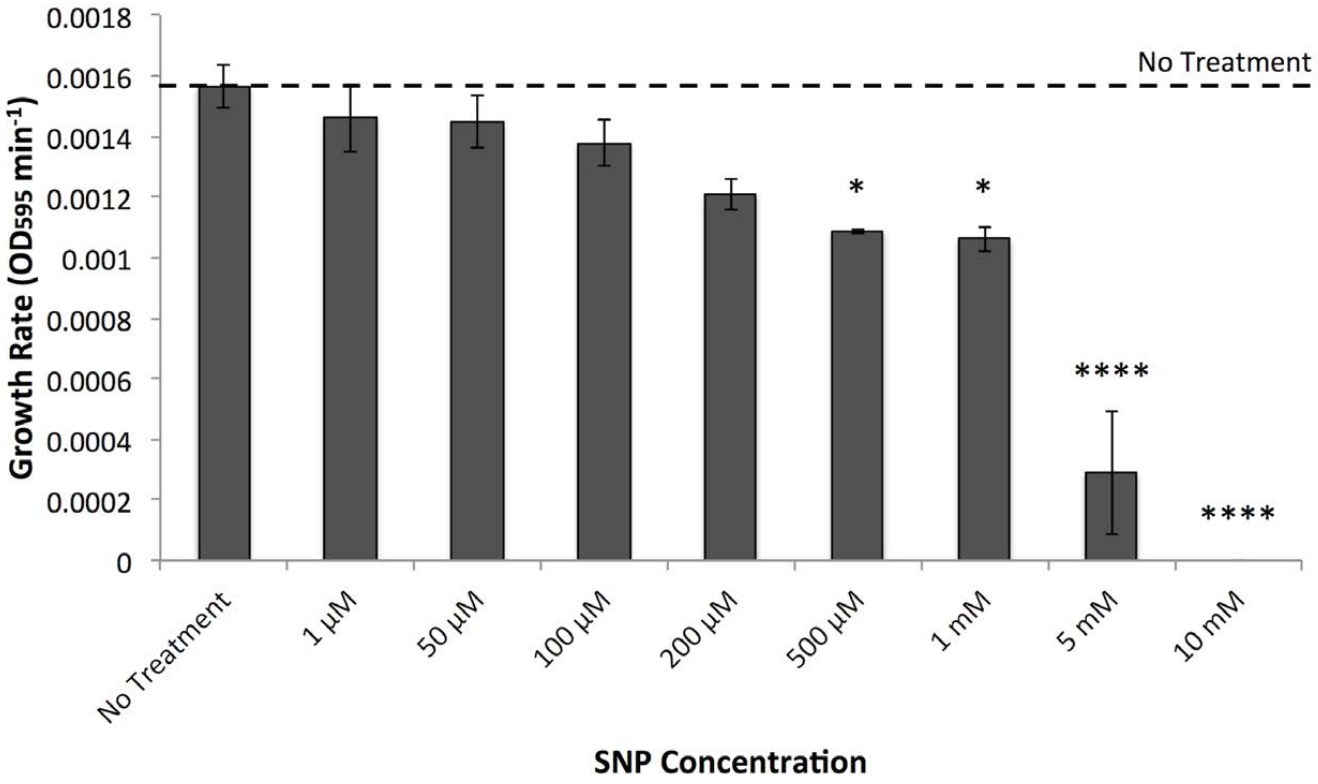
Antimicrobial Agents and Chemotherapy

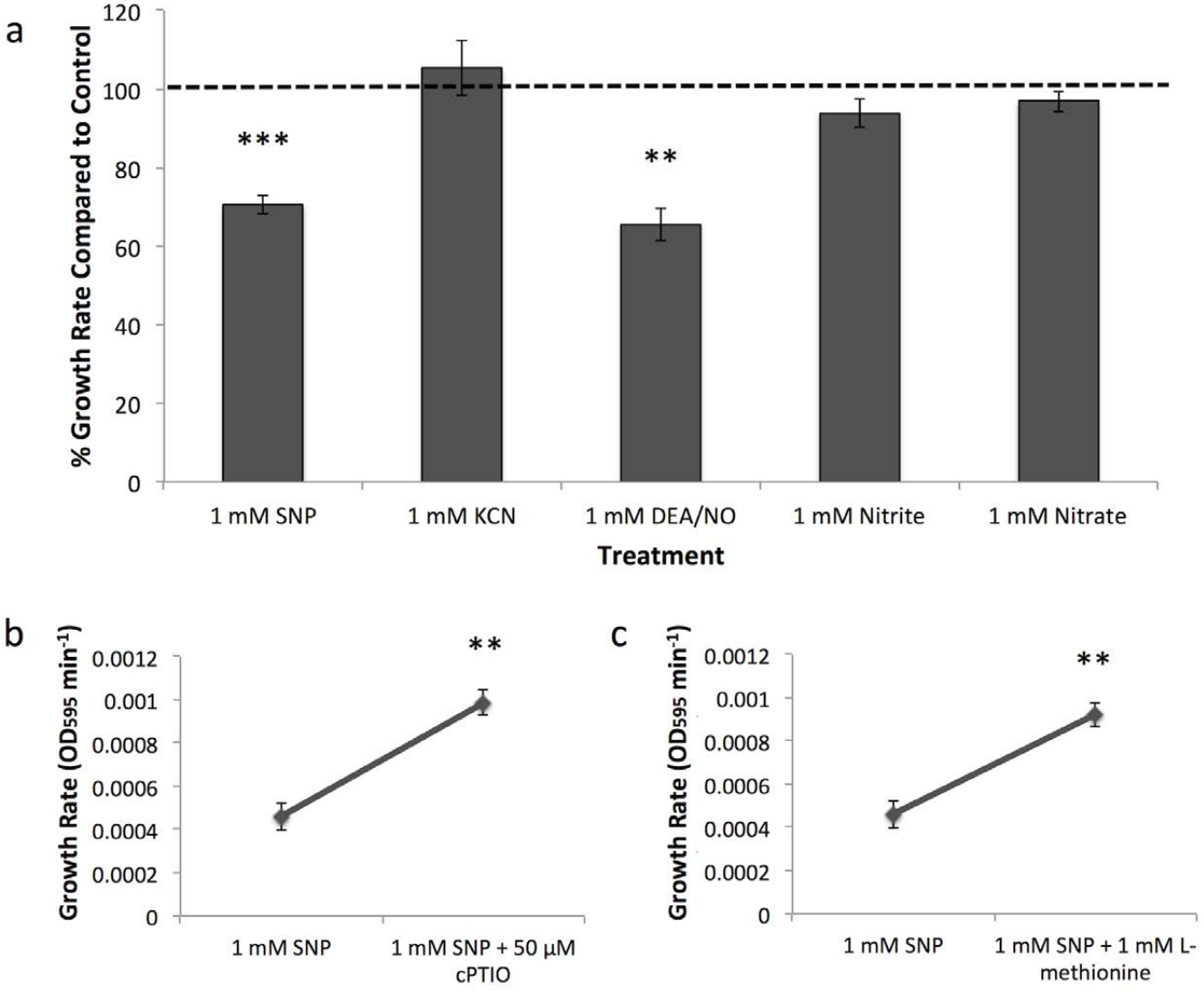
qualitatively identified 12 differentially expressed proteins following treatment. Quantitative inclusion criteria: ≥3 peptide matches, ≥50 protein score, ≥5% sequence coverage ($p<0.05$). Qualitative inclusion criteria: 2 peptide matches, ≥50 protein score, ≥5% sequence coverage ($p<0.05$). Comparative protein data with >1.3 and <0.77 ratios identified as having differential protein expression.

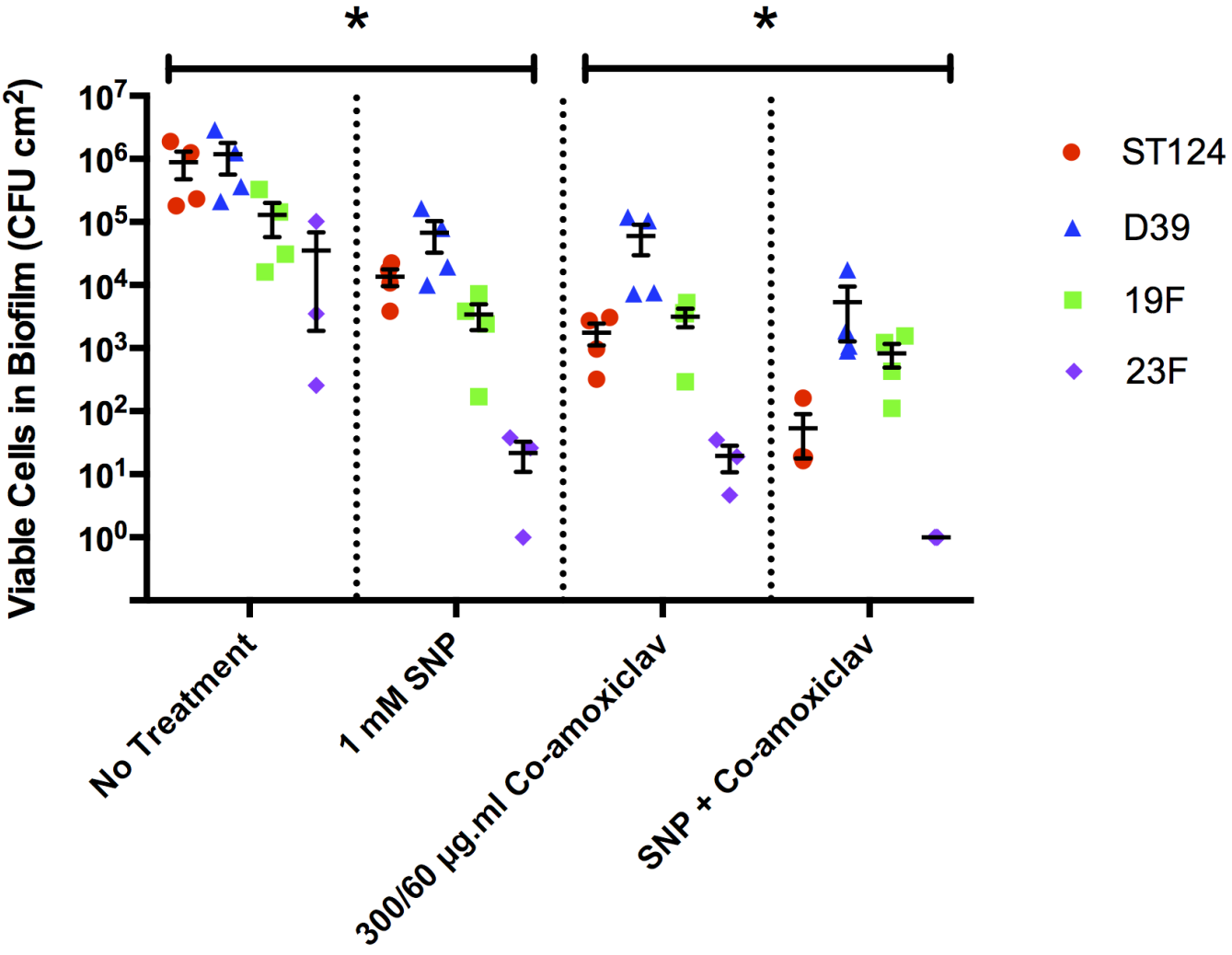


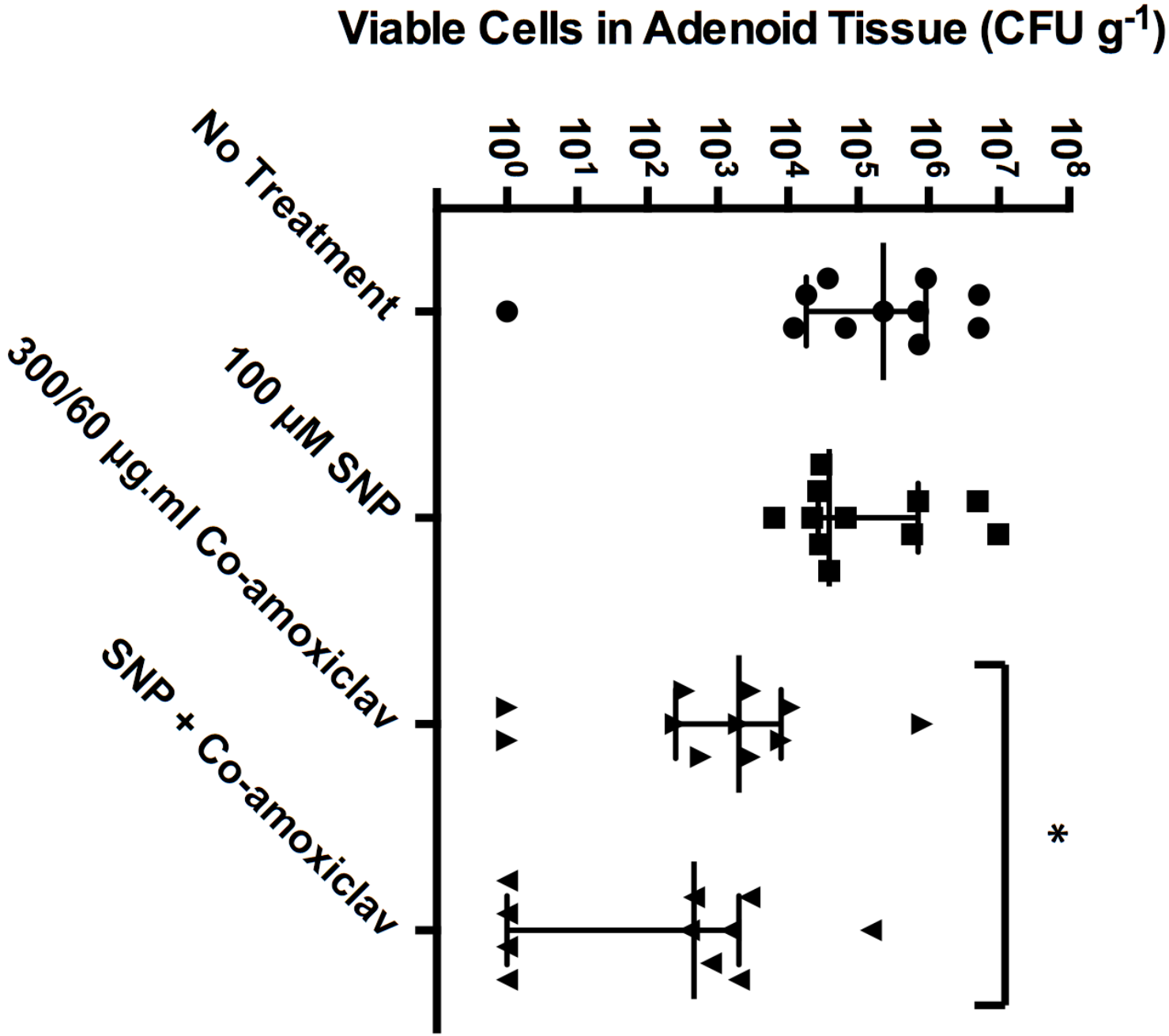


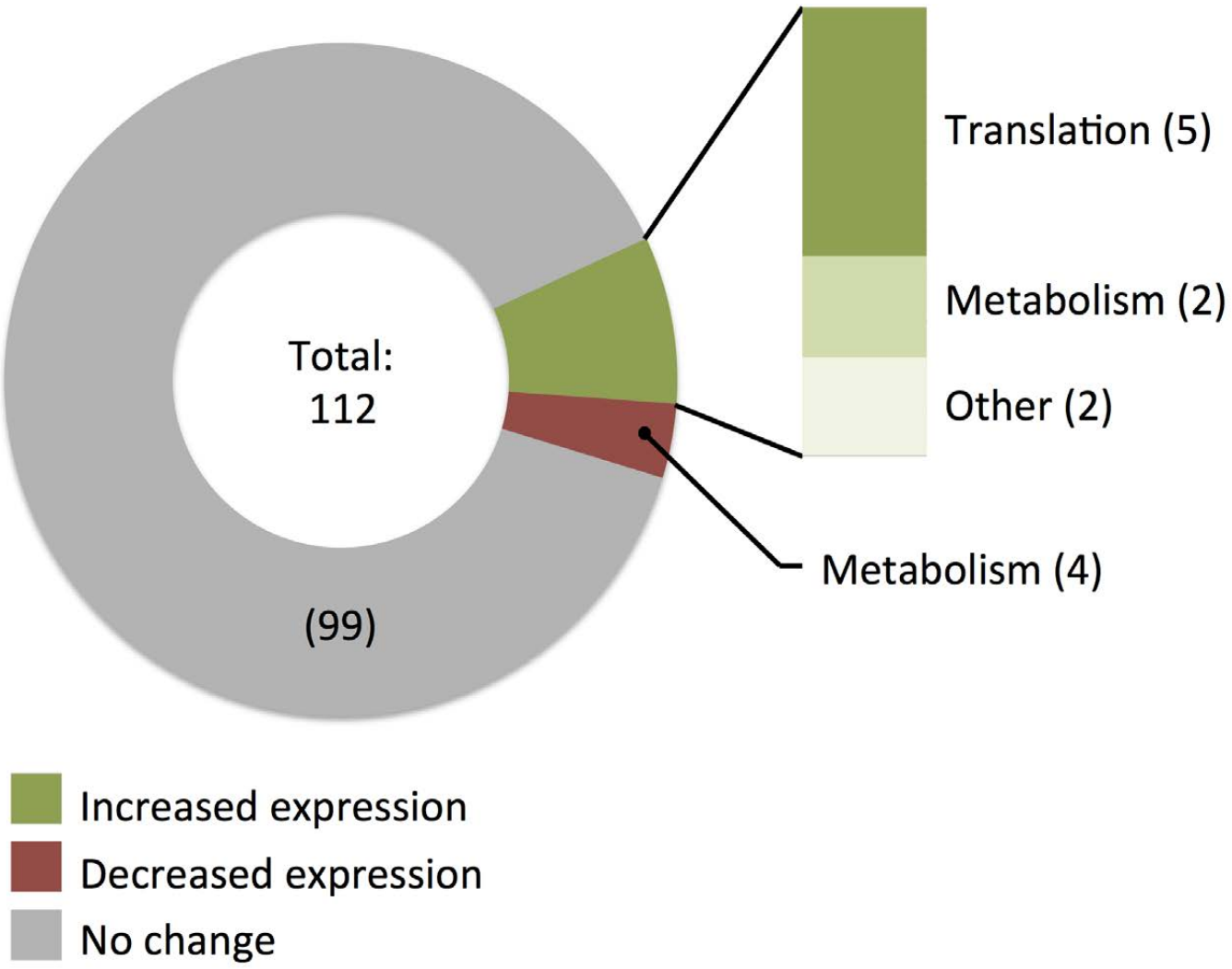









Viable Cells in Adenoid Tissue (CFU g-1)
10^0
10^1
10^2
10^3
10^4
10^5
10^6
10^7
10^8
No Treatment
100 µM SNP
300/60 µg.ml Co-amoxiclav
SNP + Co-amoxiclav
*



a

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

b

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