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1 **Low concentrations of nitric oxide modulate *Streptococcus pneumoniae* biofilm**

2 **metabolism and antibiotic tolerance**

3

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

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23

24 **Abstract**

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

47 **Introduction**

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

57 Pneumococcus is a leading pathogen in otitis media (OM), the most common
58 infection in young children and a principal reason for repeated physician visits. Upon
59 colonization with *S. pneumoniae* and the establishment of carriage in children, bacteria may
60 access the middle ear space by retrograde ascent from the nasopharynx due to the
61 presence of fluid and/or disruption of mucociliary clearance. Recurrent or chronic otitis
62 media causes much pain and morbidity at high economic cost to society (2). In spite of
63 concerns about the selection of antibiotic resistant bacteria, OM continues to be the
64 primary reason for antibiotic prescription in children (3–5). In addition, although
65 pneumococcal conjugate vaccines (PCV) have reduced vaccine type invasive pneumococcal
66 disease, PCVs have not led to a decrease in the incidence of otitis media due to
67 pneumococci, most likely due to non-vaccine type replacement (1, 6, 7). Novel treatments
68 for pneumococcal infection are therefore needed to address the problem of recurrent
69 and/or chronic infections in children.

70 Infections occur following a breach of the mucosal epithelia subsequent to
71 colonization and despite being a prerequisite for infection, little is known about how
72 pneumococci colonize and persist in the nasopharynx. However, a growing body of
73 literature suggests that bacterial biofilm development plays a prominent role in
74 colonization and disease. *In situ* investigation of paediatric middle ear biopsies indicated
75 that pneumococcal biofilms were present on the middle ear mucosal epithelium in children
76 with chronic OM but not in children without chronic OM (8, 9). *S. pneumoniae* biofilms have
77 also been identified *in situ* on adenoid mucosal epithelia from children undergoing
78 adenoidectomy for the treatment of infective (chronic OM) or inflammatory (obstructive
79 sleep apnoea - OSA) otolaryngological disease consistent with the adenoid serving as a
80 reservoir of pathogens that may contribute to infection under circumstances that favor
81 middle ear infection (10, 11). More recently, pneumococcal biofilms have been investigated
82 with animal models and epithelial cell models (12–15).

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

91 Nitric oxide (NO) is an important signaling molecule that is ubiquitous in both
92 eukaryotes and prokaryotes, bridging the boundaries between host and pathogen. In the

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

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

113 Because several studies using biofilm models have shown that low concentrations of
114 NO trigger release of bacteria from the biofilm in various bacterial species we hypothesized
115 that adjunctive NO treatment would also improve the efficacy of antibiotic killing of

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% CO₂ 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 $\sim 1.0 \times 10^7$ 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 μ M to 10 mM; diethylamine
133 NONOate (DEA/NO; sodium salt), sodium nitrate (NaNO₃), sodium nitrite (NaNO₂) 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 μ M 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% CO₂ and absorbance (OD595)

139 measured every 30 min over 2 hours using an EZ Read 400 spectrophotometer (Biochrom)
140 (n=3).

141

142 ***In vitro* biofilm experiments.** For biofilm formation mid-exponential planktonic cultures
143 grown in BHI were used to inoculate individual wells of untreated polystyrene 6-well
144 plates (1×10^8 cells per well) (Corning Incorporated, Costar, U.S.A.), and supplemented
145 with fresh BHI diluted 1:5 in distilled H₂O. Cultures were incubated at 37°C/5% CO₂ with
146 replacement of warm, fresh diluted 1:5 BHI daily for 2 to 7 days.

147 Prior to treatment medium was removed and biofilms washed twice using diluted
148 1:5 BHI. NO donor (SNP and DEA/NO) treatments were prepared fresh in diluted 1:5 BHI
149 and added to wells at final concentrations ranging from 100 nM to 1 mM. For adjuvant
150 experiments amoxicillin + clavulanic acid (AMC) was added at a final concentration of
151 300/60 µg/ml. Biofilms were incubated at 37°C/5% CO₂ for 2 hours after which the NO
152 donors or inhibitors were removed and the remaining biofilm rinsed twice with diluted 1:5
153 BHI. Biofilms were resuspended in Hank's balanced salt solution (HBSS) as described (20).
154 Briefly, biofilms were scraped and vortexed, both resuspended biofilms and removed
155 supernatants were diluted in HBSS, spot plated onto CBA plates and incubated at 37°C/5%
156 CO₂. To assess total biofilm biomass 100 µl of the resuspended biofilms were diluted 10-
157 fold in 1:5 BHI and turbidity measured by absorbance (OD₅₉₅) using a Jenway 6300
158 spectrophotometer. All assays were performed on 48 h biofilms using 2 technical replicates
159 of 2 biological replicates (n=4).

160

161 **Confocal Laser Scanning Microscopy (CLSM).** Mid-exponential planktonic cultures of
162 strain ST124 (n=3) were grown in BHI and used to inoculate 35 mm untreated glass
163 bottom CELLview cell culture dishes (Greiner Bio One, U.K.) and supplemented with fresh
164 1:5 BHI. Biofilms were grown under static conditions at 37°C/5% CO₂ for 48 h replacing
165 medium daily with fresh 1:5 BHI. Biofilms were treated with 1 mM SNP in 1:5 BHI or 1:5
166 BHI (untreated control) at 37°C/5% CO₂ for 2 hours. Treatments were removed and the
167 remaining biofilm rinsed twice with HBSS. Biofilms were stained with Live/Dead BacLight
168 Bacterial Viability Kit (Life Technologies, U.S.A.) according to manufacturer instructions.
169 Biofilms were examined immediately with an inverted Leica SP8 LSCM system using a 63x
170 oil immersion lens and sequential scanning of 1 µm sections (Leica Microsystems, Milton
171 Keynes, U.K.). To remove background eDNA staining, the Syto9 fluorescence intensity
172 threshold was set to that of planktonic pneumococci. Images were analysed using Leica LCS
173 Software.

174

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

180

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

184 replicates of 3 biological replicates. Protein extraction and iTRAQ labelling were performed
185 as described (33).

186

187 **Mass spectrometry, peak list generation, and database searching.** Mass spectrometry,
188 peak list generation and database searching were performed as previously described (33).
189 Inclusion criteria for quantitative analysis were set at ≥ 3 peptide matches, ≥ 50 protein
190 score, $\geq 5\%$ sequence coverage ($p < 0.05$). Comparative protein data with >1.3 and <0.77
191 ratios were identified as having differential expression. For qualitative identification the
192 inclusion criteria were 2 peptide matches, ≥ 50 protein score, and $\geq 5\%$ sequence coverage.

193

194 **Ex vivo adenoid experiments.** Adenoids were obtained from paediatric patients <12 years
195 of age undergoing adenoidectomy for the treatment of suspected inflammatory or infective
196 Ear, Nose and Throat (ENT) disease ($n=11$). Adenoids were collected on ice in sterile HBSS
197 + 5% fetal bovine serum (FBS; Sigma-Aldrich, U.K.), washed twice with HBSS to remove any
198 unattached bacteria, then dissected into four equal-sized sections with similar luminal
199 surface coverage. Tissue sections were normalized for weight and treated with $100 \mu\text{M}$
200 SNP alone, $300/60 \mu\text{g/ml}$ AMC alone or $100 \mu\text{M}$ SNP + $300/60 \mu\text{g/ml}$ AMC in HBSS/10%
201 FBS for 2 h at $37^\circ\text{C}/5\% \text{CO}_2$. Untreated control tissue was treated with HBSS/10% FBS
202 alone. Tissue sections were washed twice with 10 ml HBSS, macerated in 1 ml HBSS
203 through a $100 \mu\text{m}$ nylon cell strainer (Fisher Scientific, U.K.) and bacterial suspensions
204 serially diluted and spot plated onto CBA plates.

205

206 **Statistical analyses.** Statistical analysis of *in vitro* planktonic and biofilm data was
207 performed using one-way ANOVA and Tukey's multiple comparisons tests. Analysis of *ex*
208 *vivo* adenoid data was performed using a Wilcoxon Signed Ranks test. Comparative data
209 reported as $p < 0.05$ were considered statistically different.

210

211 **Results**

212 **Treatment with the NO donor SNP decreased viability of planktonic cells and the cell**

213 **population remaining within *in vitro* biofilms.** Since low-concentrations of NO have

214 been shown to result in the release or dispersal of other bacterial species from biofilms, we

215 first tested the hypothesis that low-concentrations of NO would have a similar effect on

216 pneumococcal biofilms. Established 48 hour biofilms were treated for 2 hours with a range

217 of NO concentrations generated from different concentrations of the NO-donor SNP (100

218 nM - 1 mM; Fig. 1). Measurement of biofilm biomass using turbidity, and viability using

219 colony forming unit (CFU) enumeration, respectively, indicated that treatment with low

220 concentrations of NO (100 nM to 100 μ M SNP) did not have a similar effect on 48h

221 pneumococcal biofilms. Treatment with 1 mM SNP however, resulted in a significant

222 reduction in the biomass and a 3-log reduction in the number of viable cells remaining

223 within the biofilm ($p \leq 0.001$). These results suggested that at 1mM SNP treatment may

224 either be triggering the release of *S. pneumoniae* from the biofilm or had a direct

225 bactericidal effect. To distinguish between these possibilities the number of viable cells

226 present in the biofilm supernatant was measured following treatment with 1 mM SNP.

227 Results indicated that there was a significant reduction in planktonic pneumococcal cells

228 suspended in the supernatant as well as in the biofilm following NO treatment (Fig. 2,

229 $p \leq 0.05$). Since NO treatment of biofilms formed by other bacterial species typically results
230 in increased numbers of bacterial CFUs in the supernatant (24) these data indicated that
231 treatment with higher concentrations of SNP had a direct antibacterial killing effect.
232 Furthermore, both SEM and CLSM imaging of biofilms treated with 1 mM SNP
233 demonstrated no significant change in biofilm ultrastructure following treatment
234 confirming the lack of dispersal (Fig. 3a-b, 3e-f). CLSM imaging did, however, demonstrate
235 a reduction in the number of viable cells remaining within the biofilm following treatment,
236 commensurate with the observed 3-log reduction in CFUs (Fig. 3c-d). To confirm that
237 higher NO concentrations were toxic for pneumococcus, mid-exponential planktonic
238 cultures were treated with the same range of concentrations of SNP. Cultures treated with
239 SNP concentrations between 1 μM to 10 mM for 2 hours showed a significant reduction in
240 growth with 500 μM to 5 mM SNP ($p \leq 0.05$), and complete cessation of growth with
241 concentrations greater than 5 mM (Fig. 4).

242

243 **The response of *S. pneumoniae* to treatment with SNP was mediated by NO.** Having
244 determined that both planktonic and biofilm pneumococci responded to SNP treatment, we
245 next wished to confirm that the response was indeed mediated by NO and not due to other
246 NO metabolites, intact SNP or SNP breakdown products other than NO. Treatment with 1
247 mM KCN, a control for the possible effect of cyanide anion liberation from the SNP
248 molecule, caused no reduction in pneumococcal growth confirming that the reduction in
249 viability compared with untreated bacteria was not due to cyanide toxicity ($p = 0.528$; Fig.
250 5a). Moreover, treatment with 1 mM DEA/NO, an alternative NO-donor molecule that is
251 chemically and mechanistically distinct from SNP, resulted in a significant decrease in

252 growth, similar in extent to treatment with 1 mM SNP ($p=0.013$, Fig. 5a), whilst treatment
253 with the NO-scavenger cPTIO abrogated the response to SNP ($p=0.008$, Fig. 5b). These
254 results indicated that the response to SNP treatment was NO-mediated.

255 In contrast, treatment with 1 mM nitrate ($p=0.321$) or nitrite ($p=0.078$) failed to
256 significantly reduce pneumococcal growth, indicating that the oxidative breakdown
257 products of NO were not responsible for the observed reduction in viability (Fig. 5a). Since
258 peroxynitrite (ONOO^-) is an extremely toxic molecule that can be produced by reaction of
259 NO with superoxide (O_2^-) to cause damage to DNA, proteins and lipids (24, 29), we further
260 tested whether ONOO^- toxicity might be involved in reducing pneumococcal viability
261 during SNP treatment by using the ONOO^- scavenger L-methionine. Indeed, the response to
262 SNP was also reduced by the presence of L-methionine ($p=0.005$) suggesting that
263 extracellular ONOO^- formation secondary to reaction with O_2^- might be responsible for the
264 bactericidal effects of higher concentrations of NO on pneumococcal cells (Fig. 5c).

265

266 **NO adjunctive treatment of *in vitro* pneumococcal biofilms enhanced antibiotic**
267 **effectiveness.** Others have shown that NO combined with antibiotic treatment resulted in
268 an additional reduction in the viability of biofilms in several types of bacteria (28). We
269 therefore tested whether NO treatment of pneumococcal biofilms could further reduce
270 bacterial viability when used as an adjunctive treatment in conjunction with a conventional
271 antibiotic used to treat otitis media. Serotype 2 strain D39 biofilms and biofilms from 3
272 different clinical isolates representative of serotypes 14 (ST124), 19F and 23F and based
273 on their high isolation frequency in OM (20) and high rates of antibiotic recalcitrance were

274 used to assess NO adjunctive treatment (34, 35). Established biofilms were treated with 1
275 mM SNP and 300/60 µg/ml AMC for 2 h and the viability of the remaining pneumococcal
276 biofilm bacteria assessed by CFU enumeration. Treatment of ST124, 19F and D39 biofilms
277 with the NO donor alone resulted in a 2-log reduction in viable bacteria, whereas treatment
278 of 23F biofilms resulted in a 3-log reduction (Fig. 6). AMC treatment alone resulted in a 3-
279 log reduction in ST124 and 23F biofilm viability, and a 2-log reduction in 19F and D39
280 biofilm viability (Fig. 6). Combined NO/AMC treatment, however, resulted in a 3-log
281 reduction in 19F and D39, a 5-log reduction in ST124, and complete killing of 23F when
282 compared with untreated biofilms (Fig. 6), and a significant reduction in viable
283 pneumococci in biofilms compared with antibiotic treatment alone ($p < 0.05$).

284

285 **Combined antibiotic and NO treatment enhanced *ex vivo* killing of *S. pneumoniae* on**
286 **adenoid tissue.** Adenoids have been shown to act as a reservoir for *S. pneumoniae* and
287 biofilm bacteria, and may provide a source for infection in some cases of chronic otitis
288 media (10, 11, 36). Following research ethics committee approval and informed parental
289 consent (NHS REC 09/H0501/74) we subsequently examined adenoid tissue *ex vivo* that
290 was culture positive for *S. pneumoniae* from children <12 years of age undergoing
291 adenoidectomy for the treatment of suspected inflammatory or infective ENT disease to
292 evaluate the effect of NO on *S. pneumoniae* colonized mucosal epithelia (Fig. 7). Adenoid
293 sections (n=11) were treated with 100 µM of SNP alone, 300/60 µg/ml AMC alone, or
294 treated with both NO and AMC to determine if NO adjunctive therapy increased antibiotic
295 efficacy (Fig. 7). Treatment with 100 µM of the NO-donor SNP alone did not significantly
296 reduce colonized pneumococci determined by CFUs ($p=0.722$) and AMC treatment alone

297 resulted in a 2-log reduction compared with the untreated adenoid ($p=0.005$). However,
298 similar to results with *in vitro* biofilms, combined NO and AMC resulted in a significant
299 reduction of pneumococci CFUs on *ex vivo* adenoid tissue by nearly 3 logs ($p=0.005$)
300 compared with untreated adenoid tissue and further reduced the number of CFUs
301 compared with AMC alone ($p=0.04$).

302

303 **Treatment of *in vitro* *S. pneumoniae* biofilms with NO induced a change in**

304 **translational and metabolic protein expression.** Since low dose NO treatment of 48 h
305 pneumococcal biofilms did not appear to be cytotoxic or induce dispersal we used a high-
306 throughput gel-free proteomic approach to investigate whether NO treatment induced
307 changes in protein expression to shed further light on the potential mechanisms involved.
308 Previous data from our lab demonstrated that a total of 112 proteins were differentially
309 expressed during biofilm development using iTRAQ (inclusion criteria ≥ 3 peptide matches;
310 $>5\%$ sequence coverage and a 50+ protein score; $p<0.05$) (33). Of these, 13 proteins were
311 differentially expressed in established pneumococcal biofilms treated with NO for 2 h,
312 compared with biofilms treated with HBSS alone (Fig. 8). Eighty five percent (11/13) of
313 these proteins were involved in *S. pneumoniae* translation or metabolism (Figure 9a). Five
314 ribosomal proteins, all of which demonstrated significantly decreased expression in the
315 biofilm phenotype, exhibited increased expression following NO treatment, suggesting a
316 modulation of translational capacity similar to planktonic levels (33). Additionally, 6
317 metabolism-associated proteins were differentially expressed following NO treatment: 3-
318 ketoacyl-(acyl-carrier-protein) reductase and PTS system fructose-specific II ABC
319 components were upregulated upon NO treatment, whereas arginine deiminase (ArcA), a

320 PTS system mannose specific IID component, and 2 individual alcohol dehydrogenases
321 exhibited decreased expression following NO treatment. The significant reduction in ArcA
322 expression is of particular interest given its >4-fold increase in expression during biofilm
323 growth (33). Two other proteins, a SPFH domain-containing protein and a hypothetical
324 protein were also identified as having increased expression following NO treatment. These
325 data were further supported by the qualitative identification of 12 proteins with
326 differential expression following NO treatment including 7 associated with pneumococcal
327 metabolism (Figure 9b).

328

329 Discussion

330 Consistent with other studies, *S. pneumoniae* biofilms were more tolerant to
331 antibiotic treatment than planktonic pneumococci, including an antibiotic commonly used
332 to treat otitis media (13, 20, 37). However, pneumococcal biofilm antibiotic tolerance was
333 significantly diminished (by up to 2 logs) when accompanied by adjunctive treatment with
334 a low concentration of NO. Additionally, we demonstrated biochemically that reduced
335 viability of pneumococci in planktonic and biofilm growth conditions was mediated by NO,
336 and the oxidative breakdown products of NO, nitrite and nitrate, did not mimic this effect.
337 Although NO treatment reduced *in vitro* pneumococcal biofilm CFUs, our data suggest that
338 the anti-pneumococcal effect was not due to a dispersal of bacteria. Rather, higher
339 concentrations of NO demonstrated a direct antibacterial effect on pneumococcal growth.
340 Treatment with 1 mM SNP resulted in a decrease in biofilm viability by up to 3 logs, and in
341 the number of viable cells in the surrounding supernatant. These data were commensurate
342 with CLSM and SEM imaging which demonstrated no obvious changes in biofilm

343 ultrastructure, but did reveal a significant reduction in biofilm viability. A similar response
344 was observed using the structurally distinct NO donor, DEA/NO. The reduced antibacterial
345 response in the presence of the NO scavenger cPTIO, and the lack of response to nitrite and
346 nitrate, indicated that the antimicrobial effects were indeed NO-mediated, and not
347 associated with the formation of NO_3^- and NO_2^- which have also been shown to increase
348 antibiotic efficacy in *P. aeruginosa* biofilms (38). The specificity of the NO-mediated
349 response, along with the reduction in the planktonic growth rate observed with $\geq 500 \mu\text{M}$ of
350 SNP, suggest a direct effect on growth and/or regulation of metabolism.

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

360 Pneumococcal interactions with epithelial cells have been shown to be important for
361 colonization and biofilm formation (12, 13), and pneumococci are commonly present on
362 adenoids from children with chronic OM or OSA (10, 11). We therefore used adenoid upper
363 respiratory mucosal epithelial tissue colonized with pneumococci to further determine if a
364 combination of NO and AMC might enhance pneumococcal killing using a lower
365 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

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 μ M),
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 μ M) 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 μ M. 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 deiminase 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 (O₂⁻) and NO. Compromised pneumococcal
448 superoxide dismutase (SOD) activity may lead to enhanced formation of O₂⁻ 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

455 pneumococcal metabolite. Our previous proteomic analyses indicated pyruvate oxidase,
456 which produces H₂O₂, was also markedly upregulated in pneumococcal biofilms (33).

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

462 The NO donor SNP has been widely utilized for a number of clinical applications,
463 primarily through its use as a vasodilator, however, prolonged treatment and/or high
464 doses have been suggested to pose a risk of cyanide-mediated cytotoxicity (49, 50). The
465 decomposition of SNP to cyanide has also been shown to be slow (<2.5% over 72 h) when
466 protected from direct exposure to high intensity/natural light (51). For the purpose of our
467 initial study SNP was used as a suitable NO-donor to explore the actions of NO on
468 pneumococcal biofilms since relatively low doses were applied for a short period (26, 27).
469 Furthermore, treatment with equimolar concentrations of KCN had no effect on
470 pneumococcal growth indicating that any observed responses to SNP were not the result of
471 cyanide toxicity (52). However, future studies investigating NO-mediated anti-
472 pneumococcal effects with alternative donors, such as Cephalosporin-3'-diazoniumdiolate
473 NO-Donor Prodrugs, which have been specifically designed to release NO at sites of
474 bacterial infection may offer better choices for clinical use (53).

475 The results of our study are consistent with other studies showing high
476 concentrations of NO were toxic to bacteria including pneumococcus (32). However, to our
477 knowledge our study is the first to show that: 1) planktonic and biofilm *S. pneumoniae*

478 responded differentially to low and high concentrations of NO; 2) the anti-pneumococcal
479 response was not induced by nitrite or nitrate, but was NO specific; 3) unlike other
480 bacteria, low concentrations of NO did not elicit a dispersal response by biofilm *S.*
481 *pneumoniae*; 4) a low concentration of NO altered the protein expression profile of biofilm
482 pneumococci; and 5) when accompanied by adjunctive treatment with NO, pneumococcal
483 sensitivity to antibiotic treatment was enhanced *in vitro* and *ex vivo*. These results suggest
484 that at lower concentrations, NO perturbs pneumococcal biofilm metabolism, but at higher
485 concentrations NO is toxic to *S. pneumoniae*. Targeted adjunctive NO treatment may be a
486 candidate novel therapy for reducing biofilm tolerance by pneumococcus.

487

488 **Funding Information**

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

494

495 **Acknowledgements**

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

499

500

501 **References**

- 502 1. **Moffitt KL, Gierahn TM, Lu YJ, Gouveia P, Alderson M, Flechtner JB, Higgins DE,**
503 **Malley R.** 2011. T(H)17-based vaccine design for prevention of *Streptococcus pneumoniae*
504 colonization. *Cell host & microbe* **9**:158–65.
- 505 2. **Tahtinen PA, Laine MK, Ruuskanen O, Ruohola A.** 2012. Delayed versus
506 immediate antimicrobial treatment for acute otitis media. *Pediatr. Infect. Dis. J.*
507 **31(12)**:1227-32.
- 508 3. **Vergison A, Dagan R, Arguedas A, Bonhoeffer J, Cohen R, DHooge I, Hoberman**
509 **A, Liese J, Marchisio P, Palmu AA, Ray GT, Sanders EAM, Simoes EA, Uhari M, van**
510 **Eldere J, Pelton SI.** 2010. Otitis media and its consequences: beyond the earache. *Lancet*
511 *Infect. Dis.* **10**:195-203.
- 512 4. **Van den Aardweg MT, Schilder AG, Herkert E, Boonacker CW, Rovers MM.**
513 2010. Adenoidectomy for otitis media in children. *Cochrane Database Syst. Rev.* CD007810.
- 514 5. **Boonacker CW, Rovers MM, Browning GG, Hoes AW, Schilder AG, Burton MJ.**
515 2014. Adenoidectomy with or without grommets for children with otitis media: an
516 individual patient data meta-analysis. *Health Technol. Assess.* **18**:1–118.
- 517 6. **Shak J, Ludewick H, Howery K, Sakai F, Yi H, Harvey R, Paton J, Klugman K,**
518 **Vidal J.** 2013. Novel Role for the *Streptococcus pneumoniae* Toxin Pneumolysin in the
519 Assembly of Biofilms. *mBio* **4**:e00655–13e00655–13.
- 520 7. **Casey JR, Adlowitz DG, Pichichero ME.** 2010. New patterns in the otopathogens
521 causing acute otitis media six to eight years after introduction of pneumococcal conjugate
522 vaccine. *Pediatr. Infect. Dis. J.* **29**:304–9.
- 523 8. **Hall-Stoodley L, Hu FZ, Gieseke A, Nistico L, Nguyen D, Hayes J, Forbes M,**

- 524 **Greenberg DP, Dice B, Burrows A, Wackym PA, Stoodley P, Post JC, Ehrlich GD,**
525 **Kerschner JE.** 2006. Direct detection of bacterial biofilms on the middle-ear mucosa of
526 children with chronic otitis media. *JAMA* **296**:202–11.
- 527 9. **Thornton RB, Rigby PJ, Wiertsema SP, Filion P, Langlands J, Coates HL,**
528 **Vijayasekaran S, Keil AD, Richmond PC.** 2011. Multi-species bacterial biofilm and
529 intracellular infection in otitis media. *BMC Pediatr.* **11**:94.
- 530 10. **Hoang M, Tomovic S, Nistico L, Hall-Stoodley L, Stoodley P, Sachdeva L, Berk R,**
531 **Coticchia JM.** 2009. Identification of adenoid biofilms with middle ear pathogens in otitis-
532 prone children utilizing SEM and FISH. *Int. J. Pediatr. Otorhinolaryngol.* **73**:1242–8.
- 533 11. **Nistico L, Kreft R, Gieseke A, Coticchia JM, Burrows A, Khampang P, Liu Y,**
534 **Kerschner JE, Post JC, Lonergan S, Sampath R, Hu FZ, Ehrlich GD, Stoodley P, Hall-**
535 **Stoodley L.** 2011. Adenoid reservoir for pathogenic biofilm bacteria. *J. Clin. Microbiol.*
536 **49**:1411–20.
- 537 12. **Marks L, Davidson B, Knight P, Hakansson A.** 2013. Interkingdom Signaling
538 Induces *Streptococcus pneumoniae* Biofilm Dispersion and Transition from Asymptomatic
539 Colonization to Disease. *MBio* **4**:e00438–13.
- 540 13. **Marks L, Parameswaran G, Hakansson A.** 2012. Pneumococcal Interactions with
541 Epithelial Cells Are Crucial for Optimal Biofilm Formation and Colonization In Vitro and In
542 Vivo. *Infect. Immun.* **80**:2744–2760.
- 543 14. **Weimer KE, Armbruster CE, Juneau RA, Hong W, Pang B, Swords WE.** 2010.
544 Coinfection with *Haemophilus influenzae* promotes pneumococcal biofilm formation
545 during experimental otitis media and impedes the progression of pneumococcal disease. *J.*
546 *Infect. Dis.* **202**:1068–75.

- 547 15. **Blanchette-Cain K, Hinojosa CA, Akula Suresh Babu R, Lizcano A, Gonzalez-**
548 **Juarbe N, Munoz-Almagro C, Sanchez CJ, Bergman MA, Orihuela CJ.** 2013. Streptococcus
549 pneumoniae biofilm formation is strain dependent, multifactorial, and associated with
550 reduced invasiveness and immunoreactivity during colonization. *MBio* **4**:e00745–13.
- 551 16. **Alhede M, Bjarnsholt T, Givskov M, Alhede M.** 2014. Pseudomonas aeruginosa
552 biofilms: mechanisms of immune evasion. *Adv. Appl. Microbiol.* **86**:1–40.
- 553 17. **Bakaletz L.** 2012. Bacterial biofilms in the upper airway - evidence for role in
554 pathology and implications for treatment of otitis media. *Paediatric Respiratory Reviews*
555 **13**:154159.
- 556 18. **Hall-Stoodley L, Stoodley P.** 2009. Evolving concepts in biofilm infections. *Cell.*
557 *Microbiol.* **11(7)**: 1034-43.
- 558 19. **Scherr TD, Heim CE, Morrison JM, Kielian T.** 2014. Hiding in Plain Sight: Interplay
559 between Staphylococcal Biofilms and Host Immunity. *Front. Immunol.* **5**:37.
- 560 20. **Hall-Stoodley L, Nistico L, Sambanthamoorthy K, Dice B, Nguyen D, Mershon W,**
561 **Johnson C, Hu F, Stoodley P, Ehrlich G, Post J.** 2008. Characterization of biofilm matrix,
562 degradation by DNase treatment and evidence of capsule downregulation in Streptococcus
563 pneumoniae clinical isolates. *BMC Microbiology* **8**:173.
- 564 21. **Marks L, Reddinger R, Hakansson A.** 2012. High Levels of Genetic Recombination
565 during Nasopharyngeal Carriage and Biofilm Formation in Streptococcus pneumoniae.
566 *mBio* **3**:e00200–12.
- 567 22. **Hoffmann O, Zweigner J, Smith SH, Freyer D, Mahrhofer C, Dagand E,**
568 **Tuomanen EI, Weber JR.** 2006. Interplay of pneumococcal hydrogen peroxide and host-
569 derived nitric oxide. *Infect. Immun.* **74**:5058–66.

- 570 23. **Crane BR, Sudhamsu J, Patel BA.** 2010. Bacterial nitric oxide synthases. *Annu. Rev.*
571 *Biochem.* **79**:445–70.
- 572 24. **Barraud N, Schleheck D, Klebensberger J, Webb JS, Hassett DJ, Rice SA,**
573 **Kjelleberg S.** 2009. Nitric oxide signaling in *Pseudomonas aeruginosa* biofilms mediates
574 phosphodiesterase activity, decreased cyclic di-GMP levels, and enhanced dispersal. *J.*
575 *Bacteriol.* **191**:7333–42.
- 576 25. **Gusarov I, Nudler E.** 2005. NO-mediated cytoprotection: Instant adaptation to
577 oxidative stress in bacteria. *Proceedings of the National Academy of Sciences of the United*
578 *States of America* **102**:13855–13860.
- 579 26. **Barraud N, Storey MV, Moore ZP, Webb JS, Rice SA, Kjelleberg S.** 2009. Nitric
580 oxide-mediated dispersal in single- and multi-species biofilms of clinically and industrially
581 relevant microorganisms. *Microb. Biotechnol.* **2**:370–8.
- 582 27. **Barraud N, Hassett DJ, Hwang S-HH, Rice SA, Kjelleberg S, Webb JS.** 2006.
583 Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. *J. Bacteriol.*
584 **188**:7344–53.
- 585 28. **Barraud N, Kelso MJ, Rice SA, Kjelleberg S.** 2015. Nitric oxide: a key mediator of
586 biofilm dispersal with applications in infectious diseases. *Curr. Pharm. Des.* **21**:31–42.
- 587 29. **Friedman A, Blecher K, Sanchez D, Tuckman-Vernon C, Gialanella P, Friedman**
588 **J, Martinez L, Nosanchuk J.** 2011. Susceptibility of Gram-positive and -negative bacteria to
589 novel nitric oxide-releasing nanoparticle technology. *Virulence* **2**:217221.
- 590 30. **Han G, Martinez L, Mihu M, Friedman A, Friedman J, Nosanchuk J.** 2009. Nitric
591 Oxide Releasing Nanoparticles Are Therapeutic for *Staphylococcus aureus* Abscesses in a
592 Murine Model of Infection. *PLoS ONE* **4**:e7804.

- 593 31. **Ghaffari A, Miller C, McMullin B, Ghahary A.** 2006. Potential application of
594 gaseous nitric oxide as a topical antimicrobial agent. *Nitric Oxide* **14**:2129.
- 595 32. **Kerr A, Wei X-Q, Andrew P, Mitchell T.** 2004. Nitric oxide exerts distinct effects in
596 local and systemic infections with *Streptococcus pneumoniae*. *Microbial Pathogenesis*
597 **36**:303310.
- 598 33. **Allan RN, Skipp P, Jefferies J, Clarke SC, Faust SN, Hall-Stoodley L, Webb J.** 2014.
599 Pronounced metabolic changes in adaptation to biofilm growth by *Streptococcus*
600 *pneumoniae*. *PLoS ONE* **9**:e107015.
- 601 34. **Niedzielski A, Korona-Glowniak I, Malm A.** 2013. High prevalence of
602 *Streptococcus pneumoniae* in adenoids and nasopharynx in preschool children with
603 recurrent upper respiratory tract infections in Poland - distribution of serotypes and drug
604 resistance patterns. *Med. Sci. Monit.* **19**:54-60.
- 605 35. **Mayanskiy N, Alyabieva N, Ponomarenko O, Lazareva A, Katosova L, Ivanenko**
606 **A, Kulichenko T, Namazova-Baranova L, Baranov A.** 2014. Serotypes and antibiotic
607 resistance of non-invasive *Streptococcus pneumoniae* circulating in pediatric hospitals in
608 Moscow, Russia. *Int. J. Infect. Dis.* **20**:58-62.
- 609 36. **Brook I, Shah K, Jackson W.** 2000. Microbiology of healthy and diseased adenoids.
610 *Laryngoscope* **110**:994-9.
- 611 37. **Sanchez C, Kumar N, Lizcano A, Shivshankar P, Hotopp J, Jorgensen J, Tettelin**
612 **H, Orihuela C.** 2011. *Streptococcus pneumoniae* in Biofilms Are Unable to Cause Invasive
613 Disease Due to Altered Virulence Determinant Production. *PLoS ONE* **6**:e28738.
- 614 38. **Borriello G, Richards L, Ehrlich G, Stewart P.** 2006. Arginine or Nitrate Enhances
615 Antibiotic Susceptibility of *Pseudomonas aeruginosa* in Biofilms. *Antimicrobial Agents and*

- 616 Chemotherapy **50**:382–384.
- 617 39. **Walker WT, Jackson CL, Lackie PM, Hogg C, Lucas JS.** 2012. Nitric oxide in
618 primary ciliary dyskinesia. *Eur. Respir. J.* **40**:1024–32.
- 619 40. **Lundberg, Rinder, Weitzberg, Lundberg, Alving.** 1994. Nasally exhaled nitric
620 oxide in humans originates mainly in the paranasal sinuses. *Acta physiologica Scandinavica*
621 **152**:431–2.
- 622 41. **McDougald D, Rice S, Barraud N, Steinberg P, Kjelleberg S.** 2011. Should we stay
623 or should we go: mechanisms and ecological consequences for biofilm dispersal. *Nat. Rev.*
624 *Microbiol.* **10**:39–50.
- 625 42. **McCoy LS, Xie Y, Tor Y.** 2011. Antibiotics that target protein synthesis. *Wiley*
626 *Interdiscip. Rev. RNA* **2**:209–32.
- 627 43. **Kloosterman T, Kuipers O.** 2011. Regulation of Arginine Acquisition and Virulence
628 Gene Expression in the Human Pathogen *Streptococcus pneumoniae* by Transcription
629 Regulators ArgR1 and AhrC. *Journal of Biological Chemistry* **286**:44594–44605.
- 630 44. **Schulz C, Gierok P, Petruschka L, Lalk M, Mäder U, Hammerschmidt S.** 2014.
631 Regulation of the Arginine Deiminase System by ArgR2 Interferes with Arginine
632 Metabolism and Fitness of *Streptococcus pneumoniae*. *mBio* **5**:e01858–14.
- 633 45. **Jakubovics N, Robinson J, Samarian D, Kolderman E, Yassin S, Bettampadi D,**
634 **Bashton M, Rickard A.** 2015. Critical roles of arginine in growth and biofilm development
635 by *Streptococcus gordonii*. *Mol. Microbiol.* **97**:281–300.
- 636 46. **McBride A, Borutaitė V, Brown G.** 1999. Superoxide dismutase and hydrogen
637 peroxide cause rapid nitric oxide breakdown, peroxynitrite production and subsequent cell
638 death. *Biochem. Biophys. Acta* **1454**.

- 639 47. **Stroeher UH, Kidd SP, Stafford SL, Jennings MP, Paton JC, McEwan AG.** 2007. A
640 pneumococcal MerR-like regulator and S-nitrosoglutathione reductase are required for
641 systemic virulence. *J. Infect. Dis.* **196**:1820-1826.
- 642 48. **Potter AJ, Kidd SP, McEwan AG, Paton JC.** 2010. The MerR/NmlR family
643 transcription factor of *Streptococcus pneumoniae* responds to carbonyl stress and
644 modulates hydrogen peroxide production. *J. Bacteriol.* **192(15)**:4063-4066.
- 645 49. **Friederich JA, Butterworth JF.** 1995. Sodium nitroprusside: twenty years and
646 counting. *Anesth. Analg.* **81(1)**:152-62.
- 647 50. **Ignarro LJ, Napoli C, Loscalzo J.** 2002. Nitric oxide donors and cardiovascular
648 agents modulating the bioactivity of nitric oxide: an overview. *Circ. Res.* **90(1)**:21-8.
- 649 51. **Ikeda S, Schweiss JF, Frank PA, Homan SM.** 1987. In vitro cyanide release from
650 sodium nitroprusside. *Anesthesiology.* **66**:381-385.
- 651 52. **Feelisch M.** 1998. The use of nitric oxide donors in pharmacological studies.
652 **358**:113-122.
- 653 53. **Barraud N, Kardak BG, Yepuri NR, Howlin RP, Webb JS, Faust SN, Kjelleberg S,**
654 **Rice AS, Kelso MJ.** 2012. Cephalosporin-3'-diazoniumdiolates: Targeted NO-Donor
655 Prodrugs for Dispersing Bacterial Biofilms. *Angew. Chem. Int. Ed.* **51**:9057-9060.
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662 **Figure 1: SNP treatment of *in vitro* pneumococcal biofilms reduced biofilm viability**
663 **and biomass.** 48h *S. pneumoniae* serotype 14 *in vitro* biofilms were treated with SNP for 2
664 hours, and the biomass assessed by absorbance (OD₆₀₀) and viability measured by CFU/
665 cm². A significant reduction in total biomass and the number of viable cells remaining
666 within the biofilm was observed following 1 mM SNP treatment. ***p≤0.001.

667

668 **Figure 2: SNP treatment of *in vitro* *S. pneumoniae* biofilms reduced the viable cell**
669 **population in the surrounding supernatant.** The viability of 48h *S. pneumoniae* serotype
670 14 *in vitro* biofilm and supernatant populations was measured by CFU enumeration
671 following treatment with 1 mM SNP. SNP treatment significantly reduced both the biofilm
672 and supernatant populations. *p≤0.05.

673

674 **Figure 3: *In vitro* *S. pneumoniae* biofilms treated with SNP demonstrated reduced**
675 **viability and no evidence of dispersal.** 48h *S. pneumoniae* serotype 14 *in vitro* biofilms
676 were treated with 1 mM SNP for 2 hours then imaged using confocal microscopy and
677 Live/Dead staining. 1 mM SNP-treated biofilms **(b)** demonstrated no obvious change in
678 biomass when compared with untreated biofilms **(a)**, however, a reduction in the number
679 of Syto9-stained live bacteria in the 1 mM SNP-treated biofilms **(d)** was reduced in
680 comparison with untreated biofilms **(c)**, commensurate with CFU enumeration data.
681 Scanning electron microscopy with Alcian Blue staining further demonstrated no obvious
682 changes in biofilm ultrastructure between untreated **(e)** and 1 mM SNP-treated **(f)** biofilms
683 (4,000x magnification; scale bar: 10 μm).

684

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 (OD₅₉₅) over 2 hours, and compared with the untreated growth rate. A significant
689 reduction in growth rate was observed using 500 μ M SNP, and complete cessation of
690 growth was observed with concentrations greater than 5 mM. * $p \leq 0.05$; **** $p \leq 0.0001$.

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

708 enumeration. When used separately both SNP and AMC treatment reduced the viable
709 biofilm cell population, however, combined SNP and AMC treatment resulted in a further
710 significant reduction in viability. * $p \leq 0.05$.

711

712 **Figure 7: Adjunctive treatment of *S. pneumoniae* biofilms on *ex vivo* adenoid tissue**
713 **with SNP enhanced antibiotic efficacy.** Adenoid tissue samples (n=11) were dissected
714 into four equal sections (each with a similar proportion of luminal surface) treated for 2
715 hours, and the viability of *S. pneumoniae* was measured by CFU enumeration. SNP
716 treatment alone had no significant effect on viable pneumococci (p=0.722), whereas AMC
717 treatment alone resulted in a significant reduction (p=0.005). Combined SNP and AMC
718 treatment however, resulted in enhanced antibiotic efficacy (p=0.041). * $p \leq 0.05$ (Wilcoxon
719 Signed Ranks test).

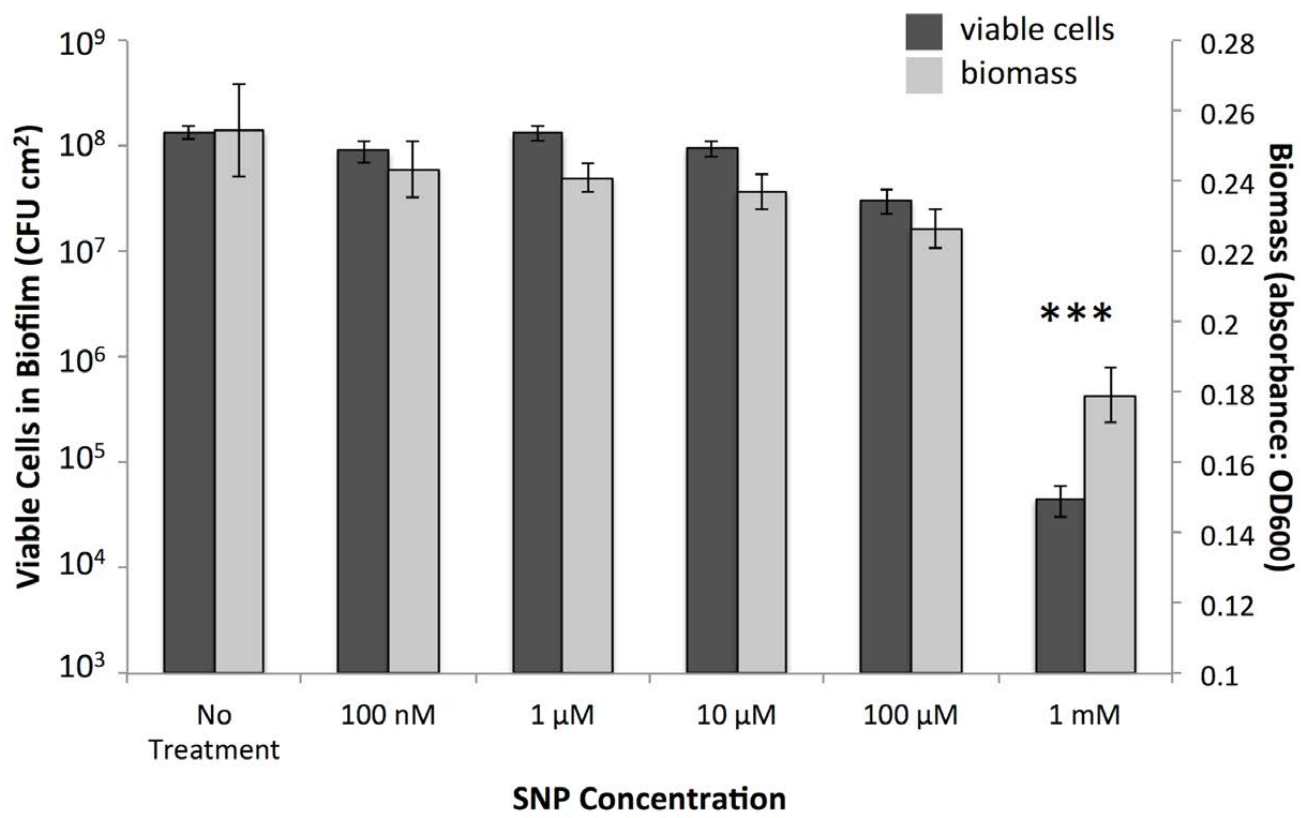
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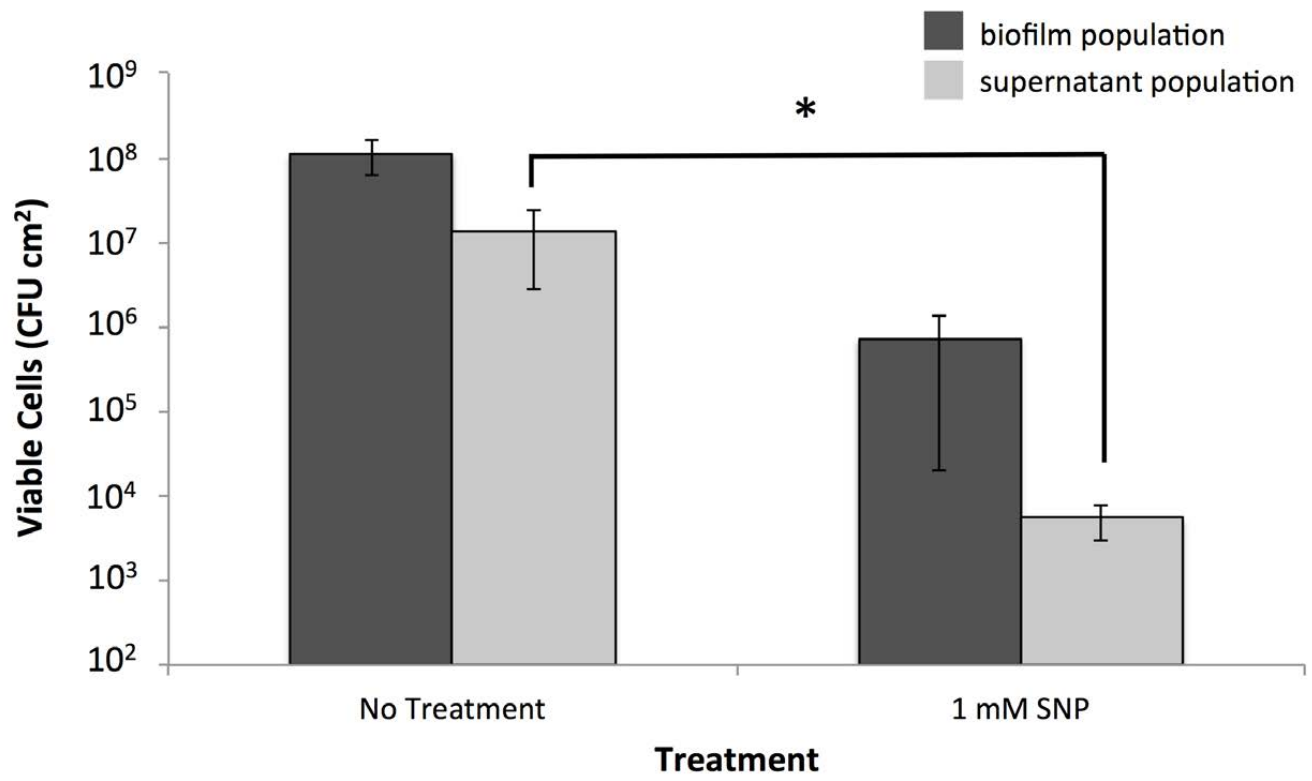
721 **Figure 8: Treatment of *S. pneumoniae in vitro* biofilms with SNP resulted in the**
722 **differential expression of a small subset of quantitatively identified proteins.**
723 Comparative iTRAQ analyses of SNP treated (100 μ M SNP/2 hours) and untreated *S.*
724 *pneumoniae* serotype 14 7-day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

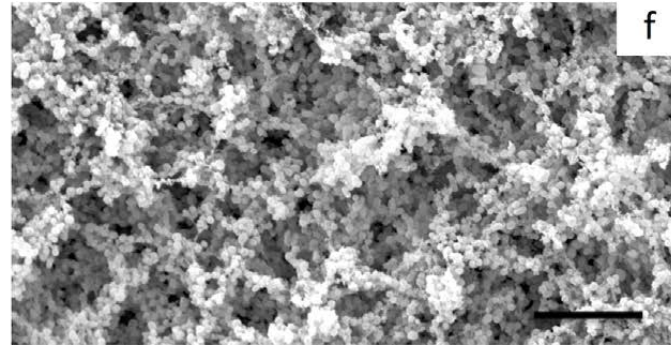
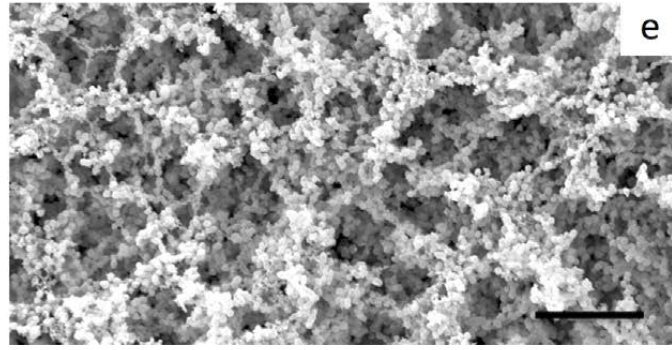
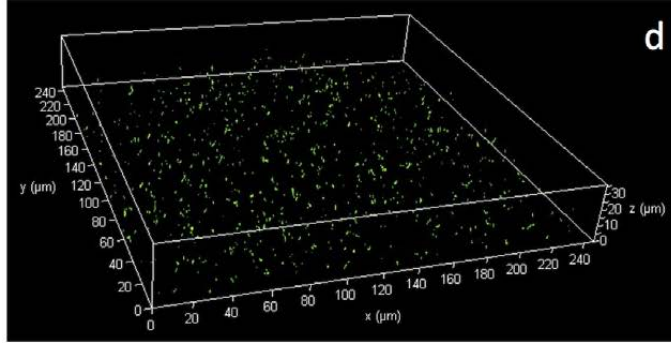
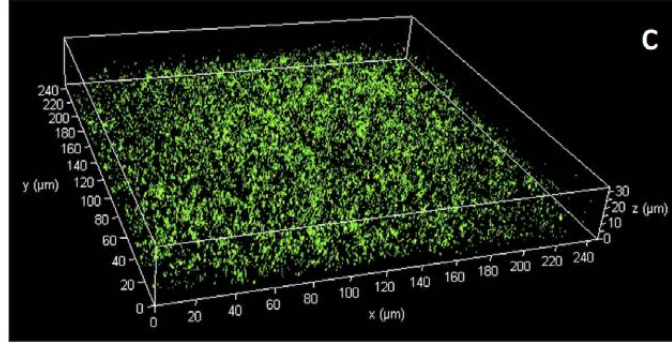
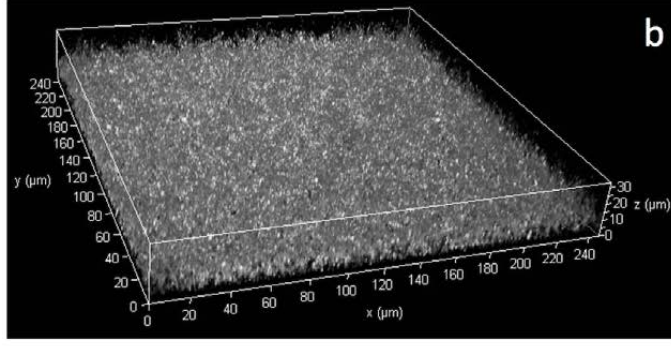
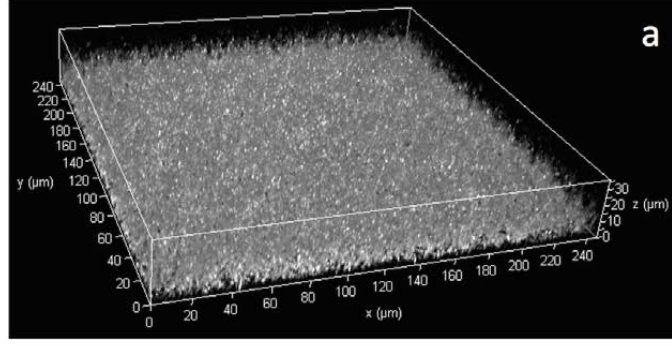
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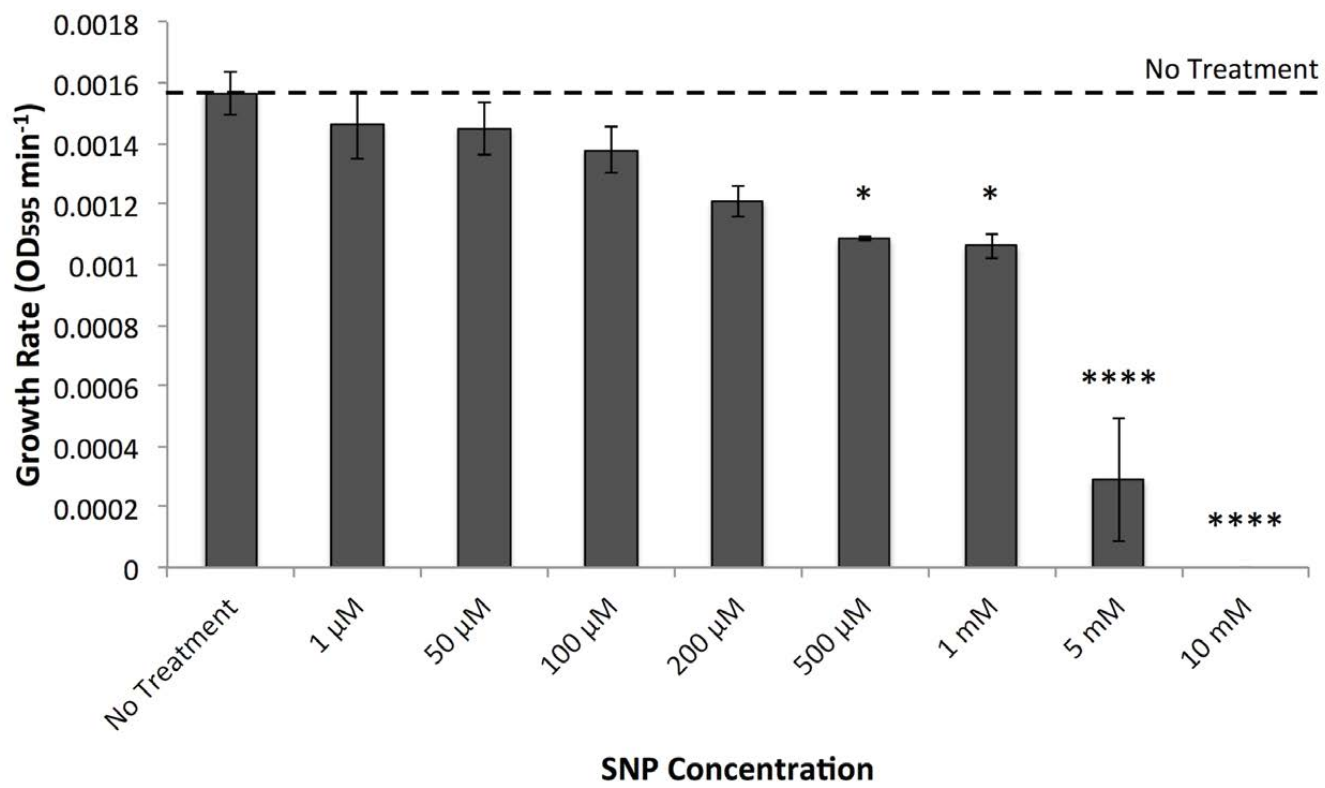
727 **Figure 9: Treatment of *S. pneumoniae in vitro* biofilms with SNP resulted in a change**
728 **in metabolic and translation protein expression levels.** Comparative iTRAQ analyses of
729 SNP treated (100 μ M SNP/2 hours) and untreated *S. pneumoniae* serotype 14 *in vitro*
730 biofilms **a)** quantitatively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

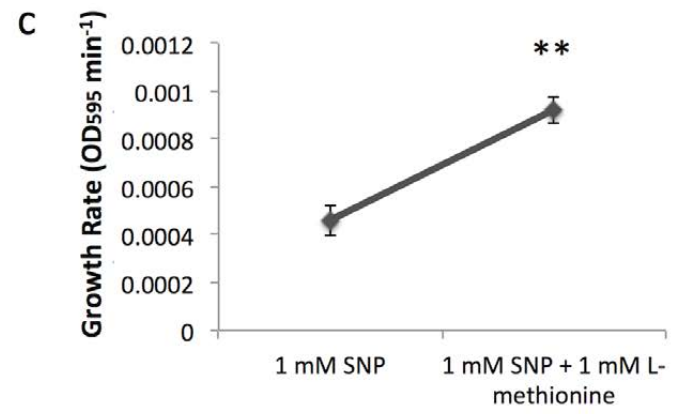
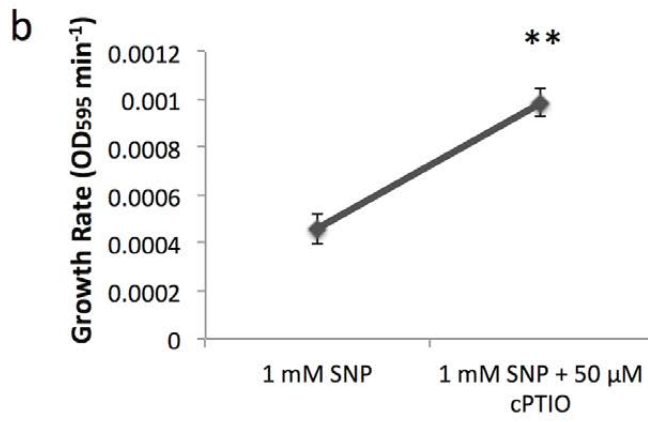
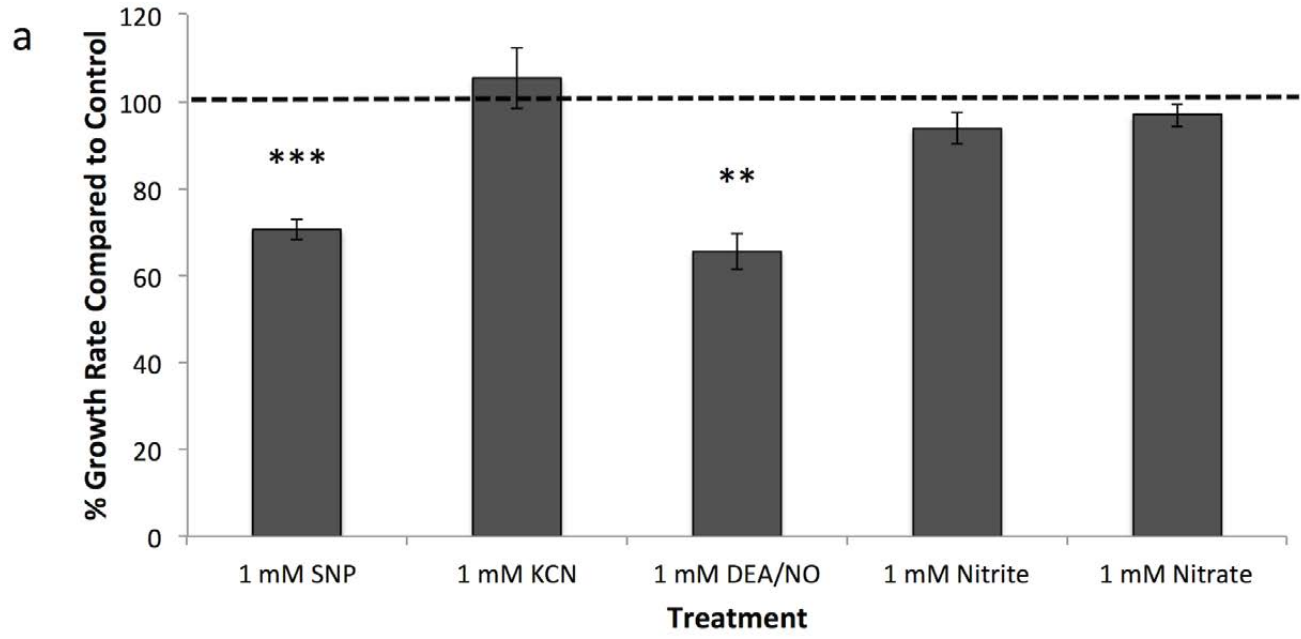
731 qualitatively identified 12 differentially expressed proteins following treatment.
732 Quantitative inclusion criteria: ≥ 3 peptide matches, ≥ 50 protein score, $\geq 5\%$ sequence
733 coverage ($p < 0.05$). Qualitative inclusion criteria: 2 peptide matches, ≥ 50 protein score,
734 $\geq 5\%$ sequence coverage ($p < 0.05$). Comparative protein data with > 1.3 and < 0.77 ratios
735 identified as having differential protein expression.

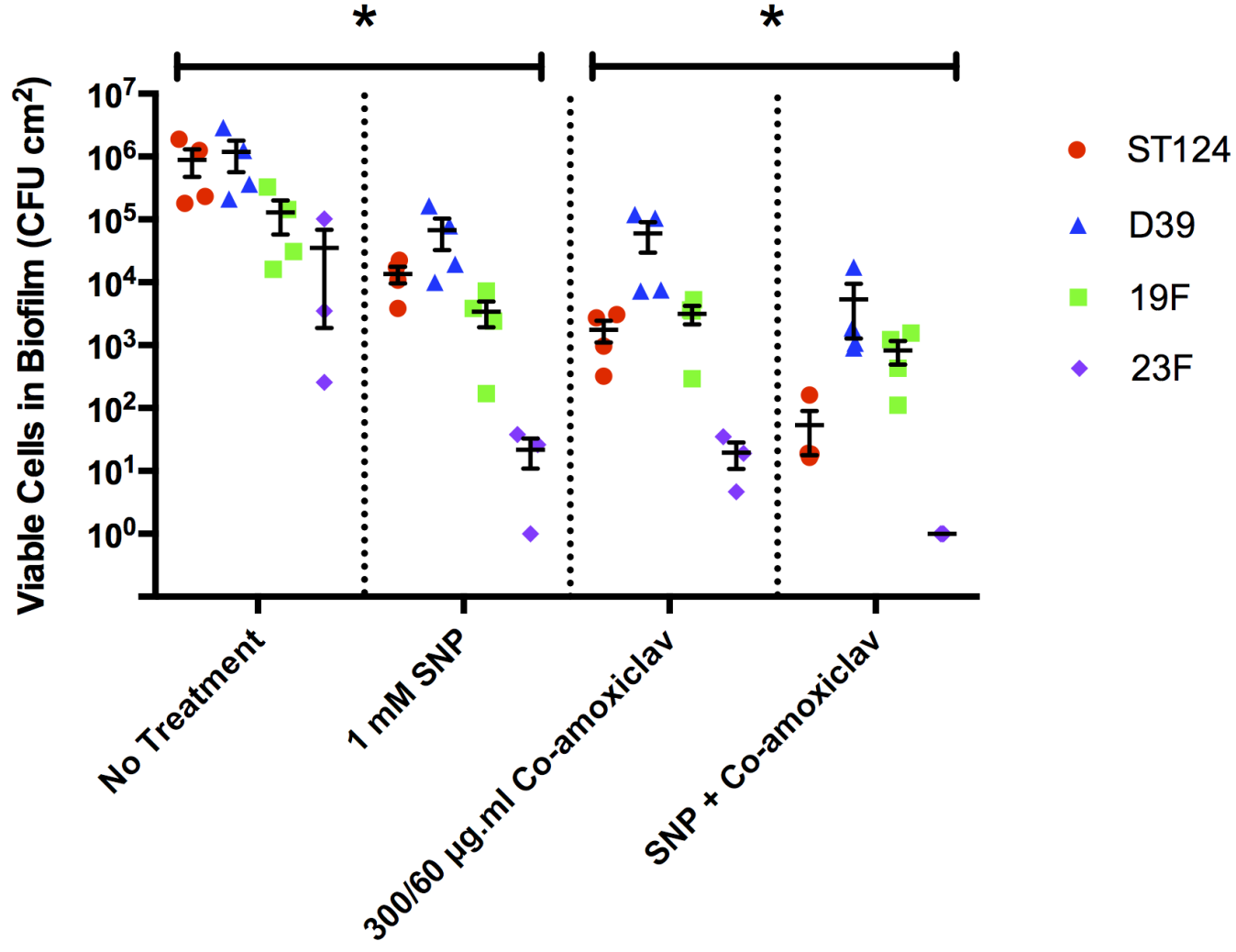


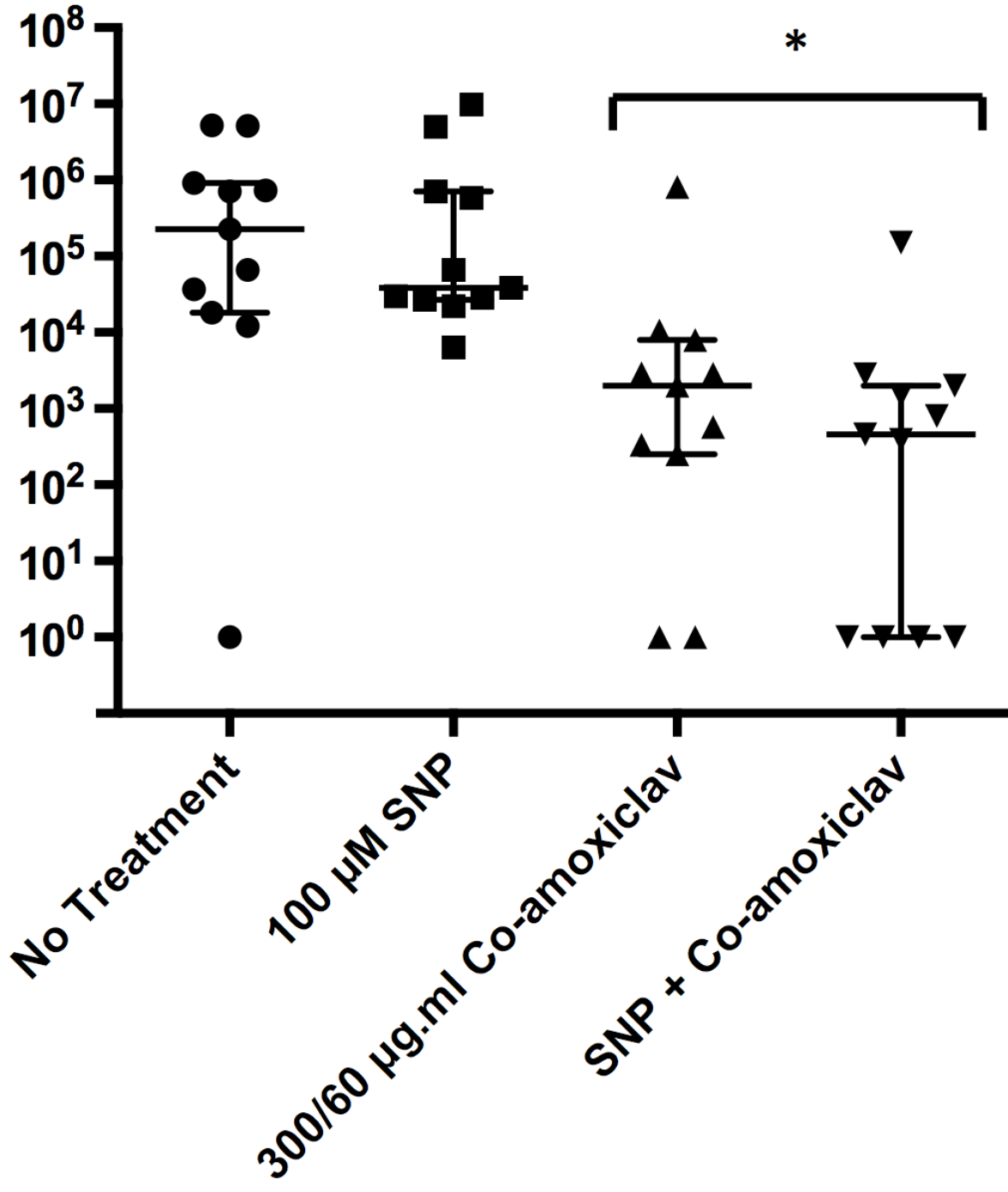


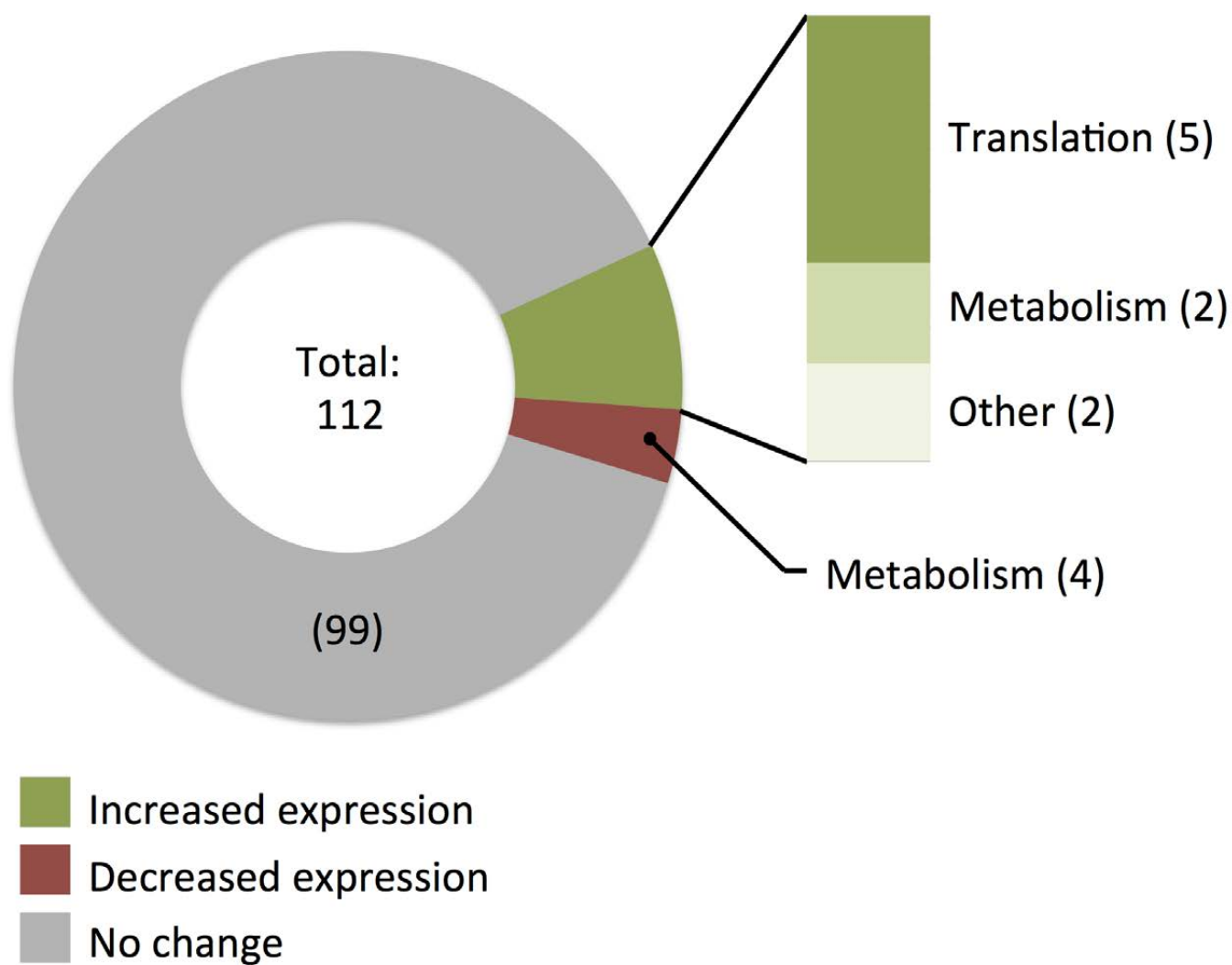








Viable Cells in Adenoid Tissue (CFU g⁻¹)



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 dehydrogenase	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