1   The bacillithiol pathway is required for biofilm formation in *Staphylococcus aureus*

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12   Running Head: Bacillithiol is required for *S. aureus* biofilms

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24   **Abstract**

25   *Staphylococcus aureus* is a major human pathogen that can cause infections that range from

26   superficial skin and mucosal infections to life threatening disseminated infections. *S. aureus* can

27   attach to medical devices and host tissues and form biofilms that allow the bacteria to evade the

28   host immune system and provide protection from antimicrobial agents. To counter host-generated

29   oxidative and nitrosative stress mechanisms that are part of the normal host responses to invading

30   pathogens, *S. aureus* utilizes low molecular weight (LMW) thiols, such as bacillithiol (BSH).

31   Additionally, *S. aureus* synthesizes its own nitric oxide (NO), which combined with its

32   downstream metabolites may also protect the bacteria against specific host responses. We have

33   previously shown that LMW thiols are required for biofilm formation in *Mycobacterium*

34   *smegmatis* and *Pseudomonas aeruginosa*. Our data show that the *bshC* mutant, which is defective

35   in the last step of the bacillithiol pathway and lacks BSH, is impaired in biofilm formation. We

36   also identify a putative *S*-nitrosobacillithiol reductase (BSNOR), similar to a *S*-nitrosomycothiol

37   reductase found in *M. smegmatis*, and show that the BSNOR mutant has reduced levels of BSH

38   and decreased biofilm formation. Our studies also show that NO plays an important role in biofilm

39   formation and that acidified sodium nitrite severely reduces biofilm thickness. These studies

40   provide insight into the roles of oxidative and nitrosative stress mechanisms on biofilm formation

41   and indicate that bacillithiol and nitric oxide are key players in normal biofilm formation in *S.*

42   *aureus*.

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44   **Importance**

45   *Staphylococcus aureus* is the most frequent cause of biofilm-associated infections in hospital

46   settings. The biofilm mode of growth allows the pathogen to escape the host immune response and

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47   is extremely difficult to combat, as biofilms are highly resistant to physical and chemical stressors.

48   As outbreaks of antibiotic-resistant bacterial strains become more commonplace, it is essential to

49   understand the pathways involved in biofilm formation in order to target this important virulence

50   factor. Low molecular weight thiols enable *S. aureus* to combat oxidative and nitrosative stress

51   mechanisms that are used by host cells to defend against infection. Our findings indicate that

52   bacillithiol and nitric oxide, which are produced by *S. aureus* to combat these host generated

53   stressors, are important for biofilm development, and that disruption of these pathways results in

54   biofilm defects. In the long term, this work may lead to new solutions to eradicate *S. aureus*

55   biofilms in the clinic.

56

57   **Introduction**

58   *S*taphylococcus aureus is a gram-positive, spherical-shaped bacteria that is a major human

59   pathogen capable of causing both superficial as well as life-threatening systemic and chronic

60   infections in humans (1, 2). *S. aureus* is also a commensal microorganism that can reside on the

61   skin, and within the nostrils, throat, perineum and axillae of 25-30% of healthy humans (3-6). One

62   *S. aureus* virulence factor that is particularly challenging to manage in hospital settings is its ability

63   to form biofilms, surface-attached communities of cells that are encased in an extracellular

64   polymeric matrix (7). These biofilms can form on both biotic and abiotic surfaces and are more

65   resistant to antimicrobial agents and the host immune response compared to their free-floating

66   (planktonic) counterparts (7-10). *S. aureus* was one of the first microbial species found to grow as

67   a biofilm on medical devices in clinical settings and is responsible for frequent episodes of

68   bacteremia and sepsis in hospital settings (11). In fact, *S. aureus* biofilms, including those formed

69   by methicillin-resistant *S. aureus* (MRSA) strains, are the leading cause of hospital-acquired

70   sepsis, commonly observed in patients with burn wounds and implanted or indwelling medical

71   devices (12-16).

72   The host immune system employs various mechanisms to recognize and counter infections

73   by *S. aureus* and other invading pathogens (17-19). Neutrophils and macrophages are among the

74   first lines of defense against epithelial invasion by *S. aureus*, leading to a release of reactive oxygen

75   species (ROS) to combat *S. aureus* colonization (20-22). In particular, activated neutrophils mount

76   an oxidative burst, where NADPH oxidase produces ROS, such as superoxide (O_2^-) and hydroxyl

77   radical ($\cdot OH$) (23, 24). Consistent with this protective oxidative burst, mouse models of chronic

78   granulomatous disease that show a severe reduction in NADPH oxidase are highly susceptible to

79   infections by *S. aureus* (25, 26). *S. aureus* proteins are particularly vulnerable to damage by ROS,

80   where ROS oxidize the thiol residues in cysteine moieties (18). Neutrophils also release cytokines

81   and chemokines, which in turn can activate macrophages at the site of *S. aureus* infection.

82   Activated macrophages release high concentrations of nitric oxide (NO), which can combine with

83   superoxide to form highly toxic anions, such as peroxynitrite ($OONO^-$) (27). These high

84   concentrations of NO and other reactive nitrogen species (RNS), which are formed by reactions of

85   NO with other oxidants, together produce nitrosative stress that is useful in eliminating invading

86   bacterial cells (24). To counter these oxidative and nitrosative host stress mechanisms, *S. aureus*

87   in turn has evolved several pathogen-derived adaptive mechanisms to survive and propagate within

88   the host (20, 28-31).

89   One pathogen-derived mechanism is the production of low molecular weight (LMW) thiols

90   that are thought to be crucial in providing protection to cytosolic proteins against ROS, other

91   reactive electrophilic species, antibiotics, and heavy metals (32). LMW thiols oxidize more slowly

92   than cysteine and can play crucial roles in redox reactions and maintaining redox homeostasis (33).

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93   Glutathione (GSH), a tripeptide, is the most abundant LMW thiol produced in cells of eukaryotes

94   and many gram-negative bacteria (34, 35); most gram-positive bacteria, however, are thought to

95   lack GSH (35). Another LMW thiol, mycothiol (MSH), was identified as the primary thiol in high

96   G+C gram-positive bacteria (36, 37), while bacillithiol (BSH) was identified as the primary LMW

97   thiol in the low G+C firmicutes (38). MSH and BSH differ considerably in structure from GSH by

98   containing an amino sugar glucosamine backbone instead of a peptide backbone. BSH biosynthesis

99   and function has been elucidated in the firmicutes *S. aureus* and *B. subtilis* (38), and *S. aureus*

100   mutants lacking BSH have been shown to display increased sensitivities and susceptibilities to

101   killing by oxidative stress (39, 40). One study demonstrated that BSH can reduce oxidants such as

102   H_2O_2 directly and that BSH may participate in a general electron relay with bacilliredoxins to

103   reduce oxidants (41).

104   LMW thiols have also been implicated in protection against nitrosative stress (42). NO can

105   react with GSH to form GSNO spontaneously, preventing its reaction with cysteine moieties (43).

106   The amount of GSNO in the cell is modulated by *S*-nitrosoglutathione reductase (GSNOR), which

107   regenerates GSH and reduces NO to ammonia, and also has a dual function as a *S*-

108   (hydroxylmethyl) glutathione dehydrogenase, participating in formaldehyde detoxification (44).

109   The role of GSNOR in virulence and pathogenesis has been elucidated in *Streptococcus*

110   *pneumoniae,* where GSNOR is required for survival in blood (44). Paradoxically, *S. aureus*

111   synthesizes its own NO using a bacterial nitric oxide synthase (SaNOS), which suggests that NO

112   may also serve some protective role for the bacterium (45). NO and its downstream metabolite,

113   such as nitrite, may protect *S. aureus* and other NOS-producing bacteria by scavenging HClO to

114   produce nitrate, which is less reactive (18). NO can also block cysteine oxidation by *S*-nitrosylating

115   exposed cysteines (46) and activating catalases that reduce the concentration of harmful H_2O_2 (18).

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116   Overall, SaNOS mutants display increased endogenous ROS and superoxide levels (47). Recent

117   studies also find that the production of NO by *S. aureus* is essential for nasal colonization and skin

118   abscess development, and mutants lacking the enzyme show decreased virulence (48-50).

119   Interestingly, at low concentrations NO can act as a signaling molecule in a dose-dependent

120   manner to modulate group activities, such as biofilm formation in numerous bacterial species (51).

121   These complex and multifactorial roles for NO at different concentrations in bacteria are analogous

122   to that in mammalian cells (51).

123   In *S. aureus*, one report found that NO-mediated signal transduction regulates biofilm

124   formation and dispersal, although the mechanistic details of the regulation are unknown (52). NO

125   and nitrite (NO_2^-) impair polysaccharide intercellular adhesion (PIA)-dependent biofilm formation

126   in *S. aureus* (53); PIA is a major constituent of the extracellular matrix of staphylococcal biofilms.

127   It was also recently shown that NO inhibits *S. aureus* virulence by disrupting intercellular

128   communication between bacterial cells by targeting proteins involved in quorum sensing (46).

129   Biofilm formation in *P. aeruginosa* is also affected by NO in a concentration dependent manner

130   and the bacteria harbor NO-responsive regulators that modulate biofilm dispersal (54, 55).

131   We previously reported that MSH and a *S*-nitrosomycothiol reductase are required for

132   biofilm formation in *Mycobacterium smegmatis* (56). We also recently reported that a *P.*

133   *aeruginosa* mutant lacking GSH is defective in biofilm formation (57). These studies indicate that

134   regardless of the structure of the LMW thiols, these thiols play important roles in biofilm formation

135   in diverse bacterial species. Here, we report a new role for BSH in *S. aureus* biofilms. We show

136   that like GSH and MSH mutants, BSH mutants are also impaired in biofilm formation. We confirm

137   that NO is involved in biofilm formation and identify a putative *S*-nitrosobacillithiol reductase in

138   *S. aureus*. Overall, both ROS and RNS stressors are encountered by *S. aureus* biofilms (23),

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139   however the detailed mechanisms by which biofilms counter these stressors and how thiols play a

140   role in response to them are unknown.

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142   **Results**

143   ***S. aureus* contains a putative *S*-nitrosobacillithiol reductase.** Recently, we described the

144   characterization of a *M. smegmatis* mutant disrupted in *S*-nitrosomycothiol reductase

145   (MSMEG_4340) (56). This mutant along with mutants lacking the LMW thiols, MSH and

146   ergothioneine (ESH), was more susceptible to *S*-nitrosoglutathione (GSNO) (56). Since MSH is

147   structurally similar to BSH, we reasoned that the *S*-nitrosobacillithiol reductase (BSNOR) would

148   be similar in sequence to the mycobacterial protein. MSMEG_4340 amino acid sequence and

149   BLASTp analysis was used to identify the protein with the most amino acid identity/similarity in

150   *S. aureus* (strain SAUSA300_FPR375). ORF SAUSA300_0055 contained the most identity at

151   35% (127/357) and similarity at 54% (193/357) with 96% coverage to MSMEG_4340 (E-value =

152    $1e^{-72}$). We obtained a *S. aureus* USA300_FPR375 LAC JE2 transposon mutant disrupted in this

153   ORF from the "Network on Antimicrobial Resistance in *Staphylococcus aureus*" through BEI

154   Resources to characterize its role in biofilm formation and response to oxidative and nitrosative

155   stressors. Under standard growth conditions in rich TSB media, the mutant grew similar to

156   wildtype (WT). Surprisingly, the mutant also grew similarly to WT when treated with GSNO and

157   sodium nitrite (Supplemental Figure 1). Since *S*-nitrosothiol reductases have a dual activity as

158   formaldehyde dehydrogenases (58, 59), growth on formaldehyde was also assessed, and we found

159   that there was no difference in growth from WT (Supplemental Figure 1).

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161 **MIC for Diamide is higher under biofilm conditions compared with planktonic growth.** We

162 determined the MIC for diamide, a thiol oxidant, DTT, a reductant, and sodium nitrite, which

163 releases NO chemically in an acidic solution at pH less than 7, to cause nitrosative stress (60) for

164 the wildtype (WT) *S. aureus* strain under both planktonic and biofilm conditions. The MIC was

165 3.91 mM, 250 mM, and 31.25 mM for diamide, DTT, and sodium nitrite, respectively for WT

166 grown under planktonic culture conditions (Table 1). The MIC for DTT and sodium nitrite was

167 the same for the WT grown under biofilm conditions (Table 1). There was an approximate two-

168 fold increase in the WT MIC for diamide under biofilm conditions, indicating that biofilms are

169 more resistant to this oxidant.

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171 **Bacillithiol levels decrease in the BSNOR mutant upon treatment with sodium nitrite.** Since

172 BSH can buffer NO by forming BSNO, we measured BSH levels upon exposure to sodium nitrite

173 in the WT and BSNOR transposon mutant. As the MIC for sodium nitrite was 31.25 mM, we chose

174 20 mM sodium nitrite as the sublethal concentration for testing the effect of sodium nitrite on BSH

175 levels. Treatment with 20 mM sodium nitrite did not cause cell death as indicated by a lack of

176 change in CFUs before or after 30 mins of sodium nitrite treatment in both WT and BSNOR mutant

177 strains. However, treatment with sodium nitrite resulted in a decrease in BSH levels from 1.21 to

178 0.81 µmol/g dry weight in the WT and a decrease in BSH levels from 1.15 to 0.62 µmol/g dry

179 weight in the BSNOR transposon mutant. There was no difference in BSH levels in the untreated

180 WT and BSNOR mutant, but the BSNOR mutant had significantly less BSH levels relative to WT

181 after exposure to sodium nitrite (Figure 1).

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183   **Sodium nitrite has no effect on *bshC* and BSNOR expression.** To determine if the decrease in

184   BSH was due to a decrease in BSH biosynthesis, *bshC* expression levels were quantified using

185   qPCR on WT cultures treated with sodium nitrite. At 5 min post-treatment, *bshC* is repressed

186   (0.47) and remains repressed even after 30 min of sodium nitrite treatment. This data indicates that

187   sodium nitrite causes repression of BSH biosynthesis through transcriptional control (Figure 2).

188   Surprisingly, BSNOR expression does not change after 5 min or 30 min of sodium nitrite exposure.

189   The positive control, the catalase *katA,* is induced three-fold (53).

190   We also checked *bshC* and BSNOR expression after treatment with oxidants (Figure 2).

191   Upon exposure to hydrogen peroxide, BSNOR is induced two-fold while *katA* is only induced

192   slightly (1.51) and *bshC* is repressed (0.55). Neither BSNOR nor *bshC* are affected by treatment

193   with the lipid peroxide, cumene hydroperoxide, and *katA* is induced weakly (1.36). Treatment

194   with the thiol oxidant, diamide, results in a slight upregulation of BSNOR (1.5), upregulation of

195   *bshC* (4-fold), and no change in *katA* expression.

196

197   **BshC and BSNOR are needed for normal biofilm development.** To determine if BshC and

198   BSNOR are needed for biofilm formation, we tested the *bshC* and BSNOR transposon mutants

199   and compared them with WT. Biofilms were allowed to develop for 24 hours, at 37°C on

200   polystyrene plates and imaged using confocal scanning laser microscopy (CSLM). Our results

201   show that either gene deletion results in a reduced biofilm depth (~46 μm) compared to the

202   wildtype biofilm (~80 μm) (Figure 3).

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204   **Diamide, DTT, and sodium nitrite reduce biofilm formation in *S. aureus.*** To determine if

205   oxidative, reductive, and nitrosative stressors can inhibit biofilm formation, we performed a

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206   biofilm MIC assay on polystyrene plates using a sustained inhibition biofilm assay (61), where the

207   compound (diamide, DTT, or nitrite) is present during cell adherence and during the period of

208   biofilm formation (24 hr). Our results show that 7.81 mM of diamide, 250 mM DTT, or 31.25 mM

209   of sodium nitrite completely inhibits wild type biofilm formation in wildtype, ΔbshC, and ΔbsnoR

210   strains (Table 1).

211   We also visualized the WT biofilm formed in the presence of sublethal concentrations of

212   diamide, DTT and sodium nitrite by CSLM. Our results indicated that even sublethal

213   concentrations of DTT (150 mM) severely reduced biofilm thickness to ~ 20 µm (Figure 4B, 4E)

214   and sublethal concentrations of diamide (1.95 mM) reduced biofilm thickness to ~35 µm (Figure

215   4C, 4F). Since acidified TSB media was used for sodium nitrite treatments, we tested whether the

216   decrease in biofilm thickness was due to the pH or sodium nitrite. Our results show that acidified

217   TSB media reduces biofilm thickness to ~45 µm compared with ~80 µm biofilms formed in

218   standard TSB media (Figure 4A, 4D and Figure 5A, 5C). However, sodium nitrite further reduced

219   biofilm thickness to ~10 µm in acidified TSB media (Figure 5B).

220

Discussion

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222   A number of studies have implicated LMW thiols in biofilm formation. Mutants defective for GSH

223   production are defective for biofilm formation in *P. aeruginosa* (57) and a mutant defective in

224   MSH production is defective for biofilm formation in *Mycobacterium smegmatis* (56). Moreover,

225   the importance of thiols may not be limited to bacterial biofilms. For example, the production of

226   GSH is upregulated in the early stages of biofilm formation by the opportunistic fungal pathogen

227   *Candida albicans* (62). Here we report that a *S. aureus* mutant disrupted in the enzyme encoding

228   for the third and last step of BSH biosynthesis, and thus lacking BSH, was significantly impaired

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229   in biofilm formation compared to the wildtype. Treatment with diamide, a thiol oxidant, also

230   resulted in impaired biofilm formation. We also treated the BSH mutant with the common

231   reductant, DTT, to see if DTT could rescue the *bshC* mutant phenotype. Our results indicate that

232   instead of complementing the mutant, the application of DTT resulted in less biofilm formation,

233   suggesting that biofilm formation specifically requires BSH. Trivedi *et al.* reported that treatment

234   with DTT resulted in an increase in the formation of biofilms and also an increase in intracellular

235   thiols in *Mycobacterium tuberculosis* (63). The contradictory findings between *M. tuberculosis*

236   biofilm formation in the Trivedi *et al.* study and *S. aureus* biofilm formation in our experiments

237   upon DTT treatment could potentially be explained by the different concentrations of DTT used

238   in the experiments (2-4 mM for *M. tuberculosis* and 200 mM for *S. aureus*), whereby high DTT

239   concentrations would likely result in oxidative stress instead of reductive stress. Our results

240   support previous studies that indicate that other reducing agents, such as β-mercaptoethanol and

241   cysteine inhibit *S. aureus* biofilm formation, although they were found not to affect the initial

242   adhesion step of biofilm formation (64).

243   Biofilm formation may be connected to the interaction of LMW thiols and NO. It is likely

244   that NO interacts with BSH during *S. aureus* biofilm formation as our results indicate that the

245   putative BSNOR mutant, which shows a decrease in biofilm formation, also has reduced BSH

246   levels after treatment with acidified sodium nitrite. It is known that NO reacts with GSH to form

247   GSNO, which may serve as a reservoir for NO or conversely sequester GSH so that it is

248   unavailable to the cell (65). NO can *S*-nitrosylate proteins directly through a radical-mediated

249   pathway or indirectly via higher oxides of NO, and may compete with *S*-glutathionylation of

250   exposed cysteines (66). Moreover, GSH can react with protein *S*-nitrosothiols leading to either

251   denitrosylaton or *S*-glutathionylation (67). Schlag *et al.*, 2007 demonstrated nitrite dependent

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252 inhibition of biofilm formation in *S. aureus* with repression of the icaADBC gene cluster,

253 mediated by IcaR (53), although acidified sodium nitrite was not used in these experiments.

254 Genes involved in DNA repair, reactive oxygen intermediates (ROI), RNS detoxification, and

255 iron homeostasis were induced, and preformed biofilms could be eradicated by the addition of

256 nitrite (53). Major *et al* further demonstrated that acidified sodium nitrite could inhibit biofilm

257 formation and kill planktonic cultures grown aerobically and anaerobically in *S. aureus*, *P.*

258 *aeruginosa* and *Burkholderia cepacia* (68). Our results also show that acidified sodium nitrite

259 causes an inhibition of biofilm formation.

260   Since levels of *S*-nitrosothiols are modulated by *S*-nitrosothiol reductase, which reduces

261 the GSNO to ammonia or other reduced nitrogen species (58), we measured biofilm formation in

262 a putative BSNOR mutant and found that this mutant is defective in biofilm formation. However,

263 the BSNOR mutant was not impaired in growth on formaldehyde or nitrosative stress. Other

264 mutants such as the MscR (dual function *S*-nitrosomycothiol reductase and formaldehyde

265 dehydrogenase) mutant in mycobacteria are impaired in biofilm formation and sensitive to these

266 stressors (56). It has been noted that *S. aureus* is particularly resistant to NO and nitrosative

267 stress as compared to other bacterial species (31). Additionally, a number of *S. aureus* genes

268 have been annotated as aldehyde dehydrogenases, such as *adhA*, which is highly upregulated

269 upon formaldehyde and methylglyoxal treatment (69). It has been suggested that *adhA* functions

270 in the LMW thiol-dependent detoxification of aldehydes, but the gene product has not yet been

271 validated as a BSNOR. Nevertheless, it is possible that there is redundancy in protection against

272 nitrosative and formaldehyde stress in *S. aureus* that leads to a lack of growth phenotypes under

273 these stressors for the BSNOR mutant.

274   We also assessed the gene expression of BSNOR in wildtype under nitrosative stress and

275   observed no differences in expression levels in the presence or absence of the nitrosative stress. In

276   *Vibrio cholerae*, GSNO reductase activity is regulated post-translationally by *S*-nitrosylation and

277   this activity can be reversed by DTT (70). *S*-nitrosylation of GSNOR1 leading to inhibition of

278   activity has also been documented in plants (71). In addition, in *Neisseria meningitidis*, S-

279   glutathionylation of the cys54 of the thioesterase catalyzing the downstream step from S-

280   formylglutathione hydrolase has been shown to decrease the activity of the enzyme (72). Together,

281   these observations suggest that BSNOR may also be regulated post-translationally, possibly

282   through *S*-bacillithiolation of the corresponding esterase in *S. aureus,* which would result in

283   increased formaldehyde levels and less biofilm formation as seen in *N. meningitidis*. Interestingly,

284   *bshC* expression is decreased under sodium nitrite and hydrogen peroxide and this likely results in

285   a concomitant decrease in *S*-bacillithiolation in *S. aureus* under these stressors.

286   Recently Gupta *et al.* identified a transcriptional regulator, BifR, which is a member of the

287   MarR protein family in *Burkholderia thailandensis* (73). Under oxidizing conditions, BifR forms

288   a disulfide linked dimer of dimers, which affects its ability to bind to promoters of several genes.

289   A mutant disrupted in BifR had enhanced biofilm formation (73). A similar redox switch

290   controlling biofilm formation may be present in *S. aureus.* In the *bshC* and the BSNOR mutant

291   strains, one would predict that treatment with sodium nitrite would lead to a decrease in BSH and

292   a more oxidized cytoplasm, which would facilitate disulfide formation of a transcriptional

293   regulator, consequently affecting expression of genes involved in biofilm formation. Additionally,

294   in the BSNOR mutant, the BSNO would not be reduced and BSH not released, causing oxidative

295   stress further affecting the regulation of biofilm formation. Whether this occurs in *S. aureus* needs

296   further study.

13

297   The data presented here indicates that the bacillithiol pathway likely interacts with NO and

298   together they play key roles in biofilm formation in *S. aureus*. Further characterization of the

299   mechanistic details of this interaction and a search for a transcriptional regulator or other proteins

300   that link these pathways will further our understanding of oxidative and nitrosative stress

301   mechanisms in biofilm formation in *S. aureus*, and may lead to the development of novel targets

302   for therapies to eliminate biofilms in the clinic.

303

304   **Materials and Methods**

305   **Strains and culture conditions.** *S. aureus* USA300 (JE2) wild-type cultures were grown from

306   glycerol stocks on TSA (Tryptic Soy Agar) (BD211825) plates and incubated at 37°C overnight.

307   Antibiotics were added when appropriate (erythromycin at 10 μg/ml for mutants *bshC (NE230)*

308   and *NE122* disrupted in SAUSA300_0055, a putative *S*-nitrosobacillithiol reductase. These

309   transposon mutants were provided by the Network on Antimicrobial Resistance in *Staphylococcus*

310   *aureus* (NARSA) for distribution by BEI Resources, NIAID, NIH. For all cultures, a single colony

311   was inoculated in TSB (Tryptic Soy Broth) (BD211825) liquid media and grown at 37°C, shaking

312   at 250 rpm, for 12 hours. All biofilms, except experiments concerning sodium nitrite, were grown

313   in TSB media supplemented with 0.75% glucose. For experiments with sodium nitrite, biofilms

314   were grown in acidified TSB media (pH 5.5) supplemented with 0.75% glucose. Biofilms were

315   grown as follows: cell cultures grown for 12 hours were diluted to a final OD_{600} of 0.2 and added

316   to all wells excluding blank (no cells added) control wells. The plate was incubated for 60 minutes

317   at 37°C under static conditions, wells were washed with PBS and 100 μl of media was added. The

318   plate was incubated for 24 hours at 37°C under static conditions. The media was aspirated, and the

319   biofilm was measured by OD_{600}. Experimental data for each concentration was obtained by

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320   subtracting the OD_{600} readings of the average blank well of each concentration from each

321   corresponding experimental well.

322

323   **Minimum inhibitory concentrations (MICs).** For planktonic MICs, DTT (Fisher scientific

324   BP17225), diamide (MP Biomedicals 0210152705) and sodium nitrite (VWR AA 14244-22) were

325   serially diluted two-fold from 1M to 61 µM in 100 µl TSB. Acidified TSB media (pH 5.5) was

326   used for sodium nitrite. Cell cultures grown for 12 hours, diluted to a final OD_{600} of 0.005 and

327   added to all wells excluding blank (no cells added) control wells. The 384-well plate (Thermo

328   242765) was incubated at 37°C for 24 hours with no shaking. Eleven replicates were performed

329   for each concentration and the MIC assay was performed in replicate. The lowest concentration at

330   which cell turbidity was not visible was determined to be the MIC.

331   For biofilm MIC, DTT, diamide and sodium nitrite were serially diluted two-fold from 1M

332   to 61.04 µM in 100 µl TSB, supplemented with 0.75% glucose or 100 µl acidified TSB (pH 5.5),

333   supplemented with 0.75% glucose (for sodium nitrite). Cell cultures grown for 12 hours were

334   diluted to a final OD_{600} of 0.2 and added to all wells excluding blank (no cells added) control wells.

335   The plate was incubated with no shaking at 37°C for 60 minutes, wells were washed with PBS and

336   100 µl of media with DTT, diamide or sodium nitrite (serially diluted twofold from 1M to 61 µM)

337   was added. The plate was incubated for 24 hours at 37°C under static conditions. The media was

338   aspirated, and the biofilm was measured by OD_{600}. Experimental data for each concentration was

339   obtained by subtracting the OD_{600} readings of average blank well of each concentration from each

340   corresponding experimental well. The blank subtracted OD_{600} values of each experimental well

341   (10 replicates per concentration) was divided by the blank subtracted OD_{600} value of control well

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342   (no DTT or diamide added) to obtain normalized values. Reported data represents mean

343   normalized value and standard deviation.

344

345   **Confocal scanning laser microscopy (CSLM) biofilm visualization.** Cell cultures grown for 12

346   hours, were diluted to a final OD_{600} of 0.2 in 4 ml in a 6-well plate (Falcon 351146). For biofilm

347   formation without added drugs, JE2, BshC and BsnoR, cells were added to TSB, supplemented

348   with 0.75% glucose. For biofilms formed in the presence of DTT or diamide, 1.95 mM diamide or

349   150 mM DTT in TSB supplemented with 0.75% glucose was used. For sodium nitrite experiments,

350   acidified TSB (pH 5.5) supplemented with 0.75% glucose and 20 mM sodium nitrite or no sodium

351   nitrite was used. The 6-well plates were incubated for 60 min at 37°C, wells were washed with

352   PBS and 4 ml of fresh media was added to each well. The plates were incubated for 24 hrs at 37°C

353   with no shaking. Biofilms were stained with 5 µM Syto9 nucleic acid stain (Thermo Fisher

354   S34854) for 1 hour at 37°C in the dark. Medium containing the stain was removed, 4 ml of PBS

355   was added, and biofilms were imaged using an LSM 700 confocal microscope with a 63x

356   objective. Images were analyzed, and biofilm thicknesses were measured using ZEN software

357   (Carl Zeiss).

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359   **Analysis of LMW thiol levels after treatment with sodium nitrite.** *S. aureus* cultures were

360   grown overnight in tryptic soy broth (TSB) before being diluted in 50 mL acidified TSB (pH 5.5)

361   to either OD_{600} 0.2, or 0.1 and incubated at 37 °C. Quadruplicates were treated with 20 mM sodium

362   nitrite once cultures reached OD_{600} 0.5 for 30 minutes at 37 °C. Immediately after treatment, 10

363   µL of culture was serial diluted in PBS by 10^{-5}, 10^{-6}, and 10^{-7}, and 50 µL was plated on tryptic soy

364   agar and incubated at 37 °C to determine if significant killing occurred. The remaining culture was

16

365 pelleted by centrifugation at 4,000 RPM for 10 minutes at 4°C and the supernatants were removed.

366 Pellets were stored at -80 °C overnight. LMW thiol analysis was performed on pellets as previously

367 described (40).

368

369 **Quantative real-time pCR (qPCR).** Expression levels of *katA*, *BSNOR* and *bshC* treated with 20

370 mM sodium nitrite for 5 min or 30 min in the *S. aureus* wildtype strain were measured by qPCR.

371 Samples were grown in acidified (pH 5.5) tryptic soy broth (TSB) until they reached an OD_{600} of

372 0.5 before treatment. Relative gene expression was calculated using the $-2^{\Delta\Delta CT}$ method ($\Delta C_t = C_t$

373 sample $- C_{t\ control}$) and reported as fold change in gene expression of each sample normalized to the

374 *gyrB* housekeeping gene relative to the untreated culture. A Mann-Whitney U test was performed

375 to determine significance $p<0.05$.

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386   **Conflict of Interest.** Clarissa J. Nobile is a cofounder of BioSynesis, Inc., a company developing

387   inhibitors and diagnostics of biofilm formation, and Megha Gulati is a consultant of BioSynesis,

388   Inc.

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573 **Tables**

574 Table 1

	MIC Planktonic			MIC Biofilm		
Strain	DTT	Diamide	Sodium Nitrite	DTT	Diamide	Sodium Nitrite
JE2	250 mM	3.91 µM	31.25 mM	250 mM	7.81 mM	31.25 mM
BshC	250 mM	3.91 µM	31.25 mM	250 mM	7.81 mM	62.50 mM
BsnoR	250 mM	3.91 µM	31.25 mM	250 mM	7.81 mM	31.25 mM

575

576

577 **Figure Legends**

578 **Figure 1 – Reduced Bacillithiol level in the BSNOR mutant after treatment with sodium**

579 **nitrite.** BSH levels measured in *S. aureus* wild type (WT) cells and BSNOR mutant using HPLC

580 analysis. BSH levels were measured in both cell types with no treatment and following treatment

581 with sublethal concentrations of sodium nitrite (20 mM). The experiment was performed in

26

582   quadruplicate and data presented represents mean and standard deviation. The right axis indicates

583   the CFU/mL count which is represented by the triangle with error bars for standard deviation. The

584   left axis indicates the bacillithiol concentrations that are represented by bars with standard

585   deviation error bars. A student t-test was performed to determine significance; p-values ≤ 0.05 (*).

586

587   **Figure 2 – Expression levels of *BSNOR*, *bshC*, and *katA* genes under various stressors.** A.

588   Expression of *BSNOR*, *bshC*, and *katA* treated with 20 mM sodium nitrite for 5 min or 30 min in

589   *S. aureus* wildtype. B. Expression of *BSNOR, katA* and *bshC* under oxidative stress (5 mM

590   diamide, 100 μM cumene hydroperoxide, and 100 μM H_2O_2 for 30 min) in *S. aureus* wildtype.

591   Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (ΔCt = Ct sample - Ct control)

592   and reported as fold change in gene expression of each sample normalized to the *gyrB*

593   housekeeping gene relative to the untreated culture. A Mann-Whitney U test was performed to

594   determine significance; p-values ≤ 0.05 (*).

595

596   **Figure 3 – Confocal scanning laser microscopy (CSLM) images of *S. aureu*s 24 hr biofilms.**

597   Images show depth (side view) and top view of biofilms formed by wild-type JE2 (A and D),

598   Δ*bshC* (B and E), and Δ*bsnoR* (C and F). Biofilms were formed on the bottom of 6-well

599   polystyrene plates and stained with 2 μM Syto9 for 1 hr in the dark. Biofilms were imaged using

600   an LSM-700 confocal microscope with a 63x objective and analyzed using ZEN software. Scale

601   bars are shown in white in each panel and represent 5 μm.

602
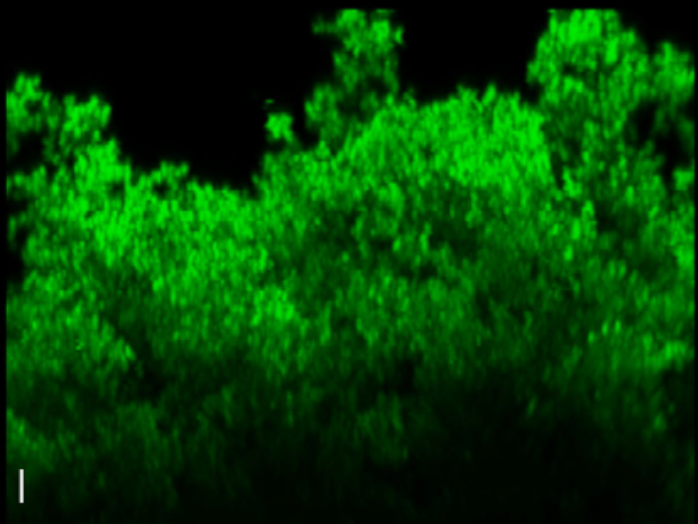
603   **Figure 4 – Effects of sublethal concentrations of DTT and diamide on *S. aureus* biofilms by**

604   **CSLM.** Images show depth (side view) and top view of biofilms formed by wild-type JE2 in TSB

27

605   media supplemented with 0.75% glucose (TSB-G) (A and D), TSB-G media and 150mM DTT (B

606   and E), and TSB-G media with 1.95 mM diamide (C and F). Biofilms were formed on the bottom

607   of 6-well polystyrene plates and stained with 2 μM Syto9 for 1 hr in the dark. Biofilms were

608   imaged using an LSM-700 confocal microscope with a 63x objective and analyzed using ZEN

609   software. Scale bars are shown in white in each panel and represent 5 μm.

610

611   **Figure 5 – Effect of sublethal concentration of sodium nitrite on *S. aureus* biofilms by CSLM.**

612   Images show depth (side view) and top view of biofilms formed by wild-type JE2 in acidified TSB

613   media supplemented with 0.75% glucose (aTSB-G) (A and C), and TSB-G media and 20mM

614   sodium nitrite (B and D). Biofilms were formed on the bottom of 6-well polystyrene plates and

615   stained with 2 μM Syto9 for 1 hr in the dark. Biofilms were imaged using an LSM-700 confocal

616   microscope with a 63x objective and analyzed using ZEN software. Scale bars are shown in white

617   in each panel and represent 5 μm.

618

619   **Supplemental Figure 1 – *S. aureus* cultures grown in nutrient rich media.** Overnight cultures

620   were grown in tryptic soy broth (TSB) at 37°C while shaking before being diluted to OD_{600} 0.05

621   in 200 uL in a 96-well plate with indicated treatments. *S. aureus* wildtype (green), *bshC*[-] (blue),

622   and *BSNOR*[-] (purple) were incubated at 37°C for 24 hrs with reads every hour. *S. aureus* was either

623   non-treated, treated with GSNO at 1 mM or 5 mM, treated with formaldehyde at 0.25 mM, 0.75

624   mM, or 1.5 mM, treated with acidified sodium nitrite pH 5.5 (20 mM), or treated with

625   methylglyoxal at 0.75 mM or 1.5 mM.

28

A

B

C

D