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2   **The impact of combinatorial stress on the growth dynamics and metabolome of *Burkholderia***

3   ***mesoacidophila* demonstrates the complexity of tolerance mechanisms**

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8   **Running title: Growth dynamics *B. mesoacidophila***

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14

15   **Abstract**

16   Aims: The recently sequenced *Burkholderia mesoacidophila* (previously *Pseudomonas*

17   *mesoacidophila*) is a soil organism and as such will be exposed to multiple concurrent stresses in the

18   natural environment. The combinatorial stress potentially experienced by microbes in soil has not

19   been investigated in detail.

20   Methods and Results: The impact of combinatorial stress on growth was investigated using tripartite

21   variables – temperature, nutritional environment and either osmotic or oxidative stress. In

22   nutritionally stringent conditions, increasing diamide concentration had no effect on growth while

23   increasing H_2O_2 concentration reduced both growth rate and maximum density. Metabolomic

24   studies with oxidative stress revealed specific (unidentified) metabolites associated with diamide

25   tolerance, and an overwhelming dominance of sugars and sugar alcohols in nutritionally stringent

26   conditions with and without the additional stressor.

27   Conclusions: Combinatorial stress tolerance is complex. Temperature had the greatest independent

28   impact on growth, while the impact of the nutritional environment played a key role in oxidative

29   stress tolerance. In nutritionally stringent conditions, the metabolome suggested different tolerance

30   mechanisms for different types of oxidative stress.

31   Significance and Impact of Study: This work demonstrates the specificity of the stress response, and

32   the need to consider multiple environmental factors to meaningfully investigate tolerance. Both

33   environmental and clinical settings subject bacteria to combinatorial stress and this should be

34   considered in the design of further studies.

35   Keywords: stress tolerance; *Burkholderia*; growth dynamics; metabolome; diamide; oxidative stress

36   **Introduction**

37   *Burkholderia mesoacidophila* (the proposed new name for the species previously called

38   *Pseudomonas mesoacidophila*) is a soil organism which belongs to the *Burkholderia cepacia* complex

39   (Bcc; Loveridge *et al.*, 2017) and is therefore of both environmental and potentially clinical interest.

40   Organisms in all ecological niches experience stressful conditions sometimes. Bacteria, as single

41   celled organisms, are both uniquely vulnerable to environmental change and exquisitely well-

42   adapted to rapid metabolic adaptation to enable survival. *B. mesoacidophila* was originally identified

43   from soil samples (Imada *et al.*, 1981), and yet there is a paucity of further information about its

44   ecology and growth dynamics. Its genome has recently been sequenced, and shows considerable

45   potential for specialised metabolite production (Loveridge *et al.*, 2017). Specialised metabolites are

46   often associated with growth in stressful conditions, but little is currently known about the stress

47   tolerances of this under-studied species and it has not yet been characterised in terms of metabolic

48   potential. As these specialised metabolites may be utilised in industrial or therapeutic settings, the

49   effect of stress on *B. mesoacidophila* is of general interest.

50   Osmotic stress and oxidative stress are experienced by bacteria in both the clinical and

51   environmental setting and must be tolerated or ameliorated. Some studies of a variety of stress

52   responses in the Burkholderiaceae have been conducted. In an elegant study of osmotic stress,

53   Behrends *et al.* (2011) showed a decrease in culture optical density and an increase of glycine-

54   betaine and/ or trehalose concentration in response to 0.5 mol l^{-1} NaCl in a range of strains of

55   *Burkholderia cenocepacia*. The study highlighted that assumptions of parity across the genus are

56   unwise as they also demonstrated a wide range of inter-strain variability. Pumirat *et al.* (2009) also

57   used a salt stress (of 150 mmol l^{-1} NaCl) to study the impact on the secretome of *Burkholderia*

58   *pseudomallei*, showing changes in proteins associated with metabolic pathways, solute transport

59   and drug resistance amongst others. The impact on growth was not reported. A comprehensive

60   study into the effects of several different stressors on survival and growth of *B. pseudomallei*

61   demonstrated how robust some members of this genus can be (Robertson *et al.*, 2010), but did not

62   combine stresses or investigate the mechanisms of survival. Pumirat *et al.* (2017) used exposure to a

63   range of salt concentrations to prime *B. pseudomallei* cultures before challenge with either high

64   temperature or oxidative stress (mediated by H_2O_2), and an increase in thermo- and oxidative stress

65   tolerance was seen. Further studies have highlighted susceptibility to oxidative stress as being a

66   function of growth temperature (Paksanont *et al.*, 2018). These last two studies highlight the

67   potential for co-ordination of stress tolerance mechanisms across different specific stress-inducing

68   conditions, and the inter-dependence of stress responses in enabling growth in challenging

69   conditions. This reinforces the need for studies utilising multiple concurrent stressors to gain a true

70   understanding of the survival ability of this hardy genus.

71   This study therefore aims to further this field by considering the impact of exposure to simultaneous,

72   multiple stressors, which is more likely to be how stress is experienced in the soil environment. The

73   stresses chosen are known in other species to exhibit some features of co-regulation (e.g. ScbR2 in

74   *Streptomyces coelicolor*, Li *et al.*, 2015; σ^B in *Listeria monocytogenes*, Liu *et al.*, 2017) and are known

75   to interact and act on some of the same pathways or processes in the cell. General oxidative stress

76   was simulated here with the addition of an increasing concentration of H_2O_2 to the media. This

77   exposes cells to free radicals and reactive oxygen species (ROS) which damage proteins, DNA and

78   lipids. A particular example of oxidative stress is a disulphide stress, caused by the addition of

79   diamide to the media. Diamide is a membrane-permeable oxidizer which targets protein cysteine

80   thiol groups, causing disulphide bridges to form, ultimately leading to both a redox imbalance and

81   potential protein misfolding (Kallifidas *et al.*, 2010). Osmotic stress is simulated by the addition of

82   KCl to the media, and the impact that has on water availability within the cell.

83   These three stresses were applied individually at a range of concentrations. Each stress condition

84   was tested in two different media conditions, one a nutrient rich environment (Luria Bertani, LB) and

85   the other a more limited nutrient broth (Czapek-Dox, CD). CD broth is a sucrose-based medium

86   containing 35 mmol l^{-1} sodium nitrate. The recently sequenced genome suggests *B. mesoacidophila*

87   has genetic capacity to assimilate and metabolise both sucrose and nitrate/nitrite, and previous

88   work has demonstrated both reduction of nitrates and utilisation of inorganic nitrogen sources

89   (Kintaka *et al*., 1981). Further work on related species suggests nitrate is a preferred nitrogen source

90   at a concentration of at least 10 mmol l^{-1} and therefore nitrogen availability is unlikely to be limiting

91   to growth (Lardi *et al*., 2015; Mangalea *et al*., 2017). It was anticipated therefore that this medium

92   would present a more stringent nutritional environment while providing both carbon and nitrogen in

93   a usable form. The experimental work was then conducted at four different temperatures ranging

94   from a warm environmental temperature (25 °C) to that associated with pathogenicity in humans

95   (40 °C). Thus the tripartite combination of nutritional availability, temperature challenge and added

96   stressor was investigated. Although liquid culture does not necessarily inform on behaviour in soil or

97   the clinic, it was used here as larger scale production and metabolite extraction would be more

98   feasible if interesting specialised metabolites were detected (although that is beyond to scope of the

99   current work). The results show that *B. mesoacidophila* can tolerate a wide range of growth

100   conditions, and that nutritional availability has a differential impact on stress tolerance.

101   **Materials and Methods**

102   **Microbial culture and plate reader set-up**

103   *B. mesoacidophila* (ATCC 31433) overnight cultures were grown in 5 ml LB low salt broth, incubated

104   at 30 °C and 150 rpm, to an optical density of 0.2 at 550 nm. 20 µl of overnight culture was added to

105   each well, with 180 µl of the appropriate test media. All plates were run for 24 hours in a

106   ThermoScientific Multiskan FC, shaken for 10 seconds prior to a reading at 550 nm every hour. Two

107   media with different nutritional content were used – low salt LB broth and CD broth (Sigma)

108   amended with KCl (Fisher) with give a total molarity of 85.5 mmol l^{-1} salt, which is equivalent to the

109   LB. Osmotic stress was induced using the addition of KCl to these baseline media to give a range of

110   total salt molarities from 85.5 mmol l^{-1} to 375 mmol l^{-1}. (Unamended CD was also used in the

111   osmotic stress experiment, which has 6.7 mmol l^{-1} KCl.) Oxidative stress was induced by the addition

112   of H$_2$O$_2$ (Fisher) at concentrations ranging from 88 µmol l^{-1} to 880 µmol l^{-1}. Disulphide stress was

5

113   induced using a range of diamide (Fisher) concentrations from 1 mmol l^{-1} to 0.125 mmol l^{-1}. Each

114   experiment was conducted at four temperatures: 25, 30, 37 and 40 °C. All experiments were

115   conducted in triplicate, with blank media controls also in triplicate for all conditions.

Statistical analysis of growth dynamics

117   Growth curves were initially visualised using Generalised Additive Mixed Models (GAMMs) with

118   cubic spline smoothing functions. Changes in optical density (the response variable) were modelled

119   over time, due to variation in both temperature stress and chemical stressor concentration (i.e.,

120   time x temperature + time x concentration). Since each of the three types of chemical stressor was

121   applied over differing ranges of concentrations, this was done separately for each stressor. This

122   analysis was performed for each growth medium (LB and CD). Replicates grouped by experimental

123   plates were modelled as random intercepts, temporal autocorrelation was modelled as a first-order

124   autoregressive process (AR1), and Gaussian residual errors were assumed (and verified by visual

125   inspection). Results were visualised graphically for each stressor (temperature and chemical

126   concentration) while averaging across the effects of the third stressor.

127   Having identified the underlying functional form of the dynamics using smoothing splines, logistic

128   growth curves were subsequently fitted to individual combinations of temperature stress and

129   chemical stressor concentration using Nonlinear Mixed-Effects Models (NLMEs) with the same

130   random effects and variance-covariance structure as described above for GAMMs. This allowed

131   estimation of averages and 95 % confidence intervals for the asymptote (growth curve plateau),

132   midpoint (time at which optical density reaches 50 % of the maximum), and scale parameter

133   (relating to the slope of the logistic curve at the midpoint, so maximum growth rate) for each

134   treatment combination. This approach permitted statistical comparison of growth dynamics

135   between treatments.

Culture for metabolite extractions

137   For metabolite extractions, cultures were inoculated with 100 μl of fresh overnight culture (as

138   described in the section above) into 5 ml CD85, CD85 with 220 μmol l^{-1} H_2O_2 or CD85 with 1 mmol l^{-1}

139   diamide. The cultures were incubated at 30 ^{o}C, 200 rpm for 20 hours. All conditions were conducted

140   in triplicate.

141   **Metabolite quenching and extraction**

142   The cultures were harvested by centrifugation at 4500 x *g* for 10 minutes at room temperature. The

143   supernatant was decanted, and the remaining cell pellet was flash frozen under liquid N_2. 1 ml

144   methanol (Fisher) was added and the samples were then sonicated in a cool water bath for 1 hour

145   with vortexing part way through to ensure good cell breakage. All samples were centrifuged at 4500

146   x *g* for 5 minutes at room temperature. The solvent layer was removed into separate tubes and

147   dried *in vacuo*.

148   **GC-MS analysis**

149   The dried samples were derivatized to increase volatility, according to the following methodology:

150   the dried samples were dissolved in 30 μl of 15 mg/ml methoxyamine in pyridine and baked for 60

151   minutes at 70 ^{o}C. 50 μl of MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide) was added, and

152   the samples were incubated at 40 ^{o}C for 90 minutes. The samples were run on an Agilent HP5975C

153   GC-MS, using a 1 μl injection volume and a Durabond DB-5 column (length 30 m, diameter 0.25 mm

154   with a 0.25 μm film) with helium as the carrier gas. The column temperature was held at 50 ^{o}C for 2

155   minutes, increased to 300 ^{o}C at 10 ^{o}C /min, then held for a further 15 minutes at 300 ^{o}C . Mass

156   spectra were acquired using an electron impact ion source with an emission voltage of 70 eV, single

157   quadrupole ion separation and detection between 50 and 600 amu. Results were analysed using

158   OpenChrom and the NIST database.

159   **Results**

160   **Growth dynamics during combinatorial stress**

161   To investigate the impact of multiple concurrent stressors on growth rate and maximum cell density,

162   growth curves were set up using LB and CD85 broth. Both media have a pH of approximately 7, so

163   acidity/alkalinity was not considered as an added variable. The 24 hour incubation time enabled

164   logarithmic growth and entry into stationary phase when the bacteria were in control conditions in

165   both media. As anticipated, LB consistently represented a nutritionally replete environment and as

166   such, supported a higher maximum cell density than CD85. This simpler medium provided a more

167   nutritionally stringent environment, appearing to provide sufficient usable carbon and nitrogen to

168   support growth but with a reduced end-point density, suggesting some growth limitation. The

169   additional stressors tested in conjunction with nutritional availability were temperature (range 25-40

170    $^{\circ}$C), salt concentration (85.5 mmol l^{-1} to 375 mmol l^{-1}) and oxidative stress (0.125 mmol l^{-1} to 1 mmol

171   l^{-1} diamide or 88 µmol l^{-1} to 880 µmol l^{-1} H$_2$O$_2$). *B. mesoacidophila* showed optimal growth at 30 $^{\circ}$C in

172   both nutritional conditions, and barely grew at higher temperatures. Thermotolerance was such a

173   strong factor that when temperature was high, neither of the other two stressors (nutrient

174   availability or chemical stressor) had an additional effect (R side panel of both Figures 1 and 2). In

175   contrast, nutrient availability was a strong predictor for maximum optical density reached at 25 $^{\circ}$C

176   and 30 $^{\circ}$C, with the limited nutritional environment limiting cell density. (At 37 $^{\circ}$C and 40 $^{\circ}$C the

177   additional nutrient availability did not overcome the effect of temperature and cultures grew equally

178   poorly.)

179   **Stress tolerance in nutritionally replete conditions**

180   In nutritionally replete conditions, while *B. mesoacidophila* was able to grow well at 25 $^{\circ}$C, 30 $^{\circ}$C

181   remained the overall optimal temperature for both growth rate and final optical density for all three

182   additional stressors (Figure 1, R panel). The salt tolerance experiments (Figure 1, top row) revealed

183   optimal growth occurred at 125 mmol l^{-1} total salt concentration. Unamended LB media contained

184   sufficient salt (85.5 mmol l^{-1}) for this never to be a limiting factor at low concentration, and salt

185   tolerance with minimal impact on growth was observed at 250 mmol l^{-1}. At 375 mmol l^{-1} a

186   substantial drop in final cell density was noted, at both 25 $^\circ$C and 30 $^\circ$C. Using an oxidative stress

187   induced by either H_2O_2 or diamide failed to impact on the growth rate or final optical density of *B.*

188   *mesoacidophila*, even at the highest concentration (880 µmol l^{-1} or 1 mmol l^{-1} respectively, Figure 1,

189   middle and bottom rows).

190   **Stress tolerance in nutritionally stringent conditions**

191   In nutritionally stringent conditions, growth was consistently best at 30 $^\circ$C across all stress conditions

192   but response to temperature as one of multiple stresses was neither consistent nor predictable

193   (Figure 2, R panel). It was noted that during combined nutritional and oxidative stress (induced by

194   either H_2O_2 or diamide) that temperature tolerance had a narrow range, with poor growth at 25 $^\circ$C,

195   37 $^\circ$C and 40 $^\circ$C (Figure 2, R panel, top and bottom rows). This was in contrast to the data obtained

196   using nutritional and osmotic stress, which suggested low level salt supplementation (85.5 mmol l^{-1}

197   and 175 mmol l^{-1}) enabled comparable growth and cell densities at 25 $^\circ$C and 30 $^\circ$C (Figure 2, R

198   panel, middle row). Growth was still poor at 37 $^\circ$C and 40 $^\circ$C, regardless of salt concentration.

199   At the optimal temperature of 30 $^\circ$C, there were differences noted in combined stress tolerance

200   based on the concentration of the chemical stressor. In osmotically challenging conditions, a great

201   deal of variation was seen, with both salt stringency and salt overload suggested in the data. At low

202   salt concentration (unamended CD has a salt concentration of 6.7 mmol l^{-1}), growth was as limited as

203   it was at high salt concentration (Figure 2, L panel, middle row). No impact on growth rate or final

204   optical density was seen with diamide supplementation (Figure 2, L panel, top row). This was in

205   contrast to the other source of oxidative stress used, H_2O_2, which showed a decrease in maximum

206   optical density with increasing concentration at 30 $^\circ$C (Figure 3).

207   **Statistical comparison of stress tolerance in the two nutritional conditions**

208   Statistical analysis of these results using the logistic growth model showed that the asymptote for

209   both media was highest at intermediate salt concentrations, confirming that the selection of

210   concentrations spanned the tolerance range for *B. mesoacidopila*.

211   When subjected to increasing diamide stress, the impact on growth rate (measured as the midpoint

212   of the curve) was small but negative, more so in the richer medium. The maximum optical density

213   achieved was consistently higher in LB. More of a surprise was the finding that in LB maximum

214   growth decreases with increasing diamide concentration, whereas this is not seen when grown in

215   CD85. Broadly speaking, diamide stress did not have a big effect on growth as part of the

216   combinatorial stress set-up, and nutritional availability had far more impact and was more limiting

217   than diamide.

218   When exposed to H_2O_2, as in the diamide treatment, the asymptote was higher in LB than CD85. In

219   contrast to the diamide data, maximum growth in H_2O_2 treated cells decreased with increasing H_2O_2

220   in CD85 but not in LB, indicating a potential reduction in H_2O_2 stress tolerance during nutritionally

221   stringent conditions. The midpoint of growth (indicative of growth rate) was similar in both media

222   and decreased with increasing H_2O_2 concentration.

223   In summary, *B. mesoacidophila* growth is reduced in CD85 compared to LB but the response to

224   oxidative stress in CD85 varies with which stressor is used, suggesting different pathways to

225   tolerance.

Metabolomics of combinatorial stress

227   The growth dynamics data were used to identify conditions in which *B. mesoacidophila* could grow

228   exponentially and enter stationary phase within a 20 hour time frame. Growth was inhibited in CD85

229   compared to LB, suggestive of a degree of nutritional stress. A total salt concentration of 85 mmol l^{-1}

230   in CD media was permissive for our growth requirements, while being sub-optimal for growth (as

231   shown in the growth dynamics experiments). The CD media amended to 85 mmol l^{-1} total salt (CD85)

232 was chosen as a baseline media, for enabling sufficient growth to sample without being optimal in

233 terms of nutritional content or salt concentration. The impact of diamide on growth in CD85 at 30 °C

234 was shown above to be negligible, while the impact of H_2O_2 was considerable. These were therefore

235 followed up at concentrations which had an impact on cell density but still allowed log growth and

236 entry into stationary phase within 20 hours. Intracellular metabolites were extracted from cells

237 grown in CD85, CD85 with 1 mmol l^{-1} diamide and CD85 with 220 μmol l^{-1} H_2O_2, and derivatised with

238 MSTFA to increase the volatility of molecules such as alcohols (including simple sugars) and

239 carboxylic acids. Derivatised samples were analysed by GC-MS to identify any differences that may

240 be associated with the tolerance of diamide and the stress induced by H_2O_2.

241

242 The majority of GC-MS peaks putatively identifiable with a NIST score of 80 or higher were sugars

243 (Figure 4 and Supplementary Information Table S1), and the remainder were sugar alcohols (glycerol

244 and xylitol) and a carboxylic acid (gluconic acid). Glycerol and glucose were detected in all samples,

245 mannose in all but one CD85 with H_2O_2 sample (Figure 5 and Supplementary information Table S2).

246 Analysis of the relative abundance of the sugars and related alcohols was performed, based on the

247 raw peak areas in the total ion current chromatogram. In all samples, simple sugars (glucose and

248 mannose) were the dominant metabolites detected, by three or four orders of magnitude. Gluconic

249 acid was detected in greater abundance than glycerol only in the CD85 samples. Diamide treatment

250 did not lead to significant changes in the concentrations of any metabolite identified, although the

251 relative proportion of xylitol to glycerol increased significantly, whereas H_2O_2 treatment led to a

252 10±2-fold increase in the amount of glycerol. The samples contained between 10 and 15 unique

253 compound peaks (excluding peaks for different derivatives of the same parent compound and

254 multiple peaks identifying the same compound, within the constraints of robust NIST identification).

255 Diamide treated samples uniquely and reproducibly contained two small peaks – one at 7.142

256 minutes, and one at approximately 53 minutes (Figure 6), neither of which was identifiable using the

257 NIST database.

258 The H_2O_2 treated samples were less consistent, perhaps suggestive of a more disordered metabolic

259 response to stress. In one sample (#2) 2 keto-I-gluconic acid was putatively identified with a NIST

260 score of 64, but not seen in either of the other replicates. The same #2 sample also putatively

261 contained butanal (NIST score 80), which was not seen in any other samples. Sample #3 uniquely

262 contained a tentatively identified 3-O-β-d-mannopyranosyl-d-glucitol (NIST score 64). None of these

263 compounds were identified in the CD85 samples or diamide samples.

264 **Discussion**

265 This work is one of few to examine the role of combinatorial stress on growth dynamics and the

266 metabolome in *Burkholderia*. Uniquely the impact of a range of each factor has been studied,

267 enabling a more global view of the impact of stress on growth. Thermo-tolerance has been shown to

268 be the strongest determinant of *B. mesoacidophila* growth, with a strong preference for 25-30 °C

269 temperature range, and extremely inhibited growth above that. This suggests this species has

270 limited human pathogenic potential due to temperature tolerance, despite being part of the Bcc. In

271 contrast to *B. pseudomallei* (Pumirat *et al.*, 2017) the presence of salt in the medium did not impact

272 on thermo-tolerance, and nor did nutritional stress. While the asymptote was lower in nutritionally

273 stringent conditions in all experiments, the salt tolerance range was comparable to that in rich

274 media and no major differences in thermo-tolerance were observed. This may be due to the stresses

275 being experienced combinatorially in this work but sequentially in the aforementioned study.

276 Tolerance of oxidative stress was variable depending on both the type of oxidative stress used and

277 the nutritional conditions. While the range of diamide concentrations used here did not show an

278 impact on *B. mesoacidophila* growth dynamics in either of the nutritional conditions or in terms of

279 an additive effect on temperature tolerance, differences were seen in the metabolome profile. Two

280   unique peaks were identified in the diamide spiked cultures which were absent in all other samples.

281   Although they remain unidentified, their presence suggests induction of tolerance pathways

282   enabling normal growth to continue. Tolerance to diamide is typically mediated by NADPH-

283   dependent thioredoxins reducing damaged disulphides (Chavarria *et al.*, 2012), but this work may

284   also indicate production of new, protective metabolites. Additionally, the relative abundance of

285   xylitol in the diamide treated cells is interesting as xylose metabolism has been shown to be

286   impacted by the presence of diamide in yeast. Hector *et al.* (2011) found that at concentrations

287   which did not impact negatively on growth, diamide treatment up-regulated genes involved in xylose

288   metabolism. This would be an interesting avenue for future work.

289   In contrast, using an oxidative stress induced by the presence of H_2O_2 did show an impact on growth

290   when under nutritionally stringent conditions. Previous work on this strain (originally identified as

291   *Pseudomonas mesoacidophila* strain SB-72310) characterised its ability to produce catalase in

292   response to H_2O_2 challenge (Kintaka *et al.*, 1981) but did not report the effect on growth. In this

293   work, when nutritionally replete the wide range of H_2O_2 concentrations used did not have an impact

294   on asymptote, although it did reduce the midpoint – indicative of a decrease in growth rate, even if

295   the same final cell density was still achieved. When grown in nutritionally stringent conditions, the

296   same effect was seen with a gradual reduction in the midpoint with increasing H_2O_2 concentration,

297   but there was also a significant decline in asymptote at the higher concentrations of H_2O_2 suggesting

298   a failure of tolerance mechanisms to mitigate against generalised oxidative damage. This, combined

299   with the metabolomics, suggests that the pathways induced to tolerate oxidative stress may be

300   different depending on the type of oxidative damage the cell experiences.

301   The mechanisms of oxidative stress tolerance pathway induction are complex with multiple

302   transcription factors identified as important (e.g. σE, Jitprasutwit *et al.*, 2014; OxyR, Si *et al.*, 2017)

303   and functional redundancy built in to the proteome (e.g. Reott *et al.*, 2009). The genome of *B.*

304   *mesoacidophila* encodes a range of predicted proteins with known roles in protection such as

305 superoxide dismutase, catalases, peroxidases and thiol peroxidases (Loveridge *et al.*, 2017). The

306 roles of glutathione and thioredoxin in protection against oxidative stress are well established

307 (Carmel-Harel and Storz, 2000) as both systems contribute to the continuance of a reduced

308 intracellular environment. Evidence suggests that within this group of protective proteins,

309 differential induction and expression can be seen on exposure to either diamide or H_2O_2 as a source

310 of stress (Somprasong *et al.*, 2012). Viewing the results presented here through the lens of this prior

311 knowledge suggests that combinatorial stress including nutritional stringency may have a disruptive

312 effect on these processes, with greater impact seen in a generalised stress response. While the cell

313 induced production of novel, possibly protective metabolites in response to diamide, the same co-

314 ordination of an effective stress response was not seen in the H_2O_2 metabolomics.

315 The metabolomic studies were conducted in a nutritionally stringent environment, with sub-optimal

316 salt concentration (85 mmol l^{-1}) and either 1 mmol l^{-1} diamide or 220 µmol l^{-1} H_2O_2, thereby

317 subjecting the cells to a tripartite combinatorial stress. The metabolites detected at the highest

318 concentration and in all samples were glycerol and glucose, with mannose detected in all but one

319 sample (CD85 with H_2O_2). Previous work has shown *B. mesoacidophila* (identified as strain SB-72310)

320 to assimilate and grow on all three of these compounds as the sole carbon source (Kintaka *et al.*,

321 1981). Glucose is the most abundant monosaccharide in biological systems, so its presence in

322 relatively high abundance is to be expected. The sugar alcohol glycerol (synonym glycerin) is an

323 intermediate in carbohydrate and lipid metabolism. It has also been identified as part of the

324 endogenous stress response in bacteria (Chen *et al.*, 2017), and conducive to thermotolerance of

325 *Pseudomonas* when applied exogenously (Srivastava *et al.*, 2008). Previous evidence also suggests

326 both glycerol and glucose may be accumulated by *Pseudomonas putida* and *Pseudomonas*

327 *fluorescens* during salt stress (Sévin *et al.*, 2015). The ubiquitous presence of glycerol in the

328 metabolome of B. mesoacidophila growing under tripartite stress is therefore unsurprising.

329 Mannose and mannose-derived compounds have been shown to be important in production of

330 protective exopolysaccharides and to confer tolerance to ROS (Ge *et al.*, 2013; Cuzzi *et al.*, 2014) and

331 contribute to osmoregulation (Smith *et al.*, 1990).

332 In summary, while models of genomic similarity may cluster species together in the Bcc, this hides a

333 wealth of diversity in terms of tolerance and ability to survive adverse conditions. Individual species

334 are worthy of investigation to understand growth dynamics and no assumptions can be made about

335 common stress tolerance pathways and processes. Combinatorial stress is likely to be encountered

336 in the natural environment, either in a human host or in soil, and studies must therefore consider

337 the impact of multiple stresses on the growth and survival of *Burkholderia* species.

341 Conflict of Interest: The authors declare no conflict of interest.

342 **References**

343 Behrends V, Bundy JG, Williams HD. Differences in strategies to combat osmotic stress in

344 *Burkholderia cenocepacia* elucidated by NMR-based metabolic profiling. Lett Appl Microbiol. 2011

345 Jun;52(6):619-25. doi: 10.1111/j.1472-765X.2011.03050.x. Carmel-Harel O, Storz G. Roles of the

346 glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and

347 *Saccharomyces cerevisiae* responses to oxidative stress. Annu Rev Microbiol. 2000;54:439-61.

348 Chavarría M, Nikel PI, Pérez-Pantoja D, de Lorenzo V. The Entner-Doudoroff pathway empowers

349 *Pseudomonas putida* KT2440 with a high tolerance to oxidative stress. Environ Microbiol. 2013

350 Jun;15(6):1772-85. doi: 10.1111/1462-2920.12069.

351   Chen MJ, Tang HY, Chiang ML. Effects of heat, cold, acid and bile salt adaptations on the stress

352   tolerance and protein expression of kefir-isolated probiotic *Lactobacillus kefiranofaciens* M1. Food

353   Microbiol. 2017 Sep;66:20-27. doi: 10.1016/j.fm.2017.03.020.

354   Cuzzi B, Herasimenka Y, Silipo A, Lanzetta R, Liut G, Rizzo R, Cescutti P. Versatility of the *Burkholderia*

355   *cepacia* complex for the biosynthesis of exopolysaccharides: a comparative structural investigation.

356   PLoS One. 2014 Apr 10;9(4):e94372. doi: 10.1371/journal.pone.0094372.

357   Ge X, Wang W, Han Y, Wang J, Xiong X, Zhang W. *Methylovorus* sp. MP688 exopolysaccharides

358   contribute to oxidative defense and bacterial survival under adverse condition. World J Microbiol

359   Biotechnol. 2013 Dec;29(12):2249-58. doi: 10.1007/s11274-013-1391-4.

360   Hector RE, Mertens JA, Bowman MJ, Nichols NN, Cotta MA, Hughes SR. Saccharomyces cerevisiae

361   engineered for xylose metabolism requires gluconeogenesis and the oxidative branch of the pentose

362   phosphate pathway for aerobic xylose assimilation. Yeast. 2011 Sep;28(9):645-60. doi:

363   10.1002/yea.1893.

364   Imada A, Kitano K, Kintaka K, Muroi M, Asai M. Sulfazecin and isosulfazecin, novel β-lactam

365   antibiotics of bacterial origin. 1981. Nature 289:590–591. https://doi.org/10.1038/289590a0.

366   Jitprasutwit S, Ong C, Juntawieng N, Ooi WF, Hemsley CM, Vattanaviboon P, Titball RW, Tan P,

367   Korbsrisate S. Transcriptional profiles of *Burkholderia pseudomallei* reveal the direct and indirect

368   roles of Sigma E under oxidative stress conditions. BMC Genomics. 2014 Sep 12;15:787. doi:

369   10.1186/1471-2164-15-787.

370   Kallifidas D, Thomas D, Doughty P, Paget MS. The sigmaR regulon of *Streptomyces coelicolor* A32

371   reveals a key role in protein quality control during disulphide stress. Microbiology. 2010 Jun;156(Pt

372   6):1661-72. doi: 10.1099/mic.0.037804-0.

373   Kintaka K, Haibara K, Asai M, Imada A. Isosulfazecin, a new beta-lactam antibiotic, produced by an

374   acidophilic pseudomonad. Fermentation, isolation and characterization. J Antibiot (Tokyo). 1981

375   Sep;34(9):1081-9.

376   Lardi M, Aguilar C, Pedrioli A, Omasits U, Suppiger A, Cárcamo-Oyarce G, Schmid N, Ahrens CH, Eberl

377   L, Pessi G. σ54-Dependent Response to Nitrogen Limitation and Virulence in Burkholderia

378   cenocepacia Strain H111. Appl Environ Microbiol. 2015 Jun 15;81(12):4077-89. doi:

379   10.1128/AEM.00694-15.

380   Li X, Wang J, Li S, Ji J, Wang W, Yang K. ScbR- and ScbR2-mediated signal transduction networks

381   coordinate complex physiological responses in *Streptomyces coelicolor*. Sci Rep. 2015 Oct 7;5:14831.

382   doi: 10.1038/srep14831.

383   Liu Y, Orsi RH, Boor KJ, Wiedmann M, Guariglia-Oropeza V. Home Alone: Elimination of All but One

384   Alternative Sigma Factor in *Listeria monocytogenes* Allows Prediction of New Roles for σB. Front

385   Microbiol. 2017 Oct 11;8:1910. doi: 10.3389/fmicb.2017.01910.

386   Loveridge EJ, Jones C, Bull MJ, Moody SC, Kahl MW, Khan Z, Neilson L, Tomeva M, Adams SE, Wood

387   AC, Rodriguez-Martin D, Pinel I, Parkhill J, Mahenthiralingam E, Crosby J. Reclassification of the

388   Specialized Metabolite Producer *Pseudomonas mesoacidophila* ATCC 31433 as a Member of the

389   *Burkholderia cepacia* Complex. J Bacteriol. 2017 Jun 13;199(13). pii: e00125-17. doi:

390   10.1128/JB.00125-17.

391   Mangalea MR, Plumley BA, Borlee BR. Nitrate Sensing and Metabolism Inhibit Biofilm Formation in

392   the Opportunistic Pathogen Burkholderia pseudomallei by Reducing the Intracellular Concentration

393   of c-di-GMP. Front Microbiol. 2017 Jul 25;8:1353. doi: 10.3389/fmicb.2017.01353.

394   Paksanont S, Sintiprungrat K, Yimthin T, Pumirat P, Peacock SJ, Chantratita N. Effect of temperature

395   on *Burkholderia pseudomallei* growth, proteomic changes, motility and resistance to stress

396   environments. Sci Rep. 2018 Jun 15;8(1):9167. doi: 10.1038/s41598-018-27356-7.

397   Pumirat P, Saetun P, Sinchaikul S, Chen ST, Korbsrisate S, Thongboonkerd V. Altered secretome of

398   *Burkholderia pseudomallei* induced by salt stress. Biochim Biophys Acta. 2009 Jun;1794(6):898-904.

399   doi: 10.1016/j.bbapap.2009.01.011.

400   Pumirat P, Vanaporn M, Boonyuen U, Indrawattana N, Rungruengkitkun A, Chantratita N. Effects of

401   sodium chloride on heat resistance, oxidative susceptibility, motility, biofilm and plaque formation of

402   *Burkholderia pseudomallei*. Microbiologyopen. 2017 Aug;6(4). doi: 10.1002/mbo3.493.

403   Reott MA, Parker AC, Rocha ER, Smith CJ. Thioredoxins in redox maintenance and survival during

404   oxidative stress of *Bacteroides fragilis*. J Bacteriol. 2009 May;191(10):3384-91. doi:

405   10.1128/JB.01665-08.

406   Sévin DC, Stählin JN, Pollak GR, Kuehne A, Sauer U. Global Metabolic Responses to Salt Stress in

407   Fifteen Species. PLoS One. 2016 Feb 5;11(2):e0148888. doi: 10.1371/journal.pone.0148888.

408   Si M, Zhao C, Burkinshaw B, Zhang B, Wei D, Wang Y, Dong TG, Shen X. Manganese scavenging and

409   oxidative stress response mediated by type VI secretion system in *Burkholderia thailandensis*. Proc

410   Natl Acad Sci U S A. 2017 Mar 14;114(11):E2233-E2242. doi: 10.1073/pnas.1614902114.

411   Smith LT, Smith GM, Madkour MA. Osmoregulation in *Agrobacterium tumefaciens*: accumulation of

412   a novel disaccharide is controlled by osmotic strength and glycine betaine. J Bacteriol. 1990

413   Dec;172(12):6849-55.

414   Somprasong N, Jittawuttipoka T, Duang-Nkern J, Romsang A, Chaiyen P, Schweizer HP,

415   Vattanaviboon P, Mongkolsuk S. *Pseudomonas aeruginosa* thiol peroxidase protects against

416   hydrogen peroxide toxicity and displays atypical patterns of gene regulation. J Bacteriol. 2012

417   Aug;194(15):3904-12. doi: 10.1128/JB.00347-12.

418   Srivastava S, Yadav A, Seem K, Mishra S, Chaudhary V, Nautiyal CS. Effect of high temperature on

419   *Pseudomonas putida* NBRI0987 biofilm formation and expression of stress sigma factor RpoS. Curr

420   Microbiol. 2008 May;56(5):453-7. doi: 10.1007/s00284-008-9105-0.

421   **Supplementary Information Legends**

422   Table1 S1. Total Ion Chromatogram data lists. All compounds with a NIST score of 60 or more have

423   been reported with a putative identification. Where apparent multiple peaks for the same

424   compound are identified, this is due to the presence of multiple derivatives.

425   Table S2. Compounds in each sample putatively identified with a NIST score of 80 or more.

426   **Figure legends**

427   Figure 1. *B. mesoacidophila* growth changes over time under a range of conditions in nutritionally

428   replete media (LB). The charts to the left show optical density over time, with concentration of the

429   particular stressor as the third axis. The charts on the right show optical density over time, with

430   temperature as the third axis. The top panels show the growth in the presence of KCl, the middle

431   panels in the presence of diamide, and the bottom panels in the presence of H_2O_2.

432   Figure 2. *B. mesoacidophila* growth changes over time under a range of conditions in nutritionally

433   stringent media (CD85). The charts to the left show optical density over time, with concentration of

434   the particular stressor as the third axis. The charts on the right show optical density over time, with

435   temperature as the third axis. The top panels show the growth response in the presence of KCl

436   concentration, the middle panels in the presence of diamide, and the bottom panels in the presence

437   of H_2O_2.

438   Figure 3. *B. mesoacidophila* growth curves in CD85 at 30 °C with an increasing concentration of H_2O_2.

439   All conditions were performed in triplicate.

440   Figure 4. Chromatogram of one of the replicates in CD85 (without additional stress) produced in

441   OpenChrom using the Savitzky-Golay smoother and Amdis peak deconvoluter. The collection of

442   peaks at A are dominated by mannose, the peaks at B by α-D-glucopyranoside.

443   Figure 5. Compounds putatively identified with a NIST score of 80 or higher, across all replicates in

444   each condition (CD85 in black, CD85 with 1 mmol l^{-1} diamide in light grey, CD85 with 220 μmol l^{-1}

445   H$_2$O$_2$ in dark grey). All sugars were identified as their derivatized forms.

446   Figure 6. Chromatogram of intracellular metabolome of *B. mesoacidophila* cells grown in CD85

447   treated with diamide showing peaks at 7.142 minutes and ~53 minutes, both of which were unique

448   to diamide treated cells. Images were generated and analysed in OpenChrom.

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