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Putrescine reduces antibiotic-induced oxidative stress as a mechanism of modulation of antibiotic resistance in *Burkholderia cenocepacia*

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Running Head: Putrescine and oxidative stress in *Burkholderia cenocepacia*

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SECTION: Mechanisms of Resistance

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Communication of antibiotic resistance among bacteria via small molecules is implicated in transient reduction of bacterial susceptibility to antibiotics, which could lead to therapeutic failures aggravating the problem of antibiotic resistance. Released putrescine from the extremely antibiotic resistant bacterium *Burkholderia cenocepacia* protects less resistant cells from different species against the antimicrobial peptide polymyxin B (PmB). Exposure of *B. cenocepacia* to sub-lethal concentrations of PmB and other bactericidal antibiotics induce reactive oxygen species (ROS) production and expression of the oxidative stress response regulator OxyR. We evaluated whether putrescine alleviates antibiotic-induced oxidative stress. The accumulation of intracellular ROS such as superoxide ion and hydrogen peroxide was assessed fluorometrically with dichlorofluorescein diacetate, while the expression of OxyR and putrescine synthesis enzymes was determined in luciferase assays using chromosomal promoter-*lux* reporter system fusions. We evaluated wild type and isogenic deletion mutant strains with defects in putrescine biosynthesis after exposure to sub-lethal concentrations of PmB and other bactericidal antibiotics. Exogenous putrescine protected against oxidative stress induced by PmB and other antibiotics, whereas reduced putrescine synthesis resulted in increased ROS generation, and a parallel increased sensitivity to PmB. Of the 3 *B. cenocepacia* putrescine synthesizing enzymes, PmB induced only BCAL2641, an ornithine decarboxylase. This study exposes BCAL2641 as a critical component of the putrescine-mediated communication of antibiotic resistance, and as a plausible target for designing inhibitors that would block the communication of such resistance among different bacteria, ultimately reducing the window of therapeutic failure in treating bacterial infections.

48 **INTRODUCTION**

49 The relentless increase in multidrug resistance, particularly intrinsic, high-level resistance,
50 undermines new treatments improving health and extending the life of patients especially of
51 those with chronic conditions (1). For example, respiratory failure secondary to chronic
52 pulmonary bacterial infection in patients with cystic fibrosis hinders the dramatic improvements
53 in survival achieved over the last several decades and remains the primary cause of death (2).
54 The emergence of growing numbers of cystic fibrosis pathogens with intrinsic, multidrug
55 resistance such as *Burkholderia cepacia* complex, *Stenotrophomonas maltophilia*,
56 *Achromobacter xylosoxidans*, and nontuberculous mycobacteria creates a further need for novel
57 therapies (2). We investigate the mechanisms of high-level intrinsic multidrug resistance using
58 *Burkholderia cenocepacia* as a model bacterium. *B. cenocepacia* is an environmental,
59 opportunistic pathogen that belongs to the *B. cepacia* complex and causes serious respiratory
60 infections in CF patients (3). These infections are associated with faster decline in lung function,
61 debilitating exacerbations and ultimately death (4-6), and they also reduce the survival of CF
62 patients after lung transplant (7).

63 While genetic mechanisms are considered the quintessential means of transfer of antibiotic
64 resistance traits among bacteria, small molecules are also capable of modulating the antibiotic
65 response of bacteria (8). The clinical outcome of antibiotic treatment does not always correlate
66 with the expectations based on *in vitro* susceptibility testing performed on individual clinical
67 isolates (9). Owing to the polymicrobial nature of many infections (10), cross-talk between the
68 different bacterial species is likely to occur during infection. Such chemical communication of
69 antibiotic resistance among bacteria may aggravate the problem of antibiotic resistance by
70 potentially causing transient reduction in the susceptibility to antibiotics, potentially leading to

71 therapeutic failures. For example, a transient increase in resistance to antimicrobial peptides by
72 exposure to host polyamines was shown for the urogenital pathogen *Neisseria gonorrhoeae* (11).
73 Identifying chemical communicators of antibiotic resistance and their mechanism of protection
74 would provide another avenue for intervention to combat the increase and spread of
75 antimicrobial resistance. Recently, we demonstrated that *B. cenocepacia* exhibits a non-genetic
76 mechanism to reduce antibiotic susceptibility that is chemically mediated by putrescine and
77 YceI, a small secreted protein of unknown function that is highly conserved in bacteria (12).
78 Putrescine is a polyamine produced by almost all living organisms (13). When released from *B.*
79 *cenocepacia*, putrescine protects less resistant cells from the same and different species from the
80 antimicrobial peptide polymyxin B (PmB) (12).

81 The mechanism of protection is partly due to the ability of putrescine to compete with PmB
82 for binding to the surface of *B. cenocepacia* (12). However, polyamines can also quench
83 oxidative species (14) and protect membranes from lipid peroxidation (15). Various classes of
84 antibiotics induce oxidative stress and increased production of reactive oxygen species (ROS)
85 (16-19). Although the specific lethal role of ROS generated in response to antibiotics remains
86 under discussion (16, 20, 21), oxidative stress constitutes a burden on the bacterial cells (22).
87 Therefore, it is conceivable that protection from oxidative stress accompanying antibiotic
88 exposure would improve the bacterial response to antibiotics, thus increasing resistance.

89 Here we show that when present at sub-lethal concentrations, PmB and other bactericidal
90 antibiotics induce oxidative stress in *B. cenocepacia*. Our findings revealed that exogenous and
91 endogenous putrescine protects against antibiotic-mediated oxidative stress. This work exposes
92 another mechanism of putrescine-mediated protection from antibiotics alongside with protection
93 of cell surface from binding of PmB previously described (12). By examining the expression

94 patterns of the different putrescine synthesizing enzymes in response to antibiotics, we
95 discovered that the ornithine decarboxylase BCAL2641 is a plausible target for designing
96 inhibitors that would block putrescine-mediated communication of antibiotic resistance among
97 different bacteria, ultimately reducing the window of therapeutic failure in treating bacterial
98 infections.
99

100 **MATERIALS AND METHODS**

101 **Strains and reagents.** Table 1 lists bacteria and plasmids used in this study. Bacteria grew in LB

102 at 37°C. Antibiotics (Sigma, St Louis, MO, USA) were diluted in water except for PmB, which

103 was diluted in 0.2% bovine serum albumin/0.01% glacial acetic acid buffer. Rifampicin was

104 dissolved in dimethyl sulphoxide (DMSO).

105 **General molecular techniques.** DNA manipulations were performed as previously

106 described (23). T4 DNA ligase (Roche Diagnostics, Laval, Quebec, Canada), Antarctic

107 phosphatase (New England Biolabs, Pickering, Ontario, Canada) and restriction endonucleases

108 (Roche or New England Biolabs) were used as recommended by the manufacturers.

109 Transformation of *Escherichia coli* GT115 was performed using the calcium chloride method

110 (24). Mobilization of plasmids into *B. cenocepacia* was conducted by triparental mating (25)

111 using *E. coli* DH5α carrying the helper plasmid pRK2013 (26). DNA amplification by

112 polymerase chain reaction (PCR) was performed using a C1000 Thermal cycler (Bio-Rad

113 Laboratories Ltd., Mississauga, Ontario, Canada) with Taq or HotStar HiFidelity DNA

114 polymerases (Qiagen, Mississauga, Ontario, Canada) and optimized for each primer pair. DNA

115 sequencing was carried out at the DNA sequencing Facility of York University, Toronto, Canada

116 or at Eurofins MWG Operon, Huntsville, Alabama, USA. The DNA sequences were analyzed

117 with the BLAST computer program and compared to the sequenced genome of *B.*

118 *cenocepacia* strain J2315.

119 **Fluorometric determination of ROS.** Overnight cultures of the parental *B. cenocepacia*

120 K56-2 and the appropriate mutants in LB medium were diluted to an optical density at 600 nm

121 (OD_{600}) of 0.1 in fresh medium. Five-ml aliquots were incubated at 37°C for 3 h at 200 rpm.

122 Antibiotics and/or putrescine were added at the specified concentrations and the cultures were

123 further incubated at 37°C for 2 h at 200 rpm. After incubation, the OD_{600} was measured and

124 aliquots containing cells equivalent to an OD_{600} of 0.4 were pelleted, washed with phosphate

125 buffered saline (PBS), and resuspended in 1 ml of PBS. Superoxide radicals and other ROS were

126 determined by diluting the obtained suspension 100 fold in 1 ml PBS and adding 2',7'-

127 dichlorofluorescein diacetate (DCF) to a final concentration of 2 µM. The reaction mixture was

128 then incubated at 37°C for 30 min with rotation. After incubation, the fluorescence was measured

129 in 200-µl aliquots placed into 96-well white plates (Microfluor-2 White, Thermo Scientific) at

130 λ_{ex}= 480 nm and λ_{em}= 521 nm, using Cary Eclipse fluorescence spectrophotometer (Varian, Inc.,

131 Mississauga, Ontario, Canada). In addition, the OD_{600} of the same suspensions were measured

132 and used to normalize the fluorescence values. Hydroxyl radical production was determined in

133 600 µl bacterial suspensions without dilution using 3'-(*p*-hydroxyphenyl) fluorescein (HPF) at a

134 final concentration of 5 µM. Fluorescence was measured at λ_{ex}= 495 nm and λ_{em}= 530 nm in 200

135 µl aliquots placed into 96-well white plates. Background fluorescence of each probe in buffer

136 control was subtracted. Autofluorescence of the bacterial suspensions, without adding the probes,

137 was measured and corrected for by subtraction from the fluorescence signals. Data were

138 normalized to the OD_{600} of the bacterial suspensions. The suspensions were protected from light

139 throughout the assays to avoid photo-oxidation.

140 **Antibiotic susceptibility testing.** Overnight cultures of the parental *B. cenocepacia* K56-2

141 and the appropriate mutants in LB medium were diluted to an optical density at 600 nm (OD_{600})

142 of 0.0008 (low inoculum) or 0.04 (high inoculum) in fresh LB medium and 0.04 in fresh M9

143 minimal medium with or without the antibiotic and incubated at 37°C with medium continuous

144 shaking in a Bioscreen C automated growth curve analyzer (MTX Lab Systems, Vienna, VA,

145 USA). Bacterial growth was assessed turbidimetrically at 600 nm.

146 ***In vitro* antioxidant activity assay.** The ability of putrescine to scavenge free radicals was
147 determined using a system of *in vitro* generation of superoxide radicals containing phenazine
148 methosulfate (PMS)-NADH as previously described (27). Briefly, the reaction mixture consisted
149 of 21 mM phosphate buffer (pH 8.3), 0.7 mM NADH, 17 μM nitro blue tetrazolium, and the
150 corresponding quantity of putrescine. The reaction was initiated by adding 4 μM PMS. The
151 reaction mixtures were mixed and the amount of formazan formation was measured immediately
152 using the spectrophotometer at 560 nm. The percentage of inhibition of formazan formation by
153 putrescine was calculated relative to the control lacking putrescine.

154 **Transcriptional fusions to *luxCDABE*.** The promoter regions from BCAL2641,
155 BCAM1111, BCAM1112 and OxyR were PCR amplified. The PCR products were digested with
156 *Eco*RI and cloned into the *Eco*RI digested and dephosphorylated pGSVTp-*lux* plasmid. The
157 orientation of the promoter region was checked by PCR and luminescence of *E. coli* GT115
158 colonies carrying the plasmids. The resulting plasmids contained the promoter region of the
159 genes of interest fused to the *luxCDABE* reporter system. The plasmids were mobilized into
160 K56-2 and the appropriate mutants by triparental mating. Transconjugants (carrying the
161 chromosomal promoter-reporter fusions) were selected on LB agar plates containing 100 μg/ml
162 of trimethoprim (Tp), 200 μg/ml ampicillin and 10 μg/ml gentamicin.

163 **Luminescence expression assays.** Overnight cultures in LB containing 100 μg/ml Tp were
164 diluted into fresh LB medium to OD_{600}=0.04. After addition of the antibiotics and/or putrescine,
165 300 μl of sample were loaded in triplicate, for each time-point, in a 100-well honeycomb
166 microtitre plate. The plates were incubated at 37°C with medium continuous shaking in a
167 Bioscreen C automated growth curve analyzer (MTX Lab Systems, Vienna, VA, USA). Growth
168 was followed by measuring the OD_{600} at 37°C every 30 min. At pre-determined time points post-

169 inoculation, the Bioscreen was paused and three 200 µl aliquots for each condition tested were

170 transferred into a flat bottom 96-well microtiter plate (Microfluor-2 White, Thermo Scientific)

171 and luminescence (in relative light units, RLU) was measured using a Fluoroskan Ascent FL

172 Microplate Fluorometer and Luminometer (Thermo Scientific, Ottawa, Ontario, Canada).

173 Expression levels of each gene of interest in the different strain backgrounds were calculated as

174 RLU/OD_{600} for each time-point.

175 **Construction of a conditional mutant.** A fragment (~300-bp) spanning the 5′ region of

176 BCAL2641 was PCR amplified, digested by *NdeI* and *XbaI* and cloned into the *NdeI* and *XbaI*

177 digested and dephosphorylated pSC200 plasmid. The plasmids were mobilized into OME12

178 (ΔBCAM1111-1112) by triparental mating. Transconjugants were selected on LB agar plates

179 containing 100 µg/ml of trimethoprim (Tp), 200 µg/ml ampicillin, 10 µg/ml gentamicin and

180 0.5% (wt/vol) rhamnose. This strategy creates conditional mutants in which the expression of the

181 targeted gene depended on the rhamnose concentration in the medium (28).

182 **Thin-layer chromatography analyses of polyamines.** The conditional mutant and the wild

183 type were grown at 37°C in M9 minimal medium supplemented with final concentrations of Tp

184 100 µg/ml and rhamnose 0.4% (wt/vol), permissive condition of expression. An aliquot of an

185 overnight culture in M9 medium with rhamnose was spun down and washed three times with

186 sterile phosphate-buffered saline (PBS), resuspended in PBS, and adjusted to an OD_{600} of 1.

187 Drops (10 µl) of undiluted suspension and 10-fold serial dilutions were plated onto M9 agar

188 plates supplemented with 0.4% (wt/vol) glucose and incubated at 37°C (non-permissive

189 condition of expression). Bacteria growing on the plates were collected, suspended in sterile

190 PBS, and the OD_{600} was adjusted to 0.1. Polyamines were extracted, derivatized to their dansyl

191 derivatives, sequentially separated on TLC silica gel plates (20×20 cm, Merck, Darmstadt,

192 Germany) in two solvent systems: I) benzene–triethylamine (20:2 v/v); II) benzene–methanol
193 (10:0.45 v/v) and visualized under ultraviolet light as previously described (12). Standard
194 solutions of putrescine, cadaverine, spermidine and spermine (0.2 mM each) were treated
195 similarly and included as controls.

196 **Catalase enzyme activity assay.** Overnight cultures of the wild type *B. cenocepacia* K56-2 in
197 LB were diluted to OD_{600}=0.04 into 30 ml fresh LB medium, with or without antibiotics, and
198 incubated at 37°C, 200 rpm for 16 h. Bacterial cells were pelleted, washed with sterile PBS and
199 resuspended in 300 µl (or less if necessary depending on bacterial inhibition of growth by
200 antibiotics) of PBS. The OD_{600} of the bacterial suspensions was measured. The catalase enzyme
201 activity was evaluated using the method described by Iwase et al. (29). Briefly, 100 µl of
202 bacterial suspension or bovine liver catalase solution at different concentrations were added in a
203 glass tube followed by the addition of 100 µl of 1% Triton X-100. Finally, 100 µl of undiluted
204 hydrogen peroxide (30%) were added to the solutions, mixed thoroughly and incubated at room
205 temperature. The height of O_2-forming foam that remained constant for 15 min in the test tube
206 was finally measured using a ruler. The catalase activity of bacterial suspensions was determined
207 using calibration curves constructed using the standard catalase solutions with different
208 concentrations and normalized to the OD_{600} of the tested suspensions.

209 **Statistical Analyses.** Unpaired student's t-tests were conducted with GraphPad Prism 5.0.

210

211 **RESULTS AND DISCUSSION**

212 **Putrescine reduces ROS production induced by PmB.** Treatment of *B. cenocepacia* K56-2
213 with 1 mg/ml PmB led to significantly increased production of intracellular ROS, as detected by
214 2',7'-dichlorofluorescein diacetate (DCF) (Fig. 1). DCF is a colorless, nonfluorescent fluorescein
215 derivative which passively diffuses into cells where the two acetate groups are cleaved by
216 intracellular esterases to yield the non-cell permeable 2', 7'- dichlorofluorescein (30). This
217 cleaved product becomes trapped within the cells and becomes oxidized by intracellular ROS
218 resulting in the formation of a highly fluorescent product; hence it is a measure of generalized
219 oxidant production rather than that of any particular reactive species (30). Lower concentrations
220 of PmB (0.5 mg/ml or less) did not alter the intracellular DCF-detectable ROS pool (data not
221 shown), whereas due to its reduced solubility in the culture medium higher concentrations of
222 PmB could not be reliably tested. Since putrescine protects *B. cenocepacia* from PmB (12), we
223 assessed whether it also alleviates PmB-induced ROS production. Compared to control cells,
224 exogenous putrescine reduced DCF-detectable ROS generation in PmB-treated bacteria (Fig. 1).
225 This effect was assessed at 2 h incubation with PmB and/or putrescine to avoid potential
226 interference from putrescine degradation or metabolic by-products at prolonged incubation
227 times. It should be noted that putrescine did not decrease the background ROS levels produced
228 by bacterial cells not exposed to PmB, but rather caused a slight but significant increase in DCF-
229 detected ROS levels compared to control cells at 20 mM (Fig. 1, white bars). We attributed these
230 results to polyamines catabolism, which also generate ROS (31).

231 To assess whether endogenous putrescine also has the ability to reduce ROS levels in PmB-
232 treated *B. cenocepacia*, we employed deletion mutants in the putrescine biosynthesis pathway.
233 Putrescine can arise through the action of either ornithine decarboxylase or arginine

234 decarboxylase (12). *B. cenocepacia* has two ornithine decarboxylase homologues, BCAL2641
235 and BCAM1111, and one arginine decarboxylase protein, BCAM1112 (Fig. 2A). The ornithine
236 decarboxylase BCAL2641 is encoded by a gene located on chromosome 1 of *B. cenocepacia*;
237 whereas both the ornithine decarboxylase BCAM1111 and the arginine decarboxylase
238 BCAM1112 are encoded by genes located adjacent to each other, but in opposite orientation, on
239 chromosome 2. In a previous study, we have shown that ΔBCAL2641 had a greater reduction in
240 the amount of secreted putrescine compared to wild type than ΔBCAM1111-BCAM1112 (12).
241 Here, we confirmed that these three enzymes are the only contributors to putrescine production
242 in *B. cenocepacia*. A conditional mutant of BCAL2641 in the ΔBCAM1111-BCAM1112
243 background did not produce detectable levels of putrescine at the non-permissive conditions of
244 expression compared to the wild type strain (Fig. 2B). With respect to the response to PmB, the
245 ornithine decarboxylase BCAL2641 was the only enzyme, among the 3 putrescine synthesis
246 enzymes, involved in resistance against PmB. ΔBCAL2641, but not ΔBCAM1111-BCAM1112,
247 had increased susceptibility to PmB compared to wild type when tested in LB medium (Fig. 2C
248 and 2D) or M9 medium (Fig. 2E). Although the growth of ΔBCAM1111-BCAM1112 was not
249 impaired in LB medium regardless of the initial inoculum size (Fig. 2C and 2D), it exhibited
250 significant reduction in growth compared to the wild type cells in M9 medium (Fig. 2E).
251 Nevertheless, this mutant did not show increased susceptibility to PmB in M9 medium in which
252 its growth was retarded (Fig. 2E). On the contrary, ΔBCAL2641 showed slight reduction in
253 growth in LB medium only at low inoculum size (Fig. 2C) but not at high inoculum size (Fig.
254 2D) or in M9 medium (Fig. 2E). This suggests that these genes involved in putrescine synthesis
255 are not functionally redundant; they seem to be stimulated under different conditions and
256 regulated differently with BCAL2641 only involved in resistance to antibiotics. Next, detection

257 of ROS by DCF was assessed after incubation of ΔBCAL2641 and ΔBCAM1111-BCAM1112
258 mutants with PmB for 16 h to allow the different enzymes to reach their maximum expression
259 levels which occurred at about 12 h in the luminescence expression assays (not shown). No
260 differences were observed in PmB-untreated cells between the wild type and the deletion
261 mutants (Fig. 3, white bars). In contrast, ΔBCAL2641 exhibited a significant increase in levels of
262 superoxide and other ROS detected by DCF in response to PmB compared to wild type, whereas
263 ΔBCAM1111-BCAM1112 produced the same level as that in the parental strain (Fig. 3).
264 Together, these results support the notion that putrescine reduces the level of PmB-induced ROS
265 production and this reduction contributes to protection of bacteria from the bactericidal effects of
266 PmB.

267 Hydroxyl radical is another ROS that may be produced upon oxidative stress. Others have
268 used hydroxyphenyl fluorescein (HPF) to fluorometrically detect hydroxyl radicals upon
269 antibiotic stress (16). Using HPF in similar experiments as above, we found a comparable pattern
270 of reduction of PmB-induced ROS by putrescine (data not shown). However, the fluorescence
271 signal detected by HPF was too low compared to that detected by DCF, and required 100-fold
272 higher inoculum than that for the DCF experiments to detect signal above the background noise
273 of fluorescence. Such high inoculum of cells led to high autofluorescence compared to the actual
274 fluorescence signal detected upon adding HPF, which was not the case with the DCF assays (Fig.
275 S1). Thus, we disregarded the results of HPF assays. Similar criticism to the use of HPF was
276 raised recently concerning the interference between the autofluorescence of cells with the actual
277 fluorescence in the presence of the probe especially upon antibiotic treatment (32).

278 Although the DCF fluorometric assay is a well established method and has many advantages
279 over other techniques developed for measurement of intracellular ROS (30), the probe may be

280 nonselective reacting with other oxidants such as hydroxyl radicals and lipid peroxides (33).
281 Hence, to provide additional evidence supporting the DCF fluorometric assays results, we
282 measured the expression of OxyR as an independent indicator of oxidative stress. OxyR belongs
283 to the LysR family of transcription factors whose regulon is involved in the cellular response to
284 oxidative stress (22). OxyR is very sensitive to ROS concentrations, it is activated at very low
285 hydrogen peroxide concentrations leading to upregulation to its regulon (34). Moreover,
286 expression of *oxyR* is upregulated in response to hydrogen peroxide using lacZ promoter fusions
287 (35); similarly, another LysR-type transcription regulator involved in the response to oxidative
288 stress is overexpressed in response to ROS (36). Therefore, we constructed derivatives of wild
289 type and mutant strains carrying an *oxyR*::*lux* promoter fusion to measure *oxyR* gene expression
290 at chromosomal levels. PmB stimulated the *oxyR* expression (Fig. 4A), which was consistent
291 with the induction of intracellular ROS detected by DCF (Fig. 1 and 3). Likewise, catalase
292 activity, regulated by OxyR (22), increased in response to PmB (Table 2). This further confirms
293 the induction of intracellular ROS in response to PmB and validates the findings of DCF
294 fluorometric and *oxyR* expression assays as measures of intracellular ROS. *oxyR* expression was
295 significantly higher in ΔBCAL2641 compared to the parental strain both in the presence or
296 absence of PmB. In contrast, no difference in *oxyR* expression between the wild type and
297 ΔBCAM1111-BCAM1112 was detected in response to PmB (Fig. 4A). No differences in the
298 growth rate of the different strains were noted in absence of PmB; whereas ΔBCAL2641 was
299 more susceptible to PmB compared to the wild type and ΔBCAM1111-BCAM1112 (Fig. S2).
300 These results follow the same pattern of ROS generated in response to PmB in the mutants
301 compared to the wild type strain (Fig. 3).

302 Next, we investigated the mechanism by which putrescine protects from oxidative stress.
303 Putrescine stimulated the expression of *oxyR* (Fig. 4A), probably as a result of a slight induction
304 of ROS accumulation as detected by DCF (Fig. 1). However, putrescine alleviated the increase in
305 *oxyR* expression in response to PmB (Fig. 4A), suggesting a protective effect against ROS.
306 Nevertheless, putrescine did not induce a statistically significant difference in growth of the wild
307 type in the presence or absence of PmB at this early time point of incubation (3 h) under the
308 conditions of this test (Fig. S2). Supporting the protective role of putrescine from oxidative
309 stress, we confirmed the antioxidant properties of putrescine by demonstrating that it could
310 scavenge superoxide radicals generated *in vitro* from a phenazine methosulfate-NADH system in
311 a concentration dependent manner (Fig. 4B). Together, the results of this section reveal a link
312 between reduced susceptibility to PmB, induction of ROS production, and expression of OxyR
313 with the intracellular level of putrescine, which can be attributed to the antioxidant properties of
314 this polyamine.
315 **Expression of the putrescine synthesis enzymes in response to PmB.** To better understand
316 the role of the different putrescine synthesizing enzymes in response to oxidative stress and
317 consequently to PmB, we investigated the expression profiles of their corresponding genes also
318 using *lux* promoter fusions as before. BCAL2641::*lux* gene expression was stimulated by
319 exposure to PmB (Fig. 5A); whereas neither BCAM1111::*lux* nor BCAM1112::*lux* fusions were
320 responsive to PmB (Fig. 5B and 5C respectively). This agrees with the behaviour of
321 ΔBCAL2641 and ΔBCAM1111-BCAM1112 mutants to PmB in terms of antimicrobial
322 resistance (Fig. 2C-2E) and ROS production (Fig. 3). Moreover, this is consistent with our
323 previous data showing increased transcription of BCAL2641, but not BCAM1111 or
324 BCAM1112, in response to PmB (12). BCAL2641 also appears to regulate by an unknown

325 mechanism the gene expression of BCAM1111 and BCAM1112 putrescine synthesis enzymes,
326 since the expression of both genes was significantly reduced in the ΔBCAL2641 background
327 (Fig. 5B and 5C, respectively). This regulation is not mediated through the action of putrescine
328 since 10 mM of putrescine did not stimulate the gene expression of BCAM1111 or BCAM1112
329 (not shown). Other indirect regulatory pathways may be involved which will require further
330 investigation. On the other hand, the gene expression of BCAL2641 increased in the absence of
331 BCAM1111 and BCAM1112 (Fig. 5A), which may explain the slight increase in survival of the
332 ΔBCAM1111-BCAM1112 when exposed to PmB shown in Fig. 2E. This might be due to
333 compensation of the reduced synthesis of putrescine by these enzymes being normally stimulated
334 by BCAL2641. Alternatively, BCAM1111 and BCAM1112 might provide feedback inhibition to
335 BCAL2641; thus their absence would lead to increased BCAL2641 gene expression. Notably,
336 the expression of BCAM1112 (RLU/OD_{600} 0.2423) is much lower than that of the other 2
337 enzymes (RLU/OD_{600} 1.4829 and 1.5585 for BCAL2641 and BCAM1111 respectively). This
338 suggests that *B. cenocepacia* does not preferentially utilize the arginine decarboxylase
339 BCAM1112. This agrees with the fact that *B. cepacia* can degrade arginine only through the use
340 of the succinyl transferase pathway, despite the possession of an arginine decarboxylase
341 homologue (37, 38). Except for ΔBCAL2641, which exhibited reduced growth in the presence of
342 PmB, no differences in growth were observed in the other strains tested regardless of PmB
343 exposure (Fig. S3). Together, our findings expose BCAL2641 as a crucial contributor of
344 putrescine synthesis in the response against antibiotics.
345 **ROS production in response to other bactericidal antibiotics.** To evaluate whether the
346 induction of oxidative stress and its amelioration by putrescine is a general phenomenon, we
347 tested other bactericidal antibiotics. Exposure of *B. cenocepacia* to gentamicin, norfloxacin,

348 ceftazidime and rifampicin led to increased ROS production as detected by DCF (Fig. S4) at sub-
349 lethal concentrations; i.e. concentrations below but more specifically near the MIC of these
350 antibiotics (Fig. S5). Putrescine reduced the antibiotic-induced elevation in ROS levels only for
351 norfloxacin and rifampicin (Fig. 6), and this correlated with induction of BCAL2641 gene
352 expression (Fig. 7A and 7B, respectively). This agrees with the contribution of BCAL2641 in
353 resistance to both antibiotics that we have previously reported (12). Moreover, *oxyR* transcription
354 was also upregulated in response to both norfloxacin and rifampicin (Fig. 7A and 7B,
355 respectively), which was reflected in an increase in the catalase activity (Table 2), supporting the
356 notion that both antibiotics lead to increased ROS production (Fig. 6 and Fig. S4). In contrast,
357 neither antibiotic affected BCAM1111 gene expression (Fig. 7), indicating that this gene and its
358 product are not directly involved in the response to antibiotic-mediated oxidative stress. It should
359 be noted that higher rifampicin concentrations resulted in great reduction in the expression of
360 BCAL2641, *oxyR*, and BCAM1111 (Fig. 7B), which might be attributed to non-specific
361 inhibition of transcription by rifampicin, especially at 512 μg/ml where expression from these
362 genes was almost completely inhibited.

363 Putrescine did not reduce ROS production generated in response to ceftazidime, but rather
364 further increased the generated ROS at 10 mM but not at 20 mM concentration of putrescine
365 (Fig. 6). Ceftazidime did not affect the expression of BCAL2641, *oxyR* or BCAM1111 (Fig. 7C),
366 and did not alter the catalase enzyme activity (Table 2). However, in a previous study we
367 reported that BCAL2641 is involved in the response of *B. cenocepacia* to ceftazidime (12). This
368 may suggest another role of BCAL2641 in the protective actions against ceftazidime not related
369 to the oxidative stress.

370 Concerning the response to gentamicin, exogenous putrescine did not affect the level of
371 gentamicin-induced superoxide anion (Fig. 6). Moreover, gentamicin did not alter the expression
372 of BCAL2641 (Fig. 7D). This agrees with the previously reported lack of involvement of this
373 enzyme in the response to gentamicin in *B. cenocepacia* (12). Furthermore, gentamicin did not
374 affect the expression of *oxyR* (Fig. 7D). However, the highest tested concentrations of
375 gentamicin did reduce the expression of both BCAL2641 and *oxyR* (Fig. 7D). Similarly,
376 gentamicin reduced the catalase enzyme activity (Table 2). Such inhibition might be due to the
377 mechanism of action of the aminoglycoside inhibiting translation and protein synthesis in
378 general, since it also inhibited the expression of BCAM1111, which consequently might have led
379 to increased ROS levels at high concentration (Fig. 6).
380 **Conclusions.** We show that: (i) sub-lethal concentrations of different bactericidal antibiotics
381 (PmB at 1 mg/ml, rifampicin at 256 and 512 μg/ml and norfloxacin at 32 and 64 μg/ml) induce
382 oxidative stress in *B. cenocepacia* that is manifested as induction of ROS formation as detected
383 by DCF, stimulation of expression of the transcription regulator OxyR involved in response to
384 oxidative stress (at antibiotic concentrations similar to or even lower than those inducing ROS
385 formation; PmB at 0.5 mg/ml, rifampicin at 16 and 32 μg/ml and norfloxacin at 16-64 μg/ml),
386 and increased catalase enzyme activity (PmB at 0.5 mg/ml, rifampicin at 16 μg/ml and
387 norfloxacin at 8 μg/ml); (ii) this response does not apply to gentamicin and ceftazidime which do
388 not induce OxyR expression or increase catalase enzyme activity, suggesting that not all
389 bactericidal antibiotics induce oxidative stress; (iii) putrescine protects against oxidative stress
390 induced by several bactericidal antibiotics (PmB, norfloxacin and rifampicin); (iv) protection by
391 putrescine correlates with increased BCAL2641 gene expression; (v) BCAL2641, in addition to
392 synthesizing putrescine, regulates the other putrescine biosynthetic enzymes BCAM1111 and

393 BCAM1112 by an unknown mechanism that does not directly involve putrescine. Together,
394 these observations suggest a model (Fig. 8) by which *B. cenocepacia* responds to antibiotic stress
395 by overproducing putrescine and in turn, this polyamine protects bacterial cells by a surface
396 effect blocking antibiotic binding (12) as well as by reducing oxidative damage.

397 Putrescine was previously shown to communicate antibiotic resistance among different
398 bacteria (12). Its increased production in *B. cenocepacia* occurs in response to a subset of
399 bactericidal antibiotics (12), which induce oxidative stress in bacterial cells at near lethal
400 concentration ranges. It is still controversial whether the generation of ROS is the cause of
401 lethality of antimicrobial agents or a consequence of antibiotic stress (16, 20, 21). However, it is
402 conceivable that the oxidative stress accompanying antibiotic treatment imposes a metabolic
403 burden on the bacterial cells at near death conditions. Thus, our results demonstrating a
404 protective role for putrescine in the response to the oxidative stress generated in *B. cenocepacia*
405 during antibiotic exposure represent another mechanism of protection from the antibacterial
406 effects of bactericidal antibiotics. This agrees with previous reports on the antioxidant properties
407 and protective effects of putrescine against antibiotic induced ROS formation in *E. coli* (17).

408 While little is known about the physiological levels of putrescine, it seems that its level
409 varies in different body sites. For example, putrescine concentration was reported to be 3 mM in
410 urine (11), whereas it was shown to be up to 0.2 mM in sputum samples from CF patients (39,
411 40). However, it is difficult to predict the local concentration of putrescine and other polyamines
412 in the lung of CF patients, as infection alters the rheology of the mucus and the lung environment
413 (41). Moreover, putrescine levels increase dramatically (by 10 fold or more) during
414 exacerbations of bacterial infections in CF patients (39, 40). Hence, the concentrations used in
415 this study could potentially resemble the physiological situation in certain body compartments.

416 Furthermore, a direct relationship exists between increased putrescine concentration during
417 infection and the proliferation of lung microbiota and specific pathogens such as *P. aeruginosa*
418 in the lungs of CF patients (40). Also, putrescine and other polyamines in genital mucosal fluids
419 increase the resistance of *N. gonorrhoeae* to antimicrobial peptides (PmB and LL-37), possibly
420 enhancing its survival during infection by reducing bacterial susceptibility to host-derived
421 antimicrobials (11). Interestingly, the expression of the ornithine decarboxylase BCAL2641 is
422 induced in *B. cenocepacia* in CF conditions compared to soil environmental like conditions
423 shown by comparative transcriptomics, underscoring the importance of putrescine, and this
424 enzyme in particular, during infection (42).

425 This study also provides new information on the regulation of the putrescine synthesis
426 enzymes. The ornithine decarboxylase BCAL2641 gene responds to the external antibiotic
427 signals, while the other ornithine decarboxylase BCAM1111 or the arginine decarboxylase
428 BCAM1112 do not. Also, BCAL2641 regulates the expression of BCAM1111 and BCAM1112
429 since their expression depends on the presence of BCAL2641. This suggests that upon antibiotic
430 stress maximal production of putrescine is required, which arises from the upregulation of
431 BCAL2641 and by maintaining the expression of the other two enzymes in a BCAL2641-
432 dependent manner. The molecular mechanism of this regulation awaits further investigation.

433 In conclusion, this study broadens our understanding on the mechanism of chemical
434 communication of antibiotic resistance mediated by putrescine. In addition, it provides a clear
435 target for the design of inhibitors targeting the ornithine decarboxylase BCAL2641 that is
436 critically implicated in this phenomenon. Such inhibitors would not only reduce the resistance to
437 antibiotics in *B. cenocepacia* but also would reduce its ability to communicate high-level
438 resistance to other less resistant bacteria.

439

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444 an Ontario Graduate Scholarship.

445

TABLE 1 Strains and Plasmids

Strain or plasmid	Relevant characteristics[a]	Source and/or reference
Strains		
Burkholderia cenocepacia		
K56-2	ET12 clone related to J2315, CF clinical Isolate, [b]BCRRC	(43)
OME11	K56-2, ΔBCAL2641	(12)
OME12	K56-2, ΔBCAM1111-ΔBCAM1112	(12)
OME49	OME12 P_{rha}::BCAL2641	This study
OME50	K56-2, $P_{BCAL2641}$::pGSVTp-*luxCDABE*; Tp^R	This study
OME51	OME12, $P_{BCAL2641}$::pGSVTp-*luxCDABE*; Tp^R	This study
OME52	K56-2, $P_{BCAM1111}$::pGSVTp-*luxCDABE*; Tp^R	This study
OME53	OME11, $P_{BCAM1111}$::pGSVTp-*luxCDABE*; Tp^R	This study
OME54	K56-2, $P_{BCAM1112}$::pGSVTp-*luxCDABE*; Tp^R	This study
OME55	OME11, $P_{BCAM1112}$::pGSVTp-*luxCDABE*; Tp^R	This study
OME56	K56-2, P_{oxyR}::pGSVTp-*luxCDABE*; Tp^R	This study
OME57	OME11, P_{oxyR}::pGSVTp-*luxCDABE*; Tp^R	This study
OME58	OME12, P_{oxyR}::pGSVTp-*luxCDABE*; Tp^R	This study
Escherichia coli		
DH5α	F^- ϕ80*lacZ* M15 *endA1 recA1 supE44 hsdR*17(r_K^- m_K^+)*deoR thi-1 nupG supE44 gyrA96relA1* Δ(*lacZYA-argF*)*U169*, λ^-	Laboratory stock
GT115	F^- *mcrA*Δ(*mrr-hsdRMS-mcrBC*) ϕ80Δ*lacZ*ΔM15 Δ*lacX74 recA1 rpsL*(StrA) *endA1*Δ*dcm uidA*(ΔMluI)::*pir-116* Δ*sbcC-sbcD*	Invivogen
Plasmids		
pRK2013	$ori_{colE1,}$ RK2 derivative, Kan^R, *mob*$^+$, *tra*$^+$	(26)
pGSVTp-*lux*	Mobilizable suicide vector containing the *lux* operon, derivative from pGSV3-*lux* (44); OriT; Tp^R	(45)
pSC200	oriR6K, P_{RhaB} rhamnose-inducible promoter, Tp^R, mob^+	(28)
pOE14	$P_{BCAL2641}$::*luxCDABE* transcriptional fusion in pGSVTp-*lux*	This study
pOE17	P_{rha}::BCAL2641 in pSC200	This study
pOE18	$P_{BCAM1111}$::*luxCDABE* transcriptional fusion in pGSVTp-*lux*	This study
pOE19,	$P_{BCAM1112}$::*luxCDABE* transcriptional fusion in pGSVTp-*lux*	This study
pOE20	P_{oxyR}::*luxCDABE* transcriptional fusion in pGSVTp-*lux*	This study

[a] Tp^R, trimethoprim resistance, Kan^R, kanamycin resistance, Tet^R, tetracycline resistance.
[b] BCRRC, *B. cepacia* Research and Referral Repository for Canadian CF Clinics.

TABLE 2 Catalase enzyme activities.

Antibiotic Concentration (µg/ml)	**Catalase Activity*** % Units/OD_{600} (SEM)	**Difference from control** (P-value)
None	100 (1.3)	Not applicable
Polymyxin B (500)	120.1 (4.2)	0.0002
Norfloxacin (8)	110.6 (1.2)	0.0012
Rifampicin (16)	134.5 (5.2)	<0.0001
Ceftazidime (32)	99.8 (12.4)	0.978 (Not significant)
Gentamicin (1000)	48.0 (7.1)	<0.0001

* Results from 2 independent experiments, n=6. r^2 of calibration curves was: 0.9644 and 0.9544.

517 **REFERENCES**

518 1. **Ejim L, Farha MA, Falconer SB, Wildenhain J, Coombes BK, Tyers M, Brown ED,**
519 **Wright GD.** 2011. Combinations of antibiotics and nonantibiotic drugs enhance
520 antimicrobial efficacy. Nat Chem Biol **7:**348-350.

521 2. **Waters V.** 2012. New treatments for emerging cystic fibrosis pathogens other than
522 *Pseudomonas*. Curr Pharm Des **18:**696-725.

523 3. **Loutet SA, Valvano MA.** 2010. A decade of *Burkholderia cenocepacia* virulence
524 determinant research. Infect Immun **78:**4088-4100.

525 4. **Corey M, Farewell V.** 1996. Determinants of mortality from cystic fibrosis in Canada,
526 1970-1989. Am J Epidemiol **143:**1007-1017.

527 5. **Speert DP, Henry D, Vandamme P, Corey M, Mahenthiralingam E.** 2002.
528 Epidemiology of *Burkholderia cepacia* complex in patients with cystic fibrosis, Canada.
529 Emerg Infect Dis **8:**181-187.

530 6. **Chen JS, Witzmann KA, Spilker T, Fink RJ, LiPuma JJ.** 2001. Endemicity and inter-
531 city spread of *Burkholderia cepacia* genomovar III in cystic fibrosis. J Pediatr **139:**643-
532 649.

533 7. **De Soyza A, Ellis CD, Khan CM, Corris PA, Demarco de Hormaeche R.** 2004.
534 *Burkholderia cenocepacia* lipopolysaccharide, lipid A, and proinflammatory activity. Am
535 J Respir Crit Care Med **170:**70-77.

536 8. **El-Halfawy OM, Valvano MA.** 2012. Non-genetic mechanisms communicating
537 antibiotic resistance: Rethinking strategies for antimicrobial drug design. Expert Opinion
538 On Drug Discovery **7:**923-933.

539 9. **Smith AL, Fiel SB, Mayer-Hamblett N, Ramsey B, Burns JL.** 2003. Susceptibility
540 testing of *Pseudomonas aeruginosa* isolates and clinical response to parenteral antibiotic
541 administration: lack of association in cystic fibrosis. Chest **123:**1495-1502.
542 10. **Peters BM, Jabra-Rizk MA, O'May GA, Costerton JW, Shirtliff ME.** 2012.
543 Polymicrobial interactions: impact on pathogenesis and human disease. Clin Microbiol
544 Rev **25:**193-213.
545 11. **Goytia M, Shafer WM.** 2010. Polyamines can increase resistance of *Neisseria*
546 *gonorrhoeae* to mediators of the innate human host defense. Infect Immun **78:**3187-3195.
547 12. **El-Halfawy OM, Valvano MA.** 2013. Chemical communication of antibiotic resistance
548 by a highly resistant subpopulation of bacterial cells. PLOS One **8:**e68874.
549 13. **Tabor CW, Tabor H.** 1984. Polyamines. Annu Rev Biochem **53:**749-790.
550 14. **Wortham BW, Patel CN, Oliveira MA.** 2007. Polyamines in bacteria: pleiotropic
551 effects yet specific mechanisms. Adv Exp Med Biol **603:**106-115.
552 15. **Johnson L, Mulcahy H, Kanevets U, Shi Y, Lewenza S.** 2012. Surface-localized
553 spermidine protects the *Pseudomonas aeruginosa* outer membrane from antibiotic
554 treatment and oxidative stress. J Bacteriol.
555 16. **Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ.** 2007. A common
556 mechanism of cellular death induced by bactericidal antibiotics. Cell **130:**797-810.
557 17. **Tkachenko AG, Akhova AV, Shumkov MS, Nesterova LY.** 2012. Polyamines reduce
558 oxidative stress in *Escherichia coli* cells exposed to bactericidal antibiotics. Res
559 Microbiol **163:**83-91.

560 18. **Kolodkin-Gal I, Sat B, Keshet A, Engelberg-Kulka H.** 2008. The communication
561 factor EDF and the toxin-antitoxin module mazEF determine the mode of action of
562 antibiotics. PLOS Biol **6:**e319.
563 19. **Choi H, Lee DG.** 2012. Synergistic effect of antimicrobial peptide arenicin-1 in
564 combination with antibiotics against pathogenic bacteria. Res Microbiol **163:**479-486.
565 20. **Keren I, Wu Y, Inocencio J, Mulcahy LR, Lewis K.** 2013. Killing by bactericidal
566 antibiotics does not depend on reactive oxygen species. Science **339:**1213-1216.
567 21. **Liu Y, Imlay JA.** 2013. Cell death from antibiotics without the involvement of reactive
568 oxygen species. Science **339:**1210-1213.
569 22. **Imlay JA.** 2008. Cellular defenses against superoxide and hydrogen peroxide. Annu Rev
570 Biochem **77:**755-776.
571 23. **Sambrook J, Fritsch EF, Maniatis T.** 1990. Molecular cloning: a laboratory manual,
572 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
573 24. **Cohen SN, Chang AC, Hsu L.** 1972. Nonchromosomal antibiotic resistance in bacteria:
574 genetic transformation of *Escherichia coli* by R-factor DNA. Proc Natl Acad Sci U S A
575 **69:**2110-2114.
576 25. **Craig FF, Coote JG, Parton R, Freer JH, Gilmour NJ.** 1989. A plasmid which can be
577 transferred between *Escherichia coli* and *Pasteurella haemolytica* by electroporation and
578 conjugation. J Gen Microbiol **135:**2885-2890.
579 26. **Figurski DH, Helinski DR.** 1979. Replication of an origin-containing derivative of
580 plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci U S
581 A **76:**1648-1652.

582 27. **Tkachenko AG, Fedotova MV.** 2007. Dependence of protective functions of
583 *Escherichia coli* polyamines on strength of stress caused by superoxide radicals.
584 Biochemistry (Mosc) **72:**109-116.
585 28. **Ortega XP, Cardona ST, Brown AR, Loutet SA, Flannagan RS, Campopiano DJ,**
586 **Govan JR, Valvano MA.** 2007. A putative gene cluster for aminoarabinose biosynthesis
587 is essential for *Burkholderia cenocepacia* viability. J Bacteriol **189:**3639-3644.
588 29. **Iwase T, Tajima A, Sugimoto S, Okuda K, Hironaka I, Kamata Y, Takada K,**
589 **Mizunoe Y.** 2013. A simple assay for measuring catalase activity: a visual approach. Sci
590 Rep **3:**3081.
591 30. **Rhee SG, Chang TS, Jeong W, Kang D.** 2010. Methods for detection and measurement
592 of hydrogen peroxide inside and outside of cells. Mol Cells **29:**539-549.
593 31. **Chou HT, Kwon DH, Hegazy M, Lu CD.** 2008. Transcriptome analysis of agmatine
594 and putrescine catabolism in *Pseudomonas aeruginosa* PAO1. J Bacteriol **190:**1966-
595 1975.
596 32. **Renggli S, Keck W, Jenal U, Ritz D.** 2013. The role of auto-fluorescence in flow-
597 cytometric analysis of *Escherichia coli* treated with bactericidal antibiotics. J Bacteriol.
598 33. **Kooy NW, Royall JA, Ischiropoulos H.** 1997. Oxidation of 2',7'-dichlorofluorescin by
599 peroxynitrite. Free Radic Res **27:**245-254.
600 34. **Aslund F, Zheng M, Beckwith J, Storz G.** 1999. Regulation of the OxyR transcription
601 factor by hydrogen peroxide and the cellular thiol-disulfide status. Proc Natl Acad Sci U
602 S A **96:**6161-6165.
603 35. **Tkachenko AG, Nesterova LY.** 2003. Polyamines as modulators of gene expression
604 under oxidative stress in *Escherichia coli*. Biochemistry (Mosc) **68:**850-856.

605 36. **Reen FJ, Haynes JM, Mooij MJ, O'Gara F.** 2013. A non-classical LysR-type
606 transcriptional regulator PA2206 is required for an effective oxidative stress response in
607 *Pseudomonas aeruginosa*. PLoS One **8:**e54479.
608 37. **Vander Wauven C, Stalon V.** 1985. Occurrence of succinyl derivatives in the
609 catabolism of arginine in *Pseudomonas cepacia*. J Bacteriol **164:**882-886.
610 38. **Stalon V, Mercenier A.** 1984. L-arginine utilization by *Pseudomonas* species. J Gen
611 Microbiol **130:**69-76.
612 39. **Grasemann H, Shehnaz D, Enomoto M, Leadley M, Belik J, Ratjen F.** 2012. L-
613 ornithine derived polyamines in cystic fibrosis airways. PLoS One **7:**e46618.
614 40. **Twomey KB, Alston M, An SQ, O'Connell OJ, McCarthy Y, Swarbreck D, Febrer
615 M, Dow JM, Plant BJ, Ryan RP.** 2013. Microbiota and Metabolite Profiling Reveal
616 Specific Alterations in Bacterial Community Structure and Environment in the Cystic
617 Fibrosis Airway during Exacerbation. PLoS One **8:**e82432.
618 41. **Laube BL, Sharpless G, Benson J, Carson KA, Mogayzel PJ, Jr.** 2013. Mucus
619 Removal Is Impaired in Children with Cystic Fibrosis Who Have Been Infected by
620 *Pseudomonas aeruginosa*. J Pediatr.
621 42. **Yoder-Himes DR, Konstantinidis KT, Tiedje JM.** 2010. Identification of potential
622 therapeutic targets for *Burkholderia cenocepacia* by comparative transcriptomics. PLoS
623 One **5:**e8724.
624 43. **Mahenthiralingam E, Coenye T, Chung JW, Speert DP, Govan JR, Taylor P,
625 Vandamme P.** 2000. Diagnostically and experimentally useful panel of strains from the
626 *Burkholderia cepacia* complex. J Clin Microbiol **38:**910-913.

627 44. **Moore RA, Reckseidler-Zenteno S, Kim H, Nierman W, Yu Y, Tuanyok A, Warawa J, DeShazer D, Woods DE.** 2004. Contribution of gene loss to the pathogenic evolution of *Burkholderia pseudomallei* and *Burkholderia mallei*. Infect Immun **72:**4172-4187.
628
629
630 45. **Bernier SP, Nguyen DT, Sokol PA.** 2008. A LysR-type transcriptional regulator in *Burkholderia cenocepacia* influences colony morphology and virulence. Infect Immun **76:**38-47.
631
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633

634 **Figure Legends**

635

636 **FIG 1.** Putrescine reduces ROS production induced by PmB in *B. cenocepacia* K56-2. ROS
637 were detected by DCF. n= 6 from 2 independent experiments.

638

639 **FIG 2.** BCAL2641 is the only putrescine synthesis enzyme in *B. cenocepacia* involved in
640 reduced susceptibility to PmB. A. Putrescine synthesis pathway in *B. cenocepacia* K56-2
641 together with the enzymes involved. ADC, arginine decarboxylase; ODC, ornithine
642 decarboxylase. B. TLC plate showing the lack of production of putrescine in ΔBCAM1111-1112
643 P_{rha}-BCAL2641 conditional mutant under non-permissive conditions. Put, putrescine; Cad,
644 cadaverine; Spd, spermidine; Spn, spermine. C-E. Sensitivity of wild type and putrescine
645 synthesis mutants ΔBCAL2641 (OME11) and ΔBCAM1111-1112 (OME12) to 2048 µg/ml PmB
646 determined turbidimetrically. n=3 from a representative experiment. C, low initial inoculum in
647 LB medium; D, high initial inoculum in LB medium; D, in M9 minimal medium.

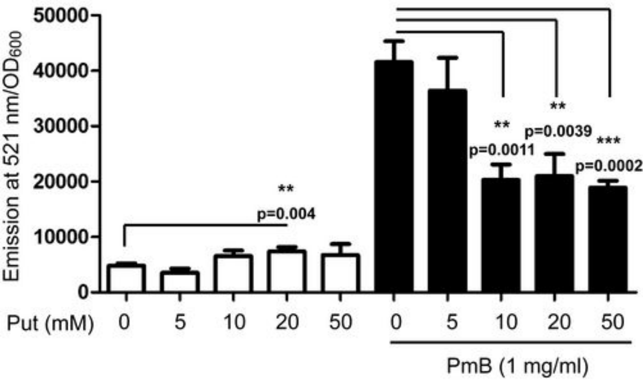
648

649 **FIG 3.** BCAL2641 is the main ornithine decarboxylase responsible for reduction of ROS
650 accumulation and reduced susceptibility to PmB. ROS production in response to 1 mg/ml PmB
651 in wild type K56-2, compared to putrescine synthesis mutants ΔBCAL2641 (OME11) and
652 ΔBCAM1111-1112 (OME12) detected by DCF. n=6 from 2 independent experiments.

653

654 **FIG 4.** A. Induction of OxyR expression as an indicator of ROS accumulation in the wild type
655 (OME56) compared to putrescine synthesis mutants ΔBCAL2641 (OME57) and ΔBCAM1111-
656 1112 (OME58) in response to 500 µg/ml PmB with or without 10 mM Put determined by
657 luciferase expression assay at 3 h. Results are shown as percentage of relative light units
658 RLU/OD_{600} relative to the OME56 control (K56-2 background). The mean RLU/OD_{600} of the
659 control is 0.09567. The percentages of OD_{600} are shown in Fig. S2. n=9 from 3 different clones.

660 * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. B. *In vitro* antioxidant activity of putrescine. n=6 from 2
661 independent experiments.
662
663 **FIG 5.** Luciferase expression assay of the different putrescine synthesizing enzymes in response
664 to 500 µg/ml PmB at 3 h. Results are shown as percentage of relative light units RLU/OD_{600}
665 relative to the control (untreated K56-2 background). The percentages of OD_{600} are shown in Fig.
666 S3. A Expression of BCAL2641 in the wild type (OME50) and ΔBCAM1111-1112 (OME51)
667 backgrounds. n=6 from 2 different clones. The mean RLU/OD_{600} of the control is 1.4829. B
668 Expression of BCAM1111 in the wild type (OME52) and ΔBCAL2641 (OME53) backgrounds.
669 n= 6 from 2 different clones. The mean RLU/OD_{600} of the control is 1.5585. C Expression of
670 BCAM1112 in the wild type (OME54) and ΔBCAL2641 (OME55) backgrounds. n= 7 from 2
671 different clones. The mean RLU/OD_{600} of the control is 0.2423. * $p<0.05$, ** $p<0.01$ and ***
672 $p<0.001$.
673
674 **FIG 6.** The role of putrescine in the bactericidal antibiotics-mediated ROS accumulation in *B.*
675 *cenocepacia* K56-2. n= 9 from 3 independent experiments. The 4 tested antibiotics alone
676 significantly ($p<0.001$) induced the accumulation of ROS compared to control cells. * $p<0.05$, **
677 $p<0.01$ and *** $p<0.001$.
678
679 **FIG 7.** Effect of different antibiotics on the expression of BCAL2641 (in OME50), *oxyR* (in
680 OME56), and BCAM1111 (in OME52) determined using a luciferase expression assay at 3 h.
681 Results are shown as percentage of relative light units RLU/OD_{600} relative to the control
682 (untreated K56-2 background). The percentages of OD_{600} are shown in Fig. S5. n= a minimum of
683 6 from at least 2 different clones. The mean RLU/OD_{600} of the control is 1.0759 for BCAL2641;
684 0.1087 for *oxyR* and 1.4723 for BCAM1111. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.
685
686 **FIG 8.** Model summarizing the role of putrescine in protecting *B. cenocepacia* from antibiotic-
687 induced stress.
688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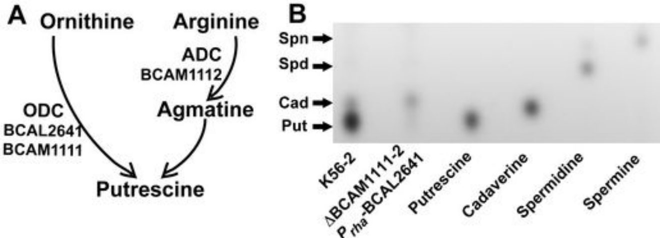
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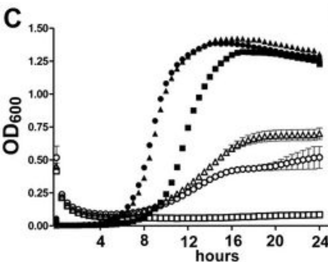
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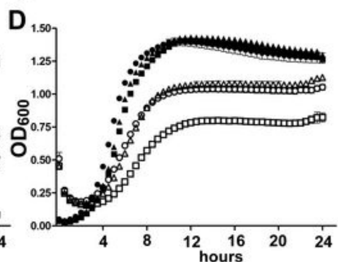
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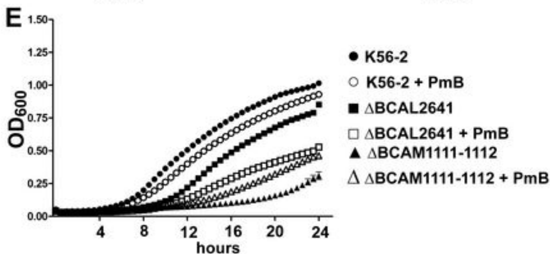
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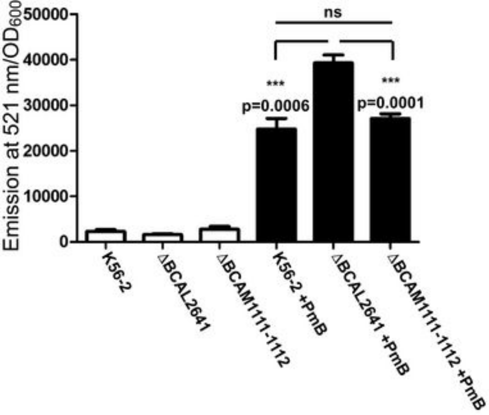
K56-2
K56-2 + PmB
ΔBCAL2641
ΔBCAL2641 + PmB
ΔBCAM1111-1112
ΔBCAM1111-1112 + PmB



Emission at 521 nm/OD600
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p=0.0006
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p=0.0001
K56-2
ΔBCAL2641
ΔBCAM1111-1112
K56-2 +PmB
ΔBCAL2641 +PmB
ΔBCAM1111-1112 +PmB

A

%RLU/OD600

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1300
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*
***
***
***

K56-2
K56-2 +Put
ΔBCAL2641
ΔBCAM1111-1112
K56-2 +PmB
K56-2 +PmB+Put
ΔBCAL2641 +PmB
ΔBCAM1111-1112 +PmB

B

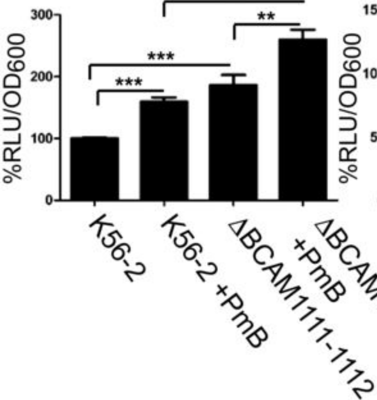
% Formazan Formation

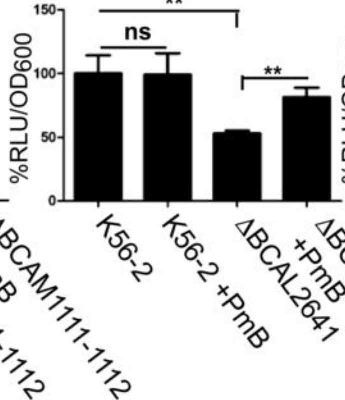
0
20
40
60
80
100

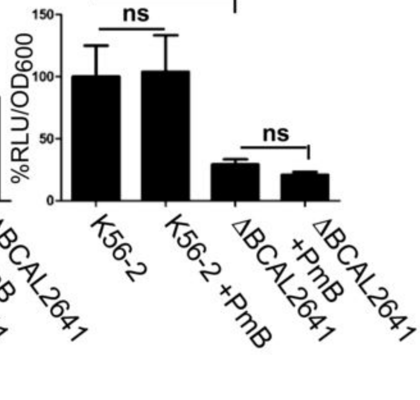
*
p=0.046
***
p<0.0001
***
p<0.0001

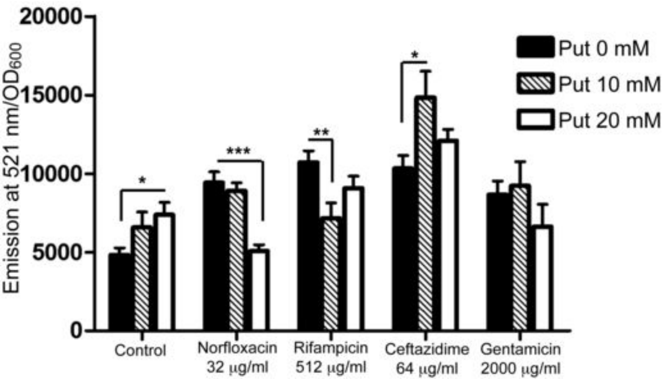
0
10
20
50
100

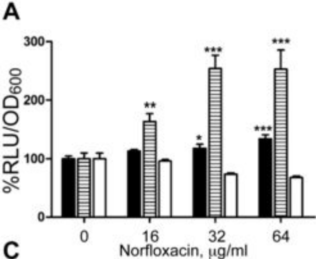
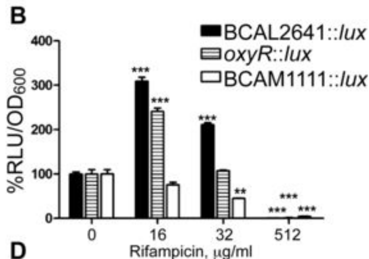
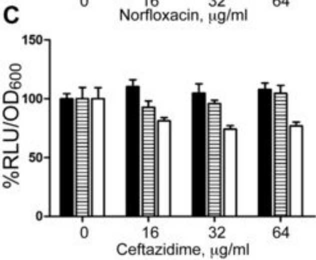
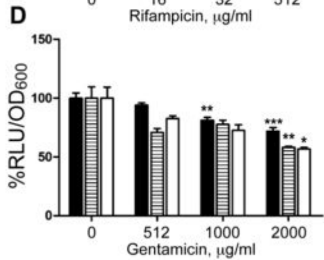
Putrescine concentration, mM


A
%RLU/OD600
300
200
100
0
***
**
***
***
K56-2
K56-2 +PmB
ΔBCAM1111-1112
ΔBCAM1111-1112 +PmB
BCAL2641::lux


B
%RLU/OD600
150
100
50
0
**
ns
**
K56-2
K56-2 +PmB
ΔBCAL2641
ΔBCAL2641 +PmB
BCAM1111::lux


C
%RLU/OD600
150
100
50
0
*
ns
ns
K56-2
K56-2 +PmB
ΔBCAL2641
ΔBCAL2641 +PmB
BCAL1112::lux


20000
15000
10000
5000
0
Emission at 521 nm/OD600
Put 0 mM
Put 10 mM
Put 20 mM
*
***
**
*
Control
Norfloxacin
32 μg/ml
Rifampicin
512 μg/ml
Ceftazidime
64 μg/ml
Gentamicin
2000 μg/ml


A
%RLU/OD600
300
200
100
0
**
***
***
*
***
0
16
32
64
Norfloxacin, μg/ml

B
BCAL2641::lux
oxyR::lux
BCAM1111::lux
%RLU/OD600
400
300
200
100
0
***
***
***
**
***
***
***
0
16
32
512
Rifampicin, μg/ml

C
%RLU/OD600
150
100
50
0
0
16
32
64
Ceftazidime, μg/ml

D
%RLU/OD600
150
100
50
0
**
***
**
*
0
512
1000
2000
Gentamicin, μg/ml

PmB
Nor
Rif

Block binding of
PmB to surface

Other mechanisms
of protection
against antibiotics
?

Ornithine

?

BCAL2641

?

?

BCAM1112

BCAM1111

?

OxyR

H_2O_2

Putrescine

Antioxidant
activity

Expression of
OxyR regulon

Antibiotic-induced
ROS