1 The DNA mimic protein BCAS0292 is involved in the regulation of
2 virulence of *Burkholderia cenocepacia*

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22 **Abstract:**

23 Adaptation of opportunistic pathogens to their host environment requires
 24 reprogramming of a vast array of genes to facilitate survival in the host. *Burkholderia*
 25 *cenocepacia*, a Gram-negative bacterium that colonizes environmental niches, is
 26 exquisitely adaptable to the hypoxic environment of the cystic fibrosis lung and
 27 survives in macrophages. *B. cenocepacia* possesses a large genome encoding multiple
 28 virulence systems, stress response proteins and a large locus that responds to low
 29 oxygen. We previously identified BCAS0292, an acidic protein encoded on replicon
 30 3. Deletion of the BCAS0292 gene resulted in altered abundance of >1000 proteins;
 31 46 proteins became undetectable while 556 proteins showed ≥ 1.5 -fold reduced
 32 abundance, suggesting BCAS0292 is a global regulator. Moreover, the Δ BCAS0292
 33 mutant showed a range of pleiotropic effects: virulence, host-cell attachment and
 34 motility were reduced, antibiotic susceptibility was altered and biofilm formation
 35 enhanced. Its growth and survival were impaired in 6% oxygen. Structural analysis
 36 revealed BCAS0292 presents a dimeric β -structure with a negative electrostatic
 37 surface. Further, the Δ BCAS0292 mutant displayed altered DNA supercoiling,
 38 implicated in global regulation of gene expression. We propose that BCAS0292 acts
 39 as a DNA-mimic, altering DNA topology and regulating the expression of multiple
 40 genes, thereby enabling the adaptation of *B. cenocepacia* to highly diverse
 41 environments.

42

43 **Introduction**

44 Pathogenic bacteria experience a broad range of stress conditions within their hosts,
 45 including changes in temperature, oxygen levels, pH, nutrient limitation and
 46 antimicrobial molecules. Bacteria respond and adapt to these stresses by
 47 reprogramming the expression of large numbers of genes. Environmental
 48 opportunistic pathogens, such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*,
 49 *Mycobacterium marinum*, *Bacillus subtilis*, *Vibrio vulnificus* and members of genus
 50 *Burkholderia* preferentially colonise environmental niches, including soil and water
 51 courses and their success as human pathogens relies on their plasticity to adapt to
 52 varying conditions. Antimicrobial resistance (AMR) ranks among the most important
 53 threats to human health. Among the most problematic AMR bacteria, the so-called
 54 ESKAPE pathogens, such as *P. aeruginosa*, are opportunistic pathogens that are
 55 highly adaptable in their ability to colonise a diverse range of niches including the
 56 human host ((De Oliveira *et al.*, 2020). Insights into the adaptability of these
 57 successful opportunistic pathogens will enable the development of alternatives to
 58 antimicrobial therapies.

59 One example of an exquisitely adaptable pathogen which colonises very diverse
 60 ecological niches ranging from contaminated soils, water sources and pharmaceutical
 61 plants to the human lung is the Gram-negative genus *Burkholderia*. These bacteria
 62 possess large genomes (~8Mb) and have enormous genetic potential for adaptation
 63 within the host. Within this genus, *Burkholderia cepacia* complex (Bcc) is a group of
 64 closely related bacteria which causes opportunistic chronic infections in people with
 65 CF (Mahenthiralingam *et al.*, 2008). The complex currently comprises 24 species
 66 (Peeters *et al.*, 2013, Bach *et al.*, 2017) which are highly resistant to different classes

67 of antibiotics (Zhou *et al.*, 2007). Some members of the complex, notably *B.*
68 *cenocepacia* and *B. multivorans* have been shown to survive in macrophages and to
69 attach to human epithelial cells (Saini *et al.*, 1999, Cullen *et al.*, 2017, Schmerk and
70 Valvano, 2013, Valvano, 2015, Caraher *et al.*, 2007). Further, *B. cenocepacia* can
71 persist in 6% oxygen by means of a co-regulated 50-gene cluster of genes, designated
72 the low-oxygen activated (*lxa*) locus (Sass *et al.*, 2013) which we have shown to be
73 upregulated during chronic infection in response to the hypoxic environment of the
74 CF lung (Cullen *et al.*, 2018).

75 Previously, we identified an immunogenic protein encoded by the BCAS0292 gene in
76 *B. cenocepacia* using serum from Bcc-colonised people with cystic fibrosis (CF)
77 (Shinoy *et al.*, 2013). This gene encodes a hypothetical protein and is located within a
78 virulence gene cluster on replicon 3 of the *B. cenocepacia* genome (Figure 1), which
79 also includes *aidA* (BCAS0293). The *aidA* gene, a paralog of BCAS0292, encodes a
80 protein that is required for virulence in *C. elegans* (Huber *et al.*, 2004). Both
81 BCAS0292 and AidA proteins have been classified as PixA proteins, a poorly
82 understood group of inclusion body proteins associated with cells in stationary phase
83 (Winsor *et al.*, 2008, Goetsch *et al.*, 2006). Both BCAS0292 and *aidA* are the most
84 highly quorum sensing regulated genes in *cepR* and *cepRcciIR* mutants (O'Grady *et*
85 *al.*, 2009). Both genes are also involved in adaptation to the host in a rat chronic
86 infection model (O'Grady and Sokol, 2011). Further, transcripts of both genes are
87 upregulated under low oxygen conditions, and BCAS0292 is also the most
88 upregulated gene in stationary phase, suggesting it may be involved in the stress
89 response of *B. cenocepacia* during anoxic and low nutrient conditions (Sass *et al.*,
90 2013). Overall, these studies suggest a role for the BCAS0292 protein in the stress
91 response of *B. cenocepacia*; however, its specific function remained unknown. In this

work, we constructed a Δ BCAS0292 deletion mutant strain to investigate its role in *B. cenocepacia* pathogenesis. We demonstrate that loss of BCAS0292 alters the abundance of more than a thousand proteins, suggesting the protein is involved in global regulation of gene expression. The deletion results in a range of pleiotropic effects in the mutant strain. We also provide evidence indicating the protein has surface characteristics comparable to known DNA mimic proteins and propose that BCAS0292 functions as a DNA mimic by altering the state of DNA supercoiling.

Results

BCAS0292 belongs to the PixA protein family. These inclusion body proteins, first identified in *Xenorhabdus nematophila*, are typically between 173 and 191 amino acids in length (Goetsch *et al.*, 2006). They are elevated in cells transitioning to stationary phase (Goetsch *et al.*, 2006), but are poorly understood. BCAS0292 has a calculated molecular weight of 19.2 kDa and a calculated pI of 4.81 and has 34 homologs in several sequenced *B. cenocepacia* strains, and in *B. anthina*, *B. cepacia*, *B. pyrrocinia* and other *Burkholderia* species (Winsor *et al.*, 2008); however, a BlastP search showed that it is not found outside *Burkholderia* (Altschul *et al.*, 1997). The BCAS0292 gene is encoded in a two-gene operon with *aidA* (O'Grady *et al.*, 2009) (Figure 1A). AidA is also classified as a PixA protein while it also shares sequence similarity with PixB of *X. nematophila* (Lucas *et al.*, 2018). The *aidA* gene is more widely distributed across *Burkholderia* species with 417 orthologs identified to date (Winsor *et al.*, 2008). AidA also shares a high level of identity with PixB proteins across Gammaproteobacteria, Alphaproteobacteria, Acinobacteria and species (Lucas *et al.*, 2018). While BCAS0292 is immunoreactive, indicating that it is expressed during human infection, we did not detect AidA in our immunoproteomic experiments

(Shinoy *et al.*, 2013). To investigate the potential function of BCAS0292 in infection, we constructed a deletion mutant of BCAS0292 in K56-2, a *B. cenocepacia* strain that is amenable to genetic manipulation and a complemented strain (referred to as Δ BCAS0292 and Δ 0292_0292, respectively) and compared their phenotypes to the wild type (WT) strain.

122

123 **Virulence of K56-2 WT and Δ BCAS0292 strains in *Galleria mellonella* infection**

124 **model.** To examine any potential role in pathogenicity, the virulence of the K56-2
125 Δ BCAS0292 mutant strain and the K56-2 WT strain were compared using the *G.*
126 *mellonella* wax moth larvae infection model. K56-2 WT is highly virulent in this
127 model, which is demonstrated by the very low mean LD₅₀ value of 1.7 CFU at 48 h.
128 Deletion of the BCAS0292 gene resulted in a moderate reduction in virulence (Figure
129 1b, $p = 0.036$) which was partially rescued in the Δ 0292_0292 complemented strain,
130 suggesting that BCAS0292 contributes to *B. cenocepacia* virulence in this model.

131

132 **Potential role in host-cell interactions.** We have previously shown that other
133 immunogenic proteins, including peptidoglycan associated lipoprotein are involved in
134 attachment to host epithelial cells (McClellan *et al.*, 2016, Dennehy *et al.*, 2017). The
135 mutant strain Δ BCAS0292 displayed 2.2-fold reduction in attachment to CFBE41o⁺
136 cells relative to the WT strain ($p = 0.0073$) (Figure 2a). Adhesion was rescued to WT
137 levels in the Δ 0292_0292 complemented strain, suggesting a role for the BCAS0292
138 protein in host cell attachment (Figure 2a). BCAS0292 is predicted to localize at the
139 inner membrane (PSort prediction tool: <http://psort1.hgc.jp/form.html>; certainty
140 0.117). Therefore, to examine if it was directly involved in host cell attachment, the
141 BCAS0292 gene was cloned into *E. coli* BL21 cells, expression induced with 1 mM

142 IPTG and cellular attachment quantified using confocal immunofluorescent
143 microscopy (Figure 2b). *E. coli* binds poorly to lung epithelial cells with roughly 2
144 cells attached per 100 epithelial cells (Figure 2c). *E. coli* BL21_BCAS0292 cells
145 showed no increase in attachment to CFBE41o⁺ cells, relative to control BL21 cells,
146 with a mean value of 1.5 bacterial cells per 100 epithelial cells. Expression of
147 BCAS0292 protein under these experimental conditions was confirmed by SDS-
148 PAGE electrophoresis (Figure 2d). The absence of an effect on host cell attachment of
149 *E. coli* expressing BCAS0292 together with the reduction in host cell attachment of
150 the Δ BCAS0292 *B. cenocepacia* mutant indicates that the protein exerts an indirect
151 role in bacterial adhesion to lung epithelial cells.

152 **Analysis of the susceptibility of K56-2 WT and Δ BCAS0292 to polymyxin B and**
153 **meropenem.** BCAS0292 expression was previously shown to be increased in
154 response to antibiotics but the encoded protein did not have a direct role in resistance
155 (Sass *et al.*, 2011). To further examine antimicrobial susceptibility, the sensitivity of
156 the Δ BCAS0292 mutant to meropenem and polymyxin B (not previously examined)
157 was measured to determine if the absence of this protein affected the susceptibility of
158 the mutant to either of these antibiotics. The Δ BCAS0292 mutant was 8-fold more
159 sensitive to polymyxin B compared to the WT and complemented strains with an MIC
160 of 12 μ g/ ml for Δ BCAS0292 compared to 96 μ g/ ml for the WT and complemented
161 strains (Figure 3a). Polymyxin B targets LPS components (Mares *et al.*, 2009) and
162 destabilises the outer membrane; suggesting that the loss of BCAS0292 may play a
163 role in the maintenance of membrane integrity. In contrast, Δ BCAS0292 was more
164 resistant to the β -lactam meropenem in comparison to the WT and complemented
165 strains with an MIC of 4 μ g/ ml for Δ BCAS0292 compared to 1.5 μ g/ ml for the WT
166 and complemented strains (Figure 3b).

167

168 **Effect on Biofilm formation and motility.** Biofilm formation is regulated by the
 169 cepIR quorum sensing system, which has been shown to regulate BCAS0292
 170 expression (O'Grady and Sokol, 2011). Therefore, we examined whether deletion of
 171 the BCAS0292 gene had any effect on biofilm formation. Δ BCAS0292 mutant cells
 172 showed significant increased biofilm formation ($p < 0.0001$) at all time points,
 173 which was partially compensated in the complemented strain at 72 h (Figure 3c),
 174 suggesting that the BCAS0292 protein repressed biofilm formation. Motility was also
 175 impaired in the mutant strain (Figure 3d). The K56-2 WT showed a mean swimming
 176 diameter of 3.55 ± 0.12 cm and 6.22 ± 0.12 cm after 24 and 48 h, respectively.
 177 Whereas the Δ BCAS0292 mutant strain showed average swimming diameters of 1.83
 178 ± 0.22 and 2.72 ± 0.23 cm after 24 and 48 h, respectively. This phenotype was not
 179 restored in the complement strain.

180 **Whole proteome analysis of WT and Δ BCAS0292 strains.** The lack of a direct
 181 effect on host cell attachment, combined with the apparent repression of biofilm
 182 formation; behaviour in response to antibiotics; and impact on motility suggested that
 183 the BCAS0292 protein has a regulatory effect. To test this hypothesis, we compared
 184 the proteome profiles of the Δ BCAS0292 mutant with WT under stationary phase
 185 conditions. Stationary-phase cultures were used for this experiments since BCAS0292
 186 expression is maximal in stationary phase (Sass *et al.*, 2013). We identified 1,933
 187 proteins showing different abundance between the strains, of which 23 proteins were
 188 unique to the Δ BCAS0292 mutant while 46 were unique to K56-2 WT cells (Table 2).
 189 Unique proteins are those which are detected in all replicates of one strain but are
 190 undetectable in all four replicates of the comparator. A volcano plot highlights that
 191 there were more proteins showing reduced abundance and that the fold-changes were

more extensive than those showing increased abundance (Figure 4a). We identified 391 proteins with significantly increased abundance by ≥ 1.5 -fold in Δ BCAS0292 while 556 proteins (59 % of the total altered proteins) showed significantly reduced abundance by ≥ 1.5 -fold. These results suggest BCAS0292 is a global positive regulator of protein expression (Figure 4a). Among the proteins showing reduced abundance in the Δ BCAS0292 mutant were proteins involved in metabolism (36%), virulence (7%), stress responses (6%), and uncharacterized proteins (35%) (Figure 4b). The fold reduction in protein abundance was substantial: 92 proteins showed reduced abundance (ranging from 3- to 276-fold) in the mutant, and 272 proteins were reduced by over 2-fold (Table 3). In contrast, among the 391 proteins with significantly increased abundance by ≥ 1.5 - fold in the Δ BCAS0292 mutant relative to the K56-2 WT strain, 58 were increased by ≥ 3.0 fold, with the greatest fold increase in abundance being only 9.8-fold (Supplemental Table S3). When proteins showing increased abundance of 3 to 9.8-fold were grouped according to their function (GO annotation) 67% had roles in metabolic processes, 19% in translation and 7% had roles in the stress response (Supplementary Table S3).

Proteins unique to either strain: Proteins that are unique to the Δ BCAS0292 mutant proteome are likely to be proteins that are repressed by BCAS0292. These proteins include RpfR, a receptor for the autoinducer BDSF and two flagellar hook-associated proteins FlgK and FlgL (Table 2). The majority of proteins that are absent in the mutant (and consequently may rely on the presence of BCAS0292 for expression) are proteins with catalytic activity (67%). In addition, 8 (17%) are classified as phage proteins. There are 10 proteins classified as oxidation/reduction proteins, of which 7 are unique to the WT strain. Four out of five transcriptional regulation proteins identified were unique to the Δ BCAS0292 mutant proteome, while the other was

217 unique to the WT strain proteome.

218 Proteins involved in metabolic functions: Proteins with roles in various metabolic
219 processes represented 36 % of the total number of proteins reduced in abundance
220 (Figure 4b). Among these proteins are a plasmid encoded NUDIX hydrolase family
221 protein (pBCA087) reduced by 62-fold (Table 3). This was one of five NUDIX
222 proteins identified that showed reduced abundance in the mutant (Supplemental Table
223 S3). In addition two Glyoxalase/bleomycin resistance protein/dioxygenase
224 superfamily proteins (BCAM2547a, BCAM0974) were reduced by 33.2-fold and 8-
225 fold in Δ BCAS0292, and 6 other proteins in this superfamily were also reduced by 6
226 fold to 1.74-fold (Table 3 and Supplementary Table S2). The majority of proteins
227 unique to the WT strain also had functions in metabolic processes (37%) (Table 2).
228 Most of these proteins function as enzymes which act as catalases, hydrolases,
229 dehydrogenases, oxidoreductases or transferases, indicating that there is a global
230 change in metabolism of these cells in the absence or presence of BCAS0292. Among
231 the proteins involved in metabolic processes that showed increased abundance were
232 111 proteins with amino acid metabolism functions (i.e. 25% of total proteins with
233 increased abundance, in comparison with only 42 (6.4%) of proteins with this
234 function showing reduced abundance (Table 3). Other proteins upregulated in the
235 metabolic processes category had synthase, transferase and cyclohydrolase activities,
236 among others, highlighting the broad range of functions carried out by proteins in this
237 category.

238

239 Proteins involved in the adaptive response: The abundance of many proteins with
240 roles in the stress response were reduced in the Δ BCAS0292 mutant, representing 6 %
241 of the total number of downregulated proteins in this group (Figure 4b). Of particular

242 interest was the reduced abundance of 19 proteins encoded on the low-oxygen
 243 activated (*lxa*) locus (BCAM0272 to 0323) in the mutant strain, highlighting the
 244 possible co-regulation of expression of proteins encoded on this gene cluster (Table 3
 245 and Supplementary Table S2). This locus has been shown to be upregulated under
 246 hypoxic conditions and in stationary phase (Sass *et al.*, 2013); and in sequential
 247 clinical *B. cenocepacia* isolates over time of chronic infection (Cullen *et al.*, 2018). In
 248 particular a two-component system response regulator encoded within the locus,
 249 BCAM0288, was undetectable in the mutant strain. Eight *lxa*-encoded proteins that
 250 showed reduced abundance proteins have stress response functions. The abundance of
 251 a small heat shock/ α -crystallin protein (BCAM0278) was dramatically reduced (276-
 252 fold) relative to K56-2 WT. There are 6 universal stress proteins (USPs) encoded on
 253 the *lxa* locus, and five of these showed reduced abundance in the Δ BCAS0292 mutant
 254 strain, with BCAM0292, being the lowest (6.5-fold). Other stress response proteins in
 255 this category were reduced in abundance from 1.5- to 2.2-fold. Three other *lxa* locus
 256 encoded proteins reduced by 2- to 6.3-fold have roles in phospholipid binding,
 257 electron transfer, storage polymer turnover and alcohol turnover. Sass *et al.* (2013)
 258 identified four other low-oxygen co-regulated gene loci and five proteins that are
 259 encoded by *lxa* co-regulated genes and these were also reduced in abundance:
 260 BCAM1480- to- 1482, another USP, BCAM1495, and an uncharacterised protein,
 261 BCAM1496. In contrast, only three *lxa*-encoded proteins BCAM0299 (a zinc-binding
 262 alcohol dehydrogenase), BCAM0300 (Metallo- β -lactamase superfamily protein) and
 263 BCAM0310 (Putative ribonucleotide reductase), were increased 1.9- to 1.6-fold.
 264 Further, there were 27 proteins with roles in stress responses showing increased
 265 abundance and all but one of these proteins were associated with DNA repair
 266 functions (Table 3).

267

268 Phage or viral proteins: Viral proteins represented 17 % of the total number of
269 proteins that were undetectable in the Δ BCAS0292 mutant, including several
270 hypothetical phage proteins, a putative phage tail tube protein (BCAS0517), a
271 putative phage tail sheath protein (BCAS0518) and a putative DNA-binding phage
272 protein (BCAS0547). Viral proteins also represented 4 % of the proteins reduced in
273 abundance (Figure 4b), including hypothetical phage proteins (BCAM1883,
274 BCAM1919 and BCAM1920) and a putative phage-related regulator (BCAL3205a),
275 which were reduced by 9.5-, 8.5-, 12.5- and 5.4-fold, respectively (Table 3).

276

277 Virulence proteins: Three T6SS secretion system proteins encoded by BCAL0337,
278 BCAL0341 and BCAL0343 (TssD, Hcp1) were also reduced in abundance by 4.2 to
279 1.6-fold in the mutant. Other virulence proteins included a zinc metalloprotease
280 (ZmpA) (BCAS0409), a known quorum sensing regulated virulence factor (Kooi *et*
281 *al.*, 2006); nematocidal AidA and a Flp pilus type assembly-related protein
282 (BCAL1529), were also reduced. Flagellar hook-associated protein 1 (HAP1) and
283 flagellar hook-associated protein 3 (HAP3) were both identified in the Δ BCAS0292
284 mutant while absent from the K56-2 WT strain, indicating that BCAS0292 represses
285 their expression. In contrast, two proteins belonging to the T3SS and T6SS families of
286 proteins, were increased in abundance in the Δ BCAS0292 strain, including a type III
287 secretion system protein (BCAM2040) and a putative type VI secretion system
288 protein TssK (BCAL0338) (Supplementary Table S3).

289

290 Uncharacterised or hypothetical proteins: BCAS0292 is identified as a hypothetical
291 protein (Winsor *et al.*, 2008) and over a third (35%) of the proteins showing reduced

abundance in the Δ BCAS0292 mutant were also classed as uncharacterized proteins or hypothetical proteins, including BCAL3228 and BCAS0636 (59.5- and 29-fold reduced abundance respectively). A putative exported protein (BCAL2401), with unknown function, was also reduced by 25.4-fold in the Δ BCAS0292 mutant strain. There are five PixA containing proteins in the sequenced J2315 genome (Winsor *et al.*, 2008) and all other PixA proteins showed reduced abundance (between 4.1 to 49.8-fold) or were undetectable in the Δ BCAS0292 mutant suggesting a co-regulation or co-dependence of these uncharacterised proteins. Three of these PixA proteins are encoded on chromosome 2, with BCAM1412, being downregulated by 4.1-fold while the proteins encoded on the adjacent genes, BCAM1413 and BCAM1414 were both absent in the Δ BCAS0292 mutant. BCAM1412 and BCAM1414 genes are both orthologs of *aidA*, while BCAM1413 is not.

Transcription and Translation. Proteins with roles in transcription represented another 6 % of the proteins reduced in abundance (55 proteins), including a HU DNA-binding protein (BCAS0551) which was reduced by a substantial 60.1-fold in the mutant. Among the 23 proteins unique to the Δ BCAS0292 mutant strain were 4 proteins (19%) with roles in transcriptional regulation and a transcription-repair-coupling factor (BCAL2017). There were only 17 proteins with roles in transcription that were increased in abundance in the mutant strain, including two DNA-directed RNA polymerase subunit proteins (RpoB and RpoC) which were increased by 3.2- and 2.9-fold. A large number of ribosomal proteins were also increased in the Δ BCAS0292 mutant (Supplemental Table S3).

Proteins associated with other functions: Membrane proteins with roles in adhesion

316 and possible roles in structure/ transport represented 4 % of total number of proteins
 317 reduced in abundance in the mutant strain (Figure 4b) including ten membrane
 318 proteins, along with other surface proteins were lost or significantly reduced (by $\geq$
 319 1.5-fold) in the Δ BCAS0292 strain (Table 3). Among these were proteins associated
 320 with roles in adhesion that were reduced by between 3.5- and 12.5-fold in the
 321 Δ BCAS0292 mutant including a maltose-binding protein (BCAL3041), putative
 322 lipoproteins (BCAM0834 and BCAM0944) and a putative membrane protein
 323 (BCAM1261. In addition, four membrane proteins were undetectable in the mutant
 324 strain, including the outer membrane assembly protein, BamE and a putative
 325 lipoprotein (BCAL2166), a putative lipoprotein (BCAL2166) and a putative
 326 membrane protein (BCAM0264) (Table 2).

327
 328 **Δ BCAS0292 mutant shows gross morphological changes.** Given the vast number
 329 of proteins with altered abundance in the mutant strain we compared the morphology
 330 of the mutant with the WT and complement strains. Unsurprisingly, the Δ BCAS0292
 331 mutant cells had a grossly altered morphology, with a highly irregular cell surface and
 332 complete loss of their rod-like shape (Figure 4c). This was reversed in the
 333 complemented strain suggesting that BCAS0292 is involved in maintaining cell
 334 structure integrity which may contribute to the reduced adherence of this strain to
 335 lung epithelial cells.

336 **Δ BCAS0292 mutant has limited growth at 6% oxygen.** Given that several proteins
 337 encoded on the *lxa* locus showed reduced abundance in the Δ BCAS0292 mutant, we
 338 compared the growth of WT and Δ BCAS0292 mutant in 6% oxygen in a controlled
 339 hypoxia chamber. The growth of the mutant strain was severely reduced under

340 hypoxic conditions ($p < 0.0001$, Figure 4D). When the bacteria from each strain were
341 plated at time intervals and CFU/ml compared, it was apparent that the survival of the
342 mutant strain was also impaired ($p = 0.0294$) following liquid culture under hypoxic
343 conditions. In contrast, survival recovered to WT levels in the complemented strain.

344

345 **Structural analysis of BCAS0292 protein.** In order to help elucidate the function of
346 BCAS0292, we analysed its structural features in solution. Following affinity
347 chromatography purification, His-tagged BCAS0292 was purified by SEC to remove
348 any contaminating proteins and protein aggregates and obtain pure fractions (Figure
349 5a). The secondary structure of rBCAS0292, purified under non-denaturing
350 conditions was determined by Circular Dichroism (CD). A peak minimum was
351 observed at 215 nm indicating that rBCAS0292 has a β -sheet secondary structure (fig.
352 5b). The purified protein was highly stable with a melting temperature (T_m) of 49 °C,
353 (Figure 5c). Light scattering was used to determine the oligomeric state of the
354 BCAS0292 protein. Rayleigh scattering showed that it has a dimeric organisation, in
355 both reducing and oxidising conditions, with a molecular weight of 39.4 ± 0.5 kDa
356 (Figure 5d). Despite several attempts, we were unable to obtain crystals of
357 BCAS0292, therefore molecular modelling was used to predict the 3D structure of the
358 protein using consensus sequence alignment with PixA inclusion body protein
359 BCAM1413 from *B. cenocepacia* described by Nocek et al., (2013) as a template
360 (PDB code 4lzk, E-value- $3.5e^{-55}$). Consistent with CD analysis (Figure 5b),
361 BCAS0292 homology model presents a structure with mostly β -folds, and only one
362 small α helix in the N-terminal region of the protein (residues 13-21) (Figure 6a).
363 Also, as experimentally proven by light scattering studies (Figure 5d), the molecule
364 has a dimeric organization, where two jelly-roll like monomers are compactly held

365 together (Figure 6a). Electrostatic potential calculations show that the entire surface of
366 the protein has a strong negative charge distribution (Figure 6b), a finding which
367 excludes BCAS0292 regulatory function occurring via interactions with DNA or
368 RNA. Instead, similar electrostatic surface distributions are a distinctive feature of
369 DNA mimicry (Wang *et al.*, 2014).

370

371 **BCAS0292 may function as a DNA mimic protein.** Given that BCAS0292 had an
372 overall negative surface charge, we hypothesized that it bound to DNA-binding
373 proteins to mediate its global regulatory activity. To corroborate this hypothesis we
374 examined whether BCAS0292 alters DNA supercoiling, an activity associated with
375 global changes in bacterial gene regulation (Shortt *et al.*, 2016, Scanlan *et al.*, 2017)
376 by investigating the impact of the presence of BCAS0292 on DNA topology.
377 BCAS0292 has been shown to be upregulated in stationary phase (Sass *et al.*, 2013),
378 therefore we compared stationary phase cultures with exponential phase cultures for
379 the WT K56-2, Δ BCAS0292 mutant and complemented strain. DNA topoisomers
380 were not as highly resolved in *B. cenocepacia* (compared with *E. coli*, Supplemental
381 Figure S1) presumably due to the very high levels of restriction modification systems
382 expressed in *Burkholderia*. There were no obvious differences in supercoiling in
383 plasmid DNA extracted from exponential phase cultures of the three strains (Figure
384 6c). In contrast, DNA extracted from stationary phase cultures of the mutant showed a
385 more relaxed DNA topology with fewer highly supercoiled topoisomers at the top of
386 the chloroquine gel relative to WT and complement strains (Figure 6c), indicating that
387 BCAS0292 expressed in stationary phase alters DNA supercoiling. Fluoroquinolones
388 target DNA gyrase and topoisomerase IV, enzymes involved in establishing and or
389 relaxing supercoiling. Consequently, any difference in DNA topology may have

390 impact on the susceptibility to fluoroquinolones between the mutant and WT. In
391 addition, the mutant clearly showed a 2-fold decrease in ciprofloxacin susceptibility,
392 which was reversed in the complemented strain (Figure 6d), further indicating that a
393 lack of BCAS0292 expression impacted on DNA topology.

394

395 Discussion

396 The adaptation of opportunistic pathogens enhancing their fitness in the host and
397 consequently their successful colonisation requires substantial rearrangement of gene
398 expression. Understanding the processes of bacterial regulation in pathogens that
399 adapt to chronic infection is crucial, particularly those that are virtually impossible to
400 eradicate. Our initial studies on host cell attachment of the *B. cenocepacia* K56-2
401 Δ BCAS0292 mutant strongly pointed towards a regulatory role for the BCAS0292
402 protein. The mutant showed reduced attachment to epithelial cells relative to the K56-
403 2 WT strain but induction of BCAS0292 expression had no impact on the adhesion of
404 *E. coli* BL21 cells to CFBE41o⁻ cells in contrast to our earlier work on peptidoglycan-
405 associated lipoprotein (Dennehy *et al.*, 2017). Consistent with this, the dramatic
406 upregulation of BCAS0292 and *aidA* genes in the presence of low oxygen or in
407 stationary phase *B. cenocepacia* cultures (Sass *et al.*, 2013), coupled with the dramatic
408 downregulation of these genes in the quorum sensing mutant strain K56-2 Δ *cepR*
409 (O'Grady and Sokol, 2011) demonstrated that both genes are highly responsive and
410 collectively suggest that BCAS0292 plays a role in the response of *B. cenocepacia* to
411 extracellular stress. Our work now demonstrates that it has a global regulatory role in
412 the expression of over a thousand proteins including those involved in stress
413 responses and metabolism. Specifically, the reduced abundance of five of the six
414 USPs and 14 other *lxa*-encoded proteins in the mutant supports the role for

BCAS0292 in the regulation of the hypoxic stress responses. Further, the impaired growth of the mutant when cultured in a tightly controlled environment of 6% oxygen also substantiates a role for BCAS0292 in the adaptation of *B. cenocepacia* to hypoxic conditions.

BCAS0292 may also play an indirect role in antibiotic susceptibility. The increased susceptibility of the Δ BCAS0292 mutant to polymyxin B, an antibiotic that targets LPS was not surprising given the impact that the absence of BCAS0292 had on the abundance over 10 membrane proteins and on cell morphology. Although meropenem is quite resistant to degradation by β -lactamases, the production of metallo- β -lactamases can facilitate meropenem resistance (Yano *et al.*, 2001); thus the increased abundance of metallo- β -lactamase (BCAM0300) likely contributed to meropenem resistance in the mutant. Similarly, the reduced virulence in the *G. mellonella* infection model of the mutant is likely due to the depressed expression of some of the virulence factors in the mutant, including ZmpA; T6SS proteins, and a flp pilus protein.

Comparison of the proteomes of the K56-2 WT and Δ BCAS0292 mutant strains highlight that discrete groups of proteins involved in other specific processes, were also affected by the deletion of BCAS0292. A recent *in silico* analysis demonstrated that *B. cenocepacia* genomes are rich in phage genomes, with 63 prophage regions identified in 16 sequenced *B. cenocepacia* databases (Roszniowski *et al.*, 2018). A total of 18 viral proteins, most of which are encoded within two clusters on chromosomes 2 and 3, were either absent or dramatically reduced in the Δ BCAS0292 mutant, indicating that there is a positive correlation between the abundance of specific viral proteins and BCAS0292 expression. The finding that eight phage proteins were among the 49 proteins (16%) that were undetectable in the

440 mutant strengthens this view. Among these 18 phage proteins are six encoded by the
 441 intact prophage Bcep-Mu which is widespread among human pathogenic *B.*
 442 *cenocepacia* strains of the ET12 lineage (Summer *et al.*, 2004) and four are encoded
 443 in a newly identified prophage region (BCAM1879 to BCAM1926), not previously
 444 reported (Roszniowski *et al.*, 2018). Finally, BCAL3205 is a helix-turn-helix
 445 transcriptional regulator associated with the control of lytic/lysogenic cycle of phages
 446 and bacterial gene expression (Aravind *et al.*, 2005). Its reduced abundance in the
 447 mutant may regulate the expression of the other phage proteins downstream. The
 448 extracellular portion of the T6SS and bacteriophage tail-associated protein complexes
 449 are both structurally and functionally similar (Zoued *et al.*, 2017). Thus, the shared
 450 impact of BCAS0292 gene deletion implicates BCAS0292 protein in the regulation of
 451 expression of these phage tail-like proteins and T6SS in *B. cenocepacia* by a common
 452 mechanism.

453 Among the proteins that were unique to the mutant (i.e. undetectable in the
 454 WT) at stationary phase was RpfR, the receptor for Burkholderia diffusible signal
 455 factor (BDSF). RpfR is involved in the synthesis and degradation of cyclic-di-GMP, a
 456 second messenger which regulates biofilm formation, biofilm dispersal, motility,
 457 extracellular protease production, antibiotic resistance and other virulence associated
 458 phenotypes. Point mutations in different *rpfR* domains resulted in mutants showing
 459 increased biofilm formation and impaired motility, consistent with the Δ BCAS0292
 460 mutant (Mhatre *et al.*, 2020). Given that the Δ BCAS0292 mutant showed increased
 461 biofilm formation and reduced motility and uniquely expressed RpfR, it is likely that
 462 the BCAS0292 protein mediates some of its pleiotropic effects via RpfR suppression.

463 Our observation that all 5 PixA proteins encoded in *B. cenocepacia* were
 464 either reduced by up to 50-fold or absent in the Δ BCAS0292 mutant, indicates that the

other PixA proteins require BCAS0292 for their complete expression. A reduction in AidA abundance in the mutant was expected, as the *aidA* gene is located in the same operon and co-regulated with BCAS0292 (O'Grady *et al.*, 2009); however, the three other *pixA* genes are located on chromosome 2 suggesting their co-regulation with BCAS0292. Studies on PixA and PixB in *X. nematophila* revealed they had no direct role in virulence or colonisation of nematodes (Lucas *et al.*, 2018); however, they may also be involved in regulation of the response to different environmental conditions in this bacterium.

Purified rBCAS0292 protein is dimeric, consistent with many functionally important proteins involved in regulation such as transcription factors (Zhanhua *et al.*, 2005, Ispolatov *et al.*, 2005), which also points towards a regulatory role for the BCAS0292 protein. The unexpected finding that BCAS0292 has a highly negatively charged surface, indicated that BCAS0292 is likely to bind to DNA or RNA binding protein(s) and may act as a DNA mimic protein, which led us to evaluate its impact on DNA topology in stationary phase. DNA supercoiling is considered a crude regulator of gene expression with variable responses to environmental signals and the potential to act right across the bacterial genome (Dorman and Corcoran, 2009). The role of DNA supercoiling in global regulation of SPI1 and SPI2 pathogenicity islands genes in *Salmonella enterica* serovar typhimurium are well described, with negative supercoiling up-regulating SPI1 genes and conferring a more invasive phenotype, while SPI2 genes upregulated by DNA relaxation are essential for survival inside the macrophage (Dorman and Corcoran, 2009, O' Croinin *et al.*, 2006). Hence DNA supercoiling discriminated between two different intracellular environments and modulated the expression of virulence genes in macrophage cells (O' Croinin *et al.*, 2006). Alterations in *C. jejuni* supercoiling also resulted in increased cellular invasion

490 and secretion (Scanlan *et al.*, 2017). Differences in DNA supercoiling between the
491 Δ BCAS0292 mutant and WT at stationary phase combined with enhanced
492 ciprofloxacin susceptibility reinforced the view that BCAS0292 can alter DNA
493 topology, suggesting this is the mechanism by which it wields its regulatory effect.

494 DNA mimic proteins generally have a molecular weight <25kDa and pI values
495 <5.0 and are characterised by having a negative charged distribution on the protein
496 surface, allowing them to mimic the negatively charged phosphate backbone of DNA
497 (Wang *et al.*, 2018) and BCAS0292 matches each of these criteria. Very few (~20)
498 have been discovered to date because, consistent with BCAS0292, they have unique
499 amino acid sequences and structures and are hard to predict using bioinformatic tools.
500 Half of the DNA mimic proteins already identified are bacterial and are involved in
501 many internal response controls (Wang *et al.*, 2014). Two were identified in *Neisseria*
502 spp, namely DMP12 and DMP19 (Wang *et al.*, 2012, Wang *et al.*, 2013). The latter,
503 DMP19, forms a dimer and has a variety of potential binding partners and is
504 suggested to prevent the interaction of *Neisseria* hypothetical transcription factor from
505 binding to its DNA binding sites (Wang *et al.*, 2012, Huang *et al.*, 2017), while
506 DMP12 is likely to maintain the stability of unbound HU-binding regions (Wang *et*
507 *al.*, 2014, Wang *et al.*, 2013). Another DNA mimic, MfpA, was identified in
508 fluoroquinolone resistant strains of *Mycobacterium tuberculosis* and functions by
509 protecting the DNA binding site of gyrase from fluoroquinolones (Hegde *et al.*,
510 2005). Ongoing pull-down analyses involving different approaches to identify
511 possible binding partners of BCAS0292 have been inconclusive to date.

512 A large number of proteins encoded on gene clusters associated with roles in
513 translation showed increased abundance in the Δ BCAS0292 mutant suggesting that
514 BCAS0292 negatively regulates or represses their expression. Taken together, this led

us to propose that BCAS0292, which is dramatically upregulated in response to hypoxic stress and in stationary phase interacts with DNA-binding proteins, altering DNA topology which globally influences transcription and expression of a vast number of genes involved virulence, host cell attachment, antibiotic susceptibility and biofilm formation (Figure 7). Recently, we showed that 20 proteins encoded on the *lxa* locus show increased abundance in late sequential chronic CF isolates relative to the earlier isolates (Cullen *et al.*, 2018). Thirteen of these proteins are also reduced in the Δ BCAS0292 mutant suggesting a common adaptive stress response in the hypoxic CF lung (Supplementary table S4). In contrast, only two proteins encoded on genes within the adjacent 37-gene *cci* pathogenicity island were significantly reduced in the Δ BCAS0292 mutant indicating that the downregulation of *lxa*-encoded genes in the mutant is specific to the BCAS0292 protein. Subsequent analysis revealed that 109 other proteins that were either absent or reduced in abundance in the mutant strain were also elevated in the late chronic infection *B. cenocepacia* isolates (Suppl Table S5). This represents 64% of the proteins with increased abundance over time of chronic infection (Cullen *et al.*, 2018) and points to a role of BCAS0292 in adaptation to chronic infection. Taken together these data suggest that this protein may contribute to the adaptation and survival of *B. cenocepacia* in the hypoxic environment of the CF lung.

In summary, we propose a mechanism whereby BCAS0292 regulates expression of multiple groups of genes which facilitate its adaptation to stress (Figure 7). In this way, it allows an environmental pathogen such as *Burkholderia* which is exquisitely successful at thriving in a wide variety of niches from the rhizosphere, to the CF lung and disinfectants. The finding that two thirds of proteins previously shown to be upregulated in chronic infection isolates suggests that BCAS0292 may

also play a role in the adaptation to the hypoxic CF lung. Future work will focus on identifying the probing the mechanism by which it mediates this effect.

Experimental procedures

Bacterial and epithelial cell culture maintenance. *B. cenocepacia* strain K56-2 was obtained from the BCCM/LMG, University of Ghent, Belgium and was routinely plated on to *B. cepacia* selective agar (BCSA) (Henry *et al.*, 1997). Bacteria were routinely grown in Luria Bertani (LB broth) at 37 °C with orbital agitation (150 rpm). The CFBE41o⁻ cell line, which is homozygous for the ΔF508 mutation, was maintained in fibronectin-vitrogen coated flasks containing minimal essential medium (MEM) with 10% FBS, 1% penicillin/ streptomycin, 1% L-glutamine and 1% non-essential amino acids and incubated in 5% CO₂ environment at 37 °C (Wright *et al.*, 2011).

Mutagenesis of *B. cenocepacia* K56-2 and complementation. Unmarked, non-polar gene deletions were constructed as previously described (Flannagan *et al.*, 2008). The deletion of BCAS0292 was confirmed by DNA sequencing of the PCR product spanning the deletion site. To complement *B. cenocepacia* K56-2_ΔBCAS0292, wild-type BCAS0292 was amplified from *B. cenocepacia* K56-2 (See primer pairs in Supplementary Table S1), digested with the restriction enzymes NdeI and XbaI and ligated into similarly digested pMH447. The complementation plasmid was introduced into the mutant by conjugation. Once transferred into the target mutant strain the complementation vector integrates into the genome at aminoglycoside efflux genes (*BCAL1674-BCAL1675*) (Hamad *et al.*, 2010). As before, pDAISce-I,

565 was then introduced, resulting in the replacement of BCAL1674-1675 by BCAS0292.
566 The complementation of BCAS0292 was confirmed by PCR and then confirmed by
567 phenotype analysis.

568

569 ***G. mellonella* infection model.** Log-phase grown bacteria were suspended to an
570 starting OD_{600nm} in 0.1 in sterile PBS and serially diluted to 10⁻⁷. Each dilution (20 µl)
571 was injected into the hindmost left proleg of *G. mellonella* larvae using a sterile
572 Terumo 0.3 ml syringe (10 larvae per group) (Costello *et al.*, 2011). An equal volume
573 of each dilution was also plated onto LB agar counted at 48 h to accurately quantify
574 bioburden injected. Ten larvae injected with PBS alone served as controls. Larvae
575 were incubated at 37 °C and examined for survival at 24, 48 and 72 h following
576 injection.

577

578 **Cloning and expression of recombinant BCAS0292.** The forward primer was
579 designed to contain the 'CACC' sequence at the beginning of the primer to introduce
580 a 5' overhang allowing directional cloning of BCAS0292 into the pET100/ D-Topo
581 vector in frame with the N-terminal 6xHis tag. PCR amplification was carried out
582 using HotStar Taq polymerase (Qiagen, Germany) and amplicons cloned into the
583 pET100/ D-Topo vector as previously described (Dennehy *et al.*, 2017). Each
584 transformation reaction was spread on to pre-warmed selective plates containing 50
585 µg/ml of ampicillin and incubated overnight at 37°C and transformants identified by
586 PCR. *E. coli* BL21 Star™ (DE3) One Shot® cells (Thermo Fisher Scientific) were
587 used for protein expression (Dennehy *et al.*, 2017). Following the confirmation of
588 recombinant BCAS0292 (rBCAS0292) expression in a pilot study, 1 L cultures of
589 BL21 cells transformed with the expression plasmid, were grown in LB-ampicillin

(100 µg/ ml) and induced with IPTG at a final concentration of 1 mM overnight at 25 °C. Bacteria were then pelleted using centrifugation at 2,500 g for 10 min. Supernatants were discarded, pellets weighed and stored at -80 °C.

593

Recombinant BCAS0292 purification. Purification of rBCAS0292 was carried out as previously described (Dennehy *et al.*, 2017). Ice-cold lysis buffer containing 6 M Guanidine-HCL, 100 mM sodium phosphate, 10 mM Tris-HCL, (pH= 8) and 1X EDTA-free protease inhibitor was added to bacterial pellets at a volume of 30 ml/ 7 g of pellet. Cells were resuspended in lysis buffer and lysed on ice with a cell disruptor (maximum output, two 30-sec pulses). Cell debris and any remaining intact cells were pelleted using centrifugation at 4,800 xg for 35 min. Supernatants were filtered through a 0.45 µM filter and incubated with a rotating HisPur Ni-NTA column (ThermoFisher, Ireland), which was pre-equilibrated with buffer A, for 1 h at 4 °C. Unbound proteins were removed by a series of washes and bound protein was subsequently eluted in two steps with 250 mM and 300 mM imidazole. Following purification, the protein concentration of each fraction was determined using the Bradford assay. Protein purity was determined by SDS-PAGE.

607

Attachment of bacteria to epithelial cells. To quantify the attachment of Bcc or BCAS0292-expressing *E. coli* strains to lung epithelial cells, CFBE41o⁺ cells were seeded in coated chamber slides 24 h prior to the 30 min incubation of individual bacterial strains at an MOI of 50:1, as previously described (Dennehy *et al.*, 2017). In the case of studies with recombinant *E. coli* strains, rBCAS0292 expression was induced in all strains (*E. coli* BL21, *E. coli* BL21_BCAS0292) with 1 mM IPTG for 3 h prior to incubation with epithelial cells. Unattached bacteria were removed by

615 washing with sterile PBS four times for five min each. The CFBE41o⁻ cells and
 616 attached bacteria were fixed in 3 % paraformaldehyde (pH=7.2) for 10 min at room
 617 temperature. Cells were gently washed once with PBS and then blocked in PBS
 618 containing 5 % BSA for one hour at room temperature. Cells were then incubated
 619 overnight at 4 °C in PBS containing 1 % BSA and either a rabbit anti-Bcc antibody
 620 (1:800 dilution) or an anti-*E. coli* FITC-conjugated antibody (1:100 dilution). The
 621 following day cells were washed three times with PBS before the addition of FITC-
 622 conjugated anti-rabbit antibody (1:500 dilution), in the case of Bcc strains, for one h
 623 in the dark. The secondary antibody was removed, and cells washed twice for 5 min
 624 each with PBS. Cells were then counterstained with DAPI and phalloidin conjugated
 625 to Alexa fluor 568 (5U/ ml) for 15 min in the dark at a concentration of 1 µg/ml in
 626 PBS. A coverslip was applied to each chamber before bacteria and cells were
 627 visualised using confocal microscopy. Bacterial attachment was counted in 20
 628 randomly selected fields for each strain and values were expressed as the number of
 629 bacteria/100 cells.

630

631 **Whole proteome analysis.** To extract whole cell lysates were extracted from
 632 stationary phase cultures of three biological replicates of K56-2 WT and the
 633 ΔBCAS0292 mutant strains. Bacterial cells were pelleted with centrifugation of 6,000
 634 g for ten min. Ice-cold lysis buffer containing 8 M Urea, 25 mM Tris-HCL, 10 mM
 635 DTT and 1 X EDTA-free protease inhibitor cocktail (pH=8.6) was added to pellets,
 636 using a volume of 8 ml/g bacterial pellet. Pellets were resuspended and cells lysed
 637 using a cell-disruptor probe set with an output power of 16, using four 30-sec bursts
 638 on ice. Lysates were then centrifuged at 14,000 xg for 15 min, supernatants
 639 transferred to fresh tubes and a Bradford assay used to determine the protein

640 concentration of each sample. DTT (1M) was added to each supernatant (10 µl/ml
641 lysate) and incubated at 56 °C for 30 min followed by 1M iodoacetamide (55 µl/ml
642 lysate) which was incubated in darkness at RT for 20 min. Lysates were then dialysed
643 in dialysis tubing with a cut-off of 3.5 kDa against 100 mM ammonium bicarbonate
644 overnight with stirring at 4 °C then the ammonium bicarbonate was replaced with
645 fresh solution and incubated for a further six hours. Trypsin (400 ng/µl) was added to
646 dialysed protein (5 µl/100 µl protein) and tubes incubated overnight at 37 °C. Aliquots
647 (20 µl) from each sample were placed in fresh tubes and samples were dried in a
648 speedy vac using medium heat and resuspended in 20 µl resuspension buffer
649 containing 0.5 % TFA in MilliQ water. Tubes were sonicated for two min and
650 centrifuged briefly. ZipTips (Millipore, Germany) were wetted with 10 µl of wetting
651 solution containing 0.1 % TFA in 80 % acetonitrile slowly five times. Equilibration
652 solution (10 µl) containing 0.1 % TFA in MilliQ water was aspirated and dispensed
653 into the ZipTip, five times before 10 µl of the resuspended sample was then aspirated
654 and dispensed slowly into the ZipTip, 15 times. Then 10 µl of the washing solution
655 containing 0.1 % TFA in MilliQ water was aspirated into the ZipTip and dispensed to
656 waste. This step was repeated five times before 10 µl of the elution solution
657 containing 0.1 % TFA in 60 % Acetonitrile was aspirated and dispensed from the
658 ZipTip to a fresh 1.5 ml tube six times. Samples were then dried under medium heat
659 and resuspended in 15 µl of loading buffer containing 0.05 % TFA in 2 % acetonitrile.
660 Tubes were sonicated for 2 min, centrifuged briefly and samples transferred into fresh
661 vials and 3 µl (1 µg protein) used for Q-Exactive analysis. All biological replicate and
662 technical replicate samples were analysed on a Thermo Scientific Q Exactive mass
663 spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography
664 system. Each sample was loaded onto an EASY-Spray PepMap RSLC C18 Column

(50 cm x 75 μ m, Thermo Scientific), and was separated by an increasing acetonitrile gradient over 120 min at a flow rate of 250 nL/min. The mass spectrometer was operated in positive ion mode with a capillary temperature of 220 °C, and with a potential of 2500 V applied to the frit. All data was acquired with the MS operating in automatic data dependent switching mode. A high resolution (140,000) MS scan (300-2000 Dalton) was performed using the Q-Exactive to select the 15 most intense ions prior to MS/MS analysis using HCD (higher-energy collisional dissociation). Protein identification and label free quantitative (LFQ) analysis was conducted using MaxQuant (version 1.2.2.5: <http://maxquant.org/>) supported by the Andromeda database search engine to correlate MS/MS data against the *B. cenocepacia* strain J2315 Uniprot database and Perseus to organise the data (Version 1.4.1.3). For protein identification the following search parameters were used: precursor-ion mass tolerance of 1.5 Da, fragment ion mass tolerance of 6 ppm with cysteine carbamidomethylation as a fixed modification and a maximum of 2 missed cleavage sites allowed. False Discovery Rates (FDR) were set to 0.01 for both peptides and proteins and only peptides with minimum length of six amino acid length were considered for identification. Proteins were considered identified when a minimum of two peptides for each parent protein was observed. LFQ intensities measured for individual runs were grouped based on their experimental treatment. The data was log2 transformed and an ANOVA ($p < 0.05$) was performed between the control and individual treatment samples to identify differentially abundant proteins. A log2 fold change ≥ 1.5 and log2 fold change ≤ -1.5 with adjusted p-value < 0.05 were considered. Proteins with statistically significant differential expression were extracted and these were used to generate maps of expression. To improve visual representation of differentially abundant proteins, mean values were generated for

each treatment and used to build the maps. A qualitative assessment was also conducted. This involved the identification of proteins that were completely absent in a specific strain. Those proteins that were completely missing from all replicates of a particular group but present in other groups were determined manually from the data matrix. These proteins are not considered statistically significant as the values for absences are given as NaN (not a number) which is not a valid value for an ANOVA analysis. However, the complete absence of a protein from a group may be biologically significant and these proteins are reported as qualitatively differentially expressed.

Bacterial cell shape analysis. Cell shape was examined according to published methods (Shiomi *et al.*, 2008). Cells were grown at 37°C in LB overnight before reinoculation and culture to achieve log growth (OD600 of 0.5 – 0.7). Optical sectioning was performed with an Olympus FV - 10 ASW confocal microscope using a Plan Apo X60 1.40 oil immersion objective lens and Olympus FLUOVIEW Ver.3.1 software. Section images were captured along the z - axis at 0.2 µm intervals.

Circular dichroism spectroscopy. Circular dichroism measurements were carried out using a Jasco J-810 spectropolarimeter equipped with a Peltier temperature control system (Model PTC-423-S). The molar ellipticity per mean residue, $[\theta](\text{deg cm}^2 \times \text{dmol}^{-1})$, $[\theta](\text{deg cm}^2 \times \text{dmol}^{-1})$ was calculated from the equation $[\theta] = [\theta]_{\text{obs}} \times \text{mrw} \times (10 \times l \times C)^{-1}$, where $[\theta]_{\text{obs}}$ is the ellipticity measured in degrees, mrw is the mean residue molecular mass (105.3 Da), C is the protein concentration in $\text{g} \times \text{l}^{-1}$ and l is the optical path length of the cell in cm. Far-UV spectra (190–260 nm) of recombinant BCAS0292 protein were

715 recorded at 293K using a 0.1 cm optical path-length cell, with a protein concentration
716 of 0.2 mg ml⁻¹. For thermal denaturation experiments, ellipticity was monitored at 222
717 nm with a temperature slope of 1°C/min from 20°C to 100°C. Melting temperature
718 (*T_m*) was calculated from the maximum of the first derivative of the unfolding curves.
719

720 **Light scattering Analysis.** Purified rBCAS0292 was analyzed by size exclusion
721 chromatography (SEC) coupled with light scattering (LS) using a DAWN MALS
722 instrument and an Optilab rEX (Wyatt Technology). Eight hundred micrograms of
723 purified rBCAS0292 was loaded into the column S75 16/60 (GE Healthcare, Italy),
724 equilibrated in 50mM TrisHCl, 150mM NaCl, 5% Glycerol, pH 7.5 and. The on-line
725 measurement of the intensity of the Rayleigh scattering as a function of the angle as
726 well as the differential refractive index of the eluting peak in SEC was used to
727 determine the weight average molar mass (MW) of eluted protein, using the Astra
728 5.3.4.14 (Wyatt Technologies, USA) software.

729
730 **Homology modelling.** The homology model structure of BCAS0292 was built after
731 consensus-based sequence comparison using HHPRED. Best model template was
732 identified as the structure of the PixA inclusion body protein from *Burkholderia*
733 *cenocepacia* (PDB code 4lzk, sequence identity 23.2%, E-value 7.2e-38). Using this
734 alignment, the homology model was built using the program MODELLER
735 (Bitencourt-Ferreira and de Azevedo, 2019). As a validation tool, an independent
736 model was computed using I-Tasser (Yang and Zhang, 2015) and the two models
737 compared. The meta-threading approach LOMETS was used to retrieve template
738 proteins of similar folds from the PDB library. The program SPICKER was used to
739 cluster the decoys based on the pair-wise structure similarity. Best confidence model

presented a C-score -0.34 (Yang and Zhang, 2015). The two approaches provided consistent models, with RMSD computed on C α atoms of 1.5 Å. Based on the estimated local accuracy of the model computed by i-TASSER, the C-terminal 10 residues were removed from the model. Electrostatic potential surface was computed using the program Chimera (Yang *et al.*, 2012).

745

Chloroquine gel electrophoresis. *B. cenocepacia* strains were transformed with pDA-12 reporter plasmid and cultured on plates containing tetracycline (150 µg/ml), ampicillin (100µg/ml) and polymyxin B (50 µg/ml). The reporter plasmids were extracted from either log or stationary phase cultures using a Qiagen Miniprep kit (ThermoFisher, Germany). The plasmids were analyzed on chloroquine gels (1.5% agarose, 10 µg/ml chloroquine) separated for 24 h at 100V, 150mA (Scanlan *et al.*, 2017). The gels were washed 8 to 12 times over 8 h to remove chloroquine, before staining with ethidium bromide (10 µg/ml) for 2 h. The gel was washed for 1 h before imaging under UV light (Scanlan *et al.*, 2017).

755

Statistical analysis. Analysis of virulence data was performed using a log rank (Mantel-Cox) test on the survival curves. Statistical analysis of host cell attachment, biofilm formation and hypoxic growth and survival were carried out by two-way ANOVA using Prism software.

760

761

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769

770 **Data Availability:** The data that support the findings of this study are available from
771 the corresponding author upon reasonable request.

772

773 References

- 774 Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., *et al.*
775 1997. Gapped BLAST and PSI-BLAST: a new generation of protein
776 database search programs. *Nucleic Acids Res*, 25, 3389-402.
- 777 Aravind, L., Anantharaman, V., Balaji, S., Babu, M. M. & Iyer, L. M. 2005. The many
778 faces of the helix-turn-helix domain: transcription regulation and beyond.
779 *FEMS Microbiol Rev*, 29, 231-62.
- 780 Bach, E., Sant'anna, F. H., Magrich Dos Passos, J. F., Balsanelli, E., De Baura, V. A.,
781 Pedrosa, F. O., *et al.* 2017. Detection of misidentifications of species from
782 the Burkholderia cepacia complex and description of a new member, the
783 soil bacterium Burkholderia catarinensis sp. nov. *Pathog Dis*, 75.
- 784 Bitencourt-Ferreira, G. & De Azevedo, W. F., Jr. 2019. Homology Modeling of
785 Protein Targets with MODELLER. *Methods Mol Biol*, 2053, 231-249.
- 786 Caraher, E., Duff, C., Mullen, T., Mc Keon, S., Murphy, P., Callaghan, M., *et al.* 2007.
787 Invasion and biofilm formation of Burkholderia dolosa is comparable with
788 Burkholderia cenocepacia and Burkholderia multivorans. *J Cyst Fibros*, 6,
789 49-56.
- 790 Costello, A., Herbert, G., Fabunmi, L., Schaffer, K., Kavanagh, K. A., Caraher, E. M.,
791 *et al.* 2011. Virulence of an emerging respiratory pathogen, genus
792 Pandoraea, in vivo and its interactions with lung epithelial cells. *J Med*
793 *Microbiol*, 60, 289-99.
- 794 Cullen, L., O'connor, A., Drevinek, P., Schaffer, K. & McClean, S. 2017. Sequential
795 Burkholderia cenocepacia Isolates from Siblings with Cystic Fibrosis
796 Show Increased Lung Cell Attachment. *Am J Respir Crit Care Med*, 195,
797 832-835.
- 798 Cullen, L., O'connor, A., McCormack, S., Owens, R. A., Holt, G. S., Collins, C., *et al.*
799 2018. The involvement of the low-oxygen-activated locus of Burkholderia
800 cenocepacia in adaptation during cystic fibrosis infection. *Scientific*
801 *Reports*, 8, 13386.
- 802 De Oliveira, D. M. P., Forde, B. M., Kidd, T. J., Harris, P. N. A., Schembri, M. A.,
803 Beatson, S. A., *et al.* 2020. Antimicrobial Resistance in ESKAPE Pathogens.
804 *Clin Microbiol Rev*, 33.
- 805 Dennehy, R., Romano, M., Ruggiero, A., Mohamed, Y. F., Dignam, S. L., Mujica
806 Troncoso, C., *et al.* 2017. The Burkholderia cenocepacia peptidoglycan-

807 associated lipoprotein is involved in epithelial cell attachment and
808 elicitation of inflammation. *Cell Microbiol*, 19.

809 Dorman, C. J. & Corcoran, C. P. 2009. Bacterial DNA topology and infectious
810 disease. *Nucleic Acids Res*, 37, 672-8.

811 Flannagan, R. S., Linn, T. & Valvano, M. A. 2008. A system for the construction of
812 targeted unmarked gene deletions in the genus *Burkholderia*. *Environ*
813 *Microbiol*, 10, 1652-60.

814 Goetsch, M., Owen, H., Goldman, B. & Forst, S. 2006. Analysis of the PixA inclusion
815 body protein of *Xenorhabdus nematophila*. *J Bacteriol*, 188, 2706-10.

816 Hamad, M. A., Skeldon, A. M. & Valvano, M. A. 2010. Construction of
817 aminoglycoside-sensitive *Burkholderia cenocepacia* strains for use in
818 studies of intracellular bacteria with the gentamicin protection assay.
819 *Appl Environ Microbiol*, 76, 3170-6.

820 Hegde, S. S., Vetting, M. W., Roderick, S. L., Mitchenall, L. A., Maxwell, A., Takiff, H.
821 E., *et al.* 2005. A fluoroquinolone resistance protein from *Mycobacterium*
822 *tuberculosis* that mimics DNA. *Science*, 308, 1480-3.

823 Henry, D. A., Campbell, M. E., Lipuma, J. J. & Speert, D. P. 1997. Identification of
824 *Burkholderia cepacia* isolates from patients with cystic fibrosis and use of
825 a simple new selective medium. *J Clin Microbiol*, 35, 614-9.

826 Holden, M. T., Seth-Smith, H. M., Crossman, L. C., Sebahia, M., Bentley, S. D.,
827 Cerdeno-Tarraga, A. M., *et al.* 2009. The genome of *Burkholderia*
828 *cenocepacia* J2315, an epidemic pathogen of cystic fibrosis patients. *J*
829 *Bacteriol*, 191, 261-77.

830 Huang, M. F., Lin, S. J., Ko, T. P., Liao, Y. T., Hsu, K. C. & Wang, H. C. 2017. The
831 monomeric form of *Neisseria* DNA mimic protein DMP19 prevents DNA
832 from binding to the histone-like HU protein. *PLoS One*, 12, e0189461.

833 Huber, B., Feldmann, F., Kothe, M., Vandamme, P., Wopperer, J., Riedel, K., *et al.*
834 2004. Identification of a novel virulence factor in *Burkholderia*
835 *cenocepacia* H111 required for efficient slow killing of *Caenorhabditis*
836 *elegans*. *Infect Immun*, 72, 7220-30.

837 Ispolatov, I., Yuryev, A., Mazo, I. & Maslov, S. 2005. Binding properties and
838 evolution of homodimers in protein-protein interaction networks. *Nucleic*
839 *Acids Res*, 33, 3629-35.

840 Kooi, C., Subsin, B., Chen, R., Pohorelic, B. & Sokol, P. A. 2006. *Burkholderia*
841 *cenocepacia* ZmpB is a broad-specificity zinc metalloprotease involved in
842 virulence. *Infect Immun*, 74, 4083-93.

843 Lucas, J., Goetsch, M., Fischer, M. & Forst, S. 2018. Characterization of the *pixB*
844 gene in *Xenorhabdus nematophila* and discovery of a new gene family.
845 *Microbiology*, 164, 495-508.

846 Mahenthiralingam, E., Baldwin, A. & Dowson, C. G. 2008. *Burkholderia cepacia*
847 complex bacteria: opportunistic pathogens with important natural
848 biology. *J Appl Microbiol*, 104, 1539-51.

849 Mares, J., Kumaran, S., Gobbo, M. & Zerbe, O. 2009. Interactions of
850 lipopolysaccharide and polymyxin studied by NMR spectroscopy. *J Biol*
851 *Chem*, 284, 11498-506.

852 McClean, S., Healy, M. E., Collins, C., Carberry, S., O'shaughnessy, L., Dennehy, R.,
853 *et al.* 2016. Linocin and *OmpW* Are Involved in Attachment of the Cystic
854 Fibrosis-Associated Pathogen *Burkholderia cepacia* Complex to Lung

855 Epithelial Cells and Protect Mice against Infection. *Infect Immun*, 84,
856 1424-37.

857 Mhatre, E., Snyder, D. J., Sileo, E., Turner, C. B., Buskirk, S. W., Fernandez, N. L., *et*
858 *al.* 2020. One gene, multiple ecological strategies: a biofilm regulator is a
859 capacitor for sustainable diversity. *bioRxiv*, 2020.05.02.074534.

860 O' Croinin, T., Carroll, R. K., Kelly, A. & Dorman, C. J. 2006. Roles for DNA
861 supercoiling and the Fis protein in modulating expression of virulence
862 genes during intracellular growth of *Salmonella enterica* serovar
863 Typhimurium. *Mol Microbiol*, 62, 869-82.

864 O'grady, E. P. & Sokol, P. A. 2011. *Burkholderia cenocepacia* differential gene
865 expression during host-pathogen interactions and adaptation to the host
866 environment. *Front Cell Infect Microbiol*, 1, 15.

867 O'grady, E. P., Viteri, D. F., Malott, R. J. & Sokol, P. A. 2009. Reciprocal regulation
868 by the CeiIR and CciIR quorum sensing systems in *Burkholderia*
869 *cenocepacia*. *BMC Genomics*, 10, 441.

870 Peeters, C., Zlosnik, J. E., Spilker, T., Hird, T. J., Lipuma, J. J. & Vandamme, P. 2013.
871 *Burkholderia pseudomultivorans* sp. nov., a novel *Burkholderia cepacia*
872 complex species from human respiratory samples and the rhizosphere.
873 *Syst Appl Microbiol*, 36, 483-9.

874 Roszniewski, B., McClean, S. & Drulis-Kawa, Z. 2018. *Burkholderia cenocepacia*
875 Prophages-Prevalence, Chromosome Location and Major Genes Involved.
876 *Viruses*, 10.

877 Saini, L. S., Galsworthy, S. B., John, M. A. & Valvano, M. A. 1999. Intracellular
878 survival of *Burkholderia cepacia* complex isolates in the presence of
879 macrophage cell activation. *Microbiology*, 145 (Pt 12), 3465-75.

880 Sass, A., Marchbank, A., Tullis, E., Lipuma, J. J. & Mahenthiralingam, E. 2011.
881 Spontaneous and evolutionary changes in the antibiotic resistance of
882 *Burkholderia cenocepacia* observed by global gene expression analysis.
883 *BMC Genomics*, 12, 373.

884 Sass, A. M., Schmerk, C., Agnoli, K., Norville, P. J., Eberl, L., Valvano, M. A., *et al.*
885 2013. The unexpected discovery of a novel low-oxygen-activated locus for
886 the anoxic persistence of *Burkholderia cenocepacia*. *Isme J*, 7, 1568-81.

887 Scanlan, E., Ardill, L., Whelan, M. V., Shortt, C., Nally, J. E., Bourke, B., *et al.* 2017.
888 Relaxation of DNA supercoiling leads to increased invasion of epithelial
889 cells and protein secretion by *Campylobacter jejuni*. *Mol Microbiol*, 104,
890 92-104.

891 Schmerk, C. L. & Valvano, M. A. 2013. *Burkholderia multivorans* survival and
892 trafficking within macrophages. *J Med Microbiol*, 62, 173-184.

893 Shinoy, M., Dennehy, R., Coleman, L., Carberry, S., Schaffer, K., Callaghan, M., *et al.*
894 2013. Immunoproteomic analysis of proteins expressed by two related
895 pathogens, *Burkholderia multivorans* and *Burkholderia cenocepacia*,
896 during human infection. *PLoS One*, 8, e80796.

897 Shiomi, D., Sakai, M. & Niki, H. 2008. Determination of bacterial rod shape by a
898 novel cytoskeletal membrane protein. *EMBO J*, 27, 3081-91.

899 Shortt, C., Scanlan, E., Hilliard, A., Cotroneo, C. E., Bourke, B. & T, O. C. 2016. DNA
900 Supercoiling Regulates the Motility of *Campylobacter jejuni* and Is Altered
901 by Growth in the Presence of Chicken Mucus. *MBio*, 7.

902 Summer, E. J., Gonzalez, C. F., Carlisle, T., Mebane, L. M., Cass, A. M., Savva, C. G., *et*
903 *al.* 2004. Burkholderia cenocepacia phage BcepMu and a family of Mu-like
904 phages encoding potential pathogenesis factors. *J Mol Biol*, 340, 49-65.
905 Valvano, M. A. 2015. Intracellular survival of Burkholderia cepacia complex in
906 phagocytic cells. *Can J Microbiol*, 61, 607-15.
907 Wang, H. C., Chou, C. C., Hsu, K. C., Lee, C. H. & Wang, A. H. 2018. New paradigm of
908 functional regulation by DNA mimic proteins: Recent updates. *IUBMB Life*.
909 Wang, H. C., Ho, C. H., Hsu, K. C., Yang, J. M. & Wang, A. H. 2014. DNA mimic
910 proteins: functions, structures, and bioinformatic analysis. *Biochemistry*,
911 53, 2865-74.
912 Wang, H. C., Ko, T. P., Wu, M. L., Ku, S. C., Wu, H. J. & Wang, A. H. 2012. Neisseria
913 conserved protein DMP19 is a DNA mimic protein that prevents DNA
914 binding to a hypothetical nitrogen-response transcription factor. *Nucleic*
915 *Acids Res*, 40, 5718-30.
916 Wang, H. C., Wu, M. L., Ko, T. P. & Wang, A. H. 2013. Neisseria conserved
917 hypothetical protein DMP12 is a DNA mimic that binds to histone-like HU
918 protein. *Nucleic Acids Res*, 41, 5127-38.
919 Winsor, G. L., Khaira, B., Van Rossum, T., Lo, R., Whiteside, M. D. & Brinkman, F. S.
920 2008. The Burkholderia Genome Database: facilitating flexible queries
921 and comparative analyses. *Bioinformatics*, 24, 2803-4.
922 Wright, C., Pilkington, R., Callaghan, M. & McClean, S. 2011. Activation of MMP-9
923 by human lung epithelial cells in response to the cystic fibrosis-associated
924 pathogen Burkholderia cenocepacia reduced wound healing in vitro. *Am J*
925 *Physiol Lung Cell Mol Physiol*, 301, L575-86.
926 Yang, J. & Zhang, Y. 2015. I-TASSER server: new development for protein
927 structure and function predictions. *Nucleic Acids Res*, 43, W174-81.
928 Yang, Z., Lasker, K., Schneidman-Duhovny, D., Webb, B., Huang, C. C., Pettersen, E.
929 F., *et al.* 2012. UCSF Chimera, MODELLER, and IMP: an integrated
930 modeling system. *J Struct Biol*, 179, 269-78.
931 Yano, H., Kuga, A., Okamoto, R., Kitasato, H., Kobayashi, T. & Inoue, M. 2001.
932 Plasmid-encoded metallo-beta-lactamase (IMP-6) conferring resistance to
933 carbapenems, especially meropenem. *Antimicrob Agents Chemother*, 45,
934 1343-8.
935 Zhanhua, C., Gan, J. G., Lei, L., Sakharkar, M. K. & Kanguane, P. 2005. Protein
936 subunit interfaces: heterodimers versus homodimers. *Bioinformation*, 1,
937 28-39.
938 Zhou, J., Chen, Y., Tabibi, S., Alba, L., Garber, E. & Saiman, L. 2007. Antimicrobial
939 susceptibility and synergy studies of Burkholderia cepacia complex
940 isolated from patients with cystic fibrosis. *Antimicrob Agents Chemother*,
941 51, 1085-8.
942 Zoued, A., Durand, E., Santin, Y. G., Journet, L., Roussel, A., Cambillau, C., *et al.*
943 2017. TssA: The cap protein of the Type VI secretion system tail.
944 *Bioessays*, 39.
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947 **Table 1: Total number of differentially expressed proteins between K56-2 WT**
 948 **and $\Delta BCAS0292$ strains**

Protein groups	Number of differentially expressed proteins
Total number of proteins identified	1,933
Total number of proteins altered by ≥ 1.5 or absent in all replicates of a either strain	1,016
Proteins unique to K56-2 WT	46
Proteins unique to $\Delta BCAS0292$	23
Proteins significantly downregulated (by ≥ 1.5 -fold) in the $\Delta BCAS0292$ strain	556
Proteins significantly upregulated (by ≥ 1.5 -fold) in the $\Delta BCAS0292$ strain	391

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Table 2: Proteins that were unique to either the ΔBCAS0292 or K56-2 WT strain, i.e proteins were absent or undetectable in the mutant strain relative to WT or in the WT strain relative to the mutant strain.

Gene	Protein name	UniProtKB ID	strain	# peptides	MW	SC
Amino acid metabolism						
BCAS0147	metF	B4EPR4_BURCI		3	41.627	12.7
BCAM0478	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]	B4E1Y0_BURCI		7	66.539	26.9
BCAM1488	Putative proline racemase	B4E1H9_BURCI		2	36.295	10.4
Antibiotic resistance						
BCAM2634a	Putative chloramphenicol resistance-related protein	B4EK89_BURCI		5	19.001	41.8
BCAL3430 ampD	N-acetylmuramyl-L-alanine amidase	B4ESW0_BURCI		3	21.514	19.4
Biosynthetic process						
BCAL0614	Uncharacterized protein	B4E937_BURCI		3	15.312	35.1
BCAL1450	Putative nucleosidase	B4E716_BURCI		4	27.578	21.1
BCAL3128 wbxF	Glycosyltransferase	B4ECE8_BURCI		2	49.489	6.9
BCAM2782	Putative acetyltransferase protein	B4ELI0_BURCI		2	20.671	27
Catalytic activity						
BCAL0667	Putative adenosylmethionine-8-amin-7-oxononanoate aminotransferase	B4E9L7_BURCI		5	49.026	17.4
BCAL0983	Pseudouridine synthase	B4EA15_BURCI		3	37.388	12.3
DNA repair						
BCAL2774	Methylated-DNA--protein-cysteine methyltransferase	B4E9H3_BURCI		3	17.101	27.6
BCAL3141	Putative Holliday junction resolvase	JRUVX_BURCI		2	16.642	14.1
BCAL0041	Primosomal protein N	B4EEZ4_BURCI		2	81.339	4.2
Electron transfer						
BCAL0330 petC	Cytochrome c1	B4E6Q2_BURCI		3	28.388	16.7
BCAM1734	Putative cytochrome C	B4ELB2_BURCI		3	45.422	14.1
Membrane protein						
BCAL2166	Putative lipoprotein	B4ED88_BURCI		4	41.467	16.8
BCAL3377 omIA	Outer membrane protein assembly factor BamE	B4EEW5_BURCI		2	27.098	15.1
BCAM0264	Putative membrane protein	B4E175_BURCI		4	41.859	12.2
BCAS0125	Uncharacterized protein	B4EPP2_BURCI		2	26.453	18.6
BCAS0321	Hypothetical glycine-rich autotransporter protein	B4EQ87_BURCI		4	382.92	1.3
Metabolic process						
BCAL1913	Putative acetoin catabolism protein	B4EAZ9_BURCI		4	38.833	21.7
BCAL2202	Putative iron-sulfur binding protein	B4EDT1_BURCI		3	52.122	11.5
BCAL3058	3-octaprenyl-4-hydroxybenzoate carboxylase	B4EBU2_BURCI		2	56.941	13.9
BCAM2096	Putative gamma-glutamylputrescine oxidoreductase	B4EFG4_BURCI		3	47.096	10.7
BCAM0067	Putative short chain dehydrogenase	B4EG11_BURCI		3	24.257	21.5
BCAM0138	Phosphoglycerate mutase family protein	B4EH38_BURCI		2	24.526	13.8
Motility						
FlgK	Flagellar hook-associated protein 1 (HAP1)	B4E8L8_BURCI		3	65.147	8.7
FlgL	Flagellar hook-associated protein 3 (HAP3)	B4E8L9_BURCI		2	42.136	8
Oxidation reduction						
BCAM0016	Tartrate dehydrogenase	B4EG02_BURCI		6	39.657	29.4
BCAL0603	Gamma-glutamyl-gamma-aminobutyraldehyde dehydrogenase	B4E926_BURCI		2	52.823	8.5
BCAL2978	NAD-dependent formate dehydrogenase alpha subunit	B4EB77_BURCI		7	107.08	12.4
BCAM0155	2-deoxy-D-glucosate 3-dehydrogenase	B4EH55_BURCI		3	28.113	18.3
BCAL1911 accA	Acetoin:2,6-dichlorophenol dioxygenase	B4EA27_BURCI		3	36.334	10.8
BCAM0585	Putative aldo/keto reductase	B4EL06_BURCI		3	31.649	19.9
BCAM1682	Putative short-chain dehydrogenase/reductase	B4EKT2_BURCI		5	42.665	20.2
BCAM1814	Putative flavin-binding monooxygenase	B4ELX4_BURCI		3	59.388	4.9
BCAM1860	2OG-Fe(II) oxygenase superfamily protein	B4EMC2_BURCI		3	26.646	16.7
BCAM2754	Putative ketoreductase	B4ELF3_BURCI		2	25.403	11.6
BCAL3430 ampD	N-acetylmuramyl-L-alanine amidase	B4ESW0_BURCI		3	21.514	19.4
Phage Protein						
BCAL2970	Hypothetical phage protein	B4EB67_BURCI		3	25.278	25
BCAM1075	Hypothetical phage protein	B4EG55_BURCI		3	15.562	32.2
BCAS0505a	Hypothetical phage protein	B4ENQ1_BURCI		3	80.076	6.2
BCAS0517	Putative phage tail tube protein	B4ENR3_BURCI		4	19.047	35.1
BCAS0518	Putative phage tail sheath protein	B4ENR4_BURCI		7	50.266	18
BCAS0524	Hypothetical phage protein	B4ENS0_BURCI		3	41.115	9.3
BCAS0527	Hypothetical phage protein	B4ENS3_BURCI		2	54.396	5.1
BCAS0547	Putative DNA-binding phage protein	B4ENU6_BURCI		4	44.578	15.6
PilA protein						
BCAM1413a	Uncharacterized protein	B4E1I9_BURCI		4	19.421	24.5
BCAM1414	Uncharacterized protein	B4E1I0_BURCI		2	20.313	17.8
proteolysis						
BCAL1267	ATP-dependent zinc metalloprotease RtsH	B4ESF4_BURCI		3	69.001	9.2
Purine/ pyrimidine metabolism						
BCAL3010	Guanosine-3,5-bis(Diphosphate) 3-pyrophosphohydrolase	B4EBP4_BURCI		3	88.067	5.2
BCAS0731	phenylhydantoinase	B4EPB9_BURCI		4	52.877	12.6
RNA processing						
BCAL2076	Putative RNA methylase protein	B4EC83_BURCI		2	27.145	23.8
cell signalling						
BCAM0288	Two-component regulatory system, response regulator protein	B4E1A0_BURCI		3	23.744	21.4
BCAM1705	Two-component regulatory system, sensor kinase protein	B4EL83_BURCI		3	70.546	5.1
BCAM0580 RpfR	Putative cyclic-di-GMP signaling protein	B4EKM4_BURCI		3	73.391	7.5
Transcription						
BCAL1721	Putative transcriptional regulator	B4E9C0_BURCI		4	26.971	30.4
BCAL2017	Transcription-repair-coupling factor	B4EC24_BURCI		3	129.1	4.6
BCAL2686	Cys regulon transcriptional activator CysB	B4E8V9_BURCI		3	34.874	19.2
BCAM0756	GtrR family regulatory protein	B4EM58_BURCI		4	26.203	24.7
BCAL1797	ArsC family regulatory protein	B4E9X8_BURCI		2	17.092	16.9
Unknown						
BCAL0384	Uncharacterized protein	B4E6V7_BURCI		2	12.862	34.8
BCAL0872	Uncharacterized protein	B4EBC3_BURCI		2	14.959	21.2
BCAM0429	Uncharacterized protein	B4E1F2_BURCI		2	13.806	26.6
BCAM0576	UPF0317 protein Bce12315_40370	Y4037_BURCI		4	27.953	23.3
BCAM2204	Uncharacterized protein	B4EGD6_BURCI		2	10.129	39.8
BCAS0664	Uncharacterized protein	B4EP58_BURCI		5	16.938	44.1
BCAS0686	Uncharacterized protein	B4EP78_BURCI		3	14.851	38.6
SC - Sequence coverage	Absent in Mutant at stationary phase - expression requires BCAS0292					
	Absent in WT at stationary phase - Repressed by BCAS0292					

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Table 3: Examples of proteins altered by >2 fold ΔBCAS0292 mutant relative to K56-2 WT cells.

BCA ID	Protein names	Fold diff	BCAM1736	Putative oxidoreductase	-3.7	Fold decrease
Stress Response			BCAM2129	2-amino-3-carboxymuconate 6-semialdehyde decarboxylase	-6.4	2 -4
BCAL0332	Putative stringent starvation protein B	-2.3	BCAM2547a	Glyoxalase/bleomycin resistance protein/dioxygenase superfamily protein	-33.2	4.1 to 11.9
BCAL0438	Putative DNA-3-methyladenine glycosylase II	+9.8	BCAM2752	NAD dependent epimerase/dehydratase	-9.5	12 to 40
BCAL1648	Putative stress-related protein	-3.2	BCAS0252	DJ-1/Pfpl family protein	-2.7	>40
BCAL2119	Universal stress protein family protein	-2.7	ClpA	Putative ATP-dependent Clp protease ATP-binding subunit	+2.9	Fold increase
BCAM0276	Universal stress protein	-2.0	HutU	Urocanate hydratase	+2.9	2-4
BCAM0277	Uncharacterized protein	-2.6	pBCA087	NUDIX hydrolase family protein	-62.0	4.1 to 10
BCAM0278	Putative heat shock protein	-276	Phage protein			
BCAM0284	Putative cytochrome c	-3.3	BCAL3205a	Putative phage-related regulator	-5.4	
BCAM0285	Uncharacterized protein	-4.3	BCAM1883	Hypothetical phage protein	-9.5	
BCAM0286	Putative alcohol dehydrogenase	-2.0	BCAM1919	Hypothetical phage protein	-8.5	
BCAM0290	Putative universal stress protein	-2.2	BCAM1920	Hypothetical phage protein	-12.5	
BCAM0292	Putative universal stress protein	-6.5	BCAS0551	HU DNA-binding protein	-60.1	
BCAM0296	Acetoacetyl-CoA reductase phbB	2.36	Signaling			
BCAM0307	Uncharacterized protein	-6.3	BCAL0208	AnsC family regulatory protein	-4.2	
BCAM0308	Uncharacterized protein	-6.3	BCAM0623	Two-component regulatory system, response regulator protein	-2.7	
BCAM0315	Putative exported protein	-3.6	BCAM2756	Two-component regulatory system, response regulator protein	-4.3	
BCAM0316	Uncharacterized protein	-2.1	CblR	Two-component regulatory system, response regulator protein	-4.3	
BCAM1495	Universal stress protein	-3.0	BCAM0176	AnsC family regulatory protein	-3.0	
DNA repair			Transcription			
BCAL3277	DNA repair protein RecN	+3.9	RpoD	RNA polymerase sigma factor RpoD	+3.8	
UvrA	UvrABC system protein A	+4.9	BCAL1011	Sigma-54 interacting response regulator	+2.1	
MutS	DNA mismatch repair protein MutS	+3.1	CheB1	Chemotaxis response regulator protein-glutamate methyltransferase	+2.9	
UvrB	UvrABC system protein B	+3.1	RpoB	DNA-directed RNA polymerase subunit β	+3.2	
DNA binding			RpoC	DNA-directed RNA polymerase subunit β	+2.9	
BCAL3297	Putative ferritin DPS-family DNA binding protein	-2.3	BCAS0191	Putative endoribonuclease	-17.4	
BCAL0122	Histone-like nucleoid-structuring (H-NS) protein	-2.1	Virulence			
BCAL2295	Putative chromosome condensation protein	-4.2	BCAL0337	Type VI secretion system protein TssL	-4.2	
BCAM1012	Putative histone-like protein	+4.2	BCAL1529	Flp pilus type assembly-related protein	-6.2	
BCAM1619	Putative DNA-binding cold-shock protein	-2.7	BCAS0293	Nematocidal protein AidA	-49.8	
GyrB	DNA gyrase subunit B	+2.1	BCAS0409	Zinc metalloprotease ZmpA	-3.3	
BCAM0626	Putative DNA-binding protein	-2.1	Uncharacterised			
BCAS0646A	Putative DNA-binding protein	-2.7	BCAL1265	Uncharacterized protein	+7.0	
Metabolic Process			BCAL2401	Putative exported protein	-25.4	
BCAL0077	Putative oxidoreductase	-2.4	BCAL3228	Uncharacterized protein	-59.5	
BCAL0436	Glyoxalase/bleomycin resistance protein/dioxygenase superfamily	-2.7	BCAM1261	Putative membrane protein	-12.5	
BCAL1910	Acetoin:2,6-dichlorophenolindophenol oxidoreductase beta subunit acoB	-3.2	BCAM1412	Uncharacterized protein	-4.1	
BCAM0974	Glyoxalase/bleomycin resistance protein/dioxygenase superfamily protein	-8.0	BCAM1480	Uncharacterized protein	-2.3	
BCAM1098	NUDIX hydrolase	-2.7	BCAM1481	Uncharacterized protein	-2.4	
BCAM1407	DJ-1/Pfpl family protein	-7.6	BCAM1482	Uncharacterized protein	-2.8	
BCAM1547	Glyoxalase/bleomycin resistance protein/dioxygenase superfamily protein	-6.0	BCAS0636	Uncharacterized protein	-29.0	
BCAM1704	2,3-butanediol dehydrogenase	+8.8	other processes			
			BCAL0702	Putative ferredoxin	-3.1	
			BCAM0843	Putative lipoprotein	-10.5	

955 **Figure Legends:**

956

957 **Figure 1. (A) BCAS0292 gene cluster.** Genetic organisation of the gene cluster encoding
 958 the BCAS0292 and AidA proteins in *B. cenocepacia* J2315 and K56-2. The direction of
 959 transcription of each gene is indicated by the grey arrows. BCAS gene designations are
 960 according to annotation of the *B. cenocepacia* J2315 genome (Holden *et al.*, 2009). (B)
 961 **Effect of BCAS0292 gene deletion on virulence.** Kaplan Meier plot showing *G.*
 962 *mellonella* survival following exposure 2 CFU of K56-2 WT, Δ BCAS0292 mutant or
 963 Δ 0292_0292 complement strain. Data collated from three independent experiments. *
 964 Statistical difference between K56-2 WT and Δ BCAS0292; $p = 0.0036$

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966 **Figure 2: Role of BCAS0292 in epithelial cell attachment.** A) Attachment of K56-2
 967 WT, Δ BCAS0292 mutant and Δ 0292_0292 complemented strain to CFBE41o⁻ cells at an
 968 MOI 50:1. Data represents the mean number of bacteria/ 100 cells for each strain with 20
 969 fields of view counted per data point in three independent experiments, ** $p = 0.0073$. B)
 970 Confocal microscopy images representing the attachment of *E. coli* BL21 control and *E.*
 971 *coli* BL21_BCAS0292 strains to CFBE41o⁻ cells. Bacteria were labelled using a primary
 972 anti-*E. coli* FITC-conjugated antibody (green). Nuclei of CFBE41o⁻ cells were
 973 counterstained with DAPI (blue) and actin stained with phalloidin conjugated with Alexa
 974 fluor 568. C) Attachment *E. coli* BL21 control and *E. coli* BL21_BCAS0292 to
 975 CFBE41o⁻ cells, using an MOI 50:1. Data represents the mean number of bacteria/ 100
 976 cells for each strain, with 20 fields of view counted per data point in three independent
 977 experiments. D) Confirmation of expression of BCAS0292, following induction in *E. coli*
 978 BL21 cells with 1mM IPTG by Coomassie Blue stained SDS PAGE gel (conditions used
 979 for Figure 1C). Lane 1: MW marker; Lane 2: *E. coli* BL21 expression control; Lane 3: *E.*
 980 *coli* BL21_BCAS0292 after incubation with IPTG (1mM) for 2 h.

981

982 **Figure 3: Effect of BCAS0292 on antibiotic sensitivity.** Images represent the
 983 susceptibility of WT, mutant and complemented strains to polymyxin B (A), (0.064-1,024
 984 μ g/ ml) or meropenem (B), (0.002-32 μ g/ ml) using E-test strips. The data shown are
 985 representative of three independent experiments. (C). Bar chart representing biofilm
 986 formation of K56-2 WT, Δ BCAS0292 and Δ 0292_0292 after 24, 48 and 72 h. Error bars
 987 represent the standard deviation from three independent experiments. * $p < 0.0001$
 988 represents a significant difference between Δ BCAS0292 and K56-2 WT strain. D)

Swimming motility of K56-2 WT and Δ BCAS0292 mutant cells 24 and 48 h after inoculation of agar plates. Images are representative of three independent experiments.

Figure 4: Effect of deletion of the BCAS0292 gene on the proteome and phenotype of *B. cenocepacia* K56-2. A) Fold change in protein abundance plotted as a volcano chart of log₂ fold change in protein abundance in the Δ BCAS0292 mutant strain relative to WT strain. Points on the negative side of the X axis represent a reduction in protein abundance. The proteins with the greatest changes in abundance are highlighted with red arrows. Dashed line represents the 1.5-fold change in abundance. B) Pie chart representing the functional GO categories of the proteins that showed abundance reduced by ≥ 1.5 fold in the Δ BCAS0292 mutant strain relative to the K56-2 WT strain. C) Effect of deletion of BCAS0292 on cell morphology as determined by phase contrast microscopy. D) Growth and survival of K56-2 strain, Δ BCAS0292 mutant and 0292_0292 complement strain in 6% oxygen. Cells were cultured in a hypoxia chamber at 6% oxygen for up to 48 h. Growth was determined by OD_{600nm}. Survival was determined by serially diluting aliquots of cultures that had been incubated at 6% oxygen in a controlled hypoxia chamber, plating at time intervals and culturing for 48h in a normoxic environment.

Figure 5: Structural analysis of rBCAS0292. A) Size-exclusion chromatogram and subsequent SDS-PAGE analysis of purified rBCAS0292 protein. B) CD analysis of purified rBCAS0292 protein to determine the secondary structure at 20 °C. A minimum was observed at 215nm, indicative of a β -sheet fold. C) Thermal gradient to determine the T_m of purified rBCAS0292, demonstrating a T_m of 49 °C. D) CD analysis of purified rBCAS0292 protein to examine the ability of the protein to re-fold. Black line: 20 °C, green line: 90 °C, red line: 20 °C following thermal gradient. This indicates that the protein aggregates following heat denaturation and is unable to restore the correct fold at 20°C. E) Light scattering analysis to determine the oligomeric state of rBCAS0292, which indicated that the protein has a dimeric organisation.

Figure 6: Molecular modelling of BCAS0292 and impact on DNA supercoiling A) Cartoon representation of the predicted 3D structure of the dimer. Consensus based sequence alignment was used to determine the predicted structure using MODELLER software. B) Analysis of the electrostatic potential of the hypothetical protein. Side view of the predicted dimer model. C) Comparison of DNA supercoiling of plasmid DNA

1024 extracted from *B. cenocepacia* K56-2, Δ BCAS0292 and Δ 0292_0292 cells cultured to
 1025 either exponential or stationary phase. Chloroquine-stained gel image is representative of
 1026 three independent experiments. D) Ciprofloxacin susceptibility of K56-2, Δ BCAS0292
 1027 and Δ 0292_0292 strains as determined by E-strips. Images representative of three
 1028 independent experiments.

1029

1030 **Figure 7:** Schematic of the proposed mechanism of regulation by BCAS0292 in response
 1031 to stress, low oxygen and stationary phase. Previous work has shown that BCAS0292 is
 1032 elevated in response to stress, low oxygen and stationary phase and elevated in late
 1033 isolates from chronically colonised patients. The present work shows that absence of
 1034 BCAS0292 reduces the abundance of ≥ 500 proteins, particularly those including those
 1035 involved in the stress response, metabolic processes, transcription cell signaling, and
 1036 virulence. In addition we have shown that the presence of BCAS0292 alters DNA
 1037 topology, providing a mechanism by which it globally regulates protein expression in
 1038 response to stress.

1039

Figure 1:

Figure 2

Figure 3

A Polymyxin B

K56-2 WT

Δ BCAS0292

Δ 0292_0292

B Meropenem

K56-2 WT

Δ BCAS0292

Δ 0292_0292

C

D

Figure 4

C

D

E

Fig 5

A

B

C

D

Fig 6

A

B

C

D

Fig 7