Environmental Microbiology (2021) **00**(00), 00–00 doi:10.1111/1462-2920.15451

# Proteomics insights into the *Burkholderia cenocepacia* phosphorus stress response

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## Summary

**The *Burkholderia cepacia* complex is a group of *Burkholderia* species that are opportunistic pathogens causing high mortality rates in patients with cystic fibrosis. An environmental stress often encountered by these soil-dwelling and pathogenic bacteria is phosphorus limitation, an essential element for cellular processes. Here, we describe cellular and extracellular proteins differentially regulated between phosphate-deplete (0 mM, no added phosphate) and phosphate-replete (1 mM) growth conditions using a comparative proteomics (LC–MS/MS) approach. We observed a total of 128 and 65 unique proteins were downregulated and upregulated respectively, in the *B. cenocepacia* proteome. Of those downregulated proteins, many have functions in amino acid transport/metabolism. We have identified 24 upregulated proteins that are directly/indirectly involved in inorganic phosphate or organic phosphorus acquisition. Also, proteins involved in virulence and antimicrobial resistance were differentially regulated, suggesting *B. cenocepacia* experiences a dramatic shift in metabolism under these stress conditions. Overall, this study provides a baseline for further research into the biology of *Burkholderia* in response to phosphorus stress.**

## Introduction

*Burkholderia cenocepacia* is a member of the *Burkholderia cepacia* complex (Bcc), a group of closely related *Burkholderia* species (Mahenthiralingam *et al*., 2005; Vial *et al*., 2011). The genus *Burkholderia* was separated from *Pseudomonas* species and validated in 1992 (Burkholder, 1950; Yabuuchi *et al*., 1992). Although first described as a plant pathogen causing onion rot, Bcc species are also opportunistic human pathogens that cause infections in patients with cystic fibrosis (CF) and chronic granulomatous disease (Mahenthiralingam *et al*., 2005; Vial *et al*., 2011). Bcc species cause under 5% of infections in CF patients, but these types of infections are feared due to easy transmission, high intrinsic resistance to antibiotics and the unpredictability of patient outcome, i.e. an asymptomatic infection or the development of fatal cepacia syndrome (Leitão *et al*., 2017).

One of the most well-studied bacterial stress responses is phosphate limitation, which has not been extensively studied in Bcc species. Phosphorus is an essential element required for many cellular processes and is a major component of membranes and genetic material. Bacteria largely acquire phosphorus as inorganic phosphate (Pi) (Wanner, 1996; Lamarche *et al*., 2008b; Crépin *et al*., 2011; Santos-Beneit, 2015). In Gram-positive bacteria, the Pho regulon is well studied in the genus *Bacillus*. The two-component system (TCS) PhoPR is known to mediate a specific response to phosphate stress, many of which are involved in teichoic acid turnover in the cell wall, a feature specific to Gram-positive bacteria (Antelmann *et al*., 2000; Prunty *et al*., 2018). In Gram-negative bacteria, however, the response to Pi stress is orchestrated by the PhoBR TCS. At high external Pi concentrations, PhoR is hypothesized to form a repression complex with the high-affinity Pi transporter, PstSCAB. However, when the environmental Pi concentration falls below a few micromolar, PhoR is liberated from this repression and can activate its transcriptional regulator, PhoB (Wanner, 1996; Lamarche *et al*., 2008b; Santos-Beneit, 2015). Genes directly controlled by PhoB are collectively termed the Pho regulon and contain a conserved Pho box in their promoter region which PhoB can bind to. In *Escherichia coli*, the Pho regulon originally comprised eight transcriptional units containing 31 genes (Wanner, 1990, 1993, 1996). However, over 47 genes have now been assigned to the

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*E. coli* Pho regulon, some of the newer genes include an AMP nucleosidase (*amn*) and a metal ion stress response gene (*yibD*) (Baek and Lee, 2006; Lamarche *et al*., 2008b). ChIP-seq analysis of PhoB has identified 19 directly regulated genes that have varying roles in metabolism, transport and transcriptional regulation (Yang *et al*., 2012). However, proteomic studies have suggested the response to Pi limitation could involve over 400 proteins (Van Bogelen *et al*., 1996). Although the soil and plant environments are known to have limited Pi availability, whether human pathogens could experience Pi stress during host invasion is not completely understood. One hypothesis is that intracellular pathogens such as *Salmonella* experience Pi limitation in the macrophage vacuole during the host immune response. This theory is supported by the expression of PhoBR and other genes involved in Pi acquisition (Eriksson *et al*., 2003; Srikumar *et al*., 2015; Choi *et al*., 2019). Such a scenario is also possible for *Burkholderia* species as they are intracellular pathogens able to survive in immune cells like macrophages where they could encounter Pi stress (Valvano, 2015). The human bronchial epithelium is also a low Pi environment and the induction of Pi acquisition genes has already been reported during *Pseudomonas aeruginosa* infections in this location (Frisk *et al*., 2004; Chugani and Greenberg, 2007).

Not only is the response to Pi stress critical for cell survival, but it is often linked to virulence and antimicrobial resistance. This has been extensively studied in pathogenic species of *E. coli* (Lamarche *et al*., 2008b; Bertrand *et al*., 2010; Yoshida *et al*., 2010; Crépin *et al*., 2011) and *Vibrio cholerae*, the human intestinal pathogen causing cholera (Von Krüger *et al*., 1999). Similarly, in the common CF pathogen, *P. aeruginosa*, Pi limitation increases swarming motility and cytotoxicity to human bronchial epithelia (Bains *et al*., 2012) whilst Pi depletion was also a trigger for the lethal red death phenotype in a *Caenorhabditis elegans* infection model (Zaborin *et al*., 2009). Proteomic studies have also shown Pi limitation increases the expression of virulence factors in many pathogens, including *Salmonella typhimurium* and *V. cholerae* (Von Krüger *et al*., 2006; Jiang *et al*., 2018). Many cell wall modifications can occur under Pi depletion, such as the remodelling of the cell membrane with non-phosphorus-containing lipids (Taylor *et al*., 1998; Zavaleta-Pastor *et al*., 2010). Changes in the cell wall due to Pi depletion can also lead to increased antimicrobial resistance. For example, the modification of the lipid A moiety of lipopolysaccharide causes increased resistance to polymyxins (Lamarche *et al*., 2008a; Crépin *et al*., 2011). Furthermore, the PstSCAB Pi transporter is known to be involved in the efflux of fluoroquinolone antibiotics (Banerjee *et al*., 2000; Li and Nikaido, 2004) and the TolC efflux pump, which is implicated in drug resistance, is upregulated in *V. cholerae* under Pi stress (Von Krüger *et al*., 2006).

In this work, we set out to identify the Pi limitation stress response in *B. cenocepacia* using comparative proteomics of the cellular and extracellular proteomes. In the last 10 years, advances in proteomic methods have facilitated studies of the exoproteome. This extracellular 'compartment' is a key area for secretion of toxins, virulence factors and degradative enzymes (Brown *et al*., 2012; Gagic *et al*., 2016). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of our data showed differential regulation of a wide range of cellular processes, particularly proteins related to phosphate regulation, virulence and antimicrobial resistance. These results not only identify the proteins that are regulated by phosphorus limitation in *Burkholderia* species, but also highlight the complexity of the Pi stress response and begin to unravel the consequences of Pi limitation on pathogenesis and antimicrobial resistance in this key human pathogen.

## Results and discussion

### *The effects of Pi stress on growth and alkaline phosphatase activity in* B. cenocepacia

To examine the *Burkholderia* Pi stress response, *B. cenocepacia* K56-2 was cultivated under both Pi-deplete (0 mM) and Pi-replete (1 mM) conditions. *Burkholderia cenocepacia* had a significantly slower growth rate under Pi depletion ($P < 0.01$, Welch *t*-test) (Fig. 1A). To ensure these cultures were experiencing Pi stress, we used alkaline phosphatase activity as an indicator of Pi limitation since these secreted proteins scavenge Pi from their environment and are known to be regulated by the PhoBR TCS (Hong *et al*., 2007; Richards and Vanderpool, 2012). *Burkholderia cenocepacia* showed a significant increase in alkaline phosphatase activity 4 h after exposure to Pi-deplete conditions ($P < 0.01$, Welch *t*-test) (Fig. 1B). These results confirmed the experimental setup was sufficient for inducing Pi limitation in *B. cenocepacia*.

### *Environmental Pi limitation causes significant shifts in cellular and extracellular proteomes in* B. cenocepacia

To begin to unravel the proteins regulated by Pi availability in *Burkholderia* species, we used comparative proteomics to study the response of *B. cenocepacia* K56-2 to Pi stress. Samples for LC–MS/MS were taken 8 h after exposure to Pi-deplete (0 mM) and Pi-replete (1 mM) conditions (Fig. 1A). The cellular proteome was analysed from pelleted cells and the extracellular proteome from

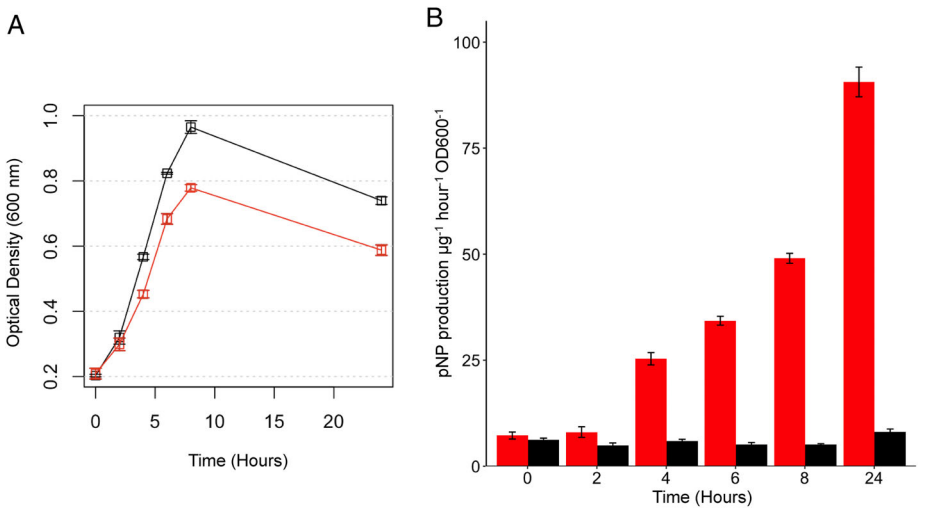


**Fig. 1.** A. Growth of *B. cenocepacia* K56-2 under Pi-deplete (0 mM, red) and Pi-replete (1 mM, black) conditions. Samples for proteomics were taken at 8 h.
B. At each growth time point, alkaline phosphatase activity was measured using the *p*NPP assay to assess the activation of the Pho regulon. Alkaline phosphate activity was measured by calculating the *p*NP production in $\mu g^{-1}\ h^{-1}$ normalized to cell density of the sample ($OD_{600}$). Error bars represent standard error of the mean of triplicate cultures. [Color figure can be viewed at wileyonlinelibrary.com]

filtered supernatant after removing cells by centrifugation. A total of 1728 and 332 proteins were detected in the cellular and extracellular proteome respectively (Suppl. Table 1). Of these proteins, 182 and 23 were significantly differentially regulated ($P \leqslant 0.05$) in Pi-deplete compared with Pi-replete conditions in the cellular and extracellular proteome respectively (Fig. 2). Twelve of these proteins were significantly differentially regulated in both the cellular and exoproteome fractions. In the cellular proteome, 63 proteins were significantly upregulated and 119 proteins were significantly downregulated ($P \leqslant 0.05$, Fig. 2A). In the exoproteome, 11 proteins were significantly upregulated and 12 proteins were significantly downregulated ($P \leqslant 0.05$, Fig. 2B; Suppl. Table 1).

In terms of abundance, 13 proteins made up at least 1% of the total cellular proteome in either Pi-deplete or Pi-replete conditions, two of which were significantly differentially regulated (Table 1). Noticeably, PstS showed the largest difference in relative abundance (7.02%) in Pi-deplete compared with Pi-replete conditions. PstS is the periplasmic substrate-binding protein of the high-affinity Pi transporter, PstSCAB (Rosenberg *et al*., 1977; Torriani, 1990; Yu*an et al*., 2006a; Yuan *et al*., 2006b). PstSCAB expression has been shown to be controlled by PhoB in many bacterial species and is a well-characterized member of the Pho regulon (Rosenberg *et al*., 1977; Yamada *et al*., 1989; *Yuan et al*., 2006a; Yuan *et al*., 2006b; Lubin *et al*., 2016). Therefore, this sharp increase in PstS abundance in our results confirms the activation of the Pi stress response. In the extracellular proteome, 17 proteins made up at least 1% of the total exoproteome in either Pi-deplete or Pi-replete conditions, none of which, however, were significantly differentially regulated (Table 1). Noticeably, FliC, a flagellar filament structural protein accounted for >20% of the total exoproteome.

### *Proteins classified by clusters of orthologous groups and KEGG analysis further demonstrate the shift occurring in metabolism under Pi stress*

Pi stress elicited a major change in the cellular and extracellular proteome with a total of ~200 proteins being significantly affected (Fig. 2). To gain a better understanding of the metabolic pathways affected by Pi limitation, significantly differentially regulated proteins (Fig. 2) were subjected to KEGG and Clusters of Orthologous Groups (COG) analysis (Table 2 and Suppl. Table 2 respectively). KEGG analysis highlights protein functions, whereas COG analysis gives a general overview of the cellular processes each protein contributes to. In the KEGG analysis (Table 2), there were a large number of enzymes upregulated and downregulated in both fractions of the Pi-deplete proteome compared with the Pi-replete proteome. The most striking result was the high number of downregulated transporters in the Pi-deplete cellular proteome. A deeper analysis into the function of transporters using the KEGG identifier ko02000 (Table 2) revealed four of the upregulated proteins comprised the PstSCAB Pi transporter, whereas the downregulated transporters were involved in amino acid, sulfur, carbon, nitrogen and iron transport (Fig. 3).

Other observations in the KEGG analysis showed three proteins related to TCSs were upregulated (Table 2, Suppl. Table 2), including PhoB and PhoR, the TCS for the Pi stress response and BCAM1199, the homologue of which is involved in chemotaxis in *P. aeruginosa* (Joshi *et al*., 2019). The only TCS downregulated in response to Pi stress was the response regulator CheY that is involved in chemotaxis in *B. cenocepacia* (Bazzini *et al*., 2011). Proteins related to genetic information processing were mostly downregulated with a few exceptions, such as mRNA

**Table 1.** Proteins detected in the cellular and exoproteomes comprising at least 1% of the total cellular or exoproteome in either Pi-deplete (0 mM) or Pi-replete (1 mM) conditions.

| $P \leqslant 0.05$ | Log2-fold change) | Locus tag (gene name) | Annotation | Sequence length | Relative abundance (%) | | |
|---|---|---|---|---|---|---|---|
| | | | | | +Pi | -Pi | Difference |
| *Cellular proteome* | | | | | | | |
| + | +3.054 | BURCENK562V_C1990 (*pstS*) | Phosphate ABC transporter substrate-binding protein, PhoT family | 344 | 0.92 | 7.94 | +7.02 |
| | +0.946 | BURCENK562V_C0963 (*phaP*) | Phasin family protein | 188 | 2.33 | 4.67 | +2.34 |
| | +0.343 | BURCENK562V_C5730 (*groL1*) | Chaperonin GroEL | 546 | 1.92 | 2.53 | +0.61 |
| | +0.103 | BURCENK562V_C1183 (BPSL2096) | Peroxiredoxin, alkyl hydroperoxide reductase subunit C | 182 | 3.11 | 3.48 | +0.37 |
| | +0.370 | BURCENK562V_C0143 (*sodB*) | Superoxide dismutase, Fe-Mn family | 192 | 0.75 | 1.01 | +0.26 |
| | +0.284 | BURCENK562V_C2988 (*mdh*) | Malate dehydrogenase, NAD | 328 | 0.96 | 1.21 | +0.25 |
| | +0.024 | BURCENK562V_C6532 (*tufA1*) | Translation elongation factor 1A, EF-1A/EF-Tu | 396 | 3.01 | 3.19 | +0.18 |
| | −0.156 | BURCENK562V_C5942 (*ompA*) | OmpA-OmpF porin, OOP family | 222 | 1.90 | 1.78 | −0.12 |
| | −0.104 | BURCENK562V_C2161 (*hupA)* | HU family DNA-binding protein | 92 | 4.24 | 4.10 | −0.14 |
| | −0.323 | BURCENK562V_C3041 (BPSS1680) | DNA-binding protein | 149 | 2.24 | 1.86 | −0.38 |
| | −0.803 | BURCENK562V_C5678 (BPSL2748) | 1-Cys peroxiredoxin | 212 | 1.36 | 0.81 | −0.55 |
| | −1.026 | BURCENK562V_C7254 (*aidA*) | Inclusion body protein | 167 | 1.98 | 1.01 | −0.97 |
| + | −3.292 | BURCENK562V_C2529 (*groL5*) | Chaperonin GroEL | 546 | 2.28 | 0.24 | −2.04 |
| *Extracellular proteome* | | | | | | | |
| | +0.849 | BURCENK562V_C6831 (*fliC*) | Flagellin | 384 | 21.76 | 43.19 | +21.43 |
| | +2.330 | BURCENK562V_C7127 (*zmpA*) | ZmpA-like peptidase, Metallopeptidase, MEROPS family M04 | 565 | 0.24 | 1.32 | +1.08 |
| | −0.018 | BURCENK562V_C6346 | Protein of unknown function (DUF4399) | 178 | 1.78 | 1.94 | +0.16 |
| | −0.064 | BURCENK562V_C4415 (*livK1*) | L-leucine-binding protein/L-isoleucine-binding protein/L-valine-binding protein | 372 | 0.96 | 1.01 | +0.05 |
| | −0.514 | BURCENK562V_C1183 (BPSL2096) | Peroxiredoxin (alkyl hydroperoxide reductase subunit C) | 182 | 1.08 | 0.83 | −0.25 |
| | −0.476 | BURCENK562V_C4051 (BCAM1931) | Outer membrane protein, porin | 359 | 1.23 | 0.97 | −0.26 |
| | −0.254 | BURCENK562V_C1023 (*ppiA*) | Peptidyl-prolyl cis-trans isomerase A, cyclophilin A | 191 | 3.41 | 3.15 | −0.26 |
| | −0.746 | BURCENK562V_C6611 (BCAL0305) | Phospholipid transport system substrate-binding protein | 210 | 1.23 | 0.81 | −0.42 |
| | −0.609 | BURCENK562V_C1663 (BCAL1577) | Hypothetical protein | 315 | 1.57 | 1.14 | −0.43 |
| | −0.564 | BURCENK562V_C6135 (BCAL1092) | Iron(III) transport system substrate-binding protein | 347 | 1.89 | 1.41 | −0.48 |
| | −0.183 | BURCENK562V_C1990 (*pstS*) | Phosphate ABC transporter substrate-binding protein, PhoT family | 344 | 19.70 | 19.13 | −0.57 |
| | −1.016 | BURCENK562V_C0461 (BPSL0466) | D-methionine transport system substrate-binding protein | 271 | 1.48 | 0.81 | −0.67 |
| | −0.904 | BURCENK562V_C0550 (*dsbA*) | Thiol:disulfide interchange protein DsbA | 212 | 1.75 | 1.03 | −0.72 |
| | −1.772 | BURCENK562V_C4473 (*zmpB*) | Fungalysin metallopeptidase, M36 | 570 | 1.48 | 0.48 | −1.00 |
| | −1.682 | BURCENK562V_C6106 (BCAL1065) | Amino acid ABC transporter substrate-binding protein, PAAT family | 257 | 1.67 | 0.57 | −1.10 |
| | −1.501 | BURCENK562V_C1576 (BCAL1657) | Monosaccharide ABC transporter substrate-binding protein, CUT2 family | 316 | 1.32 | 0.51 | −1.31 |
| | −0.652 | BURCENK562V_C2355 (*gltI*) | L-glutamate-binding protein/L-aspartate-binding protein | 297 | 8.82 | 6.18 | −2.64 |

Changes in the phosphate-deplete condition were compared with the phosphate-replete condition. Significance ($P \leqslant 0.05$) is related to the log2-fold change of their relative abundance in proteomes.

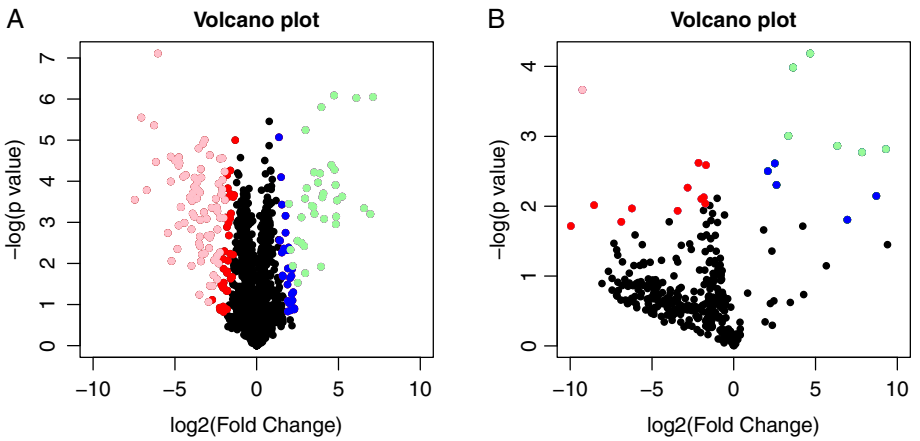


**Fig. 2.** Protein expression fold changes when comparing *B. cenocepacia* K56-2 grown in Pi-deplete (0 mM) to Pi-replete (1 mM) conditions.
A. Cellular proteome expression changes.
B. Exoproteome expression changes. Upregulated and downregulated proteins are shown in green/blue and red/pink respectively. Pink/green and blue/red represent $P \leqslant 0.01$ and $P \leqslant 0.05$ respectively. Black proteins are not significantly different. [Color figure can be viewed at wileyonlinelibrary.com]

synthesis and ribosome biogenesis. Results from the KEGG analysis give the overall impression of metabolic adaptation occurring in response to Pi stress in *B. cenocepacia*. A similar adaptation has been described in other bacterial species such as *Salmonella*, *Corynebacterium* and *Streptomyces* whereby primary metabolic processes including transcription, amino acid metabolism and protein synthesis have previously been shown to decrease in Pi-limited environments (Ishige *et al*., 2003; Novotna *et al*., 2003; Rodríguez-García *et al*., 2007; Jiang *et al*., 2018). As seen in both analyses (Table 2, Suppl. Table 2), a large fraction of the differentially regulated proteins were unclassified, i.e. encoding hypothetical proteins with unknown function.

### *Proteins related to Pi regulation are significantly upregulated under Pi depletion*

Out of 63 and 11 significantly upregulated cellular and extracellular proteins respectively, 22 and 6 were identified as being involved in Pi regulation (Table 3). In *B. cenocepacia*, the PhoBR TCS forms a putative operon with the high-affinity Pi transporter PstSCAB and the regulator PhoU, which were all upregulated under Pi stress in the cellular proteome (Fig. 4A; Table 3). Other classic members of the *E. coli* Pho regulon include PsiA (a.k.a. PhoA, an alkaline phosphatase) and PsiF (a protein of unknown function) which form an operon in *E. coli* (Wanner, 1996; Luu *et al*., 2018). PhoA homologues (BCAM0389 and BCAM0390, 31% and 32% identity respectively) were upregulated in both fractions of the proteome (Table 3), whereas the PsiF homologue (BURCENK562V_C1248) was not significantly differentially expressed and does not form an operon with the PhoA homologues. *Burkholderia cenocepacia* also has two homologues of the PhoH protein (BURCENK562V_C0301 and BURCENK562V_C1266, 48% and 36% identity respectively), a protein of unknown function (Wanner, 1996; Goldsmith *et al*., 2011). However, neither protein was significantly differentially regulated under Pi stress. *Burkholderia cenocepacia* has homologues for the UgpBAECQ operon involved in *sn*-glycerol-3-phosphate (G3P) uptake (BURCENK562V_C6584-C6588), however, they were not significantly regulated (Suppl. Table 1). *Burkholderia cenocepacia* does not have homologues for the other three members of the *E. coli* Pho regulon, which are the phosphonate uptake/degradation operon, PhnC-PhnP, the porin, PhoE and PsiE, a protein of unknown function (Wanner, 1996). Therefore, only nine of the cellular upregulated proteins (Table 3) can be linked to the classic *E. coli* Pho regulon.

Other significantly differentially expressed proteins in *B. cenocepacia* include Ppk/Ppk2 encoding polyphosphate kinases involved in the storage of Pi, PhnA encoding a phosphonoacetate hydrolase involved in releasing Pi and acetate from phosphonoacetate, and GlpQ, a glycerophosphoryl diester phosphodiesterase involved in releasing Pi from deacetylated phospholipids (Nocek *et al*., 2008; Gilbert *et al*., 2009; Racki *et al*., 2017; Jorge *et al*., 2018). Homologues of the following upregulated proteins (Table 3) are induced under Pi starvation in other bacteria: PlcN, BPSL1375 (OlsB), BCAM1663, BCAM0451, BCAL1116, BPSL1190 and BCAL2364. The majority of these proteins are involved in increasing the availability of Pi by degrading phospholipids or extracellular DNA (eDNA) (Korbsrisate *et al*., 2007; Gangaiah *et al*., 2009; Mulcahy *et al*., 2010; Zavaleta-Pastor *et al*., 2010; Diercks *et al*., 2015; Ball *et al*., 2016; Wilton *et al*., 2018). BCAL1083, encoding a putative alkaline phosphatase, is significantly overexpressed during Pi starvation in *B. cenocepacia* K56-2. Interestingly, this gene was shown to be highly induced in a polymyxin B-sensitive mutant of *B. cenocepacia* K56-2 (Loutet *et al*., 2011). Similarly, three putative phospholipases (BCAM0164, BCAM2729 and BCAM0408) are also highly overrepresented in *B. cenocepacia* K56-2

**Table 2.** Significantly, differentially regulated proteins in the Pi-deplete compared with the Pi-replete condition ($P \leqslant 0.05$, identified in Fig. 2) were assigned KEGG BRITE identifiers using BlastKOALA (version 2.2).

| KEGG BRITE reference hierarchy identifier | Number of proteins identified | | | | | |
|---|---|---|---|---|---|---|
| | Cellular only | | Extracellular only | | Both fractions | |
| | Upregulated | Downregulated | Upregulated | Downregulated | Upregulated | Downregulated |
| Protein families: Metabolism | | | | | | |
| ko01000 Enzymes | 25 | 38 | 2 | 3 | 4 | 1 |
| ko01001 Protein kinases | 1 | – | – | – | – | – |
| ko01002 Peptidases and inhibitors | 2 | 1 | – | 1 | – | – |
| ko01004 Lipid biosynthesis proteins | 1 | – | – | – | – | – |
| ko01005 Lipopolysaccharide biosynthesis proteins | 1 | – | – | – | – | – |
| ko01007 Amino acid–related enzymes | 2 | 1 | – | – | – | – |
| ko01011 Peptidoglycan biosynthesis and degradation proteins | – | 1 | – | – | – | – |
| ko00194 Photosynthesis proteins | 1 | – | – | – | – | – |
| Protein families: Signalling and cellular processes | | | | | | |
| ko02000 Transporters | 8 | 25 | – | – | – | 2 |
| ko02022 Two-component system | 3 | 1 | – | – | – | – |
| ko02035 Bacterial motility proteins | 1 | 2 | – | 2 | – | – |
| ko02042 Bacterial toxins | 1 | – | 2 | – | – | – |
| ko02048 Prokaryotic defence system | – | 1 | – | – | – | – |
| ko04147 Exosome | – | 1 | – | – | 2 | – |
| ko04812 Cytoskeleton proteins | 1 | 1 | – | – | – | – |
| ko01504 Antimicrobial resistance genes | – | 1 | – | – | – | – |
| ko00537 Glycosylphosphatidylinositol (GPI)-anchored proteins | – | – | – | – | 2 | – |
| Protein families: Genetic information processing | | | | | | |
| ko03000 Transcription factors | – | 2 | – | – | – | – |
| ko03009 Ribosome biogenesis | 1 | – | – | – | – | – |
| ko03011 Ribosome | – | 1 | – | – | – | – |
| Ko03016 Transfer RNA biogenesis | – | – | – | 1 | – | – |
| ko03019 Messenger RNA biosynthesis | 2 | 1 | – | – | – | – |
| ko03021 Transcription machinery | 1 | – | – | – | – | – |
| ko03029 Mitochondrial biogenesis | – | 2 | – | – | – | – |
| ko03036 Chromosome and associated proteins | 1 | 2 | – | – | – | – |
| ko03110 Chaperones and folding catalysts | – | 4 | – | – | – | – |
| Unclassified | 18 | 48 | – | 4 | 5 | – |

The number of proteins associated with each KEGG BRITE identifier was calculated for each fraction of the proteome.

during Pi starvation, one of which (BCAM0408) has been shown previously to be highly expressed in a clinical *B. cenocepacia* isolate (IST439) obtained from a CF patient who died from cepacia syndrome (Mira *et al*., 2011; Stone *et al*., 2014).

Using the upstream DNA sequences of the upregulated extracellular proteins identified in *B. cenocepacia* K56-2, Multiple Em for Motif Elicitation analysis was undertaken to detect a putative consensus sequence for a *Burkholderia* Pho box (Suppl. Fig. 1). The promoter region of *pstS* from *Burkholderia thailandensis* was also included, which has been shown previously to bind to the PhoB protein in a gel shift assay (Nowak-Lovato *et al*., 2012). The consensus *Burkholderia* Pho box sequence was identified as follows: 5′-C(TC)GTCACNNNN(GT)(TC)TTC(AC)(TG)-3′. Interestingly, this Pho box differs from the Pho box consensus sequences described in other bacterial species, e.g. *E. coli* Pho box: 5′-CTGTCATNNNNCTGT(CA)A(CT)-3′ (Wanner, 1993; Yuan *et al*., 2006a; Yuan *et al*., 2006b; Diniz *et al*., 2011).

### *Differential expression of putative virulence factors occurs in response to Pi limitation*

Apart from PhoB and PstSCAB, a total of 11 and 18 unique proteins involved in virulence were upregulated and downregulated respectively (Table 4). The following proteins, previously identified as playing a

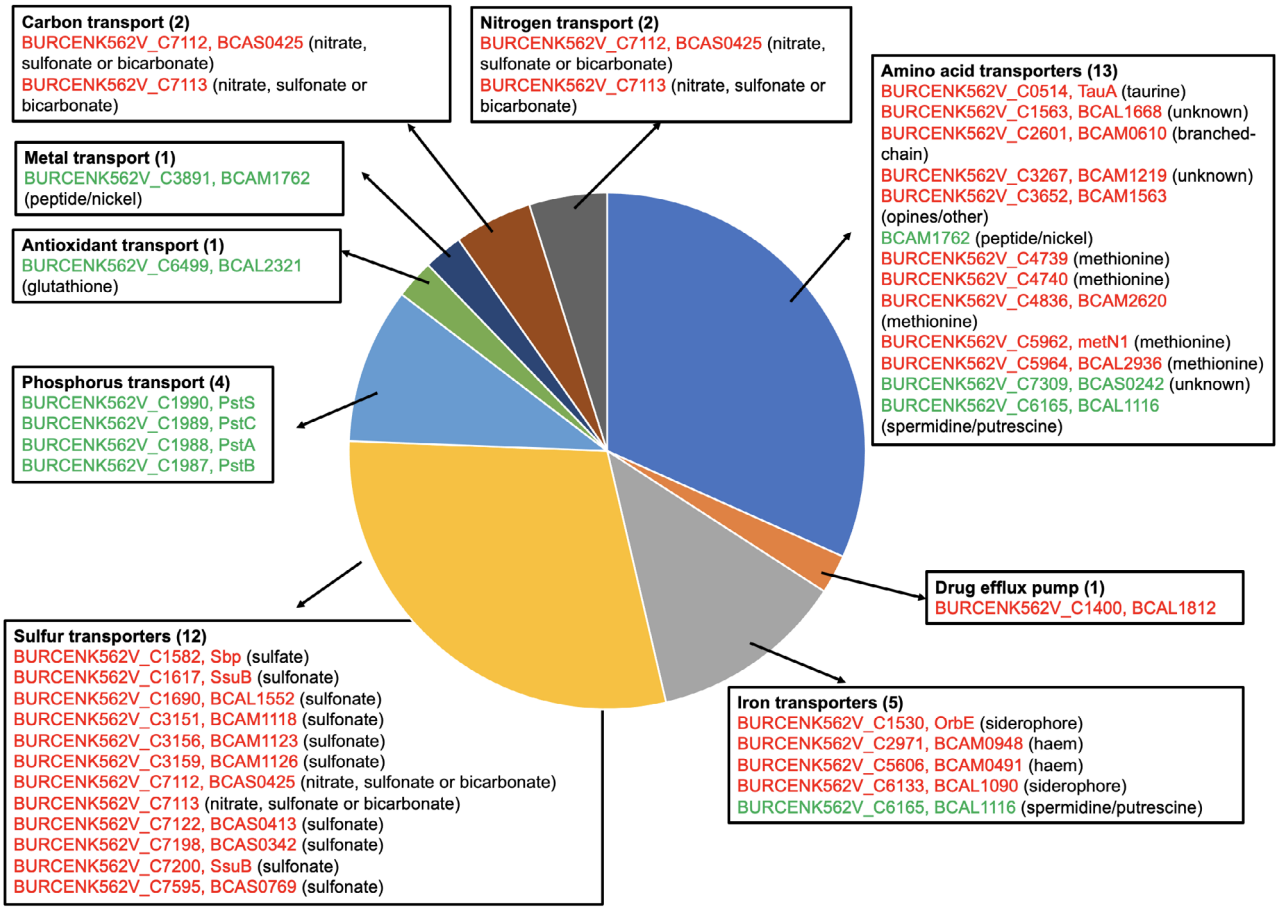


**Fig. 3.** Cellular processes related to each transporter identified in the KEGG analysis (Table 2). Transporters highlighted in red are down-regulated and those in green are upregulated in Pi-deplete compared with Pi-replete conditions. Some proteins have putative roles in more than one cellular process. Specific substrates related to each protein are shown in brackets. [Color figure can be viewed at wileyonlinelibrary.com]

role in Pi regulation (see section above), also have a role in virulence: GlpQ, BPSL1375, Ppk, BCAM0451, BCAM2720, PlcN and BCAM0408. For respiratory pathogens like *B. cenocepacia*, pulmonary surfactant can act as a source of Pi as it is comprised of a mixture of lipids and proteins. Between ~70% and 80% of the phospholipid portion of pulmonary surfactant is phosphatidylcholine (PC) which can be degraded by phospholipases produced by the lung tissues, converting PC into glycerophosphocholine (GPC) (Batenburg, 1992; Hurley and McCormick, 2008). In the respiratory pathogen *Mycoplasma pneumoniae*, GPC is imported into the cell and degraded by GlpQ to release glycerol-3-phosphate which can then be used for many cellular processes, such as phospholipid synthesis. GlpQ is required for pathogenicity against HeLa cells due to its indirect role in hydrogen peroxide formation (Schmidl *et al*., 2011). A similar observation was found in *Streptococcus pneumoniae* whereby cytotoxicity was compromised in GlpQ mutants infecting human epithelial cells (Chuang *et al*., 2015). Ppk was shown in *Burkholderia pseudomallei* to be important for swarming and swimming motility, as well as biofilm formation (Tunpiboonsak *et al*., 2010). PlcN and BCAM0408 homologues were shown to cause cytotoxicity in HeLa cells and the BCAM2720 homologue was significantly upregulated in a hamster model for melioidosis (Tuanyok *et al*., 2006; Korbsrisate *et al*., 2007; Mira *et al*., 2011; Stone *et al*., 2014). BCAM0451 is a homologue of PA3909 (EddB) in *P. aeruginosa* encoding a DNase which is secreted to degrade eDNA. It has been shown that EddB can be used as a defence mechanism to evade the host immune system, specifically neutrophil extracellular traps. These are DNA lattices containing granular proteins with antimicrobial activity (Wilton *et al*., 2018). OlsB (BPSL1375) is an enzyme in the first step of creating Pi-free ornithine lipids (OLs), which has been well studied in *B. cenocepacia* (Vences-Guzmán *et al*., 2012). In *P. aeruginosa*, OlsB overexpression leads to an increase in biofilm production and better evasion of host immune responses (Kim *et al*., 2018). These results suggest upregulated putative Pho regulon members are not only

**Table 3.** Significantly differentially expressed proteins in the Pi-deplete compared with the Pi-replete condition ($P \leqslant 0.05$, identified in Fig. 2) associated with phosphate metabolism.

| $P \leqslant 0.05$ | Log2 (fold change) | Locus tag (gene name) | Annotation | Sequence length | Relative abundance (%) | | |
|---|---|---|---|---|---|---|---|
| | | | | | +Pi | −Pi | Difference |
| *Cellular proteome* | | | | | | | |
| + | +2.150 | BURCENK562V_C1978 (*olsB*) | Ornithine-acyl [acyl carrier protein] *N*-acyltransferase | 268 | 0.0016 | 0.0072 | +0.0056 |
| ++ | +7.112 | BURCENK562V_C6451 (BPSL1190) | Glycosyltransferase involved in cell wall biosynthesis | 344 | 0.0006 | 0.0851 | +0.0845 |
| ++ | +6.949 | BURCENK562V_C6126 (BCAL1083) | Hypothetical protein | 654 | 0.0021 | 0.2756 | +0.2735 |
| ++ | +5.228 | BURCENK562V_C5565 (BCAM0451) | Hypothetical protein | 604 | 0.0004 | 0.0158 | +0.0154 |
| ++ | +4.915 | BURCENK562V_C5497 (BCAM0389) | Alkaline phosphatase | 472 | 0.0006 | 0.0184 | +0.0178 |
| ++ | +4.863 | BURCENK562V_C5498 (BCAM0390) | Alkaline phosphatase | 463 | 0.0004 | 0.0123 | +0.0119 |
| ++ | +4.824 | BURCENK562V_C3463 (*glpQ*) | Glycerophosphoryl diester phosphodiesterase | 381 | 0.0010 | 0.0286 | +0.0276 |
| ++ | +4.758 | BURCENK562V_C1985 (phoB) | Two-component transcriptional regulator, winged helix family | 235 | 0.0023 | 0.0639 | +0.0616 |
| ++ | +4.739 | BURCENK562V_C1987 (*pstB*) | Phosphate ABC transporter ATP-binding protein, PhoT family | 282 | 0.0072 | 0.2011 | +0.1939 |
| ++ | +4.563 | BURCENK562V_C6086 (*plcN*) | Phospholipase C | 714 | 0.0019 | 0.0463 | +0.0444 |
| ++ | +4.116 | BURCENK562V_C1983 (*ppk*) | Polyphosphate kinase | 687 | 0.0007 | 0.0130 | +0.0123 |
| ++ | +3.455 | BURCENK562V_C1984 (*phoR*) | PAS/PAC sensor signal transduction histidine kinase/histidine kinase | 439 | 0.0036 | 0.0411 | +0.0375 |
| ++ | +3.054 | BURCENK562V_C1990 (*pstS*) | Phosphate ABC transporter substrate-binding protein, PhoT family | 344 | 0.9185 | 7.9420 | +7.0235 |
| ++ | +2.987 | BURCENK562V_C1986 (*phoU*) | Phosphate uptake regulator | 234 | 0.0576 | 0.4757 | +0.4181 |
| ++ | +2.969 | BURCENK562V_C6165 (BCAL1116) | Putative spermidine/putrescine transport system substrate-binding protein | 342 | 0.0009 | 0.0072 | +0.0063 |
| ++ | +2.903 | BURCENK562V_C1988 (*pstA*) | Phosphate ABC transporter membrane protein 2, PhoT family | 292 | 0.0009 | 0.0070 | +0.0061 |
| ++ | +2.737 | BURCENK562V_C3379 (*ppk2*) | Polyphosphate kinase 2, PA0141 family | 316 | 0.0009 | 0.0065 | +0.0056 |
| ++ | +2.732 | BURCENK562V_C1989 (*pstC*) | Phosphate ABC transporter membrane protein 1, PhoT family | 328 | 0.0009 | 0.0060 | +0.0051 |
| + | +2.157 | BURCENK562V_C5264 (BCAM0164) | Histidine phosphatase superfamily (branch 2) | 542 | 0.0005 | 0.0024 | +0.0019 |
| + | +2.124 | BURCENK562V_C4699 (*phnA*) | Phosphonoacetate hydrolase | 436 | 0.0006 | 0.0028 | +0.0022 |
| + | +1.656 | BURCENK562V_C6452 (BCAL2364) | UDP-2,3-diacylglucosamine pyrophosphatase, LpxH | 312 | 0.0009 | 0.0029 | +0.0020 |
| + | +1.542 | BURCENK562V_C3774 (BCAM1663) | Calcineurin-like phosphoesterase | 561 | 0.0007 | 0.0022 | +0.0015 |
| *Extracellular proteome* | | | | | | | |
| + | +8.719 | BURCENK562V_C3774 (BCAM1663) | Calcineurin-like phosphoesterase | 561 | 0.0001 | 0.0356 | +0.0355 |
| ++ | +7.842 | BURCENK562V_C5497 (BCAM0389) | Alkaline phosphatase | 472 | 0.0001 | 0.0274 | +0.0273 |
| + | +6.944 | BURCENK562V_C5498 (BCAM0390) | Alkaline phosphatase | 463 | 0.0001 | 0.0194 | +0.0193 |
| ++ | +4.676 | BURCENK562V_C5264 (BCAM0164) | Histidine phosphatase superfamily (branch 2) | 542 | 0.0020 | 0.0577 | +0.0557 |
| ++ | +3.631 | BURCENK562V_C5565 (BCAM0451) | Hypothetical protein | 604 | 0.0727 | 0.9928 | +0.9201 |
| ++ | +3.334 | BURCENK562V_C4951 (BCAM2720) | Phospholipase C | 779 | 0.0014 | 0.0159 | +0.0145 |
| + | +2.611 | BURCENK562V_C6126 (BCAL1083) | Hypothetical protein | 654 | 0.0307 | 0.2068 | +0.1761 |

*(Continues)*

**Table 3.** Continued

| $P \leqslant 0.05$ | Log2 (fold change) | Locus tag (gene name) | Annotation | Sequence length | Relative abundance (%) | | |
|---|---|---|---|---|---|---|---|
| | | | | | +Pi | −Pi | Difference |
| + | +2.513 | BURCENK562V_C3463 (BCAM1396) | Glycerophosphoryl diester phosphodiesterase | 381 | 0.0010 | 0.0061 | +0.0051 |
| + | +2.082 | BURCENK562V_C5518 (BCAM0408) | Phospholipase C | 815 | 0.0010 | 0.0045 | +0.0035 |

Significance is related to the log2-fold change of their relative abundance in proteomes.
$+P \leqslant 0.05$; $++P \leqslant 0.01$.

involved in Pi regulation but have other direct or indirect roles in virulence.

There are at least six other proteins upregulated under Pi limitation that are not *bona fide* members of the classic Pho regulon (Table 4). CpdB is a well-characterized phosphodiesterase in *E. coli* involved in degrading non-transportable organophosphates, which is regulated by carbon source availability (Wanner, 1996). In avian pathogenic *E. coli* and *Salmonella enterica*, CpdB is thought to be important for long-term colonization of avian tissues (Liu *et al*., 2015, 2017). However, CpdB is not involved in virulence in a mouse model for the pathogen *Yersinia enterocolitica* (Trülzsch *et al*., 2001). This suggests it may only be a specific virulence factor for avian infections through the gut. The homologue of BCAM1283 in *Burkholderia mallei*, BMAA0553, was discovered to be a novel virulence factor that interferes with the host cytoskeleton arrangement and has other roles in signalling and adhesion (Memišević *et al*., 2013, 2015; Bozue *et al*., 2016). Proteases BPSL0627 and BCAL0849 are likely to be involved in host tissue degradation, though BPSL0627 has yet to be characterized in any species of bacteria (Drevinek *et al*., 2008). The putative citrate synthase, BCAS0207, is required for optimal virulence and biofilm formation in *B. cenocepacia* and is less virulent in an alfalfa infection model (Subramoni *et al*., 2011). Finally, BCAM1453 is a putative LysR family transcriptional regulator which typically regulates genes involved in quorum sensing and virulence (Maddocks and Oyston, 2008). However, its role in *B. cenocepacia* is unknown.

Although there are a large number of upregulated virulence factors, our results also show many virulence factors are downregulated (Table 4). This includes many members of the siderophore (ornibactin) gene cluster (BURCENK562V_C1523 to BURCENK562V_C1535, Table 5) suggesting a reduction in the amount of siderophore produced under Pi limitation (Agnoli *et al*., 2006). Another LysR transcriptional regulator, BCAL3178, is downregulated, which is involved in the regulation of the Hmq operon, a quorum-sensing system in many *Burkholderia* species (Coulon *et al*., 2019). Some of the proteins downregulated are involved in defence against macrophages. For example, BPSS1342 is homologous to MAV2054 in *Mycobacterium avium*, which induces macrophage apoptosis (Lee *et al*., 2016). BCAM0276, a putative universal stress protein, is highly expressed during intracellular infection. However, its role in protection from macrophage attack is not fully understood (Tolman and Valvano, 2012). Another downregulated protein is an acid phosphatase (BCAM1576) whose homologue in *B. thailandensis* (BTH_I320) shows similarity to a phospholipase C (PlcH) from *P. aeruginosa* which is a hemolytic exotoxin (Franco *et al*., 2018). The final cellular downregulated protein, BCAM0580, has been characterized in *B. cenocepacia* as RfpR, a key regulator of cyclic-di-GMP signalling. This type of signalling upregulates a range of virulence factors involved in biofilm formation, swarming motility and protease activity (Kumar *et al*., 2018; Richter *et al*., 2019). In the extracellular fraction, five proteins are downregulated, three of which are part of the flagellum (FlgL, FliD1 and FliK). Flagella are important for motility as well as adhesion to host cells, both of which are important for virulence (Williams *et al*., 1996; Ryan *et al*., 2005; Hanuszkiewicz *et al*., 2014). Indeed, when swarming assays were performed, *B. cenocepacia* K56-2 had decreased motility in Pi-deplete conditions (Fig. 4B), likely due to the decrease in flagellar proteins. Another exoprotein involved in adhesion/motility is BCAL1681, part of an operon involved in the assembly of the pilus machinery (Inhülsen *et al*., 2012). Lastly, BCAL1722 is an exported chitinase that most likely plays a role in plant virulence (O'Grady *et al*., 2009; Burtnick *et al*., 2014).

Overall, these results suggest Pi limitation stimulates a shift in virulence factor expression when *B. cenocepacia* experiences Pi stress. In previous studies, although some virulence factors such as swarming motility, exopolysaccharide production and host colonization are known to be increased during Pi stress, many suggest virulence is overall decreased under Pi limitation (Rüberg *et al*., 1999; Von Krüger *et al*., 1999; Lamarche

**Table 4.** Significantly differentially expressed proteins in the Pi-deplete compared with the Pi-replete condition ($P \leqslant 0.05$, identified in Fig. 2) associated with virulence.

| $P \leqslant 0.05$ | Log2 (fold change) | Locus tag (gene name) | Annotation | Sequence length | Relative abundance (%) | | |
|---|---|---|---|---|---|---|---|
| | | | | | +Pi | −Pi | Difference |
| *Cellular proteome* | | | | | | | |
| ++ | +5.228 | BURCENK562V_C5565 (BCAM0451) | Hypothetical protein | 604 | 0.0004 | 0.0158 | +0.0154 |
| ++ | +4.824 | BURCENK562V_C3463 (*glpQ*) | Glycerophosphoryl diester phosphodiesterase | 381 | 0.0010 | 0.0286 | +0.0276 |
| ++ | +4.563 | BURCENK562V_C6086 (*plcN*) | Phospholipase C | 714 | 0.0019 | 0.0463 | +0.0444 |
| ++ | +4.116 | BURCENK562V_C1983 (*ppk*) | Polyphosphate kinase | 687 | 0.0007 | 0.0130 | +0.0123 |
| ++ | +4.079 | BURCENK562V_C0530 (*cpdB*) | 2,3-Cyclic-nucleotide 2-phosphodiesterase/3-nucleotidase | 689 | 0.0003 | 0.0047 | +0.0044 |
| ++ | +4.010 | BURCENK562V_C0366 (BCAL0849) | Putative metalloprotease | 253 | 0.0010 | 0.0167 | +0.0157 |
| ++ | +3.513 | BURCENK562V_C3335 (BCAM1283) | Calcineurin-like phosphoesterase | 487 | 0.0007 | 0.0080 | +0.0073 |
| ++ | +2.954 | BURCENK562V_C3526 (BCAM1453) | Transcriptional regulator, LysR family | 319 | 0.0006 | 0.0052 | +0.0046 |
| + | +2.150 | BURCENK562V_C1978 (BPSL1375) | Ornithine-acyl [acyl carrier protein] *N*-acyltransferase | 268 | 0.0016 | 0.0072 | +0.0056 |
| + | +1.429 | BURCENK562V_C7355 (BCAS0207) | Citrate synthase | 414 | 0.0058 | 0.0164 | +0.0106 |
| + | +1.366 | BURCENK562V_C0351 (BPSL0627) | Protease I | 193 | 0.0147 | 0.0395 | +0.0248 |
| + | −1.612 | BURCENK562V_C5692 (BCAL3178) | Transcriptional regulator, LysR family | 327 | 0.1359 | 0.0463 | −0.0896 |
| + | −1.751 | BURCENK562V_C2564 (BCAM0580) | Diguanylate cyclase/ phosphodiesterase | 667 | 0.0016 | 0.0005 | −0.0011 |
| + | −1.764 | BURCENK562V_C3669 (BCAM1576) | Acid phosphatase | 554 | 0.0213 | 0.0065 | −0.0148 |
| + | −1.817 | BURCENK562V_C1530 (*orbE*) | Putative ATP-binding cassette transporter | 581 | 0.0052 | 0.0015 | −0.0037 |
| ++ | −2.331 | BURCENK562V_C1524 (*orbF*) | Formyl transferase | 279 | 0.0083 | 0.0017 | −0.0066 |
| ++ | −2.417 | BURCENK562V_C1528 (*orbJ*) | Amino acid adenylation domain-containing protein | 1669 | 0.0009 | 0.0002 | −0.0007 |
| ++ | −2.593 | BURCENK562V_C1523 (*orbL*) | Protein *N*-acetyltransferase, RimJ/ RimL family | 338 | 0.0064 | 0.0011 | 0.0053 |
| ++ | −3.451 | BURCENK562V_C1535 (*orbG*) | Taurine dioxygenase, alpha-ketoglutarate-dependent | 335 | 0.0101 | 0.0010 | −0.0091 |
| ++ | −3.476 | BURCENK562V_C5387 (BCAM0276) | Nucleotide-binding universal stress protein, UspA family | 156 | 0.0211 | 0.0020 | −0.0191 |
| ++ | −3.948 | BURCENK562V_C1529 (*orbI*) | Non-ribosomal peptide synthase domain TIGR01720/amino acid adenylation domain-containing protein | 3234 | 0.0024 | 0.0002 | −0.0022 |
| ++ | −4.482 | BURCENK562V_C2521 (BPSS1342) | Major membrane protein 1 family protein | 310 | 0.4929 | 0.0230 | −0.4699 |
| ++ | −5.254 | BURCENK562V_C1526 (*pvdA*) | L-ornithine N5-oxygenase | 458 | 0.0220 | 0.0006 | −0.0214 |
| *Extracellular proteome* | | | | | | | |
| ++ | +6.330 | BURCENK562V_C0530 (*cpdB*) | 2,3-Cyclic-nucleotide 2-phosphodiesterase/3-nucleotidase | 689 | 0.0001 | 0.0067 | +0.0066 |
| ++ | +3.631 | BURCENK562V_C5565 (BCAM0451) | Hypothetical protein | 604 | 0.0727 | 0.9928 | +0.9201 |
| ++ | +3.334 | BURCENK562V_C4951 (BCAM2720) | Phospholipase C | 779 | 0.0014 | 0.0159 | +0.0145 |
| + | +2.513 | BURCENK562V_C3463 (BCAM1396) | Glycerophosphoryl diester phosphodiesterase | 381 | 0.0010 | 0.0061 | +0.0051 |
| + | +2.082 | BURCENK562V_C5518 (BCAM0408) | Phospholipase C | 815 | 0.0010 | 0.0045 | +0.0035 |
| + | −1.740 | BURCENK562V_C1500 (BCAL1722) | Chitinase family 18 | 451 | 0.9304 | 0.3070 | −0.6234 |

*(Continues)*

**Table 4.** Continued

| $P \leqslant 0.05$ | Log2 (fold change) | Locus tag (gene name) | Annotation | Sequence length | Relative abundance (%) | | |
|---|---|---|---|---|---|---|---|
| | | | | | +Pi | −Pi | Difference |
| + | −1.835 | BURCENK562V_C0662 (*flgL*) | Flagellar hook-associated protein 3, FlgL | 411 | 0.2751 | 0.0850 | −0.1901 |
| + | −1.977 | BURCENK562V_C6832 (*fliD1*) | Flagellar hook-associated protein 2 | 502 | 0.0197 | 0.0055 | −0.0142 |
| + | −3.431 | BURCENK562V_C1546 (BCAL1681) | Hypothetical protein | 473 | 0.0537 | 0.0055 | −0.0482 |
| + | −6.226 | BURCENK562V_C0711 (BCAL0529) | Hook-length control protein, FliK | 450 | 0.0077 | 0.0001 | −0.0076 |

Significance is related to the log2-fold change of their relative abundance in proteomes.
$+P \leqslant 0.05$; $++P \leqslant 0.01$.

**Table 5.** Significantly differentially expressed proteins in the Pi-deplete compared with the Pi-replete condition ($P \leqslant 0.05$, identified in Fig. 2) associated with antimicrobial resistance.

| $P \leqslant 0.05$ | Log2 (fold change) | Locus tag (gene name) | Annotation | Sequence length | Relative abundance (%) | | |
|---|---|---|---|---|---|---|---|
| | | | | | +Pi | −Pi | Difference |
| *Cellular proteome* | | | | | | | |
| ++ | +4.116 | BURCENK562V_C1983 (*ppk*) | Polyphosphate kinase | 687 | 0.0007 | 0.0130 | +0.0123 |
| ++ | +3.931 | BURCENK562V_C3621 (BCAM1540) | Isoquinoline 1-oxidoreductase, beta subunit | 739 | 0.0004 | 0.0065 | +0.0061 |
| + | +2.150 | BURCENK562V_C1978 (*olsB*) | Ornithine-acyl [acyl carrier protein] *N*-acyltransferase | 268 | 0.0016 | 0.0072 | +0.0056 |
| + | +1.857 | BURCENK562V_C3815 (BCAM1700) | Protein *N*-acetyltransferase, RimJ/RimL family | 400 | 0.0009 | 0.0035 | +0.0026 |
| + | +1.578 | BURCENK562V_C6450 (*dagK*) | Diacylglycerol kinase | 177 | 0.0013 | 0.0041 | +0.0028 |
| + | −1.796 | BURCENK562V_C1400 (BCAL1812) | Multidrug efflux pump subunit, AcrB | 1048 | 0.0011 | 0.0003 | −0.0008 |
| ++ | −2.868 | BURCENK562V_C4836 (BCAM2620) | D-methionine transport system substrate-binding protein | 268 | 0.2400 | 0.0342 | −0.2058 |
| ++ | −3.766 | BURCENK562V_C3648 (BCAM1559) | Acetyltransferase (GNAT) family protein | 186 | 0.0249 | 0.0019 | −0.0230 |
| ++ | −5.253 | BURCENK562V_C5411 (BCAM0300) | Metallo-beta-lactamase family protein | 467 | 0.0164 | 0.0004 | −0.0160 |
| *Extracellular proteome* | | | | | | | |
| + | −2.821 | BURCENK562V_C4836 (BCAM2620) | D-methionine transport system substrate-binding protein | 268 | 0.5332 | 0.0832 | −0.4500 |

Significance is related to the log2-fold change of their relative abundance in proteomes.
$+P \leqslant 0.05$; $++P \leqslant 0.01$.

*et al*., 2008b; Bertrand *et al*., 2010; Crépin *et al*., 2011; Bains *et al*., 2012). For example, PhoB has been shown to regulate operons involved in cyclic di-GMP production, a key signalling factor for quorum sensing and biofilm formation. Pi stress stimulates a decrease in c-di-GMP leading to reduced biofilm formation (Haddad *et al*., 2009; Pratt *et al*., 2009). Interestingly, here we show that RpfR (BCAM0580) is downregulated under Pi stress, RfpR being a key regulator of c-di-GMP in *Burkholderia* (Richter *et al*., 2019). However, further experimentation is required to confirm whether this protein would lead to a decreased amount of c-di-GMP and a decrease in virulence under Pi limitation. Despite the suggestion PhoB activation results in decreased virulence, the deletion of PhoB in *Salmonella* causes hypervirulence which in turn decreases *Salmonella* survival in macrophages (Choi *et al*., 2019). Pyocyanin production (a toxin) in *P. aeruginosa* is stimulated under Pi limitation and is PhoB-dependent, specifically when iron is also present in the media (Jensen *et al*., 2006). From these examples, it appears that the Pho regulon can stimulate some aspects of virulence whilst indirectly inactivating others and overall has a very complex role to play in bacterial virulence, which could be specific to the pathogen.

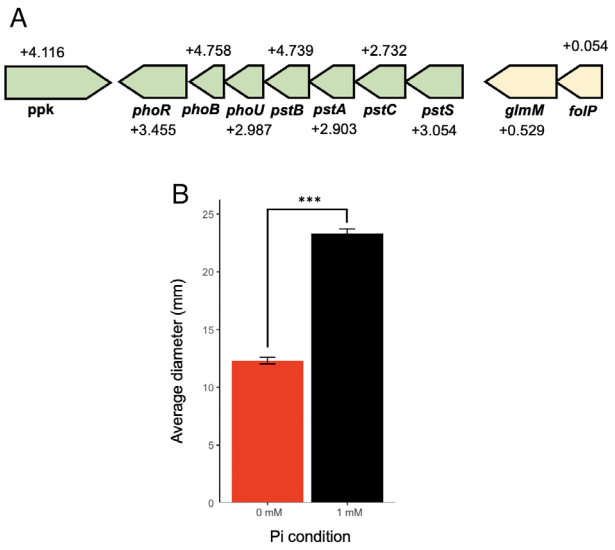


**Fig. 4.** A. Fold change (log2) in protein expression between Pi-deplete and Pi-replete conditions for the phosphate transporter (PstSCAB) and two-component system (PhoBR) and their neighbouring genes. Proteins are colour-coded for significantly upregulated (green) and non-significantly regulated (yellow).
B. Diameter of swarming colonies of *Burkholderia cenocepacia* under Pi-replete and Pi-deplete conditions. $N = 5$; $^{***}P < 0.001$. [Color figure can be viewed at wileyonlinelibrary.com]

### *Putative antimicrobial resistance proteins are differentially regulated in response to Pi limitation in* B. cenocepacia

*Burkholderia* species are well-studied for their high tolerance to many different classes of antibiotics and antimicrobial peptides, as well as their ability to produce antifungals (Vial *et al*., 2007; Podnecky *et al*., 2015; Regan and Bhatt, 2019). Following our results indicating a shift in expression of putative virulence factors as a response to Pi deprivation, we similarly investigated whether the significantly differentially regulated proteins may be also involved in bacterial resistance to antimicrobials (Table 5). OlsB (BPSL1375) and Ppk have been mentioned previously for their roles in virulence and Pi storage. However, they also have roles in resistance. OlsB plays a role in producing OLs under Pi-stress conditions and the resulting change in lipid membrane composition confers resistance to antimicrobial peptides in *P. aeruginosa* (Kim *et al*., 2018). A *ppk* mutant in *Campylobacter jejuni* was more susceptible to a wide range of antibiotics including erythromycin, ciprofloxacin and cefotaxime (Gangaiah *et al*., 2009). Overexpression of diacylglycerol kinase (DagK) in *Acinetobacter baumannii* resulted in increased resistance to colistin through an unknown mechanism (Cafiso *et al*., 2019). The homologue of BCAM1700 in *Klebsiella pneumoniae* (KPN_00437) is thought to be associated with the functionality of SHV-11, a beta-lactamase (Miryala *et al*., 2020). Finally, the homologue of BCAM1540 in *Pseudomonas putida* (PP_2478) may be involved in tolerance to the toxic compound, 2,4,6-Trinitrotoluene (Fernández *et al*., 2009).

There were also five proteins downregulated under Pi limitation related to antimicrobial resistance (Table 5). One was detected in both the cellular and extracellular proteome, BCAM2620, a putative lipoprotein that may confer resistance of *B. cenocepacia* to attack by *P. aeruginosa* (Bernier *et al*., 2018). BCAL1812 is the homologue of MexK in *P. aeruginosa*, one part of an efflux pump involved in expelling triclosan and tetracycline from the cell (Chuanchuen *et al*., 2002; Godoy *et al*., 2010). The homologue of BCAM1559, AK961_03495, in *Serratia marcescens* is an anti-toxin protein thought to be involved in protection against non-ribosomal peptides (Matteoli *et al*., 2018). The *B. thailandensis* homologue for BCAM0300 (BTH_II0462) is a putative metallo-beta-lactamase (Wagley *et al*., 2014). These results indicate a shift in AMR in response to Pi limitation and suggest that the consequences of this AMR shift could be as complex as the virulence changes we also observed under these conditions.

## Conclusions

Inorganic phosphate (Pi) is an essential nutrient for life with many environments containing low Pi concentrations, including soil, oceans and some human host environments. As such, all bacteria require a stress response mechanism for when they experience Pi stress. In the Bcc species, *B. cenocepacia* K56-2, we identified a total of 182 and 23 proteins that are significantly differentially expressed in the cellular and exoproteome respectively. KEGG analysis highlighted the drastic upregulation and downregulation of different protein classes under Pi limitation, suggesting there is a major shift in cellular metabolism under this condition. Our comparisons have highlighted proteins of the Pho regulon which play a complex role in virulence, as well as other proteins involved in nutrient transport and antimicrobial resistance. Our results begin to unravel the phosphate stress response in Bcc species and serve as a baseline for further research into the biology of phosphorus metabolism in *Burkholderia*.

## Experimental procedures

### *Cultivation of* B. cenocepacia

*Burkholderia cenocepacia* K56-2 was obtained from the DSMZ culture collection (Germany) and routinely cultured in lysogeny broth (LB) and LB agar (1.5% wt./vol.) at 37°C. A modified minimal A medium, which has been

used in similar experiments with *Pseudomonas* species, was used to induce phosphate stress (Lidbury *et al*., 2016). This comprised Na-Succinate 5.4 g $L^{-1}$, NaCl 200 mg $L^{-1}$, $NH_4Cl$ 450 mg $L^{-1}$, $CaCl_2$ 200 mg $L^{-1}$, KCl 200 mg $L^{-1}$, $MgCl_2$ 450 mg $L^{-1}$, $FeCl_2$ 10 mg $L^{-1}$, $MnCl_2$ 10 mg $L^{-1}$, 10 mM Hepes buffer at pH 7 and 0.01% (wt./vol.) yeast extract. $Na_2HPO_4$ was used as the phosphate source at a final concentration of 1 mM (phosphate-replete condition) and 0 mM (phosphate-deplete condition). All components were dissolved in deionized water and filter sterilized (through a 0.22 μm pore size filter) before use. For proteomic analysis, a single colony of *B*. *cenocepacia* was grown in LB overnight at 37°C in triplicate. Cells were pelleted at 4000 rpm for 10 min at 4°C and gently re-suspended in modified minimal A media with the appropriate phosphate source. The optical density of *B*. *cenocepacia* cultures was measured at 600 nm ($OD_{600}$) across a 24-h growth period.

### *Alkaline phosphatase assay*

Alkaline phosphatase activity was measured to determine whether cultures were experiencing phosphate stress. At each time point, samples were incubated in the dark with *p*-nitrophenol phosphate (*p*NPP) at a final concentration of 1 mM or with Tris buffer (pH 8) at a final concentration of 10 mM as a control. After 1 h, cells were pelleted at 13 000 rpm for 10 min. Yellow *p*-nitrophenol (*p*NP) supernatant was measured at 405 nm in triplicate (FLUOstar Omega plate reader, BMG Labtech). These measurements were compared with a *p*NP standard curve to calculate the amount of *p*NP produced in 1 h, normalized to cell density ($OD_{600}$).

### *Peptide isolation of cellular fraction*

Isolation of peptides from the cellular fraction was carried out as previously described (Zadjelovic *et al*., 2020). After 8 h of growth in phosphate-replete/deplete conditions, biological triplicates of cells were collected by centrifugation at 4000 rpm at 4°C for 10 min. The cell pellet was collected and re-suspended in 3:1 water:LDS sample buffer (LDS with 1% beta-mercaptoethanol (vol./vol.)). Cells were lysed at 95°C with repeated vortexing. 20 μl of each sample was run on a NuPAGE 10% (wt./vol.) Bis-Tris protein gel for 5 min before Coomassie staining. Each protein gel band was excised and cut into 1 mm pieces before being de-stained with 50% (vol./vol.) ethanol, 50 mM ammonium bicarbonate (ABC). Gel pieces were dehydrated with 100% (vol./vol.) ethanol, followed by alkylation with 10 mM Tris(2-carboxyethyl)phosphine, 40 mM 2-chloroacetamide and 50 mM ABC at 70°C for 5 min with shaking. Gel pieces were washed with 50% (vol./vol.) ethanol 50 mM ABC and dehydrated with 100% (vol./vol.) ethanol. Proteins were digested with trypsin overnight and peptides were extracted using repeated 10 min sonication. Peptides were precipitated using a speed vacuum concentrator for 3 h at 45°C and re-suspended in 2% acetonitrile with 0.1% trifluoroacetic acid.

### *Isolation of peptides from the extracellular fraction*

Isolation of peptides from the exoproteome was based on a previously published protocol (Christie-Oleza and Armengaud, 2010). After 8 h of growth in phosphate-replete/deplete conditions, biological triplicates of cells were collected by centrifugation at 4000 rpm at 4°C for 10 min. The supernatant was further filtered (0.22 μm pore size) before exoproteins were precipitated. A final concentration of 0.015% (wt./vol.) sodium deoxycholate (DOC) was added to the filtered supernatant. After a 10 min incubation, a final concentration of 3% (wt./vol.) trichloroacetic acid (TCA) was added followed by a 30 min incubation on ice. Proteins were pelleted at 4000 rpm at 4°C for 15 min. Pellets were re-suspended in water and the addition of DOC and TCA was repeated. Proteins were pelleted at 8000 rpm for 10 min at 4°C, then re-suspended in 1:1 ethanol:ether. Samples were further centrifuged at 13 000 rpm for 15 min at 4°C, after which the pellets were left to dry at room temperature overnight. Peptide isolation continued by SDS-PAGE and trypsin digestion, as described for the cellular fraction.

### *Proteomics analysis*

Extracted peptides were analysed by nanoLC-ESI-MS/MS using the Ultimate 3000 RSLCnano system and Thermo Orbitrap Fusion mass spectrometer (Dionex/Thermo Fisher Scientific). The proteome used for peptide analysis was concatenated from protein sequences of the *B*. *cenocepacia* K56-2 Valvano genome from the JGI IMG genome portal (Genome ID: 2562617106). Further data analysis was carried out using MaxQuant and Perseus software (Tyanova *et al*., 2016a; Tyanova *et al*., 2016b). Peptides without triplicate measures were filtered out. From these data sheets, BlastKOALA was used to assign KEGG to significantly differentially expressed proteins. Clusters of Orthologous Groups of proteins were assigned manually from IMG/M. Details of these methods can be found in the Supplementary Materials.

### *Swarming assays*

Overnight cultures of *B*. *cenocepacia* in LB medium were collected by centrifugation at 4000 rpm for 10 min at 4°C. Pellets were re-suspended in 10 ml of modified minimal

A media to $OD_{600}$ of 0.15 with a final concentration of 0 or 1 mM $Na_2HPO_4$ for the Pi-deplete or replete conditions respectively. These cultures were incubated at 37°C at 180 rpm until cultures reached mid-exponential phase (0.6–0.8 $OD_{600}$). Agarose plates for the swarming assay were made using modified minimal A media with 0.4% (wt./vol.) agarose (Sigma Aldrich) for swarming with no added phosphate. Each plate had 20 ml of medium with agarose and the plates were dried for 30 min in the centre of a laminar flow hood. On each plate, a 5-μl droplet of bacterial culture was plated on the surface. Plates were incubated for 18 h at 37°C before the diameter (mm) of the swarm was measured.

**Acknowledgements**

We are very grateful to the University of Warwick's Proteomics Research Technology Platform for processing the LC–MS/MS samples. We acknowledge the BBSRC MIBTP (BB/M01116X/1) doctoral training centre and MRC doctoral training centre for funding to H.S. and R.A.J. respectively.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Supplementary Table 1.** The list of proteins that are detected in the cellular proteome and extracellular proteome.

**Appendix S1:** Supplementary material.