2-Aminoethylphosphonate utilization in *Pseudomonas putida* BIRD-1 is controlled by multiple master regulators

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Summary

Bacteria possess various regulatory mechanisms to detect and coordinate a response to elemental nutrient limitation. In pseudomonads, the two-component system regulators CbrAB, NtrBC and PhoBR, are responsible for recognizing cellular response to carbon (C), nitrogen (N) and phosphorus (P) respectively. Phosphonates are reduced organophosphorus compounds produced by a broad range of biota and typified by a direct C-P bond. Numerous pseudomonads can use the environmentally abundant phosphonate species 2-aminoethylphosphonate (2AEP) as a source of C, N, or P, but only PhoBR has been shown to play a role in 2AEP utilization. On the other hand, utilization of 2AEP as a C and N source is considered substrate inducible. Here, using the plant-growth-promoting rhizobacterium *Pseudomonas putida* BIRD-1 we present evidence that 2AEP utilization is under dual regulation and only occurs upon depletion of C, N, or P, controlled by CbrAB, NtrBC, or PhoBR respectively. However, the presence of 2AEP was necessary for full gene expression, i.e. expression was substrate inducible. Mutation of a LysR-type regulator, termed AepR, upstream of the 2AEP transaminase-phosphonatase system (PhnWX), confirmed this dual regulatory mechanism. To our knowledge, this is the first study identifying coordination between global stress response and substrate-specific regulators in phosphonate metabolism.

Introduction

The three most essential nutrients for bacterial cells, by quantity, are carbon (C), nitrogen (N) and phosphorus (P). These nutrients provide energy, building blocks for anabolism and primary and secondary metabolism, and maintain cellular homeostasis. In the environment, the ratio of these nutrients fluctuates and the concentration of any one can become limiting for cellular growth (Shimizu, 2014). To maximize resources and conserve energy bacteria possess ‘sensors’ to detect the relative depletion of a given nutrient (Groisman, 2016). These sensors can detect signals either internal or external to the cell and are usually two-component regulatory systems, which coordinate a cellular response to nutrient depletion via the simultaneous regulation of numerous genes (regulon). Two-component regulators typically consist of a histidine protein kinase sensor protein, which detects the stress signal, and a response regulator, which, once activated by phosphorylation by the sensor protein, acts as a transcription factor to drive transcription of its associated regulon (Zschiedrich et al., 2016). In pseudomonads, the two-component regulators CbrAB (Nishijyo et al., 2001), NtrBC (Li and Lu, 2007) and PhoBR (Monds et al., 2006; Lidbury et al., 2016) detect and coordinate a response to C, N and P stress respectively (Fig. 1). Of these, PhoBR is widespread among bacteria (Santos-Beneit, 2015), NtrBC is found primarily in Proteobacteria (Leigh and Dodsworth, 2007), and CbrAB has thus far been found only in the *Pseudomonadaceae* (Nishijyo et al., 2001; Valentini et al., 2014; Monteagudo-Cascales et al., 2019). Bacteria use inorganic phosphate (Pi) as their preferred source of P, and as such PhoBR coordinates the response to exogenous depletion of Pi (threshold in *Escherichia coli* = 4 μM) (Wanner, 1996). In pseudomonads, albeit with a degree of intra-genus variation, PhoBR regulates the synthesis of multiple systems.
related to the efficient acquisition of organic and inorganic P compounds (Lidbury et al., 2016). Collectively, these include alkaline phosphatases (Monds et al., 2006), phosphodiesterases targeting both lipid headgroups and nucleotides (Bains et al., 2012; Lidbury et al., 2016), pathways required for phosphonate degradation (Bains et al., 2012; Lidbury et al., 2016; Lidbury et al., 2017), the high-affinity phosphate ATP-binding cassette (ABC) transporter, pstSCAB (Monds et al., 2006) and the ptx and htx operons, which transport and oxidize phosphate and hypophosphite respectively (White and Metcalf, 2004). Additionally, PhoBR is involved in pathogenicity in Pseudomonas aeruginosa, with defects in swarming motility and cytotoxicity observed in phoB and phoR mutants even under phosphate replete conditions (Bains et al., 2012). Likewise, ammonium is the preferred source of N for pseudomonads, and during times of ammonium scarcity NtrBC induces the ammonium transporter AmtB as well as expression of genes required for scavenging N from alternative N sources, such as amino acids and nitrate/nitrite (Li and Lu, 2007; Zhang and Rainey, 2008). NtrBC also controls the expression of genes involved in dinitrogen fixation in diazotrophs, e.g. the rhizobacterium P. stutzeri (Zhimin et al., 2021). An ntrBC mutant of P. aeruginosa PA14 also shows defects in both swarming motility and biofilm formation, and pathogenicity is impacted due to poor dissemination within a murine host (Alford et al., 2020). Pseudomonads appear to use TCA cycle intermediates as their preferred C source (Rojo, 2010), and use a catabolite repression system to control the synthesis of non-preferential C source transporters/catabolic enzymes. This catabolite repression occurs post-transcriptionally, using the Crc/Hfq system (Moreno et al., 2015). This system binds and sequesters mRNA, preventing its translation. In response to low C availability, CbrB drives the expression of crcZ (Sonnleitner et al., 2009), and in P. putida also crcY (Moreno et al., 2012; Garcia-Mauriño et al., 2013). cbrAB mutants of P. aeruginosa and P. fluorescens are thus unable to use some C sources, such as histidine and arginine (Li and Lu, 2007; Zhang and Rainey, 2008). PhoBR also interacts with C, N, sulfur, and iron regulatory networks to coordinate gene activation under various nutrient limiting growth conditions, across different bacteria (Santos-Beneit, 2015). Furthermore, cbrAB:ntrBC double mutants are unable to use some N sources that ntrBC single mutants can, indicating the connection between these regulons (Li and Lu, 2007; Zhang and Rainey, 2008). A cbrA mutant also shows defects in swarming motility but enhanced biofilm formation and
cytotoxicity (Yeung et al., 2011). Thus, PhoBR, NtrBC and CbrAB can co-regulate the same gene sets, particularly those linked with survival through adaptation to varying environmental conditions.

Phosphonates are organophosphorus molecules that contain a direct C-P bond (Villarreal-Chiu et al., 2012). While less common than the C-oxygen (O)-P ester bond found in other organic P molecules, phosphonates are found in a wide range of environments including freshwater lakes (Cade-Menun et al., 2006), marine systems (Clark et al., 1998; Kolowith et al., 2001; Young and Ingall, 2010) and soils (Tate and Newman, 1982; Turner et al., 2004). The vast majority of phosphate biosynthesis reactions begin with the isomerisation of phosphoenolpyruvate to phosphonopyruvate via phosphoenolpyruvate mutase (PepM) (Yu et al., 2013). Using pepM as a marker gene, phosphate biosynthesis is ubiquitous in marine, soil and gut microbiomes (Villarreal-Chiu et al., 2012; Peck and van der Donk, 2013; Yu et al., 2013; Ju et al., 2014; Chin et al., 2016). Consequently, phosphonate catabolism genes are also prevalent in distinct environments. 2-aminoethylphosphonate (2AEP) is considered the most abundant phosphonate in nature (White et al., 2021) although to our knowledge in situ analytical estimates are lacking. Our previous work identified three transporters responsible for 2AEP uptake in the soil rhizobacteria Pseudomonas putida BIRD-1 (hereafter BIRD-1) (Murphy et al., 2021). Two of these are ABC transporters, AepXVW and AepSTU, whilst the third, AepP, is a member of the organophosphatase: phosphate antiporter subfamily of major facilitator transporters (Lemieux et al., 2005; Law et al., 2009). AepSTU was constitutively synthesized and only played a minor role in facilitating 2AEP uptake as a sole P source, whilst AepXVW was the major transporter facilitating 2AEP uptake during P limiting growth conditions but played no role in 2AEP uptake under N limitation. AepP was essential for 2AEP uptake under N limitation but could facilitate growth on 2AEP as a sole P source in the absence of a functional AepXVW and AepSTU. BIRD-1 possesses a single phosphonate catabolism system, a two-enzyme complex containing the 2AEP-pyruvate transaminase (PhnW) and the phosphonoacetaldehyde hydrolase (PhnX) otherwise known as phosphonatase (Jiang et al., 1995; Kim et al., 2002), which is essential for 2AEP catabolism in BIRD-1 (Murphy et al., 2021). The abundance of aepX and genes encoding 2AEP-specific catabolic enzymes throughout marine systems suggests that catabolism, and thus also biosynthesis of 2AEP, occurs at a greater rate than that of other phosphonates (Murphy et al., 2021).

In various bacteria regulation of 2AEP transport and subsequent intracellular catabolism is under the control of PhoBR, such as the phnCDEFHIJKLMNOP operon encoding C-P lyase in Escherichia coli or the phnSTU- phnWX operon of Salmonella enterica (Jiang et al., 1995). In other bacteria, regulation is thought to be substrate inducible or phosphate-insensitive (Temam and Quinn, 1998; Cooley et al., 2011; Chin et al., 2018). Substrate induction through LysR-type regulators has been demonstrated for phosphonoacetate (PhnR) (Kulakova et al., 2001) and phosphonoalanine (PalR) (Kulakova et al., 2009). LysR-type regulators (referred to as AepR), suggested to play an analogous role in the regulation of 2AEP degradation (Borisova et al., 2011), are found as part of 2AEP operons though these are not closely related to either PhnR or PalR (Murphy et al., 2021). An aepR homologue has been shown to be essential to complement an E. coli C-P lyase mutant with phnWX (Martinez et al., 2010), suggesting a role in substrate induction. However, substrate induction does not preclude other forms of regulation, and the differential expression of the 2AEP transporters of BIRD-1 suggests other regulatory mechanisms (Murphy et al., 2021). Here, we investigate the regulation of 2AEP utilization (transport and catabolism) within BIRD-1 using a combination of bacterial genetics, proteomics and promoter reporter assays. These data reveal a dual mechanism of regulation, whereby gene expression is substrate inducible, demonstrated by mutation of aepR upstream of phnWX, but requires coordination by the master regulators CbrAB, NtrBC and PhoBR, under C, N and P limitation respectively.

Results

PhoBR is essential for growth on 2-aminoethylphosphonate as a sole source of P

We have previously shown that BIRD-1 synthesizes distinct transport systems to facilitate growth on 2AEP as either the sole N or P source (Murphy et al., 2021). In response to Pi-limitation, BIRD-1 synthesizes AepXVW (2AEP transporter) and PhnWX (the 2AEP-pyruvate transaminase-phosphonatase system) in a PhoBR-dependent manner (Lidbury et al., 2016). This suggested a role for master regulators in the utilization of 2AEP as the sole P source, and potentially, N and C sources. Mutation of phoBR eliminated growth of BIRD-1 on 2AEP as the sole P source (Fig. 2A), suggesting that phoBR is essential for 2AEP utilization when used as a sole P source. Mutation of phoBR had no effect on growth when 2AEP was the sole N source (Fig. 2B). This is consistent with the synthesis of another 2AEP transporter (AepP) during growth on 2AEP as the sole N source (Murphy et al., 2021) and suggests PhnWX is also regulated by the N-stress response regulator NtrBC. Taken together, this
sustains PhoBR is essential for 2AEP utilization as the sole P source by tightly controlling the synthesis of PhnWX, despite the fact that a basal level of AepP is expressed in this condition (Murphy et al., 2021).

*NtrBC and CbrAB are involved in the utilization of 2AEP as either a sole N or sole C source*

Given that mutation of *phoBR* did not affect growth on 2AEP as the sole N source, we predicted that the master regulator of the N-stress response, NtrBC, may also be involved. Generation of a Δ*ntrBC* mutant revealed NtrBC was required for efficient 2AEP utilization (Fig. 2B) but did not affect 2AEP utilization as the sole P (Fig. 2A) or sole C source (Fig. 2C). Generation of a Δ*phoBR*:Δ*ntrBC* double master regulator mutant further delayed growth on 2AEP as the sole N source (Fig. 2B) and again eliminated growth on 2AEP as the sole P source (Fig. 2A). Mutation of the genes encoding either the 2AEP antiporter (*aepP*) or phosphonatase (*phnWX*) in the Δ*ntrBC* background (Δ*phoBR*:Δ*aepP* and Δ*ntrBC*:Δ*phnWX*) resulted in complete elimination of growth on 2AEP as the sole N source, suggesting 2AEP partially induces both AepP and PhnWX synthesis in the Δ*ntrBC* mutant at a level sufficient to support partial growth (Fig. S1).

Wild-type Δ*phoBR* and Δ*ntrBC* were able to grow on 2AEP as the sole C source (Fig. 2C and D). Therefore, we predicted that the C stress-response master regulator CbrAB also interacted with AepP and PhnWX. Indeed, mutation of *cbrAB* in both the wild-type background (Δ*cbrAB*) and the Δ*ntrBC* mutant (Δ*ntrBC*:Δ*cbrAB*) severely inhibited growth on 2AEP as the sole C source at comparable amounts (Fig. 2C). Δ*cbrAB* showed no defect in growth on 2AEP as the sole N or P source (Fig. S2). We also confirmed AepP, and not AepXVW, is the sole transporter responsible for 2AEP utilization as the sole C source (Fig. 2D) as well as an N source (Murphy et al., 2021). However, given poor growth still occurs despite mutation of *cbrAB*, both AepP and PhnWX must still be synthesized, as they are essential for growth on 2AEP as the sole C source (Fig. 2D), again suggesting 2AEP partially induces both AepP and PhnWX synthesis in Δ*cbrAB*, similar to induction in Δ*ntrBC* (Fig. S1). Collectively, these data suggest that both NtrBC and CbrAB likely interact with the *aepP* and/or the *phnWX* promoters, directly or indirectly. However, whilst this interaction is not essential
for growth on 2AEP as either the sole N or sole C source it is necessary for efficient growth on 2AEP under these conditions.

*N* limitation alone does not induce synthesis of 2AEP utilization proteins

To determine if AepP and PhnWX are synthesized in response to N limitation in the absence of 2AEP, as observed under P limitation (Lidbury et al., 2016), we performed comparative proteomics. Total protein was extracted from BIRD-1 wild-type and Δ*ntrBC* cells grown overnight on 5.6 mM NH₄⁺ (High N) or 1.5 mM NH₄⁺ (Low N) (Fig. 3). In total, across all strains and conditions, 1421 proteins were identified of which 60 were differentially synthesized (FDR corrected *p* < 0.05, log₂ fold change >2, Tables S2–S4) between wild-type High and Low N (Table 1). Thirty-three of these proteins were significantly enriched (*p* < 0.05) in wild-type Low N compared to High N. Comparison between wild-type and mutant Low N proteomes suggested 16 of these were induced by NtrBC, whilst the others responded to N limitation independently of this master regulator. The putative Ntr regulon was found to include several ABC transport and catabolism proteins for various organic nitrogen compounds, including amino acids, ethanolamine, glycine betaine and urea (Table 1). Furthermore, the synthesis of several proteins linked to N metabolism was significantly downregulated in Δ*ntrBC* irrespective of nitrogen status (Fig. S3, Table 1). Whilst low level PhoBR-dependent synthesis of AepXVW and PhnWX occurred in BIRD-1 in response to P limitation (Lidbury et al., 2016), neither were detected in the wild-type (or *ntrBC* mutant) proteome during N-limitation. This is despite the fact NtrBC is required for efficient growth on 2AEP as the sole N source (Fig. 2B).

2AEP is necessary for full transcriptional activation of transport and catabolic genes

So far, our data suggested that a nutrient limitation response mediated through master regulators is required for efficient 2AEP catabolism. To investigate if this was the only mechanism of transcriptional regulation the promoter regions of both 2AEP transporters (AepXVW and AepP) and the phosphonatase pathway (PhnWX) were cloned into the lacZ fusion plasmid pBIO1878 (Todd et al., 2012; Lidbury et al., 2014). Three reporter plasmids, pBIO-aepXVW-pr, pBIO-aepP-pr and pBIO-phinWX-pr were transformed into BIRD-1 wild-type and Δ*phoBR*, Δ*ntrBC* and ΔcbrAB mutants. β-galactosidase assays were performed using crude cell extracts with ortho-nitrophenyl-β-galactopyranoside as the substrate. As some strains were incapable of growth on 2AEP, cultures were grown in replete media prior to washing and re-suspension in fresh media and subsequently incubated in the various nutrient conditions for 5 h prior to assaying.

Despite the role of each master regulator in 2AEP utilization, β-galactosidase assays clearly revealed that the presence of 2AEP was required for promoter activation in all our various reporter strains (Fig. 4). For pBIO-aepXVW-pr and pBIO-phinWX-pr a functional PhoBR was essential for full activation of β-galactosidase activity when 2AEP was used as the sole P source (Fig. 4A and

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B). In contrast, while for pBIO-aepP-pr and pBIO-phnWX-pr a functional NtrBC was essential for full activation of β-galactosidase activity when 2AEP was used as the sole N source (Fig. 4C and D), induction still occurred in the ΔntrBC mutant. Similarly, for pBIO-aepP-pr and pBIO-phnWX-pr a functional CbrAB was essential for full activation of β-galactosidase activity when 2AEP was the sole C source (Fig. 4E and F), induction still occurred in the ΔcbrAB mutant. Thus, complete expression from all three promoter regions requires substrate induction in the presence of 2AEP, and nutrient stress alone is not sufficient.

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2AEP does not induce full expression from aminoethylphosphonate operons

To determine whether 2AEP itself or a metabolite of 2AEP catabolism is responsible for activating the three aminoethylphosphonate operons, the reporter plasmids pBIO-aepXVW-pr, pBIO-aepP-pr and pBIO-phnWX-pr were transformed into BIRD-1 ΔphnWX, which is incapable of growth on 2AEP as either the sole P, N (Murphy...
et al., 2021), or C source (Fig. 2D). However, as the ΔphnWX mutant possesses intact 2AEP transporters import of 2AEP should still happen. Relative to the wild-type, induction by 2AEP from all aminoethylphosphonate operons was significantly reduced, but still occurred in the ΔaepRWX mutant (Fig. 5). As such, it is likely that 2AEP, as well as an, as yet, unidentified compound contributes towards the induction of these three operons (i.e. aepXVW, aepP, phnWX).

AepR<sub>RVW</sub> is required for efficient growth on 2AEP and substrate-induction from the phnWX promoter

To investigate whether the AepR proteins, encoded next to aminoethylphosphonate operons in BIRD-1 and other organisms (Murphy et al., 2021), are responsible for full activation of 2AEP-utilization genes, a mutant of the aepR encoded next to phnWX (hereafter ΔaepR<sub>RVW</sub>) was constructed. This aepR homologue was chosen as phnWX is essential for growth on 2AEP as the sole P, N, or C source, and as such ΔaepR<sub>RVW</sub> can provide insight into all three growth conditions. Whilst growth on 2AEP as the sole P source at 1.5 mM showed no difference between wild-type and ΔaepR<sub>RVW</sub> (Fig. S4), ΔaepR<sub>RVW</sub> showed slower growth compared to the wild-type on 2AEP as the sole P source at 0.1 mM (Fig. 6A). When grown on 2AEP as the sole N (1.5 mM) or C source (2.5 mM), ΔaepR<sub>RVW</sub> again showed a growth defect relative to that of the wild-type (Fig. 6B and C). Collectively, this suggests that AepR<sub>RVW</sub> is involved in, but not essential for, the expression of phnWX when 2AEP is the sole P, N or C source.

To determine if this growth defect was due to the role of AepR<sub>RVW</sub> in substrate inducible activation of phnWX, we transformed ΔaepR<sub>RVW</sub> with the reporter plasmid pBIO-phnWX-pr. In this mutant, induction of β-galactosidase activity from pBIO-phnWX-pr was significantly impaired in comparison with wild-type cells when 2AEP was the sole P, N, or C source (Fig. 6D–F). In fact, only ΔaepR<sub>RVW</sub> cells incubated with 2AEP as a C source produced β-galactosidase activity above the control reading (Fig. 6F). In summary, AepR<sub>RVW</sub> is required for full (substrate-inducible) activation of phnWX and subsequent growth on 2AEP as the sole P, N, or C source, but not essential.

Discussion

The regulation of 2AEP uptake and catabolism has previously been considered to be either under the control of PhoBR, the master regulator of P stress (Villarreal-Chiu et al., 2012; Lidbury et al., 2016), or otherwise substrate inducible (Ternan and Quinn, 1998). Here, we present evidence that, in BIRD-1, two other master regulators, NtrBC and CbrAB, which coordinate the response to N and C stress respectively, are also involved in the regulation of 2AEP uptake and catabolism, as their absence impairs growth on 2AEP as their corresponding nutrient source. Previous evidence of PhoBR-independent 2AEP catabolism in other Pseudomonas spp. does not rule out a role for these master regulators (Ternan and Quinn, 1998). We saw no evidence of interaction between these master regulators in the use of 2AEP as the sole P source, where PhoBR alone was essential. Neither was there any evidence of interaction between NtrBC and CbrAB in the use of 2AEP as the sole N or C source, demonstrated by the double mutants showing no differences in phenotype to the respective single master regulator mutants, unlike utilization of some other N and C containing compounds (Li and Lu, 2007; Zhang and Rainey, 2008). However, we cannot rule out an interaction between PhoBR and NtrBC/CbrAB in the use of 2AEP as the sole N or C source respectively. On the other hand, a role for such master regulators in 2AEP metabolism cannot be assumed in other bacteria, such as marine Alphaproteobacteria. Indeed, there is evidence consistent with substrate-inducible regulation in both laboratory-cultivated (Cooley et al., 2011; Chin et al., 2018) and environmental marine bacteria independent of nutrient concentration (Murphy et al., 2021). However, the exact mechanism or mechanisms of regulation in these organisms remains to be determined. Additionally, we showed that in BIRD-1 transcriptional activation of the aepP, aepXVW and phnWX promoters was substrate-inducible in that nutrient stress alone did not drive measurable enzyme activity in the BIRD-1 reporter strains. Nevertheless, our data clearly identify an interaction between the nutrient stress response regulators and substrate-inducible production of PhnWX and 2AEP transporters.

This study also demonstrated an important, but non-essential, role for the LysR-like AepR whose gene is located adjacent to the phnWX operon in BIRD-1. Both growth and β-galactosidase activity from the phnWX promoter is severely curtailed in the aepR<sub>RVW</sub> mutant, and is only detectable above background levels when 2AEP is present as the sole C source. This low-level expression is consistent with our previous proteomics data, which revealed AepX, PhnW and PhnX are synthesized at low levels in Pi-limited cells (Lidbury et al., 2016). As such AepR<sub>RVW</sub>, alongside the other AepR homologues possessed by BIRD-1 (AepR<sub>XVW</sub> and AepR<sub>PS</sub>) (Murphy et al., 2021), provide candidates for substrate-induction regulators. We speculate that each AepR regulator interacts only with its adjacent operon, given that neither could compensate for AepR<sub>RVW</sub>. We therefore present a model of aminoethylphosphonate regulation in BIRD-1 in Fig. 7. Our finding that nutrient limitation in the absence
of 2AEP was insufficient to drive complete expression from either the aepP or phnWX promoter regions in BIRD-1 agrees with the N limitation proteomics data we present here and our previous proteomics data (Murphy et al., 2021), that shows the presence of 2AEP in addition to N limitation is required to detect synthesis of PhnWX.

Fig. 5. Promoter reporter assays for 2AEP-utilization operons in the P. putida BIRD-1 wild-type or ΔphnWX mutant. β-galactosidase activity (n = 3) was driven from the aepXVW promoter (pBIO-aepXVW-pr) (A) or the phnWX promoter (B). Wild-type and ΔphnWX backgrounds are shown, under nutrient replete (Pi), deplete (-P), or 2AEP as sole P source (P Source) conditions. β-galactosidase activity (n = 3) driven from the aepP promoter (pBIO-aepP-pr) (C), and the phnWX promoter (pBIO-phnWX-pr) (D), in wild-type and ΔphnWX backgrounds, under nutrient replete (NH₄), deplete (-N), 2AEP as sole N source (N Source) conditions. β-galactosidase activity (n = 3) driven from the aepP promoter (pBIO-aepP-pr) (E) and the phnWX promoter (pBIO-phnWX-pr) (F), in wild-type and ΔphnWX backgrounds, under nutrient replete (succinate), deplete (-C), 2AEP as sole C source (C Source) conditions. 2AEP was used at a concentration of 1.5 mM for all β-galactosidase experiments. Error bars denote the standard deviation of the mean.
limitation in BIRD-1, we did not detect these in Pseudo-
monas fluorescens SBW25 (Lidbury et al., 2016). Collec-
tively, our combined data demonstrate a role for
substrate inducible expression, controlled by AepR, in
combination with nutrient limitation, controlled by either
PhoBR, NtrBC, or CbrAB.

Given that β-galactosidase activity from all ami-
nophosphonate promoters is reduced in a ΔphnWX strain
that is incapable of 2AEP catabolism (Murphy et al., 2021),
it is likely that a breakdown product of 2AEP is
at least partially) responsible for substrate induction
via AepR proteins. We hypothesize that phospho-
acetaldehyde is the most likely candidate mole-
cule, as, like AepR homologues (Murphy et al., 2021),
this intermediate is common to both PhnWX and
PhnWAY pathways of 2AEP breakdown (Villarreal-Chiu
et al., 2012), as well as the pathway of 1-OH-2AEP
degradation by PbfA (Zangelmi et al., 2021). Indeed, the
gene (pbfA) encoding PbfA is located in both phnWX and
phnWAY operons (Zangelmi et al., 2021). Phos-
phonoacetaldehyde is only produced during phosphonate
degradation, unlike the final breakdown products alanine,
acetaldehyde/acetate and phosphate.

2AEP can act as a replacement headgroup for classical
phospholipids (Mukhamedova and Glushenkova, 2000;
Kafarski, 2019) and can also form part of bacterial polysac-
charides (Baumann et al., 1992; Vinogradov et al., 2001).
However, the source of 2AEP in the rhizosphere is unclear.
Ecologically important soil protists (Gao et al., 2019; Xiong
et al., 2020) produce abundant phosphonolipids
(Mukhamedova and Glushenkova, 2000; Kafarski, 2019).
Plants also synthesize phosphonates (Wieczorek et al., 2021),
though the importance of this to the rhizo-
sphere has yet to be determined. Additionally, genomic
evidence suggests soil bacteria are also a major source of
2AEP (Li and Horsman, 2022). Moreover, the abundance

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Dual regulatory mechanisms for 2AEP utilization

of 2AEP transporters and degradation pathways in bacterial meta-omics datasets suggests 2AEP production is ubiquitous (Murphy et al., 2021). We speculate that 2AEP acquisition provides a clear advantage during nutrient limiting growth conditions which are frequently observed in plant rhizospheres (Bell et al., 2014; Cui et al., 2018), by expanding the metabolic repertoire of BIRD-1 to utilize substrates associated with the plant microbiome (Kuramae et al., 2020; Akinola et al., 2021). Indeed, 2AEP catabolism may present a key nutrient driving plant–Pseudomonas interactions and partially explain why this genus forms abundant components of rhizosphere, rhizoplane and root endophyte communities (Robinson et al., 2016; Rathore et al., 2017). Importantly, utilization of 2AEP as sole N, or C and N source (Ternan and Quinn, 1998; Murphy et al., 2021) releases bioavailable Pi into the surrounding

Fig. 7. A proposed model of the interactions between the master regulators, PhoBR, NtrBC and CbrAB, and AepR regulators of Pseudomonas putida BIRD-1 with 2AEP as the sole nutrient source and expression from aminoethylphosphonate promoters. Conditions under which full expression occurs are shown with filled line arrows, conditions under which partial expression occurs are shown with dashed line arrows, conditions under which no expression occurs are shown with blunt-ended arrows.

A. The aminoethylphosphonate operons of BIRD-1.

B. PhoBR together with 2AEP is required for expression from the phnWX and aepXVW promoters. Substrate induction occurs via AepRwx at the phnWX promoter, and likely through the homologous AepRwx at the aepXVW promoter. In the absence of PhoBR, substrate-induction is not sufficient for expression at either promoter, and no growth occurs. Substrate induction via AepRwx at the phnWX promoter is not essential for growth but is required for efficient utilization of 2AEP.

C. NtrBC together with 2AEP is required for complete expression from the aepP and phnWX promoters. Substrate induction occurs via AepRwx at the phnWX promoter, and likely through the homologous AepRwx at the aepP promoter. In the absence of NtrBC, substrate induction drives expression at both promoters, albeit to a lesser extent, allowing for slower growth. Substrate induction via AepRwx at the phnWX promoter is not essential for growth but is required for efficient utilization of 2AEP.

D. CbrAB together with 2AEP is required for complete expression from the phnWX promoter. However, 2AEP as the sole C source is sufficient for full expression from the aepP promoter. Substrate induction occurs via AepRwx at the phnWX promoter, and likely through the homologous AepRwx at the aepP promoter. In the absence of NtrBC, substrate induction drives expression at both promoters, albeit to a lesser extent, allowing for slower growth. Substrate induction via AepRwx at the phnWX promoter is not essential for growth but is required for efficient utilization of 2AEP. N.B. arrows do not necessarily indicate direct interactions between regulators and promoter regions.

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environment, which may be an important process in recycling Pi in the rhizosphere.

The putative Ntr regulon elucidated by our proteomics dataset has much in common with previous transcriptomics datasets retrieved from *P. putida* KT2440 (Hervás et al., 2008) and *P. aeruginosa* PA14 (Alford et al., 2020). Both strains possess homologues of aepP and phnWX, neither of which was differentially transcribed in either strain (Hervás et al., 2008; Alford et al., 2020). In agreement with Hervás et al. (2009) we found that the AmtB ammonium channel and the sole pseudomonad PII protein GlnK, which are transcribed from a single operon, were significantly downregulated in the ntrBC mutant under low N conditions. However, it was also significantly downregulated under high N conditions in the ntrBC mutant. To our knowledge, this is the first time this has been demonstrated. Indeed, examination of the raw dataset showed neither AmtB nor GlnK was detected in the ntrBC mutant under either high or low N conditions. This is in keeping with the suggestion by Hervás et al. that glnK and amtB expression may be sensitive to low concentrations of phosphorylated NtrC, and high levels of GlnK, induced by NtrBC, serve to quickly deactivate Ntr regulon activity if sufficient N is obtained (Hervás et al., 2009). The absence of transaminase (PhnW) and phosphonatase (PhnX) from this dataset is interesting given that NtrBC is required for proper growth on 2AEP under N limited conditions. By conducting β-galactosidase assays we discovered that the presence of 2AEP is required, and that global omics approaches can miss regulon members which require substrate induction. Differences in β-galactosidase expression in the ΔntrBC and ΔcbrAB mutants compared to wild-type are small compared to what would be expected given the differences in growth in these strains, suggesting a discrepancy between protein activity and gene transcription. The mechanism behind this discrepancy is unclear. However, the involvement of post-transcriptional mechanisms of regulation, such as catabolite repression (Moreno et al., 2012; Moreno et al., 2015), cannot be ruled out, especially given the interaction between CbrAB and catabolite repression (García-Maurín et al., 2013). Equally, it remains to be established whether RNA chaperones such as Crc/Hfq (Moreno et al., 2015) interact with mRNAs in the BIRD-1 phosphonate operons.

In summary, we demonstrate that BIRD-1 requires the two-component master regulators of nutrient stress PhoBR, NtrBC and CbrAB to optimally use 2AEP as the sole P, N, or C source respectively. However, the presence of 2AEP, as well as an as-yet-unidentified metabolite, is required for complete expression of the phosphonate operons and maximal growth. Through mutagenesis of AepR<sup>WXX</sup>, we also identified a role for the LysR-like regulators found adjacent to aminophosphonate operons in bacteria, driving substrate induction at their adjacent operons. Thus, BIRD-1 employs a twofold regulatory strategy for 2AEP transport and catabolism comprising nutrient stress responses via PhoBR, NtrBC, or CbrAB, as well as substrate induction via AepR.

**Experimental procedures**

**Bacterial strains and growth conditions**

*P. putida* BIRD-1 was maintained on Luria–Bertani (LB) agar plates (1.5% wt./vol.) at 30°C. Mutants were also maintained on LB agar plates but with the addition of the appropriate antibiotic (see below). For all growth curves and proteomics experiments, *P. putida* BIRD-1 strains were grown in modified minimal media A (Lidbury et al., 2016) using 20 mM sodium succinate as the sole C source or 1.5 mM KH₂PO₄ as the sole P source, where applicable. Under high N conditions, 5.6 mM NH₄Cl was used as the sole nitrogen source, whilst under low N conditions 1.5 mM NH₄Cl was used. 2AEP was added at a final concentration of 1.5 mM when used as sole N or sole P source, and at 7.5 mM when used as the sole C source. Culture experiments were either performed in a FLUOstar Omega 96-well plate reader using Sarstedt 96-well plates, incubated at 30°C with 200 rpm shaking, or in 100 ml flasks incubated at 30°C with 180 rpm shaking.

**Generation of mutants and lacZ expression strains**

Mutants were generated according to the protocol detailed in Lidbury et al. (2016) and Murphy et al. (2021). Briefly, regions of genomic DNA corresponding to the ends of the genes targeted for knockout were cloned into pk18mobsacB together with a gentamicin resistance cassette using the HiFi DNA assembly kit (New England Biolabs). *Escherichia coli* S17.1 λ pir cells were transformed by electroporation and used to mobilize plasmids by conjugation (18 h at 30°C). Transconjugants were selected with gentamicin (50 μg ml⁻¹), using chloramphenicol (10 μg ml⁻¹) as counter selection. Single crossovers were confirmed by PCR and double crossovers were selected by plating on LB with gentamicin and 10% wt./vol. sucrose, with the exception of the ΔcbrAB mutants where minimal media A plates with gentamicin and 10% wt./vol. sucrose were used. Homologous recombinants were confirmed using PCR and Sanger sequencing. Promoter fusion plasmids were created using pBio1878 (Todd et al., 2012). Briefly, promoter regions (250 bp prior to, but not including, the translation start site) were
amplified using PCR primers containing restriction sites, subcloned into pGEM-T easy vectors (Promega), purified by restriction digest and gel purification, and ligated into restriction-digested pBIO1878 using T4 DNA ligase (Promega). Again, plasmids were electroporated into E. coli S17.1 λ pir, and mobilized into P. putida strains via conjugation. Transconjugants were selected on LB containing spectinomycin (50 μg ml⁻¹) and tetracycline (20 μg ml⁻¹), using chloramphenicol (10 μg ml⁻¹) as counter selection, prior to PCR confirmation. A full list of primers and plasmids used in this study is presented in Supplementary Table 1.

Proteomics sample preparation and experimental analysis

To identify differentially synthesized proteins in wild-type versus ΔntrBC strains under high and low N conditions (n = 3), total protein was collected by pelleting cells after overnight growth. Prior to this, to account for differences in growth yield between high and low N conditions, OD(600) readings were taken and volumes were adjusted so approximately equal cells were sampled for each condition. Cells were re-suspended in 1 ml Tris–HCl (20 mM, pH 7.5) and disrupted by sonication. Protein concentrations were determined using a Bradford assay and equivalent quantities (~20 μg) of each sample were loaded into lithium dodecyl sulfate buffer (Abcam) onto a 4%–20% Bis-Tris sodium dodecyl sulfate (SDS) precast gel (Abcam) for a short (~1 cm) run. Following Coomassie Blue staining to ensure protein had been loaded correctly, gel slices were destained using 50% wt./vol. ethanol in 50 mM ammonium bicarbonate, dehydrated with 100% ethanol, reduced and alkylated with Tris-2-carboxyethylphosphine and iodoacetamide, washed again with 50% wt./vol. ethanol in 50 mM ammonium bicarbonate, dehydrated in 100% ethanol and digested overnight with trypsin. Peptides were extracted using 25% acetonitrile 5% formic acid, vacuum dried and re-suspended in 2.5% acetonitrile 0.05% trifluoroacetic acid and analysed using an Orbitrap Fusion Ultimate 3000 RSLCNano system (Thermo Scientific) in electrospray ionization mode at the University of Warwick Proteomics Research Technology Platform.

Tandem mass spectrometry (MS/MS) files were searched against the P. putida protein sequence database (NC_017530.1) using MaxQuant (Tyanova et al., 2016a) with default settings. Label-free quantification (LFQ) was used for quantification. As done previously (Murphy et al., 2021) Perseus 1.6.15.0 (Tyanova et al., 2016b) was used to analyse the data and identify differentially synthesized proteins using Log₂ LFQ values and t-tests using FDR adjusted p-values. Proteins absent from more than two replicates in any condition were discarded, whilst remaining missing values were replaced with inputs from a normal distribution using default parameters. This dataset is provided as a supplementary.

β-Galactosidase assays

Assays were performed as described by Miller (1972). Briefly, cultures were incubated with replete nutrients overnight, prior to centrifugation and re-suspension in fresh media (performed twice). Once the requisite nutrients for each condition were added, cultures were then incubated at 30°C for 5 h with shaking at 180 rpm, at which point the OD(600) was measured. Cells were then twice centrifuged and re-suspended in Z buffer (8.54 g L⁻¹ Na₂HPO₄, 5.5 g L⁻¹ NaH₂PO₄·H₂O, 0.75 g L⁻¹ KCl, 0.25 g L⁻¹ MgSO₄·7H₂O, pH 7.0). Two drops of chloroform and one drop of 0.1% wt./vol. SDS were added to 1 ml sample, and samples vortexed briefly to permeabilize the cells. Samples were briefly pre-incubated at 30°C and 200 μl of 4 g L⁻¹ 2-Nitrophenyl-β-D-galactopyranoside was added. Samples were incubated until colour became apparent, with time recorded. Reactions were stopped with 500 μl 1 M Na₂CO₃, and samples were incubated at 30°C for 5 min, before centrifugation to remove cell debris. OD(420) was measured and β-galactosidase activity was calculated as Miller units according to the formula activity = (OD(420) × 1000)/(OD(600) × time × volume).

Acknowledgements

We thank Dr Andrew Bottrill and Dr Cleidiane Zamprionio of the Warwick Proteomics Research Technology Platform for their assistance in generating the mass-spectrometry dataset. This study was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) under project codes BB/L026074/1 and BB/T009152/1 linked to The Soil and Rhizosphere Interactions for Sustainable Agri-ecosystems (SARISA) programme and a Discovery Fellowship (IL), respectively, and the European Research Council (ERC) under the European Union’s Horizon 2020 Research and Innovation program (Grant agreement no. 726116).

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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:

**Appendix S1**: Supporting Information.