The 2-methylcitrate cycle and the glyoxylate shunt in *Pseudomonas aeruginosa* are linked through enzymatic redundancy.

Andre J. Wijaya[1#], Stephen K. Dolan[1,2#*], Michael Kohlstedt[3], Lars Gläser[3], Paul Brear[1], Stephen Geddis[4], Christoph Wittmann[3], David R. Spring[4], and Martin Welch[1*]

[1] Department of Biochemistry, University of Cambridge, United Kingdom

[2]Department of Genetics and Biochemistry, Eukaryotic Pathogens Innovation Center, Clemson University, Clemson, USA

[3]Institute of Systems Biotechnology, Saarland University, Saarbrücken, Germany

[4]Department of Chemistry, University of Cambridge, United Kingdom

#Contributed equally

*Authors to whom correspondence may be addressed.

Martin Welch: mw240@cam.ac.uk

Stephen Dolan: skdolan@clemson.edu

Running title: *P. aeruginosa* AceA has 2-methyl*iso*citrate lyase activity

Significance: Here we demonstrate that the glyoxylate shunt enzyme, *iso*citrate lyase, also has 2-methyl*iso*citrate lyase activity, which has implications for the development of antibacterial agents that target propionate catabolism.

Keywords: *Pseudomonas aeruginosa*; enzyme promiscuity; glyoxylate shunt; 2-methylcitrate cycle; 2-methyl*iso*citrate lyase; *iso*citrate lyase.

ABSTRACT

The 2-methylcitrate cycle (2-MCC) and the glyoxylate cycle are central metabolic pathways in *Pseudomonas aeruginosa*, enabling the organism to utilize organic acids such as propionate and acetate during infection. Here, we show that these cycles are linked through enzymatic redundancy, with *iso*citrate lyase (AceA) exhibiting secondary 2-methyl*iso*citrate lyase (2-MICL) activity. Furthermore, we use a combination of structural analyses, enzyme kinetics, metabolomics, and targeted mutation of $PrpB_{Pa}$ to demonstrate that whereas loss of PrpB function impairs growth on propionate, the promiscuous 2-MICL activity of AceA compensates for this by mitigating the accumulation of toxic 2-MCC intermediates. Our findings suggest that simultaneous inhibition of PrpB and AceA could present a robust antimicrobial strategy to target *P. aeruginosa* in propionate-rich environments, such as the cystic fibrosis airways. Our results emphasize the importance of understanding pathway interconnections in the development of novel antimicrobial agents.

INTRODUCTION

Short chain fatty acids such as propionate are abundant in certain infection scenarios, and readily accumulate to mM concentrations (1). The metabolism of short chain fatty acids is becoming increasingly topical, especially given their potent immune-modulatory activity (2). The 2-methylcitrate cycle (2-MCC) is one of the main metabolic pathways used by bacteria to metabolize propionate. The 2-MCC can also be regarded as a propionate detoxification pathway (3–5). The enzyme 2-methyl*iso*citrate lyase (2-MICL, PrpB) catalyzes the last step of the 2-MCC. Its function is to cleave the toxic intermediate, 2-methyl*iso*citrate (2-MIC), into pyruvate and succinate, enabling these metabolic precursors to feed directly into central metabolism (**Figure 1A**). This step has been suggested as a potential point of antimicrobial intervention for several microorganisms (6–8).

The prominent Gram-negative human pathogen, *Pseudomonas aeruginosa* utilizes organic acids such as propionate as a carbon source during infection (9). We previously showed that the methylcitrate synthase, PrpC, which catalyzes the first step of the 2-MCC, is a possible target for antimicrobial development. However, PrpC has structural features which are shared with citrate synthase from other bacterial and mammalian systems, potentially making targeting more complex (10). In the current work, we characterize the X-ray crystal structure of the final enzyme in the 2-MCC, PrpB (2-methyl*iso*citrate lyase) from *P. aeruginosa* (hereafter, $PrpB_{Pa}$). We further use a combination of enzyme kinetic analyses, reverse genetics, and metabolomics to demonstrate how the organism responds when the 2-methyl*iso*citrate lyase activity is lost (e.g., due to mutation of $prpB_{Pa}$, or due to chemical inhibition of the enzyme). Finally, we show that a $prpB_{Pa}$ deletion mutant is partially protected from the toxic impact of 2-MIC accumulation through the secondary activity of *aceA*-encoded

*iso*citrate lyase (ICL), which conventionally functions in the glyoxylate cycle (**Figure 1A**). This new knowledge into the structural and biochemical properties of $PrpB_{Pa}$ provides valuable insights into how best to target this pathway and block growth of *P. aeruginosa* during infection.

RESULTS

Catalytic properties and regulation of *P. aeruginosa* PrpB. To directly assess the enzymatic activity of $PrpB_{Pa}$, we purified this enzyme to investigate its specificity and kinetic properties *in vitro*. The *prpB*$_{Pa}$ gene was cloned and overexpressed (with a cleavable His_6 tag) in *Escherichia coli* and purified to homogeneity. The purified enzyme was then assayed for *threo*-2-methyl*iso*citrate lyase (2-MICL) activity. Using a lactate dehydrogenase (LDH) coupled assay (11) we determined the kinetic parameters of $PrpB_{Pa}$ for 2-MIC. Here, the pyruvate generated as a result of $PrpB_{Pa}$ activity is converted to lactate by the LDH, with concomitant oxidation of NADH. However, we noted kinetics consistent with substrate inhibition, which we reasoned was due to the LDH coupling reaction since LDH activity is known to be vulnerable to this (12). Hence, the kinetic parameters were calculated assuming substrate inhibition, using GraphPad Prism 6. We found that DTT was essential for optimal activity of $PrpB_{Pa}$ *in vitro*; 10 min pre-incubation with DTT was sufficient to achieve 95% activation (assuming 100% activity following overnight incubation with the thiol, **Figure 1B**). Calculated kinetic parameters for $PrpB_{Pa}$ with for 2-methyl*iso*citrate as a substrate were K_M = 632 ± 158 μM, k_{cat} = 48.2 ± 4.2 s^{-1}, and k_{cat}/K_M = 7.6 x 10^4 M^{-1} s^{-1}. The PrpB enzymes from *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Aspergillus nidulans* have uniformly lower K_M values (19 μM, 19 μM, and 31 μM, respectively) than $PrpB_{Pa}$, but variable k_{cat} values (12 s^{-1}, 74 s^{-1}, and 198 s^{-1},

respectively). We do not know the reason for these disparities, since in most other respects (activation by thiols, structural similarity, behaviour in solution etc), $PrpB_{Pa}$ appears very similar to PrpB from these other species.

We next examined whether $PrpB_{Pa}$ might be inhibited by common central metabolic intermediates or other low molecular weight compounds. This is relevant because the analogous glyoxylate shunt enzyme (ICL_{Pa}) is subject to robust allosteric regulation (13). To do this, we screened the activity of $PrpB_{Pa}$ against a panel of metabolites (**Figure S1**). [Note that, due to their interaction with LDH, a number of potential regulators could not be tested using this assay, including NADH, NADPH, phospho*enol*pyruvate, pyruvate, ATP, and ADP]. Of the potential physiological regulators that we tested, α-*keto*glutarate, *cis*-aconitic acid, and D-glyceraldehyde 3-phosphate were all potent inhibitors of $PrpB_{Pa}$ (**Figure S1**). Maleic acid was a non-physiological inhibitor. We also found, in agreement with the literature, that 3-nitropropionic acid was also a potent inhibitor of $PrpB_{Pa}$ ($P < 1x10^{-4}$) (14).

The 2-MCC methylcitrate synthase (PrpC) from *P. aeruginosa* catalyzes the condensation of oxaloacetate and propionyl-CoA. In a parallel reaction, the TCA cycle enzyme, citrate synthase (GltA), catalyzes the condensation of oxaloacetate and acetyl-CoA. We recently showed that $PrpC_{Pa}$ also possesses robust secondary citrate synthase activity (10). Since methyl*iso*citrate lyase and *iso*citrate lyase both catalyze biochemically similar steps in the 2-methylcitrate and glyoxylate cycles, respectively, this made us wonder whether $PrpB_{Pa}$ might also catalyze the ICL reaction and cleave *iso*citrate to yield succinate and glyoxylate. However, even at the highest concentration of *iso*citrate (500 μM) and $PrpB_{Pa}$ (6.2 μM) tested, we could not detect any glyoxylate formation arising from the cleavage of *iso*citrate (**Figure 1C**), even following incubation of the reaction mixtures overnight (*data not shown*). We conclude that $PrpB_{Pa}$ does

not possess ICL activity. We note that PrpB from *E. coli* and *Aspergillus fumigatus* also shows similar specificity for methyl*iso*citrate over *iso*citrate (11, 15).

Structure of $PrpB_{Pa}$. To gain insights into the possible structural bases for these kinetic data, we used X-ray crystallography to solve the structure of $PrpB_{Pa}$ in both the apo-form, and in the presence of bound Mg^{2+}/pyruvate. The pyruvate was introduced by crystal soaking in the presence of an equimolar (30 mM each) solution of pyruvate and succinate. Mg^{2+} was not included in the soak, or in the crystallization buffer, so was presumably acquired as a contaminant in these buffers, or was present in the purified enzyme preparations. The crystal structure of apo-$PrpB_{Pa}$ was solved by molecular replacement (MR) method using the structure of *E. coli* PrpB as the search template (PDB ID: 1MUM). For the Mg^{2+}/pyruvate bound $PrpB_{Pa}$, apo- $PrpB_{Pa}$ was used as the MR template. The structures were solved to 1.8 Å for apo-$PrpB_{Pa}$ and 1.76 Å for holo- $PrpB_{Pa}$.

In the asymmetric unit, like all the characterized PrpB and ICL structures to date, $PrpB_{Pa}$ formed a tetrameric (dimer of dimers) quaternary structure (13, 16, 17) (**Figure 2A**). This also agrees with our analytical ultra-centrifugation (AUC) data which suggest a tetrameric assembly of $PrpB_{Pa}$ in solution (**Figure S2**). All four protomers in each of the apo- and holo-structures had an almost identical conformation (RMSD < 0.18 Å). Additionally, no significant changes were seen in protomer conformation when comparing the apo- and holo- $PrpB_{Pa}$ structures (RMSD 0.19 Å). In both structures, the electron density for most of the amino acid residues was clearly resolved. However, electron density for the first and last 7 amino acid residues at the C and N termini, and 11 residues around the active site loop (residues 121-131, circled in **Figure 2B**) were missing. Even with the product and cofactor bound (Mg^{2+}/pyruvate), the active site loop of $PrpB_{Pa}$ remained unresolved, suggesting that this loop

is intrinsically flexible in these conditions. This was also reported for the *Salmonella enterica* serovar Typhimurium PrpB structure (18). The overall model stereochemistry in the Ramachandran plot (19) showed that 99.6% of the amino acids were within the preferred/allowed regions.

$PrpB_{Pa}$ belongs to the pyruvate/phospho*enol*pyruvate (PEP) kinase-like superfamily (IPR015813). All members of this family, except the *keto*pantoate hydroxymethyl transferase, contain a central TIM-barrel fold with an extensive dimer interface involving helix swapping. **Figure 2A** highlights an example of this helix swapping involving α11 on protomers A and B in the $PrpB_{Pa}$ structure.

Several structures of PrpB homologs in both apo- and holo- form from *E. coli*, *S. enterica* serovar Typhimurium, and *Burkholderia pseudomallei* have been solved PDB (*E. coli* PDB ID: 1MUM, 1OQF, 1XG3, 1XG4; *S. enterica* PDB ID: 1O5Q, 1UJQ; *B. pseudomallei* PDB ID: 3EOO). The amino acid sequences and structures of all these PrpB homologs are similar to that of $PrpB_{Pa}$ (sequence identity > 65%, Cα RMSD < 1.5 Å). Unsurprisingly, the catalytic site residues are conserved; the detailed catalytic mechanism of PrpB has been proposed previously by Liu *et al*. (17). Although not resolved in the structure(s) here, $PrpB_{Pa}$ contains the canonical PrpB motif (KRCGH, residues 123-128, highlighted in **Figure 2B**) (20). Multiple conformations of this catalytic loop region have been reported previously (16, 17). We note that this loop region is slightly different from the analogous one in ICL enzymes, which contain a conserved KKCGH motif (13, 18).

The active site of the ICL superfamily (which includes PrpB) contains an essential Mg^{2+}, which is crucial for enzymatic activity. In our holo-$PrpB_{Pa}$ structure, the Mg^{2+} and pyruvate could be modelled with confidence (**Figure 2C**). Mg^{2+} was bound in a negatively charged pocket

159 comprised by the side chains of D60, D87, D89 and E117. The Mg^{2+} was hexahedrally-
160 coordinated by three water molecules, two oxygen atoms from the pyruvate, and by the side
161 chain of D87 (**Figure 2C**). The binding of Mg^{2+} in $PrpB_{Pa}$ did not change the Cα geometry of
162 D87. This contrasts with the situation in *E. coli* PrpB, where Mg^{2+} binding has been reported
163 to shift the ϕ and ψ angle of D87 into an unfavourable region of the Ramachandran plot (19).

164 **Structural comparison of *P. aeruginosa* PrpB and ICL**. *P. aeruginosa* ICL is encoded by *aceA*.
165 We previously solved the X-ray crystal structure of $AceA_{Pa}$ (13). This allowed us to compare
166 the tertiary structures of $PrpB_{Pa}$ and $AceA_{Pa}$. The superimposed structures are shown in **Figure**
167 **3A**. Despite the relatively low amino acid sequence identity (36%) between $PrpB_{Pa}$ and $AceA_{Pa}$,
168 their core structural folds were very similar. However, $AceA_{Pa}$ is significantly larger (58.9 kDa)
169 than $PrpB_{Pa}$ (32.1 kDa), and $AceA_{Pa}$ contains an additional β-loop-β motif and an α-helical
170 bundle that are absent from the structure of $PrpB_{Pa}$.

171 The inner core αβ TIM barrel of $PrpB_{Pa}$ and $AceA_{Pa}$ contains the binding site for pyruvate and
172 glyoxylate (respectively), and some of the residues that interact with these reaction products
173 are conserved between these proteins (Y45, R160; $PrpB_{Pa}$ numbering), as well as in AceA from
174 other organisms such as *Mycobacterium tuberculosis* (**Figure 3B**). Interestingly, the position
175 occupied by Mg^{2+} in $PrpB_{Pa}$ is occupied by a Ca^{2+} ion in $AceA_{Pa}$. Additionally, we note that the
176 binding mode of pyruvate and glyoxylate in $PrpB_{Pa}$ and in *M. tuberculosis* AceA ($AceA_{Mtb}$),
177 respectively, is identical, whereas in $AceA_{Pa}$, the glyoxylate is rotated by almost 90° and
178 translated by 4.3 Å (**Figure 3B**). In the $PrpB_{Pa}$ structure, the pyruvate interacts with the enzyme
179 *via* the hydroxyl oxygen atoms of Y45 and S47, the peptide bond nitrogen from G48 and G49,
180 and a guanidino group nitrogen from R160. By contrast, in $AceA_{Pa}$, the glyoxylate interacts
181 with the enzyme *via* the carboxylate side chains of D117, D186, and E213, and *via* the hydroxyl

oxygen atom of S217 (PDB: 6G1O). The binding mode of pyruvate and glyoxylate in $PrpB_{Pa}$ and $AceA_{Pa}$, respectively, is therefore distinct.

A possible dual function for $AceA_{Pa}$. We have previously noted that the enzymes of the glyoxylate shunt (*iso*citrate lyase and malate synthase (GlcB)) are both highly-expressed during growth of *P. aeruginosa* on propionate as a sole carbon source (10). This high-level expression of $AceA_{Pa}$ and $GlcB_{Pa}$ is likely due to RccR-mediated de-repression of the glyoxylate cycle genes (21). Nevertheless, and in spite of the robust expression of $AceA_{Pa}$ and $GlcB_{Pa}$, very little carbon was fluxed through the glyoxylate shunt during growth on propionate (10). Although $PrpB_{Pa}$ lacks *iso*citrate lyase activity (**Figure 1**), this observation raised the possibility that $AceA_{Pa}$ might also play a role in the 2-MCC. We therefore decided to evaluate whether $AceA_{Pa}$ could catalyse the cleavage of 2-MIC (the reaction normally carried out by $PrpB_{Pa}$). ICL from other bacterial and fungal sources have been previously shown to exhibit some 2-MICL activity, although to our knowledge, the functional significance (if any) of this potential pathway crosstalk has not been investigated before. Although its activity was lower than that of $PrpB_{Pa}$, purified $AceA_{Pa}$ demonstrated robust 2-methyl*iso*citrate lyase (2-MICL) activity *in vitro* (**Figure 4A,B**). Using the LDH coupled reaction, the specificity constant (k_{cat}/K_M) of $AceA_{Pa}$ for 2-methyl*iso*citrate was calculated to be 3.4×10^3 M^{-1} s^{-1} (*cf*. 7.6×10^4 M^{-1} s^{-1} for $PrpB_{Pa}$). This clear secondary activity suggested that under some circumstances, $AceA_{Pa}$ may play a physiologically relevant role during *P. aeruginosa* propionate catabolism; a role that is normally masked by $PrpB_{Pa}$ functionality.

Growth of *prpB*-deficient *P. aeruginosa* is inhibited in the presence of propionate. To examine the interplay between $PrpB_{Pa}$ and $AceA_{Pa}$ in more detail, we first generated a *prpB* deletion mutant in PAO1 (a "wild type" *P. aeruginosa* strain used in most laboratories). The

205 Δ*prpB* mutant was unable to grow on propionate as a sole carbon source, but was able to
206 grow in a manner indistinguishable to the wild type progenitor on glucose, succinate, or
207 acetate as the sole carbon source (**Figure S3**). We next examined growth of the Δ*prpB* mutant
208 (and, as a control, the wild type progenitor (PAO1), a Δ*aceA* mutant, and a Δ*prpC* mutant) on
209 a rich medium (LB) in the presence of increasing concentrations of propionate (**Figure 4C**). To
210 our surprise, the Δ*prpB* was more tolerant of propionate compared with the Δ*prpC* mutant,
211 and grew visibly better than the latter, especially at intermediate concentrations of
212 propionate. This was surprising because we expected the Δ*prpB* mutant to accumulate 2-MIC,
213 which is toxic to the cell. However, the same argument applies to the Δ*prpC* mutant, which
214 would also be expected to accumulate toxic intermediates (principally, propionyl-CoA). We
215 therefore wondered whether the relative growth advantage of the Δ*prpB* mutant was simply
216 a reflection of the differential toxicity of the accumulated intermediates in each mutant, or
217 whether the 2-MICL activity of $AceA_{Pa}$ might help to mitigate toxicity in the Δ*prpB* background.
218 To test this, we made a Δ*prpB* Δ*aceA* double mutant.

219 **A Δ*prpB* Δ*aceA* double mutant is hyper-sensitive to propionate**. The Δ*prpB* Δ*aceA* double
220 mutant grew normally on succinate, glucose, and LB, but exhibited no growth on either
221 propionate or acetate as a sole carbon source (**Figure S3**). In addition, the Δ*prpB* Δ*aceA*
222 mutant exhibited an even greater growth defect than the Δ*prpC* mutant on LB-agar
223 supplemented with exogenous propionate. These data suggested that $AceA_{Pa}$ does indeed
224 help to rescue *P. aeruginosa* from intoxication by self-produced 2-MCC intermediates, and
225 further, that the intermediates which accumulate in the Δ*prpB* mutant are more toxic than
226 those which accumulate in the Δ*prpC* mutant. This “enhanced susceptibility” phenotype could

be complemented back to Δ*prpB* levels by expression of $aceA_{Pa}$ *in trans* in the Δ*prpB* Δ*aceA* double mutant.

To explore the 2-MCC metabolites responsible for the observed propionate-dependent toxicity in the mutants in more detail, we used LC-MS to quantify the 2-MCC pathway intermediates in the wild type progenitor (PAO1), the Δ*prpC* mutant, and the Δ*prpB* mutant following addition of propionate (5 mM) to cultures grown in MOPS-succinate medium (**Figure 4D**). After 3 h exposure to propionate, the Δ*prpB* mutant had accumulated approximately 10-fold more 2-MIC than either the wild type or the Δ*prpC* mutant. This propionate spiking experiment was also attempted for the Δ*prpB* Δ*aceA* double mutant. However, these cells ceased growth and died so rapidly following propionate exposure that cell pellets could not be obtained for intracellular metabolomics. Instead, we grew the wild type and the Δ*prpB* Δ*aceA* double mutant in MOPS succinate to an OD_{600} of 1.0, and then spiked the cultures for just 1 h with 5 mM propionate. The Δ*prpB* Δ*aceA* double mutant accumulated over 1000-fold more 2-MIC and 2-MC compared with the wild type (**Figure 4E**). The intracellular concentration of 2-MIC and 2-MC was comparable in the wild type in both the 1 h and 3 h samplings (**Figure 4D,E**). Together, these data suggest that growth of the Δ*prpB* Δ*aceA* double mutant is rapidly compromised following exposure to propionate, likely due to the accumulation of intracellular 2-MIC and 2-MC.

DISCUSSION

The 2-MCC is required for infection by many human pathogens and may thus be an important conditionally-essential target for antimicrobial development in *P. aeruginosa* (4, 5, 22–26). However, our understanding of precisely how propionate is metabolized by this organism

remains limited (27). Predicting how microbes evolve and adapt upon antimicrobial challenge is notoriously challenging, especially given that these processes are frequently species or even strain specific. Moreover, there is a pervading practice of extrapolating metabolic principles between organisms, in spite of the ever-growing body of evidence suggesting that individual species are often "wired up" very differently relative to the handful of model organisms to which they are often compared (28, 29).

Using a combination of biochemical, structural, and reverse genetics approaches, we have carried out a comprehensive examination of the final step in propionate assimilation by *P. aeruginosa* – catalyzed by the 2-methyl*iso*citrate lyase, PrpB. Our data show that $PrpB_{Pa}$ exhibits a strict preference for cleaving 2-MIC, whereas the analogous enzyme in the glyoxylate shunt, $AceA_{Pa}$, is able to catalyze the cleavage of both *iso*citrate and 2-MIC, albeit, the latter with somewhat lower catalytic efficiency.

The precise residues involved in the selectivity of $AceA_{Pa}$ and $PrpB_{Pa}$ for their respective substrates remains an open question. Simply changing the K**R**CGH motif of PrpB into the K**K**CGH motif present in ICLs did not lead to methyl*iso*citrate lyase activity in *E. coli* PrpB (11). A more comprehensive phylogenetic and structural approach hinted that the residues L521 and S523 in *A. fumigatus* PrpB may be critical for substrate selectivity. Mutation of these residues to their counterparts in *A. fumigatus* ICL (L521F, S523T and the L521F S523T double mutation) yielded proteins that retained methyl*iso*citrate lyase activity, but which also had detectable (albeit, low level) ICL activity (15). However, it is clear that simple substitutions of this type lead to only minor changes in specificity, and that a comprehensive mutagenesis campaign will be required to resolve the issue.

We found that the absence of the *prpB* prevents *P. aeruginosa* from growing on propionate as a sole carbon source. Thus, one would predict that specifically inhibiting $PrpB_{Pa}$ would also lead to rapid cessation of growth on propionate. However, we further show that a *P. aeruginosa* Δ*prpB* mutant is shielded from the toxic impact arising from accumulation of 2-MCC intermediates through substrate promiscuity of the *iso*citrate lyase, $AceA_{Pa}$. Surprisingly, in addition to 2-MIC accumulation, we also detected a significant increase in both 2*R*, 3*S*-2MC and 2*S*, 3*S*-2MC in the Δ*prpB* and the Δ*prpB* Δ*aceA* double mutant. These data suggest that the 2-MCC blockage in these mutants leads to a build-up of precursor metabolites beyond those directly upstream of the $PrpB_{Pa}$-catalyzed reaction. This may also contribute to the observed toxicity. The precise cellular targets of 2-MIC or 2-MC (if these are indeed the inhibitory agents) remain to be discovered for *P. aeruginosa*. In spite of extensive efforts, resistance to exogenous propionate exposure could not be selected for in the Δ*prpB* Δ*aceA* double mutant, suggesting that the toxic pathway intermediates likely act through multiple pathways, making the evolution of resistance extremely challenging.

Our data show that, through enzyme promiscuity, *P. aeruginosa* encodes a 'fail-safe mechanism' which prevents the excessive accumulation of pathway intermediates upon impairment of $PrpB_{Pa}$ function (**Figure 5**). It is unclear if this is by "evolutionary design" or is a fortuitous secondary activity. However, it is conceivable that *P. aeruginosa* has bolstered its defences around this particular 2-MCC reaction, especially considering the immediate consequences of blocking 2-methyl*iso*citrate cleavage. Taken together, our data suggest that it may be necessary to target both $PrpB_{Pa}$ and $AceA_{Pa}$ simultaneously if propionate-dependent toxicity is to be developed as an antimicrobial target at this step in the 2-MCC. For example, one obvious mechanism of bypassing $PrpB_{Pa}$ inhibition would be to constitutively activate

295 *aceA*$_{Pa}$ expression (e.g., through mutation of the gene encoding RccR) (21, 30). Consistent
296 with this, and with the known accumulation of propionate in the cystic fibrosis (CF) airways,
297 *rccR* loss of function mutations have been detected in *P. aeruginosa* CF isolates (9, 31).

298 The current work highlights the importance of dissecting pathway interconnections in
299 microbial metabolism, and that microbial metabolism is inherently highly flexible (38). The
300 combination of near-atomic resolution of the PrpB$_{Pa}$ structures described in this work,
301 alongside the existing structural data for *P. aeruginosa* AceA, provides a clear structural
302 template for targeting these key metabolic nodes.

303

304 **EXPERIMENTAL PROCEDURES**

305 **Growth conditions**. *P. aeruginosa* strain PAO1 was routinely grown in lysogeny broth (LB
306 Lennox) (Oxoid Ltd) at 37°C with shaking at 250 rpm. The strains used in this study are listed
307 in **Table S1**. Strains were cultured in MOPS (morpholinepropanesulfonic acid) media with the
308 relevant carbon sources, as indicated. Cell growth was monitored as optical density in a
309 spectrophotometer (BioSpectrometer®, Eppendorf) at a wavelength of 600 nm (OD_{600}).

310 **Cloning, overexpression and purification**. Primers used in this study is listed in **Table S2**.
311 PrpB$_{Pa}$ and AceA$_{Pa}$ (PA0796 and PA2634, respectively) were PCR-amplified from PAO1 gDNA
312 template and the amplicons were cloned into pET-19m for over-expression. The proteins
313 were over-expressed with a Tobacco Etch Virus (TEV) protease-cleavable N-terminal
314 hexahistidine-tag. Cultures of *E. coli* containing plasmid (pET-19M-*prpB* or pET-19M-*aceA*, as
315 appropriate) were grown at 37°C (with good aeration at 200 shaking) to an OD_{600} of 0.6.
316 Isopropyl-β-D-thiogalactoside (IPTG) was then added to a final concentration of 1 mM to

induce protein expression and the temperature was decreased to 16°C. After overnight incubation, the cells were harvested by centrifugation (3430 × *g*, 30 min, 4°C) and the cell pellets were stored in -80°C until use. The frozen cell pellets were thawed and resuspended in 20 mL of ice-cold lysis buffer (50 mM Tris HCl pH 7.5, 300 mM NaCl, 10 mM imidazole and 5% v/v glycerol). The cells were lysed to completion by sonication on ice. Cell debris was removed by centrifugation (15000 × *g*, 30 min, 4°C) and the supernatant was filtered through a 0.45 μm membrane (Satorius). The filtered sample was loaded onto a 5 mL Ni-NTA Superflow Cartridge (Qiagen) and $PrpB_{Pa}$ and $AceA_{Pa}$ were purified as previously described (10). The purified samples were dialysed against 2 x 1 L of storage buffer (50 mM Tris HCl pH 7.5, 100 mM NaCl, and 5% v/v glycerol) for 16 h at 4°C. Hexahistidine tagged TEV protease was added (1:100 ratio of protease:sample) in the dialysis step. The TEV protease and cleaved His_6 tags were removed by Ni-NTA (Qiagen) chromatography. The purified tag-free proteins were then concentrated by ultrafiltration, flash frozen in liquid nitrogen, and stored at -80°C.

Enzyme assays. The 2-methyl*iso*citrate lyase (2-MICL) activity was measured as follows. Each reaction mixture contained 50 mM HEPES pH 7.5, 2.5 mM $MgCl_2$, 5 mM DTT, 1 unit of rabbit muscle LDH (Sigma), 250 μM NADH and the indicated concentration of *threo*-2-methyl*iso*citrate (2-MIC). The *threo*-2-methyl*iso*citrate was synthesized in-house and was confirmed by [^{1}H] NMR to be a 99% pure racemic mixture of (*2R*, *3S*)- and (*2S*, *3R*)-2-methyl*iso*citrate. Before the reaction was initiated, the substrate and buffer were pre-incubated at 37°C for 5 min. Reactions were initiated by the addition of $PrpB_{Pa}$ (final concentration of 311 nM unless otherwise stated) or $AceA_{Pa}$ (concentration as indicated) and the A_{340} was monitored using a BioSpectrometer® (Eppendorf) for 1 min at 37°C. Reaction rates were calculated assuming an NADH extinction coefficient of 6200 M^{-1} cm^{-1}.

For the inhibitor screening, metabolites were tested at 1 mM final concentration. $PrpB_{Pa}$ was pre-incubated with each putative regulator for 5 min before the reactions were initiated. Relative activity was measured by comparing the reaction with and without the addition of the putative regulator.

The *iso*citrate lyase (ICL) assay of $PrpB_{Pa}$ and $AceA_{Pa}$ was measured using a phenylhydrazine-based assay. The reaction mixture contained 25 mM imidazole pH 7.0, 10 mM EDTA, 5 mM $MgCl_2$, 4 mM phenylhydrazine and 4 mM DL *iso*citric acid. Reactions were initiated by the addition of $AceA_{Pa}$ or $PrpB_{Pa}$ at the indicated final concentration, and A_{324} was monitored using BioSpectrometer® (Eppendorf) for 1 min.

Protein crystallization. Crystallization conditions were screened using the sitting drop vapor diffusion technique using a stock solution of protein containing ca. 13-15 mg mL^{-1} purified $PrpB_{Pa}$. Protein drops were generated using an automated nanolitre liquid handler mosquito® HTS (TTP LabTech). Optimization conditions were determined using dragonfly® discovery system (TTP LabTech). $PrpB_{Pa}$ crystals were obtained in a 1:1 ratio of protein and reservoir solution (100-200 mM Tris-HCl pH 8.5, 20-30 % (w/v) PEG 4000, 100-250 mM $LiSO_4$ and 5 mM DTT). Crystals were grown for 9-15 days at 19°C. Crystals were cryoprotected with 25% (v/v) glycerol and 75% (v/v) reservoir solution, mounted in nylon loops (Hampton Research), and flash frozen in liquid nitrogen prior to data collection. For soaking, a solution of pyruvate and succinate (30 mM of both) was added to the crystal-containing drops and left for 3 h prior to mounting.

X-ray diffraction, structure determination and refinement. Diffraction data were collected remotely on beamline MX-I03 at the Diamond Light Source Synchrotron (DLS, Didcot, UK). The parameters for the data collection were as follows: wavelength 0.97629 Å, omega (Ω)

start: 0°, Ω oscillation: 0.15°, total oscillation: 180°, total images: 1200, exposure time: 0.050 s. Diffraction images were processed using Xia2 DIALS (32) The structure was determined by molecular replacement using Phaser (33) with the atomic coordinates of PrpB from *E. coli* (PDB entry: 1MUM) as the search model. Automated refinement was performed using Refmac5 and PHENIX.refine (34). Manual modelling and refinement were performed in COOT (35). Data collection and refinement statistics are listed in **Table S3**. Donor-acceptor distances for hydrogen bonds were in the 3.2–2.2 Å range. The PDB codes for the apo-PrpBPa and $PrpB_{Pa}/Mg^{2+}$/pyruvate structures are 6T4V and 6T5M, respectively.

Analytical ultracentrifugation. Analytical ultracentrifugation-sedimentation velocity (AUC-SV) was done in the Department of Biochemistry (University of Cambridge) Biophysics Facility. Samples were dialyzed overnight at 4°C against a buffer solution containing 100 mM NaCl and 50 mM Tris-HCl pH 7.5 to remove traces of glycerol. Data were collected using an An60Ti analytical rotor (Beckman Coulter) in a Beckman Optima XL-I ultracentrifuge with absorbance and interference optical detection systems. Protein solution (400 µL volume, concentration approximately 1 mg mL^{-1}) and the reference solution (protein-free dialysate) were added to the Epon (epoxy) double-sector centrepieces. All samples were sedimented at 40,000 rpm and 20°C. Absorbance data (A_{280}) were collected in intervals of 2 min and interference scans were taken every 1 min. The viscosity and density of the buffer used in the experiments were estimated using SEDNTERP. Data analysis was conducted using SEDFIT.

Construction of in-frame *P. aeruginosa* PAO1 deletion mutants. The flanking regions approximately 800 bp upstream and downstream of the desired genes were PCR-amplified (primers in **Table S2**). The upstream and downstream regions were then overlapped amplified by PCR. The fragments were then cloned into the suicide vector, pEX19Gm, using Gibson

assembly as described previously (10). The resulting deletion plasmid was introduced into *P. aeruginosa* PAO1 by electroporation and transconjugants were selected on LB plates containing 50 µg mL^{-1} gentamicin. Deletion mutants were identified following SacB-mediated sucrose counter-selection and confirmed by PCR.

LC-MS analysis of 2-MCC intermediates. The intracellular accumulation of (2*R*,3*S*)-2-methylcitrate, (2*S*, 3*S*)-2-methylcitrate – the two physiologically occurring diastereomers of methylcitrate – and 2-methyl*iso*citrate was measured in cell extracts of the indicated mutant strains using LC-MS as described previously (36, 37). Briefly, cells from 8 mL cultures grown to OD_{600} = 2 were pelleted and re-suspended in 200 µL "supercool" ultra-pure water (0°C) and 1 mL quenching-extraction buffer (95% acetonitrile, 25 mM formic acid, -20°C). The mixture was vortexed and kept on ice for 10 min, before being clarified by centrifugation at 0°C. The supernatants were transferred into 3 mL of ultra-pure water, before being snap-frozen in liquid nitrogen and lyophilized (Alpha 3-4 LSCbasic, Christ, Germany). The freeze-dried samples were diluted in 500 µL pre-cooled resuspension buffer (25 mM ammonium formate, pH 3.0, 2% methanol, 4°C) and immediately analysed by LC-MS (QTRAP 6500+ (AB Sciex, Darmstadt, Germany) coupled to an HPLC system (Agilent Infinity 1290)). Aliquots (5 µL volume) were separated at 25°C on a C18 column (VisionHT C18 HighLoad, 1.5 µm, 100x2mm) using 0.4% formic acid in ultrapure water as eluent A and a 1:1 mixture of acetonitrile and methanol as eluent B. Flow rate was set to 200 µL min^{-1} with a gradient of A as follows: at 0 min 95%, at 5 min 80%, at 5.5 min 95% and at 8 min 95%. Detection was performed in multiple reaction monitoring (MRM) mode with *m/z* of 204.8 (Q1) > 125.0 (Q3) for 2-MC and 2-MIC. MS voltages were optimized for the target compounds. Commercial standards were used for identification, tuning and quantification.

Data availability

Coordinates and X-ray crystallographic data for the apo-PrpBPa and $PrpB_{Pa}/Mg^{2+}$/pyruvate structures have been deposited in the PDB. The PDB codes are 6T4V and 6T5M, respectively.

Supporting Information

This article contains supporting information.

Acknowledgements

Joshua Western is thanked for technical assistance.

Author contributions

AW, SKD, MK, LG and SG carried out the experimental work. PB assisted with the crystallographic data analyses. MW, CW, SKD and DRS secured funding for the work. SKD and AJW drafted the manuscript, MW edited the draft. All authors contributed to the final draft.

Funding information

This work was funded by a grant (BB/M019411/1) from the BBSRC to MW. CW acknowledges support by the German Federal Ministry for Education and Research (BMBF) through the grants “Bio2Nylon” (FKZ 03V0757) and “LignoValue” (FKZ 031B0344A), as well as funding from the German Research Foundation (DFG) for the project “ePseudomonas” (project no. 445908911) as part of the priority program eBiotech (SPP 2240). SKD was supported by a Herchel Smith Postdoctoral Fellowship. Some elements of this work were supported by an EMBO Short Term Fellowship to SKD (7293-2017). Research in the DRS laboratory is supported by the Engineering and Physical Sciences Research Council, the Biotechnology and Biological Sciences Research Council and the Medical Research Council.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

REFERENCES

1. Ciarlo E, Heinonen T, Herderschee J, Fenwick C, Mombelli M, Le Roy D, Roger T. 2016. Impact of the microbial derived short chain fatty acid propionate on host susceptibility to bacterial and fungal infections in vivo. Sci Rep 6:37944.

2. Kim CH. 2023. Complex regulatory effects of gut microbial short-chain fatty acids on immune tolerance and autoimmunity. Cell Mol Immunol 20:341–350.

449 3. Rocco CJ, Escalante-Semerena JC. 2010. In Salmonella enterica, 2-methylcitrate blocks
450 gluconeogenesis. J Bacteriol 192:771–778.

451 4. Dolan SK, Wijaya A, Geddis SM, Spring DR, Silva-Rocha R, Welch M. 2018. Loving the
452 poison: the methylcitrate cycle and bacterial pathogenesis. Microbiology 164:251–259.

453 5. Muñoz-Elías EJ, Upton AM, Cherian J, McKinney JD. 2006. Role of the methylcitrate cycle
454 in Mycobacterium tuberculosis metabolism, intracellular growth, and virulence. Mol
455 Microbiol 60:1109–1122.

456 6. Quinonez CG, Lee JJ, Lim J, Odell M, Lawson CP, Anyogu A, Raheem S, Eoh H. 2022. The
457 Role of Fatty Acid Metabolism in Drug Tolerance of Mycobacterium tuberculosis. mBio
458 13:e0355921.

459 7. Brock M. 2005. Generation and phenotypic characterization of Aspergillus nidulans
460 methylisocitrate lyase deletion mutants: methylisocitrate inhibits growth and
461 conidiation. Appl Environ Microbiol 71:5465–5475.

462 8. Horswill AR, Dudding AR, Escalante-Semerena JC. 2001. Studies of propionate toxicity in
463 Salmonella enterica identify 2-methylcitrate as a potent inhibitor of cell growth. J Biol
464 Chem 276:19094–19101.

465 9. Flynn JM, Niccum D, Dunitz JM, Hunter RC. 2016. Evidence and Role for Bacterial Mucin
466 Degradation in Cystic Fibrosis Airway Disease. PLOS Pathogens 12:e1005846.

467 10. Dolan SK, Wijaya A, Kohlstedt M, Gläser L, Brear P, Silva-Rocha R, Wittmann C, Welch M.
468 2022. Systems-Wide Dissection of Organic Acid Assimilation in Pseudomonas aeruginosa
469 Reveals a Novel Path To Underground Metabolism. mBio 13:e02541-22.

11. Grimek TL, Holden H, Rayment I, Escalante-Semerena JC. 2003. Residues C123 and D58 of the 2-methylisocitrate lyase (PrpB) enzyme of Salmonella enterica are essential for catalysis. J Bacteriol 185:4837–4843.

12. Reed MC, Lieb A, Nijhout HF. 2010. The biological significance of substrate inhibition: a mechanism with diverse functions. Bioessays 32:422–429.

13. Crousilles A, Dolan SK, Brear P, Chirgadze DY, Welch M. 2018. Gluconeogenic precursor availability regulates flux through the glyoxylate shunt in Pseudomonas aeruginosa. Journal of Biological Chemistry 293:14260–14269.

14. Schloss JV, Cleland WW. 1982. Inhibition of isocitrate lyase by 3-nitropropionate, a reaction-intermediate analogue. Biochemistry 21:4420–4427.

15. Müller S, Fleck CB, Wilson D, Hummert C, Hube B, Brock M. 2011. Gene acquisition, duplication and metabolic specification: the evolution of fungal methylisocitrate lyases. Environ Microbiol 13:1534–1548.

16. Grimm C, Evers A, Brock M, Maerker C, Klebe G, Buckel W, Reuter K. 2003. Crystal structure of 2-methylisocitrate lyase (PrpB) from Escherichia coli and modelling of its ligand bound active centre. J Mol Biol 328:609–621.

17. Liu S, Lu Z, Han Y, Melamud E, Dunaway-Mariano D, Herzberg O. 2005. Crystal structures of 2-methylisocitrate lyase in complex with product and with isocitrate inhibitor provide insight into lyase substrate specificity, catalysis and evolution. Biochemistry 44:2949–2962.

490 18. Simanshu DK, Satheshkumar PS, Savithri HS, Murthy MRN. 2003. Crystal structure of
491 Salmonella typhimurium 2-methylisocitrate lyase (PrpB) and its complex with pyruvate
492 and Mg(2+). Biochem Biophys Res Commun 311:193–201.

493 19. Ramachandran GN, Ramakrishnan C, Sasisekharan V. 1963. Stereochemistry of
494 polypeptide chain configurations. J Mol Biol 7:95–99.

495 20. Brock M, Fischer R, Linder D, Buckel W. 2000. Methylcitrate synthase from Aspergillus
496 nidulans: implications for propionate as an antifungal agent. Mol Microbiol 35:961–973.

497 21. Dolan SK, Pereira G, Silva-Rocha R, Welch M. 2020. Transcriptional regulation of central
498 carbon metabolism in Pseudomonas aeruginosa. Microb Biotechnol 13:285–289.

499 22. Gould TA, van de Langemheen H, Muñoz-Elías EJ, McKinney JD, Sacchettini JC. 2006.
500 Dual role of isocitrate lyase 1 in the glyoxylate and methylcitrate cycles in
501 Mycobacterium tuberculosis. Mol Microbiol 61:940–947.

502 23. Feng J, He L, Xiao X, Chen Z, Chen C, Chu J, Lu S, Li X, Mylonakis E, Xi L. 2020.
503 Methylcitrate cycle gene MCD is essential for the virulence of Talaromyces marneffei.
504 Med Mycol 58:351–361.

505 24. Upton AM, McKinney JD. 2007. Role of the methylcitrate cycle in propionate
506 metabolism and detoxification in Mycobacterium smegmatis. Microbiology (Reading)
507 153:3973–3982.

508 25. Textor S, Wendisch VF, De Graaf AA, Müller U, Linder MI, Linder D, Buckel W. 1997.
509 Propionate oxidation in Escherichia coli: evidence for operation of a methylcitrate cycle
510 in bacteria. Arch Microbiol 168:428–436.

26. Limenitakis J, Oppenheim RD, Creek DJ, Foth BJ, Barrett MP, Soldati-Favre D. 2013. The 2-methylcitrate cycle is implicated in the detoxification of propionate in Toxoplasma gondii. Mol Microbiol 87:894–908.

27. Eoh H, Rhee KY. 2014. Methylcitrate cycle defines the bactericidal essentiality of isocitrate lyase for survival of Mycobacterium tuberculosis on fatty acids. Proc Natl Acad Sci U S A 111:4976–4981.

28. Zheng EJ, Andrews IW, Grote AT, Manson AL, Alcantar MA, Earl AM, Collins JJ. 2022. Modulating the evolutionary trajectory of tolerance using antibiotics with different metabolic dependencies. Nat Commun 13:2525.

29. Santos-Lopez A, Marshall CW, Scribner MR, Snyder DJ, Cooper VS. 2019. Evolutionary pathways to antibiotic resistance are dependent upon environmental structure and bacterial lifestyle. Elife 8:e47612.

30. Campilongo R, Fung RKY, Little RH, Grenga L, Trampari E, Pepe S, Chandra G, Stevenson CEM, Roncarati D, Malone JG. 2017. One ligand, two regulators and three binding sites: How KDPG controls primary carbon metabolism in Pseudomonas. PLoS Genet 13:e1006839.

31. Hwang W, Yong JH, Min KB, Lee K-M, Pascoe B, Sheppard SK, Yoon SS. 2021. Genome-wide association study of signature genetic alterations among pseudomonas aeruginosa cystic fibrosis isolates. PLoS Pathog 17:e1009681.

32. Winter G, Lobley CMC, Prince SM. 2013. Decision making in xia2. Acta Crystallogr D Biol Crystallogr 69:1260–1273.

532 33. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. 2007.
533 Phaser crystallographic software. J Appl Crystallogr 40:658–674.

534 34. Afonine PV, Grosse-Kunstleve RW, Echols N, Headd JJ, Moriarty NW, Mustyakimov M,
535 Terwilliger TC, Urzhumtsev A, Zwart PH, Adams PD. 2012. Towards automated
536 crystallographic structure refinement with phenix.refine. Acta Crystallogr D Biol
537 Crystallogr 68:352–367.

538 35. Emsley P, Cowtan K. 2004. Coot: model-building tools for molecular graphics. Acta
539 Crystallogr D Biol Crystallogr 60:2126–2132.

540 36. Monostori P, Klinke G, Richter S, Baráth Á, Fingerhut R, Baumgartner MR, Kölker S,
541 Hoffmann GF, Gramer G, Okun JG. 2017. Simultaneous determination of 3-
542 hydroxypropionic acid, methylmalonic acid and methylcitric acid in dried blood spots:
543 Second-tier LC-MS/MS assay for newborn screening of propionic acidemia,
544 methylmalonic acidemias and combined remethylation disorders. PLoS One
545 12:e0184897.

546 37. Assessment of methylcitrate and methylcitrate to citrate ratio in dried blood spots as
547 biomarkers for inborn errors of propionate metabolism | Scientific Reports.
548 https://www.nature.com/articles/s41598-019-48885-9.

549 38. Contextual Flexibility in Pseudomonas aeruginosa Central Carbon Metabolism during
550 Growth in Single Carbon Sources. Dolan SK, Kohlstedt M, Trigg S, Vallejo
551 Ramirez P, Kaminski CF, Wittmann C, Welch M. mBio 11:10.1128/mbio.02684-19.

552

FIGURE LEGENDS

Figure 1. Biochemical characterization of recombinant $PrpB_{Pa}$. **(A)** Schematic of the reactions catalyzed by $PrpB_{Pa}$ and AceA (ICL_{Pa}). [Note that, as shown later in this report, ICL_{Pa} also catalyzes the methyl*iso*citrate lyase reaction.] **(B)** Shows a plot of reaction velocity *vs* substrate (2-methyl*iso*citrate, 2-MIC) concentration in samples containing untreated $PrpB_{Pa}$ (black triangles) or $PrpB_{Pa}$ pre-treated with 5 mM DTT for 10 min (red circles). Blue squares show the kinetic behaviour of $PrpB_{Pa}$ that had been pre-treated overnight with DTT. (**C)** *Iso*citrate lyase activity of $PrpB_{Pa}$ and ICL_{Pa} (as indicated) measured using a phenylhydrazine-based assay. ICL_{Pa} (1 µg, 170 nM) could catalyse the cleavage of *iso*citrate into glyoxylate and succinate, whereas no detectable *iso*citrate lyase activity was associated with $PrpB_{Pa}$ at any

574 of the concentrations tested (ranging from 311 nM to 6.2 µM, as indicated). The data are
575 representative of two independent experiments, each performed in triplicate.

576

577 **Figure 2.** Crystal structure of $PrpB_{Pa}$. (A) Shows the homotetramer (dimer of dimers)
578 quaternary structure from $PrpB_{Pa}$. The protomers are coloured red (protomer A), blue
579 (protomer B), green (protomer C), and magenta (protomer D). Note the swapping of α11 from
580 protomers A and B. (B) Monomer of apo-$PrpB_{Pa}$ coloured rainbow (blue to red) from the N to
581 C terminal. The missing active site loop (residue 121-131) is circled in red and indicated with
582 red arrows. (C) Close-up view around the Mg^{2+}/pyruvate binding site. The Mg^{2+} (green sphere)
583 is hexahedrally-coordinated by three water molecules, two oxygen atoms from pyruvate, and
584 the carboxyl group of D87. The pyruvate (PYR, deep blue) and amino acid (cyan) residues are
585 shown as sticks. Water molecules (W) are shown as orange spheres. Polar interactions are
586 shown with a dashed line. The electron density map ($2F_o$-F_c) around the Mg^{2+} and pyruvate is
587 shown in white and is contoured at 1.5σ.

588

589 **Figure 3.** The tertiary structures of $PrpB_{Pa}$ and $AceA_{Pa}$ are conserved. (**A**) Superimposed
590 secondary structures of $PrpB_{Pa}$ (cyan) and $AceA_{Pa}$ (magenta, PDB 6G1O). Note that the
591 secondary structures of the proteins are very similar, even though the sequence identity is
592 relatively low (36%). (**B**) Pyruvate (Pyr)/glyoxylate (Glx) binding site of $PrpB_{Pa}$ (cyan), $AceA_{Pa}$
593 (magenta) and $AceA_{Mtb}$ (green, PDB no. 1F8I). Some of the active site residues are conserved
594 (Y45, R160; $PrpB_{Pa}$ numbering). In $PrpB_{Pa}$ and $AceA_{Mtb}$, the binding mode of pyruvate and
595 glyoxylate is identical, whereas in $AceA_{Pa}$ the departing product molecule is displaced.

596 Additionally, $PrpB_{Pa}$ and $AceA_{Mtb}$ contain bound Mg^{2+} (MG), whereas $AceA_{Pa}$ contains a bound
597 Ca^{2+} (CA).

598

599 **Figure 4.** The secondary 2-methyl*iso*citrate lyase (2-MICL) activity of AceA blunts 2-MIC
600 toxicity in *P. aeruginosa*. (A) Relative 2-MICL activity of the indicated concentrations of $PrpB_{Pa}$
601 and $AceA_{Pa}$ measured using the LDH-coupled reaction (1 mM 2-MIC). Full (100%) activity of
602 $PrpB_{Pa}$ is set at 100%. The data are representative of two independent experiments, each
603 performed in triplicate. (B) Reaction velocity *vs* substrate (2-methyl*iso*citrate, 2-MIC)
604 concentration. $AceA_{Pa}$ (blue symbols and line) and $PrpB_{Pa}$ were pre-incubated with 5 mM DTT
605 prior to assaying. The $PrpB_{Pa}$ data are the same as those shown in Figure 1A. The data are
606 representative of two independent experiments, each performed in triplicate. (C) Growth of
607 the wild-type (WT) and deletion mutants of *P. aeruginosa* on complex medium (LB-Lennox)
608 agar containing increasing amounts of propionate (0-20 mM, as indicated). The data are
609 representative of three independent experiments. (D) Accumulation of intracellular 2-MCC
610 intermediates in the wild-type (PAO1), a Δ*prpC* mutant and a Δ*prpB* mutant following 3 h
611 exposure to propionate (5 mM)**.** The measured intermediates were (2*R*,3*S*)-2-methylcitrate,
612 (2*S*, 3*S*)-2-methylcitrate, and 2-methylisocitrate. Note the log-scale on the y-axis. The data are
613 representative of biological triplicates. (E) Accumulation of intracellular 2-methylcitrate
614 intermediates in the wild-type (PAO1), and in a Δ*prpB* Δ*aceA* double mutant following 1 h
615 exposure to propionate (5 mM)**.** The data are representative of biological triplicates.

616

617 **Figure 5. Summary of the interplay between $PrpB_{Pa}$ and $AceA_{Pa}$ in the *P. aeruginosa* 2-MCC**
618 **and glyoxylate shunt.** During *P. aeruginosa* growth in the presence of propionate, the 2-MCC

619 is activated and catabolizes the organic acid. In the final step of the 2-MCC, the 2-
620 methyl*iso*citrate lyase, PrpB, cleaves 2-methyl*iso*citrate (2-MIC) to yield pyruvate (PYR) and
621 succinate (SUC). Inhibition of $PrpB_{Pa}$ results in the accumulation of 2-MCC intermediates and
622 subsequent moderate growth inhibition in the presence of propionate. However, the loss of
623 $PrpB_{Pa}$ activity can be partially compensated by the secondary 2-methyl*iso*citrate lyase activity
624 of the glyoxylate shunt enzyme, *iso*citrate lyase ($AceA_{Pa}$), which presumably lowers 2-MIC
625 (and other toxic intermediates) levels in the cell and maintains viability. $AceA_{Pa}$ is highly-
626 expressed during growth on propionate as a sole carbon source. Consequently, a dual-
627 purpose inhibitor, which simultaneously targets both PrpB and AceA could offer potential as
628 an antimicrobial agent that blocks *P. aeruginosa* growth in infection scenarios where short
629 chain fatty acids such as propionate are abundant.

630