Contextual flexibility in *Pseudomonas aeruginosa* central carbon metabolism during growth in single carbon sources

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Abstract

Pseudomonas aeruginosa is an opportunistic human pathogen, particularly noted for causing infections in the lungs of people with cystic fibrosis (CF). Previous studies have shown that the gene expression profile of *P. aeruginosa* appears to converge towards a common metabolic program as the organism adapts to the CF airway environment. However, at a systems level, we still have only a limited understanding of how these transcriptional changes impact on metabolic flux. To address this, we analysed the transcriptome, proteome and fluxome of *P. aeruginosa* grown on glycerol or acetate. These carbon sources were chosen because they are the primary breakdown products of airway surfactant, phosphatidylcholine, which is known to be a major carbon source for *P. aeruginosa* in the CF airways. We show that the flux of carbon through central metabolism is radically different on each carbon source. For example, the newly-recognised EDMP cycle plays an important role in supplying NADPH during growth on glycerol. By contrast, the EDMP cycle is attenuated during growth on acetate, and instead, NADPH is primarily supplied by the isocitrate dehydrogenase(s)-catalyzed reaction. Perhaps more importantly, our proteomic and transcriptomic analyses reveal a global remodelling of gene expression during growth on the different carbon sources, with unanticipated impacts on aerobic denitrification, electron transport chain architecture, and the redox economy of the cell. Collectively, these data highlight the remarkable metabolic plasticity of *P. aeruginosa*; a plasticity which allows the organism to seamlessly segue between different carbon sources, maximising the energetic yield from each.

Importance

Pseudomonas aeruginosa is an opportunistic human pathogen, well-known for causing infections in the airways of people with cystic fibrosis. Although it is clear that *P. aeruginosa* is metabolically well-adapted to life in the CF lung, little is currently known about how the organism metabolises the nutrients available in the airways. In this work, we use a combination of gene expression and isotope tracer (“fluxomic”) analyses to find out exactly where the input carbon goes during growth on two CF-relevant carbon sources, acetate and glycerol (derived from the breakdown of lung surfactant). We find that carbon is routed (“fluxed”) through very different pathways during growth on these substrates, and that this is accompanied by an unexpected remodelling of the cell’s electron transfer pathways. Having access to this “blueprint” is important because the metabolism of *P. aeruginosa* is increasingly being recognised as a target for the development of much-needed antimicrobial agents.

Introduction

Pseudomonas aeruginosa (Pa) is an opportunistic pathogen. This cosmopolitan microbe has become one of the most frequent causative agents of nosocomial infection [1]. It is also well-known for colonising the airways of cystic fibrosis (CF) patients; a spatially and chemically heterogeneous environment characterised by gradients of oxygen and nutrients. To survive in this niche, Pa must therefore overcome numerous challenges [2,3]. Indeed, recent studies have suggested that CF-adapted *P. aeruginosa* exhibits distinct physiological adaptations, including a tailored preference for specific carbon sources, increased requirement for oxygen, and decreased fermentation [4]. Moreover, and despite extensive studies into the physiology and metabolism of *P. aeruginosa*, we still lack a clear understanding of how the growth and assimilation of carbon is controlled in this organism. Uncovering these processes is central to developing future treatment strategies.

Carbon source utilisation by *P. aeruginosa* is hierarchical, and the organism displays marked diauxy during growth on mixed carbon sources [5]. *In vitro*, the preferred carbon source of domesticated *P. aeruginosa* strains includes tricarboxylic acid cycle intermediates and amino acids. Intriguingly, and unlike many enteric bacteria, glucose is not especially favoured, even though it is present at millimolar concentrations in CF secretions [6]. Instead, adapted CF airway isolates seem to prefer glycerol during exponential growth. The precise reason for this is unclear, as glycerol *per se* is not abundant in CF sputum [4]. However, the surfactant phosphatidylcholine (PC) is abundant in the CF airways, and *P. aeruginosa* is known to secrete lipases which can cleave PC to yield phosphorylcholine, glycerol, and long-chain fatty acids (FAs) such as palmitate. The liberated glycerol is then metabolised through the action of enzymes

encoded by the *glp* operon, whereas the FAs are iteratively degraded by β -oxidation to yield acetyl-CoA. The acetyl moiety is then shuttled into the TCA cycle and glyoxylate shunt to generate energy and gluconeogenic precursors for biomass production, respectively. Other sources of short chain fatty acids in the CF airways have also been recently identified [7]. Indeed, acetate derived from tracheobronchial mucin breakdown by anaerobes has been reported at concentrations in excess of 5 mM in CF sputum, and reconstitution of the CF airway microbiota in mucin-containing medium *in vitro* leads to >30 mM acetate accumulating [7]. Glycerol and acetate metabolism therefore occupy an important crossroads in *P. aeruginosa* pathophysiology [8]. Glycerol is also a preferred carbon source for alginate synthesis by CF isolates of *P. aeruginosa* and promotes the appearance of mucoid variants when present at high concentrations [9].

Despite the obvious importance of these substrates for pathophysiology, we still do not know how glycerol and acetate impact on the metabolism, redox balance, and gene expression profile of *P. aeruginosa*. Much of what we think we know has been gleaned by extrapolation from other bacterial species, yet those species often occupy very different niches compared with *P. aeruginosa*, and display different substrate preferences. To redress this, in the current work, we use a combination of 'omics approaches (transcriptomics, proteomics, and [^{13}C] fluxomics), coupled with reverse genetics, to systematically investigate the pathway(s) of carbon assimilation during growth on acetate and glycerol. Surprisingly, not only do these different carbon sources lead to a “rewiring” of central metabolism; they also give rise to profound changes in the expression of pathogenicity-associated functions including denitrification, redox balance mechanisms and the electron transport chain. These data

underline the striking metabolic flexibility of *P. aeruginosa*, which allows this organism to carry out efficient, real-time free energy conservation, a trait which is likely to aid its ability to proliferate in diverse environmental conditions.

Results

Comparative transcriptomic, proteomic and fluxomic analysis of PAO1 cultured on glycerol or acetate as a sole carbon sources reveals global changes in central carbon metabolism.

We first examined the growth characteristics of *P. aeruginosa* during cultivation on acetate and glycerol as single carbon sources. This revealed that *P. aeruginosa* grows more slowly in glycerol (growth rate, $\mu_{\max} = 0.37 \pm 0.01 \text{ h}^{-1}$) than it does in acetate ($\mu_{\max} = 0.80 \pm 0.01 \text{ h}^{-1}$), glucose ($\mu_{\max} = 0.88 \pm 0.05 \text{ h}^{-1}$) or succinate ($\mu_{\max} = 0.87 \pm 0.05 \text{ h}^{-1}$) (Figure S2). To investigate this further, we examined the transcriptome, proteome and fluxome of *P. aeruginosa* during the assimilation of glycerol and acetate. Our analysis was carried out on cells grown to mid-exponential phase ($\text{OD}_{600} = 0.5$) in baffled shake flasks containing MOPS-buffered media. This allowed us to elucidate specific impact of carbon source utilization on *P. aeruginosa* metabolism and physiology without the confounding factors of nutrient and oxygen limitation that accompany entry into the stationary phase.

Acetate and glycerol have different entry points into *P. aeruginosa* central carbon metabolism, and are also thought to have distinct effects on redox metabolism [10–13]. We therefore anticipated a carbon source-specific impact on the expression of enzymes (and corresponding fluxes) involved in the relevant pathways. These pathways are summarised in Figure 1.

RNA-Seq transcriptomic analyses on quadruplicate biological replicates yielded quantification of the mRNA levels from 5578 genes. After normalisation and statistical analysis (Figure S1 A-C), we identified 389 genes displaying increased expression on acetate, and 364 genes that displayed increased expression on glycerol (p -value ≤ 0.01 , $\log_2$ fold change ≥ 2 or ≤ -2) (File S1). A selection of these modulated genes were verified using promoter-luciferase transcriptional fusions (Figure S3) [14].

A complementary proteomic analysis reproducibly detected a total of 3921 proteins across three replicates for each condition. To our knowledge, this is the most comprehensive (in terms of coverage) transcriptome and proteome analysis carried out on *P. aeruginosa* to date. Following normalisation and statistical analysis (Figure S1), we identified 429 proteins that showed increased abundance during growth on acetate, and 402 proteins displaying increased expression on glycerol (p -value ≤ 0.01 , $\log_2$ fold change ≥ 1 or ≤ -1) (File S1). There was a clear relationship between the transcriptome and proteome fold-changes, particularly with respect to central carbon metabolism (Figure 2, File S3).

To allow for a more detailed analysis of the 'omic alterations between these conditions, we used the proteomaps web service [15] to illustrate the statistically significant changes (p -value ≤ 0.01 , $\log_2$ fold change ≥ 1 or ≤ -1) as Voronoi tree maps. This provided a global overview of the proteomic consequences of growth in each carbon source. As shown in Figure 3 most of the proteomic changes were centred around 'central carbon metabolism', 'biosynthesis', 'signalling and cellular process' and 'energy metabolism'.

Several recent studies have analysed the metabolic interactions of clinically-relevant bacteria within their host environment [16,17] and metabolic differences between mutant strains [18]. However, published fluxome studies for *Pseudomonas* species are scarce. In addition, all *P. aeruginosa* metabolic flux analysis (MFA) studies to date have been carried out using glucose as a sole carbon source [19–22], yet this substrate is not thought to play a major role during CF airway colonization [6,23,24]. To provide insight into the absolute metabolic fluxes of *P. aeruginosa* during growth on acetate and glycerol, we carried out a ^{13}C fluxome analysis. This was done by measuring the mass isotopomer distributions of proteinogenic amino acids and cell carbohydrates (glycogen, glucosamine) using three separate tracers per carbon source (*Materials and Methods*) [25]. The calculated relative fluxes for the wild-type cultured on labelled glycerol or acetate are shown in Figure 4. Comparison of the flux maps, in combination with the proteomic/transcriptomic data, generated an unparalleled insight into the central carbon metabolic network(s) of *P. aeruginosa*.

Glycerol metabolism. During growth on glycerol, there was strong induction of the glycerol uptake system at both the proteomic and transcriptomic level (Figure 2, File S1). Glycerol is assimilated through the process of uptake (GlpF), phosphorylation (GlpK) and dehydrogenation (GlpD) to yield dihydroxyacetone phosphate (DHAP) [26–28]. Commensurate with this, the expression of all three enzymes/transporters (and their corresponding transcripts) was strongly stimulated during growth on glycerol.

Once synthesised, DHAP has two possible fates. One is anabolic. Here, DHAP is isomerised to generate glyceraldehyde 3-phosphate (G3P) through the action of triose phosphate isomerase (TpiA). DHAP and G3P are subsequently converted into fructose 1,6-bisphosphate (F1,6BP),

thence to fructose 6-phosphate (F6P) and finally, to glucose 6-phosphate (G6P) through the action of Fda, Fbp and Pgi, respectively. This route is a necessary prerequisite for the generation of hexose sugars for cell wall synthesis and biomass production. The alternative fate of DHAP is catabolic. Here, G3P is converted to 1,3-bisphosphoglycerate for glycolytic energy production [29]. However, recent analyses of carbon fluxes in *Pseudomonas putida* suggests that the seemingly distinct anabolic and catabolic fates of DHAP/G3P may be more closely intertwined than previously thought, and that a proportion of the triose phosphate carbon skeletons are recycled rather than continuing to pyruvate *via* the lower glycolytic reactions [30]. This “EDEMP cycle” incorporates elements of the Entner-Doudoroff (ED) pathway, Embden-Meyerhof-Parnas (EMP) pathway, and Pentose Phosphate (PP) pathway. In glucose-grown *P. aeruginosa* and *P. putida* the EDEMP cycle operates in a markedly asymmetrical manner, with most (ca. 90%) of the flux proceeding through the ED pathway catalysed reactions; glucose → gluconate → 6-phosphogluconate → 2-keto-3-deoxy-6-phosphogluconate → G3P/pyruvate [25]. Intriguingly, and in spite of the anticipated demand for hexose synthesis, our data indicate that during growth on glycerol this asymmetry is retained, since the enzymes catalysing the “catabolic” reactions (Zwf, Pgl, Edd and Eda) are strongly up-regulated, whereas the enzymes catalysing the “anabolic” reactions (TpiA, Fda, Fbp and Pgi) remain relatively unaffected. We also note that glucokinase (Glk) and gluconokinase (GntK) were up-regulated during growth on glycerol. Intriguingly, this indicates that even in the absence of glucose, glycerol stimulates expression of the full ED glycolytic pathway [31].

Consistent with the proteomic/transcriptomic data, when *P. aeruginosa* was grown on glycerol, the fluxomics indicated that about 15% of the triose phosphates (G3P + DHAP) were diverted

into the EDEMP cycle to form hexose phosphates [30]. Hexose generation is necessary for the synthesis of fructose 6-phosphate (F6P) and glucose 6-phosphate (G6P), required for biomass production. This recycling may also have a secondary function, since it generates NADPH through the action of glucose 6-phosphate dehydrogenase (Zwf). NADPH is a source of reducing agent for anabolism, and for dealing with oxidative insult [30]. However, the majority of the glycerol-derived carbon was oxidized to pyruvate through reactions in the lower half of the ED pathway. This notwithstanding, a significant amount of pyruvate was also generated from TCA-derived malate *via* the action of malic enzyme, feeding the so-called “pyruvate shunt”. The pyruvate shunt (a cyclical series of reactions converting malate → pyruvate → oxaloacetate → malate) generates NADPH for anabolism at the cost of consuming one equivalent of ATP. *P. aeruginosa* lacks phosphogluconate dehydrogenase activity (a major source of NADPH in many other species), so this apparently futile cycling reaction may be of major importance for growth. Indeed, $> \frac{2}{3}$ of the carbon in the malate pool was shuttled back to pyruvate through the action of malic enzyme, with $< \frac{1}{3}$ being converted to oxaloacetate *via* the malate dehydrogenase reaction. Overall, glycerol metabolism seems to be characterised by operation of the EDEMP cycle, net catabolism of G3P to pyruvate, and cycling of the latter through the pyruvate shunt.

Acetate metabolism. Growth on acetate as a sole carbon source elicited a similarly informative set of changes. There was a strong induction of the glyoxylate shunt enzymes (AceA and GlcB), which are known to be essential for growth on acetate [32,33], and of enzymes directly involved in acetate activation (such as AcsA, AckA and Pta) and acetate uptake (PA3234) [34]. The TCA cycle-associated enzymes were almost all up-regulated during growth on acetate, as was the membrane-bound malate-quinone oxidoreductase, MqoB (Figure 2). Unlike many

bacteria, *P. aeruginosa* does not encode a soluble NADH-producing malate dehydrogenase, and MqoB directly donates the abstracted electrons to the membrane quinone pool [35].

Carbon fluxes in acetate-grown cultures were very different from those observed in glycerol-grown cells. Firstly, net flux through the lower reactions of the ED pathway was in the gluconeogenic direction. Second, the flow of carbon through the EDEMP cycle was much lower than that observed in glycerol, and flux terminated at the gluconeogenic end-point, G6P. The absence of flux through the G6P dehydrogenase (Zwf) reaction following this point is significant, since this reaction is often thought of as a major source of NADPH for anabolism. Third, and compounding this, the extent of carbon cycling through the NADPH-producing pyruvate shunt was low. Fourth, most of the carbon for anabolism was derived from oxaloacetate rather than malate. Presumably, this may reflect the increased rate of conversion of malate to oxaloacetate by the acetate-induced malate:quinone oxidoreductase (MqoB). However, perhaps the most notable difference was that during steady-state growth on acetate, around $\frac{1}{3}$ of the carbon reaching the TCA cycle-glyoxylate shunt branchpoint was redirected into the glyoxylate shunt. [In glycerol-grown cells, only around 3% of the carbon reaching this branchpoint was redirected to the glyoxylate shunt.] The glyoxylate shunt serves to supply the cell with malate and thence (following the MqoB-catalyzed conversion) also the gluconeogenic precursor, oxaloacetate. In an elegant feedback loop, high levels of oxaloacetate (such as would accumulate if the NADPH supply for anabolism was limiting) stimulate the activity of one of the isocitrate dehydrogenase isozymes, IDH, thereby restoring flux through the TCA cycle [36]. One outcome of this is that NADPH levels become replenished because the IDH-catalyzed reaction is a major source of this coenzyme *in vivo*. This presumably relieves the limitation on oxaloacetate usage in anabolism.

At the steady state, our flux data show that around $\frac{2}{3}$ of the carbon flowing through the branchpoint is fluxed through the isocitrate dehydrogenases.

Our flux data show that no carbon passes through the NADPH-generating steps of the ED pathway during growth on acetate. So far, we have primarily framed our assessment of these observations around the need for NADPH in anabolism. However, it has not escaped our attention that NADPH limitation may impact on infection too. Several earlier workers have suggested that flux through the ED pathway may confer an additional benefit on *P. aeruginosa* by providing sufficient reducing power (NADPH) to counteract host-mediated oxidative stress [37,38]. Whereas our data neither confirm nor refute this notion, we show here that four major NADPH-supplying reactions are accessible, depending on the substrate; transhydrogenation (NADH $\rightarrow$ NADPH), the EDEMP cycle, the pyruvate shunt (malic enzyme), and the isocitrate dehydrogenase(s)-catalysed reaction.

Beyond central metabolism – growth in acetate induces extensive remodelling of the electron transport chain.

Aerobic respiration re-oxidizes NADH, thereby generating energy, maintaining redox homeostasis, and ensuring continued oxidative metabolism [39] [40]. Bacteria are known to coordinate the composition of the electron transport chain (ETC), and in particular, levels of the terminal oxidases, according to their metabolic needs [41]. The ETC of *P. aeruginosa* contains three NADH dehydrogenases; the multi-subunit proton pumping NDH-1 (encoded by *nuoA-N* (PA2637-PA2649)), the HQNO-resistant proton pump Nqr (encoded by *nqrA-F* (PA2994-2999)), and a single-subunit flavoenzyme which does not participate in ion translocation (NDH-2,

encoded by *ndh*, PA4538) [42]. All three dehydrogenases displayed increased expression during growth on acetate, particularly at the protein level (file S1).

A characteristic feature of the *P. aeruginosa* respiratory chain is its use of high-affinity terminal oxidases, even during aerobic growth. This unusual wiring of the *P. aeruginosa* ETC is thought to drive the formation of a microaerobic environment; a trait that may give the pathogen a fitness advantage during infection [43]. Expression of the terminal oxidases in *P. aeruginosa* is directly or indirectly controlled by a two-component system, RoxS-RoxR. The kinase, RoxS, is thought to sense the redox status of the respiratory chain, either by titrating the redox status of the ubiquinone/ubiquinol (UQ) pool or by responding to electron flux through the terminal oxidases. UQ acts as the electron donor for complex III, the quinol oxidases (Cyo), and the cyanide-insensitive terminal oxidase, CIO. Complex III transfers electrons to a c-type cytochrome, which then acts as the electron donor for the terminal cytochrome oxidases Cox, Cco1, or Cco2. Cco1 is constitutively expressed at high levels, whereas Cco2 is thought to support growth under low oxygen tension (~2% O₂), as it is regulated by the anaerobic sensor, Anr [44]. The 'omics data indicated that Cco1, Cco2, Cyo and complex III subunits (encoded by PA4429-PA4431) are highly-expressed during growth on acetate, whereas the Cox oxidase was more highly-expressed during growth on glycerol. This may be a strategy to limit oxidative stress, as Cox has a high H⁺/e⁻ ratio and can extract more energy per unit of carbon [45,46]. The greater expression of Cox during growth on glycerol may be a consequence of RoxSR-mediated inhibition of *cox* gene expression during growth on acetate (File S1) [47].

Levels of the soluble pyridine nucleotide transhydrogenase, Sth (PA2991), were increased ca. 3-fold during growth on acetate. Pyridine nucleotide transhydrogenases catalyse the reversible reduction of either NAD^+ or NADP^+ by NADPH or NADH (respectively). The primary physiological role of Sth is thought to be in the NAD^+ -dependent re-oxidation of NADPH [48]. Re-oxidation of excess NADPH is likely to be important during growth on acetate, as extensive catabolism of this substrate through the TCA cycle generates more NADPH than is required for biosynthesis [49]. Interestingly, the NAD(P) transhydrogenase encoded by *pntAA* and *pntAB* was not differentially expressed during growth on acetate or glycerol, suggesting that Sth is the main transhydrogenase used by *P. aeruginosa* in these conditions [48].

As noted earlier, *P. aeruginosa* cultured in glycerol has a significantly slower growth rate than cells grown in acetate, glucose or succinate (Figure S2). This lower growth rate may result in a lower metabolic demand, meaning these cells do not require high expression of terminal oxidases and other ETC components. By contrast, higher growth rates may result in accumulation of NADH due to the rate-limitations inherent in ETC-dependent re-oxidation. This is because faster growing cells are known to increase their length [53], suggesting that they are likely have lower surface-to-volume ratios. This limits the membrane's physical capacity for accommodating respiratory complexes, and thereby also limits NADH re-oxidation [50]. To investigate this possibility further, we examined the length of exponentially-growing *P. aeruginosa* cells grown in MOPS media with various carbon sources. To do this, we introduced an eGFP-expressing plasmid (pMF230) to PAO1 and cultured the strain in MOPS medium containing different carbon sources [51]. When cells reached an OD_{600} of 0.5, they were fixed and examined by fluorescence microscopy. As shown in Figure S6 (and File 3), the slower-

growing cells in MOPS-glycerol were indeed significantly shorter than cells grown in acetate, glucose or succinate [52]. This supports the notion that *P. aeruginosa* cell size (and hence, surface-to-volume ratio) is dependent on growth rate.

Induction of the denitrification apparatus in *P. aeruginosa* during aerobic growth

When O₂ is limiting, *P. aeruginosa* can use nitrate as an alternative electron acceptor. This is made possible by the presence of nitrate reductase (NAR) which can accept electrons from the UQ pool, and nitrite reductase (NIR), which receives electrons *via* complex III and cytochrome c [53]. Denitrification is thought to be an important pathway to support the anoxic growth of *P. aeruginosa* in the CF airways [54,55] and nitrate is a known component of human body fluids, arising from diet and NO auto-oxidation. Indeed, NO₃⁻ concentrations in the CF airways have been reported between 73 µM – 792 µM, with an average of 348 µM [56]. Molecular oxygen is therefore not essential for growth in this environment.

The gene clusters encoding the dissimilatory nitrate reductases (*nar* and *nap*) and those encoding nitrous oxide (N₂O) utilisation (*nos*) are unlinked, whereas the genes encoding nitrite respiration (*nir*) and nitric oxide respiration (*nor*) are adjacent. In addition to the enzymes above, the denitrification operons also harbour genes for ancillary functions such as cofactor synthesis, transport and protein maturation [53]. Both the proteomic and transcriptomic analyses showed activation of the denitrification pathways following growth in MOPS-acetate (File S1). This was surprising, since the cultures were harvested during exponential growth in baffled, well-aerated conical flasks without added nitrate. This is an important point, because denitrification in *P. aeruginosa* is known to be under the control of the master regulator Dnr. In

turn, Dnr is under the control of the anaerobic transcriptional regulator Anr. The latter contains an oxygen sensitive $[4\text{Fe-4S}]^{2+}$ cluster and is thought to be active only under conditions of oxygen limitation. In contrast, Dnr is a nitric oxide (NO) sensor, and contains a ferrous heme. Dnr protein expression was up-regulated 2.8-fold during growth on acetate compared with glycerol (File S1). Previous work has shown that, in addition to Dnr-mediated regulation, the *narGHJ* operon is also activated by the NarXL two-component system. The NarX and NarL proteins were up-regulated 2-fold during growth on acetate. Taken together, these data indicate that in spite of the presence of molecular oxygen and the absence of added nitrate, growth on acetate strongly induces the denitrification apparatus in comparison with glycerol. We conclude that the denitrification apparatus and components required for aerobic respiration are expressed simultaneously during growth on acetate [57].

***P. aeruginosa* cellular NAD(P)(H) ratios change in a carbon source dependent manner**

By summing the fluxes through reactions that either produce or consume NADPH or ATP, we were able to calculate the relative contributions of different metabolic reactions to the redox (NADPH) and energy (ATP) balances during growth on each carbon source (Figure 5). This revealed that cells grown on glycerol obtain their NADPH from a variety of reactions, whereas acetate-grown cells are heavily-reliant on NADPH derived from the isocitrate dehydrogenase(s) reaction. Moreover, most of the NADPH generated during growth on acetate was used in anabolism. With regards to ATP, cells grown on glycerol were effectively “over-supplied” with this reagent, maintaining a substantial “operating surplus” of ATP. In contrast, the supply of ATP in cells grown on acetate was essentially identical to the metabolic demand. The most likely reason for this is the 2 x ATP ‘cost’ of acetate uptake and activation [58]. During growth on

glycerol, the cell generates 2.6 moles of ATP per mole of C, whereas just 1.1 moles of ATP are generated on acetate. This “energy deficit” may explain why the cell induces additional mechanisms to maximise energy production during growth on acetate (e.g., high affinity electron acceptors, increased denitrification, increased Sth transhydrogenase expression etc (File S1)).

We anticipated that the faster growth rate (Figure S2) and TCA cycle driven metabolism (Figure 2) during growth on acetate would result in an increased rate of respiration, and that this might drive the NAD(P)H/NAD(P)⁺ ratio towards a more reduced state [50]. This was indeed the case (Figure S2) indicating that carbon source alone is sufficient to alter the intracellular redox economy of *P. aeruginosa*. We next investigated what might be driving this increased NADH/NAD⁺ ratio during growth on acetate. Microorganisms display an elevated NADH/NAD⁺ ratio when NADH re-oxidation is slowed by limitation of electron acceptors [59], or when the metabolism of certain carbon sources outpaces the capacity of the *P. aeruginosa* ETC to re-oxidise the coenzyme. This may explain why cells grown in acetate appear to scavenge for alternative electron acceptors (such as nitrate, as indicated by the increased expression of the denitrification apparatus). To determine if aerobic denitrification might be used by *P. aeruginosa* to correct the reduced status of its redox pool, we therefore examined the impact of nitrate addition on the NADH/NAD⁺ ratio. As shown in Figure 7, the addition of 20 mM nitrate to MOPS-acetate cultures decreased the NADH/NAD⁺ ratio during exponential growth. Absolute quantitation of the NADH and NAD⁺ levels (Figure S2) confirmed that the total NAD(H) and NADP(H) pools were similar in the presence and absence of added nitrate. To confirm that this drop in the NADH/NAD⁺ ratio was due to aerobic denitrification, we generated a deletion

mutant in the master transcriptional regulator of denitrification, *dnr*. As expected, nitrate reductase (NirS) expression was abolished in the *dnr* mutant, and also in an *anr* mutant (Anr controls the expression of *dnr*) (Figure S4). Crucially, and consistent with findings from other laboratories showing that nitrate addition has little impact on the NADH:NAD⁺ ratio in mutants defective in denitrification [60], the *dnr* mutant was also defective in nitrate-dependent re-oxidation of NADH and NADPH (Figure 7). We conclude that aerobic denitrification during growth on acetate impacts on the redox status of the NAD(P)H/NAD(P)⁺ pool.

Discussion

Several earlier studies have described the systems-level cellular adjustments that accompany the growth of industrially-relevant model organisms such as *E. coli*, *Saccharomyces cerevisiae*, *B. subtilis*, *C. glutamicum*, *B. succiniciproducens*, and *P. putida* in single carbon sources [61–66]. However, and although the accrued metabolic models have made a valuable contribution towards our “textbook understanding” of microbial metabolism, it is clear that they can be only loosely extrapolated to other organisms, such as *P. aeruginosa* [67]. Surprisingly, a comparative ‘omics study has yet to be carried out for *P. aeruginosa* grown on carbon sources relevant to infection. Overall, carbon preference remains poorly understood for *P. aeruginosa*, even though it is known to have a profound impact on virulence-associated phenotypes, including toxin production, biofilm formation and growth rate. In this work, we rectify this by developing a high-resolution global map of *P. aeruginosa* metabolism during growth on two infection-relevant carbon sources, acetate and glycerol.

Our data indicate a clear relationship between transcript and protein changes in *P. aeruginosa* for the growth regimens tested [20,36,68]. These expression data enabled us to establish which metabolic pathways are active in each growth condition, thereby providing an experimentally-supported framework for interpreting the fluxomic data. This notwithstanding, the fluxomics highlighted the possibility of additional layers of regulatory complexity involved in fine-tuning of central carbon metabolism. For example, growth on acetate results in the expression of three enzymes (the isocitrate dehydrogenases, ICD and IDH, and the isocitrate lyase, ICL), all of which compete for a shared substrate, isocitrate. These enzymes are known to exhibit radically different catalytic and regulatory properties compared with their *E. coli* counterparts [36]. In spite of this, our fluxomic analysis revealed a remarkably similar flux partitioning at the branchpoint between the TCA cycle and the glyoxylate shunt in both organisms [36].

In addition to alterations in central carbon metabolism, we also noted substantial remodelling of the ETC composition during growth in acetate compared with glycerol. For example, we noted a substantial increase in the expression of terminal oxidases (*cyo*, *cco1* and *cco2*) during growth on acetate, and increased *cox* expression during growth on glycerol. This suggests that different terminal oxidases are employed in different growth conditions, allowing metabolism to be optimally adjusted for energy generation [69]. Interestingly, growth on mucus has been previously shown to stimulate *Cyo* expression and to repress *Cco2* expression [70]. Our data suggest that these observations may be driven by metabolism of one or more mucus-derived compounds.

The ETC alterations that we observed included strong aerobic induction of the denitrification machinery during growth on acetate. This was unexpected, since denitrification is usually

associated with oxygen limitation and microaerobic growth. The signal which leads to this remodelling of the ETC remains to be elucidated. However, we speculate that these changes in ETC architecture and composition may be a mechanism to restore redox balance (NADH:NAD⁺ ratio) in the cell [71]. Acetate-grown cells accumulate NADH, so activating alternative mechanisms (such as the denitrification apparatus) to oxidise NADH may be a homeostatic attempt to regain an optimal cellular redox status. Consistent with this, the addition of nitrate to acetate-grown *P. aeruginosa* did lead to a more oxidized NADH:NAD⁺ ratio. Moreover, a mutant (Δdnr) defective in denitrification, was unable to maintain optimal redox homeostasis. However, growth was not affected in the Δdnr mutant, perhaps suggesting that the organism also has additional (possibly compensatory) mechanisms to deal with redox dysregulation [37,72].

Aerobic denitrification has been recently suggested to function as a ‘bet-hedging’ strategy to anticipate nitrate availability and respond to abrupt anoxia [73,74]. The oxygen-limited microenvironment in the CF airways is thought to result in *P. aeruginosa* growth by microaerobic respiration [75]. One possibility is that hybrid respiration (a combination of aerobic respiration and denitrification) may well be a predominant mechanism of survival in these conditions [76]. Genes under the control of the transcriptional regulator Anr are also abundant in *P. aeruginosa* RNA extracted from CF sputum [77–79].

A key fundamental question arises from this study – how does *P. aeruginosa* activate the denitrification system during growth in aerobic conditions? Until now, induction of the denitrification machinery has been largely attributed to the activity of Anr (and its subordinate denitrification-specific regulator, Dnr). Anr dimerization is thought to be dependent on the

formation of an oxygen-labile $[4\text{Fe-4S}]^{2+}$, which is destabilised and disassociates in the presence of oxygen, abolishing Anr activity. However, there is accruing evidence that Anr may be active even in the presence of oxygen. For example, Jackson *et al.*, have shown that choline catabolism leads to aerobic expression of the Anr regulon, suggesting that Anr activity can be sustained in the presence of oxygen. Also, Anr overexpression in well-aerated cultures increases the levels of *dnr* transcript 29-fold [80]. Furthermore, the expression of *nir* and *nar* genes has been shown to increase in late exponential phase cultures compared with stationary phase cultures of PA14 [81]. This mobilisation of the Anr regulon in the presence of oxygen suggests that Anr can modulate gene expression without fully intact metallo-centres or dimers. This may be to assist the cells during the transition to conditions of low oxygen tension [80].

Finally, our data demonstrate that *P. aeruginosa* grows more rapidly on acetate than it does in glycerol, both in terms of specific growth rate (measured as optical density) and cell length (Figure S6). In order to maintain redox homeostasis in such conditions, cells will often switch to “overflow metabolism” whereby partially oxidised metabolic intermediates such as acetate are excreted [59,82]. At first glance, overflow metabolism seems wasteful, although it does allow them to maximise their growth rate [83]. It is tempting to speculate that aerobic denitrification in *P. aeruginosa* may also be a form of ‘overflow’ metabolism in which the alternate electron acceptor nitrate is utilised alongside oxygen in an effort to maintain redox homeostasis.

450

451 **Materials and Methods**

452 **Growth Conditions**

453 Unless otherwise indicated, *P. aeruginosa* strain PAO1 [84] was routinely grown in lysogeny
 454 broth (LB LENNOX) (Oxoid Ltd) at 37°C with shaking (250 rpm). The strains used in this study are
 455 listed in Table S1. The overnight pre-cultures were started from separate clonal source colonies
 456 on streaked LB agar plates. Strains were cultured in MOPS (morpholinepropanesulfonic acid)
 457 media with the relevant carbon sources [85]. Cell growth was monitored as optical density in a
 458 spectrophotometer at a wavelength of 600 nm (OD₆₀₀). A previously determined conversion
 459 factor of 0.42 g CDW per OD unit was used to calculate biomass specific rates and yields from
 460 the obtained OD₆₀₀ values [21].

461 **Transcriptomics (RNA-Seq)**

462 PAO1 was grown in 40 mL MOPS with acetate or glycerol as sole carbon sources
 463 (quadruplicate), 37°C with shaking (250 rpm) in baffled flasks (500 mL). An aliquot (5 mL) of
 464 culture was harvested from each sample at OD₆₀₀ of 0.5 (exponential growth) and added to an
 465 equal volume of RNeasy lysis buffer. Samples were then sedimented in an Eppendorf 5810R centrifuge
 466 at 3220 × *g* for 15 min (4°C) and the pellets were stored at −80°C. Total RNA was isolated as
 467 described in [86], followed by phenol-chloroform-isoamyl alcohol extraction and ethanol
 468 precipitation. Ribosomal RNA (rRNA) was then depleted from each sample (5 µg each) using the
 469 bacterial Ribo-Zero rRNA Removal Kit (Illumina). The integrity of the RNA was evaluated using
 470 an RNA 6000 Nano LabChip and an Agilent 2100 Bioanalyzer (Agilent Technologies, Germany).

Eight indexed, strand-specific cDNA libraries were prepared, and samples were sequenced on an Illumina HiSeq 2000 with a 51 bp single-end read length (GATC Biotech, Germany). The sequencing data are deposited at ArrayExpress (accession number E-MTAB-8374).

Reads mapping and annotations

The resulting FASTQ files were mapped to the PAO1 genome obtained from the Pseudomonas Genome Database (PDG) (<http://www.pseudomonas.com/>) using TopHat v.2.0.3 [37], Bowtie v.0.12.8 [38] with a ~97% success rate to generate SAM files. The sequence reads were adaptor clipped and quality trimmed with trimmomatic [87] with default parameters. Transcript abundance and differential gene expression were calculated with the program Cufflinks v.2.0.1 [88]. Gene expression levels were normalized using fragments per kilobase of exon per million mapped reads (FPKM) report values. Genes were considered as induced or repressed, only when their log₂ fold change was >1 or <-1, respectively, and their *p*-value was <0.01 (File S1, Figure S1).

Quantitative proteomic analysis

P. aeruginosa cells (OD₆₀₀ = 0.5, 30 mL) were harvested in identical growth conditions as the transcriptomics section described above. Pellets were resuspended in lysis buffer (100 mM Tris-HCl, 50 mM NaCl, 10% (v/v) glycerol, 1 mM tris(2-carboxyethyl)phosphine (TCEP), pH 7.5) with cOmplete Mini protease inhibitor cocktail (Roche). Following three rounds of sonication (3 × 10 sec) on ice, supernatants were clarified by sedimentation (21130 × *g*, 15 min, 4°C) in an Eppendorf 5424R centrifuge. Aliquots (100 µg) of each sample was reduced with TCEP, alkylated with iodoacetamide and labelled with Tandem Mass Tags (TMTs). TMT labelling was

performed according to the manufacturer's protocol
(<https://www.thermofisher.com/order/catalog/product/90110>).

LC-MS/MS

Dried fractions from the high pH reverse-phase separations were resuspended in 30 µL of 0.1% (v/v) formic acid (14 combined fractions). LC-MS/MS experiments were performed using a Dionex Ultimate 3000 RSLC nanoUPLC (Thermo Fisher Scientific Inc, Waltham, MA, USA) system and a Lumos Orbitrap mass spectrometer (Thermo Fisher Scientific Inc, Waltham, MA, USA). Peptides were loaded onto a pre-column (Thermo Scientific PepMap 100 C18, 5 mm particle size, 100 Å pore size, 300 mm i.d. x 5 mm length) from the Ultimate 3000 auto-sampler with 0.1% (v/v) formic acid for 3 min at a flow rate of 10 µL/min. Separation of peptides was performed by C18 reverse-phase chromatography at a flow rate of 300 nL/min using a Thermo Scientific reverse-phase nano Easy-spray column (Thermo Scientific PepMap C18, 2 mm particle size, 100 Å pore size, 75 mm i.d. x 50 cm length). Solvent A was water + 0.1% formic acid and solvent B was 80% (v/v) acetonitrile, 20% water + 0.1% formic acid. The linear gradient employed was 2-40% B in 93 min (total LC run time was 120 min including a high organic wash step and column re-equilibration).

The eluted peptides were sprayed into the mass spectrometer by means of an Easy-Spray source (Thermo Fisher Scientific Inc.). All m/z values of eluting peptide ions were measured in an Orbitrap mass analyser, set at a resolution of 120,000 and were scanned between m/z 380-1500 Da. Data dependent MS/MS scans (Top Speed) were employed to automatically isolate and fragment precursor ions by collision-induced dissociation (CID, Normalised Collision Energy

(NCE): 35%) which were analysed in the linear ion trap. Singly charged ions and ions with unassigned charge states were excluded from being selected for MS/MS and a dynamic exclusion window of 70 sec was employed. The top 10 most abundant fragment ions from each MS/MS event were then selected for a further stage of fragmentation by Synchronous Precursor Selection (SPS) MS3 [89] in the HCD high energy collision cell using HCD (High energy Collisional Dissociation, (NCE: 65%). The m/z values and relative abundances of each reporter ion and all fragments (mass range from 100-500 Da) in each MS3 step were measured in the Orbitrap analyser, which was set at a resolution of 60,000. This was performed in cycles of 10 MS3 events before the Lumos instrument reverted to scanning the m/z ratios of the intact peptide ions and the cycle continued.

Proteomic Data Analysis

Proteome Discoverer v2.1 (Thermo Fisher Scientific) and Mascot (Matrix Science) v2.6 were used to process raw data files. Data was aligned with the UniProt *Pseudomonas aeruginosa* (5584 sequences) the common repository of adventitious proteins (cRAP) v1.0.

The R package MSnbase [90] was used for processing proteomics data. Protein differential abundance was evaluated using the Limma package [91]. Differences in protein abundances were statistically determined using the Student's *t*-test with variances moderated by Limma's empirical Bayes method. *P*-values were adjusted for multiple testing by the Benjamini Hochberg method [92]. Proteins were considered as increased or decreased in abundance, only when their $\log_2$ fold change was >1 or <-1 , respectively, and their *p*-value was <0.01 . The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE (55) partner repository with the data set identifier PXD015615.

Construction of Luciferase reporter strains

Translational reporter constructs were made by fusing the upstream promoter sequences with *luxCDABE* using the primers listed in Table S1. Purified PCR products were digested and directionally ligated into the multiple cloning site of the pUC18T-mini-Tn7T-lux-Gm plasmid [14]. The mini-Tn7-lux element were integrated into the PAO1 chromosome by electroporation along with the helper plasmid pTNS2 as previously described [93].

Luciferase-Promoter Assay

Luciferase and OD₆₀₀ readings were measured using a BMG Labtech FLUOstar Omega microplate reader. Strains were cultured in MOPS media with the relevant carbon sources (100 µL) in 96 well microplates (Greiner bio-one, F-Bottom, Black), covered with gas permeable imaging seals (4titude - 4ti-0516/96). Luciferase expression was assessed every 30 min (Gain = 3600) for up to 24 hr. Growth was assessed by taking OD readings at 600 nm simultaneously with the luminescence readings. Luciferase readings were expressed as relative luminometer units (RLU) normalised to OD₆₀₀ in order to control for growth rate differences across the selected carbon sources.

¹³C fluxomics

Starter cultures were grown in LB medium. For the second and main cultures, PAO1 was grown in MOPS minimal media with 60 mM acetate or 40 mM glycerol as the sole carbon source (120 mM carbon). For ¹³C flux experiments, naturally labelled acetate and glycerol was replaced with three separate tracers per carbon source to maximise dataset resolution and to accurately determine substrate uptake. Naturally labelled glycerol was substituted with [1,3-¹³C₂] glycerol

(99%), [2-¹³C] glycerol (99%) and an equimolar mixture of [U-¹³C₃] glycerol (99%) (Cambridge Isotope Laboratories, Inc., Andover, MA, USA) and naturally labelled glycerol. Naturally labelled acetate was substituted with 99% [1-¹³C] sodium acetate, [2-¹³C] sodium acetate or a molar 1:1 mixture of [U-¹³C₂] sodium acetate obtained from Sigma-Aldrich (Poole, Dorset, UK) and natural sodium acetate.

Starter cultures were prepared by inoculating LB medium with a loop of freshly plated PAO1. After 6 hr of incubation, 50 µL of cell suspension was transferred to a second culture of MOPS minimal medium. Subsequently, exponentially growing cells were used as inoculum for main cultures, and PAO1 was cultured in 25 mL of minimal medium in 250 mL baffled shake flasks (200 rpm, 37°C) in an orbital shaker (Aquatron, Infors AG, Switzerland). These growth conditions were selected to ensure sufficient aeration during cultivation. As shown previously for *P. aeruginosa* PAO1, the oxygen level was maintained above 80% of saturation under identical growth conditions [21]. In cultures incubated with ¹³C-tracer, the inoculum level was always kept below 1% (initial OD < 0.02) of the final sampled cell concentration to exclude potential interference of non-labelled inoculum on subsequent calculation of flux [94].

Mass isotopomer labelling analysis of proteinogenic amino acids, mass isotopomer labelling analysis of cell sugar monomers (glucose and glucosamine), metabolic reaction network and flux calculation were carried out as described in [25].

Quantification of substrates and products

Acetate and glycerol, as well as organic acids (citric acid, α-ketoglutaric acid, gluconic acid, 2-ketogluconic acid, pyruvic acid, succinic acid, lactic acid, formic acid, fumaric acid) were

quantified in filtered culture supernatants (Costar® Spin-X® 0.22 µm) using isocratic high-performance liquid chromatography (Agilent 1260 Infinity series, Aminex HPX-87H column at 65°C and a flow rate of 0.5 mL min⁻¹) equipped with RI and UV detector (210 nm) with 50 mM H₂SO₄ as eluent [95]. Concentrations were determined from commercial standards which were analysed on the same run. These data were then used to calculate specific uptake and formation rates and yields for acetate, glycerol and secreted by-products, respectively (File S2).

Calculation of redox cofactor and ATP balances

NADPH, NADH and FADH₂: Total production of reduced cofactors was determined by summing up all cofactor-forming fluxes considering substrate-dependent cofactor specificities [30,96,97]. Anabolic NADPH requirements and anabolically produced NADH were estimated from the biomass composition [37,98] and measured specific growth rates. Surplus NADPH was considered to be converted into NADH *via* the activities of a soluble (SthA, PA2991) and a membrane-bound, proton-translocating (PntAB, PA0195-PA0196) pyridine nucleotide transhydrogenases [48].

ATP: Production of ATP *via* substrate-level phosphorylation was calculated by adding all ATP-producing fluxes and subtracting all ATP-consuming fluxes. Acetate uptake was considered *via* acetyl-CoA synthase (ACS) consuming 2 mol of ATP per mol acetate catabolized [58]. The ATP synthesized from NADH and FADH₂ *via* oxidative phosphorylation in the respiratory chain was estimated assuming a P/O ratio of 1.875 for NADH [48,98,99] and 1.0 for FADH₂ [100], respectively. The ATP demand was calculated by adding up the requirements for anabolism estimated from biomass composition, observed specific growth rates, and non-growth

associated maintenance (NGAM) needs [48,98,99]. ATP surplus represents available ATP to fulfil growth-associated maintenance and other cellular ATP-consuming tasks.

NAD(P)(H) extraction

P. aeruginosa PAO1 cultures were grown in MOPS media containing a single carbon source (40 mM acetate, 15 mM glucose, 30 mM glycerol, or 30 mM succinate) at 37°C with shaking at 250 rpm, using a culture volume of 150 mL in a 2 L baffled Erlenmeyer flask. For each NAD(P)(H) extraction, 1.8 mL of culture was removed and immediately added to 7.5 mL ice-cold 100% methanol followed by centrifugation at $3220 \times g$ for 14 min at 4°C to obtain a cell pellet. The pellet was resuspended in 0.2 M HCl for NAD(P)⁺ or 0.2 M NaOH for NAD(P)H extraction, before incubation at 52.5°C for 10 min followed by incubation on ice for 5 min. HCl or NaOH was then neutralised by the dropwise addition of 0.1 M NaOH or 0.1 M HCl, respectively, whilst vortexing at low speed. The mixture was then centrifuged for 5 min at $15,800 \times g$ and 135 µL of the supernatant was removed for immediate NAD(P)(H) measurement or storage at -80°C. Samples were stored for a maximum of 1 week before measurement.

NAD(P)(H) measurement

NAD(P)(H) concentrations were measured using an enzyme cycling assay in a 96-well microtiter plate (Thermo Scientific 167008) as described in [101]. A reagent master mix was prepared containing 2 volumes 1 M bicine (pH 8.0), 1 volume 100% ethanol, 1 volume 40 mM EDTA (pH 8.0), 1 volume 4.2 mM thiazolyl blue, 2 volumes 16 mM phenazine ethosulfate, and 1 volume dH₂O. The reagent mix was incubated at 30°C and primed to injectors in a BMG LABTECH FLUOstar Omega microplate reader. Aliquots (15 µL) of NAD(P)(H) extracts were added to

individual wells of a 96-well microtiter plate, which was then incubated in the microplate reader at 30°C. Reagent master mix (80 μ L) was added *via* the microplate reader injector (300 μ L s⁻¹) and vigorously mixed (200 rpm, 3 sec) before static incubation for 10 min. Immediately before measurement, a solution of alcohol dehydrogenase (1 mg mL⁻¹ in 0.1 M bicine) was prepared for NAD(H) measurement or glucose 6-phosphate dehydrogenase (0.1 mg mL⁻¹ in 0.1 M bicine) for NAD(P)(H) measurement and primed to a second injector. To start the reaction each well was injected (300 μ L s⁻¹) with 5 μ L of enzyme solution, followed by vigorous mixing (200 rpm, 1 sec). The absorbance at 570 nm was then recorded every 30-60 sec for 20 min, with vigorous shaking (200 rpm, 1 sec) before each read. Slopes from plots of absorbance over time were calculated for NAD(P)H and NAD(P)⁺, which were then used to calculate ratios.

Colony Forming Unit (CFU) enumeration

Alongside each NAD(P)(H) extraction, an aliquot from the same culture was removed, serially diluted and plated onto LB agar using the single plate-serial dilution spotting method described in [102], and colonies were then grown overnight at 37°C.

Western-blot analysis

The cultures were grown aerobically to an OD₆₀₀ of 0.5 in MOPS minimal medium supplemented with the indicated carbon sources. The samples were centrifuged at 3220 $\times g$ for 10 min. Equal amounts of protein were resolved on a 12% SDS—polyacrylamide gel. The proteins were blotted onto a nitrocellulose membrane, which was blocked with 5% (w/v) dried skimmed milk in TBS buffer. The membranes were probed with mouse anti-NirS [103] and with

a IRDye® 680RD and IRDye® 800CW Goat anti-mouse IgG secondary antibodies (925-68070).
Bands were visualized on an Odyssey Infrared Imaging System (LI-COR Biosciences).

Fluorescence microscopy

Fluorescence microscopy experiments to determine bacteria length were performed on a custom-built microscope based on an Olympus (Center Valley, PA) IX-73 frame with a 100 × 1.49 NA oil objective lens (Olympus UAPON100XOTIRF) and a 488-nm laser (Coherent Sapphire 488-300 CW CDRH). Samples were imaged using epi-illumination and images were relayed onto an Andor iXon Ultra 897 camera by a 1.3 × magnification Cairn Twincam image splitter (the second port of the image splitter was not used during these experiments). The resulting pixel width on the sample was measured to be 118 nm. For each field of interest, 100 frames at 100 msec exposure time were captured in a 256 × 256 pixel² region.

Bacterial size analysis

The micrographs captured for this study exhibited typically one or two bacteria over a 30 mm × 30 mm² area. The raw data were segmented and filtered for analysis using a MATLAB script included in the Supplementary Information. The data were first segmented: the MATLAB function `adaptthresh` was used to binarize the data using an adaptive threshold. This was deemed good enough as a segmentation step, since the bacteria were sparsely distributed over a dark background in a single frame. The MATLAB `regionprops` function was used to extract the area, major axis length (length of the major axis of a fitted ellipse to the detected blob shape), eccentricity, and centroid of each segmented blob. A filtering step was implemented to only

include detected shapes with major axis lengths between 1.7 and 4.5 μm (14 and 38 pixels),
corresponding to the possible size ranges for the bacteria.

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680 **References**

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Figure Legends

Figure 1; Biochemical pathways involved in central carbon catabolism in *P. aeruginosa* PAO1.

The metabolic network was constructed around six main metabolic blocks, identified with different colours: (i) the peripheral pathways, that encompass the oxidative transformation of glucose, acetate and glycerol (orange); (ii) the Embden-Meyerhoff-Parnas pathway (EMP, non-functional in *P. aeruginosa* due to the absence of 6-phosphofructo-1-kinase, purple); (iii) the pentose phosphate pathway (PPP, red); (iv) the Entner-Doudoroff pathway (EDP, green); (v) the tricarboxylic acid cycle and glyoxylate shunt (blue); and (vi) anaplerotic and gluconeogenic bioreactions (grey). Figure adapted from [30].

Figure 2; Comparison between protein and transcript fold-changes for selected *P. aeruginosa*

enzymes involved in central carbon metabolism. The figure shows the log₂ fold-changes in protein and transcript levels in (i) the peripheral pathways, that encompass the oxidative transformation of glucose, acetate and glycerol and the corresponding phosphorylated derivatives of these metabolites (orange); (ii) EMP pathway (non-functional, due to the absence of a 6-phosphofructo-1-kinase activity) (purple); (iii) the pentose phosphate (PP) pathway (red); (iv) the upper ED pathway (green); (v) the tricarboxylic acid cycle and glyoxylate shunt (blue); and (vi) anaplerotic and gluconeogenic bioreactions (grey). RNA-Seq and proteomic data are shown in File S1. Correlation plots are shown in File S3.

Figure 3: Growth on different carbon sources primarily affects metabolism. Illustration of the statistically significant changes ($p\text{-value} \leq 0.01$, fold change ≥ 1 or ≤ -1) during growth on glycerol and acetate as Voronoi tree maps using the proteomaps web service [15]. Most of the proteomic changes were centred around ‘metabolism’, notably ‘central carbon metabolism’, ‘biosynthesis’, ‘signalling and cellular process’ and ‘energy metabolism’. Pathway assignment was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) data set. Proteome alterations which could not be assigned to a specific pathway (uncharacterised/hypothetical proteins) are illustrated as ‘Not Mapped’.

Figure 4; *In vivo* carbon flux distributions in central metabolism of *P. aeruginosa* PAO1 during growth on glycerol (A) or acetate (B) as sole carbon sources. Flux is expressed as a molar percentage of the average glycerol ($9.2 \text{ mmol g}^{-1} \text{ h}^{-1}$) or acetate ($30.4 \text{ mmol g}^{-1} \text{ h}^{-1}$) uptake rate, calculated from the individual rates in File S2. Anabolic pathways from 11 precursors to biomass are indicated as filled blue triangles. The flux distributions with bidirectional resolution (i.e., net and exchange fluxes), including the drain from metabolic intermediates to biomass and confidence intervals of the flux estimates, are provided in File S2. In agreement with previous studies of flux in *P. aeruginosa* and *Pseudomonas fluorescens*, we found no evidence for significant metabolite export during exponential growth in minimal media [19,21,22]. The errors given for each flux reflect the corresponding 90% confidence intervals. The full flux data sets are presented in Supporting Information File S2. Colours qualitatively indicate fluxomic correlation with changes on the protein/transcript level during growth in acetate (light green or red $\rightarrow$ significant up- or down-regulation (respectively); dark green or red $\rightarrow$ less significant up- or down-regulation).

Figure 5; Quantitative analysis of NADPH supply and demand (redox) for glycerol (A) and acetate (B) grown *P. aeruginosa*. ATP (energy metabolism) supply and demand for glycerol (A) and acetate (B) grown *P. aeruginosa*. 6). Reactions linked to NADPH (A-B) and ATP (C-D) metabolism were calculated from the obtained fluxes (Fig. 4). They are given as absolute fluxes ($\text{mmol g}^{-1} \text{h}^{-1}$) and are related to the specific carbon uptake rate (see File S2). G6PDH; glucose 6-phosphate dehydrogenase, MAE; malic enzyme, ICDH; isocitrate dehydrogenase, Ox-P; oxidative phosphorylation, NGAM; non-growth associated maintenance needs.

Figure 6; Maximal measured NADH:NAD⁺ and NADPH:NADP⁺ ratios in *P. aeruginosa* grown in the indicated sole carbon sources. Exponentially-growing cells in MOPS-glycerol have a significantly lower NADH:NAD⁺ ratio ($p < 0.01$) compared with cells grown in MOPS-acetate, MOPS-glucose or MOPS-succinate, and a significantly lower NADPH:NADP⁺ ratio compared with cells grown in MOPS-glucose ($p = 0.0064$). Total NAD(P)(H) concentrations at each time-point and in each carbon source are shown in Figures S2 and S4. The data were analysed using GraphPad Prism (v 6.01) using t-test statistical analysis (MOPS-glycerol *versus* MOPS-acetate, MOPS-glucose or MOPS-succinate).

Figure 7; Coenzyme re-oxidation is impaired in a *dnr* mutant. The NADH:NAD⁺ (A and B) and NADPH:NADP⁺ (C and D) ratios were measured in cultures of wild-type PAO1 (A and C) and in cultures of an isogenic Δdnr mutant (B and D). Cultures were grown in MOPS-acetate -/+ 20 mM KNO₃, as indicated. Corresponding CFUs are shown in Figure S4. The data were analysed using GraphPad Prism (v 6.01) using t-test statistical analysis at 5h growth. Nitrate addition to PAO1 wild-type resulted in a significant reduction in the NADH:NAD⁺ ratio ($p = 0.0017$, indicated by

1108 **) and the NADPH:NADP⁺ ratio (p = 0.0202, indicated by *). Nitrate addition to a Δdnr mutant
1109 did not significantly alter either ratio (p = 0.8065, p = 0.1862).

1110 **Figure S1;** Transcriptomic analysis (CummeRbund) and proteomic analysis (LIMMA Package) of
1111 MOPS-acetate *versus* MOPS-glycerol grown *P. aeruginosa*. A; Principal component analysis
1112 (PCAplot) of RNA-seq data. BS; Scatterplot matrix of gene and isoform level expression
1113 (csScatterMatrix). C; A scatter plot comparing the FPKM values from the two conditions
1114 (csScatter). E; Heatmap and hierarchical clustering of proteomic data. F; Principal component
1115 analysis of proteomic data. G; Pairwise comparison of proteomic data.

1116 **Figure S2;** NADH:NAD⁺ ratios alongside colony forming units (CFUs) from cells grown in MOPS
1117 A; Acetate, B; Glycerol, C: Glucose, D; Succinate. NADPH:NADP⁺ ratios alongside colony forming
1118 units (CFUs) from cells grown in MOPS E; Acetate, F; Glycerol, G: Glucose, H; Succinate. Total
1119 NADP(H) concentrations detected over multiple time points from cells grown in MOPS I;
1120 Acetate, J; Glycerol, K: Glucose, L: Succinate. Total NAD(H) concentrations detected over
1121 multiple time points from cells grown in MOPS M; Acetate, N; Glycerol, O: Glucose, P:
1122 Succinate. Three biological replicates per time-point.

1123 **Figure S3;** Luciferase detection in *P. aeruginosa* PAO1 wild-type carrying chromosomal
1124 promoter: *lux* fusions for the above promoter regions, grown on various carbon sources, MOPS-
1125 acetate, MOPS-glucose, MOPS-glycerol, MOPS-succinate. A; *aceA*, B; *glcB*, C; *cco1*, D; *cco2*, E;
1126 *cox*, F; *cyo*, G; *glpD*, H; *nir*. Values normalised to OD₆₀₀ (RLU/OD₆₀₀). Three biological replicates
1127 per sample. Data analysed in GraphPad Prism (V 6.01) using t-test statistical analysis (MOPS
1128 glycerol *versus* acetate, glucose or succinate).

1129 **Figure S4;** A; Colony forming units (CFUs) B; NAD(H) concentrations, and C: NADP(H)
 1130 concentrations extracted from PAO1 wild-type grown in MOPS-acetate +/- 20 mM KNO₃. Three
 1131 biological replicates per time-point. D; Colony forming units (CFUs) E; NAD(H) concentrations, and F:
 1132 NADP(H) concentrations extracted from PAO1 WT and Δdnr cells grown cells grown in MOPS-acetate +/-
 1133 20 mM KNO₃. Three biological replicates per time-point.

1134 **Figure S5;** Western blot showing; (A) Expression of the Anr/Dnr-regulated denitrification
 1135 enzyme NirS during exponential growth of *P. aeruginosa* in MOPS-glucose, MOPS-succinate and
 1136 MOPS-acetate. NirS is not expressed in a Δanr or Δdnr mutant. The isocitrate dehydrogenases
 1137 (ICD and IDH) were used as loading controls. (B) Expression of NirS during exponential growth
 1138 of *P. aeruginosa* in MOPS-acetate and MOPS-acetate + sodium nitrate (20 mM). Sodium nitrate
 1139 (20 mM) is capable of inducing NirS expression in the WT and in a $\Delta roxSR$ mutant, but not in a
 1140 Δanr or Δdnr mutant. Three biological replicates per sample. The Δanr , Δdnr and $\Delta roxR$ lanes
 1141 are representative of the triplicates.

1142 **Figure S6;** Bacterial cell length as determined by fluorescence microscopy, PAO1 carrying the
 1143 pMF230 eGFP-expressing plasmid. Cells grown in MOPS media with acetate, glucose, glycerol
 1144 or succinate as the sole carbon sources. Actual data points shown in File S3.

1145 **Table S1;** A; Oligonucleotide primers used in this study. B; Bacterial strains and plasmids used in
 1146 this study [14,51,84,104].

1147 **File_S1;** Full transcriptomics (tab A) and proteomics (tab B) of MOPS-acetate *versus* MOPS-
 1148 glycerol grown *P. aeruginosa*.

1149 **File_S2;** ^{13}C fluxomics data of MOPS acetate and MOPS glycerol grown *P. aeruginosa*, including
 1150 calculations for anabolic demand (A), OpenFLUX SimVector files (B), reaction network (C),
 1151 goodness of fit (D) and metabolic fluxes (E).

1152 **File_S3;** Correlation plot calculations to illustrate the log2 fold-change differences between the
 1153 transcriptomic, proteomic and fluxomic data (A-C) and cell size data (D).

Figure 1; Biochemical pathways involved in central carbon catabolism in *P. aeruginosa* PAO1.

	Key	Enzyme(s)	PAO1 Gene Name(s) and PA number(s)
PERIPHERAL PATHWAYS	1	Glucose dehydrogenase	PA2290 (Gcd)
	2	Gluconate 2-dehydrogenase	PA2265 (Gad)
	3	Gluconate kinase	PA2321 (GntK)
	4	2-Ketogluconate kinase	PA2261 (KguK)
	5	2-Ketogluconate-6-P- reductase	PA2263 (KguD)
	6	Acetyl-CoA synthetase / Acetate kinase and Phosphotransacetylase	PA0887 (AcsA) / PA0836 (AckA), PA0835 (Pta)
	7	Glycerol Kinase	PA3582 (GlpK)
	8	sn-Glycerol-3-phosphate dehydrogenase	PA3584 (GlpD)
	9	Glycerol-3-phosphate dehydrogenase	PA1614 (GpsA)
EDP	10	6-Phosphogluconate dehydratase	PA3194 (Edd)
	11	2-Keto-3-deoxy-6-Phosphogluconate aldolase	PA3181 (Eda)
PPP	12	Glucose-6-P-1-dehydrogenase	PA5439, PA3183 (Zwf)
	13	6-Phosphogluconolactonase	PA3182 (Pgl)
	14	Ribulose-5-P-3-epimerase	PA0607 (Rpe)
	15	Ribose-5-P- isomerase	PA0330 (RpiA)
	16	Transketolase	PA0548 (TktA)
	17	Transaldolase B	PA2796 (Tal)
EMPP	18	Glucokinase	PA3193 (Glc)
	19	Glucose-6-P- isomerase	PA4732 (Pgi)
	20	Fructose-1,6-bisphosphatase	PA5110 (Fbp)
	21	Fructose-1,6-P-2 aldolase	PA0555 (Fda)
	22	Triose phosphate isomerase	PA4748 (TpiA)
	23	Glyceraldehyde-3-P- dehydrogenase	PA3195 (GapA), PA3001, PA2323 (GapN)
	24	Phosphoglycerate kinase	PA0552 (Pkg)
	24	Phosphoglycerate mutase	PA5131 (Pgm)
	25	Phosphopyruvate hydratase / Enolase	PA3635 (Eno)
KREBS CYCLE AND GLYOXYLATE SHUNT	25	Pyruvate Kinase	PA4329 (PykA), PA1498 (PykF)
	26	Phosphoenolpyruvate synthase	PA1770 (PpsA)
	27	Pyruvate dehydrogenase	PA5015 (AceE), PA5016 (AceF), PA4152 (AcoC), PA1587 (LpdG)
	28	Citrate synthase	PA1580 (GltA), PA0795 (PrpC)
	29	Aconitate synthase	PA1562 (AcnA), PA1787 (AcnB)
	30	Isocitrate dehydrogenase	PA2623 (Icd), PA2624 (Idh)
	31	2-Ketoglutarate dehydrogenase	PA1585 (SucA), PA1586 (SucB), PA4829 (Lpd3)
	31	Succinyl-coenzyme A synthetase	PA1588 (SucC), PA1589 (SucD)
	32	Succinate dehydrogenase	PA1581 (SdhC), PA1582 (SdhD), PA1583 (SdhA), PA1584 (SdhB)
ANAPLEUROSIS GLUCONEOGENESIS	33	Fumarate hydratase	PA4470 (FumC1), PA0854 (FumC2), PA4333 (FumA)
	34	Malate: quinone oxidoreductase	PA3452 (MqoA), PA4640 (MqoB)
	35	Isocitrate lyase	PA2634 (AceA)
	36	Malate synthase	PA0482 (GlcB)
	37	Malic enzyme	PA3471 (MaeA), PA5046 (MaeB)
	38	Pyruvate carboxylase	PA5435/PA5436 (PC)
	39	Phosphoenolpyruvate carboxylase	PA3687 (Ppc)
	40	Phosphoenolpyruvate carboxykinase	PA5192 (PckA)

Figure 2; Comparison between protein and transcript fold-changes for selected *P. aeruginosa* enzymes involved in central carbon metabolism

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Key	Gene	PA01	Protein Description	Log2(FC)_Protein	Log2(FC)_RNA
1	<i>gcd</i>	PA2290	Gcd, glucose dehydrogenase	-1.09	-1.03
2	<i>gnd</i>	PA2265	Gnd, gluconate dehydrogenase	-0.91	-0.28
3	<i>gntK</i>	PA2321	GntK, gluconokinase	-1.93	-1.30
4	<i>kguK</i>	PA2261	KguK, probable 2-ketogluconate kinase	N/A	-0.82
5	<i>kguD</i>	PA2263	KguD, 2-hydroxyacid dehydrogenase	0.57	-0.12
6	<i>acsA</i>	PA0887	AcsA, acetyl-coenzyme A synthetase	2.47	4.69
	<i>ackA</i>	PA0836	AckA, acetate kinase	0.6	0.51
	<i>pta</i>	PA0835	Pta, phosphate acetyltransferase	0.52	1.02
7	<i>glpK</i>	PA3582	GlpK, glycerol kinase	-3.93	-5.31
8	<i>glpD</i>	PA3584	GlpD, glycerol-3-phosphate dehydrogenase	-3.4	-7.64
9	<i>gpsA</i>	PA1614	GpsA, glycerol-3-phosphate dehydrogenase	0.25	0.25
10	<i>edd</i>	PA3194	Edd, phosphogluconate dehydratase	-3.53	-4.21
11	<i>eda</i>	PA3181	Eda, 2-keto-3-deoxy-6-phosphogluconate aldolase	-4.27	-4.62
12	<i>PA5439</i>	PA5439	glucose-6-phosphate dehydrogenase	0.15	0.21
	<i>zwf</i>	PA3183	Zwf, glucose-6-phosphate 1-dehydrogenase	-3.91	-6.01
13	<i>pgl</i>	PA3182	Pgl, 6-phosphogluconolactonase	-3.1	-5.74
	<i>rpe</i>	PA0607	Rpe, ribulose-phosphate 3-epimerase	0.82	0.66
14	<i>rpiA</i>	PA0330	RpiA, ribose 5-phosphate isomerase	-0.06	-0.17
15/16	<i>tktA</i>	PA0548	TktA, transketolase	-0.05	0.03
17	<i>tal</i>	PA2796	Tal, transaldolase	-0.63	0.29
18	<i>glk</i>	PA3193	Glk, glucokinase	-2.1	-2.87
19	<i>pgi</i>	PA4732	Pgi, glucose-6-phosphate isomerase	-0.11	0.12
20	<i>fbp</i>	PA5110	Fbp, fructose-1,6-bisphosphatase	0.14	-0.05
21	<i>fda</i>	PA0555	Fda, fructose-1,6-bisphosphate aldolase	0.07	-0.09
22	<i>tpiA</i>	PA4748	TpiA, triosephosphate isomerase	0.2	0.20
23	<i>gapA</i>	PA3195	GapA, glyceraldehyde 3-phosphate dehydrogenase	-3.76	-4.67
	<i>PA3001</i>	PA3001	glyceraldehyde-3-phosphate dehydrogenase	1.34	1.07
	<i>gapB</i>	PA2323	GapB, glyceraldehyde-3-phosphate dehydrogenase	-2.79	-2.49
	<i>pgk</i>	PA0552	Pgk, phosphoglycerate kinase	0.86	0.88
24	<i>pgm</i>	PA5131	Pgm, phosphoglycerate mutase	0.13	0.20
	<i>eno</i>	PA3635	Eno, enolase	0.43	0.56
25	<i>pykA</i>	PA4329	PykA, pyruvate kinase	0.55	0.71
26	<i>ppsA</i>	PA1770	PpsA, phosphoenolpyruvate synthase	0.32	0.20
27	<i>aceE</i>	PA5015	AceE, pyruvate dehydrogenase	-2.24	-0.51
	<i>aceF</i>	PA5016	AceF, dihydrolipoamide acetyltransferase	-2.01	-0.19
	<i>acoC</i>	PA4152	AcpC, hydrolase	-0.04	-0.21
	<i>lpdG</i>	PA1587	Lpd, dihydrolipoamide dehydrogenase	0.98	0.98
28	<i>glcA</i>	PA1580	GlcA, citrate synthase	1.64	0.95
	<i>prpC</i>	PA0795	PrpC, citrate synthase 2	0.52	1.31
29	<i>acnA</i>	PA1562	AcnA, aconitate hydratase 1	-0.9	-0.19
	<i>acnB</i>	PA1787	AcnB, aconitate hydratase 2	2.16	1.96
30	<i>icd</i>	PA2623	Icd, isocitrate dehydrogenase	-0.26	1.29
	<i>ldh</i>	PA2624	Ldh, isocitrate dehydrogenase	2.34	1.79
31	<i>sucA</i>	PA1585	SucA, 2-oxoglutarate dehydrogenase (E1)	1.45	1.01
	<i>sucB</i>	PA1586	SucB, dihydrolipoamide succinyltransferase (E2)	1.25	1.01
32	<i>sucC</i>	PA1588	SucC, succinyl-CoA synthetase beta chain	0.05	0.67
	<i>sucD</i>	PA1589	SucD, succinyl-CoA synthetase alpha chain	0.06	0.72
	<i>sdhC</i>	PA1581	SdhC, succinate dehydrogenase (C subunit)	1.46	0.60
	<i>sdhD</i>	PA1582	SdhD, succinate dehydrogenase (D subunit)	0.78	0.64
	<i>sdhA</i>	PA1583	SdhA, succinate dehydrogenase (A subunit)	1.31	0.85
	<i>sdhB</i>	PA1584	SdhB, succinate dehydrogenase (B subunit)	1.06	1.04
33	<i>fumC1</i>	PA4470	FumC1, fumarate hydratase	0.13	-0.53
	<i>fumC2</i>	PA0854	FumC2, fumarate hydratase	-0.94	0.04
	<i>fumA</i>	PA4333	FumA, probable fumarase	1.35	0.64
34	<i>mqaA</i>	PA3452	MqaA, malate:quinone oxidoreductase	-0.82	-1.52
	<i>mqaB</i>	PA4640	MqaB, malate:quinone oxidoreductase	1.82	1.65
35	<i>aceA</i>	PA2634	AceA, isocitrate lyase	4.47	3.51
36	<i>glcB</i>	PA0482	GlcB, malate synthase G	2.56	1.98
37	<i>maeA</i>	PA3471	MaeA, malic enzyme	0.29	0.19
	<i>maeB</i>	PA5046	MaeB, Malic enzyme	-1.35	-1.10
38	<i>oadA</i>	PA5435	PC, pyruvate carboxylase	-1.25	-0.93
	<i>accC</i>	PA5436	PC, pyruvate carboxylase	-1.1	-0.75
39	<i>ppc</i>	PA3687	Ppc, phosphoenolpyruvate carboxylase	-0.42	0.10
40	<i>pckA</i>	PA5192	PckA, phosphoenolpyruvate carboxykinase	1.32	0.96

Figure 3: Growth on different carbon sources primarily affects metabolism.

Figure 5; Quantitative analysis of NADPH supply and demand (redox) for glycerol (A) and acetate (B) grown *P. aeruginosa*.

Figure 6; Maximal measured NADH:NAD⁺ and NADPH:NADP⁺ ratios in *P. aeruginosa* grown in the indicated sole carbon sources.

Figure 7; Coenzyme re-oxidation is impaired in a *dnr* mutant.