Redox stress reshapes carbon fluxes of *Pseudomonas putida* for cytosolic glucose oxidation and NADPH generation

by

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Abstract

The soil bacterium and metabolic engineering platform *Pseudomonas putida* tolerates high levels of endogenous and exogenous oxidative stress, yet the ultimate reason of such property remains unknown. To shed light on this question, NADPH generation routes—the metabolic currency that fuels redox stress responses—were assessed when *P. putida* KT2440 was challenged with H₂O₂ as proxy of oxidative conditions. ¹³C-tracer experiments, metabolomics and flux analysis, together with inspection of physiological parameters and measurement of enzymatic activities, revealed a substantial flux reconfiguration under oxidative stress. In particular, periplasmic glucose processing was rerouted to cytoplasmic oxidation, and cyclic operation of the pentose phosphate pathway led to significant NADPH fluxes, exceeding biosynthetic demands by ~50%. This NADPH surplus, in turn, fuelled the glutathione system for H₂O₂ reduction. These properties not only contribute to the tolerance of *P. putida* to environmental stresses, but they also highlight the value of this host for harsh biotransformations.

Introduction

Oxidative stress is an unavoidable consequence of aerobic cell respiration, characterized by the systemic manifestation of reactive oxygen species (ROS) followed by activation of multiple responses deployed to detoxify reactive intermediates¹⁻⁴. Peroxides and free radicals damage virtually all macromolecular components of the cell (e.g. proteins, lipids and DNA^{5,6}), and mechanisms to prevent (or repair) the damage exerted by ROS are key components of virtually all biological systems⁷. Long-term transcriptional responses that scavenge ROS, for instance, appear to be conserved across species⁸. Model microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae* have been shown to activate coordinated transcriptional responses that include up-regulation of ROS-quenching superoxide dismutases, catalases and glutathione/glutaredoxin recycling systems⁹⁻¹². Similarly, mammalian cells display long-term anti-oxidative responses for ROS detoxification and, depending on the severity of oxidative stress, initiate either pro-survival gene expression strategies to support NADPH formation¹³, ROS clearance and (in most cases) DNA repair¹⁴, or they commit cell death programs^{15,16}. However, until transcriptionally-regulated defence mechanisms become fully operational, cell survival depends on the basal activity of the above-mentioned enzymes and availability of antioxidants (e.g. reduced glutathione^{17,18}) to scavenge at least part of the ROS generated by respiration or direct oxidative damage. Besides transcriptional responses, metabolic programmes are rapidly deployed upon exposure of bacteria, yeast and mammalian cells to oxidative stress¹⁹⁻²², thereby providing reducing power to fuel ROS detoxifying enzymes²³. However, metabolic mechanisms to counteract oxidative stress in non-model organisms remain poorly studied.

Environmental bacteria are constantly exposed to different types of stress in the niches they colonize. *Pseudomonas putida*, a strictly-aerobic, Gram-negative soil bacterium characterized by a remarkable metabolic versatility²⁴⁻²⁷, is equipped with the enzymatic machinery needed to catabolize both natural and recalcitrant aromatic compounds besides sugars and organic acids²⁸. While many of the scenarios where *P. putida* meets such environmental pollutants are oxidative *per se*, metabolic pathways for their biodegradation often involve harsh redox reactions prone to generate ROS endogenously²⁹. The adaptability of *P. putida* KT2440 (type strain of this species³⁰) to adverse conditions, including exposure to aromatic substrates that cause endogenous oxidative stress, is wired to a unique metabolic architecture. Glucose catabolism in *P. putida* relies on the Entner-Doudoroff (ED) pathway³¹, which

starts with 6-phosphogluconate (6PG) as the substrate, formed *via* separate and converging routes for hexose phosphorylation (in the cytoplasm) or oxidation (in the periplasm)³². Additionally, the *EDEMP* cycle, a combination of enzymes from the ED, the pentose phosphate (PP) and the (incomplete) Embden-Meyerhof-Parnas (EMP) pathways³³, processes hexoses phosphate to feed trioses into the lower catabolism, i.e. downwards phosphoenolpyruvate (PEP), pyruvate (Pyr) and the tricarboxylic acid (TCA) cycle. The metabolic network of *P. putida* KT2440 and biochemical reactions considered in this study are summarized in **Supplementary Fig. S1** and **Supplementary Table S1** respectively. In addition to catabolic processing of sugars, the operation of the EDEMP cycle entails redirecting part of the trioses phosphate back to hexoses phosphate *via* the gluconeogenic activity of the EMP branch of the cycle³⁴. As such, cyclic sugar catabolism contributes to a slight catabolic overproduction of NADPH in glucose cultures³³. The EDEMP cycle has been also shown to be important for the hierarchical consumption of sugars and aromatic compounds³⁵. While the main sink of NADPH is the anabolic use of reducing power for biomass formation, this redox cofactor is also key to counterfeit oxidative stress since it serves as the reductant for several ROS detoxifying enzymes and mechanisms³⁶, e.g. glutathione regeneration. However, the connection between the operation of central carbon metabolism and oxidative stress responses remained elusive thus far.

In this work, the reshaping of the central metabolism in *P. putida* KT2440 in response to sub-lethal doses of oxidative stress elicited by exposure to hydrogen peroxide (H₂O₂) has been explored. The experimental strategy encompassed the use ¹³C-tracer experiments, metabolomics and metabolic flux analysis, combined with determination of physiological parameters, *in vitro* measurement of key enzymatic activities and assessment of glutathione-mediated ROS detoxifying mechanisms. The results revealed adaptations in the operation of the EDEMP cycle, concomitant with a decrease in the flux through sugar oxidation pathways for glucose processing, which helps replenishing the NADPH pool upon oxidative challenges. These metabolic adjustments are discussed in the light of the lifestyle of *P. putida* (and related environmental bacteria) and the value of this species as robust platform for hosting redox-demanding biotransformations.

Results

Quantitative physiology analysis of *P. putida* exposed to sub-lethal doses of oxidative stress. In order to capture the metabolic effects of oxidative stress in *P. putida* KT2440 without compromising cell viability, the experimental design (summarized in **Fig. 1a**) entailed exposure of the cultures, grown in M9 minimal medium with glucose until mid-exponential phase [optical density at 600 nm (OD_{600}) of ca. 0.5], to H_2O_2 at 1.5 mM. Cultures with different ^{13}C -tracers ([1- ^{13}C]-, [6- ^{13}C]- and [U- ^{13}C]-glucose, all of them added at 20 mM) were grown in parallel to resolve the relative contribution of cyclic EDMP, PP and ED pathway to sugar processing. At 1.5 h after the oxidative stress treatment, samples were collected to assess quantitative physiology parameters, flux ratio analysis and to perform *in vitro* biochemical assays. This experimental setup resulted in similar growth patterns in the absence and presence of the oxidative stress agent (**Fig. 1b**), and neither the specific growth rate (μ) nor the final cell density (plateauing at ca. 10 h of cultivation) were significantly affected by the addition of H_2O_2 to the cultures. Quantitative analysis of physiology parameters confirmed that this was the case (**Fig. 1c**), with μ , the specific rate of glucose uptake (q_s) and the yield of biomass on substrate ($Y_{X/S}$) being essentially the same in control and H_2O_2 -treated experiments. The formation and secretion of organic acids to the extracellular milieu, a typical feature of glucose-grown *P. putida*, decreased significantly in the presence of the stressor. *P. putida* typically oxidizes glucose in the periplasm into gluconate and 2-ketogluconate³¹, which are then taken up by the cell³⁷. The about halved concentrations of the two products of sugar oxidation in H_2O_2 -treated cultures indicates a decrease in periplasmic oxidation upon exposure to stressful conditions. The concentration of both acids peaked during exponential growth, and the corresponding molar yields of gluconate and 2-ketogluconate on glucose in control experiments were $y_{G/S} = 0.31 \pm 0.09$ and $y_{K/S} = 0.12 \pm 0.02$ C-mol C-mol⁻¹, respectively. In the presence of oxidative stress, $y_{G/S}$ and $y_{K/S}$ were 0.19 ± 0.08 and 0.05 ± 0.01 C-mol C-mol⁻¹, suggestive, again, of a redistribution of metabolic fluxes between oxidative (i.e. glucose $\rightarrow$ gluconate $\rightarrow$ 2-ketogluconate) or phosphorylative [i.e. glucose $\rightarrow$ glucose-6-phosphate (G6P)] processing routes of the carbon source (**Supplementary Fig. S1**). Since a shift from periplasmic to cytoplasmic glucose oxidation would entail higher NADPH formation, we next looked for direct evidence of increased intracellular fluxes through these pathways.

PP pathway metabolite pools increase upon exposure of *P. putida* to oxidative stress. To assess the adaptations within the metabolic network of *P. putida* upon exposure to oxidative stress, the relative abundance of key metabolites was quantified by LC-MS/MS. Out of the metabolites measured, the species that showed the most significant changes in terms of abundance were intermediates of the PP pathway, the EMP route and the TCA cycle—albeit in opposite directions (**Fig. 2a**). In particular, the relative abundance of 6PG (key metabolic node where oxidative and phosphorylative pathways for glucose processing converge; **Supplementary Fig. S1**), ribose-5-phosphate (R5P), ribulose-5-phosphate (Ru5P), xylulose-5-phosphate (Xu5P) and sedoheptulose-7-phosphate (S7P) augmented significantly in H₂O₂-stressed cells, ranging from a 2- (Xu5P) to 7.6-fold (Ru5P) increase. At the same time, the relative abundance of glycolytic intermediates of the EMP route decreased roughly by half (e.g. fructose-6-phosphate, F6P) and up to 70% (e.g. Pyr) when cells were exposed to oxidative stress. Intermediates of the TCA cycle shared the same fate, and their relative abundance was reduced at least by 60% (e.g. isocitrate, ICT) and up to ca. 90% (e.g. 2-ketoglutarate, KG; succinate, SUC; and malate, MAL) in stressed cells. The changes in the relative abundance of other metabolites in the biochemical network of *P. putida* KT2440 upon exposure to the oxidative stress agent are listed in **Supplementary Data 1**. Taken together, these results indicate increased fluxes through the PP pathway coupled to a decrease in the catabolic activities of the EMP route and the TCA cycle during oxidative stress. However suggestive, these metabolomic data cannot be used to infer relative or net fluxes, and flux ratio analysis was employed to piece together the contribution of fluxes within the upper metabolism of *P. putida* to the pool of glycolytic intermediates.

Increased cyclic operation of PP but not ED pathway upon exposure to oxidative stress. The relative contributions of reverse (i.e. gluconeogenic) flux from triose phosphates or through the PP pathway to the hexose phosphate pool was elucidated by using relative flux ratios derived from [1-¹³C₁]- and [6-¹³C₁]-glucose-labeling experiments. Calculation of relative ratios around the hexose node was possible given that the reaction catalyzed by 6-phosphofructo-1-kinase is absent in *P. putida*^{38,39}, and by considering the reactions mediated by GntZ (6PG dehydrogenase) and Edd (6PG dehydratase) to be irreversible. In particular, using [6-¹³C₁]-glucose as a tracer enables the resolution of fluxes from the cyclic PP and ED pathways back to hexose phosphates because the C6 position is maintained in both routes and can lead to double-labeled hexose phosphate molecules. Flux ratio analysis supported the

notion that fluxes through the PP pathway significantly responded to H₂O₂ addition (**Fig. 2b**), in particular in the oxidative branch of this metabolic pathway.

Experiments conducted in the presence of [1-¹³C₁]-glucose suggested that the contribution of glucose kinase (Glc, v_3 in **Supplementary Fig. S1**) to the G6P pool was not significantly affected by oxidative stress, whereas the fraction of 6PG from G6P via G6P dehydrogenase (Zwf, v_7 in **Fig. S1**) increased by at least 3.5-fold (as deduced from experiments conducted with either [6-¹³C₁]- or [U-¹³C₆]-glucose) in stressed *P. putida*. The increase was even more pronounced for the flux ratio reflecting the fraction of F6P from the PP pathway (4.5-fold higher in cultures treated with H₂O₂ than in control experiments). The full list of flux ratios, including those for reactions in lower catabolism, is presented in **Supplementary Table S2**, and the GC-MS data are given in **Supplementary Data 2**. These results provided further evidence for a flux rerouting from periplasmic glucose oxidation to the NADPH-generating PP pathway as a direct metabolic response to oxidative stress. We next quantified net fluxes as the basis for accurate estimation of NADPH production in response to oxidative stress.

Redistribution of net fluxes in *P. putida* as a response to sub-lethal oxidative stress. Combining the quantitative physiology data (**Fig. 1c**) with the flux ratios as additional constraints (**Fig. 2b** and **Supplementary Table S2**), we estimated net fluxes through the network (**Fig. 3**, and **Supplementary Table S3**). Since the relative contribution of the direct phosphorylation of gluconate to 6PG and that of the indirect (NADPH-dependent) route through 2-ketogluconate could not be resolved from ¹³C-labeling data, we determined the *in vitro* enzyme activities of gluconate kinase (GnuK, i.e. direct phosphorylation of the sugar) and 2-ketogluconate-6-phosphate reductase (KguD, i.e. 6PG from 2-ketogluconate) as a proxy. GnuK and KguD activities in cell-free extracts of *P. putida* KT2440 were similar under control and stress conditions, and we thus used a GnuK/KguD activity ratio of 0.87/0.13 to constrain the flux split at the gluconate branch point.

Under control conditions, glucose was processed mostly through the periplasmic pathway with about 90% of the sugar uptake rate channeled via Gcd (**Fig. 3a**). Most of the gluconate fed the 6PG node, with a very high flux through the ED route and a ca. 10% recycling of trioses phosphate through the EDMP cycle. Upon exposure to H₂O₂, *P. putida* substantially re-routed its carbon fluxes (**Fig. 3b**). Periplasmic glucose oxidation decreased, and instead much more glucose was imported into the

cytoplasm and converted to 6PG through the NADP⁺-dependent Zwf. Since the glucose uptake rate was essentially identical in the two experimental conditions, this large relative flux change reflects a likewise large change in absolute fluxes. In particular, a major change in flux distribution was detected in the non-oxidative branch of the PP pathway. Exposure of the cells to H₂O₂ increased the GntZ flux by more than 6-fold, which fed the pools of other pentoses phosphate (as suggested by the measurement of their relative abundance increase, **Fig. 2**). The backward flux in this route fed the F6P and T3P node, resulting in carbon recycling *via* glucose-6-phosphate isomerase (Pgi, in the gluconeogenic F6P → G6P direction) and Zwf (G6P → 6PG). Accordingly, fluxes through Pgi and Zwf in H₂O₂-treated cells increased by 5.2- and 4.5-fold, respectively. The ED route was still the predominant catabolic pathway for 6PG under oxidative stress, albeit with a slight decrease in the flux values as compared to control conditions. Gluconeogenic fluxes *via* fructose-1,6-bisphosphate aldolase (Fda) and fructose-1,6-bisphosphatase (Fbp) had a small increase (1.5-fold) in cultures exposed to oxidative stress, and flux through the lower, catabolic branch of the EMP pathway (from glyceraldehyde-3-phosphate to PEP and Pyr) was reduced by ca. 10% under oxidative stress. The diminished input of the ED and the EMP catabolism under oxidative stress propagated into a relatively low flux towards acetyl-CoA (i.e. Pyr dehydrogenase) and fluxes within the TCA cycle. No significant activity of the glyoxylate shunt could be detected under any of the experimental conditions tested. Anaplerotic routes within the Pyr shunt, typical of glucose-grown *P. putida*, had a likewise lower activity in H₂O₂-stressed cells. The impact of oxidative stress-mediated re-arrangement of carbon fluxes on redox metabolism was investigated next.

Re-arrangement of net fluxes in *P. putida* under oxidative stress increases NADPH supply.

Considering that the flux through Zwf and GntZ, two of the major NADP⁺-dependent dehydrogenases in the biochemical network, showed a significant increase under oxidative stress, the overall net NADPH production increased substantially. This reconfiguration presumably matches the increased NADPH demand to regenerate glutathione (and other anti-oxidants) for H₂O₂ reduction. To assess this impact quantitatively, the net rate of NADPH production (R_{NADPH}) was calculated according to $\sum_i (r_i^F - r_i^C)$, where r_i^F and r_i^C represent the rates of NADPH formation and consumption, respectively, of all major dehydrogenases potentially carrying significant flux in the biochemical network (see **Supplementary Fig. S1**). Zwf, GntZ, Gap, Icd, Mdh and MaeB were considered as potential inputs to NADPH formation for this analysis, whereas biomass formation and the KguD activity were included as NADPH sinks. The values of r_i were derived from the actual previously calculated net fluxes, and the cofactor specificity of

all dehydrogenases under both saturating, *in vitro* conditions and non-saturating, *quasi in vivo* conditions (**Supplementary Table S4**) was used to derive the r_i values as previously indicated. By combining these parameters, R_{NADPH} was estimated for *P. putida* in both control and H_2O_2 -treated experiments (**Fig. 4**). When using cofactor specificities determined under saturating, *in vitro* conditions, NADPH formation and consumption in control experiments added up (i.e. $R_{\text{NADPH}} = 0$, **Fig. 4a**), whereas adjusting these specificities according to non-saturating, *quasi in vivo* determinations revealed a slight catabolic overproduction of reducing power characteristic of strain KT2440 ($R_{\text{NADPH}} = 1.3 \text{ mmol g}_{\text{CDW}}^{-1} \text{ h}^{-1}$, **Fig. 4b**). In H_2O_2 -stressed cells, R_{NADPH} was > 0 irrespective of the cofactor specificity used to calculate the individual r_i values (**Figs. 4c and 4d**). In particular, when applying the specificity coefficients derived from non-saturating, more realistic *quasi in vivo* determinations, R_{NADPH} increased by 3.6-fold (**Fig. 4d**) as compared to the control condition (**Fig. 4b**). Taken together, these results expose a ca. 50% surplus in NADPH formation under oxidative stress conditions that becomes available to counteract ROS accumulation.

To ensure that our NADPH balance estimates are not biased by key assumptions, we assessed the sensitivity of net NADPH formation as a function of the GnuK/KguD activity ratio at the gluconate node and the NADP^+ -specificity of the dehydrogenase reaction catalyzed by Zwf. The Zwf activity of *P. putida* KT2440 is represented by three isozymes (i.e. Zwf, ZwfA and ZwfB; **Supplementary Table S1**). Even when we have determined NADP^+ and NAD^+ specificities for the overall Zwf reaction with *in vitro* assays, it is likely that the individual isozymes may have different cofactor preferences, which would have a direct effect on the overall redox balance if expressed differently. Irrespective of the cofactor preferences (0.672 and 0.937 either under saturating conditions or *quasi in vivo* conditions, respectively; see **Supplementary Table S4**), R_{NADPH} was relatively more responsive to changes in the GnuK/KguD flux ratio in control, non-stressed experiments (**Figs. 4e and 4f**). The opposite was true for H_2O_2 -treated cells, with a strong dependence of R_{NADPH} on the output of the Zwf flux (**Figs. 4g and 4h**) mainly because the relative flux through Zwf increased in comparison to the control experiment. These calculations demonstrate that the NADPH surplus observed in stressed cells largely stems from the NADP^+ -specificity of Zwf, with the GnuK/KguD flux ratio playing a minor role on R_{NADPH} values.

Altered expression of enzymes in glucose catabolism during oxidative stress. In agreement with the relative flux distribution (**Fig. 3**), there was a significant decrease in the activities of enzymes within the peripheral reactions for glucose oxidation under oxidative stress conditions (**Fig. 5a**). The observed decrease was particularly significant in the case of the specific Gad activity (gluconate → 2-ketogluconate), which was roughly halved in cells exposed to H₂O₂. Hexose kinase activity, accordingly, increased by 2-fold. The activity of the two NADPH producing dehydrogenases of the PP pathway was more significant, with the specific Zwf and GntZ activities increasing by 4.7- and 9.2-fold, respectively. The *in vitro* activities of two enzymes of the ED pathway, in contrast, remained unchanged within the experimental error in control and H₂O₂-stressed cells. All these results are consistent with the previous observations underline the importance of PP pathway for NADPH production when cells are challenged with an oxidative stress agent. We next explored the connection between catabolic NADPH overproduction and mechanisms that counteract oxidative stress such as the glutathione-dependent redox system.

The glutathione system of *P. putida* links H₂O₂-induced overproduction of NADPH to ROS quenching. Thiols play several roles in bacteria, including maintenance of the redox balance, fighting ROS and nitrogen reactive species, and detoxification of other toxins and stress-inducing factors. In most organisms, the major thiol is the tripeptide glutathione (GSH, γ-L-glutamyl-L-cysteinylglycine). The formation of GSH from L-glutamate and L-cysteine in strain KT2440 is catalyzed by GshAB (glutamate-cysteine ligase and glutathione synthase, respectively; **Fig. 5b**). The glutathione cycle, connecting the reduced (GSH) and the oxidized (GS-SG) forms of the thiol, acts as a first-line reductant of ROS (**Fig. 5b**) via glutathione peroxidases (e.g. PP_0777 and PP_1686) and glutaredoxins. Glutathione reductase (Gor, PP_3819) then replenishes the GSH pool by using NADPH as the reducing currency. Since the glutathione system of *P. putida* has not been explored thus far from a biochemical point of view, the cellular content of both GSH and GS-SG was assessed by means of a biochemical assay in both control and H₂O₂-stressed cultures (**Fig. 5c**). Cells exposed to H₂O₂ had a 1.6-fold higher total content of glutathione (i.e. GSH and GS-SG) than control experiments, and the composition of the total pool changed significantly upon exposure to oxidative stress. The GSH/GS-SG molar ratio, which reflects the fraction of reduced thiol in the cells, increased by 3-fold in H₂O₂-stressed *P. putida*. This observation suggests that the reduced form of glutathione can be sufficiently replenished by the cellular

NADPH sources upon an oxidative stress—directly linking the increased NADPH turnover with the pool of antioxidants as known from well-studied species.

Discussion

Environmental microorganisms are constantly exposed to a variety of physicochemical perturbations that require dynamic and concerted responses to ensure cell survival⁴⁰. In this context, *P. putida* KT2440 provides a unique experimental system to explore the interplay between stress and metabolic adaption. This understanding has not only a fundamental biological interest, but also a practical one in view of the growing demand of robust *chassis* for synthetic biology and metabolic engineering⁴¹. In this sense, the results of this study strengthen the notion that network-wide adaption of metabolic fluxes^{42,43} accompanies (and plausibly, enables) what has been traditionally described—mostly at a genetic and regulatory level—as *general stress response*^{44–47}. Metabolic adaption to oxidative stress has been recently revisited by Christodoulou *et al.*²³, demonstrating how reserve flux capacity in the PP pathway empowers *E. coli* to rapidly respond to H₂O₂ by increased flux through Zwf and Gnd. It was postulated that inhibition of Zwf caused by NADPH (either by a competitive, allosteric or combined mechanism) is lifted under these conditions—releasing ‘reserve’ flux through the oxidative PP pathway. Moreover, the activity of glyceraldehyde-3-phosphate is lowered due to ROS-mediated damage, which causes a metabolic *jam* in the metabolite pools of the PP route as previously shown in yeast⁴⁸. Product inhibition of Zwf is lowered due to NADPH drainage to counteract oxidative stress⁴⁹, and the sudden enlargement of the 6PG pool is channelled *via* Gnd. Recently, such an NADPH replenishing mechanism catalysed by Zwf and Gnd has been recognized as a general strategy in enteric bacteria, yeast and mammalian cells⁵⁰. Here, we asked how *P. putida*, which does not have a linear, EMP-based glycolysis and processes glucose mostly through the conversion of the sugar into gluconate, reacts to oxidative stress. Since glycolysis is not an alternative glucose degradation route and therefore *P. putida* cannot alter the split ratio between the EMP and PP pathways, we hypothesized that the EDEMP cycle could contribute to increased NADPH production under oxidative stress *via* the PP pathway branch.

Metabolite changes and flux analysis revealed that the lower (EMP) glycolysis and the PP pathway were affected by H₂O₂ as compared with untreated controls. In a simplified model of the upper pathways for sugar processing (**Fig. 6a**), the output of NADPH is largely governed by the fluxes through

Zwf and GntZ—that, in turn, respond to the split between oxidative conversion of glucose into gluconate or phosphorylation into G6P, and the recycling activity of the EDEMP cycle. As such, the overall NADPH turnover is dependent on r_s (**Fig. 6b**), and is further modulated by the fraction of glucose oxidized in the bacterial periplasm. Consequently, a low activity of the EDEMP cycle contributes to NADPH formation when cells grow in the absence of any oxidative stress. Upon an oxidative challenge, the backward flux from the PP and ED routes to F6P increases >3 fold, and the ED flux through lower glycolysis decreases due to re-arrangement. The jump in the activities through the cyclic PP pathway is most remarkable, as *P. putida* maintains very low fluxes within this metabolic block under normal growth conditions⁵¹. Such adaption mechanism entails a significant increase in the activities of enzymes needed for a cyclic operation that burn carbon into CO₂ to generate NADPH. The overall biomass yield is nevertheless similar to that under control conditions because CO₂ production by cellular respiration (i.e. pyruvate dehydrogenase and TCA cycle) is reduced. Remarkably, *P. putida* deploys a survival strategy that differs from that observed in the close relative species *P. fluorescens*, where production of α -ketoacids and ATP formation prevails under oxidative stress *via* several converging metabolic mechanisms⁴⁶.

Increasing evidence points to glutathione peroxidase as a key survival mechanism in stressed bacteria⁵²⁻⁵⁴—and *P. putida* seems to be no exception. Glutathione peroxidase-dependent reduction of ROS requires a continuous supply of NADPH for regeneration⁵⁵. Upon oxidative stress, the glutathione-based detoxification of ROS drains a lot of NADPH that must be replenished *via* the mechanisms explained above. Importantly, the results of this study reflect a situation where the immediate, early metabolic response (which in *E. coli* takes place within the first 10 s after exposure to the stress agent) is blended with early transcriptional responses (e.g. activation of inducible defence mechanisms, which take several minutes to occur). Experimental evidence indicates that there is a multi-layered array of defence strategies against oxidative stress in *P. putida*, including (i) several catalases and superoxide dismutases^{56,57}, (ii) ferredoxin-NADP⁺ oxido-reductases⁵⁸, (iii) polyphosphate-dependent NAD⁺ and NADH kinases⁵⁹ and (iv) stress-induced transhydrogenation activities⁶⁰. However important, these mechanisms can be classified as (relatively) late responses to oxidative insults as they mostly depend on transcriptional regulation, and the re-arrangement of metabolic fluxes to ensure NADPH supply appears to represent a first line of defence as indicated in other organisms⁶¹.

Finally, the data presented in this work highlights the value of *P. putida* as a chassis for synthetic biology-inspired metabolic engineering. In one hand, the flexibility to reconfigure fluxes that supply NADPH helps counteracting ROS resulting from harsh redox reactions to become excessively mutagenic, increasing genetic and genomic stability of engineered catabolic modules⁶². On the other hand, stress-quenching mechanisms enable *P. putida* to sustain diversification of catabolic pathways (e.g. for novel aromatic substrates) involving interim, faulty reactions that create ROS due to substrate misrecognition⁶³. In either circumstance, an abundant, conditional NADPH supply of is a key feature to ensure cell survival and durability in the scenarios indicated herein. Together with the capability of *P. putida* KT2440 to adapt to stressful, industrial-scale conditions⁶⁴, these properties are being exploited to enlarge the panoply of microbial platforms for industrial and environmental whole-cell biocatalysis.

Methods

Bacterial strain and culture conditions. Wild-type *P. putida* strain KT2440 was used throughout this study⁶⁵, and it was routinely grown in LB medium⁶⁶. Quantitative physiology experiments were carried out in M9 minimal medium containing 6 g L⁻¹ Na₂HPO₄, 3 g L⁻¹ KH₂PO₄, 1.4 g L⁻¹ (NH₄)₂SO₄, 0.5 g L⁻¹ NaCl, 0.2 g L⁻¹ MgSO₄·7H₂O and 2.5 mL L⁻¹ of a trace elements solution⁶⁷. Either natural or ¹³C labelled glucose at 20 mM was used as the sole carbon source. Growth was estimated by measuring the OD₆₀₀ after diluting the culture with 9 g L⁻¹ NaCl. Correlation factors between cell dry weight (CDW) and OD₆₀₀ were determined in batch cultures⁶⁸. All cultures were started with an isolated colony from an LB medium plate, suspended in 5 mL of M9 minimal medium in a test tube. After an 18-h incubation, this culture was used to inoculate fresh medium at an OD₆₀₀ of 0.01. Working cultures were cultivated in 250-mL Erlenmeyer flasks containing medium up to one-fifth of their nominal volume. H₂O₂ was used as an oxidative stress agent at 1.5 mM, added at OD₆₀₀ = 0.5 (mid-exponential phase). Cultures were incubated for an additional 1.5 h, and samples were taken for analyses as explained below.

Determination of physiological parameters. Regression analysis was applied during exponential growth to calculate [i] maximum specific growth rate (μ), [ii] biomass yield on substrate ($Y_{X/S}$), [iii] specific rate of glucose consumption (q_S), and [iv] molar yield of organic acids on glucose ($Y_{P/S}$). CDW was quantified by harvesting cells by fast filtration in pre-weighed nitrocellulose filters (0.45 μ m), washed twice with 9 g L⁻¹ NaCl, and dried at 105°C to a constant weight. Glucose was assayed using a

commercial kit from R-Biopharm AG (Darmstadt, Germany). Gluconate and 2-ketogluconate were measured in culture supernatants as described previously⁶⁰.

Determination of relative metabolite concentrations and ¹³C-labeling patterns by LC-MS/MS.

Cultures were grown on either 100% [1-¹³C₁]-glucose or 100% [6-¹³C₁]-glucose as the sole carbon source. The biomass corresponding to 0.5-0.6 mg of CDW was collected in triplicates by fast centrifugation (13,000×g, 30 s, -4°C). Bacterial pellets were immediately frozen in liquid N₂. Samples were then extracted three times with 0.5 mL of 60% (v/v) ethanol buffered with 10 mM ammonium acetate (pH = 7.2) at 78°C for 1 min. After each extraction step, the biomass was separated by centrifugation at 13,000×g for 1 min. The three liquid extracts were pooled and dried at 120 μbar, and stored at -80°C thereafter. Samples were re-suspended in 20 μL of MilliQ water, distributed in sealed 96-well microtiter plates, and injected into a Waters Acquity UPLC (Waters Corp., Milford, MA, USA) with a Waters Acquity T3 column (150 mm × 2.1 mm × 1.8 μm, Waters Corp.) coupled to a Thermo TSQ Quantum Ultra triple quadrupole instrument (Thermo Fisher Scientific Inc., Waltham, MA) with electrospray ionization. ¹³C-Labeling patterns of free intracellular metabolites were determined for dihydroxyacetone phosphate (DHAP), F6P, FBP, G6P, 6PG, PEP, R5P, Ru5P, S7P, X5P and Pyr as described previously⁶⁹. All other metabolites are listed in **Supplementary Data 1**. Relative abundance was calculated as the total ion count of all measured ¹³C isotopes for each metabolite. The raw data of labeling experiments is available in **Supplementary Data 1**.

Determination of ¹³C-labeling patterns by GC-MS. Cultures were grown on either 100% [1-¹³C₁]-glucose or a mixture of 20% (w/w) [U-¹³C₆]-glucose and 80% (w/w) naturally-labelled glucose, and 5 mL aliquots of cell broth were harvested by centrifugation at 1,200×g and -4°C for 10 min. Bacterial pellets were washed twice with 1 mL of 9 g L⁻¹ NaCl, hydrolyzed in 1 mL of 6 M HCl for 24 h at 110°C, and desiccated overnight at 85°C under a constant air stream. The hydrolyzate was dissolved in 50 μL of 99.8% (w/v) dimethyl formamide and subsequently transferred into a new tube. For sample derivatization, 30 μL of *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide was added to the biomass hydrolyzate and incubated at 85°C for 60 min. The ¹³C-labeling patterns of proteinogenic amino acids were determined on a 6890N Network GC system with a 5975 inert XL mass selective detector (Agilent Technologies Inc., Santa Clara, CA, USA) as described previously⁷⁰. The raw GC-MS data from four independent experiments is presented in **Supplementary Data 2**.

Metabolic flux ratio analysis. Mass distribution vectors of the proteinogenic amino acids were corrected for the natural abundance of all stable isotopes, and the relative metabolic flux ratios [i] oxaloacetate (OAA) from Pyr, [ii] glyoxylate shunt, [iii] PEP from OAA, and [iv] the lower and upper bound for Pyr from malate were calculated using the Fiat Flux software⁷¹. The mass distribution vectors of the free intracellular metabolites were corrected for the natural abundance of all stable isotopes using MatLab (The Mathworks Inc., Natick, MA, USA). Novel relative flux ratios were defined and calculated as described previously³³. The fraction of G6P originating from glucose was estimated using data from the experiments using 100% [1-¹³C₁]-glucose, whereas 6PG from G6P was calculated using data from either 100% [1-¹³C₁]-glucose or 100% [6-¹³C₁]-glucose experiments. Formation of F6P through the PP pathway was estimated using data from 100% [6-¹³C₁]-glucose experiments, and Pyr through the ED pathway was assessed with data from 100% [1-¹³C₁]-glucose experiments. The metabolic model used for net-flux analysis was based on a master reaction network with 45 reactions and 33 metabolites. Fluxes were calculated using [i] the stoichiometric reaction matrix, [ii] constraints accounting for the ratios from FiatFlux analysis and additionally for the ratios in the initial steps of glucose catabolism as described above, [iii] physiological data, and [iv] precursor requirements for biomass. The experimentally-determined relative flux ratios were translated into constraints as follows (the reaction numbers, v_x , are defined in **Fig. S1** in the Supplementary Information). The fraction of G6P originating from glucose (a) was estimated as $a = v_3/(v_3 + v_{16})$; the fraction of F6P originating through the PP pathway (b) was calculated as $b = (v_{14} + v_{15})/(v_{14} + v_{15} + v_{17})$; and the fraction of 6PG originating from G6P (c) was determined as $c = v_7/(v_4 + v_6 + v_7)$. For this ratio, the values from the experiments using data from either 100% [1-¹³C₁]-glucose or [6-¹³C₁]-glucose were averaged. The fraction of Pyr originating through the ED pathway (d) was determined as $d = v_9/(v_9 + v_{22} + v_{33} + v_{34})$. The upper and lower bounds of Pyr originating from malate (e and f , respectively) were obtained according to $e \geq v_{34}/(v_9 + v_{22} + v_{33} + v_{34})$ and $f \leq v_{34}/(v_9 + v_{22} + v_{33} + v_{34})$. The fraction of OAA originating from Pyr (g) was obtained following $g = v_{32}/(v_{30} + v_{32})$. Finally, the fraction of Pyr originating from OAA (h) was derived from $h = v_{33}/(v_9 + v_{22} + v_{33} + v_{34})$. The determined linear system of mass balances, flux ratios, quantitative physiology data, and biomass requirements was then solved with the *fmincon* function from MatLab using the Netto module from FiatFlux to obtain the net metabolic fluxes as described previously⁷¹. The values for all the fluxes within the network are provided in **Table S3** in the Supplementary Information.

Preparation of cell-free extracts and *in vitro* enzymatic assays. Cell-free extracts were prepared from cells harvested by centrifugation from an appropriate culture volume at 4,000×g at 4°C for 10 min. Pellets were suspended in 1 volume of 10 mM phosphate-buffered saline (PBS, pH = 7.5, and previously refrigerated) containing 10 mM 2-mercaptoethanol and centrifuged again. Cells were finally resuspended in 0.3-0.5 volume of the same buffer and sonicated intermittently for 6 min in an ice bath. Sonicated cells were centrifuged at 7,500×g at 4°C for 30 min, and the total protein concentration in cell extracts was measured by the Bradford method. The activities of Edd, Eda, Glk, Gcd, and Gad were assayed using standard protocols^{72,73}. The activity of Zwf and Gnd were assayed under both saturating and non-saturating, *quasi in vivo* conditions using concentrations reflecting intracellular experimentally determined abundance of cofactors and substrates. For those reactions in which more than one enzyme catalyzes the corresponding transformation (e.g., Zwf, for which there are three isozymes in *P. putida* KT2440), the total activity is reported. In the latter case, the concentrations of the substrates (experimentally determined in cell-free extracts of glucose-grown KT2440 by means of LC-MS/MS), were 1.2 mM G6P and 2.4 mM 6PG.

Glutathione quantification. Glutathione (both oxidized and reduced forms) was quantified in cells harvested from 25 mL-culture samples using a modification of the procedure described by Michie *et al.*⁷⁴. Cells were pelleted by centrifugation (4,000×g at 4°C for 10 min) and suspended in 2 mL of cold PBS. The cell suspension was mixed 1:1 (v/v) with a freshly-prepared 10% (w/v) 5-sulfosalicylic acid solution, incubated on an ice bath for 10 min and then sonicated. For the measurement of total glutathione, 40 µL of Tris(2-carboxyethyl)phosphine-HCl was added to an equal volume of the cell-free extract. For the measurement of reduced glutathione, 40 µL of H₂O were used instead. Samples were centrifuged at 15,600×g for 5 min after treatment, and 25 µL of the supernatant fluid was added to 100 µL of a buffer containing 200 mM *N*-ethylmorpholine and 20 mM NaOH. Following the addition of 50 µL of 0.5 N NaOH to this mixture, glutathione was derivatized by addition of 10 µL of 10 mM naphthalene-2,3-dicarboxaldehyde. The mixture was incubated at room temperature for 30 min, and the fluorescence intensity of the resulting naphthalene-2,3-dicarboxaldehyde–glutathione conjugate was measured at an excitation wavelength ($\lambda_{\text{excitation}}$) = 472 nm and an emission wavelength ($\lambda_{\text{excitation}}$) = 528 nm. A standard curve was constructed with known amounts of freshly-prepared naphthalene-2,3-dicarboxaldehyde–glutathione conjugate.

Chemicals and enzymes. [1-¹³C₁]-Glucose and [6-¹³C₁]-glucose were purchased from Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA), and [U-¹³C₆]-glucose was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Other chemicals and enzymes used for *in vitro* assays were obtained from Sigma-Aldrich Co. and Merck KGaA (Darmstadt, Germany). H₂O₂ solutions were freshly prepared from a 30% (w/w) stock solution (Sigma-Aldrich Co.).

Data availability. Metabolite levels and ¹³C-labelling data are available in the Supplementary Information files. Other datasets generated and analyzed in this study are available from the corresponding authors on request.

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Author contributions. P.I.N. and V.D.L. conceived the study and wrote the manuscript with input from all other authors. P.I.N., T.F., M.C. and A.S.P. carried out the quantitative physiology and tracer experiments. P.I.N. and A.S.P. measured the in vitro enzymatic activities. T.F. performed metabolomics and fluxome analysis. All authors contributed to the discussion of the experimental results.

Additional information. Supplementary Information accompanies this paper online.

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FIGURES

Figure 1. Experimental overview and quantitative physiology parameters for *P. putida* KT2440 cultures.

a. Overview of the experimental design. All experiments were conducted at least in biological triplicates. OD₆₀₀, optical density measured at 600 nm. **b.** Typical growth curves for control (Ctrl.) experiments and H₂O₂-stressed cultures. Note that samples were taken 1.5 h after addition of H₂O₂. **c.** Physiological parameters of *P. putida* KT2440 batch cultures. All values represent the mean at least three biological replicates. The specific growth rate (μ) and the specific rate of carbon uptake (q_s) were determined during exponential growth by linear regression of log-transformed OD data. The yield of biomass on substrate ($Y_{X/S}$) was calculated at 24 h, after glucose was completely exhausted. The extracellular concentration (concn.) of organic acids reported is the maximal reached during the whole culture period. CDW, cell dry weight.

Figure 2. Metabolite levels in *P. putida* KT2440 under oxidative stress.

a. Relative abundance of selected metabolites, grouped according to the biochemical block they belong to (i.e. PP pathway, EMP pathway and TCA cycle). Relative metabolite abundance is expressed as the ratio between H_2O_2 -induced oxidative stress and control (Ctrl.) conditions, derived from summed ion abundance of all isotopes (counts). Data from experiments in the presence of $[1-^{13}C_1]$ - and $[6-^{13}C_1]$ -glucose (Glc.) are averaged. Bars represent the mean value of the abundance $\pm$ standard deviations of triplicate measurements from at least two independent experiments. Single (*) and double asterisks (**) identify significant differences at $P < 0.05$ and $P < 0.01$, respectively, as assessed by the Student's t test.

b. Changes in selected metabolic flux ratios in upper metabolism upon exposure of the cells to H_2O_2 . The ^{13}C -labeled substrate used in each experiment is indicated. Bars represent averages from three independent experiments, and standard deviations were calculated using the covariance matrices of the respective mass distribution vectors by applying the Gaussian law of error propagation.

c. Relative pathway contribution to the G6P and F6P pools. The contribution of each of the metabolic pathways to the sugar phosphate pool under oxidative stress conditions is indicated with different colours. All abbreviations in this figure are as indicated in the legend to **Fig. S1** in the Supplementary Information. CDW, cell dry weight.

Figure 3. *In vivo* carbon flux distribution in glucose-grown *P. putida* KT2440 obtained from ratio-constrained flux balance analysis.

All fluxes, calculated under control conditions (a) and in the presence of H_2O_2 -induced oxidative stress (b), were normalized to the specific glucose uptake rate (q_s), and the width of each arrow is scaled to the relative flux. Dashed lines indicate that no significant flux through the corresponding biochemical step was detected under the conditions tested. Abbreviations used for the metabolic intermediates and the main metabolic blocks within the biochemical network are given in the legend to **Fig. S1** in the Supplementary Information. CDW, cell dry weight.

Figure 4. Dynamic NADPH balance in *P. putida* KT2440 upon oxidative stress.

Overall NADPH balances and sensitivity analysis for the wild-type strain KT2440 under control (a, b, e and f) or H₂O₂-induced oxidative stress conditions (c, d, g and h) using cofactor specificities of major dehydrogenases under saturating conditions (left column) or *quasi in vivo* conditions (right column). NADPH formation was determined from carbon fluxes through redox cofactor-dependent reactions (Fig. 3 and Table S1 in the Supplementary Information) multiplied by experimentally-determined relative cofactor specificities. NADPH consumption was calculated from requirements for biomass production and KgdD activity. NADPH formation (green) and consumption (red) rates are individually indicated (a–

d). Dependence of the overall rates of NADPH turnover on the relative GnuK/KguD flux ratio and the cofactor specificity of Zwf derived from saturating conditions or *quasi in vivo* conditions (e–h). Selected values for the net NADPH balance are given for the experimentally-determined NADP⁺ specificity of Zwf (0.937), and the calculated net rate for each conditions is indicated with a red dot. CDW, cell dry weight.

Figure 5. *In vitro* analysis of key enzymatic activities and glutathione metabolism.

a. Enzyme activity ratios were calculated from the specific activity for each of the indicated reactions assessed under H₂O₂-induced oxidative stress and control (Ctrl.) conditions. Each bar represents the mean value of the corresponding ratio $\pm$ standard deviations of triplicate measurements from at least two independent experiments, and the horizontal dashed line indicates a ratio = 1 (i.e. no changes in enzymatic activities across conditions). Single (*) and double asterisks (**) identify significant differences at $P < 0.05$ and $P < 0.01$, respectively, as assessed by the Student's t test. Circled numbers identify the enzymes in the biochemical network of **Fig. S1** in the Supplementary Information. **b.** Glutathione metabolism in *P. putida* KT2440. The key activities involved in biosynthesis and recycling of the reduced (GSH) and oxidized (GS-SG) forms of glutathione are indicated along with the corresponding PP identifiers. **c.** Enzymatic determination of total glutathione and the fraction of the oxidized and reduced form. Bars represent the mean value of the corresponding parameter $\pm$ standard deviations of duplicate measurements from at least three independent experiments, and the asterisk (*) identifies significant differences between stressed cells and control conditions at $P < 0.05$ as assessed by the Student's t test. CDW, cell dry weight.

Figure 6. The architecture of central carbon metabolism in *P. putida* enables rapid supply of NADPH upon oxidative stress.

a. Schematic representation of the upper metabolism of *P. putida* KT2440. Several biochemical reaction have been lumped to illustrate the main routes for carbon circulation (see **Fig. S1** in the Supplementary Information for details and abbreviations). Note that the total rate of carbon uptake (q_s) is split between glucose phosphorylation and oxidation to gluconate, such that $q_s = r_p + r_{ox}$. The overall cycling flux of trioses phosphate towards hexoses phosphate is indicated as r_c and the flux through the PP pathway shunt is termed r_s . Cofactors other than NADPH have been omitted in the drawing for the sake of clarity. **b.** Functional relationship between r_{NADPH} , the rate of NADPH formation within the simplified metabolic network of panel **a**, and the fluxes through the oxidative loop for glucose processing and the PP pathway shunt. All (arbitrary) values are given as a fraction of q_s , and the experimental conditions tested in this work are indicated with red dots (Ctrl., control conditions). **c.** General model for flux distribution in the upper metabolic domain of *P. putida*. Under normal growth conditions, glucose is processed mostly through its oxidative conversion to gluconate, and the EDMP cycle provides intermediates for biomass, with a very low flux through the PP pathway. Upon oxidative stress conditions (exerted either by endogenous or external perturbations), a rapid increase of fluxes via the PP pathway shunt replenishes the intermediates within upper metabolism and provides a direct

- 1 source of NADPH that can be coupled to anti-oxidant defence mechanisms against reactive oxygen
- 2 species (ROS). Note that fluxes predominant under each condition are highlighted.