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Metabolic and regulatory rearrangements underlying glycerol metabolism in *Pseudomonas putida* KT2440

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Summary

While the natural niches of the soil bacterium *Pseudomonas putida* are unlikely to include significant amounts of free glycerol as a growth substrate, this bacterium is genetically equipped with the functions required for its metabolism. We have resorted to deep sequencing of the transcripts in glycerol-grown *P. putida* KT2440 cells to gain an insight into the biochemical and regulatory components involved in the shift between customary C sources (e.g. glucose or succinate) to the polyol. Transcriptomic results were contrasted with key enzymatic activities under the same culture conditions. Cognate expression profiles revealed that genes encoding enzymes of the Entner–Doudoroff route and other catabolic pathways, e.g. the gluconate and 2-ketogluconate loops, were significantly downregulated on glycerol. Yet, the compound simultaneously elicited a gluconeogenic response that indicated an efficient channelling of C skeletons back to biomass build-up through the glyoxylate shunt rather than energization of the cells through downwards pathways, i.e. tricarboxylic acid cycle and oxidative phosphorylation. The simultaneous glycolytic and gluconeogenic metabolic regimes on glycerol, paradoxical as they seem, make sense from an ecological point of view by favouring prevalence versus exploration. This metabolic situation was accompanied by a considerably low expression of stress markers as compared with other C sources.

Introduction

Pseudomonas putida KT2440 is a ubiquitous Gram-negative, soil resident and plant root-associated saprophytic bacterium endowed with a notable metabolic versatility as well as a remarkable tolerance to many organic compounds (Nelson *et al*., 2002; Martins dos Santos *et al*., 2004). These physiological and metabolic traits make this microorganism an attractive agent for biocatalysis in a number of biotechnological and environmental applications (Puchałka *et al*., 2008; Nikel, 2012; Poblete-Castro *et al*., 2012). Yet, the limited knowledge of its central biochemical network when using low-cost substrates, e.g. in industrial settings, hampers the use of *P. putida* in such endeavours. Glucose catabolism in this bacterium takes place through the Entner–Doudoroff (ED) pathway, as the absence of a 6-phosphofructokinase prevents the processing of hexoses through a *bona fide* Embden–Meyerhof–Parnas (EMP) route (Vicente and Cánovas, 1973a,b; Clarke, 1982; Velázquez *et al*., 2004; del Castillo *et al*., 2007). In contrast, the use of glycerol as a C source for *P. putida* has been somewhat overlooked. Being a by-product of the biodiesel industry, glycerol is currently one of the most promising growth substrates for empowering whole-cell biocatalysis and production of new biomaterials (da Silva *et al*., 2009; Pettinari *et al*., 2012). This state of affairs makes this simple and abundant polyol an attractive C source for microbial-based processes, such as the production of polyhydroxyalkanoates and other bulk chemicals (Murarka *et al*., 2008; Ashby *et al*., 2012; Escapa *et al*., 2012; Gomez *et al*., 2012; Ruiz *et al*., 2012).

Glycerol catabolism has been studied in much detail in *Escherichia coli*. Uptake of the compound is mediated by a facilitator (GlpF) that fosters a diffusion reaction (Sweet *et al*., 1990), particularly at low substrate concentrations. Once inside the cell, glycerol is phosphorylated by glycerol kinase (GlpK) to *sn*-glycerol-3-*P* (G3P), which can no longer diffuse out of the cell (Lin, 1976). This type of transport system has also been reported in several other Gram-negative and Gram-positive bacteria, such as *Pseudomonas aeruginosa* (Schweizer and Po, 1996), *Streptomyces clavuligerus* (Baños *et al*., 2009) and *Bacillus subtilis* (Beijer *et al*., 1993). Once inside and phosphorylated, glycerol can meet different fates depending on the growth conditions. In *E. coli*, respiratory metabolism is typically mediated by an adenosine triphosphate (ATP)-dependent GlpK and the aerobic/anaerobic respiratory G3P dehydrogenases (G3PDH) GlpD/GlpABC (Lin, 1976). Glycerol dehydrogenase (GldA) next catalyses the oxidation of glycerol to dihydroxyacetone, that subsequently enters into the central biochemical network

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Table 1. Growth parameters[a] for batch cultures of *Pseudomonas putida* KT2440 carried out on different C sources.

C source[b]	Lag phase[c] (h)	μ^d (h^{-1})	$q_S{}^d$ (mmol g^{-1} h^{-1})	$Y_{X/S}{}^e$ (g g^{-1})
Glucose	1.2 ± 0.5	0.68 ± 0.05	6.49 ± 0.09	0.48 ± 0.01
Succinate	0.4 ± 0.2	0.72 ± 0.11	4.81 ± 0.25	0.37 ± 0.08
Glycerol	18.6 ± 2.3	0.46 ± 0.02	3.95 ± 0.34	0.59 ± 0.06

a. Values shown represent the mean of the corresponding parameter ± standard deviation of triplicate measurements from at least five independent experiments.
b. Each C source was amended in order to provide 60 mM C atoms (i.e. 10 mM glucose, 15 mM succinate and 20 mM glycerol).
c. The extension of the lag phase was analytically obtained from growth parameters as detailed by Dalgaard and Koutsoumanis (2001).
d. The specific growth rate (μ) and the specific C uptake rate (q_S) were determined during exponential growth.
e. The yield of biomass on substrate ($Y_{X/S}$) was determined 24 h after each culture started to grow exponentially.

as dihydroxyacetone-*P*. The *glp* system of *E. coli* is regulated by the repressor GlpR, which is induced by G3P (Schweizer *et al*., 1985). In sum, the biochemical and regulatory features of glycerol metabolism in *E. coli* are mostly understood. In contrast, the question on the genes and enzymes activated by glycerol in *P. putida* and how they are expressed under different growth conditions remains largely unanswered.

In this work, we have adopted a transcriptomic approach for interrogating *P. putida* KT2440 on the genes and pathways that are turned on and off when cells are grown on glycerol as the only C source. To this end, we have exploited the wealth of information resulting from the massive deep sequencing of mRNA extracted from *P. putida* cells grown in conditions in which the C source is the only variable (Kim *et al*., 2013). By comparing expression profiles in glycerol versus those in typically glycolytic (i.e. glucose) or gluconeogenic (i.e. succinate) substrates, we could determine exactly the type of physiological regime that *P. putida* deploys for consumption of the compound at stake. Specifically, the genomic and biochemical evidence revealed that cells growing on glycerol display a metabolic mode characterized by (i) a simultaneous operation of glycolytic and gluconeogenic routes, (ii) a very efficient conversion of substrate into biomass, (iii) the operation of specific components of the respiratory chain, and (iv) a remarkably low level of physiological stress. These features not only make sense from an ecological point of view but also strengthen the value of glycerol as a promising C source of choice for a suite of applications involving *P. putida* as the biocatalyst.

Results and discussion

Physiology of P. putida *KT2440 growing on different C sources*

The biochemical network of *P. putida* is mostly geared for the aerobic oxidation of hexoses, e.g. glucose, through the ED pathway (Conway, 1992; Velázquez *et al*., 2004; del Castillo *et al*., 2007; Chavarría *et al*., 2013). This route yields glyceraldehyde-3-*P* (GAP) and pyruvate, later transformed into acetyl-coenzyme A (CoA) by the pyruvate dehydrogenase complex and finally oxidized in the tricarboxylic acid (TCA) cycle (Vicente and Cánovas, 1973a; Clarke, 1982; Lessie and Phibbs, 1984). Reducing equivalents generated in these pathways are used as electron donors for the respiratory chain, which ultimately leads to ATP synthesis. As the situation is less clear when cells grow on glycerol, we set out to compare some kinetic and physiological parameters of *P. putida* KT2440 in batch cultures carried out in M9 minimal medium with C sources known to impose distinct metabolic regimes (Table 1). To this end, glucose and succinate were tested alongside glycerol as reference conditions for entirely glycolytic or gluconeogenic modes, respectively, and the growth behaviour and substrate consumption of *P. putida* KT2440 were followed throughout the incubation period. Cells growing on glycerol had the slowest proliferation among all the conditions tested. Specifically, growth rates were reduced by *ca*. 40% in respect to succinate, the C source that promoted the fastest growth. In contrast, the biomass yields followed the opposite trend, being the highest in glycerol cultures (1.2- and 1.6-fold higher in glucose and succinate, respectively). Accordingly, the specific rate of C uptake followed the same tendency as the specific growth rate. A noticeable feature of glycerol cultures was an unusually long lag phase, which was consistently ≥ 12 h. Cells using glucose had a comparatively lesser delay (*ca*. 1.5 h) before starting noticeable growth. In contrast, virtually no lag phase was observed on succinate. This last quality is indicative of the default expression of enzymes for succinate catabolism within the TCA cycle, as previously suggested for *P. aeruginosa* (Tiwari and Campbell, 1969; Lessie and Phibbs, 1984). Interestingly, the extended lag phase observed in glycerol cultures was somewhat independent of the C source on which cells used as the inoculum were pregrown (data not shown), suggesting regulatory mechanisms beyond a mere transcriptional and/or biochemical adaptation to the polyol. Taken together, these results revealed substantial physi-

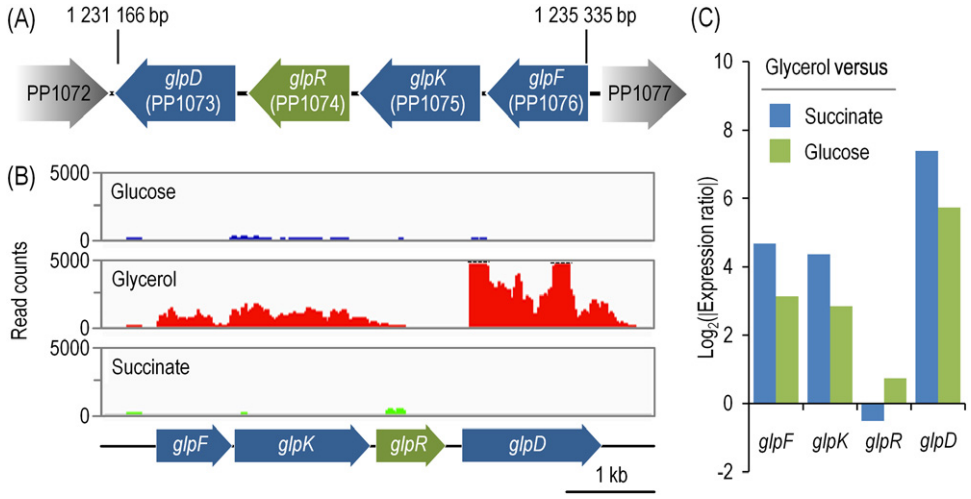

Fig. 1. Expression pattern of the *glp* genes in *Pseudomonas putida* KT2440 analysed at the single-nucleotide level.
A. Genetic organization and genomic coordinates of the *glp* locus. The genomic region encompasses *glpF* [PP1076, a major intrinsic protein (MIP) family channel protein], *glpK* (PP1075, glycerol kinase), *glpR* (PP1074, a DeoR family transcriptional regulator) and *glpD* (PP1073, glycerol-3-*P* dehydrogenase; Nelson *et al*., 2002). Catabolic genes are shown in blue, and the gene encoding the GlpR regulator is highlighted in green. The entire cluster is flanked upstream by PP1072, which encodes an uncharacterized hypothetical protein, and downstream by PP1077, which encodes an YbaK/EbsC-type protein (prolyl-tRNA editing protein). Note that the elements in this outline are not drawn to scale.
B. Sequence coverage plots for samples taken during mid-log phase from cultures developed on glucose, glycerol or succinate as the sole C source. The representative region shown below the plot charts is a *ca*. 7-kb genome segment surrounding the *glp* gene cluster, and the specific genes belonging to the *glp* locus are indicated. Note the monocistronic nature of the *glpD* transcript, particularly evident when cells were grown on glycerol.
C. Relative expression levels of the *glp* genes. The bars represent the mean value of the expression level for each gene within the cluster observed in cells grown on glycerol as the C source as normalized to the expression level observed on either succinate or glucose cultures. Differences in the pairwise comparison among C sources were significant as judged by the corresponding false discovery rate values (Benjamini *et al*., 2001).

ological differences among the conditions assayed and prompted us to investigate their genetic and biochemical bases.

Transcriptional analysis of the glp regulon of P. putida *KT2440 at the single-nucleotide resolution*

Using as a reference the widely studied *glp* regulon of *E. coli*, the genes deemed essential for glycerol metabolism in *P. putida* are encoded in a genomic cluster that includes *glpF* (PP1076, a major intrinsic protein with a channel function), *glpK* (PP1075, GlpK), *glpR* (PP1074, a transcriptional regulator belonging to the DeoR family) and *glpD* (PP1073, the main G3PDH; Fig. 1A). Other genes with plausible roles in glycerol processing and metabolism, and annotated as such in the *Pseudomonas* database (Winsor *et al*., 2011), are *gpsA* [PP4169, encoding a NAD(P)$^+$-dependent G3PDH] and several glycerol/G3P acyl transferases (see discussion later).

As a first step in the analysis of the transcriptional response of *P. putida* KT2440 to growth on glycerol as the sole C source, we analysed the genome-wide transcription of this microorganism by deep RNA sequencing (van Vliet, 2010). In a first approach, we focused on the transcription of the *glp* gene cluster at the single nucleotide resolution (Fig. 1B). Previous *in silico* predictions had suggested that the promoters of the *glp* regulon share a common regulatory motif in different Gram-negative bacteria (Freedberg and Lin, 1973; Schweizer and Po, 1996; Danilova *et al*., 2003) and that *glpR*, *glpK* and *glpF* might form an operon (Mao *et al*., 2009). However, the sequence coverage plots demonstrated that this is not the case, as two distinct transcriptional units were clearly identified; one of them encompassed *glpF* and *glpK*, whereas *glpD* was independently transcribed. RNA sequencing thus provides a definite answer to the results of Wang and Nomura (2010) and Escapa and colleagues (2012) on this same question. We next compared the relative expression levels of each of the *glp* genes with the C sources indicated. As shown in Fig. 1C, we did not detect noteworthy differences in *glpR* expression under any condition, indicating that this regulator of the *glp* cluster is not affected by the C source available in the cultures. In contrast, the expression levels of *glpF*, *glpK* and *glpD* were expectedly higher when cells were grown on glycerol as compared with glucose or succinate. The transcriptional ratio of each of these genes (i.e. the mRNA level observed in cells grown on glycerol versus the other C sources) was slightly higher for the glycerol versus succinate comparison than for the glycerol versus glucose comparison.

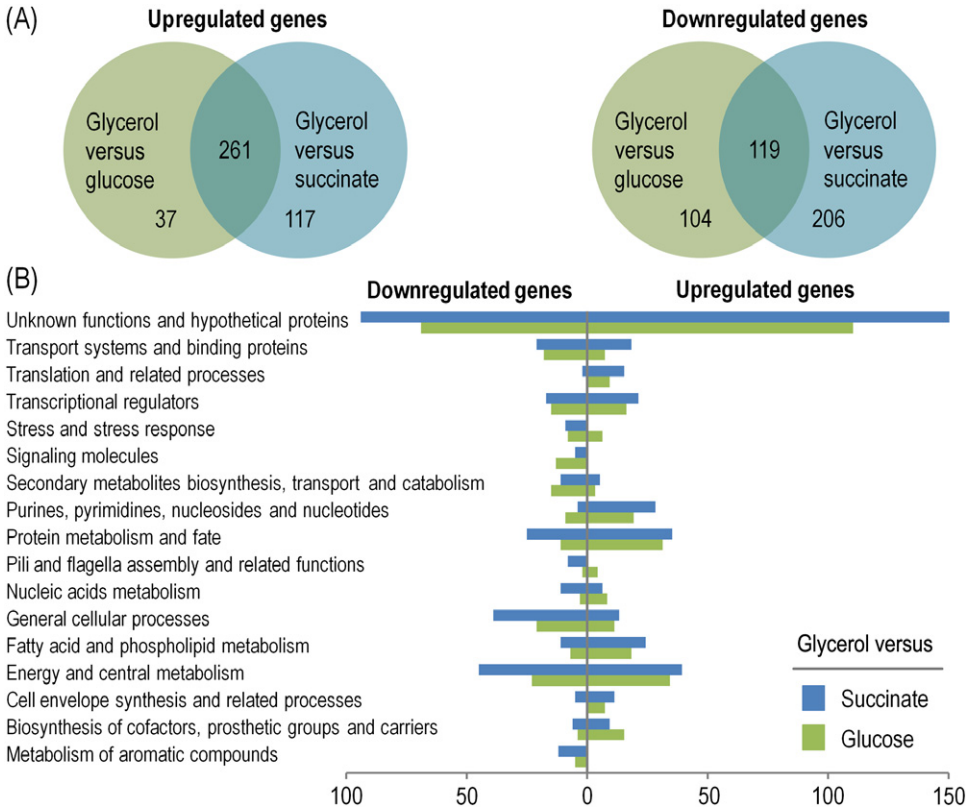

Fig. 2. Number and functional classification of genes differentially expressed in *Pseudomonas putida* KT2440 grown on glycerol.
A. Upregulated and downregulated genes in cells grown on glycerol as compared with glucose or succinate cultures. Venn diagrams show the overlap between upregulated and/or downregulated genes for each pairwise comparison among different C sources.
B. Breakdown of transcriptional responses of *P. putida* KT2440 to growth on glycerol according to different functional categories. Each plot indicates the type of physiological role(s) and the total number of genes with increased or decreased expression within that category in cells grown on glycerol (see also Tables S3–S6 in the Supporting Information). The overall score in each case is a descriptor of the engagement of the roaming transcriptional machinery with promoters that express genes belonging to different functional categories.

Growth on glycerol alters the transcription of a large number of genes encoding both metabolic and non-metabolic tasks

In order to assemble a catalogue of biological functions beyond those encoded in the *glp* genes but still specifically linked to growth on glycerol, we surveyed transcripts that went up or down by ≥ twofold in comparison with the other C sources. A total of 37 and 117 genes were significantly upregulated in the presence of glycerol as compared with glucose or succinate, respectively, while 261 of the transcripts corresponding to genes that increased with glycerol were shared in respect to either control condition (Fig. 2A). By the same token, 104 and 206 genes were downregulated in glycerol, with 119 genes shared by both control conditions. Differences were thus more profuse in the comparison glycerol versus succinate than in the glycerol versus glucose counterpart, hence mirroring the transcriptional fingerprint of the *glp* cluster. A gross classification of the collection of upregulated and downregulated genes is summarized in Fig. 2B (see also Tables S3–S6 in the Supporting information for a complete list). The coarse patterns of such pairwise comparisons (i.e. glycerol versus succinate or glycerol versus glucose) followed the same trend. The largest group of genes associated with growth on glycerol corresponded to hypothetical proteins and proteins with unknown functions. These were followed at a distance by genes related to energy and central metabolism. Supplementary Results and Discussion contains a thorough analysis of the transcripts and their cognate predicted functions that were found associated to the glycerol-growth mode. They encompass a number of genes for central and peripheral metabolic routes, respiratory pathways, transcriptional factors, two-component systems and different types of stress responses. Important pieces of information can be distilled from such analyses, as detailed later.

Stress responses and respiratory functions in glycerol-grown P. putida *KT2440*

One conspicuous feature of the transcriptomic data was the consistently lower expression of virtually all stress descriptors in glycerol cultures. Only *dsbE* (encoding a

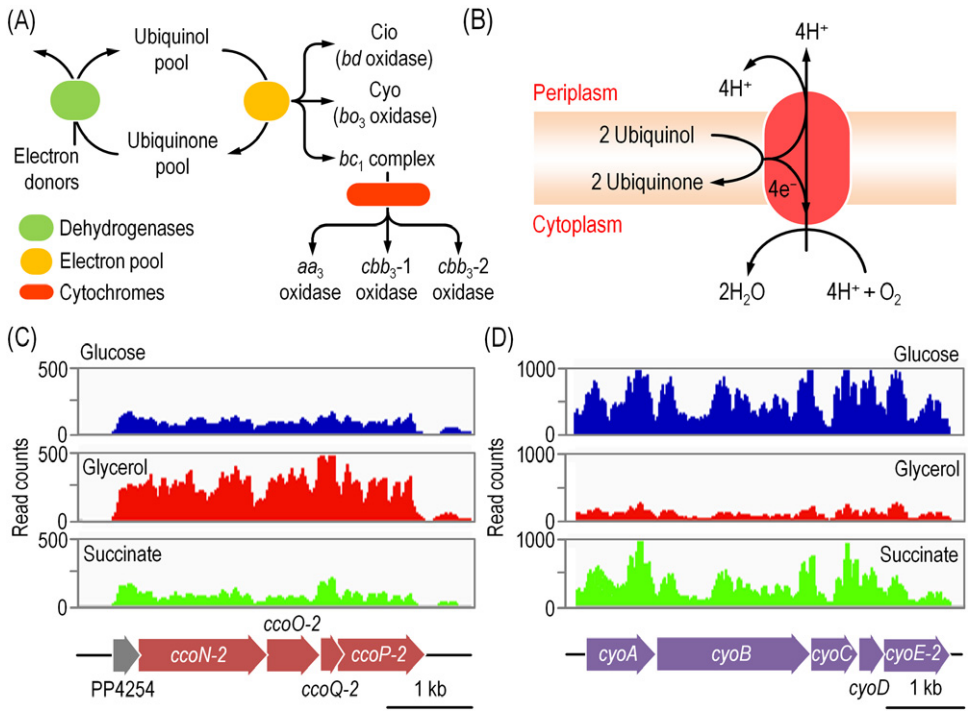

Fig. 3. Organization of the respiratory chain in *Pseudomonas putida* KT2440 and transcriptional landscape of the genes encoding its components.
A. Simplified scheme of the electron transport chain, adapted from Ugidos and colleagues (2008) and Follonier and colleagues (2013). Different electron donors first load their cargo into the ubiquinone pool within the cell membrane and ubiquinol molecules then transfer electrons from the electron pool to either the terminal ubiquinol oxidases Cio or Cyo, or to the bc_1 complex. In the latter case, electrons are fed to the terminal oxidases aa_3, cbb_3-1, or cbb_3-2 via cytochromes as the intermediate electron carriers.
B. Proposed activity of the cbb_3-1 and cbb_3-2 terminal oxidases, which are directly fed by ubiquinol as the electron donor. The proposed H^+ pumping stoichiometry is depicted along with electron transfer to O_2.
C. Sequence coverage plots for samples taken during mid-log phase from cultures developed on glucose, glycerol or succinate as the sole C source. The representative region shown below the plot charts is a genome segment surrounding the PP4254-PP4258 gene cluster, and the specific genes encoding components of the cbb_3-2 terminal oxidase are indicated (PP4254 encodes an hypothetical protein).
D. Sequence coverage plots for a genome segment encompassing the PP0812-PP0816 gene cluster. The specific genes encoding components of the Cyo terminal oxidase, schematically shown in panel B, are indicated.

thiol-disulfide oxidoreductase) and *cspD* (a cold-shock protein) were found to increase in the presence of the polyol. In contrast, any other gene related to the general stress response of *P. putida* KT2440 (Domínguez-Cuevas *et al.*, 2006; Reva *et al.*, 2006; Velázquez *et al.*, 2006) was downregulated, e.g. *groEL* (encoding the molecular chaperone *par excellence*), *groES* (a co-chaperonin), *htpG* (heat shock protein 90), *cspA* (cold-shock protein), *grpE* and *ibpA* (heat-shock proteins), and *dnaK* and *dnaJ* (molecular chaperones). It is possible that the metabolism of the polyol is intrinsically less stressful than its counterparts, i.e. glucose and succinate, partly because of the slower growth rate of cells in glycerol (see discussion earlier). Slow growth is normally accompanied by reduced respiratory activity (Neidhardt *et al.*, 1990), which would also correlate with lower generation of reactive oxygen species. Yet, it is also likely that the compound itself acts as a protective agent (Tekolo *et al.*, 2010; Lin *et al.*, 2013), thereby exerting a general defensive effect against other stress agents. Along this line, Pocard and colleagues (1994) suggested that a number of compatible solutes, such as glucosylglycerol and *N*-acetylglutaminylglutamine amide, conferred stress resistance to glycerol-grown *P. mendocina*. Such a plausible cross-protection phenomenon adds to the value of glycerol as an industrial-scale substrate for growth of *P. putida*.

The regulation of respiratory pathways on glycerol also warrants especial attention. *Pseudomonas putida* KT2440 possesses five different terminal oxidases (Fig. 3A), differing in redox potential and regulation, O_2 affinity and H^+ pumping efficiency. The oxidases repertoire comprises a cyanide-insensitive oxidase (Cio, a *bd*-type oxidase), cytochrome *o* : ubiquinol oxidase (Cyo, a bo_3-type oxidase), cytochrome aa_3 oxidase, cytochrome cbb_3-1 oxidase and cytochrome cbb_3-2 oxidase (Morales *et al.*, 2006; Ugidos *et al.*, 2008; Winsor *et al.*, 2011; Follonier *et al.*, 2013). Cio and Cyo receive electrons directly from the ubiquinol pool within the periplasmic space (Fig. 3B),

whereas cytochromes aa_3, ccb_3-1 and cbb_3-2 process electrons channelled through the bc_1 complex. Four genes involved in cytochrome *c* oxidase maturation and assembly were upregulated in cells grown on glycerol, namely, *ccmF*, *ccmE* and *ccmD* (cytochrome *c* biogenesis proteins), and *ccmC* (an heme exporter protein). These genes are arranged as an operon (PP4322-PP4325), along with PP4319, PP4320 and *dsbE*. The *ccoS* gene, involved in cytochrome *c* oxidase maturation, was only upregulated in glycerol as compared with succinate. Another operon that showed high expression levels on glycerol as compared with the two control conditions was PP4255-PP4257, encompassing the functional subunits of the cytochrome cbb_3-2 oxidase [*ccoN-2* (subunit I), *ccoO-2* (subunit II), *ccoQ-2* (undefined component) and *ccoP-2* (subunit III)] (Fig. 3C). Single-nucleotide plots certify that PP4254-PP4257 form a single transcriptional unit. The isoforms of these cytochrome *c* oxidase components, encoded by PP0103, PP0104 and PP0106, also showed high expression levels on glycerol. Interestingly, only the genes encoding oxidase components (i.e. catalysing the final electron transfer to O_2) showed expression differences as compared with, for instance, genes encoding ubiquinol: cytochrome *c* reductase components, or the multiple nicotinamide adenine dinucleotide, reduced form (NADH)/succinate dehydrogenase enzymes of *P. putida* KT2440. We detected a strong repression of the genes than encode Cyo, the cytochrome bo_3:ubiquinol oxidase complex of *P. putida* KT2440 (Fig. 3B). In particular, *cyoA* (subunit II), *cyoB* (subunit I), *cyoC* (subunit III) and *cyoD* (cytochrome *o*:ubiquinol oxidase protein) were downregulated on glycerol as compared with both glucose and succinate (Fig. 3D). Again, the continuous pattern of transcription at the single-nucleotide level accredits an operon-like arrangement. The redox status of the respiratory components is supposed to be significantly affected by nutritional conditions and respiratory stressors as well as O_2 availability (Arai, 2011). Yet, from our present results, it is tempting to speculate that the oxidation status of the C source could be the relevant signal that ultimately dictates the choice of a terminal oxidase over the others, as hinted by van der Werf and colleagues (2006), and a catabolite repression phenomenon cannot be ruled out (Dinamarca *et al*., 2002). Recent insights on the catabolite repression mechanism in *P. putida* provide a clue on how this regulation could take place. Sensing availability and quality of different C sources in the culture medium, mediated by the CbrAB two-component signal transduction system (García-Mauriño *et al*., 2013), is transmitted to Crc via the concerted action of the small regulatory RNAs CrcY and CrcZ (Moreno *et al*., 2012). This regulatory loop provides the means for fine-tuning adjustments of the respiratory activity depending on the C source available. Whether these regulatory signals exert their influence on the respiratory chain via the environmental conditions themselves or through the activation of regulatory proteins, such as Anr (Ugidos *et al*., 2008), remains to be elucidated. In any case and beyond these overarching effects, the transcriptome revealed unexpected insights on the way glycerol metabolism is wired to the rest of the biochemical network of this bacterium as disclosed later.

The transcriptome of P. putida *KT2440 reveals a multi-tiered organization of glycerol metabolism*

The qualitative distribution of upregulated and downregulated genes within the upper catabolic pathways in cells grown on glycerol is schematically shown in Fig. 4. As shown in this figure and in the Supporting information, default expression of genes for conversion of trioses into hexoses, i.e. *fda* (fructose-1,6-P_2 aldolase) and *fbp* (fructose-1,6-bisphosphatase), was noticeable and basically constant in *P. putida* through all the C sources tested, while the transcription of *pgi* (PP1808, encoding one out of the two glucose-6-*P* isomerases of *P. putida* KT2440) went up in glycerol. Expression of this gene set suggested a considerable upwards rerouting of C skeletons coming from glycerol into hexoses (Heath and Gaudy, 1978; McCowen *et al*., 1981; Lessie and Phibbs, 1984). In contrast, expression of ED pathway genes was downregulated in glycerol as compared with both glucose and succinate (Table 2). Transcription of genes for interconversions within the gluconate and 2-ketogluconate loops was slightly downregulated as well, with the exception of PP3623, a putative gluconate 2-dehydrogenase. As expected for this transcriptional shutdown of the gluconate loop, no gluconate was detected in supernatants of cultures developed on glycerol (in cultures carried out on glucose, in contrast, the gluconate concentration reached *ca*. 5 mM during the mid-exponential phase of growth). The transcriptional arrest of these *peripheral* biochemical loops hints to a considerable saving of C by avoiding the synthesis of by-products from central intermediates. Along the same line, we also noticed a high, glycerol-dependent expression of genes encoding gluconeogenic functions involving C2 (e.g. acetate or acetaldehyde) and C3 (e.g. propanoate) compounds, as well as components of the glyoxylate shunt (Kornberg, 1966; Neidhardt *et al*., 1990). But, at the same time, we observed a remarkable transcription of *acnA* (aconitate hydratase), which catalyses the conversion *cis*-aconitate → D-*threo*-isocitrate within the TCA cycle, accompanied by the concomitant downregulation of PP4012 (a $NADP^+$-dependent isocitrate dehydrogenase), and *sucD* and *sucC* (α and β subunits of succinyl-CoA synthetase, respectively). These variations added to the notion of a considerable recycling of C via the glyoxylate shunt and a restrained functioning of the TCA cycle. Consistently, *lpdG* (PP4187, the dihydrolipoamide

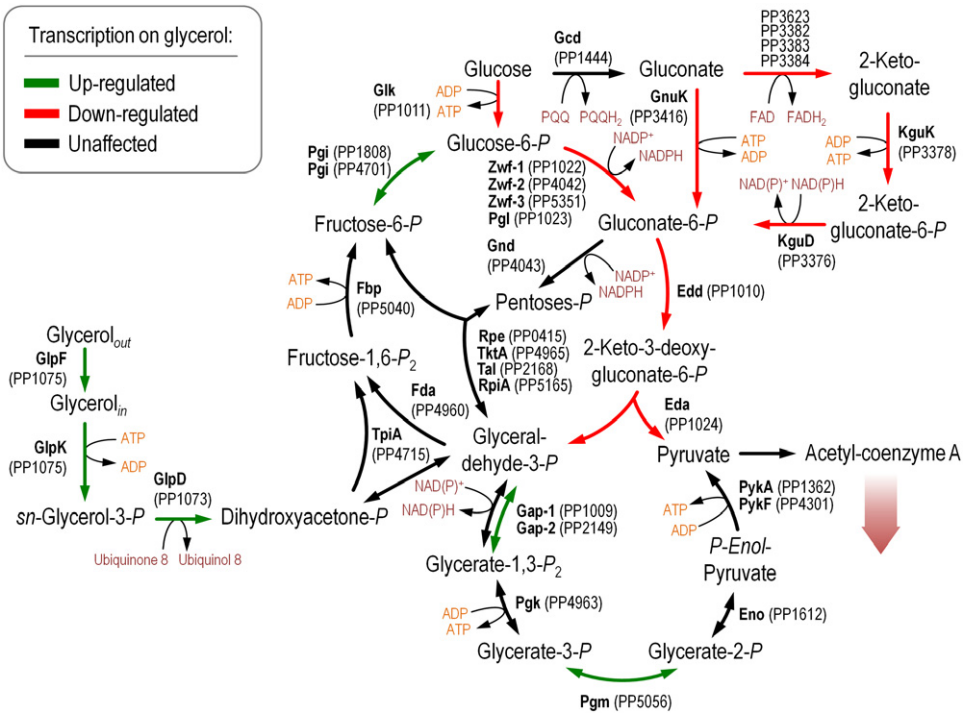

Fig. 4. Genes within the upstream central C metabolism in *Pseudomonas putida* KT2440 affected by growth on glycerol. The biochemical network schematically sketches the main bioreactions involved in C processing along with the enzymes catalysing the corresponding conversions. In some cases, reactions have been lumped to simplify the diagram, and only some isoforms of these enzymes are shown. Further metabolism of acetyl-coenzyme A via the tricarboxylic acid cycle is indicated by a wide shaded arrow. Genes encoding enzymes involved in these bioreactions transcriptionally affected in cells grown on glycerol as compared with the glucose condition are highlighted in different colours according to whether they were significantly upregulated (green), downregulated (red) or remained unaffected (black).

dehydrogenase component of 2-ketoglutarate dehydrogenase) was also downregulated in glycerol as compared with succinate, a further indication of a repressed TCA cycle. Interestingly, a similar metabolic behaviour was recently described for *E. coli* JM101 when growing on glycerol (Martínez-Gómez *et al*., 2012), and the authors observed a strong dependence of this C scavenging mechanism on acetate and indole formation.

Taken together, the results of the transcriptional landscape associated to growth on glycerol suggest a metabolic status characterized by (i) a partial gluconeogenic C recycling process, (ii) a low activity of the enzymes within the TCA cycle and loops conducive to by-products, and (iii) a high activity of the glyoxylate shunt that would prevent loss of C as CO_2. These predictions, derived from the mere analysis of the deep mRNA sequencing data, were then tested experimentally as explained later.

Glycerol catabolism in P. putida *KT2440 involves GlpK and a membrane-bound G3PDH*

As mentioned earlier, the pathway proposed for glycerol catabolism in *P. putida* KT2440 starts with the phosphorylation of the triol substrate followed by oxidation of

Table 2. Expression of selected genes belonging to central catabolic pathways on different C sources.

Gene	PP number[a]	Function(s)	Expression level on glycerol [$\log_2$(IExpression ratiol)] normalized to: Succinate	Glucose
zwf-1	PP1022	Glucose-6-*P* 1-dehydrogenase	2.771	– 2.216
pgl	PP1023	6-Phosphogluconolactonase	3.059	– 2.435
edd[b]	PP1010	Phosphogluconate dehydratase	1.136	– 2.235
eda[b]	PP1024	Ketohydroxyglutarate aldolase/ ketodeoxyphosphogluconate aldolase	2.481	– 2.852

a. As annotated by Nelson and colleagues (2002).
b. Genes encoding the components of the ED pathway.

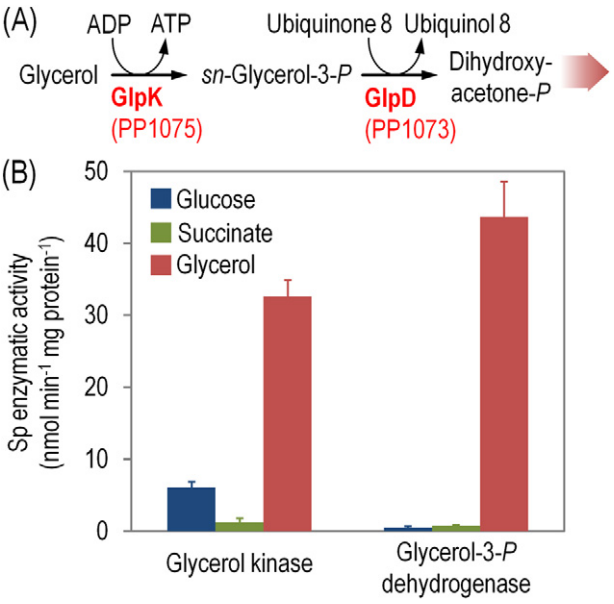

Fig. 5. Biochemical characterization of enzymes involved in glycerol catabolism in *Pseudomonas putida* KT2440.
A. Biochemical pathway proposed for glycerol phosphorylation and oxidation. Upon entrance of glycerol into the cytoplasm (mediated by GlpF, the glycerol facilitator), it is used as a substrate for GlpK (glycerol kinase). The product of this transformation is then oxidized in a ubiquinone-dependent reaction catalysed by the membrane-bound GlpD (*sn*-glycerol-3-*P* dehydrogenase). Dihydroxyacetone-*P* formed therein enters into the central carbohydrate metabolic pathways through the activity of TpiA (triosephosphate isomerase, indicated in this outline as a wide shaded arrow). ADP, adenosine diphosphate.
B. *In vitro* quantification of specific (Sp) enzymatic activities of cells grown on M9 minimal medium added with either glucose, succinate or glycerol. Cells were harvested in mid-exponential phase (OD_{600} *ca*. 0.5) and the activity of GlpK and GlpD was determined in the cell-free extract and in a membrane-enriched fraction, respectively, as detailed in the Experimental procedures section. Each bar represents the mean value of the corresponding enzymatic activity ± standard deviation of duplicate measurements from at least three independent experiments. Differences in the pairwise comparison among C sources were significant ($P < 0.05$) as evaluated by ANOVA.

G3P (Fig. 5A). In order to gain some insight in the biochemistry of the process on the background of the mRNA sequencing data, we examined the enzymatic activities involved in such a pathway *in vitro* using cell-free and membrane-enriched extracts of cells growing alternatively on glycerol, glucose or succinate. As shown in Fig. 5B, we detected high levels of GlpK and GlpD activities in samples from glycerol cultures, which is consistent with the transcriptional pattern of the cognate genes (see discussion earlier). The corresponding biochemical activities peaked 27- and 55-fold higher, respectively, when cells were cultured on glycerol-containing M9 minimal medium as compared with the succinate-grown counterparts, where the activity of the same enzymes was just residual. A similar tendency in fold induction was observed when comparing activities in cultures carried out with glycerol versus glucose. The differences in biochemical fold induction as compared with those detected at the transcriptional level observed in our study can be better understood if the post-transcriptional regulation of GlpK is brought into consideration (Lin, 1976). This regulatory pattern includes catabolite repression, specific allosteric inhibition by the intracellular levels of fructose-1,6-P_2 and inducer exclusion that concertedly modulate enzymatic activity in a transcriptional-independent fashion (Holtman *et al.*, 2001). Moreover, Applebee and colleagues (2011) recently studied different adaptive *glpK* mutants of *E. coli* and found no proportional correlation between *glp* transcriptional levels and GlpK activity (and, consequently, in metabolic fitness). In any case, it is worth of notice that GlpK activity was still detectable at a relatively high level (6.1 ± 0.8 nmol min^{-1} mg $protein^{-1}$) in cells growing under an entirely glycolytic regime, as compared with that observed under a gluconeogenic regime (1.2 ± 0.6 nmol min^{-1} mg $protein^{-1}$). This result is in full agreement with the transcriptional pattern of the *glp* genes as shown in Fig. 1C.

Even though there is only one *bona fide* GlpK activity encoded in the genome of *P. putida* KT2440 (Nogales *et al.*, 2008; Winsor *et al.*, 2011), there are other genes that could encode the cognate dehydrogenase activity apart of the orthologous *glpD* co-transcribed with the other *glp* genes (Fig. 1). One of them is *gpsA* (PP4169), a soluble, $NAD(P)^+$-dependent G3PDH that could participate in oxidation of G3P instead of (or in addition to) GlpD. To examine this issue, we quantified different types of G3PDH activity in cell-free and membrane-enriched extracts. The G3PDH activity was recovered almost entirely in the membrane-enriched fraction, accounting for > 80% of the total activity and therefore suggesting that no soluble G3PDH operates in *P. putida* KT2440 when cells are grown on glycerol. Separate biochemical assays were carried out using the cell-free extract and in the presence of either NAD^+ or $NADP^+$ to test different electron acceptors, but no significant soluble G3PDH activity was observed in these trials (data not shown). This observation substantiated that the membrane-bound GlpD enzyme is the predominant (if not the only) G3PDH activity involved in glycerol catabolism in *P. putida* KT2440. What is then the role of GpsA? PP4169 is highly homologous to the enterobacterial GpsA protein, also termed *biosynthetic* G3PDH (Clark *et al.*, 1980). In *E. coli,* this enzyme is known to foster the reaction dihydroxyacetone-*P* → G3P (Bell and Cronan, 1975), thus playing a predominantly gluconeogenic role by providing G3P as a building block. In any case, GpsA is unlikely to participate in glycerol catabolism by *P. putida*, although it could be connected to the biosynthesis of glycerophospholipids (Rühl *et al.*, 2012) in the same fashion explained earlier for the cognate enzyme in *E. coli*.

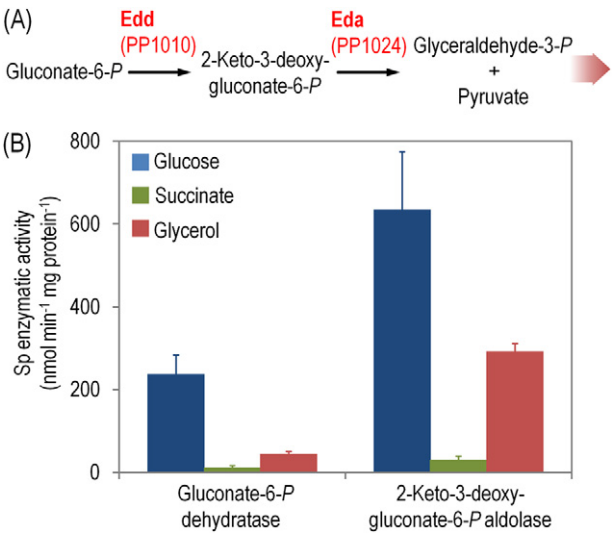

Fig. 6. Biochemical characterization of the Entner–Doudoroff pathway in *Pseudomonas putida* KT2440 grown on different C sources.
A. Biochemical sequence proposed for gluconate-6-*P* processing through the Entner–Doudoroff pathway. Enzymes involved in these conversions (Edd, gluconate-6-*P* dehydratase, and Eda, 2-keto-3-deoxygluconate-6-*P* aldolase) are shown on top of the reaction they catalyse. Note that the final metabolic currency of this biochemical sequence is a equimolar mixture of glyceraldehyde-3-*P* and pyruvate, that are further processed within the lower catabolic pathways (indicated in this outline as a wide shaded arrow).
B. *In vitro* quantification of specific (Sp) enzymatic activities in cells grown on M9 minimal medium added with either glucose, succinate or glycerol. Cells were harvested in mid-exponential phase (OD_{600} *ca.* 0.5), and the Edd and Eda activities were determined in the cell-free extract as detailed in the Experimental procedures section. Each bar represents the mean value of the corresponding enzymatic activity ± standard deviation of triplicate measurements from at least three independent experiments. Differences in the pairwise comparison among C sources were significant ($P < 0.05$) as evaluated by ANOVA.

The ED pathway is essential for glycerol utilization by P. putida *KT2440*

As mentioned earlier, the ED pathway is the main catabolic route used by *P. putida* KT2440 for processing glucose and fructose (Conway, 1992; Velázquez *et al.*, 2004; del Castillo *et al.*, 2007; Chavarría *et al.*, 2013). This route involves the sequential activity of gluconate-6-*P* dehydratase (Edd, PP1010) and 2-keto-3-deoxygluconate-6-*P* aldolase (Eda, PP1024), which convert gluconate-6-*P* into GAP and pyruvate (Fig. 6A). Yet, it is unclear whether the ED pathway is still active when cells are grown on non-sugar substrates, e.g. glycerol. RNA sequencing revealed a significant decrease in the expression of the *edd* and *eda* genes on glycerol as compared with glucose (Table 2), which would suggest *a priori* that the entire ED pathway has a low level of activity when the polyol is used as the C source. But, somewhat intriguingly, the same *edd* and *eda* transcripts were unexpectedly higher on glycerol than on succinate, which would establish a hierarchy in the expression level of the ED pathway in the order glucose>glycerol>succinate. A quite similar trend was observed for the expression of genes encoded in the same transcriptional unit in which *edd* and *eda* are located, i.e. *zwf-1* and *pgl* (Table 2). This scenario was confirmed when the Edd and Eda activities were directly tested *in vitro*. As shown in Fig. 6B, the highest level of either enzymatic activity was observed in glucose-grown cells, peaking at 238 ± 46 and 635 ± 141 nmol min^{-1} mg $protein^{-1}$ for Edd and Eda, respectively. These values were 5.3- and 2.2-fold higher, respectively, than those observed on glycerol. On the other hand, the enzymatic activities in succinate were way below that on the other two substrates and can be considered just marginal (Fig. 6B). Taken together, these figures argue for an active role of the ED pathway in glycerol catabolism or at least in the associated physiological state. But, which could this role be? Inspection of the metabolic map of Fig. 4 suggests that *P. putida* would still grow on glycerol even in the absence of an ED pathway. To test this prediction, we cultured an *eda*::mini-Tn*5* mutant of *P. putida* KT2440 in M9 minimal media containing glycerol, glucose or succinate as the only C source as explained before. Specific growth rates in glycerol and succinate were very slow but still detectable ($\mu = 0.21 \pm 0.05$ and 0.34 ± 0.02 h^{-1}, respectively), whereas the control culture with glucose did not grow at all, as expected for such an essential pathway when using this hexose as the substrate. In contrast, the *eda*::mini-Tn*5* strain grew in rich lysogeny broth (LB) medium at a similar growth rate as wild-type *P. putida* KT2440 (data not shown). Previous work from our laboratory has indicated that the ED pathway activity is not only necessary for the direct processing of hexoses in *P. putida* KT2440 but also to generate high levels of NADPH necessary to endure oxidative stress (Chavarría *et al.*, 2013). It is thus plausible that while non-strictly required for producing the bulk of biomass-building intermediates from glycerol, the ED route is still necessary to fulfil the cellular needs of redox cofactors (i.e. NADPH), even when the *modus operandi* of this metabolic block does not act in the standard, downwards glycolytic mode.

A $NADP^+$-dependent GAP dehydrogenase (PP3443) contributes to glycerol metabolism in P. putida *KT2440*

Because of the reversibility of the oxidation step of GAP into glycerate-1,3-P_2, the enzyme GAP dehydrogenase (GAPDH) plays a pivotal role in the EMP pathway acting either on its downwards mode (glycolysis) and in gluconeogenesis (Ruiz-Amil *et al.*, 1969; Vicente and Cánovas, 1973b; Lessie and Phibbs, 1984; Fuhrer *et al.*, 2005). This biochemical step lies at the very core of both glycolytic and gluconeogenic metabolic pathways in most microorganisms, somewhat deciding the direction in which the C

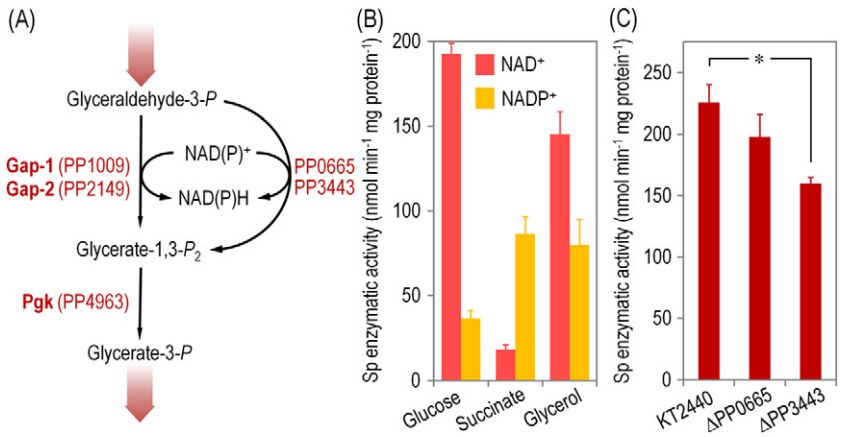

Fig. 7. Biochemical characterization of the glyceraldehyde-3-*P* node in *Pseudomonas putida* KT2440.
A. Biochemical sequence proposed for glyceraldehyde-3-*P* processing. Enzymes involved in these conversions are shown beside the reaction they catalyse. Note that the first biochemical step is catalysed by the glyceraldehyde-3-*P* dehydrogenases Gap-1 and Gap-2, and two other possible isoforms of these enzymes, encoded by PP0665 and PP3443. All the reactions are conventionally written in the catabolic direction, and the wide shaded arrows indicate the connection of this series of biochemical reactions with the rest of the central carbohydrate metabolic pathways.
B. *In vitro* quantification of specific (Sp) enzymatic activities of cells grown on M9 minimal medium added with either glucose, succinate or glycerol. Cells were harvested in mid-exponential phase (OD_{600} *ca.* 0.5), and the NAD^+- or $NADP^+$-dependent activity of glyceraldehyde-3-*P* dehydrogenase was determined in the cell-free extract, as detailed in the Experimental procedures section. Each bar represents the mean value of the corresponding enzymatic activity ± standard deviation (SD) of duplicate measurements from at least three independent experiments.
C. *In vitro* quantification of the *total* specific (Sp) activity of glyceraldehyde-3-*P* dehydrogenase in *P. putida* KT2440 and their isogenic ΔPP0665 and ΔPP3443 derivatives determined in cells grown on glycerol. Each bar represents the mean value of the corresponding enzymatic activity ± SD of duplicate measurements from at least three independent experiments, and the asterisk mark (*) identifies a significant difference with $P < 0.05$ (ANOVA).

flow proceeds (Neidhardt *et al.*, 1990). *Pseudomonas putida* KT2440 encodes two *bona fide* GAPDH isozymes, i.e. Gap-1 (PP1009) and Gap-2 (PP2149), which are easily identified given their similarity to the same enzyme counterparts in related microorganisms (Winsor *et al.*, 2011). The metabolic node of *P. putida* that encompasses GAPDH is schematically shown in Fig. 7A. In the available genome annotation of this bacterium (Nelson *et al.*, 2002) and on the basis of information reported previously for *P. aeruginosa* (Rivers and Blevins, 1987), the Gap-1 and Gap-2 isoenzymes are suggested to differ in their cofactor dependence, but this important biochemical trait has not been so far investigated. Comparisons with other bacteria do not clarify the point. In *E. coli*, one GAPDH form called GapA (which is more similar to eukaryotic counterparts) is required for downwards glycolysis, but Epd, the second enzyme (also termed GapB) is not and seems to play an entirely glyconeogenic role (Boschi-Muller *et al.*, 1997; Seta *et al.*, 1997). In contrast, conversion of GAP into glycerate-1,3-P_2 in *B. subtilis* is catalysed by two specialized isoenzymes that are dedicated to either catabolism (GapA, NAD^+ dependent) or anabolism (GapB, $NADP^+$-dependent) (Fillinger *et al.*, 2000). On this background, we set out to ascertain what GAPDH activities were operating in *P. putida* KT2440 cells growing on glycerol, what genes encoded them and what are their cofactors *in vitro*.

The cofactor dependence of the *total* GAPDH activity in cell-free extracts was first studied to determine the coarse role of this biochemical step on glycerol utilization, either in the downwards, catabolic (i.e. NAD^+ dependent) or upwards, anabolic (i.e. $NADP^+$ dependent) direction. The *in vitro* enzymatic activities were assessed in cells grown on either glucose, succinate or glycerol, and the reaction mixture for activity determinations was separately amended with NAD^+ or $NADP^+$ in order to investigate the cofactor dependence (Fig. 7B). Although we detected significant levels of $NADP^+$-dependent activity in all the conditions tested, the NAD^+-dependent activity was by far the predominant form of GAPDH present in cell-free extracts from glucose and glycerol (accounting for *ca.* 84% and 65% of the total activity, respectively). The opposite trend was verified in cells growing on succinate: the $NADP^+$-dependent GAPDH activity attained *ca.* 83% of the total enzymatic activity detected (which in turn was half of that observed on either glucose or glycerol), thus accrediting a pivotal role of the GAPDH step in furnishing intermediates through gluconeogenesis. The similarity in the GADPH activity levels on glucose and glycerol can be better understood by considering that both the ED pathway and the polyol catabolism produce GAP as an end metabolite, which meets a similar metabolic fate irrespective of its origin. Cells growing on glycerol also had a significant level of $NADP^+$-dependent GAPDH activity, exposing a highly

amphibolic nature of this biochemical step when *P. putida* is cultured on the triol. In other words, the GADPH-catalysed biochemical step had a NAD^+ dependence compatible with downwards functioning when cells grew on glucose, but the same activity was almost entirely dependent on $NADP^+$, i.e. acting in the upwards direction, when succinate was used as the C source. Glycerol thus determined a cofactor dependence for GADPH that lie in between that observed with glucose or succinate, demonstrating that this enzyme plays an amphibolic role under such growth condition. This situation is entirely compatible with the transcriptional landscape in cells growing on glycerol, namely, a significant rerouting of C skeletons through gluconeogenic pathways in addition to high expression of catabolic genes.

What are then the GAPDH enzymes relevant for glycerol-dependent growth of *P. putida* KT2440? Inspection of the transcriptomes, as shown in Tables S3–S6, expose a significant increase in *gap-2* transcripts in glycerol-grown cells, what suggested a role of the encoded GAPDH in metabolism of the compound. Transcription of *gap-1* did not vary on glycerol as compared with the other C sources, but we detected changes in the expression of a previously unassigned open reading frame (ORF) (PP3443) with similarity to GAPDHs. The MetaCyc platform (Caspi *et al*., 2012) also suggests that there is a fourth ORF (PP0665), encoding the same enzymatic activity in *P. putida* KT2440, the transcription of which showed a slight increase on glycerol as compared with succinate. In sum, there are four potential GAPDH enzymes in *P. putida* KT2440, and three of them (*gap-2*, PP3443 and PP0665) were transcriptionally affected by growth on glycerol. Most interestingly, currently available metabolic models of *P. putida* KT2440 predict a prominent role of Gap-2 in downwards carbohydrate catabolism (see for instance, Puchałka *et al*., 2008), but the contribution of the products encoded by PP3443 and PP0665 is not at all clear. In fact, the possible role of PP3443 and/or PP0665 on glycerol metabolism was not anticipated – thus opening the relevant question of whether the products encoded in these loci play a role in glycerol processing.

Based on this premise, are the secondary GAPDH enzymes encoded by these two ORFs involved in glycerol processing? As a first step to answer this question, we constructed ΔPP0665 and ΔPP3443 single knock-out mutants in the *P. putida* KT2440 background, and their growth parameters were evaluated in batch cultures in M9 minimal medium using glycerol as the C source. Both mutants exhibited specific growth rates and final biomass concentrations similar to those of the wild-type strain (data not shown), although the ΔPP3443 mutant had a 22% lower specific growth rate than *P. putida* KT2440 [$P < 0.05$, analysis of variance (ANOVA)], suggesting a relevant role of the product encoded by PP3443 in glycerol utilization. We also analysed the contribution of the PP3443 and PP0665 products to the *total* GAPDH activity *in vitro*. In doing so, the levels of activity were compared in the wild-type strain and its isogenic ΔPP0665 and ΔPP3443 derivatives (Fig. 7C) when cells were grown on glycerol. No significant differences were observed in the NAD^+-dependent activity among the strains assayed (data not shown), but a 30% lower $NADP^+$-dependent activity was registered in the ΔPP3443 mutant. This result accredits the role of the PP3443 product as the source of a GAPDH activity relevant for glycerol metabolism in *P. putida* KT2440, and its cofactor dependence points to a probable gluconeogenic role.

Conclusion

The transcriptional and biochemical evidence presented earlier reveals both similarities and divergences in the use of glycerol by *P. putida* with respect to other bacteria. The most salient features include (i) upregulation of glycerol catabolic genes, (ii) downregulation of alternative routes for C processing, (iii) activation of a gluconeogenic response, and (iv) concomitant slow-down of the activities through the TCA cycle and the gluconate/2-gluconate loops. The glycerol-consuming physiological mode seems therefore to favour biomass build-up all the while preventing loss of C as CO_2 or acidic by-products. The considerable lag phase in cultures with the polyol could indicate a substantial rearrangement of the whole metabolic network prior to reaching an optimum for growth on this substrate. It is revealing that such a long take-off and the ensuing slow proliferation rate (Table 1) is accompanied by a general decrease of stress and a very efficient (if late) conversion of substrate into biomass, accompanied by a differential expression of components of the respiratory chain (Fig. 3C and D). This raises interesting questions on the relationships between growth rate, stress and general fitness in bacteria. It has been suggested that microorganisms are subjected to the general biological principle of *caloric restriction*, i.e. highly energetic C substrates lead to transient fast growth but also to physiological stress and relative loss of individual reproductive capacity (Anderson *et al*., 2003; Skinner and Lin, 2010). The results presented here are consistent with such a perspective: by avoiding to overrun the reactions within the TCA cycle and external metabolic loops, and by recycling C equivalents back to biomass building intermediates, cells may grow slower in glycerol and be less energized. Yet, under these circumstances, the metabolism would also be less stressful, and the population as a whole should be eventually more successful in terms of final numbers. This situation was reflected in the swimming motility of *P. putida* KT2440, a coarse descriptor of the energy load of the cells (Blair, 1995), grown on the

different substrates tested (Fig. S1 in the Supporting information). It is tempting to speculate that this is in itself an evolutionary trait that makes cells to tune the balance prevalence versus niche exploration in a fashion dependent on the available C sources. From a more practical point of view, the data reported accredits the value of glycerol as a growth substrate for *P. putida* KT2440 that merges its abundance and affordability along with its physiological benefits in the same lot.

Experimental procedures

Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this study are listed in Table S1 of the Supporting information. *Escherichia coli* and *P. putida* strains were routinely grown at 37 and 30°C, respectively, in LB medium (containing 10 g l^{-1} tryptone, 10 g l^{-1} NaCl and 5 g l^{-1} yeast extract). *Escherichia coli* DH5α and CC118λ*pir* were used for cloning procedures and plasmid maintenance. M9 minimal medium, containing 6 g l^{-1} Na_2HPO_4, 3 g l^{-1} KH_2PO_4, 1.4 g l^{-1} $(NH_4)_2SO_4$, 0.5 g l^{-1} NaCl, 0.2 g l^{-1} $MgSO_4{\cdot}7H_2O$ and 2.5 ml l^{-1} of a trace elements solution as previously described (Abril *et al*., 1989; Nikel and de Lorenzo, 2013), was used for the physiological characterization of the strains. Unless otherwise noted, cultures were grown in 250 ml Erlenmeyer flasks containing medium up to one-fifth of their nominal volume with rotary shaking at 170 r.p.m. For *E. coli* cultivations, 5 mg l^{-1} thiamine·HCl was also added to M9 minimal medium. Growth was estimated by measuring the optical density at 600 nm (OD_{600}), assessed in an Ultrospec 3000 *pro* UV/Visible Spectrophotometer (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). All solid media used in this work contained 15 g l^{-1} agar, and kanamycin (50 μg ml^{-1}) or ampicillin (500 μg ml^{-1} for *P. putida* and 150 μg ml^{-1} for *E. coli*) were added when appropriate as filter-sterilized solutions. For the physiological characterization of the strains, RNA extraction and measurements of enzymatic activity, *P. putida* strains were pregrown overnight in M9 minimal medium containing 15 mM succinate as the sole C source. Cells were collected and washed twice with M9 minimal medium without any added C source, and concentrated to an OD_{600} of *ca*. 2. This cell suspension was diluted 100-fold in fresh M9 minimal medium containing either 10 mM glucose, 15 mM succinate or 20 mM glycerol. Each culture was incubated at 30°C until mid-exponential phase was reached (OD_{600} of *ca*. 0.5). Cells were promptly harvested at this point and processed as detailed later.

DNA manipulation and sequencing and mutant construction

Standard techniques were used for DNA manipulations. Oligonucleotides were synthesized by Sigma-Aldrich Co. (St. Louis, MO, USA). Polymerase chain reactions (PCR) were set up using either *Taq* DNA polymerase (Promega, Madison, WI, USA) or Phusion high-fidelity DNA polymerase (New England BioLabs, Ipswich, MA, USA) based on experimental requirements and used according to the manufacturers' instructions. Restriction endonucleases and other DNA modifying enzymes were purchased from New England BioLabs and were used according to the manufacturers' specifications. DNA fragments were isolated from agarose gels using a NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Sanger sequencing was performed by Secugen SL (Madrid, Spain). *Pseudomonas putida* knock-out mutants were constructed as per the protocol described by Martínez-García and de Lorenzo (2011), as detailed in the Supporting information.

RNA manipulation and deep sequencing of transcripts

Total RNA was extracted by using the RNeasy kit (QIAGEN, Inc., Valencia, CA, USA), and RNase-free DNase (QIAGEN) treatment was performed during the isolation procedure to eliminate any residual DNA in the preparation. Quality of RNA samples was evaluated in an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). RNA library construction and sequencing were carried out by BGI (Shenzhen, China), using the Illumina mRNA sequencing sample preparation kit (cat. #RS-930–1001) and the Illumina HiSeq™ 2000 system (Illumina, Inc., San Diego, CA, USA). Reads generated by the sequencing machines were cleaned and mapped to the database of *Pseudomonas* genes sequences (NCBI reference sequence NC_002947, version NC_002947.3) using the SOAP2 software (version 2.21) (Li *et al*., 2009), and the resulting alignment was visualized using the IGV software (Thorvaldsdóttir *et al*., 2013). Fold changes and *P* values were calculated as described by Audic and Claverie (1997). As the RNA sequencing data generated corresponded to a single biological sample for each C source, *P* values were corrected and expressed as false discovery rate values (Benjamini *et al*., 2001). Genes with false discovery rates ≤ 0.001 and absolute fold change larger than two were considered as differentially expressed. The detailed procedures and the complete set of raw data generated in these experiments will be made available in a separate study (Kim *et al*., 2013).

Preparation of cell-free extracts and enzymatic assays

All the enzyme activity determinations were carried out during mid-exponential phase cultures (i.e. corresponding to an OD_{600} of *ca*. 0.5). Cell-free extracts were prepared starting from 50 ml of culture broth and centrifuging it at 4000 r.p.m. for 10 min. All the following procedures were carried out at 4°C as previously described (Nikel and de Lorenzo, 2013). Cells were washed in 150 mM NaCl and subsequently in 100 mM phosphate-buffered saline (PBS, pH = 7.4). The washed pellets were suspended in 500 μl of 100 mM PBS (pH = 7.4), supplemented with 1.5 mM 2-mercaptoethanol, and disrupted by ultrasonic treatment (10×15 s treatments with 30 s pauses between each round). The mixture was centrifuged at 10 000 r.p.m. for 5 min, and the supernatant was collected and kept on ice. This crude extract was separated into soluble and particulate (i.e. membrane enriched) fractions by centrifugation at 29 500 r.p.m. for 2.5 h at 4°C. Sedimented particulate fractions were homogenized in ice-cold 100 mM PBS (pH = 7.4). These washed membrane-enriched sediments were finally centrifuged at 8500 r.p.m. for

20 min, and the supernatant fluids were collected. Washed membranes obtained by this procedure contained most of the detectable G3PDH activity (see text for details). All other enzyme activities assayed were localized in the soluble fraction of crude extracts. Enzyme activities were normalized by determining the total cell protein concentration using a Bradford-based protein assay purchased from Sigma-Aldrich Co. Unless otherwise indicated, we used an extinction coefficient (ε_{NADH}) of 6.22 mM^{-1} cm^{-1}, representing the difference between the extinction coefficients of NAD(P)H and $NAD(P)^+$. One unit of enzyme activity was defined as the quantity of enzyme that catalysed the formation of 1 μmol of the corresponding product during the time indicated and at 30°C. Detailed protocols for the *in vitro* determination of GlpK, G3PDH, gluconate-6-*P* dehydratase (Edd), 2-keto-3-deoxygluconate-6-*P* aldolase (Eda) and GAPDH activities are given in detail in the Supporting information.

Analytical procedures

Succinate concentration was determined using a succinic acid assay kit from Megazyme International (Wicklow, Ireland) according to the manufacturer's protocol, adjusting the volumes to a final assay volume of 1 ml, by a coupled enzymatic assay. Succinate was first converted to succinyl-CoA with the concomitant production of adenosine diphosphate (ADP), which pyruvate kinase consumes in the formation of pyruvate. Pyruvate, produced stoichiometrically with respect to succinate, was reduced to lactate by lactate dehydrogenase. The associated decrease in NADH concentration was spectrophotometrically monitored at 340 nm. Glucose, glycerol, acetate and gluconate were assayed in culture supernatants using commercial kits from R-Biopharm AG (Darmstadt, Germany), as per the manufacturer's instructions. Mock assays were conducted for the glucose, succinate and glycerol assays by spiking the reaction mixture or M9 minimal medium with different amounts of the C sources. Biomass yields (calculated 24 h after the cells started to grow exponentially), specific rates of growth and C consumption during exponential growth, and the extension of the lag phase were derived from growth parameters for each culture condition as described elsewhere (Dalgaard and Koutsoumanis, 2001; Fuhrer *et al*., 2005; Nikel and de Lorenzo, 2012; Chavarría *et al*., 2013; Nikel *et al*., 2013).

Statistical analysis

All the physiological and biochemical experiments reported were independently repeated at least twice (as indicated in the corresponding figure legend), and the mean value of the corresponding parameter ± standard deviation is presented. The level of significance of the differences when comparing results was evaluated by means of ANOVA, with $\alpha = 0.05$, or through the false discovery rate values as noted earlier.

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Supporting information

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

Fig. S1. Swimming motility of *Pseudomonas putida* KT2440 on different C sources.
Table S1. Bacterial strains and plasmids used in this study.
Table S2. Oligonucleotides used in this study.
Table S3. List of upregulated genes in cells grown on glycerol as compared with glucose.
Table S4. List of upregulated genes in cells grown on glycerol as compared with succinate.
Table S5. List of downregulated genes in cells grown on glycerol as compared with glucose.
Table S6. List of downregulated genes in cells grown on glycerol as compared with succinate.