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

by

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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 down-regulated on glycerol. Yet, the compound simultaneously elicited a gluconeogenic response that indicated an efficient channeling 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 favoring prevalence *versus* exploration. This metabolic situation was accompanied by a considerably low expression of stress markers as compared to 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 (Puchalka *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 endeavors. 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 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 ATP-dependent glycerol kinase (GlpK) and the aerobic/anaerobic respiratory G3P dehydrogenases (G3PDH) GlpD/GlpABC (Lin, 1976). Glycerol dehydrogenase (GldA) next catalyzes the oxidation of glycerol to dihydroxyacetone, that subsequently enters into the central biochemical network 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*., submitted). 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 shown below reveals 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 Pseudomonas 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 Entner-Doudoroff (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 behavior 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 $\geq$ 12 h. Cells using glucose had a comparatively lesser delay (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 pre-grown (data not shown), suggesting regulatory mechanisms beyond a mere transcriptional and/or biochemical adaptation to the polyol. Taken together, these results revealed substantial physiological differences among the conditions assayed, and prompted us to investigate their genetic and biochemical bases.

Transcriptional analysis of the glp *regulon of* Pseudomonas 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, glycerol kinase), *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 below).

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 analyzed 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 *et al*. (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 to 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.

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 $\geq$ 2-fold in comparison to the other C sources. A total of 37 and 117 genes were significantly up-regulated in the presence of glycerol as compared to 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 down-regulated 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 up-regulated and down-regulated genes is summarized in Fig. 2B (see also Tables S3-S6 in the Supplementary Information for a complete list). The coarse patterns of such pair-wise 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 below.

Stress responses and respiratory functions in glycerol-grown Pseudomonas 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 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 down-regulated, 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 above). Slow growth is normally accompanied by reduced respiratory activity (Neidhardt *et al.*, 1990), which would also correlate with lower generation of reactive oxygen species. But 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 *et al*. (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. *P. 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 channeled through the bc_1 complex. Four genes involved in cytochrome *c* oxidase maturation and assembly were up-regulated 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 to succinate. Another operon that showed high expression levels on glycerol as compared to 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., catalyzing the final electron transfer to O_2) showed expression differences as compared to, for instance, genes encoding ubiquinol:cytochrome *c* reductase components, or the multiple 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 down-regulated on glycerol as compared to 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 *et al*. (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 below.

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

The qualitative distribution of up- and down-regulated 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 Supplementary 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 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 re-routing 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 down-regulated in glycerol as compared to both glucose and succinate (Table 2). Transcription of genes for interconversions within the gluconate and 2-ketogluconate loops were slightly down-regulated 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 developed on glucose, in contrast, the gluconate concentration reached *ca*. 50 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 catalyzes the conversion *cis*-aconitate → D-*threo*-isocitrate within the TCA cycle, accompanied by the concomitant down-regulation 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 dehydrogenase component of 2-ketoglutarate dehydrogenase) was also down-regulated in glycerol as compared to succinate, a further indication of a repressed TCA cycle. Interestingly, a similar metabolic behavior 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 below.

Glycerol catabolism in Pseudomonas putida *KT2440 involves glycerol kinase and a membrane-bound G3P dehydrogenase*

As mentioned above, the pathway proposed for glycerol catabolism in *P. putida* KT2440 starts with the phosphorylation of the triol substrate followed by oxidation of 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 above). The corresponding biochemical activities peaked 27- and 55-fold higher, respectively, when cells were cultured on glycerol-containing M9 minimal medium as compared to 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 to 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-bisphosphate, and inducer exclusion -that concertedly modulate enzymatic activity in a transcriptional-independent fashion (Holtman *et al*., 2001). Moreover, Applebee *et al*. (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 to 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 above for the cognate enzyme in *E. coli*.

The Entner-Doudoroff pathway is essential for glycerol utilization by Pseudomonas putida *KT2440*

As mentioned above, 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 to 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 nmol and 635 ± 141 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 (μ = 0.21 ± 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 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 fulfill 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 glyceraldehyde-3-P *dehydrogenase (PP3443) contributes to glycerol metabolism in* Pseudomonas 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 flow proceeds (Neidhardt *et al.*, 1990). *P. 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 catalyzed 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-catalyzed 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 re-routing 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 (Supplementary Information), 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 to the other C sources, but we detected changes in the expression of a previously unassigned 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 to 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, Puchalka *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 ($p < 0.05$, ANOVA), suggesting a relevant role of the product encoded by PP3443 in glycerol utilization. We also analyzed 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 above reveals both similarities and divergences in the use of glycerol by *P. putida* with respect to other bacteria. The most salient features include [i] up-regulation of glycerol catabolic genes, [ii] down-regulation 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 favor 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 re-arrangement 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 over-run 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 Supplementary 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 above 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 Supplementary Information. *E. coli* and *P. putida* strains were routinely grown at 37ºC and 30ºC, respectively, in LB medium (containing 10 g l^{-1} tryptone, 10 g l^{-1} NaCl, and 5 g l^{-1} yeast extract). *E. 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 rpm. 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 pre-grown 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 30ºC until mid-exponential phase was reached (OD_{600} of *ca*. 0.5). Cells were promptly harvested at this point and processed as detailed below.

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). PCR reactions 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). *P. putida* knock-out mutants were constructed as per the protocol described by Martínez-García and de Lorenzo (2011), as detailed in the Supplementary 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 2 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*., submitted).

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 4,000 rpm 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 rpm 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 rpm 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 8,500 rpm 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 catalyzed 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 glycerol kinase, G3PDH, gluconate-6-*P* dehydratase (Edd), 2-keto-3-deoxygluconate-6-*P* aldolase (Eda), and GAPDH activities are given in detail in the Supplementary 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 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 ± SD is presented. The level of significance of the differences when comparing results was evaluated by means of analysis of variance (ANOVA), with $\alpha = 0.05$; or through the false discovery rate values as noted above.

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TABLES

Table 1. Growth parameters[a] for batch cultures of *Pseudomonas putida* KT2440 developed 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 ± SD 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.

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

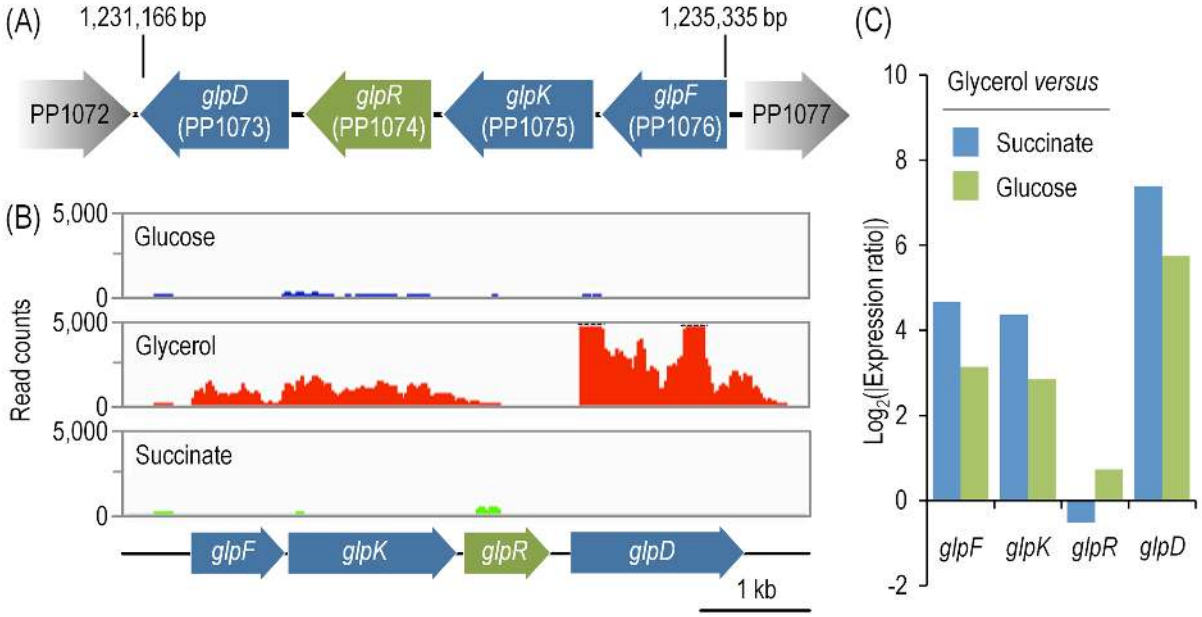
Gene	PP number[a]	Function(s)	Expression level on glycerol [$\log_2$(|Expression ratio|)] normalized to:	
			Succinate	Glucose
zwf-1	PP1022	Glucose-6-*P* 1-dehydrogenase	2.771	−2.216
pgl	PP1023	6-Phosphogluconolactonase	3.059	−2.435
edd[b]	PP1010	Phosphogluconate dehydratase	1.136	−2.235
eda[b]	PP1024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

[a] As annotated by Nelson *et al*. (2002).

[b] Genes encoding the components of the ED pathway.

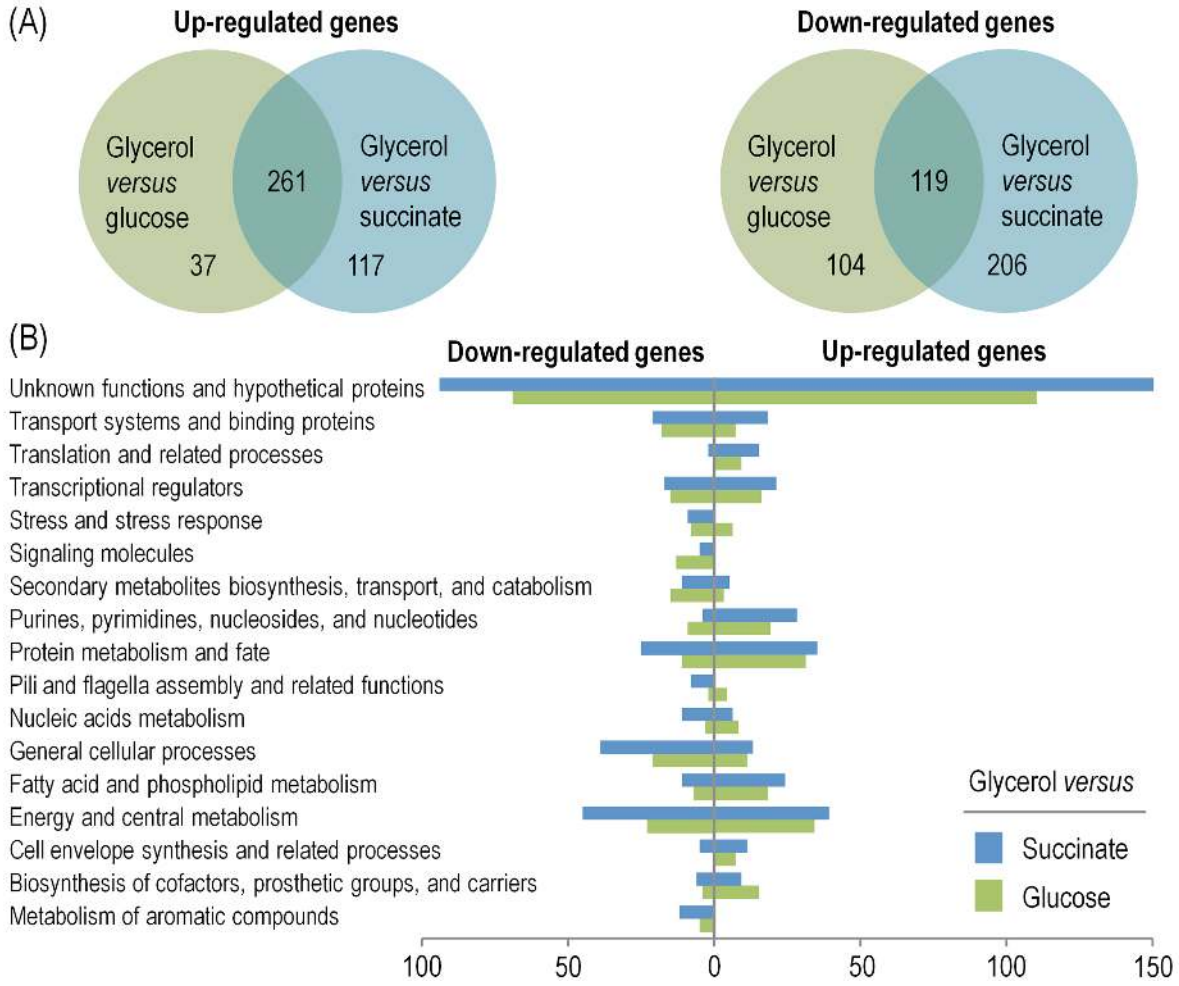
FIGURES

Fig. 1. Expression pattern of the *glp* genes in *Pseudomonas putida* KT2440 analyzed at the single-nucleotide level.



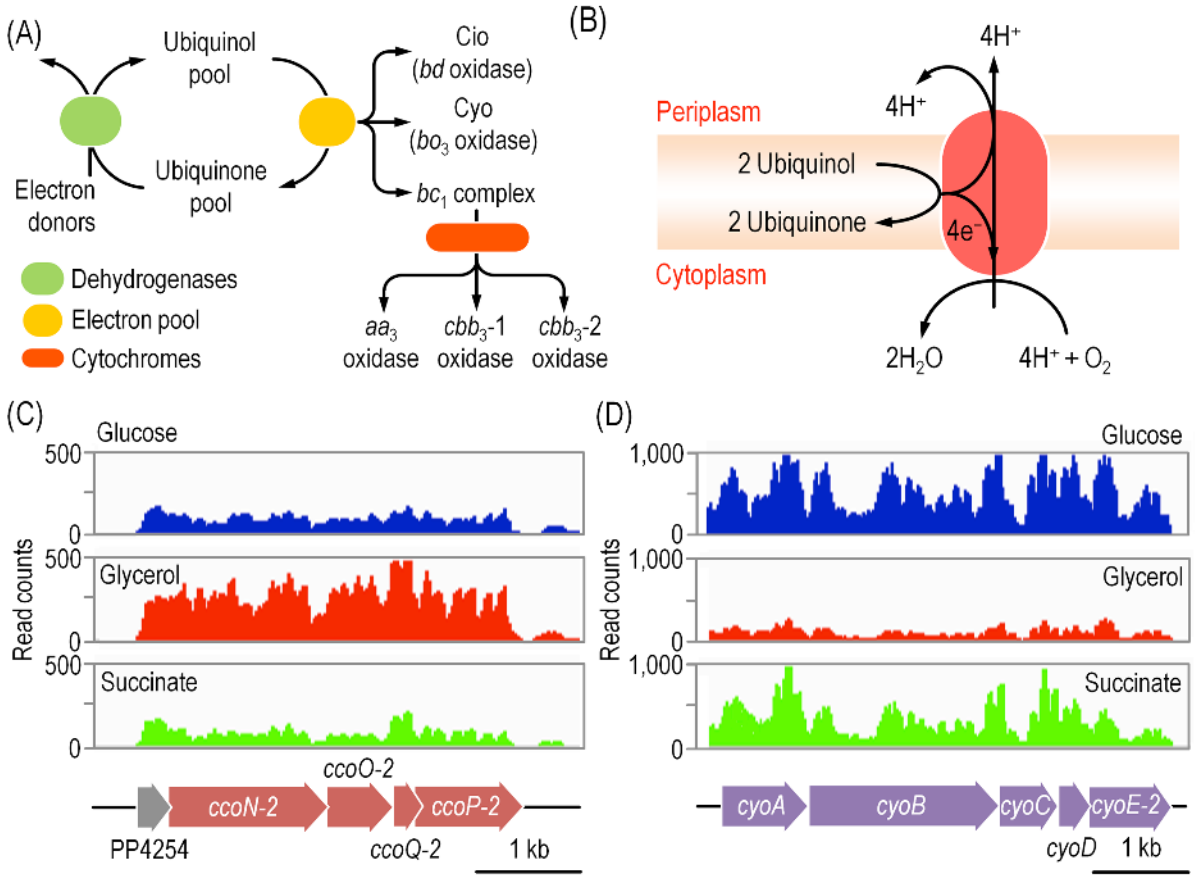
(A) Genetic organization and genomic coordinates of the *glp* locus. The genomic region encompasses *glpF* [PP1076, a major intrinsic protein (MIP) family channel protein], *glpK* (PP1075, glycerol kinase), *glpR* (PP1074, a DeoR family transcriptional regulator), and *glpD* (PP1073, glycerol-3-*P* dehydrogenase; 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 pair-wise comparison among C sources were significant as judged by the corresponding false discovery rate values (Benjamini *et al.*, 2001).

Fig. 2. Number and functional classification of genes differentially expressed in *Pseudomonas putida* KT2440 grown on glycerol.



(A) Up- and down-regulated genes in cells grown on glycerol as compared to glucose or succinate cultures. Venn diagrams show the overlap between up- and/or down-regulated genes for each pair-wise 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 Supplementary 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.

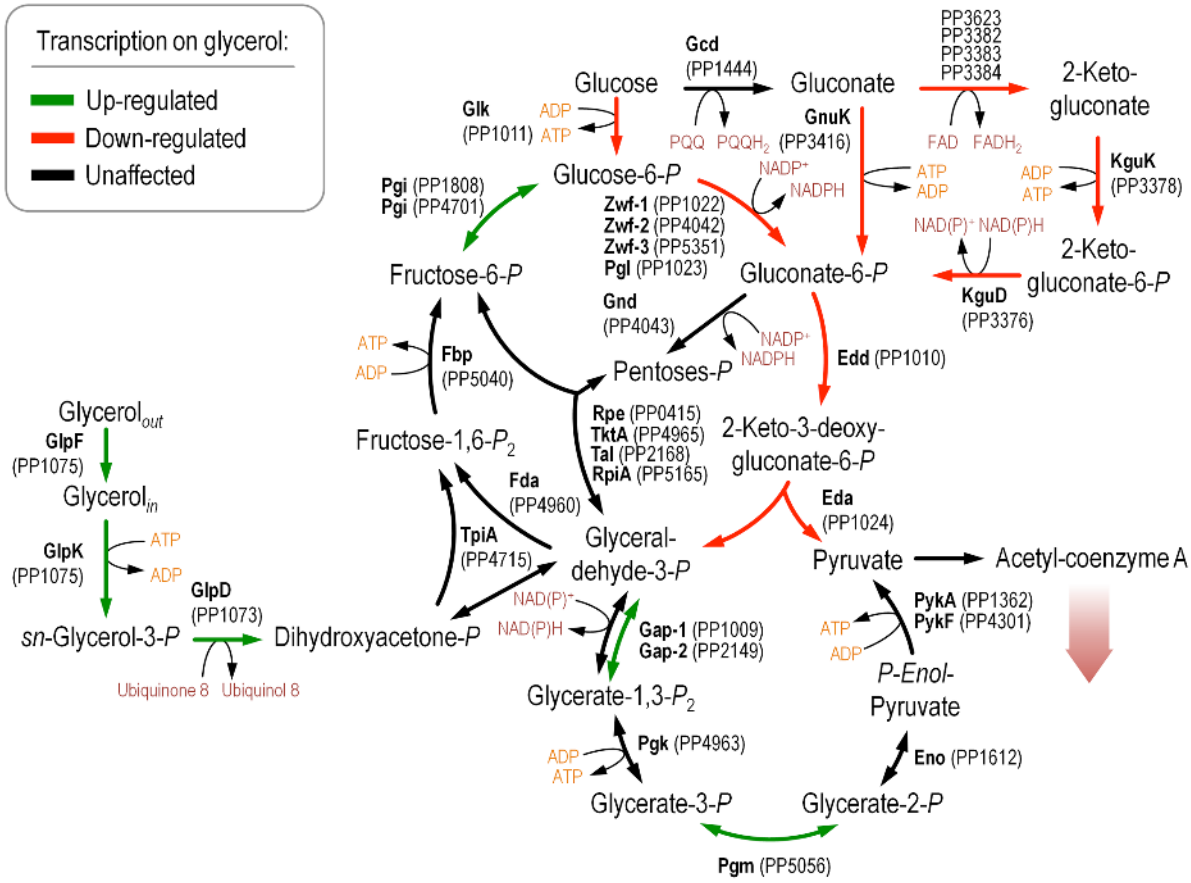
Fig. 3. Organization of the respiratory chain in *Pseudomonas putida* KT2440 and transcriptional landscape of the genes encoding its components.



(A) Simplified scheme of the electron transport chain, adapted from Ugidos *et al*. (2008) and Follonier *et al*. (2013). Different electron donors firstly 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

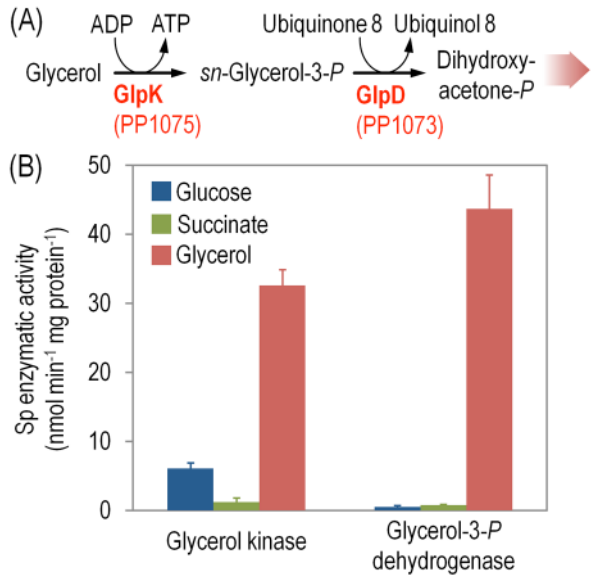
specific genes encoding components of the Cyo terminal oxidase, schematically shown in panel B, are indicated.

Fig. 4. Genes within the upstream central C metabolism in *Pseudomonas putida* KT2440 affected by growth on glycerol.



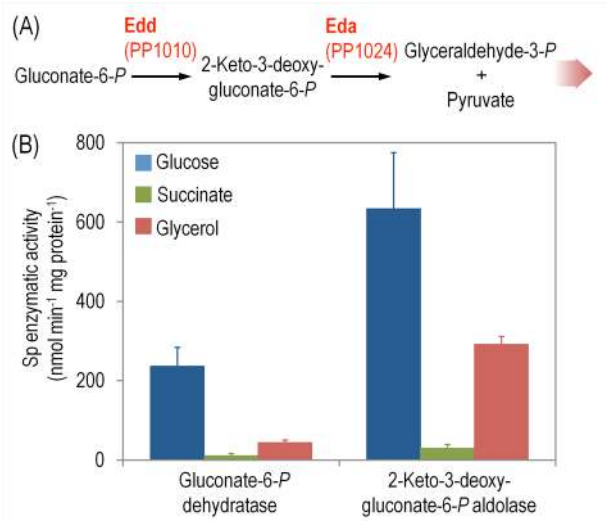
The biochemical network schematically sketches the main bioreactions involved in C processing along with the enzymes catalyzing the corresponding conversions. In some cases, reactions have been lumped to simplify the diagram, and only some isoforms of these enzymes are shown. Further metabolism of acetyl-coenzyme 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 to the glucose condition are highlighted in different colors according to whether they were significantly up-regulated (green), down-regulated (red), or remained unaffected (black).

Fig. 5. Biochemical characterization of enzymes involved in glycerol catabolism in *Pseudomonas putida* KT2440.



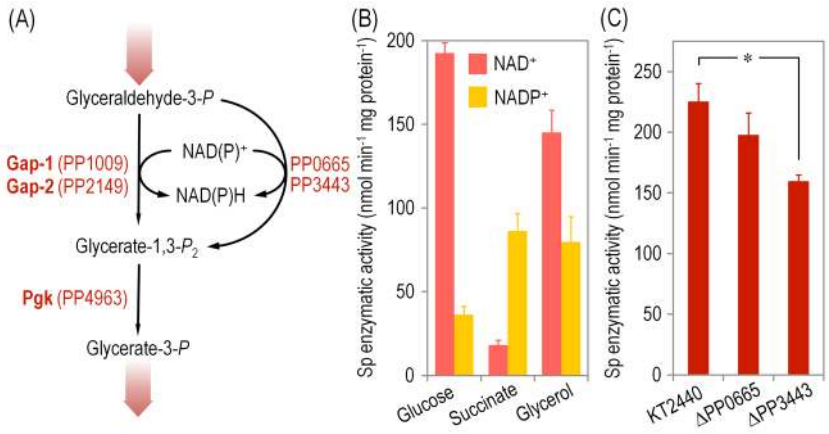
(A) Biochemical pathway proposed for glycerol phosphorylation and oxidation. Upon entrance of glycerol into the cytoplasm (mediated by GlpF, the glycerol facilitator), it is used as a substrate for GlpK (glycerol kinase). The product of this transformation is then oxidized in a ubiquinone-dependent reaction catalyzed 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). (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 ± SD of duplicate measurements from at least three independent experiments. Differences in the pair-wise comparison among C sources were significant ($p < 0.05$) as evaluated by ANOVA.

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 catalyze. 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 ± SD of triplicate measurements from at least three independent experiments. Differences in the pair-wise comparison among C sources were significant ($p < 0.05$) as evaluated by ANOVA.

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 catalyze. Note that the first biochemical step is catalyzed 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 ± SD of duplicate measurements from at least three independent experiments. (B) *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).

Supporting Information for

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

by Nikel *et al.*

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mutant construction

Pseudomonas putida knock-out mutants were constructed as per the protocol described by Martínez-García and de Lorenzo (2011). Appropriate oligonucleotides (see sequences in Table S2) were used to amplify *ca.* 500-bp flanking regions of either PP0665 (using oligonucleotides PP0665-TS1-F and PP0665-TS1-R, and PP0665-TS2-F and PP0665-TS2-R) or PP3443 (using oligonucleotides PP3443-TS1-F and PP3443-TS1-R, and PP3443-TS2-F and PP3443-TS2-R) by PCR. The resulting *ca.* 1-kbp amplification products (obtained by sewing PCR with the external oligonucleotides) were cloned into the suicide vector pEMG, giving rise to plasmids pEMGΔPP0665 and pEMGΔPP3443 (Table S1). These suicide plasmids were isolated from *Escherichia coli* CC118λ*pir* and electroporated individually in *P. putida* KT2440, which was subsequently transformed with the helper plasmid pSW-I as a source of the homing endonuclease I-*Sce*I (Wong and Mekalanos, 2000). Merodiploid clones were grown overnight in 5 ml of LB medium containing 500 μg ml^{-1} ampicillin and 15 mM sodium 3-methylbenzoate (as an inducer of the I-*Sce*I−mediated recombination) and plated onto LB agar medium. Isolated colonies were re-streaked onto either LB agar or the same medium containing 50 μg ml^{-1} kanamycin to check for the loss of the co-integrated plasmid. Kanamycin-sensitive clones were analyzed by colony PCR to identify clones in which either PP0665 or PP3443 had been deleted (data not shown). Finally, plasmid pSW-I was eliminated after three consecutive passes in LB medium. Elimination of the helper plasmid was verified in all cases by colony PCR amplification using the oligonucleotides pair pSW-F and pSW-R (Martínez-García and de Lorenzo, 2011).

In vitro *determination of enzymatic activities*

Glycerol kinase and glycerol-3-*P* dehydrogenase were assayed essentially as described by McCowen *et al*. (1981), with the modifications proposed by Williams *et al*. (1994). In brief, glycerol kinase activity was determined by measuring the reduction of NAD^+ in the presence of an externally added glycerol-3-*P* dehydrogenase. The assay mixture contained (in a final volume of 1.0 ml): 2.5 mM ATP, 3 mM glycerol, 0.5 mM NAD^+, 0.8 M hydrazine monohydrate buffer (pH = 10.4), 125 mM glycine buffer (pH = 8.5), 2 mM $MgCl_2$, 4.5 U α-glycerol-3-*P* dehydrogenase from rabbit muscle (Sigma-Aldrich Co.), and 80-200 μl of the cell-free extract. The reaction began with the addition of the cell-free extract to all the other reagents, and readings were taken every 10 s for 5 min at 340 nm. Glycerol-3-*P* dehydrogenase was determined by measuring the rate of reduction of 2,6-dichlorophenol-indophenol in an assay mixture that contained (in a final volume of 1.0 ml): 70 mM phosphate buffered saline (pH = 7.5), 10 mM KCN, 30 mM sodium D,L-glycerol-3-*P*, 75 μM 2,6-dichlorophenol-indophenol (DCPIP), 350 μM phenazine methosulfate, and 20-100 μl of the membrane-enriched fraction obtained from the cell-free extract. The reaction began with the addition of the extract to all the other reagents, and readings were taken every 15 s for 15 min at 600 nm. One unit of glycerol-3-*P* dehydrogenase activity was defined as the amount of enzyme that reduces 1 μmole of 2,6-dichlorophenol-indophenol min^{-1} under these assay conditions. An extinction coefficient (ε_{DCPIP}) of 2.1 mM^{-1} cm^{-1}, representing the difference between the extinction coefficients of oxidized and reduced DCPIP, was used in this assay.

The enzymes of the Entner-Doudoroff pathway were assayed by a modification of previously published protocols (Vicente and Cánovas, 1973; Baumann and Baumann, 1975; Stephenson *et al*., 1978; Ponce *et al*., 2005). Gluconate-6-*P* dehydratase (Edd) was assayed in a two-step reaction. Briefly, the assay mixture (in a final volume of 0.1 ml) contained: 50 mM Tris·HCl buffer (pH = 7.5), 10 mM $MgCl_2$, 10 μM gluconate-6-*P*, and a suitable amount of the cell-free extract. After a 5 min incubation at room temperature, the mixture was diluted with the same reaction buffer up to 2 ml and heated for 2 min at 95ºC. The assay mixture was centrifuged (14,000 rpm, 10 min, room temperature), and the supernatant solution was assayed for pyruvate formation as explained below. 2-Keto-3-deoxygluconate-6-*P* aldolase (Eda) was assayed in a similar manner: the reaction mixture (2 ml) contained: 50 mM Tris·HCl buffer (pH = 7.5), 10 mM $MgCl_2$, 0.15 mM 2-keto-3-deoxygluconate-6-*P*, and a suitable amount of the cell-free extract.

Following a 5 min incubation at room temperature, the reaction mixture was heated and centrifuged as explained for the gluconate-6-*P* dehydratase assay, and the supernatant solution was assayed for pyruvate as follows. The reaction mixture consisted of 0.2 ml of the corresponding supernatant and 1.8 ml of a solution containing 50 mM Tris·HCl (pH = 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM NADH, and 0.5 U L-lactate dehydrogenase from bovine heart (Sigma-Aldrich Co.). Incubation was done at 37°C with intermittent shaking until the decrease in absorbance (measured at 340 nm) ceased. The amount of pyruvate formed was calculated using the molar extinction coefficient of NADH.

Glyceraldehyde-3-*P* dehydrogenase was measured by using the protocol described by Tiwari and Campbell (1969), as modified by Rivers and Blevins (1987). The assay mixture contained (in a final volume of 1.0 ml): 100 mM Tris·HCl (pH = 7.5), 6 mM D,L-glyceraldehyde-3-*P*, 10 mM cysteine·HCl, 15 mM NaH_2AsO_4, 20 mM NaF, 0.35 mM NAD^+ or $NADP^+$, and 50-150 μl of the cell-free extract. The glyceraldehyde-3-*P* and cysteine·HCl solutions were neutralized just before use. The reaction mixture without glyceraldehyde-3-*P* was incubated at 30°C for 1 min and the reaction was initiated by adding the substrate. The reduction of either NAD^+ or $NADP^+$ was followed by taking readings every 10 s for 15 min at 340 nm.

Swimming assay

P. putida KT2440 was grown overnight on M9 minimal medium containing the corresponding C source, cells were concentrated to an optical density at 600 nm = 3.0 in fresh medium, and a 2-μl aliquot of the resulting cell suspension was laid onto the surface of soft M9 minimal medium agar plates [containing 0.3% (w/v) agar] added with either 10 mM glucose, 15 mM succinate, or 20 mM glycerol. Petri dishes were incubated at 30ºC and the maximum diameter of the bacterial layer recorded after 48 h.

Table S1. Bacterial strains and plasmids used in this study.

Bacterial strain or plasmid	Relevant characteristics[a]	Source or reference
Escherichia coli		
DH5α	Cloning host; Φ80*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U169*	Hanahan and Meselson (1983)
CC118λ*pir*	Cloning host; *araD139* Δ*(ara-leu)7697* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB*(Rif^R) *argE*(Am) *recA1*, λ*pir* lysogen	Herrero *et al.* (1990)
Pseudomonas putida		
KT2440	Wild-type strain, spontaneous restriction-deficient derivative of strain mt-2 cured of the TOL plasmid pWW0	Bagdasarian *et al.* (1981)
KT2440 *eda*::mini-Tn*5*	Same as KT2440, but *eda*::mini-Tn*5*, Km^R	Duque *et al.* (2007)
KT2440 ΔPP0665	Same as KT2440, but ΔPP0665	This work
KT2440 ΔPP3443	Same as KT2440, but ΔPP3443	This work
Plasmid		
pEMG	Suicide plasmid; *oriR6K*, *lacZ*α flanked by two I-*Sce*I restriction sites, Km^R	Martínez-García and de Lorenzo (2011)
pEMGΔPP0665	Same as pEMG, but carrying an EcoRI-BamHI 1,136-bp deletion sequence for PP0665	This work
pEMGΔPP3443	Same as pEMG, but carrying an EcoRI-BamHI 1,082-bp deletion sequence for PP3443	This work
pSW-I	Helper plasmid; *oriRK2*, *xylS* *Pm* → I-*Sce*I (transcriptional fusion of the I-*Sce*I coding sequence to *Pm*), Ap^R	Wong and Mekalanos (2000)

[a] Antibiotic markers: Rif, rifampicin; Km, kanamycin; Ap, ampicillin.

Table S2. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5' → 3')[a]	Comments
PP0665-TS1-F	AAA AGA ATT CGC GCG GGC TTG GTA GGT TTC	Incorporates an EcoRI site
PP0665-TS1-R	CAT GAA CGT TGC TCC TCA GGC C	
PP0665-TS2-F	GGC CTG AGG AGC AAC GTT CAT GTG AGG GGG AGG TTA CCT CGA TGA TC	Generates an overlapping stretch for sewing (cross-over) PCR
PP0665-TS2-R	TTT TGG ATC CAG GGG CTG GAG CAC TGG GAG AG	Incorporates a BamHI site
PP3443-TS1-F	AAA AGA ATT CTC GCA CTT TGC TGG CCA GCA G	Incorporates an EcoRI site
PP3443-TS1-R	CAT GGG TAC TGC ACC TCA CAT CAA AGG	
PP3443-TS2-F	CCT TTG ATG TGA GGT GCA GTA CCC ATG TGA CCG CCG CGC TTA TAG ACA ATC	Generates an overlapping stretch for sewing (cross-over) PCR
PP3443-TS2-R	TTT TGG ATC CGT CAG GTC CTC TTT GTG GGT GGA CAC	Incorporates a BamHI site

[a] Sites for restriction enzymes recognition are underlined, and complementary sequences used to anneal amplicons by means of sewing (cross-over) PCR are shown in red.

SUPPLEMENTAL RESULTS AND DISCUSSION

A complete description of the transcriptional landscape of *P. putida* KT2440 cells growing on the three C sources, in addition to the pathways and functions discussed in the main text of this study, is given below. For the sake of clarity, we separate the discussion according to the functional classification of genes differentially expressed on glycerol as compared to either glucose or succinate, pinpointing to relevant differences among C sources where adequate.

Central catabolic pathways

We firstly focused on the regulatory pattern of genes belonging to the upper catabolic pathways. The qualitative distribution of up- and down-regulated genes within the upper catabolic pathways in cells grown on glycerol is schematically shown in Fig. 3. As discussed in the main text, components of the ED pathway were down-regulated in glycerol as compared to the other C sources (see also Table 2). The transcription level of genes encoding enzymes of the pentose phosphate pathway did not show significant differences among the C sources tested, which would translate into a similar functionality of this pathway among different metabolic regimes.

Several genes encoding gluconeogenic functions, especially from C2 compounds (such as acetate or acetaldehyde) or C3 compounds (such as propanoate), and components of the glyoxylate shunt were particularly active on glycerol. Among these functional categories, we detected high transcription levels of *acsA*, *acsB*, and PP2351 [encoding acetyl-coenzyme A (CoA) synthetases], *actP* (acetate permease), PP2421 (an acetyltransferase), PP1412 (enoyl-CoA hydratase), and *aceA* (isocitrate lyase, the first committed step in the glyoxylate shunt) on glycerol as compared to both glucose and succinate cultures. In *E. coli*, *acsA* and *actP* (which form part of the RpoS regulon), are known to be over-expressed under conditions of hexose starvation (Gimenez *et al.*, 2003; Flores *et al.*, 2008; Sigala *et al.*, 2009). If this trait can be extrapolated to our present conditions, it would imply that *P. putida* cells grown on glycerol undergo a C starvation phenomenon compatible. This condition, in turn, is fully compatible with the catabolic restrain observed in the components of the tricarboxylic acid (TCA) cycle (see below and main text).

In close connection with the notion of C saving and partial C recycling *via* the glyoxylate shunt, *acnA* (encoding aconitate hydratase, which catalyzes the conversion *cis*-aconitate → D-*threo*-isocitrate within the TCA cycle) reached high expression levels on glycerol as compared to both control conditions, accompanied by the concomitant down-regulation of PP4012 (a $NADP^+$-dependent isocitrate dehydrogenase) and *sucD* and *sucC* (α and β subunits of succinyl-CoA synthetase, respectively). Likewise, *lpdG* (PP4187, encoding the dihydrolipoamide dehydrogenase component of 2-ketoglutarate dehydrogenase) was down-regulated on glycerol as compared to succinate, a further indication of a repressed TCA cycle. Activation of genes involved in acetate synthesis pathways and genes belonging to the glyoxylate shunt was also observed in *E. coli* cells grown on glycerol (Martínez-Gómez *et al.*, 2012), and the phenomenon was explained by the formation of acetate through the activity of PoxB (pyruvate oxidase). Acetate would then be recycled back to *P-enol*-pyruvate/pyruvate *via* the combined activity of the glyxoxylate shunt enzymes and PckA (*P-enol*-pyruvate carboxykinase, catalyzing the conversion oxaloacetate → *P-enol*-pyruvate). In accordance with such a gluconeogenic regime, *maeB* [encoding the malic enzyme (Chavarría *et al.*, 2012)] was strongly repressed in glycerol-grown *P. putida*, whereas the transcription of both *accC-2* and *oadA* (A and B subunits of pyruvate carboxylase, respectively; which convert oxaloacetate into pyruvate) were significantly up-regulated, a trait especially evident in the glucose *versus* succinate comparison. Over-expression of *pgm* (PP5056), the product of which catalyzes the highly reversible glycerate-3-*P* ↔ glycerate-2-*P* conversion, was also observed in cultures developed on glycerol. In the whole, the transcriptional landscape on glycerol reveals a metabolic situation which would involve a partial gluconeogenic C recycling process, with a low activity of the enzymes within the TCA cycle and a high activity of the glyoxylate shunt, thereby allowing for C conservation (*viz.*, avoiding C loss as CO_2 in the steps catalyzed by isocitrate lyase and 2-ketoglutarate dehydrogenase).

Peripheral catabolic pathways and secondary metabolism

Several alcohol dehydrogenases were up-regulated in cells grown on glycerol (*e.g.*, *qedH*, PP1661, PP2679, PP2680, PP2682, PP3839, and PP4037) as compared to both glucose and succinate. According to sequence similarities as compared with other alcohol dehydrogenases (Nelson *et al.*, 2002; Winsor *et al.*, 2011), some of the thereby encoded products might generate acetate from pyruvate, which would be in line with recycling of C2 units *via* gluconeogenesis from acetate. Despite

these prominent transcriptional results related to C2 units synthesis and recycling, we failed to detect any acetate in supernatants of cultures developed on any of the C sources used (data not shown). In addition to these features, the utilization of glycerol by *E. coli* elicits the activation of acetaldehyde/alcohol dehydrogenases as a result of a more reduced intracellular redox state under these conditions when compared to the use of more oxidized C sources (Nikel *et al.*, 2008a,b).

Some genes encoding amino acid metabolism and transport functions were activated, such as *aroF-2* (that encodes one of the enzymes that catalyze the conversion of erythrose-4-*P* into shikimate, the main precursor of aromatic amino acids), *argC* (*N*-acetyl-γ-glutamyl-*P* reductase, involved in the biosynthesis of arginine and ornithine), *arcD* (an arginine/ornithine antiporter), and *aapJ* (an amino acid ABC transporter substrate-binding protein). Degradation of some amino acids to C2-C3 subunits was evidenced by the strong activation of *mmsB* [3-hydroxyisobutyrate dehydrogenase, a key step in valine degradation to (*S*)-3-amino-2-methylpropanoate], *bkdB* (a branched-chain α-keto acid dehydrogenase subunit E2), and *lpdV* (a dihydrolipoamide dehydrogenase). The two former enzymes can produce acetaldehyde from pyruvate, and they might participate in glycine and/or 2-oxoisovalerate degradation. These degradation pathways might provide the gluconeogenic precursors needed for C recycling, as evidenced by the transcriptional activation of genes encoding acetyl-CoA synthetases and components of the glyoxylate shunt on glycerol.

Methione, proline, and aspartate synthesis seemed to be negatively affected by growth on glycerol, as evidenced by the strong repression of *metE* (which encodes 5-methyltetrahydropteroyltriglutamate-homocysteine *S*-methyltransferase), *metK* (*S*-adenosylmethionine synthetase, involved in methionine processing), *metH* (B_{12}-dependent methionine synthase), *aspA* (aspartate ammonia-lyase, that interconverts fumarate and aspartate), *proC-1* [encoding a pyrroline-5-carboxylate reductase, which catalyzes the conversion (*S*)-1-pyrroline-5-carboxylate + NAD(P)H + 2 H^+ → proline + $NAD(P)^+$], and *ansA* (encoding a type II L-asparaginase, which catalyzes the transformation asparagine + H_2O → aspartate + H^+ + NH_4^+).

Again, these metabolic features fit well to the proposed C-saving metabolic scheme elicited by down-regulating the expression of genes committed to amino acid synthesis, while activating pathways for amino acid breakdown and generation of C2 and C3 precursors from them. In the particular case of

methionine and proline biosynthesis pathways, the down-regulation of the cognate genes could also respond to an altered redox state, as these biosynthetic sequences consume a great deal of reducing equivalents (mainly in the form of NADPH) (Hondorp and Matthews, 2004; Chavarría *et al.*, 2013). In accordance with the concept of special needs for redox balance on glycerol, *ppnK* (PP2012, encoding a inorganic polyphosphate/ATP-NAD^+ kinase), was also over-expressed in glycerol-grown cells.

General regulatory functions

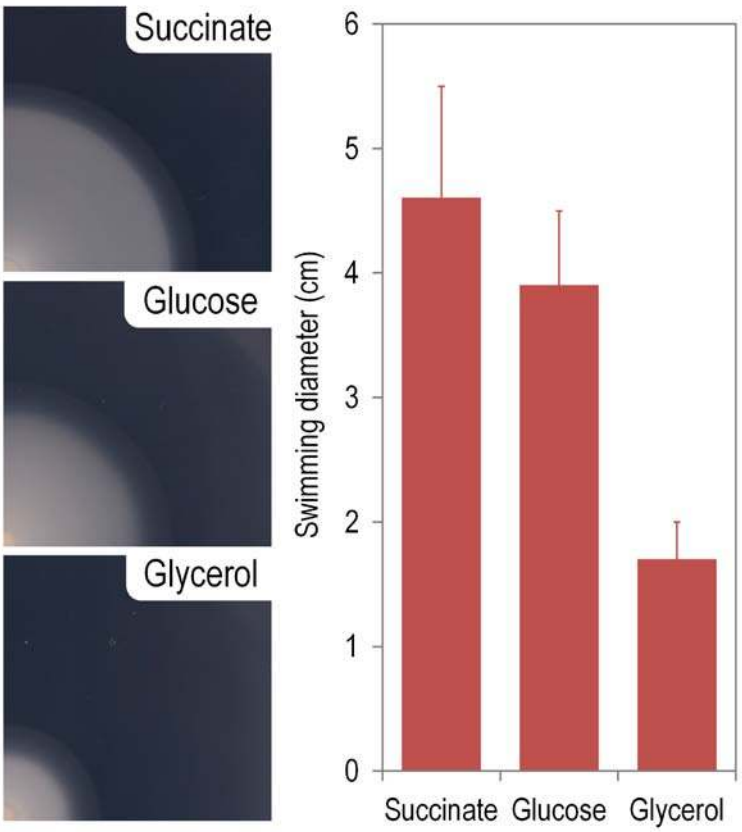
The most conspicuous difference observed in the transcription level of regulators was the repression of *hexR* and *gltR-2* on glycerol as compared to glucose. HexR is a regulator of the *zwf-1*/*pgl*/*edd* and *eda*/*glk*/*gltR-2*/*gltS* operons operons in *P. putida* KT2440 (Petruschka *et al.*, 2002; del Castillo *et al.*, 2007; del Castillo *et al.*, 2008), and it was shown to act as a transcriptional repressor of these genes (Daddaoua *et al.*, 2009); the signal molecule to release it from DNA probably being 2-keto-3-deoxygluconate-6-*P* (Kim *et al.*, 2008). GltR-2 is a positive transcriptional regulator that controls glucose uptake systems (namely, the OprB porins and the *gtsABCD* operon encoded by PP1015-1018). In turn, *gltR-2* is subjected to transcriptional control of HexR itself (del Castillo *et al.*, 2008). This pattern of transcription of glucose-related regulators is to be expected when cells are grown on non-permissive substrates, and the repression of *edd*, *eda*, *glk*, *zwf-1*, *oprB-1*, and PP1015-PP1018 observed on glycerol is in agreement with the expected transcription pattern governed by both HexR and GltR-2.

The *csrA* (sensor protein belonging to a putative two-component C storage regulator) and *ihfA* (α subunit of integration factor) genes were also repressed on glycerol. Although the role of Csr was not hitherto explored in *P. putida* KT2440, in *E. coli* this system is composed of the small CsrA protein and the two non-coding regulatory RNAs encoded by *csrB* and *csrC* (Romeo *et al.*, 1993; Romeo, 1998). CsrA is an RNA-binding protein that could prevent translation of target mRNA by binding to a site near the Shine-Dalgarno sequence, thus blocking ribosome binding and facilitating mRNA decay. CsrA has also been shown to act as a positive regulator by stabilizing and subsequently increasing the translation of certain target mRNAs. A similar behavior in transcription levels was observed for *ptxS*. This transcriptional regulator was originally described in *P. aeruginosa* (Colmer and Hamood, 1998), and the same protein in *P. putida* seems to play a relevant role in the modulation of C metabolism (Daddaoua *et*

al., 2010). The *phoP*/*phoQ* genes (encoding a winged helix family two-component transcriptional regulator and an integral membrane-sensor signal transduction histidine kinase, respectively) also followed the same trend. The activity of the PhoP/PhoQ system of *P. putida* was found to be necessary to withstand stressful conditions exerted by urea (Reva *et al*., 2006), whereas in *P. aeruginosa* the same system displays a central role in virulence (Gellatly *et al*., 2012).

Only two regulatory proteins had their cognate genes up-regulated in cells grown on glycerol, namely, *agmR* (a transcriptional regulator belonging to the LuxR family) and *algB* (a transcriptional regulator belonging to the Fis family). Schweizer (1991) reported that AgmR can complement the GlpR-related functions in *P. aeruginosa* cells deficient in *glpR* [although it was later shown that GlpR acts as a repressor (Schweizer and Po, 1996), whereas AgmR seems to act as an activator of the transcription of the *glp* genes]. In *P. putida*, AgmR is involved in the activation of genes encoding enzymes needed for the utilization of alcohols as the C source (Vrionis *et al*., 2002). More recently, Fernández *et al*. (2012) exposed the role of AgmR as a key player in the chloramphenicol resistance phenotype in *P. putida* KT2440, and *agmR* actually seems to be a pleiotropic gene. In contrast, the regulatory duties of AlgB in *P. aeruginosa* seem to be circumscribed to the activation of genes needed for alginate production (Goldberg and Ohman, 1987; Ma *et al*., 1998), and a similar role would be expected in *P. putida* KT2440 as judging by the high sequence similarity (Nelson *et al*., 2002; Winsor *et al*., 2011). Although the exact hierarchy of transcriptional regulation cannot be ascertained based solely in the results discussed herein, it is significant that the most relevant regulators of C utilization in *P. putida* were repressed in cells grown on glycerol, in line with the C saving strategy delineated before.

Fig. S1. Swimming motility of *Pseudomonas putida* KT2440 on different C sources.



One of the cell traits that consumes more intracellular ATP is swimming, as the motion of the flagelar motor puts away a considerable amount of molecules of this energy currency (Blair, 1995). In order to explore the coarse relationship between C source utilization and energy load, we ran swimming tests of the wild-type *P. putida* strain in semi-solid agar [0.3% (w/v)] plates using different C sources. Cells growing on glycerol had the lowest flagellar activity, followed by those grown on glucose and on succinate. The graded effect of the ATP pool on swimming is in line with the proposed principle of *caloric restriction*, i.e., highly-energetic C substrates normally 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). In the bar chart shown to the right of the figure, each bar represents the mean value of the swimming diameter ± standard deviation of duplicate measurements from at three independent experiments.

Table S3. List of up-regulated genes in cells grown on glycerol as compared to glucose.

Name or PP number	Fold-change (log_2) induction
PP2673	11.858
qedH	10.957
PP2675	10.632
PP2676	10.213
PP2663	10.001
PP2680	9.779
PP2677	9.553
PP2662	9.480
PP2669	9.012
PP2670	8.824
PP2664	8.296
PP2671	7.984
PP2681	7.908
PP2678	7.878
PP2672	7.611
PP2668	7.504
PP2667	7.423
PP3176	7.299
PP2666	6.421
PP2679	5.862
glpD	5.748
acsA	5.394
PP2260	5.067
agmR	4.991
PP2422	4.768
PP3178	4.446
pqqC	4.313
PP4870	4.282
pqqB	4.128
PP2682	4.021
PP1640	3.732
PP0056	3.604
PP2006	3.598
PP2007	3.584
pqqE	3.503
PP0375	3.492
PP2733	3.486
PP0057	3.452
PP4556	3.387
cfa	3.382
aceA	3.347

aroF-2	3.328
PP2683	3.274
pqqD	3.273
PP3179	3.215
PP2264	3.193
glpF	3.141
PP0711	3.076
PP3402	3.069
PP0108	2.988
PP4793	2.965
PP2421	2.875
PP4555	2.869
PP4554	2.869
PP1660	2.860
glpK	2.856
PP1144	2.848
PP1742	2.823
PP1659	2.797
PP1991	2.791
PP2730	2.742
folE	2.739
PP2731	2.712
PP1661	2.705
PP2732	2.700
wecB	2.665
PP4858	2.615
actP	2.584
PP2359	2.564
PP5241	2.558
PP5323	2.552
PP3621	2.523
PP0105	2.513
PP2735	2.512
arcD	2.498
PP3770	2.496
PP3580	2.495
argI	2.492
PP0298	2.488
PP0104	2.485
PP2655	2.479
arcA	2.458
PP3420	2.454
PP0288	2.411
arcC	2.379

PP2360	2.372
csuC	2.372
PP0536	2.366
PP4553	2.344
PP1849	2.331
PP2736	2.311
PP3444	2.308
PP3622	2.288
PP3698	2.269
mexE	2.234
PP2353	2.215
dsbE	2.155
PP4521	2.153
PP0107	2.147
PP2874	2.142
PP0106	2.140
PP2511	2.140
ccmE	2.125
PP0806	2.112
mexF	2.067
PP3421	2.047
ccmD	2.038
ccoQ-2	2.033
csuD	2.033
csuE	2.033
PP3683	2.023
PP3401	2.020
PP4034	2.015
PP3623	2.004
PP2729	1.950
PP4624	1.944
PP2358	1.943
ccmF	1.943
ccoO-2	1.939
PP2886	1.926
PP2569	1.895
PP5092	1.895
PP1841	1.881
PP1632	1.861
oprN	1.861
PP0699	1.852
phaG	1.847
PP2219	1.844
argC	1.838

PP2259	1.825
PP2737	1.791
PP2970	1.791
PP0683	1.774
PP0103	1.769
PP3419	1.739
PP0589	1.737
ccoN-2	1.712
PP1364	1.709
PP2022	1.697
PP3443	1.695
PP4038	1.694
PP2357	1.693
PP1395	1.687
PP2351	1.673
ccoP-2	1.673
prpD	1.658
PP0905	1.649
PP3127	1.617
PP1396	1.598
PP3126	1.590
PP1072	1.577
PP4319	1.577
PP0350	1.558
PP4957	1.546
PP2728	1.534
PP1412	1.530
PP0092	1.525
PP4867	1.522
PP0998	1.505
PP4282	1.493
PP4037	1.489
PP3569	1.488
PP2941	1.484
PP3697	1.479
PP2661	1.476
PP3156	1.473
PP5204	1.470
PP5341	1.466
PP2326	1.462
aapJ	1.452
hemO	1.438
PP3954	1.435
PP2660	1.421

cspD	1.411
PP2861	1.410
PP3142	1.408
gad	1.403
acnA	1.392
PP2575	1.391
exsB	1.376
PP3431	1.371
PP3128	1.365
hupA	1.364
PP3629	1.350
PP4959	1.348
fusA	1.343
xdhB	1.338
PP4054	1.336
PP2705	1.332
PP2738	1.329
PP0907	1.317
glgX	1.313
PP4035	1.308
PP1369	1.306
ccmC	1.305
PP3624	1.297
PP3691	1.291
PP0906	1.289
PP2853	1.288
PP3119	1.280
pgi	1.280
PP0700	1.279
PP3081	1.275
PP1110	1.275
PP0102	1.274
mmsA-2	1.273
PP3130	1.270
PP2578	1.261
PP3668	1.261
bkdB	1.258
PP1121	1.253
PP3611	1.251
PP2563	1.245
acsB	1.245
malQ	1.244
PP0903	1.242
PP2097	1.241

PP2927	1.236
glgA	1.229
PP2124	1.219
est	1.218
PP2572	1.217
PP3135	1.214
PP3796	1.197
PP4851	1.196
PP2942	1.190
PP0255	1.188
PP3137	1.187
lpdV	1.187
PP4647	1.186
PP3856	1.185
PP3449	1.183
PP4685	1.183
PP3518	1.182
PP3441	1.180
PP3014	1.174
PP4056	1.171
PP2374	1.170
PP0713	1.169
nfrB	1.169
PP2426	1.167
PP4863	1.161
PP3440	1.157
PP5191	1.153
ppnK	1.151
PP3414	1.149
PP5007	1.147
PP1091	1.146
PP0201	1.145
PP2553	1.145
PP1983	1.145
PP0922	1.144
PP3765	1.144
PP0412	1.141
PP2719	1.139
PP1840	1.138
tnpA	1.133
PP4033	1.128
PP1931	1.125
PP2381	1.124
PP1225	1.122

PP3020	1.117
PP2118	1.115
galE	1.115
PP4504	1.110
PP2720	1.108
gap-2	1.107
PP3134	1.105
PP4958	1.100
PP4053	1.099
PP4617	1.095
PP4864	1.093
mdh	1.087
PP5008	1.082
PP2133	1.078
PP3955	1.069
PP1244	1.061
PP1370	1.054
PP0805	1.052
PP5319	1.052
mmsB	1.051
PP2373	1.051
PP2945	1.051
PP3138	1.050
PP3847	1.050
pgm	1.050
PP1934	1.049
PP4564	1.044
PP1631	1.044
PP0685	1.039
PP0712	1.038
PP2900	1.037
PP2344	1.034
PP0801	1.033
PP0357	1.029
alkB	1.025
PP2342	1.020
flgC	1.016
PP1115	1.012
PP1687	1.007
gcdH	1.007
PP3242	1.002
PP4509	1.002

Table S4. List of up-regulated genes in cells grown on glycerol as compared to succinate.

Name or PP number	Fold-change (log_2) induction
PP2673	21.218
PP2672	18.602
qedH	11.208
PP2675	10.918
PP2676	9.985
PP2677	9.965
PP2680	9.944
PP2662	9.876
PP2663	9.657
PP2670	9.361
PP2669	9.304
PP2664	8.730
PP2681	8.371
PP2671	8.366
PP2678	8.315
PP2668	7.962
PP3176	7.437
glpD	7.391
PP2679	7.341
PP2667	7.183
PP2422	5.861
PP2666	5.310
acsA	5.246
PP1640	5.125
glpF	4.674
pqqB	4.670
PP1991	4.662
PP3178	4.590
PP2260	4.589
pqqC	4.519
agmR	4.465
argC	4.452
glpK	4.377
PP0806	4.236
PP0699	4.229
PP1659	4.200
PP3402	4.055
PP4556	4.014
PP0108	3.785
PP2007	3.766
PP3444	3.749

PP2682	3.731
PP0375	3.710
pqqE	3.675
PP2006	3.659
PP1661	3.637
PP2421	3.443
PP3179	3.411
PP5323	3.369
PP2359	3.365
PP2360	3.265
PP1742	3.234
pqqD	3.231
PP0711	3.226
PP4793	3.217
PP4319	3.183
actP	3.165
PP1660	3.115
csuC	3.071
pgl	3.059
wecB	3.024
arcD	3.011
PP1144	3.010
PP2874	3.009
PP3621	2.994
PP0536	2.989
PP4555	2.988
phaG	2.950
PP4554	2.902
mexE	2.901
PP2683	2.879
PP2264	2.856
PP3698	2.840
PP3580	2.774
zwf-1	2.771
PP0105	2.769
PP2446	2.765
PP0057	2.765
PP4858	2.760
PP2357	2.760
PP4837	2.747
PP2022	2.733
oprN	2.731
csuE	2.698
arcA	2.655

PP3622	2.596
PP2578	2.596
PP0905	2.580
PP1849	2.566
PP4038	2.547
PP3419	2.545
PP0298	2.532
PP4282	2.516
mexF	2.505
PP2353	2.492
eda	2.481
PP0056	2.453
PP2886	2.453
PP3683	2.450
csuD	2.429
PP2731	2.392
argI	2.384
pgi	2.381
PP0104	2.373
PP0288	2.369
PP5241	2.357
PP4553	2.353
PP3420	2.344
PP3421	2.321
PP2575	2.319
PP1396	2.282
gdhA	2.277
ccoO-2	2.272
PP3401	2.262
PP3623	2.260
PP5008	2.253
ccmD	2.251
arcC	2.251
PP3119	2.205
PP2720	2.202
PP0106	2.197
aceA	2.195
PP3156	2.157
PP0922	2.121
PP5092	2.109
PP4521	2.096
PP2655	2.095
PP2769	2.094
PP2569	2.081

PP1632	2.078
PP2358	2.063
PP0803	2.051
PP0102	2.039
PP4037	2.034
ccmF	2.013
PP3770	2.000
aroF-2	1.998
PP4034	1.994
exsB	1.982
PP2732	1.958
PP2326	1.948
PP4836	1.946
PP1395	1.945
ccmE	1.931
PP2733	1.928
PP2941	1.905
PP4035	1.903
PP3629	1.891
ccoP-2	1.882
PP3624	1.876
PP1840	1.873
PP1244	1.872
PP2259	1.833
acnA	1.826
PP1364	1.814
PP2097	1.803
PP2970	1.800
PP3691	1.799
PP3128	1.786
ccoN-2	1.781
PP2118	1.769
PP3440	1.757
PP0107	1.753
PP1115	1.743
dsbE	1.740
PP0804	1.740
PP3127	1.739
folE	1.739
PP1225	1.734
PP2927	1.722
PP2660	1.706
PP1759	1.694
PP0589	1.685

PP4617	1.683
PP1072	1.682
gad	1.678
xdhB	1.677
gap-2	1.666
PP2351	1.665
prpD	1.662
PP3697	1.650
PP0103	1.644
PP3081	1.641
PP1412	1.640
PP1121	1.638
PP2373	1.631
PP1110	1.626
PP4061	1.617
tnpA	1.617
mdh	1.614
PP3414	1.614
PP3431	1.608
PP0805	1.608
PP1369	1.605
PP2942	1.590
PP1687	1.574
PP2511	1.569
PP1112	1.558
PP3569	1.555
ccmC	1.550
PP0683	1.541
est	1.535
PP2661	1.535
PP4959	1.533
PP3126	1.531
PP1447	1.518
PP4564	1.511
PP2945	1.510
PP4870	1.508
sda-3	1.507
ccoQ-2	1.492
PP1690	1.483
PP2450	1.482
PP0713	1.474
PP1841	1.471
PP5007	1.471
PP3130	1.470

PP4259	1.459
PP4054	1.448
PP2730	1.448
PP2219	1.445
PP3853	1.441
PP0906	1.438
PP0255	1.436
PP2713	1.420
PP2573	1.419
PP3518	1.416
PP3668	1.409
PP1111	1.407
PP3545	1.407
PP0714	1.404
PP2984	1.386
PP3142	1.385
PP2381	1.374
PP4576	1.360
PP3137	1.357
PP5131	1.352
PP0907	1.350
accC-2	1.350
PP2827	1.347
PP0125	1.347
PP4493	1.341
petA	1.337
oadA	1.337
galE	1.334
PP4070	1.332
PP3020	1.329
PP1458	1.324
PP2426	1.322
PP3604	1.321
PP4405	1.319
PP0998	1.318
PP2705	1.317
PP2861	1.315
alkB	1.315
PP0215	1.315
fusA	1.314
PP3140	1.306
xdhA	1.301
PP4958	1.297
PP2124	1.294

PP1819	1.293
PP1122	1.288
cspD	1.287
gmk-1	1.283
PP3141	1.280
fleR	1.277
PP3312	1.276
PP2342	1.276
PP1143	1.274
PP5191	1.267
PP2554	1.266
PP3581	1.265
adhA	1.263
PP0763	1.261
PP2344	1.261
PP3441	1.260
PP4053	1.252
ccoS	1.251
PP5247	1.250
PP1493	1.249
proC-1	1.245
PP2853	1.245
PP4118	1.236
PP3837	1.235
PP1091	1.229
pgm	1.228
PP4207	1.227
PP0712	1.226
PP1931	1.224
PP2572	1.223
PP5341	1.223
obgE	1.215
PP2374	1.214
PP3449	1.208
PP2187	1.200
PP1370	1.196
PP3242	1.188
PP2133	1.187
PP1113	1.187
PP4957	1.178
PP1478	1.176
PP2553	1.175
PP3139	1.174
PP3136	1.172

PP2563	1.170
PP2291	1.169
PP3765	1.161
PP0201	1.159
PP1510	1.158
PP3131	1.150
glgX	1.148
trpF	1.148
PP2437	1.147
PP4589	1.144
PP1192	1.141
edd	1.136
PP3833	1.133
mqo-1	1.128
PP2292	1.124
PP1868	1.124
glgA	1.120
gltD	1.120
PP3133	1.112
PP1983	1.112
PP4362	1.108
PP3443	1.105
PP3134	1.100
PP3135	1.098
PP4763	1.097
PP3834	1.096
PP3844	1.095
PP4033	1.094
PP3810	1.093
PP2425	1.092
PP1087	1.091
PP2899	1.090
PP5204	1.085
PP1199	1.085
PP2595	1.085
gcdH	1.082
PP2900	1.081
PP2719	1.079
PP1154	1.074
PP2441	1.073
PP4363	1.072
oprB-2	1.072
PP3559	1.069
PP1004	1.068

pabC	1.067
PP3138	1.065
glk	1.063
cfa	1.057
PP2995	1.056
PP2021	1.056
PP3014	1.054
iscA	1.047
PP0092	1.047
PP4138	1.046
PP2943	1.043
gltB	1.041
gcvP-2	1.040
PP2685	1.037
PP2729	1.034
PP0665	1.034
PP0801	1.032
PP3610	1.029
gcp	1.029
cysI	1.025
petB	1.024
malQ	1.021
mqo-3	1.014
PP0482	1.013
PP4056	1.012
PP2307	1.011
PP4647	1.011
PP3438	1.006
PP2370	1.005
PP0566	1.005
PP2345	1.002
nfrB	1.002

Table S5. List of down-regulated genes in cells grown on glycerol as compared to glucose.

Name or PP number	Fold-change (log_2) repression
PP1016	−6.708
PP3379	−6.611
PP1018	−6.597
kguD	−6.257
kguK	−6.201
PP3332	−6.165
PP1017	−6.057
PP1015	−5.932
PP3785	−5.670
PP3782	−5.584
PP3784	−5.554
PP3783	−5.457
PP2699	−5.304
PP1014	−5.299
metE	−5.164
PP3382	−4.961
PP3781	−4.934
PP3384	−4.878
gntP	−4.809
PP4637	−4.735
PP3383	−4.728
endA-2	−4.688
PP3788	−4.640
PP2697	−4.565
PP3786	−4.482
metR-2	−4.368
oprB-1	−4.192
PP3787	−4.162
PP1020	−4.142
PP3418	−4.053
PP3377	−3.849
PP1249	−3.786
gnuK	−3.542
dapF	−3.512
PP2389	−3.375
PP3789	−3.363
PP4636	−3.342
ansA	−3.281
PP2388	−3.109
aspA	−2.931
metK	−2.909

eda	−2.852
oprH	−2.805
PP3780	−2.771
hexR	−2.658
PP2877	−2.635
oprG	−2.570
PP0153	−2.525
mqo-2	−2.491
proC-1	−2.487
rarD-3	−2.485
PP0868	−2.472
PP3777	−2.451
pgl	−2.435
PP4012	−2.391
serA	−2.385
ssuE	−2.383
PP2387	−2.381
PP3340	−2.308
metF	−2.302
PP4966	−2.290
PP3335	−2.283
ptxS	−2.271
PP3331	−2.235
edd	−2.235
cyoD	−2.225
zwf-1	−2.216
PP5274	−2.214
cyoB	−2.183
oprC	−2.172
PP2052	−2.148
PP3330	−2.138
PP0319	−2.126
PP3775	−2.125
PP0318	−2.096
PP4604	−2.013
PP1788	−2.004
PP5392	−2.000
PP3779	−1.989
cyoC	−1.980
oprE	−1.953
PP3898	−1.948
PP4178	−1.919
PP4315	−1.917
glk	−1.894

cyoA	−1.874
PP1246	−1.874
PP3904	−1.865
PP1247	−1.856
groEL	−1.831
PP1245	−1.813
PP4104	−1.805
gltR-2	−1.786
cyoE-2	−1.769
groES	−1.762
PP3924	−1.758
PP3705	−1.744
PP3443	−1.743
PP3110	−1.716
htpG	−1.710
PP3107	−1.691
PP0087	−1.664
PP1833	−1.658
PP1013	−1.657
PP3339	−1.625
maeB	−1.617
metH	−1.610
cspA-2	−1.588
hupB	−1.553
PP3106	−1.541
glyA	−1.535
PP5304	−1.517
rmlC	−1.514
trpB	−1.478
PP2928	−1.470
PP3108	−1.470
PP3104	−1.458
PP2258	−1.443
PP1098	−1.428
PP2298	−1.427
secB	−1.425
trpA	−1.409
dnaK	−1.407
PP0871	−1.406
PP2474	−1.395
PP4770	−1.394
PP3405	−1.393
phnA	−1.370
rmlB	−1.354

PP1057	−1.347
atpC	−1.342
phoP	−1.337
PP2304	−1.332
PP1065	−1.308
lon-1	−1.300
PP0799	−1.299
cyoups2	−1.296
hslU	−1.292
PP3886	−1.283
PP0423	−1.279
dapB	−1.278
Int	−1.271
PP5303	−1.265
PP3105	−1.263
sucD	−1.262
PP1056	−1.250
PP3662	−1.244
phoQ	−1.241
PP4633	−1.231
grpE	−1.228
PP1518	−1.226
gnuK	−1.221
PP1789	−1.220
PP4839	−1.217
PP1530	−1.214
PP3336	−1.212
rnhB	−1.212
rmlD	−1.211
PP0021	−1.210
PP2700	−1.206
PP3988	−1.205
mreD	−1.198
csrA	−1.187
sucC	−1.180
tpx	−1.173
ibpA	−1.169
PP3368	−1.169
hslV	−1.164
PP2099	−1.151
lpxB	−1.149
PP0596	−1.146
PP3415	−1.142
PP3406	−1.136

secF	−1.129
PP0636	−1.119
PP1069	−1.116
rmlA	−1.109
PP3699	−1.104
PP4834	−1.102
PP0673	−1.102
cycA	−1.097
hprA	−1.095
glmM	−1.090
mmsA-1	−1.089
glnA	−1.089
PP2170	−1.084
PP5054	−1.081
PP3437	−1.080
PP5059	−1.076
pyrC	−1.069
fxsA	−1.068
PP4148	−1.065
secD	−1.061
PP2541	−1.059
PP2527	−1.057
PP3407	−1.053
cyoups1	−1.052
PP4473	−1.046
PP3885	−1.045
PP5167	−1.043
infA	−1.041
PP1068	−1.041
PP1204	−1.040
PP5066	−1.040
PP4316	−1.039
oprL	−1.037
dnaJ	−1.035
PP3404	−1.031
PP4502	−1.024
PP2182	−1.022
PP4149	−1.022
PP3154	−1.019
PP0242	−1.019
hisF	−1.017
fadB	−1.010
PP4669	−1.009
atpG	−1.009

PP0721	−1.007
PP5353	−1.006
PP0770	−1.004
PP4146	−1.003
oadA	−1.001

Table S6. List of down-regulated genes in cells grown on glycerol as compared to succinate.

Name or PP number	Fold-change (log_2) repression
dctA	−6.133
aspA	−5.540
PP0883	−5.096
PP1249	−4.952
ansA	−4.788
PP2389	−3.495
PP4182	−3.475
PP2388	−3.269
PP1788	−3.241
PP0153	−3.189
PP4012	−3.030
PP0318	−2.957
cspA-2	−2.913
oprH	−2.897
dppA	−2.793
PP1400	−2.767
PP3332	−2.755
oprE	−2.736
PP3924	−2.690
PP3904	−2.603
PP4104	−2.534
PP1057	−2.422
PP0319	−2.395
PP5274	−2.375
PP3365	−2.356
PP4013	−2.342
PP0596	−2.339
PP5312	−2.308
PP2052	−2.244
PP3898	−2.231
PP2387	−2.208
PP2474	−2.202
PP3705	−2.151
PP1071	−2.138
PP0884	−2.119
PP1068	−2.116
PP5279	−2.113
mmsA-1	−2.061
tpx	−2.059
PP0087	−2.029
PP4633	−2.006

PP4447	−1.998
secB	−1.971
PP0799	−1.962
PP1518	−1.942
glcB	−1.939
PP1069	−1.934
rmlC	−1.926
maeB	−1.898
PP4109	−1.886
PP1789	−1.884
PP3989	−1.862
trx-2	−1.862
phnA	−1.851
PP4315	−1.850
PP1246	−1.831
PP1245	−1.830
PP0544	−1.828
PP1070	−1.827
PP0423	−1.822
PP0639	−1.817
rmlB	−1.814
PP0152	−1.812
PP5303	−1.800
PP0150	−1.792
livG	−1.785
braD	−1.785
PP0428	−1.776
PP1961	−1.775
PP1081	−1.760
PP1065	−1.745
groES	−1.736
PP3886	−1.709
cyoA	−1.702
PP4604	−1.698
PP0182	−1.684
PP1006	−1.682
PP3909	−1.680
PP0017	−1.677
PP3662	−1.672
PP0829	−1.671
cyoD	−1.663
PP1936	−1.658
PP3107	−1.658
hupB	−1.652

PP0636	−1.639
PP5304	−1.638
groEL	−1.638
PP1247	−1.632
PP3988	−1.626
oprB-1	−1.608
PP2298	−1.607
sucD	−1.604
cyoB	−1.603
phoP	−1.592
PP4178	−1.589
PP1530	−1.559
PP4448	−1.555
aruG	−1.544
PP3106	−1.540
PP4467	−1.526
rmlA	−1.525
PP1008	−1.523
PP4637	−1.522
PP4502	−1.520
PP0225	−1.517
PP3598	−1.511
PP5195	−1.505
glnA	−1.501
hslV	−1.485
PP0021	−1.484
PP1067	−1.482
PP2080	−1.477
acpP	−1.472
PP1790	−1.471
ampD	−1.471
PP4632	−1.469
cyoC	−1.467
PP0909	−1.466
PP1161	−1.462
PP4248	−1.460
PP5022	−1.456
sucC	−1.456
PP5278	−1.455
braG	−1.454
PP1142	−1.453
PP5280	−1.443
livM	−1.441
PP2969	−1.439

apaG	−1.438
PP5096	−1.435
pssA-2	−1.435
PP4148	−1.433
phoQ	−1.432
PP1056	−1.429
PP3186	−1.426
PP3612	−1.425
PP0282	−1.414
PP1960	−1.413
PP4693	−1.411
aapJ	−1.409
serB	−1.400
rmlD	−1.392
PP3024	−1.392
metY	−1.387
PP2304	−1.381
PP4770	−1.376
pet18	−1.374
trpC	−1.365
PP0203	−1.358
PP3154	−1.351
serA	−1.350
PP2491	−1.350
PP1941	−1.344
PP3067	−1.339
dppF	−1.338
PP5196	−1.337
PP0788	−1.336
PP2275	−1.334
PP1204	−1.330
PP0291	−1.322
PP2499	−1.322
PP2099	−1.319
PP4146	−1.317
PP1442	−1.317
fumC	−1.316
PP2174	−1.316
PP1833	−1.309
PP3104	−1.307
PP5054	−1.304
PP3319	−1.304
aapP	−1.302
hupN	−1.298

grpE	−1.296
PP3523	−1.291
PP3885	−1.289
PP0545	−1.289
PP0242	−1.285
PP4985	−1.282
PP1389	−1.281
PP5317	−1.276
PP1398	−1.273
hprA	−1.273
PP4732	−1.270
PP1324	−1.269
aapQ	−1.267
fklB-2	−1.264
PP0283	−1.258
PP3923	−1.256
mioC	−1.251
PP3105	−1.249
PP5163	−1.248
PP0418	−1.248
PP0281	−1.245
atpC	−1.244
PP0112	−1.243
PP0871	−1.241
PP3717	−1.240
PP1060	−1.237
PP2189	−1.235
gloA	−1.232
PP4242	−1.231
PP1424	−1.230
fadD	−1.229
pyrR	−1.227
dsrE	−1.225
rnt	−1.225
PP5337	−1.224
PP0680	−1.223
PP0251	−1.220
PP2432	−1.218
PP5353	−1.217
carA	−1.215
hsdS	−1.213
PP1305	−1.211
fadB	−1.210
PP1959	−1.210

aruF	−1.208
PP0770	−1.207
PP5188	−1.201
argD	−1.201
htpG	−1.200
PP1066	−1.199
pyrB	−1.197
hslU	−1.196
PP1922	−1.192
algY	−1.188
dppD	−1.187
PP3108	−1.183
glpE	−1.178
PP1084	−1.177
PP2950	−1.176
PP4779	−1.166
Int	−1.164
PP5183	−1.164
PP0007	−1.162
PP0285	−1.158
csrA	−1.158
PP0151	−1.158
PP5118	−1.158
PP4202	−1.155
PP5263	−1.154
PP4787	−1.152
PP1826	−1.152
PP4720	−1.145
PP4424	−1.143
PP2824	−1.139
dnaK	−1.138
PP0945	−1.136
PP1576	−1.135
atpG	−1.132
PP1689	−1.132
hyi	−1.131
PP0721	−1.130
PP2182	−1.127
cspA-1	−1.125
PP5165	−1.123
priA	−1.123
PP4147	−1.118
crp	−1.116
PP0534	−1.110

PP1691	−1.110
fxsA	−1.109
PP4316	−1.108
PP4022	−1.106
PP1253	−1.105
cstA	−1.104
codA	−1.104
PP3025	−1.102
atpH	−1.101
lon-1	−1.097
PP0772	−1.096
PP3491	−1.096
PP0985	−1.090
astD	−1.089
galU	−1.086
PP4588	−1.083
trpE	−1.083
lpdG	−1.083
PP0369	−1.083
ppa	−1.080
PP4460	−1.080
proC-2	−1.079
PP5066	−1.078
smpB	−1.077
PP3699	−1.073
pyrC	−1.072
PP4871	−1.072
trpD	−1.070
PP4305	−1.070
PP0244	−1.068
PP1787	−1.068
PP4669	−1.061
PP3704	−1.061
holC	−1.055
PP0162	−1.053
PP1309	−1.053
PP1474	−1.051
rpsP	−1.050
PP2121	−1.041
PP0913	−1.040
PP0673	−1.039
PP1322	−1.036
PP3896	−1.036
PP4149	−1.031

PP2181	−1.029
secF	−1.027
fliP	−1.026
oprQ	−1.024
PP5184	−1.023
bioF	−1.023
ihfA	−1.023
PP3409	−1.022
dipZ	−1.021
nhaB	−1.018
PP0227	−1.018
PP2652	−1.018
PP0641	−1.017
PP5055	−1.013
dppB	−1.009
glyQ	−1.004
PP1487	−1.004
PP3437	−1.000
carB	−1.000

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