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Analysis of *Pseudomonas putida* growth on non-trivial carbon sources using transcriptomics and genome-scale modeling.

Isotta D'Arrigo¹, João G. R. Cardoso¹, Maja Rennig¹, Nikolaus Sonnenschein¹, Markus J. Herrgård¹, Katherine S. Long^{1,2}

¹The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Building 220, Kemitorvet, DK-2800 Kgs. Lyngby, Denmark

²Address correspondence to: KSL long@aya.yale.edu Phone: +45 21799208

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Originality-significance statement

The bacterium *Pseudomonas putida* is characterized by a large metabolic repertoire and general robustness towards stress that enable it to thrive in a variety of environmental niches. This metabolic versatility is explored in this work by investigation of the uptake and assimilation mechanisms of four different sole carbon sources in the well-characterized *P. putida* strain KT2440 using a combination of RNA-sequencing and genome-scale metabolic modeling. Candidate porin and transporter genes identified via transcriptomic data were verified experimentally with transposon mutants, where delayed growth supports their involvement in citrate, ferulic acid and serine uptake. This information was used to improve the genome-scale model of *P. putida* KT2440 that identified and confirmed the active catabolic pathways for each carbon source. This study highlights the utility of expression profiles to advance understanding of adaptation to different environments and metabolic features important for cell factory development.

Summary

Pseudomonas putida is characterized by a versatile metabolism and stress tolerance traits that allow the bacterium to cope with different environmental conditions. In this work, the mechanisms that allow *P. putida* KT2440 to grow in the presence of four sole carbon sources (glucose, citrate, ferulic acid, serine) were investigated by RNA sequencing (RNA-seq) and genome-scale metabolic modeling. Transcriptomic data identified uptake systems for the four carbon sources, and candidates were subjected to preliminary experimental characterization by mutant strain growth to test their involvement in substrate assimilation. The OpdH and BenF-like porins were involved in citrate and ferulic acid uptake, respectively. The citrate transporter (encoded by PP_0147) and the TctABC system were important for supporting cell growth in citrate; PcaT and VanK were associated with ferulic acid uptake; and the ABC transporter AapJPQM was involved in serine transport. A genome-scale metabolic model of *P. putida* KT2440 was used to integrate and analyze the transcriptomic data, identifying and confirming the active catabolic pathways for each carbon source. This study reveals novel information about transporters that are essential for understanding bacterial adaptation to different environments.

Introduction

Pseudomonas putida is an ubiquitous, rod-shaped Gram-negative bacterium that colonizes several environmental niches (Nelson *et al.*, 2002; Timmis, 2002; Martins dos Santos *et al.*, 2004). Its versatile metabolism allows the bacterium to thrive in many different environments and to synthesize a broad range of compounds of industrial relevance, features that make *P. putida* an interesting platform for microbial cell factory development (Poblete-Castro *et al.*, 2012; Tiso *et al.*, 2014; Loeschcke and Thies, 2015; Molina-Santiago *et al.*, 2016). One of the best characterized *P. putida* strains (Regenhardt *et al.*, 2002) is KT2440, a plasmid-free derivative of *P. putida* mt-2 (Nakazawa, 2002). Its genome was sequenced in 2002 (Nelson *et al.*, 2002) and recently re-annotated (Belda *et al.*, 2016).

P. putida KT2440 is able to exploit a wide variety of nutrients and tolerate stressful conditions (Martins dos Santos *et al.*, 2004). The bacterium preferably uses organic acids and amino acids as sole carbon sources (Vílchez *et al.*, 2000; Lugtenberg *et al.*, 2001; Revelles *et al.*, 2007; La Rosa, Behrends, *et al.*, 2015; La Rosa, Nogales, *et al.*, 2015). However, *P. putida* can readily assimilate glucose by the Entner-Doudoroff (ED) pathway (Nelson *et al.*, 2002; La Rosa, Nogales, *et al.*, 2015), activating the glycolytic regime when entering carbon metabolism. In contrast to glucose, citrate enters carbon metabolism directly at the tricarboxylic acid (TCA) cycle. With citrate as carbon source the metabolism flows through the gluconeogenic route (Nogales *et al.*, 2008; Puchałka *et al.*, 2008). Aromatic compounds are also good sources of energy for *P. putida* KT2440, by the activation of the aromatic catabolic pathways. From this group of compounds, ferulic acid has a production relevance as it is the precursor of the flavor compound vanillin, and *P. putida* KT2440 has been shown to efficiently degrade ferulic acid and convert it to vanillin, increasing the potential of this bacterium in production applications (Plaggenborg *et al.*, 2003; Graf and Altenbuchner, 2014). Ferulic acid is processed through the protocatechuate pathway, entering the TCA cycle through succinate and acetyl-CoA (Jiménez *et al.*, 2002). Finally, *P. putida* is able to grow

with amino acids as carbon sources (Moreno *et al.*, 2009). Differently from other amino acids, metabolism of serine generates toxic intermediates (de Lorenzo *et al.*, 2014), and as a result serine is extremely toxic for many microorganisms including *E. coli* (Raskó and Alföldi, 1971; Newman and Walker, 1982). However, *P. putida* KT2440 is able to grow on serine as sole carbon source (Nogales *et al.*, 2008; Moreno *et al.*, 2009), an ability that highlights an interesting resistance trait of *P. putida*.

Transcriptomic analysis by RNA sequencing (RNA-seq) technology facilitates the understanding of bacterial adaptation to different environments by highlighting differentially expressed genes and RNA regulatory mechanisms. The transcriptome of *P. putida* KT2440 growing in different conditions has been investigated in several studies that focused on the role of regulatory RNAs (Frank *et al.*, 2011; D'Arrigo *et al.*, 2016; Bojanovic *et al.*, 2017) and regulation of metabolic pathways (Kim *et al.*, 2013; Nikel *et al.*, 2013, 2015; Molina-Santiago *et al.*, 2017). Genome-scale metabolic models (GEMs) are also important tools in systems and synthetic biology to describe the known metabolic reaction portfolio of a given organism and how it is linked to the genotype (McCloskey *et al.*, 2013). Over the past decade, scientists have built and improved GEMs of *P. putida* KT2440, and these models have been used to investigate metabolism on a wide-range of substrates (Nogales *et al.*, 2008; Puchalka *et al.*, 2008; Sohn *et al.*, 2010; Oberhardt *et al.*, 2011; Belda *et al.*, 2016). However, during the most recent effort to improve these models, a discrepancy between the *in silico* predictions and *in vivo* growth data was identified (Belda *et al.*, 2016). Only 21% of the substrates supporting growth on BIOLOG plates were predicted correctly in the *in silico* analysis. This discrepancy is mostly caused by the lack of identified transporter proteins for specific compounds. By adding the required transport reactions to the model without specifying the underlying genes, the authors could predict 75% of the growth-enabling substrates (Belda *et al.*, 2016). This shows how important it is to identify and confirm transport systems in order to generate predictive models.

In this work, the transcriptome of *P. putida* KT2440 growing on four sole carbon sources, glucose, citrate, ferulic acid, and serine, was explored. Evidence pointing towards the involvement of the most relevant uptake systems identified from the RNA-seq data was obtained using transposon mutants of genes encoding porins and inner membrane systems possibly involved in citrate, ferulic acid and serine transport. The GEM of *P. putida* KT2440 was updated using the transcriptomic data and experimental evidence from transposon mutants. The improved model accounts for missing genes, corrected reversibility and the ferulic acid degradation pathway. This study highlights the importance of exploring expression profiles to advance understanding of how *P. putida* behaves in different environments and identify metabolic features important for the development of the bacterium as a cell factory.

Results and Discussion

P. putida growth on four different sole carbon sources

To gain insights into the metabolic versatility of *P. putida*, RNA-seq experiments were performed on cells growing in minimal media supplemented with either glucose, citrate, ferulic acid or serine as sole carbon sources. The central aim was to explore different bacterial metabolic states resulting from the use of carbon sources entering at different points of central metabolism. To this end, the four substrates were chosen as representatives of different classes of organic compounds (carbohydrate, tricarboxylic acid, aromatic acid, and amino acid).

The growth of *P. putida* KT2440 cells in the presence of the four sole carbon sources was monitored by measuring OD₆₀₀ at different time points (Fig. 1, File S1, and File S2). Cells were harvested in exponential phase and total RNA was extracted and used for preparation of strand-specific cDNA libraries that were sequenced on the Illumina HiSeq platform (File S1,

File S2, and Table S1). The RNA-seq data has been deposited in the GEO Database under accession number GSE118554.

Differential gene expression reveals insights into uptake systems of the four carbon sources

The reads were mapped onto the *P. putida* KT2440 genome and used for differential expression analysis (File S1). In this work growth on glucose was considered the control condition, as cells were grown easily and faster compared with the other three carbon sources (Fig. 1). Moreover, the transcriptome and metabolism of *P. putida* growing on glucose have been extensively investigated in other studies (del Castillo *et al.*, 2007; Kim *et al.*, 2013; Nikel *et al.*, 2015). Therefore, gene expression levels in citrate, ferulic acid and serine were compared with the glucose condition, and genes were considered differentially expressed when they exhibited a p -value ≤ 0.05 and fold change greater than or equal to 2 (File S1). Expression changes in the genes related to uptake systems will be discussed in the following text, while other differentially expressed genes in the four conditions are included in File S2.

Glucose. Comparison of gene expression levels in citrate, ferulic acid, and serine with the glucose condition showed that 47 genes were consistently upregulated in glucose versus the other three carbon sources (Table S2). As expected, the gene encoding the outer membrane porin OprB-1 (PP_1019), the genes encoding the ABC transporter (PP_1015-18), the gluconate transporter GntP (PP_3417), and the 2-ketogluconate transporter KguT (PP_3377) were significantly induced. The porin has been proposed to be involved in the transport of glucose to the periplasmic space in *P. aeruginosa* and *P. putida* (Saravolac *et al.*, 1991; Wylie and Worobec, 1995; del Castillo *et al.*, 2007), while the ABC transporters GntP and KguT are responsible for the transport of glucose into the cytoplasm directly as glucose, or alternatively as gluconate or 2-ketogluconate after oxidation in the periplasmic space (Martins dos Santos *et al.*, 2004; del Castillo *et al.*, 2007; Nikel *et al.*, 2015). Related to that, the dehydrogenase

genes (PP_3382, PP_3383, PP_3384), involved in the periplasmic oxidation of glucose to 2-ketogluconate (del Castillo *et al.*, 2007, 2008), were also upregulated.

Citrate. A total of 391 genes were differentially expressed (265 upregulated and 126 downregulated; see Table S3) when comparing growth on citrate with growth on glucose. A total of 34 genes were related to transporters, of which 22 were upregulated on citrate (Table S4). Among the highly expressed genes on citrate were the gene encoding a predicted porin (PP_1419), the gene cluster for the tricarboxylate transporter TctABC (PP_1418-16), and the gene encoding the putative citrate transporter (PP_0147) suggesting their possible role in citrate transport.

The predicted porin shares a 65% amino acid similarity with the OpdH porin of *P. aeruginosa* (PA0755), and therefore, the PP_1419 gene product will subsequently be referred to as OpdH. Moreover, the *opdH* gene has the same genomic organization in *P. aeruginosa* and *P. putida*, with the porin gene located directly upstream of the tricarboxylate transporter cluster *tctABC*. OpdH has been shown previously to be involved in tricarboxylate (*cis*-aconiate, isocitrate and citrate) transport in *P. aeruginosa* (Tamber *et al.*, 2006, 2007). The TctABC system, a member of the Tripartite Tricarboxylate Transporter (TTT) family, has been shown to bind fluorocitrate, citrate, isocitrate and *cis*-aconitate in *Salmonella enterica* serovar Typhimurium through the TctC subunit (Sweet *et al.*, 1979, 1984; Ashton *et al.*, 1980; Somers *et al.*, 1981; Widenhorn *et al.*, 1988; Winnen *et al.*, 2003). Tct systems have also been functionally characterized in *Bordetella pertussis* (Antoine *et al.*, 2003) and *Corynebacterium glutamicum* (Brocker *et al.*, 2009), and showed the same function of citrate uptake. The putative citrate transporter (PP_0147) belongs to the CitMHS family of transporters, whose prototypes CitM and CitH were first identified in *B. subtilis* and shown to bind citrate in a complex with a bivalent metal ion (Mg^{2+} or Ca^{2+}) (Boorsma *et al.*, 1996; Li and Pajor, 2002; Warner and Lolkema, 2002). Other functionally characterized systems of the CitMHS family have been described for *Streptococcus mutans* (Korithoski *et al.*, 2005), *Enterococcus faecalis* (Blancato

et al., 2006), *Streptomyces coelicolor* (Lensbouer *et al.*, 2008), and *Corynebacterium glutamicum* (Brocker *et al.*, 2009).

Ferulic acid. During growth on ferulic acid, 237 genes (145 upregulated and 92 downregulated) were differentially expressed compared with glucose (Table S5). Interestingly, 22 transporter-related genes were differentially expressed, of which 16 were upregulated in cells growing on ferulic acid (Table S6). The gene encoding the BenF-like porin (PP_1383), the transporter encoded by PP_3740, the PcaK transporter (PP_1376), and the PcaT metabolite/H⁺ symporter (PP_1378), were significantly induced during growth on ferulic acid, and may be involved in transport of the compound into the cells.

The *benF*-like porin gene (PP_1383) is one of the twenty *benF*-like genes related to the benzoate-specific porin gene *benF* (PP_3168) in *P. putida* KT2440. The annotated *benF* gene is part of an operon involved in benzoate catabolism and regulated by *benR*. It has been proposed that the BenF porin may contribute to the flux of benzoate through the membrane (Cowles *et al.*, 2000; Nishikawa *et al.*, 2008; Parthasarathy *et al.*, 2010). The PP_3740 gene (here referred to as the *vanK* gene because of its homology with *vanK* from *Acinetobacter* sp. ADP1 (Jiménez *et al.*, 2002)), as well as the *pcaK* and *pcaT* genes, encode predicted inner membrane transport proteins from the aromatic catabolic pathways of *P. putida* KT2440, and they belong to the major facilitator superfamily (MFS) of transporters (Jiménez *et al.*, 2002). *P. putida* PcaK catalyzes the accumulation of *p*-hydroxybenzoate and protocatechuate, while benzoate is not a PcaK-substrate (Harwood *et al.*, 1994; Nichols and Harwood, 1997). Similar to PcaK, VanK also has specificity for *p*-hydroxybenzoate and protocatechuate in *Acinetobacter* sp. ADP1. However, the complete set of compounds transported by PcaK and VanK remains to be elucidated (D'Argenio *et al.*, 1999). No functional characterization has been performed for PcaT, which remains a candidate β -ketoadipate transporter (Harwood and Parales, 1996; Jiménez *et al.*, 2002). The *vanK* gene (PP_3740) is localized near the *vanAB* genes (PP_3736-37) (metabolic genes, see below), while the *pcaK* and *pcaT* genes are

included in a gene cluster (from PP_1376 to the *benF*-like porin PP_1383) that was entirely upregulated in the present data (see below).

Serine. For cells grown on serine there were 928 differentially expressed genes (557 upregulated and 371 downregulated) (Table S7) when compared with cells grown on glucose. A total of 56 transporter-related genes were differentially expressed and 43 of them were upregulated in cells growing on serine (Table S8). The *oprD* gene (PP_1206), as well as *aapJ* (PP_1297), *aapQ* (PP_1298), and *aapP* (PP_1300) encoding the three components of the ABC transporter AapJQMP, were found to be upregulated on serine, suggesting their participation in amino acid transport.

The porin OprD shows a 51% amino acid similarity with the homologous porin in *P. aeruginosa* (PA0958) that has been shown to play a role in the transport of basic amino acids and carbapenems, such as imipenem (Trias and Nikaido, 1990; Huang and Hancock, 1996; Ochs *et al.*, 1999, 2000; Pirnay *et al.*, 2002; Chevalier *et al.*, 2007; Li *et al.*, 2012; Shen *et al.*, 2015). AapJQMP is a general polar amino acid transporter, whose *aapJQMP* operon consists of four genes encoding a periplasmic binding protein, permease, inner membrane protein and ATP binding subunit, respectively. AapJQMP is homologous to AapJQMP of *Rhizobium leguminosarum* (51% to 79% amino acid similarity), which can transport a wide range of amino acids (Walshaw and Poole, 1996).

Transposon mutants exhibit delayed growth on sole carbon sources

To confirm the involvement of the identified porins and inner membrane uptake systems in carbon source assimilation, the mini-Tn5 transposon mutant strains of *P. putida* KT2440 were tested in a microtiter plate growth assay. Nine transposon mutants (defined as *mut::interrupted gene*) (Table S9) were obtained from the available collection at the Pseudomonas Reference Culture Collection (PRCC) (Duque *et al.*, 2007; Fernández *et al.*, 2012). The mutant strains examined have insertions in a gene of interest possibly involved in

citrate, ferulic acid or serine uptake including *opdH* (PP_1419), citrate transporter (PP_0147), *tctA* (PP_1416), *benF*-like porin (PP_1383), *pcaT* (PP_1378), *vanK* (PP_3740), *aapJ* (PP_1297), *aapQ* (PP_1298), and *aapM* (PP_1299).

Mutant strains were grown in all four carbon sources and all mutants showed growth curves (rates and yields) similar to those of the KT2440 wild type (wt) strain when grown in the presence of glucose as sole carbon source (data not shown), confirming the expression data and that the tested uptake systems are not involved in glucose assimilation. However, all mutants showed a delayed growth (long lag phase) compared with KT2440 wt when grown in the presence of the specific carbon sources (citrate, ferulic acid or serine) associated with the disrupted gene (Fig. 2). During growth in citrate, the mutant with a disrupted citrate transporter (PP_0147) gene showed a longer lag phase compared with the mutant with disrupted *tctA* (Fig. 2A), leading to the conclusion that the citrate transporter may be more active in citrate transport than the TctABC system. During growth in ferulic acid, larger effects were observed for strains with disrupted *benF*-like porin and *pcaT* genes compared to the strain with a disrupted *vanK* gene (Fig. 2B). Finally regarding growth on serine, insertions in genes encoding three subunits of the transporter (AapJ, AapQ, AapM), severely affected the growth of *P. putida* (Fig. 2C), revealing a possible major role for the AapJQMP system in serine transport.

Growth curves similar to the KT2440 wt strain were observed for the 6 mutants with insertions in the *benF*, *pcaT*, *vanK*, *aapJ*, *aapQ*, or *aapM* genes when they were grown in the presence of citrate as sole carbon source, supporting the idea that the uptake systems tested are condition specific (data not shown). However, mutants with insertions in *opdH*, *tctA*, citrate transporter PP_0147, *aapJ*, *aapQ*, or *aapM* genes, showed a delayed growth when grown in the presence of ferulic acid. Similar outcomes were observed for mutants with insertions in *opdH*, *tctA*, citrate transporter PP_0147, *benF*, *pcaT*, or *vanK* genes when growth in serine as sole carbon source (data not shown). These effects might underline a possible role

of the uptake systems also in other carbon sources, or more likely be a general consequence (fitness cost) of mutant growth in two less favorable conditions (ferulic acid, serine, Fig. 1). However, the fact that mutants with insertions in *opdH*, *tctA*, or citrate transporter PP_0147 genes, mutants with insertions in *benF*, *pcaT*, or *vanK* genes, and mutants with insertions in *aapJ*, *aapQ*, or *aapM* genes showed a delayed growth in citrate, ferulic acid, and serine, respectively (Fig. 2), strongly supports the notion of their involvement as uptake systems for these carbon sources.

The observed delayed growth of the mutant strains compared with KT2440 wt shows that the introduction of a transposon insertion in a gene codifying a specific porin or an inner membrane transporter subunit affects bacterial growth in the presence of the carbon source specifically related to the mutated uptake component. This can be explained by the fact that the insertion may directly disrupt a gene or a locus important for the uptake of the carbon source. Another possibility is that the insertion may have a polar effect on the downstream operon, altering the downstream operon indirectly and the consequent assimilation of the carbon source. Concerning the latter, an analysis of the operons including the genes investigated with transposon mutants follows using the *Pseudomonas* Genome Database (Winsor *et al.*, 2016) and DOOR: Database of prOkaryotic OpeRons (Mao *et al.*, 2014).

Regarding the citrate-related transposons, *opdH* is a putative single-gene operon situated upstream of the single-gene operon *tctC* (PP_1418), while *tctA* (PP_1416) is found in the downstream operon with *tctB* (PP_1417) and a membrane protein (PP_1415). Beside the direct effect of disrupting the porin OpdH, the transposon insertion in *opdH* may also contribute to its delayed growth effect by affecting the expression of the downstream tricarboxylate transporter subunit TctC, while the effect of the transposon insertion in *tctA* directly affects the proper functionality of the Tct transporter. The citrate transporter PP_0147 gene is in an operon with the downstream *ygdQ* (PP_0146) gene that encodes a putative transport protein, where both genes are upregulated in the citrate condition (Table S4). Thus,

transposon insertion in the citrate transporter PP_0147 gene may also affect the expression of the YdgQ protein.

In the case of ferulic acid, the *benF*-like porin (PP_1383) and *vanK* (PP_3740) genes are single-gene operons, situated upstream of the predicted porin gene *pcaP* (PP_1382, also upregulated in ferulic acid, see below) and the porin-like protein gene *galP-IV* (PP_3739), respectively. The *pcaT* (PP_1378) gene is the first gene in an operon that includes the three genes *pcaB* (PP_1379), *pcaD* (PP_1380), and *pcaC* (PP_1381) that are involved in the aromatic catabolic pathway (Jiménez *et al.*, 2002) and upregulated in our data (see below). The transposon insertion in the *benF*-like porin (PP_1383) may result in altered functionality of itself and PcaP and thereby contribute to the growth delay. Based on the genomic location and its upregulation in the ferulic acid condition, we cannot exclude a possible role of PcaP in ferulic acid uptake. Transposon insertion in *vanK* (PP_3740) may also alter the downstream gene product GalP-IV, although no upregulation of the gene is observed in our data during ferulic acid growth. The transposon insertion in *pcaT* may directly influence the functionality of the transporter PcaT and indirectly impair ferulic acid metabolism by altering the expression of the metabolic genes in the same operon.

Finally, the serine-related transposons, *aapJ* (PP_1297), *aapQ* (PP_1298), and *aapM* (PP_1299), together with *aapP* (PP_1300), are part of the same operon codifying the ABC amino acid transporter. Insertions in these genes affect the functionality of the transporter itself.

Overall, these results suggest that all the uptake systems tested here are likely involved in supporting growth on the specific carbon source or that the tested genes are part of a genomic locus relevant for supporting the growth. Nevertheless, more studies are needed to confirm the roles of the identified uptake systems, evaluate polarity effects in the operons, characterize the mechanism of action as well as identify additional porins and transporters involved in the assimilation of the studied carbon sources. The information acquired in this

work will serve as the starting point for further detailed studies to address the specificity, role, and implication of the tested uptake systems in *P. putida* metabolic versatility and production.

The metabolic pathways of the carbon sources explored using the genome-scale metabolic model constrained with transcriptomic data

Transcriptomic data were used to implement the iJP962 GEM of *P. putida* KT2440 (Oberhardt *et al.*, 2011). The iJP962 GEM was updated by including the newly identified transporters and carbon source utilization pathways identified by the transcriptomic analysis (File S1). The transcriptomic data were also used to constraint the model iJP962 GEM (Oberhardt *et al.*, 2011) and analyze the metabolic pathways for the assimilation of the four carbon sources (Fig. S1). The effects of transcriptional regulation in predicted flux distributions was investigated by comparing the optimal metabolic flux distribution calculated using parsimonious Flux Balance Analysis (pFBA) (Lewis *et al.*, 2010), and the flux distribution predicted by constraining the model with RNA-seq data using the GIMME (Gene Inactivity Moderated by Metabolism and Expression) method (Becker and Palsson, 2008) (File S1). Flux distributions obtained with pFBA are solely constrained by the stoichiometry of the reactions and the uptake rates. The method assumes optimal pathway usage for the sole purpose of cell growth. On the other hand, GIMME reduces the number of active fluxes that are not consistent with the expression data. This is achieved by defining a minimum value for expression, at which the reactions are no longer active.

Glucose. As mentioned above, glucose can be taken up by three mechanisms; a) direct transport of glucose via the ABC transporter (PP_1015-17); b) periplasmic conversion of glucose into gluconate by quinoprotein glucose dehydrogenase (*gcd*, PP_1444), followed by transport (PP_3417) into the cytoplasm; and c) further periplasmic conversion of gluconate into 2-ketogluconate and then transport into the cytoplasm. The published experimental ¹³C flux analysis (Fig. S2) (Nikel *et al.*, 2015) and the unregulated flux distributions predicted by

pFBA (Fig. S3) show glucose primarily being converted to gluconate in the periplasm. However, the *gcd* gene required to convert glucose into gluconate in the periplasm is expressed at a very low level in our transcriptomic data. Consequently, the regulated flux distributions determined by GIMME (Fig. S4) uses solely the first mechanism described above with glucose transport as the first step, which is less efficient than the experimentally observed mechanism.

P. putida KT2440 lacks the 6-phosphofructokinase enzyme and has an incomplete glycolytic pathway for hexose assimilation. However it is able to assimilate glucose by the ED route (Nelson *et al.*, 2002; Martins dos Santos *et al.*, 2004; Fuhrer *et al.*, 2005; del Castillo *et al.*, 2007; Velázquez *et al.*, 2007; Chavarría *et al.*, 2013). As expected, the transcriptomic data confirm an upregulation of genes related to the ED pathway (Fig. S1A – red box). The genes encoding the three cytoplasmic kinase enzymes GlK (PP_1011), GnuK (PP_3416), KguK (PP_3378) catalyzing the phosphorylation of glucose, gluconate, and 2-ketogluconate, respectively, were induced during growth in glucose. The genes *zwf-1* (PP_1022), *pgl* (PP_1023), *kguD* (PP_3376), and the key ED genes *edd* (PP_1010) and *eda* (PP_1024) were also upregulated.

Citrate. Differently from glucose, the unregulated flux distribution by pFBA (Fig. S5) and the regulated flux distribution by GIMME (Fig. S6) agree in the citrate condition, both allowing the use of either the citrate (PP_0147) or TctABC transporters as uptake systems. Upon entry into the cells, citrate as a carbon source is directly metabolized in the TCA cycle (Fig. S1D – green box). Genes involved in the TCA cycle (isocitrate dehydrogenase *icd*/PP_4011, succinate dehydrogenase *sdhD*/PP_4192, malate:quinone oxidoreductases *mgo-3*/PP_2925, isocitrate lyase *aceA*/PP_4116), beta-oxidation (*fadB*/PP_2136 and *fadE*/PP_1893), and lipid metabolism (PP_1689, PP_4021, PP_4817, PP_5003) were upregulated on citrate.

Ferulic acid. Ferulic acid is metabolized by the protocatechuate pathway into succinate and acetyl-CoA (Fig. S1C – purple box) and finally enters the TCA cycle. The genes encoding the

feruloyl-CoA synthase Fcs (PP_3356), the *p*-hydroxycinnamoyl CoA hydratase/lyase Ech (PP_3358), and the vanillin dehydrogenase Vdh (PP_3357), responsible for the conversion of ferulic acid to vanillate, and the genes for the VanAB enzymes (PP_3736-37) to transform vanillate into protocatechuate, were highly induced in ferulic acid. These findings agree with previous studies describing ferulic acid metabolism via a non- β -oxidative pathway (Gasson *et al.*, 1998; Narbad and Gasson, 1998; Venturi *et al.*, 1998; Overhage *et al.*, 1999; Jiménez *et al.*, 2002; Plaggenborg *et al.*, 2003; Calisti *et al.*, 2008; Graf and Altenbuchner, 2014). The PP_3354 and *aat* (PP_3355) genes were also upregulated and have been suggested as an alternative route by Overhage *et al.* (Overhage *et al.*, 1999). The gene cluster (PP_1376-83) was also upregulated and includes: the *pcaF* (PP_1377) gene encoding the beta-ketoadipyl CoA thiolase, the *pcaT* (PP_1378) symporter gene (see above text), three metabolic genes *pcaB* (PP_1379), *pcaD* (PP_1380), *pcaC* (PP_1381), the *pcaP* (PP_1382) predicted porin, and the *benF*-like porin (PP_1383) (see above). Two other clusters of *pca* genes, *pcaGH* (PP_4655-56) and *pcaIJ* (PP_3951-52), were also upregulated. These *pca* gene clusters are involved in the aromatic catabolic pathway of protocatechuate in *P. putida* KT2440 leading to succinyl-CoA and acetyl-CoA (Harwood *et al.*, 1994; Harwood and Parales, 1996; Nichols and Harwood, 1997; Parke *et al.*, 2000, 2001; Jiménez *et al.*, 2002). Both the unregulated (Fig. S7) and the regulated (Fig. S8) flux predictions show the flux to be carried by either branch of the ferulic acid degradation pathway and using all three transporters identified with the transcriptomic data (PcaK, PcaT, VanK, see above).

Serine. For growth on serine, the unregulated (Fig. S9) and regulated (Fig. S10) flux predictions differ primarily in the transport mechanism they assume. The ABC transporter (AapJQMP) newly identified here is used in the regulated flux distribution due to the low expression level of the passive serine symporter (PP_3589). Active transport should have a higher uptake rate and that is a competitive advantage in environments where cells are competing for carbon sources.

Growth on serine as sole carbon source affected the expression of several metabolic genes related to serine utilization (Fig. S1B – blue box). Differently from the catabolism of the other amino acids, which usually involves aminotransferases to produce α -ketoacids and afterwards decarboxylases (Blatt *et al.*, 1966), serine is mainly dehydrated to pyruvate by the enzyme L-serine dehydratase, as the typical transamination event produces hydroxypyruvate, a very reactive and toxic molecule (Schneider and Stephens, 1990; Fiedler *et al.*, 2002; Duggleby, 2005; Baykal *et al.*, 2006; Karsten and Cook, 2009). Indeed, the gene encoding the serine dehydratase Sda-3 (PP_3144), for the conversion of serine into pyruvate, was highly induced in serine conditions, as well as serine O-acetyltransferase (PP_3136). On the other hand, other genes that use serine in biosynthetic pathways including the tryptophan synthase genes *trpA* (PP_0082) and *trpB* (PP_0083), and the serine/glycine hydroxymethyltransferase *glyA* (PP_0671) gene were downregulated.

The results obtained by integration of transcriptomic data with metabolic models demonstrate that flux and transcriptome changes, especially for carbon source-specific uptake pathways, are consistent with each other as has been previously observed in *E. coli* (Lewis *et al.*, 2010). However, gene expression level alone is not necessarily a good predictor of metabolic fluxes for specific reactions (Machado and Herrgård, 2014) as is the case with the glucose uptake pathway. Furthermore, the data presented here also confirms that the expression of the majority of genes in central carbon metabolism is not strongly affected by carbon source (Sudarsan *et al.*, 2014). Finally, here we show how transcriptomic data can be beneficial to implement the GEM model. The expanded *P. putida* GEM model, improved by the addition of missing genes, transporter information and metabolic pathways, offers a valid tool to exploit metabolic reactions and can now be used for designing cell factories utilizing other carbon sources than glucose such as aromatics.

Concluding remarks

In conclusion, this work provides a description of *P. putida* KT2440 growth in glucose, citrate, ferulic acid and serine as sole carbon sources by using transcriptomic analysis and an updated metabolic model. The results reported here highlight the importance of uptake systems in supporting growth in specific carbon sources and in improving the construction of metabolic modeling. Overall, this work contributes to a better understanding of *P. putida* metabolic versatility and adaptation to different environments by the identification and preliminary characterization of uptake systems involved in citrate, ferulic acid and serine utilization. The information acquired in this work will serve as the starting points for future metabolic engineering strategies as well as detailed studies to address the specificity and the role of these transporters. Finally, the results reported here provide essential knowledge for the development of the bacterium as an efficient cell factory.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Figure legends

Figure 1. Growth curves of *P. putida* KT2440. Cells were grown in M9 minimal medium in the presence of glucose (0.5% (w/v)), citrate (10 mM), ferulic acid (10 mM), or serine (10 mM) as sole carbon sources. After overnight growth in 5 mL of M9 medium supplemented with one of the carbon sources (30° C, shaking at 250 rpm), cultures were diluted in 100 mL fresh M9 medium in 250 mL Erlenmeyer flasks. Cell growth, carried out at 30° C with shaking at 250 rpm, was monitored by measuring optical density at 600 nm. Growth rates in the carbon sources are: glucose μ_{MAX} (h⁻¹) 0.55±0.01, citrate μ_{MAX} (h⁻¹) 0.40±0.05, ferulic acid μ_{MAX} (h⁻¹) 0.36±0.01, and serine μ_{MAX} (h⁻¹) 0.16±0.01. Arrows indicate the cell harvest points. Error bars represent the standard deviations of three biological replicates. Glucose and citrate growth curves were determined previously in D'Arrigo *et al.*, 2016.

Figure 2. Growth curves with transposon mutant strains grown in microtiter plate. The mutant mini-Tn5 strains (mut::*interrupted gene*) were grown in a 96-well plate in the presence of M9 medium supplemented with one of the carbon sources. The growth of mutants that are affected when grown in a specific condition are shown. Growth was monitored by measuring optical density at 630 nm every 10 min in a kinetic assay in a microtiter plate. Growth curves are the result of the average of three biological replicates. Growth on citrate (A), ferulic acid (B), and serine (C).