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Global transcriptional response of solvent-sensitive and solvent-tolerant *Pseudomonas putida* strains exposed to toluene

Carlos Molina-Santiago[1]; Zulema Udaondo[1,3]; María Gómez-Lozano[2]; Soren Molin [2]; Juan Luis Ramos [1,3]

[1]Department of Environmental Protection, Consejo Superior de Investigaciones Científicas, C/ Profesor Albareda 1, E-18008, Granada, Spain

[2]Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Hørsholm, Denmark.

[3]Current address: Abengoa Research, Calle Energía Solar 1, Building E. Campus Palmas Altas, E-41014, Sevilla, Spain.

Corresponding author

Juan L. Ramos

Abengoa Research, Calle Energía Solar 1, Building E. Campus Palmas Altas, E-41014, Sevilla, Spain.

Phone: +34954937111 e-mail: juan.ramos@abengoa.com

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Abstract

Pseudomonas putida strains are generally recognized as solvent tolerant, exhibiting varied sensitivity to organic solvents. Pan-genome analysis has revealed that 30% of genes belong to the core-genome of *Pseudomonas*. Accessory and unique genes confer high degree of adaptability and capabilities for the degradation and synthesis of a wide range of chemicals. For the use of these microbes in bioremediation and biocatalysis, it is critical to understand the mechanisms underlying these phenotypic differences. In this study, RNA-seq analysis compared the short- and long-term responses of the toluene-sensitive KT2440 strain and the highly-tolerant DOT-T1E strain. The sensitive strain activates a larger number of genes in a higher magnitude than DOT-T1E. This is expected because KT2440 bears one toluene tolerant pump, while DOT-T1E encodes three of these pumps. Both strains activate membrane modifications to reduce toluene membrane permeability. The KT2440 strain activates the TCA cycle to generate energy, while avoiding energy-intensive processes such as flagellar biosynthesis. This suggests that KT2440 responds to toluene by focusing on survival mechanisms. The DOT-T1E strain activates toluene degradation pathways, using toluene as source of energy. Among the unique genes encoded by DOT-T1E is a 70kb island composed of genes of unknown function induced in response to toluene.

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Introduction

The use of microorganisms for the production of added-value chemicals can be achieved using a number of different microbial chassis that, when equipped with the appropriate genes, function as efficient biocatalysts (Nikel *et al.*, 2016). Currently, synthetic biology allows the construction of complex pathways for synthesis of chemicals that are difficult to make via chemical synthesis. However, the toxicity of some of the substrates or chemicals produced represents an important bottleneck for industrial production. To increase productivity, the fields of synthetic biology and bioengineering need to better define the mechanisms underlying microbial tolerance to toxic compounds.

Solvent tolerant microorganisms represent promising platforms for the biosynthesis of aromatic compounds such as phenylalanine and 2-phenylethanol (Molina-Santiago *et al.*, 2016), *p*-hydroxybenzoate (Ramos-Gonzalez *et al.*, 2001), catechols (Neumann *et al.*, 2004) and anthranilate (Kuepper *et al.*, 2015), among others. Production of these compounds has been achieved using genetically engineered bacterial and yeast strains, such as *E. coli*, *Clostridium* and *Saccharomyces* strains.

Pseudomonas putida strains generally possess superior tolerance to aromatic compounds than other microbes, which makes them attractive as model platforms (Vargas-Tah and Gosset, 2015; Molina-Santiago *et al.*, 2016). Strains of the *P. putida* species are metabolically versatile, and the genomes of a number of strains are now available (Nelson *et al.*, 2002; Matilla *et al.*, 2011; Li *et al.*, 2012; Tao *et al.*, 2012; Udaondo *et al.*, 2013). Genetic analyses revealed that the genomes of *P. putida* species encode a wide variety of oxidoreductases, hydrolases and thiolases that can be exploited as basic modules for the design of chemical synthesis pathways (Nelson *et al.*, 2002; Wu *et al.*, 2011; Udaondo *et al.*, 2013; Udaondo *et al.*, 2015; Belda *et al.*, 2016).

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Furthermore, pan-genome analysis of *P. putida* revealed that most of the catabolic potential of this species is associated with sets of accessory and unique genes, indicating that different strains have different catabolic potential, and that a case by case analysis is required to achieve a synthetic pathway with the minimal number of heterologous genes.

Toluene and its derivatives (*i.e.*, chloro-toluenes and xylenes) are toxic chemicals that can be used as raw materials for the biosynthesis of other aromatic compounds of industrial interest, such as methyl- and chloro-catechols, substituted semialdehydes, *p*-hydroxybenzoate, and others (Neumann *et al.*, 2004; Verhoef *et al.*, 2010; Kongpol *et al.*, 2014; Verhoef *et al.*, 2014). Biosynthesis requires that the metabolic potential of the strains, as well as their tolerance to toxic chemicals are taken into account. *Pseudomonas putida* strains such as S12, and DOT-T1E thrive in the presence of high concentrations of toxic compounds (toluene, styrene, etc.) (Isken and de Bont, 1996; Molina *et al.*, 2011; Ramos *et al.*, 2011; Tao *et al.*, 2011; Udaondo *et al.*, 2012; Ramos *et al.*, 2015); while other strains are only able to tolerate toluene when supplied at low concentrations, as is the case for KT2440 (Timmis, 2002; Segura *et al.*, 2003).

Despite the differences that *P. putida* strains exhibit to toluene resistance, a number of biochemical, physiological and genetic studies have identified common mechanisms for their toluene tolerance, for example: decrease in cell membrane permeability (Keweloh and Heipieper, 1996; Heipieper *et al.*, 2001; Heipieper *et al.*, 2003; Bernal *et al.*, 2007); the action of chaperones to refold proteins denatured by toxic compounds (Segura *et al.*, 2005; Domínguez-Cuevas *et al.*, 2006; Volkers *et al.*, 2006); different ABC transporters; and efflux pumps of the RND (resistance-nodulation cell division) family, which extrude toxic chemicals (Ramos *et al.*, 1998; Segura *et al.*, 2003; Molina-Santiago *et al.*, 2014). While these common mechanisms can be seen in most strains,

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highly-tolerant strains, such as DOT-T1E and S12, encode and express three pumps (TtgABC, TtgDEF and TtgGHI) (Rojas *et al.*, 2003; Rodriguez-Herva *et al.*, 2007). In contrast, the sensitive-toluene strain KT2440 only encodes TtgABC; while strains such as *P. putida* F1 (Phoenix *et al.*, 2003) exhibits an intermediate level of resistance and express two Ttg pumps (TtgABC and TtgDEF).

Advances in the understanding of transcriptional responses to toluene have been based on the use of microarrays (Rhee *et al.*, 2004; Domínguez-Cuevas *et al.*, 2006; Kobayashi *et al.*, 2011), specific fusions of promoters to *'lacZ* (Ramos-Gonzalez *et al.*, 2005; Busch *et al.*, 2010) and cytoplasmic proteomics (Segura *et al.*, 2005; Volkers *et al.*, 2006). However, in depth quantitative global analyses are lacking. In the last few years, transcriptomic technologies have emerged as powerful tools to fill the gaps left by classical molecular biology and microbiology approaches. RNA sequencing (RNA-seq) technology has been established as a way to reference existing data and to improve our knowledge of global transcriptomic responses under different conditions because it provides a view of the set of genes induced and repressed under specific growth or environmental conditions (Wang *et al.*, 2009).

In this study, RNA-seq analyses of the toluene-sensitive KT2440 strain and the toluene-tolerant DOT-T1E strain were carried out to shed light on the mechanisms involved in the short- and long-term toluene responses at a transcriptional level. We have identified between 250 and 650 mRNAs that are differentially expressed under different conditions in the two strains. Our results demonstrate that the magnitude of the response to toluene in the sensitive KT2440 strain is higher than in the solvent-tolerant strain, though differential expression of chaperones, efflux pumps, membrane proteins and TCA cycle proteins was observed in both strains as common toluene-induced survival strategies. Other functions were strain-specific, such as the repression of flagella in

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order to save energy (in KT2440) or the induction of the TOD pathway (in DOT-T1E) for the degradation and use of toluene.

Results

Experimental strategy

In order to better define the phenotypic differences in toluene tolerance between KT2440 and DOT-T1E we compared the transcriptional global response of these strains using RNA-seq analysis. To this end, we exposed cells to either a long-term exposure or a short-term exposure. For the long-term exposure, cells were grown in M9 minimal medium supplemented with glucose as a carbon source and exposed to low concentrations of toluene supplied through the vapor phase for several hours until they reached late-exponential phase (long-term exposure). For the short-term exposure, the needed volume of toluene to reach a concentration of 5 mM was quickly flushed into the culture when the cells had reached the mid-exponential growth phase (OD ≈ 0.5); the flasks were then hermetically closed and incubated at 30ºC for an hour before harvesting cells. It should be noted that 100% cells of both strains survived a sudden addition of 5 mM toluene. After either short- or long-term toluene exposure, RNA was isolated and the samples were sequenced using the Illumina HiSeq platform. A total of 73 M reads were obtained, of which 68 M reads mapped to the *P. putida* KT2440 or DOT-T1E genomes.

Bioinformatic analyses were carried out to compare the global transcriptomic expression of cells exposed to toluene *vs* cells growing in M9 minimal medium with glucose as a carbon source (control condition). After short-term toluene exposure, we found 280 and 661 mRNAs differentially expressed genes in DOT-T1E and KT2440

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strains, respectively (Figure 1). After long-term toluene exposure, 418 and 411 mRNAs were differentially expressed in DOT-T1E and KT2440 strains, respectively. In all cases, the number of induced genes represented 60-70% of total of differentially expressed genes, with the rest being repressed genes. In general, the magnitude of differential expression in response to toluene when compared with the control conditions was higher in KT2440 than in DOT-T1E, suggesting that the effect provoked by toluene was more intense in the sensitive KT2440 strain than in the highly tolerant strain. In both strains, the magnitude of expression levels was higher in the short-term response than in the long-term response (Figure 1).

Common transcriptional short-term response of P. putida strains to toluene

To study the short-term transcriptional response of *P. putida* DOT-T1E and KT2440 to sudden toluene shock, we analyzed the functions of the differentially expressed transcripts based on gene ontology (GO) terms. We found that in both strains around 57% of the mRNAs differentially expressed (165 mRNAs in DOT-T1E; 370 mRNAs in KT2440) were associated with GO terms. Analyses of the mRNAs that had been associated with GO terms was carried out using WEGO (Ye *et al.*, 2006)—a tool that classifies the functions of the genes into three main categories: cellular components, molecular functions and biological processes (Figure 2). The greatest mRNA changes observed in both strains were categorized under the terms "cell parts" (GO:0044464), which include genes involved with ribosomal and respiratory complex function; "membranes" (GO:0016020, GO:0044425); and "biosynthetic and cellular metabolic processes" (GO:0009058, GO:0044237).

On closer analysis of the terms associated with mRNA changes, we were able to identify other statistically over-represented terms that were strain specific. Over-

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represented terms specific to DOT-T1E included "intracellular parts"; which include hydrolases, ribonucleases, chaperones and proteases; "non-membrane-bounded organelles", which refers mainly to ribosomal subunit proteins, ribonucleoprotein complexes and ribosome structures. For KT2440, over-represented terms were more often related to membrane functions (*e.g.*, outer membrane proteins, respiratory chain complexes, transporters, fatty acids and lipids metabolism). These functions are known to be related with a decrease in cell permeability to aromatic hydrocarbons (Sikkema *et al.*, 1995; Domínguez-Cuevas *et al.*, 2006). The same experiment conducted under a short-term toluene exposure in KT2440 added terms for transferases, amino acid biosynthesis and DNA and RNA metabolic processes. Thus, this comparison suggests that toluene responses in sensitive KT2440 strain of *P. putida* are centered on decreasing membrane permeability to toxic compounds, while in the tolerant DOT-T1E strain, the response is centered on internal metabolic changes.

Common transcriptional long-term response of P. putida strains to toluene

During long-term exposure toluene, only 40% of the differentially expressed mRNA was associated with at least one GO term (175 genes for DOT-T1E; 166 genes for KT2440). Functions over-represented in the long-term response of KT2440 and DOT-T1E to toluene were similar to those seen during short-term toluene exposure (membranes and cell parts, biosynthetic and metabolic processes); although, during the long-term exposure regimen, there were more statistically significant differences between the two strains. In DOT-T1E, changes were seen in GO terms associated with intracellular and primary metabolic processes and nucleotide binding, along with changes seen in the short-term response, such as ribonucleoprotein complex functions.

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KT2440 showed changes in GO terms related to membranes and molecular functions such as transferases and membrane transporters (Figure 3).

KEGG pathway analysis of differentially expressed genes in the presence of toluene

To elucidate which metabolic genes are differentially expressed between the two strains, the results were mapped using the KEGG pathway database. In the analysis of KT2440, we found 48 pathways with repressed genes and 85 pathways with induced genes. Eight pathways were identified as significantly enriched (*P*-value <0.05) (marked with an asterisk in Figure 4A). Of the most represented pathways (shown in Figure 4A) were those related with flagellar assembly, ribosome function, bacterial secretion systems and chemotaxis. This suggests that energy-intensive pathways were shut down to redirect energy to functions related to survival. On the other hand, the pathways that were highly induced were those relating to metabolism and biosynthesis of amino acids, biosynthesis of lipopolysaccharides and other membrane proteins (i.e., ß-lactam resistance proteins), and biosynthesis of fatty acids. This confirms that KT2440 activates genes involved in lowering membrane permeability to toxic compounds and the biosynthesis of membrane proteins.

In DOT-T1E, analyses of KEGG pathways identified repressed genes that were involved in 61 pathways, and induced genes within 50 pathways. In Figure 4B, the asterisk indicates the pathways that were significantly enriched. Repressed pathways were related to ribosome formation, RNA degradation, lysine degradation, central metabolism and amino acid metabolism. As expected, the results showed that toluene and aromatic compound degradation pathways were induced in DOT-T1E, which bears chromosomal *tod* genes for toluene degradation. In DOT-T1E, induced pathways also included those involved in the extrusion of compounds via RND efflux pumps outside

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the cells, e.g., which are classified within the group "bacterial secretion sytems". Another group of highly induced pathways were those involved in fructose, mannose, *D*-glutamine and *D*-glutamate metabolism (Figure 4B). The results support that tolerance to toluene in *P. putida* DOT-T1E involves active extrusion of the solvent and reprogramming of metabolism.

It should be noted that in both KT2440 and DOT-T1E strains, genes involved with phenylalanine degradation were induced; in particular, those related with the metabolism of phenylacetate. Previously, this set of genes has been found to be induced in response to chloramphenicol (Fernández *et al.*, 2012) and formaldehyde (Roca *et al.*, 2008) and it has been hypothesized that their role is related to survival, rather than phenylalanine metabolism, although this remains to be confirmed.

At a pathway level, differences between both strains are clearly marked by the ability of DOT-T1E to degrade toluene. This strain induces pathways involved in the adaptation to and use of toluene as a carbon source by changing its carbon metabolism to degrade this compound, while reducing the intracellular concentration of toluene and minimizing its negative effects. *P. putida* KT2440, the toluene sensitive strain, focuses on inducing modifications to the cell membrane in order to minimize the entry of toxic compounds. Taken together, these results demonstrate that toluene-resistant and toluene-sensitive strains orchestrate differential responses that depend upon their specific genetic repertoire.

Specific gene expression changes in P. putida toluene sensitive and tolerant strains

As described above, we identified differences between the toluene response in two *P. putida* strains through analyses of the functions of induced and repressed pathways. We took this further by comparing the main similarities and differences in the toluene

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response between strains at the single gene level (Suppl. Tables 1-4). We focused on those genes that were identified as involved in the toluene response, such as chaperones, translation machinery, efflux pumps, membrane proteins, as well as proteins involved in motility, central metabolism, lipid metabolism/modification and the degradation of aromatic compounds.

Chaperones

While both *P. putida* strains showed differential expression of chaperones in response to toluene, the chaperone specific genes induced and repressed in each strain were different. In KT2440, heat-shock proteins and chaperones were induced, particularly after the short-term toluene exposure, and to a lesser extent after long-term exposure. These induced genes include *dnaK*, *grpE*, *htpX*, *htpG*, *lon-1*, *lon-2*, *hslV* and *hslU*. In DOT-T1E, the following genes were repressed after short- and long-term exposure: *dnaK*, *dnaJ*, *groES* and *groEL*. The results obtained for the DOT-T1E strain do not match with those reported in proteomic analyses by Segura *et al.* (2005).

Translation machinery

As expected, in response to toluene, cells increase tRNA synthesis for the formation of new proteins. We found that tRNAs were induced in both strains (with the highest inductions observed for methionine tRNAs), although for DOT-T1E, tRNAs were only statistically different after long-term (but not short-term) toluene exposure (Suppl. Table 5). We also analyzed the expression of genes that comprise ribosomal subunits and found that they were repressed in DOT-T1E, while only negligible changes were observed in KT2440.

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Efflux pumps

Efflux pumps of the RND family represent one of the key mechanisms through which *P. putida* extrudes solvents from the cell. In DOT-T1E, these pumps include TtgABC, TtgDEF and the pGRT1 plasmid-encoded TtgGHI. In KT2440, the TtgDEF and TtgGHI efflux pumps are absent, and our analyses revealed that, in response to toluene shock, genes induced included the transcriptional regulator TtgR (up to 25-fold) and the TtgABC efflux pump (between 4- to 7-fold for the three genes). These results suggest that the overexpression of this efflux pump is critical in the first response of KT2440 to toluene, which is in consonance with the exacerbated solvent-sensitivity of a DOT-T1E mutant-deficient in this efflux pump (Duque *et al.*, 2001). In the long-term response, the induction of TtgR was significant but lower (4.44-fold). Our analysis also identified that previously uncharacterized efflux pumps are also induced in the short- and long-term in KT2440. In DOT-T1E, two RND pumps (T1E_0179 and T1E_ 3619) were also induced that have yet to be characterized in the context of toluene tolerance (Tables 2 and 3).

Membrane proteins and lipids

mRNA expression changes within genes involved in the cell membrane were observed. In the two toluene conditions and strains tested, many lipoproteins and membrane proteins were upregulated or downregulated. For example, in KT2440, genes PP_2191, and PP_1737 were induced. In DOT-T1E, genes T1E_0703, T1E_2037 and T1E_4262 were induced. Of the particular interest was the extreme 1069-fold induction of T1E_4262 versus the control condition. This gene is annotated as a membrane protein involved in aromatic hydrocarbon degradation though its exact function is unknown. In both strains, other differentially expressed genes included membrane channel forming porins (*oprJ*, *oprL*, *oprB*, *oprD*) and various secretion systems (*emrA*, *hlyD* and *tolC* of

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secretion system type I). In KT2440, membrane proteins involved in the generation of energy, such as cytochromes and proteins involved in iron uptake (TonB receptors, ExbB, ExbD) (Suppl. Tables 2, 3, 4, and 5).

Central metabolism

Short- and long-term exposures to toluene provoked varied transcriptional responses in genes involved in central metabolism. In the case of DOT-T1E, genes related to the citrate cycle or the glycolysis were repressed (*i.e.*, *glcD*, *sucC*, *sucD*, *lpdG*, *maeB*, *fda*, T1E_2929, *paaA*, etc.) (Table 4); while in KT2440, genes involved in glucose uptake and central metabolism were induced (PP_3278, PP_0795, PP_0793, PP_3443, PP_1444) (Table 5). These results support the hypothesis that, in response to toluene exposure, KT2440 activates central metabolism in order to obtain energy for the production of membrane proteins, while DOT-T1E represses central metabolism to activate other secondary pathways involved in the degradation of aromatic compounds.

As previously described (Ramos *et al.*, 1998; Segura *et al.*, 2012; Volkers *et al.*, 2015; Yang *et al.*, 2016), one of the central characteristics of *P. putida* strains such as DOT-T1E is that they bear genes encoding proteins involved in the degradation of toluene and other aromatic compounds. In this study we detected the induction of all of the genes involved toluene degradation in DOT-T1E (from T1E_4262 to T1E_4274), confirming that cells sensed toluene. The induction of the TOD pathway is part of the 'first response' to toluene (See Suppl. Fig 1), although *per se* toluene degradation is not a critical feature (Ramos *et al.*, 2002; Heipieper *et al.*, 2007). In KT2440, no aromatic degradation pathways were significantly induced in response to toluene, in agreement with its inability to metabolize this aromatic hydrocarbon.

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Flagella biosynthesis

Transcriptomic analyses of KT2440 the strain in response to toluene revealed that genes involved in flagella synthesis were repressed, with the strongest repression occurring under short-term toluene exposure (Table 6 and Suppl. Figure 2). This repression is likely related to the heightened levels of energy required to mount a response in the toluene-sensitive strain. In contrast to what happens KT2440, in DOT-T1E, genes involved in flagella formation were not found to be differentially expressed in any of the conditions tested.

Other genes

Transcriptomic analyses also revealed mRNA changes to a number of other genes of interest. For example, in DOT-T1E, toluene provoked the induction of a genomic region that spans 70 kb (from 1504914 to 1610036). This region is a genomic island that is only present in DOT-T1E and not in KT2440, which could encode proteins involved in toluene tolerance. Further experiments are required to confirm the exact role that this region plays (Suppl. Tables 2 and 3).

Discussion

Segura *et al.* (2003) grouped *Pseudomonas* strains into three main groups according to their ability to tolerate aromatic compounds: sensitive strains, medium tolerant strains, and highly tolerant strains. In this study we decided to study the transcriptional response of two closely related *P. putida* strains which, at the same time, are two clearly different strains in regard to toluene tolerance, the KT2440 strain considered toluene-sensitive, and the DOT-T1E strain which is a highly toluene-tolerant strain. Furthermore, we considered two different scenarios; short-term response to toluene provoked by a

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sudden shock of toluene when cells were in the exponential phase, and a long-term response provoked by sustained growth in the presence of toluene. Our results showed a stronger transcriptional short-term response and a higher mean fold-change with a larger number of differentially expressed genes in KT2440 than in DOT-T1E. Under long-term exposure conditions, the number of genes differentially expressed was similar in both strains although the mean fold-change was again higher in KT2440. This set of results shows that the toluene-sensitive strain KT2440 responded more aggressively to toluene than the tolerant strain, and that the magnitude of the response was higher at the transcriptional level in the short-term than in the long-term.

The transcriptomic results showed common responses to toluene that were independent of the specific mechanisms and characteristics of each strain. Bioinformatic analyses allowed us to identify the differentially expressed genes that form part of the core-genome of *P. putida* (Udaondo *et al.*, 2015). Of the total 899 differentially expressed genes in KT2440, 60% (541 of 899) were found to be in the core-genome; on the other hand, the same analysis in DOT-T1E showed that only 46% of the genes (266 of 570) were part of the core-genome. These non-core genes may be part of the differential response. The pathways represented by the differentially expressed genes of the core-genome, were similar in KT2440 and DOT-T1E and were those involved in cellular mechanisms such as translation and protein synthesis. These specifically showed induction of tRNAs and repression of ribosome subunits both in the short-term and in the long-term, although induction was more pronounced in the short-term. Furthermore, the results indicated a key role for modifications and synthesis of membrane lipids; we observed an induction of many genes involved in membrane formation, an example of which is the DOT-T1E, T1E_4262 gene which was 1069 times higher when compared with the control condition. However, we observed opposed responses in KT2440 and

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DOT-T1E regarding the set of genes involved in the fatty acid synthesis and degradation. In the case of KT2440 these pathways were induced, what correlates with the deep changes that occurred at the membrane level in this strain in the presence of toluene. The need to make new proteins due to the negative effect of the toluene provoked the induction of pathways involved in the synthesis of amino acids in the two *P. putida* strains tested (e.g. synthesis of arginine, methionine, valine, isoleucine, cysteine, leucine, etc).

Accessory genes appeared to be critical for the tolerance to toluene in DOT-T1E, since pathways required for toluene degradation were not in the core-genome, demonstrating that accessory functions of DOT-T1E can confer unique abilities, such as the degradation of aromatic compounds such as toluene. In this sense, a genomic island present only in DOT-T1E and not in KT2440 showed a high induction of almost all of its genes.

The specific transcriptional response observed in the toluene-sensitive KT2440 strain highlighted the critical role of different genes and functions involved in the tolerance to this compound. For example, we observed the induction of many chaperones and stress proteins involved in the correct re-folding of affected proteins, and this, together with the induction of the synthesis of amino acids could point to the requirement to synthesize new proteins. Furthermore, due to the need of energy to mend the damage invoked by the presence of toluene, high energy-cost mechanisms such as flagellar assembly were completely repressed.

The response of DOT-T1E to toluene clearly demonstrated the critical role of aromatic degradation pathways that are active in this strain. In this sense, the presence of toluene caused a high level of induction of the toluene degradation pathway (TOD) and other secondary pathways for the degradation of intermediates of toluene degradation.

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Furthermore, because DOT-T1E is able to degrade toluene and use it as a carbon source, this also caused a deep transcriptional change in central metabolism; many genes of the TCA cycle were repressed, while others were activated so that toluene could be used as a carbon and energy source.

One of the main toluene tolerance mechanisms in *P. putida* strains are efflux pumps, theses have previously been well documented in many studies (Kieboom *et al.*, 1998; Isken and De Bont, 2000; Rojas *et al.*, 2001). TtgABC, TtgDEF and TtgGHI are the three critical efflux pumps involved in the extrusion of toluene and other aromatics in DOT-T1E. These efflux pumps have previously been shown to act synergistically in the extrusion of toxic chemicals (Molina-Santiago *et al.*, 2014). Therefore, it is not surprising that in KT2440, which only carries TtgABC, we observed that this pump was highly induced, most likely because this pump is the main one involved in the extrusion of toluene. In DOT-T1E strain, we observed that the differential expression of the efflux pumps was low. The obvious explanation is that DOT-T1E has three active efflux pumps, along with other mechanisms that help to increase its tolerance and reduce the presence of toluene inside the cells and as such the initial induction does not need to be as drastic as in KT2440.

In this study we compared the different transcriptional responses of the toluene-sensitive strain KT2440, and the highly toluene-tolerant DOT-T1E to short- and long-term exposure to toluene. Our results provide a better understanding of the differences and benefits of each of these strains as potential microbial cell factories for the production of aromatic compounds. These findings provide an important step forward, as many of the model microorganisms currently used in biocatalyst processes are not tolerant to the compounds produced.

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Experimental procedures

Growth conditions and RNA isolation

Single colonies of *P. putida* strains DOT-T1E and KT2440 were grown overnight in Luria–Bertani (LB) medium at 30°C. Overnight cultures were then diluted to a starting OD_{600} of 0.01 in M9 minimal medium (Abril *et al.*, 1989) and 50 ml aliquots were dispersed into separate 250 ml Erlenmeyer flasks and incubated with shaking at 200 rpm in an orbital shaker. In the case of long-term response experiments, toluene was supplied in the vapor phase when cultures were initiated and cells were collected when cultures reached advanced mid-exponential phase ($OD_{600} \approx 0.8$). For short-term response experiments, a non-lethal toluene concentration was quickly added to the cultures to reach a final concentration of 5 mM when they were at the early exponential phase ($OD_{600} \approx 0.5$) and cells were collected one hour later. All the assays were performed in duplicate. RNA isolation was done as previously described (Gómez-Lozano *et al.*, 2014; Molina-Santiago *et al.*, 2015). Harvested cells were mixed immediately with 0.2 volumes of STOP solution (95% [v/v] ethanol, 5% [v/v] phenol) and pelleted by centrifugation. Subsequently, total RNA was extracted with Trizol (Invitrogen). Removal of DNA was carried out by treatment with DNase I (Fermentas) in combination with the RNase inhibitor RiboLock (Fermentas). The integrity and quality of total RNA was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies).

Removal of 23S, 16S and 5S rRNAs

The 23S, 16S and 5S rRNAs were removed by subtractive hybridization using the MICROBExpress kit (Ambion) with modifications (Gómez-Lozano *et al.*, 2014). Capture oligonucleotides complementary to the rRNAs were designed specifically for

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P. putida DOT-T1E. Compared with the standard protocol, 25% more capture oligonucleotides and magnetic beads were used. Removal of rRNA was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies).

Library preparation and RNA sequencing

The sequencing libraries were prepared using the TruSeq Stranded mRNA Sample Preparation kit (Illumina). Each library was prepared with RNA isolated from cells grown in duplicate for each condition. After each step, the samples were validated using an Agilent 2100 Bioanalyzer (Agilent Technologies) and the final concentration was measured using a Qubit 2.0 Fluorometer (Invitrogen). The libraries were sequenced with the Illumina HiSeq2000 platform using a single-end protocol and read lengths of 100 nt.

Data analysis

Computational analysis of RNA-seq data were carried out using Rockhopper 1.3.0 (McClure *et al.*, 2013), including read mapping, normalization, quantification of transcript abundance and sRNA identification. The reads were mapped onto the annotated *P. putida* DOT-T1E (NC_018220), pGRT1 plasmid (NC_015855) and *P. putida* KT2440 (NC_002947) reference genomes. An analysis of variance (ANOVA) was performed on the average expression of the mRNAs to determine those with differential expression between the two conditions tested (P-value < 0.05 and two-fold change). Heatmaps and hierarchical cluster analysis were created using R packages based on expression levels (P-value < 0.05) and the fold change had to exceed 2 or to be less than -2.

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Gene ontology and KEGG pathway analysis

Functional annotation of differentially expressed genes was performed using Gene Ontology (GO) (Ashburner *et al.*, 2000) terms using BLAST (Altschul *et al.*, 1990) against UniProt database (UniProt-GO) (Suzek *et al.*, 2007). The GO terms were exported to WEGO GO plotting tool (Ye *et al.*, 2006) and categorized using level 3 of the GO lineage.

KEGG pathway analysis were performed using KOBAS 2.0 (KEGG Orthology Based Annotation System) (Xie *et al.*, 2011), a web server for annotation and identification of enriched pathways.

RNA-seq data accession number

The sequence reads have been deposited in the GEO database under accession no. GSE86321.

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Conflict of interest

Authors declare no conflict of interest.

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Legends

Figure 1. Differentially expressed genes in DOT-T1E and KT2440 strains after short-term and long-term toluene exposure. Numbers within the columns indicate the mean of the fold-change in each strain and in each condition.

Figure 2. Functional categorization of differentially expressed genes after short-term response to toluene in gene ontology (GO). The results are summarized for the three main categories: biological process, molecular function, and cellular component. Significant changes are marked with an asterisk (*).

Figure 3. Functional categorization of differentially expressed genes after long-term response to toluene in gene ontology (GO). The results are summarized in three main categories: biological process, molecular function, and cellular component. Significant changes are marked with an asterisk (*).

Figure 4. KEGG pathway representation of the main differentially repressed (black) and induced (grey) genes in the toluene response of A) KT2440 and B) DOT-T1E strains. The X axis represents the percentage of induced or repressed genes in a given pathway with respect to the total number of genes in the corresponding KEGG pathway. Statistically different pathways are indicated with an asterisk (*).

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Table 1. Fold-change values of the chaperones induced and repressed in DOT-T1E and KT2440 strains.

Table 2. Efflux pumps differentially expressed in the presence of toluene in DOT-T1E.

Table 3. Efflux pumps differentially expressed in the presence of toluene in KT2440.

Table 4. Genes induced and repressed involved in central metabolism of DOT-T1E.

Table 5. Genes induced and repressed involved in central metabolism of KT2440.

Table 6. Genes induced and repressed involved in flagellar biosynthesis and assembly of KT2440.

Supplementary Material

Suppl. Figure 1. Toluene degradation pathway in *Pseudomonas putida* DOT-T1E. Differentially expressed genes at each step of the pathway are indicated in bold.

Suppl. Figure 2. Flagellar assembly model. Genes differentially and statistically repressed in the short-term response of KT2440 are indicated in red. Other genes in KT2440 are marked in green. Colorless genes are not present in KT2440 strain.

Suppl. Table 1. Genes differentially expressed in the presence of toluene after long-term exposition of DOT-T1E.

Suppl. Table 2. Genes differentially expressed in the presence of toluene after short-term exposition of DOT-T1E.

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Suppl. Table 3. Genes differentially expressed in the presence of toluene after long-term exposition of KT2440.

Suppl. Table 4. Genes differentially expressed in the presence of toluene after short-term exposition of KT2440.

Suppl. Table 5. tRNAs and ribosomal proteins induced and repressed in DOT-T1E and KT2440 strains in the presence of toluene.

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Figure 1

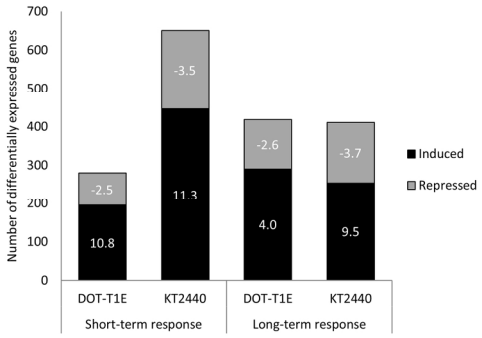

Figure 1

190x142mm (300 x 300 DPI)

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Figure 2

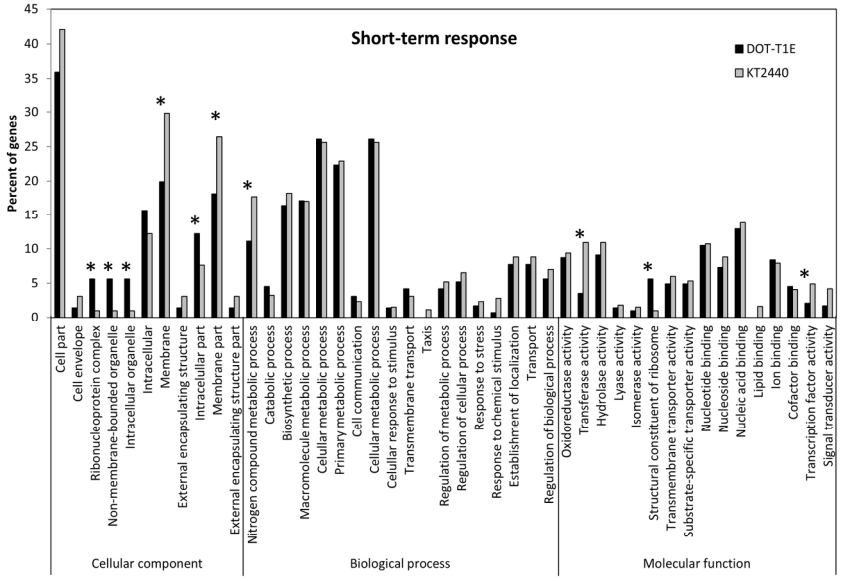

Figure 2

190x142mm (300 x 300 DPI)

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Figure 3

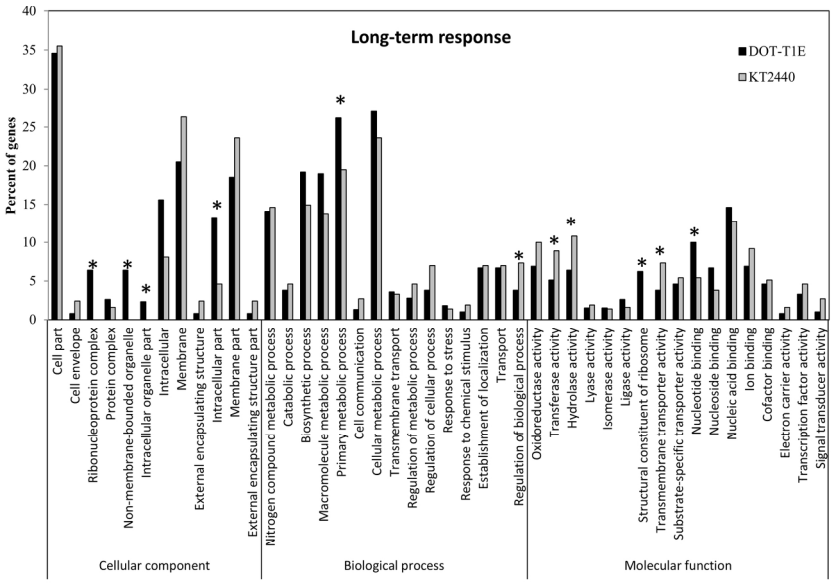

Figure 3

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Figure 4

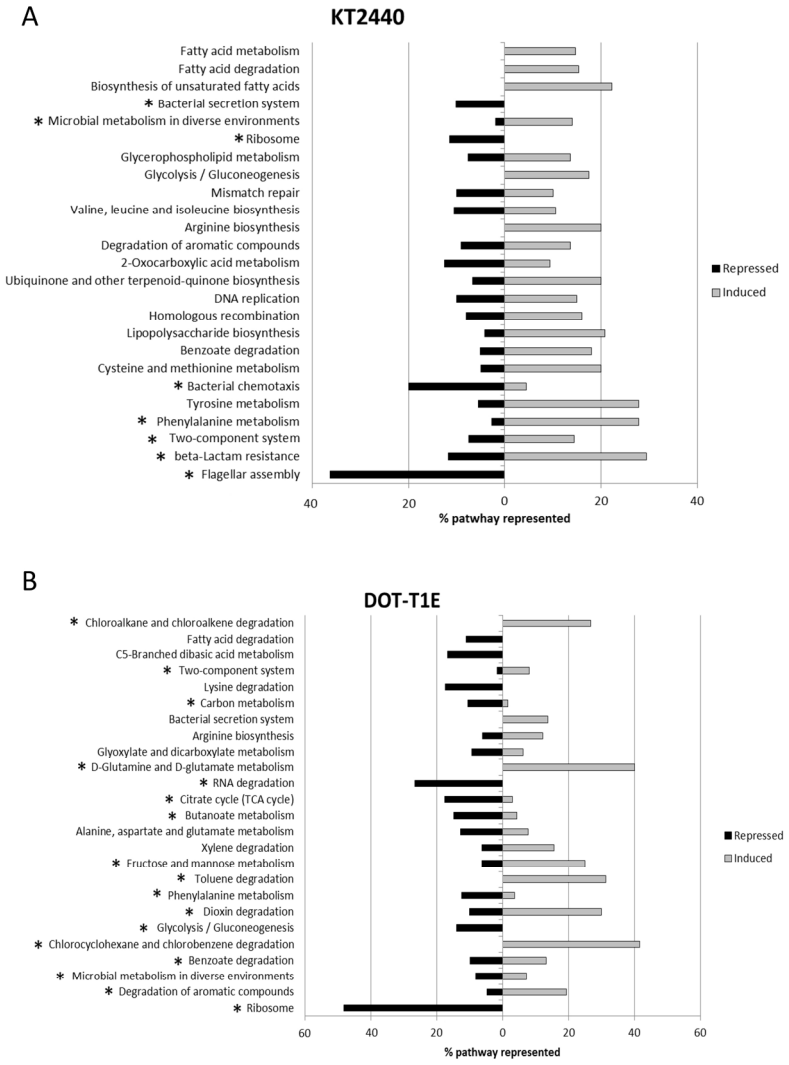

Figure 4

254x338mm (300 x 300 DPI)

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Table 1. Fold-change values of the chaperones induced and repressed in DOT-T1E and KT2440 strains. Genes were considered as induced or repressed when fold-changes were up to 2.

	DOT-T1E		KT2440	
Gene	**Long-term**	**Short-term**	**Long-term**	**Short-term**
dnaK	-3.19	-2.41	1.16	2.73
dnaJ	-2.64	-1.98	1.31	1.65
groES	-4.60	-2.88		
groEL	-2.55	-1.89		
htpX			2.65	10.49
htpG			1.21	2.75
grpE			1.40	3.02
hslV			-1.40	3.38
hslU			1.11	4.18
lon-1			2.34	5.65
lon-2	-1.57	-1.30	1.18	2.33

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Table 2. Efflux pumps differentially expressed in the presence of toluene in DOT-T1E.

Gene	Description	Long-term	Short-term
T1E_2276	RND family efflux transporter MFP subunit	2.03	
T1E_3619	RND efflux system outer membrane lipoprotein	2.00	
T1E_4281	efflux pump periplasmic linker protein (SepA)	-55.00	
T1E_5521	outer membrane efflux protein OprC (TtgF)	2.83	
T1E_0244	HTH-type transcriptional regulator (TtgR)	-2.96	
T1E_5089	RND family efflux transporter MFP subunit	-4.00	
T1E_4382	potassium efflux system protein		2.47
T1E_3612	RND family efflux transporter MFP subunit		3.20
T1E_1282	RND family efflux transporter MFP subunit		3.50
T1E_0483	major facilitator family transporter	2.07	
T1E_5652	major facilitator family transporter	4.66	

Table 3. Efflux pumps differentially expressed in the presence of toluene in KT2440.

Gene	Description	Long-term	Short-term
PP_2885	major facilitator superfamily transporter	-2.93	
PP_5330	major facilitator family transporter	2.07	
PP_1684	major facilitator superfamily transporter	4.02	
PP_2392	major facilitator family transporter	4.88	
PP_0805	TolC family type I secretion outer membrane protein	5.00	9.00
PP_2837	major facilitator family transporter	5.00	
PP_0701	MFS efflux transporter	2.34	2.07
PP_0569	MATE efflux family protein	4.17	
PP_2818	multidrug efflux RND transporter MexD	2.67	
PP_1112	major facilitator superfamily protein		2.39
PP_5262	multidrug efflux protein NorA		2.81
PP_1273	NodT family RND efflux system outer membrane lipoprotein		4.92
PP_0804	protein secretion ABC efflux system, permease and ATP-binding protein		-2.89
PP_1384	NodT family RND efflux system outer membrane lipoprotein (TtgC)		5.65
PP_1385	hydrophobe/amphiphile efflux-1 (HAE1) family transporter (TtgB)		7.76
PP_1386	RND family efflux transporter MFP subunit (TtgA)		4.93
PP_1387	TetR family transcriptional regulator (TtgR)	4.44	25.35
PP_0179	RND efflux transporter		5.63
PP_1684	major facilitator superfamily transporter		3.51
PP_5072	major facilitator family transporter		3.28
PP_2647	major facilitator family transporter		4.43
PP_0715	NodT family RND efflux system outer membrane lipoprotein		3.50

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Table 4. Genes induced and repressed involved in central metabolism of DOT-T1E.

Gene	Description	Long-term	Short-term
T1E_0140	glycolate oxidase subunit glcD		4.92
T1E_0157	malate dehydrogenase	-2.10	-2.60
T1E_0425	succinyl-CoA synthetase subunit alpha	-2.02	-2.28
T1E_0426	Succinyl-CoA ligase [ADP-forming] subunit beta	-2.48	-2.83
T1E_0427	Dihydrolipoamide dehydrogenase	-2.34	-2.68
T1E_0429	2-oxoglutarate dehydrogenase E1 component	-2.55	-2.58
T1E_0433	succinate dehydrogenase, cytochrome b556 subunit		-2.08
T1E_0178	glutamine synthetase	-2.59	-2.15
T1E_1150	Fructose-bisphosphate aldolase	-2.61	-2.22
T1E_2929	PEP phosphonomutase and related enzymes-like protein	2.13	2.33
T1E_5595	phenylacetate-CoA oxygenase subunit PaaA	-5.88	-4.7
T1E_5598	phenylacetate-CoA oxygenase, PaaJ subunit		3.5

Table 5. Genes induced and repressed involved in central metabolism of KT2440.

Gene	Description	Long-term	Short-term
PP_3443	glyceraldehyde-3-phosphate dehydrogenase	7.05	9.73
PP_0793	phosphoenolpyruvate-protein phosphotransferase	9.69	5.85
PP_0795	PTS system fructose subfamily transporter subunit IIC	6.52	4.31
PP_1444	glucose dehydrogenase	9.88	9.19
PP_3274	phenylacetate-CoA oxygenase/reductase subunit PaaK		72.50
PP_3278	phenylacetate-CoA oxygenase subunit PaaA	6.50	208.00
PP_3279	phenylacetate-CoA ligase		4.83
PP_3280	beta-ketoadipyl CoA thiolase		19.00
PP_3282	3-hydroxyacyl-CoA dehydrogenase		26.40
PP_3283	enoyl-CoA hydratase		130.00
PP_3284	enoyl-CoA hydratase		31.11
PP_3285	phenylacetic acid degradation protein PaaY		14.64

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Table 6. The repression of genes involved in flagellar assembly and biosynthesis in KT2440 in response to toluene.

Gene	Description	Long-term	Short-term
PP_4358 (FliM)	flagellar motor switch protein FliM		-4.66
PP_4359 (FliL)	flagellar basal body protein FliL		-4.57
PP_4365 (FliJ)	flagellar biosynthesis chaperone		-9.33
PP_4366 (FliI)	flagellum-specific ATP synthase		-2.80
PP_4367 (FliH)	flagellar assembly protein H		-3.58
PP_4369 (FliF)	flagellar MS-ring protein		-2.93
PP_4370 (FliE)	flagellar hook-basal body protein FliE	-3.86	-16.76
PP_4376 (FliD)	flagellar cap protein FliD		-4.51
PP_4382 (FlgJ)	flagellar rod assembly protein FlgJ		-3.46
PP_4383 (FlgI)	flagellar basal body P-ring biosynthesis protein FlgA		-4.46
PP_4385 (FlgG)	flagellar basal body rod protein FlgG		-3.70
PP_4386 (FlgF)	flagellar basal body rod protein FlgF		-4.02
PP_4391 (FlgB)	flagellar basal-body rod protein FlgB	-3.90	-269.00
PP_4395 (FlgM)	anti-sigma-28 factor FlgM	-4.24	-3.01
PP_4396	FlgN family protein	-2.65	-4.11

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