**Pseudomonas putida** as a platform for the synthesis of aromatic compounds

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Aromatic compounds such as L-phenylalanine, 2-phenylethanol and trans-cinnamate are aromatic compounds of industrial interest. Current trends support replacement of chemical synthesis of these compounds by ‘green’ alternatives produced in microbial cell factories. The solvent-tolerant *Pseudomonas putida* DOT-T1E strain was genetically modified to produce up to 1 g l−1 of L-phenylalanine. In order to engineer this strain, we carried out the following stepwise process: (1) we selected random mutants that are resistant to toxic phenylalanine analogues; (2) we then deleted up to five genes belonging to phenylalanine metabolism pathways, which greatly diminished the internal metabolism of phenylalanine; and (3) in these mutants, we overexpressed the *pheA* gene, which encodes a recombinant variant of PheA that is insensitive to feedback inhibition by phenylalanine. Furthermore, by introducing new genes, we were able to further extend the diversity of compounds produced. Introduction of histidinol phosphate transferase (PP_0967), phenylpyruvate decarboxylase (*kdc*) and an alcohol dehydrogenase (*adh*) enabled the strain to produce up to 180 mg l−1 2-phenylethanol. When phenylalanine ammonia lyase (*pal*) was introduced, the resulting strain produced up to 200 mg l−1 of trans-cinnamate. These results demonstrate that *P. putida* can serve as a promising microbial cell factory for the production of L-phenylalanine and related compounds.

INTRODUCTION

Aromatic compounds are, currently, mainly produced from fossil and other nonrenewable resources using processes that involve toxic precursors, such as benzene or toluene, high temperatures and complex catalysts (Nijkamp et al., 2005; Pugh et al., 2014). At present, there is a worldwide demand for environmentally friendly and sustainable production of chemicals, such as aromatic compounds, based on microbial cell factories that use renewable substrates and wastes as carbon sources (Achmon et al., 2014; Jenck et al., 2004; Notarnicola et al., 2012).

Industry is showing interest in the development of engineered strains that can produce building blocks from sugars, glycerol and lignocellulose for the synthesis of new added-value chemicals. In this study, we focused on the production of L-phenylalanine – an amino acid that is used as a dietary supplement for humans and animals. L-Phenylalanine is also used as a building block for the synthesis of aspartame, an artificial sweetener: poly-gamma-glutamic acid, which is used in the synthesis of biodegradable nanoparticles for pharmaceutical and biomedical applications. The worldwide production of L-phenylalanine was estimated to be more than 30 000 tons per year in 2014 (Li et al., 2010).

L-Phenylalanine is produced in bacteria and in eukaryotic organisms through the shikimate pathway. This pathway transforms phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P), from central carbon metabolism, into chorismate, the ultimate common precursor for the biosynthesis of L-phenylalanine, L-tyrosine and L-tryptophan (Ding...
et al., 2014; Maeda & Dudareva, 2012; Rodríguez et al., 2014; Zhang et al., 2013) (Fig. 1a).

Currently, the majority of the manufactured phenylalanine is produced through bacterial fermentation by genetically manipulated Escherichia coli and Corynebacterium glutamicum strains. A key feature of the process is deregulation (mainly by the elimination of the feedback inhibition by phenylalanine) of 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase and chorismate mutase prephenate dehydratase (PheA), as well as the increased availability of precursors PEP and E4P (Báez-Viveros et al., 2007; Berry, 1996; Bongaerts et al., 2001).

L-Phenylalanine is a precursor for the synthesis of a number of aromatic compounds, including trans-cinnamate and 2-phenylethanol (2-PE) (Achmon et al., 2014; Dueñas-Sánchez et al., 2014; Noda et al., 2011). Trans-cinnamic acid is an aromatic compound that is used in the manufacture of flavours (as precursor of methyl, ethyl and benzyl esters for perfumes), dyes and pharmaceuticals, as well as a precursor of the sweetener aspartame. Although trans-cinnamate is produced in nature by plants and fungal species (Cochrane et al., 2004; Nijkamp et al., 2005), its current bulk production is via chemical synthesis (Burt, 2004; Miyamoto et al., 2004; Nijkamp et al., 2005; Noda et al., 2011). Biological approaches have also been performed by using phenylalanine ammonia lyase (PAL) enzyme genes from different microorganisms (Nijkamp et al., 2005; Noda et al., 2011). The pal genes encode enzymes that catalyze nonoxidative deamination of phenylalanine to cinnamic acid. 2-PE is an aromatic alcohol with a rose-like smell that can be extracted from natural flowers, such as rose, hyacinth, narcissus and jasmine, and is currently chemically synthesized from benzene and styrene oxide. 2-PE is valuable in the cosmetic and perfumery industries and is also used as an aromatic additive and preservative in the food industry (Achmon et al., 2014; Etschmann et al., 2002; Stark et al., 2002). Biological production of 2-PE can be achieved through different pathways, including the phenylacetaldehyde synthase pathway; the phenylethylamine pathway; and the Ehrlich pathway, which has been extensively studied and functionally inserted into both E. coli and Saccharomyces cerevisiae strains (Fig. 1c) (Achmon et al., 2014; Cui et al., 2011; Etschmann et al., 2003; Hazelwood et al., 2008; Zhang et al., 2014).

![Fig. 1. Pathways for L-phenylalanine, 2-phenylethanol and trans-cinnamate production in Pseudomonas putida DOT-T1E.](image-url)
Biosynthesis of aromatic compounds

Biological production of aromatic compounds is often limited by the intrinsic toxicity of these compounds to the producer strain, which leads to decreased productivity and increased industrial costs. *Pseudomonas putida* DOT-T1E is a strain that is highly tolerant to toluene, p-xylene and styrene (Ramos et al., 1995). This strain holds great potential for biotechnological applications because its genomic sequence has been well characterized and because various genetic tools have been developed to introduce and express foreign genes (Udaondo et al., 2013). For this reason, we propose that *P. putida* DOT-T1E is an ideal platform for production of L-phenylalanine, trans-cinnamate and 2-PE (Fig. 1c).

In this study, we have constructed a *P. putida* DOT-T1E L-phenylalanine producer strain through a combination of genetic strategies, including the use of a toxic analogue of phenylalanine, chemical mutagenesis, elimination of L-phenylalanine feedback inhibition via removal of the PheA R-domain, the site-directed deletion of key genes involved in phenylalanine metabolism and the introduction and overexpression of new genes. The sum of the three approaches led to a strain that exhibits an exceptional ability to produce and accumulate phenylalanine. Finally, building on this strain as a foundation, we have devised a way to produce other compounds through the introduction of additional genes. By introducing histidinol phosphate transerase (PP_0967), phenylpyruvate decarboxylase (kdc) and an alcohol dehydrogenase (adh), the strain produced 2-PE. When phenylalanine ammonia lyase (pal) was introduced, the strain was able to produce trans-cinnamate. Taken together, these results strongly support the notion that DOT-T1E could serve as an efficient microbial cell factory for production of aromatic compounds.

**METHODS**

**Chemicals.** Chemicals used in this study include L-phenylalanine (98.5%, Sigma-Aldrich), trans-cinnamic acid (99%, Sigma-Aldrich), 2-PE (99%, Sigma-Aldrich), methanol (99.8%, Fisher Chemicals), acetic acid (99.8%, VWR Prolabo Chemicals), phenylacetalddehyde (90%, Sigma-Aldrich), p-fluoro-DL-phenylalanine (PPF) (98%, Sigma-Aldrich) and ethyl methanesulfonate (EMS) (Sigma-Aldrich).

**Bacterial strains, plasmids, culture media and growth conditions.** Strains and plasmids used in this work are listed in Table 1, and oligonucleotides are shown in Table S1 (available in the online Supplementary Material). *P. putida* strains were grown routinely at 30 °C in LB medium or M9 minimal medium (Sambrook & Russell, 2001), amended with 1.5% (v/v) glucose as a carbon source (except when other concentrations are specified in the text). *E. coli* cells were grown on LB at 37 °C. Growth was determined by following the OD600 of the cultures in tubes for 48 h. Antibiotics were added, when needed, to reach the following final concentrations: 100 µg ml⁻¹ ampicillin for *E. coli* and 500 µg ml⁻¹ for *P. putida*, 25 µg ml⁻¹ kanamycin, 50–100 µg ml⁻¹ streptomycin and 10 µg ml⁻¹ rifampicin. When indicated, 40 µg ml⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 1 mM isopropyl-β-D-1-thiogalactopyranoside or 1–5 mM 3-methylbenzoate (3-MB) were added to the medium.

**Genome editing.** Chromosomal deletions were performed using the I-SceI methodology (Martínez-García & de Lorenzo, 2011, 2012) in which upstream and downstream segments of the homologous DNA were separately amplified and then joined to the previously digested I-SceI-bearing pEMG vector (Fig. S1a) using Gibson Assembly Master Mix (Gibson et al., 2009). Plasmids were electroporated into *P. putida* CM12 (or CM12 mutants) carrying the I-SceI expression plasmid pSW-I as described by Martínez-García & de Lorenzo (2012). Cointegrate constructions were resolved by induction of I-SceI expression with 5 mM 3-MB (Fig. S1a). Kanamycin-sensitive clones were analysed by PCR and Southern blot (Fig. S1c) to verify the deletions. Plasmid pSW-I was cured after growth without selective pressure and its loss was confirmed by both sensitivity to 500 µg ml⁻¹ ampicillin and colony PCR screening as described by Martínez-García & de Lorenzo (2012).

**Chromosomal DNA isolation and Southern blot analysis.** DNA from *P. putida* DOT-T1E and its mutants were prepared using DNeasy Blood & Tissue Kit (Qiagen). The digoxigenin-labelled probe for checking deleted strains was generated by DIG DNA labelling PCR using *P. putida* DOT-T1E chromosomal DNA as a template and the same primers that were used for the construction of the different pEMG plasmids described above (Table S1). Chromosomal DNA was digested with specific restriction enzymes, resolved on a 1% agarose gel and transferred to a nylon membrane using a VacuGene XL system. Hybridization and development of Southern blots were performed according to the manufacturer’s recommendations.

**Vector constructions.** Genes involved in 2-PE and trans-cinnamic acid production were synthesized (GenScript) in plasmid pUC57. The plasmid for 2-PE production (pPE1) was based on the Ehrlich pathway (Hazelwood et al., 2008) and involved three steps: the first step is the deamination of phenylalanine into phenylpyruvate, implemented by the PP_0967 gene product of *P. putida* KT2440; the second step is the decarboxylation of phenylpyruvate into phenylacetalddehyde for which the kdc gene (phenylpyruvate decarboxylase) from *Rhodospirillum rubrum* was used. Finally, conversion of phenylacetalddehyde into 2-PE was obtained by overexpression of the adh gene T1E_5478 (alcohol dehydrogenase) of *P. putida* DOT-T1E. Each of the genes was preceded by the pET28 (Studier & Moffatt, 1986) promoter region and the corresponding Shine-Dalgarno (SD) sequences, and promoter-sd-gene construction (4.5 kb) was released from pUC57 using KpnI and SacI restriction enzymes, cloned into pSEVA438 and expressed in *P. putida* CM12-5 strain (Silva-Rocha et al., 2013).

For the construction of the trans-cinnamic production plasmid (pPAL1), we syntethized a phenylalanine ammonia lyase (pal) gene (1.7 kb) based on the sequence of the corresponding enzyme from *Nostoc punctiforme*. The gene was codon optimized and was preceded by the g1OL SD sequence (Olins & Kangwala, 1989) and flanked by enzyme restriction sites (NotI and KpnI). The synthetic pal gene was cloned into pSEVA238 plasmid (Silva-Rocha et al., 2013) and expressed in *P. putida* CM12-5 using the XylS/Pm system present in pSEVA238 that was induced by 3-MB when culture growth was at the early exponential phase (OD600 nm 0.3–0.4).

For the deletion of the R-domain of PheA (involved in feedback inhibition in the presence of phenylalanine), oligonucleotides containing restriction sites were designed to amplify the gene without its R-domain (Table S1). The pheA (gene 860 bp) was PCR amplified with Phusion DNA polymerase and *P. putida* DOT-T1E chromosomal DNA as a template incorporating the SD sequence in the primers. The appropriate fragments were cloned into pHBL plasmid and, then, digested, extracted with KpnI and HindIII, cloned into pSEVA238 (pPHE1) and finally expressed in CM12-5 strain using the XylS/Pm system present in pSEVA238 that was induced by 3-MB when culture growth was at the early exponential phase (OD600 nm 0.3–0.4).

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Table 1. Strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Strains</th>
<th>Characteristics</th>
<th>References</th>
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<tr>
<td>P. putida DOT-T1E</td>
<td>Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Ramos et al. (1998)</td>
</tr>
<tr>
<td>P. putida DOT-T1E-8</td>
<td>DOT-T1E derivative after PFP resistance evolution, Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>CM12</td>
<td>DOT-T1E-8 derivative after random mutagenesis, Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>CM12-5</td>
<td>CM12 derivative after directed mutagenesis, ΔT1E_0122, ΔT1E_3356, ΔT1E_4057, ΔT1E_1753, ΔT1E_1616, Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>Cloning host for pSEVA238-15Scel plasmids; F-A-endA1 glnX44(AS) thiE1 recA1 relA1 spoT1 gyrA96(NalR) rbc1 deoR nupG 80/lacZAM15 Δ(argF-lac)U169 hadR17(rK- mK+)</td>
<td>Grant et al. (1990)</td>
</tr>
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<td>DH5α Apir</td>
<td>Cloning host for the integrative plasmids pSEVA12S; Apir lysogen of DH5α</td>
<td>Hanahan &amp; Meselson (1980)</td>
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**Plasmids**

- pSEVA238: Expression vector; oriV(pBBR), xylS, Pm, Km<sup>R</sup>
- pPHE1: pSEVA238 derivative carrying phi<sup>R</sup> gene from P. putida DOT-T1E
- pPAL1: pSEVA238 derivative carrying pal genes from Nostoc punctiforme and Streptomyces maritima
- pSEVA438: Expression vector; oriV(pBBR), xylS, Pm, Sm<sup>R</sup>
- pPE1: pSEVA438 derivative carrying PP_0968 from P. putida KT2440, kdc from Rhodospirillum rubrum, and T1E_5478 from P. putida DOT-T1E
- pSW-1: Helper plasmid used for deletions; oriV(RK2), xylS, Pm→I-Sclr; Ap<sup>R</sup>
- pEMG: Plasmid used for deletions; oriV(RK6), lacZa fragment with two flanking I-Sclr recognition sites; Km<sup>R</sup>
- pEMG0122: pEMG bearing a 1-kb TS1-TS2 insert for deleting the T1E_0122 gene
- pEMG3356: pEMG bearing a 1-kb TS1-TS2 insert for deleting the T1E_3356 gene
- pEMG1753: pEMG bearing a 1-kb TS1-TS2 insert for deleting the T1E_1753 gene
- pEMG1616: pEMG bearing a 1-kb TS1-TS2 insert for deleting the T1E_1616 gene
- pEMG4057: pEMG bearing a 1-kb TS1-TS2 insert for deleting the T1E_4057 gene

For the plasmid constructions, transformation in E. coli DH5α cells, or in E. coli CC118 Ap<sup>R</sup> cells in the case of pEMG plasmids, was performed. Once the vectors were obtained, plasmids were electroporated in P. putida strains using standard protocols.

**Minimum inhibitory concentration assays.** Minimum inhibitory concentration (MIC) assays were performed in triplicate in liquid M9 minimal medium in the presence or in the absence of 2-PE, phenylacetaldehyde and trans-cinnamic acid using the twofold serial dilution test according to the guidelines of the Clinical and Laboratory Standards Institute (2003). The highest concentration tested for each compound was L-phenylalanine (50 mg ml<sup>-1</sup>), 2-PE (5 mg ml<sup>-1</sup>), phenylacetaldehyde (60 mg ml<sup>-1</sup>) and trans-cinnamic acid (15 mg ml<sup>-1</sup>). The MIC was determined as the lowest concentration of antibiotic that inhibited growth by >90 %.

**Isolation of PFP-resistant mutants.** For the isolation of PFP-resistant mutants, P. putida DOT-T1E was initially grown in M9 minimal medium supplemented with 1 mg l<sup>-1</sup> of this toxic analogue. Overnight cultures were reincubated in fresh M9 minimal medium supplemented with increasing concentrations of PFP until no growth was obtained (25 mg l<sup>-1</sup>). Previous to each inoculation, samples were spread on solid M9 minimal medium supplemented containing the toxic analogue, and colonies were picked. From this procedure, 30 independent colonies were selected for further characterization.

**EMS mutagenesis of P. putida CM12.** P. putida CM12 overnight cultures were used as preinoculum. For selection of the optimal EMS concentration, different exponential phase cultures were treated with EMS concentrations ranging from 0 to 192 mM, for 45 min at 30 °C. Cultures were subsequently washed twice with PBS and fresh LB was added for cell recovery (2 h at 30 °C). Finally, cultures were serially diluted on plates and incubated at 30 °C for 24 h, and colonies were enumerated. We found that 0.35 mM EMS killed 85–90% of the initial number of cells. All mutagenesis assays were performed using this EMS concentration and different concentrations of PFP were added in the cell recovery step for overproducer phenylalanine clone selection.

**Analytical methods.** The production of L-phenylalanine and its conversion into 2-PE was determined using an Agilent 1260 Infinity high-performance liquid chromatographer (HPLC) with a Zorbax Eclipse Plus C18 column coupled to a UV detector. The solvent composition in the case of phenylalanine and 2-PE measurement changed from 99 % water and 1 % acetonitrile to 70 % water and 30 % acetonitrile for 10 min followed by a 2 min hold time. Then, the solvent composition changed to 100 % acetonitrile for 2 min and finally returned to the initial conditions within 3 min followed by 3 min hold time. For trans-cinnamic acid measurement, methanol and water supplemented with 3 % acetic acid were used as solvents. The composition changed from 95 % water-acetic acid and 5 % methanol-acetic acid to 20 % and 80 %, respectively, within 8 min followed by a 2 min hold time. Then, the mobile phase changed to the initial condition in a 4 min linear gradient, followed by 3 min hold time. Under these conditions, the eluent was monitored at 215 nm for L-phenylalanine and 2-PE and at 280 nm for trans-cinnamate. Elution times of L-phenylalanine, trans-cinnamate and 2-PE were 4 min, 8 min and 11.5 min, respectively. Samples (20 μl) were injected for analysis at a total constant flow rate of 1.0 ml min<sup>-1</sup> and constant column temperature of 45 °C for L-phenylalanine and 2-PE or 25 °C for trans-cinnamic.
acid. Glucose analysis was performed using an RID detector and an anion exchange column (Aminex HPX-87H; BioRad) on the same HPLC system. The column was eluted with 0.005 M H₂SO₄ at a constant flow rate of 0.6 ml min⁻¹ and glucose was detected at 8.9 min. We have used supernatant of cultures for all the analyses performed.

RESULTS

Selection of a suitable host strain capable of tolerating high concentrations of aromatic compounds

One of the main hurdles to the production of aromatic compounds in bacterial strains is their low tolerance towards these chemicals. In this study, we decided to use P. putida DOT-T1E, a strain that tolerates high concentrations of aromatic hydrocarbons (Ramos et al., 1998; Rojas et al., 2001), as the host for the production of L-phenylalanine, 2-PE and trans-cinnamate. We initially analysed the tolerance of P. putida DOT-T1E to l-phenylalanine, 2-PE, phenylacetaldehyde – an intermediate in 2-PE biosynthesis – and trans-cinnamate using MIC assays (Table 2). The results indicated that the strain tolerated higher concentrations than E. coli DH5α.

Development of a phenylalanine overproducing P. putida DOT-T1E strain

P. putida DOT-T1E does not accumulate L-phenylalanine during growth on glucose (data not shown), and, therefore, we used four complementary strategies for the development of a P. putida DOT-T1E strain able to produce and accumulate phenylalanine: (I) isolation of clones resistant to PFP; a toxic analogue of phenylalanine; (II) chemical mutagenesis using EMS to improve phenylalanine production; (III) site-directed analogue of phenylalanine; (II) chemical mutagenesis using PFP; a toxic analogue of phenylalanine (to avoid its degradation); and (IV) removal of the R-domain of PheA to avoid feedback inhibition by phenylalanine, which in turn should lead to increased phenylalanine production by increasing the metabolic flux through the shikimate pathway.

After exposing P. putida DOT-T1E to increasing concentrations of PFP, a toxic analogue of L-phenylalanine (see Methods and Fig. S1b), we obtained several spontaneous mutants capable of tolerating up to 22 mg l⁻¹ PFP that were able to accumulate up to 40 mg l⁻¹ L-phenylalanine. One of these mutants, named P. putida DOT-T1E-8, was selected for further study (Fig. 2). P. putida DOT-T1E-8 was subjected to EMS mutagenesis (Fig S1b) and clones resistant to 40 mg l⁻¹ PFP were selected and tested for L-phenylalanine accumulation. P. putida CM12 produced the highest amount (250 mg l⁻¹) of L-phenylalanine in the culture medium after 24 h of growth (Fig. 2) and as such was chosen for further analysis.

Computational analysis of L-phenylalanine metabolism suggested that L-phenylalanine production and accumulation could be increased by blocking L-phenylalanine degradation pathways in the CM12 strain. KEGG analysis allowed us to identify up to five genes encoding enzymes involved in four pathways required for L-phenylalanine catabolism (Fig. 1b), namely, T1E_0122 and T1E_3356 (two 4-hydroxyphenylpyruvate dioxygenases involved in transformation of phenylpyruvate to 2-hydroxyphenylpyruvate), T1E_4057 (phenylalanine 4-monoxygenase involved in transformation of phenylalanine to tyrosine), T1E_1753 (catalase peroxidase that carries out the conversion of phenylalanine to 2-phenylacetamide) and T1E_1616 (aldehyde dehydrogenase that transforms phenylacetaldelyde to phenylacetate). The genes mentioned above were sequentially deleted from P. putida CM12 using the pEMG plasmid as described in Martínez-García & de Lorenzo (2012) and deletions were confirmed by PCR and Southern blot (see Methods and Fig S1a–c). The method mediates the seamless excision of genomic DNA segments of variable sizes with virtually no acquisition of additional mutations. First, we obtained a CM12 single mutant strain in the T1E_4057 gene; this strain was later mutated in T1E_1753 gene, obtaining a double mutant strain. Both single and double mutants showed small improvements in phenylalanine accumulation. Subsequently, the T1E_0122 and T1E_3356 genes of the CM12 double mutant were simultaneously deleted obtaining two triple mutants that did not show any difference in growth or phenylalanine accumulation between them but improved phenylalanine accumulation in respect to the double mutant strain. The triple mutant deleted in T1E_3356 was used as a host for a new deletion, namely, T1E_0122, obtaining a quadruple mutant. Finally, the T1E_1616 gene was knocked out, obtaining the cell factory strain P. putida CM12-5, which was able to produce around 340 mg l⁻¹ L-phenylalanine after 48 h of growth (Fig. 2), confirming that a combination of mutations could remarkably improve the phenylalanine accumulation of the CM12 strain.

To further enhance the L-phenylalanine production in P. putida CM12-5, we overexpressed genes in the shikimate pathway (Fig. S1b). One of the main steps in shikimate biosynthesis involves the bifunctional enzyme PheA, which contains two catalytic domains (chorismate mutase and prephenate dehydratase) as well as one R-domain that is

Table 2. MIC of P. putida DOT-T1E and E. coli strains to L-phenylalanine, 2-PE, phenylacetaldehyde and trans-cinnamate

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (mg ml⁻¹)</th>
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<tr>
<td></td>
<td>L-Phenylalanine</td>
</tr>
<tr>
<td>P. putida DOT-T1E</td>
<td>25</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>12.5</td>
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have consumed all ammonium in the medium. To test this, we added higher ammonium concentrations in cultures with 5% glucose; however, though we did not obtain higher phenylalanine amounts or higher glucose consumption, we did notice an increase in tyrosine concentration proportional to ammonium in the medium.

**P. putida CM12-5 as a host strain for production of bioproducts**

In an endeavour to make a *P. putida* strain for future utilization as an industrial platform, we engineered *P. putida* CM12-5 for the production of 2-PE and trans-cinnamate. For 2-PE production, we decided to use the pPE1 plasmid where PP_0967 of *P. putida* KT2440, *kdc* from *R. rubrum* and *adhl* (T1E_5478) from *P. putida* DOT-T1E were overexpressed (Fig. 1c). Although the second step is the only one not encoded on the *P. putida* DOT-T1E genome, we decided to overexpress all the genes of the pathway to improve 2-PE production. Expression of these genes in *P. putida* CM12-5(pPE1) was 60-fold higher than in *P. putida* CM12(pPE1) (Fig. 4a).

For the production of trans-cinnamate, we cloned the *pal* gene from *N. punctiforme* in pSEVA238 to construct the pPAL1 plasmid. *P. putida* CM12-5(pPAL1) was able to produce up 190 mg l\(^{-1}\) trans-cinnamate directly from glucose after 24 h of growth (Fig. 4b); as shown in the figure; the amount of L-phenylalanine in the culture medium was never higher than 50 mg l\(^{-1}\) demonstrating that the L-phenylalanine produced from glucose is being converted into trans-cinnamate.

### Optimization of culture conditions

We noted that when the preinoculum used was prepared from cultures at exponential phase of growth (OD\(_{600\text{ nm}}\) ~0.3–0.5), phenylalanine production reached concentrations twice as high as those in which the preinoculum were stationary phase cells (Fig. 3).

In addition, we tested the conversion of glucose into L-phenylalanine when different glucose concentrations were added to the medium. When the medium contained 1.5% (v/v) glucose, it was completely consumed [and in fact it was almost completely consumed in the case of 2.5% (v/v) glucose]. In these cases, phenylalanine concentrations of 930 mg l\(^{-1}\) were reached after 48 h of incubation of *P. putida* CM12-5 (pPHE1). Higher glucose concentrations did not improve L-phenylalanine production [although it was maintained in cultures with up to 10% (v/v) glucose concentration] (Tables 3 and S2), and growth diminished suggesting that the presence of high concentrations of glucose could exert some metabolic inhibition.

In an attempt to increase phenylalanine production and glucose consumption, we hypothesized that CM12-5 could produce up 190 mg l\(^{-1}\) trans-cinnamate directly from glucose after 24 h of growth (Fig. 4b); as shown in the figure; the amount of L-phenylalanine in the culture medium was never higher than 50 mg l\(^{-1}\) demonstrating that the L-phenylalanine produced from glucose is being converted into trans-cinnamate.

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DISCUSSION

The volatility of crude oil production and distribution, together with the need to create a sustainable society, has prompted initiatives towards bio-based industries. With the recent commercialization of lignocellulosic sugar production technologies, the concept of an integrated biorefinery—a facility that extracts carbohydrates from renewable sources to convert them into multiple products—is being considered by certain industries. Up until now, the main efforts have been focused on the production of a few compounds such as succinic, lactic and fumaric acids, polyhydroxyalkanoates and other linear compounds (PNNL and NREL, 2014), while limited effort has been applied to the biological production of aromatic compounds (Ramos et al., 2016).

One of the problems facing the biological production of aromatic compounds is their inherent toxicity towards the biocatalyst. Choosing the right chassis for implementation of synthetic routes is of utmost importance for the success of the industry, as productivity is key to the economics of the project. *P. putida* are equipped with numerous traits that allow them to endure harsh conditions and, as such, have been proposed as a new bacterial platform for whole-cell biocatalysis (http:www/empowerputida). *P. putida* DOT-T1E has been well characterized for its high tolerance towards aromatic compounds (Ramos et al., 1998; Udaondo et al., 2013). We have now demonstrated the superior performance of this strain regarding tolerance to the products of interest, namely, L-phenylalanine, 2-PE and trans-cinnamate, when directly compared with *E. coli* strains that have normally been used in industry to produce these kinds of compounds.

As previously demonstrated with the production of trans-cinnamic acid and *p*-hydroxycinnamic acid in *P. putida* S12 (Nijkamp et al., 2005, 2007; Vargas-Tah & Gosset, 2015; Weber et al., 1993), chemical mutagenesis in the presence of high *m*-fluoro-phenylalanine concentrations, a toxic analogue to L-phenylalanine, led to the isolation of mutants able to accumulate higher amounts of L-phenylalanine and, in consequence, to produce higher concentrations of this amino acid. A combination of different strategies such as selection rounds in PFP and the use of EMS chemical mutagenesis allowed us to obtain *P. putida* CM12, a strain able to accumulate up to 250 mg l⁻¹ of L-phenylalanine. Although we did not study the molecular basis of this accumulation, it is likely that the combination of higher L-phenylalanine production together with the reduction in

Table 3. Phenylalanine production, glucose consumption and growth of *P. putida* CM12-5(pPHE1) at M9 minimal medium supplemented with different glucose concentrations (1.5, 2.5, 5, 10, 15 % v/v) after 48 h of incubation, using a preinoculum growth up to exponential phase

<table>
<thead>
<tr>
<th>Initial glucose (%)</th>
<th>Phenylalanine (mg l⁻¹)</th>
<th>Glucose consumption (%)</th>
<th>Growth (OD₆₀₀ nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 %</td>
<td>870±25</td>
<td>1.5±0</td>
<td>7.16±0.21</td>
</tr>
<tr>
<td>2.5 %</td>
<td>915±9</td>
<td>1.94±0.01</td>
<td>8.30±0.18</td>
</tr>
<tr>
<td>5 %</td>
<td>915±15</td>
<td>1.85±0.01</td>
<td>8.72±0.16</td>
</tr>
<tr>
<td>10 %</td>
<td>930±20</td>
<td>3.42±0.04</td>
<td>5.97±0.53</td>
</tr>
<tr>
<td>15 %</td>
<td>650±6</td>
<td>5.00±1.08</td>
<td>2.81±0.06</td>
</tr>
</tbody>
</table>
flow towards some of the metabolic pathways for L-phenylalanine utilization accounted for the higher accumulation. Inhibition of the metabolic pathways that convert L-phenylalanine into other compounds was achieved by consecutive genetic deletions and/or generation of knockouts. This strategy provided strain P. putida CM12-5 showing a L-phenylalanine production 45% higher than its parental strain (P. putida CM12). In addition to the improvement in L-phenylalanine production, there was also a remarkable increase in phenylalanine accumulation, as can be observed by differences obtained at 48 h of growth between CM12-5 and its parental strain CM12 (Fig. 3). Further optimization by introducing a pheAbr into the strain, even without the deletion of the chromosomal pheA gene and improving growth conditions, allowed the production of almost 1 g l⁻¹ of L-phenylalanine under optimized conditions, similar levels to those described for the genetically engineered E. coli NST-74 strain (Mckenna & Nielsen, 2011). The importance of genetic deletions is highlighted since the CM12 strain overexpressing pheAbr gene did not increase phenylalanine accumulated levels in supernatants compared with CM12 strain (see Fig. 3). It should be noticed that phenylalanine production is influenced by the growth state of cells and cell density of the inoculum, corroborating that the metabolic state of the cells for production is a critical parameter in the future industrial process. Although optimization of strains for L-phenylalanine production by selection in PFP and elimination of enzyme feedback repression have been performed previously (Dueñas-Sánchez et al., 2014; Liu et al., 2014), to our knowledge, this is the first time that these optimizations were combined with the deletion of several genes involved in L-phenylalanine degradation. In future, further strain optimization will be focused on increasing central metabolism to enhance PEP and E4P levels (Vargas-Tah & Gosset, 2015). It is also interesting to note that an excess of glucose in the medium can cause growth inhibition in the chassis strain and therefore increasing the rate of glucose metabolism can relieve this inhibition and improve L-phenylalanine production. Concentrations of phenylalanine, 2-PE and trans-cinnamate obtained in this study at a laboratory scale are promising, showing values in the range of other experiments performed with bacterial strains and with the clear advantage of the high resistance of P. putida to the chemicals being produced. Future scale-up of the processes will show the potential of P. putida CM12-5 as a reference host for the production of phenylalanine and its derivates.

Tyrosine was produced in the experiments where minimal medium was supplemented with different ammonium concentrations, although we had deleted TIE_4057 that is involved in transformation of phenylalanine to tyrosine. Computational analyses and information obtained from the P. putida KT2440 mutant collection (Duque et al., 2007) allowed us to find an alternative pathway for tyrosine biosynthesis encoded by a bifunctional cyclohexadienyl dehydrogenase/3-phosphoshikimate 1-carboxyvinyltransferase gene (TIE_3019) that is involved in the shikimate pathway as well as in the transformation of prephenate to tyrosine.

Currently, a major trend in the chemical industry is the implementation of microbial-based processes for the production of both bulk and fine chemicals; in many cases, the biosynthetic routes are not yet competitive with petroleum-based chemistry. The development of hosts with improved properties such as high tolerance towards aromatic compounds and which are amenable to targeted redesign will reduce time to market, increase yields, and constitute an attractive production system.

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REFERENCES


Biosynthesis of aromatic compounds


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