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Dynamic flux regulation for high-titer anthranilate production by plasmid-free, conditionally-auxotrophic strains of *Pseudomonas putida*

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**ABSTRACT**

Anthranilate, an intermediate of the shikimate pathway, is a high-value aromatic compound widely used as a precursor in the production of dyes, fragrances, plastics and pharmaceuticals. Traditional strategies adopted for microbial anthranilate production rely on the implementation of auxotrophic strains—which requires aromatic amino acids or complex additives to be supplemented in the culture medium, negatively impacting production costs. In this work, we engineered the soil bacterium *Pseudomonas putida* for high-titer, glucose-dependent anthranilate production by repurposing elements of the Esa quorum sensing (QS) system of *Pantoaea stewartii*. The *P*esaS promoter mediated a self-regulated transcriptional response that effectively knocked-down the expression of the *trpDC* genes. Next, we harnessed the synthetic QS elements to engineer a growth-to-anthranilate production switch. The resulting plasmid-free *P. putida* strain produced the target compound at $3.8 \pm 0.3$ mM in shaken-flask cultures after 72 h—a titer >2-fold higher than anthranilate levels reported thus far. Our results highlight the value of dynamic flux regulation for the production of intermediate metabolites within highly-regulated routes (such as the shikimate pathway), thereby circumventing the need of expensive additives.

1. Introduction

Aromatic compounds comprise a vast group of chemicals, typically containing six-carbon conjugated cyclic structures, with multiple applications in the chemical, food, cosmetic, materials and pharmaceutical industries. Most aromatics currently in the market are derived either from benzene, toluene and xylene (molecules obtained from petroleum, with a 120 M€ global market projected by 2024). Microbial production of aromatics, using abundant and readily-available carbon feedstocks, has emerged as a promising alternative to address the need for renewable and sustainable processes to supply growing market demands (Aversich and Kramer, 2018; Kogure and Inui, 2018; Lee and Wendisch, 2017; Liu et al., 2020; Noda and Kondo, 2017; Schwanemann et al., 2020; Thompson et al., 2015). Microbial production of aromatics offers the formation of the aromatic proteinogenic amino acids tryptophan (L-Trp), tyrosine (L-Tyr) and phenylalanine (L-Phe). As hinted above, biological synthesis of anthranilate allows for the use of renewable carbon sources (e.g. glucose and other sugars) and circumvents the formation and disposal of toxic by-products associated to the customary chemical synthesis approaches—yet bio-based anthranilate production still suffers from several drawbacks that limit its widespread adoption. Traditional metabolic engineering strategies designed for microbial anthranilate production faced the issue of balancing fluxes within a core pathway that generates both the product of interest and three essential amino acids (the synthesis of which drains anthranilate). Deleting or

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anthranilate using these strategies, including Escherichia coli (Balderas-Hernández et al., 2009), Corynebacterium glutamicum (Luo et al., 2019; Walter et al., 2020), Saccharomyces cerevisiae (Kuivanen et al., 2021) and Pseudomonas putida (Kuepper et al., 2015). P. putida is an ubiquitous Gram-negative soil bacterium used as a platform for biotechnological and bioremediation applications (Calero and Nikel, 2019; Kivisaar, 2020; Nikel and de Lorenzo, 2018; Sánchez-Pascual et al., 2019; Volk et al., 2020a; Volke and Nikel, 2018; Weimer et al., 2020). Owing to its distinct tolerance to toxic substrates and products, together with a versatile metabolism, P. putida (and related Pseudomonas species) attracted attention as a host for aromatic bioproduction (Schwanemann et al., 2020)—including de novo biosynthesis from renewable resources (e.g. sugars), biotransformations in single- and bi-phasic fermentation setups, metabolic funneling of lignin-derived substrates and upcycling of aromatic monomers. Studies on de novo production of aromatic compounds report the construction and testing of microbial cell factories that require addition of supplements—typically, amino acids—to the culture medium.

Dynamic regulation strategies could be useful to circumvent the use of auxotrophic strains during microbial production of aromatic compounds (Gupta et al., 2017; Shen et al., 2019a, 2021). The shikimate pathway is particularly amenable to apply this type of synthetic regulation, since it encompasses both the target product(s) and essential biomass building-blocks (Li et al., 2020). Hence, the entry into a production phase, characterized by accumulation of the target aromatic compound and limited microbial growth, could be controlled by dynamically down-regulating the expression of key genes within the pathway of interest (Gupta et al., 2017; Shen et al., 2019b). Likewise, the use of dynamic regulation prevents the need of inducible promoters for overexpression of heterologous genes and prevents the metabolic burden frequently observed when these proteins are produced (Min et al., 2017; Tan and Prather, 2017). Genetic circuits based on quorum-sensing (QS) elements provide a pathway-independent system for rebalancing metabolism along the fermentation process (Ge et al., 2020; Wu et al., 2020b). In its natural context, QS is a cell density-dependent process that coordinates gene expression, thereby regulating various cellular processes in bacteria. Several proteobacteria, for instance, detect and respond to a chemical signal, an acylated homoserine lactone (AHL), in a concentration-dependent manner. These molecules are typically produced by an AHL synthase homologous to LuxI from Vibrio fischeri (Fuqua et al., 1996). This signal is sensed by an AHL-responsive transcription regulator homologous to LuxR. Thus, gene expression is altered in relation to the cell density such that multiple physiological processes (e.g. bioluminescence, virulence, and pigments or capsule production) are modulated at the population-wide level (Waters and Bassler, 2005). Synthetic QS genetic circuits have been implemented in a reduced number of model microorganisms, e.g. E. coli, Bacillus subtilis and S. cerevisiae (Cui et al., 2019; Dinh et al., 2020; Dinh and Prather, 2019; Gupta et al., 2017; Kim et al., 2017; Long et al., 2020; Shen et al., 2021; Wu et al., 2020a; Yang et al., 2021)—and engineering this type of regulatory layers in non-traditional production hosts could multiply their value for bioproduction of compounds (e.g. aromatics) that are difficult to produce in other microorganisms.

In this work, we have engineered P. putida KT2440 to produce anthranilate from glucose under culture conditions that require no additional nutritional supplements typically associated with aromatic biosynthesis. By harnessing elements of the Esa QS system from the Gram-negative bacterium Pantoea stewartii (formerly known as Erwinia stewartii), we implemented a set of synthetic QS circuits and characterized their behavior both in E. coli and P. putida. In this QS system, the EsaR regulatory protein can act as either transcriptional activator or repressor, allowing for a versatile transcriptional response depending on the regulator/promoter combination. The information was used to engineer an inducer-independent genetic switch operating at the level of the TrpD node, resulting in a conditionally-auxotrophic P. putida strain that accumulated anthranilate to the highest titer reported so far in a
minimal medium using sugars as the feedstock.

2. Materials and methods

2.1. Bacterial strains and general cultivation conditions

All bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* DH5α was routinely used for cloning and plasmid maintenance. During genetic manipulations, *E. coli* and *P. putida* strains were grown in lysogeny broth (LB) medium (10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract and 5 g L⁻¹ NaCl) in an orbital shaker at 200 rpm at 37 °C and 30 °C, respectively. For gene expression, QS circuit characterization and anthranilate production experiments, wild-type and engineered strains of both *E. coli* and *P. putida* were grown in M9 minimal medium (Green and Sambrook, 2012) supplemented with 20 mM glucose as the sole carbon source (MaxQ™ 8000 incubator; Thermo Fisher Scientific, Waltham, MA, USA) and the relevant additives indicated in each case. For L-Trp–auxotrophic *P. putida* strains (i.e. carrying a ΔtrpDC deletion), L-Trp was supplemented to the M9 minimal medium at 0.1 mM (Kueper et al., 2015). When appropriate, antibiotics were also added at the following final concentrations: kanamycin (Km), 50 µg L⁻¹; streptomycin (Str), 100 µg L⁻¹; and gentamicin (Gm), 10 µg L⁻¹. Bacterial growth was estimated by measuring the optical density at 600 nm (OD₆₀₀) in a Genesy 20 spectrophotometer (Thermo Fisher Scientific). Unless otherwise indicated, shaken-flask cultures were set in 250-ml Erlenmeyer flasks containing 50 ml of M9 minimal medium supplemented with 20 mM glucose as the sole carbon source. Samples were taken periodically and explained in the text.

2.2. DNA manipulation and sequencing

DNA manipulations and other molecular biology techniques were essentially performed as described by Green and Sambrook (2012). Oligonucleotides, double-stranded DNA fragments (gBlocks™) and codon-optimized synthetic genes were purchased from Integrated DNA Technologies (IDT; Leuven, Belgium) and their sequences are present in the Supplementary Data (Tables S1 and S2). DNA amplification was performed on a C1000 Touch™ Thermal Cycler (Bio-Rad Corp., Hercules, CA, USA) using Phusion Hot Start DNA polymerase or Phusion Hot Start II DNA polymerase purchased from Thermo Fisher Scientific. DNA fragments were purified with NucleoSpin™ gel and PCR clean-up kits (Macherey-Nagel, Düren, Germany). Restriction enzymes and T4 DNA Ligase were obtained from Thermo Fisher Scientific, and they were used according to the supplier’s specifications. USER assembly was performed essentially as described by Nouri-Eldin et al. (2010) with the USER enzyme from New England BioLabs (NEB; Ipswich, MA, USA). Likewise, Gibson assembly was performed as described by Gibson et al. (2009) with the Gibson Assembly Master Mix supplied by NEB. Plasmid DNA was prepared and purified with a NucleoSpin™ plasmid EasyPure kit (Macherey-Nagel). Chemically-competent *E. coli* cells were prepared using the Mix & Go E. coli transformation kit from Zymo Research (Irvine, CA, USA). DNA amplification from single bacterial colonies (i.e. colony PCR) was performed with OneTaq 2 × Master Mix (NEB). Electrocompetent *P. putida* cells were prepared by washing twice the biomass from an overnight culture of *P. putida* (in LB medium) with a filter-sterilized 300 mM sucrose solution (Choi et al., 2006). All cloned inserts and DNA fragments were confirmed by Sanger DNA sequencing (Eurofins Genomics, Ebersberg, Germany). Deletion and insertional *P. putida* mutants (Table 1) were obtained by antibiotic-free allelic exchange assisted by curable plasmids as previously described (Volke et al., 2020b; Wirth et al., 2020). Other molecular biology techniques were followed as described elsewhere (Kozawa et al., 2021; Niel et al., 2008, 2009; Ruiz et al., 2006). An extended description of plasmid construction procedures and other DNA manipulation techniques can be found in the Supplementary Data.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference</th>
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<tr>
<td><strong>Table 1</strong> Strains and plasmids used in this work.</td>
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<th>Strain or plasmid</th>
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<tbody>
<tr>
<td>DH5α</td>
<td>Cloning host; F⁻, endA1 glnX44(AS) thyI-recA1 reaD1 spoT1 gyr96(Str⁻) rbcL-deor nptII FΦΔOOC2λW2153 Δ(ergF- lenc)U169 hsdR17 R (Km,Rc)</td>
<td>Messeholm and Yuan (1968)</td>
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<tr>
<td>pSEVA221</td>
<td>Cloning vector; standard multiple cloning site (MCS), oriR7(K2); Km⁺</td>
<td>Silva-Rocha et al. (2013)</td>
</tr>
<tr>
<td>pSEVA441</td>
<td>Cloning vector; standard MCS, oriV (pR01600; ColEl); Str⁺</td>
<td>Silva-Rocha et al. (2013)</td>
</tr>
<tr>
<td>pSEVA631</td>
<td>Cloning vector; standard MCS, oriV (pBRB1); Gm⁺</td>
<td>Silva-Rocha et al. (2013)</td>
</tr>
<tr>
<td>pSEVA4413</td>
<td>Expression vector; PrΔWT/MCS (for constitutive gene expression), oriV (pR01600/ColEl); Str⁺</td>
<td>Silva-Rocha et al. (2013)</td>
</tr>
<tr>
<td>pBG25</td>
<td>Integration plasmid; Trp⁻ transposon arms flanking a PrΔ(KCG2)→mefGFP cassette, oriRf(k8); Km⁺ Gm⁻</td>
<td>Zobel et al. (2015)</td>
</tr>
<tr>
<td>pUC19-ExaQ6</td>
<td>Derivative of cloning vector pUC (Pridmore, 1987) harboring the codon-optimized synthetic genes esai and esaiT⁻</td>
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<td>pSensor1</td>
<td>Derivative of vector pSEVA221 carrying a PrΔWT→esaiT⁻ cassette; Km⁺</td>
<td>This work</td>
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<tr>
<td>pSensor3</td>
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<tr>
<td>pResponse1</td>
<td>Derivative of vector pSEVA631 carrying PrΔWT→gfp; Gm⁻</td>
<td>This work</td>
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<tr>
<td>pResponse2</td>
<td>Derivative of vector pSEVA631 carrying PrΔWT→gfp; Gm⁺</td>
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<tr>
<td>pResponse3</td>
<td>Derivative of vector pSEVA631 carrying PrΔWT→gfp(λVA); Gm⁻</td>
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<tr>
<td>pResponse4</td>
<td>Derivative of vector pSEVA631 carrying PrΔWT→gfp(λVA); Gm⁺</td>
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<td>pRespT4</td>
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<td>pRespT4D1</td>
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<td>pRespT6</td>
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<td>pRespT6D1</td>
<td>Derivative of vector pSEVA631 for dynamic regulation of the TrpD node, carrying PrΔWT→trpD(LVA) rps; Gm⁻</td>
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(continued on next page)
2.3. Bacterial growth profiling in 96-well microtiter plates and fluorescence assays

For standard growth experiments during calibration and characterization of synthetic QS circuits, bacterial cells were grown in 96-well microtiter plates (flat-bottom, Greiner Bio-One, Frickenhausen, Germany) using M9 minimal medium containing 20 mM glucose as the sole carbon source. L-Trp was supplemented at 0.1 mM when cultivating auxotrophic strains (i.e. carrying a ΔpDC deletion) as indicated in the text. Growth kinetics were followed by OD_{595} measurements with light path correction every 5 min in a Synergy™ MX microtiter plate reader (BioTek Instruments Inc., Winooski, VT, USA). For fluorescence experiments, cells were grown in 96-well black/clear bottom microtiter plates. Both bacterial growth (estimated as the OD_{600}) and the GFP fluorescence (F_{ex}=485 nm, F_{em}=516 nm) were continuously measured every 5 min. Fluorescence readings (in arbitrary units, A.U.) were normalized to the bacterial growth (A.U. OD_{600}).

2.4. Quantitative PCR (qPCR) assays and proteomic analysis

Bacterial strains were grown for until mid-exponential phase (i.e. when the cultures reached an OD_{600} of ca. 0.4–0.6) in shaken-flasks as explained above. Samples were collected at this point, and the bacterial biomass was washed and resuspended in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na_{2}HPO_{4} and 2 mM KH_{2}PO_{4}, pH = 7.4) to contain 1 × 10^{9} cells (either E. coli or P. putida) per sample and the cell suspension was stored at –20 °C afterwards. qPCR amplifications were performed in 96-well optical reaction plates using a QuantStudio™ 5 Dx real-time PCR system (Thermo Fisher Scientific), with the following conditions: 50 °C for 2 min, 95 °C for 10 min; and 40 cycles of 95 °C for 15 s, 60 °C for 1 min and 72 °C for 30 s, using the oligonucleotides indicated in Table S1 in the Supplementary Data. The results of the qPCR assays were analyzed with the built-in QuantStudio™ design and analysis software. Each individual experiment included a non-template control (i.e. a mixture of primers), and was carried out in three technical replicates for sample. Data were evaluated using the 2^{-ΔΔC_{T}} method (Livak and Schmittgen, 2001), and a comparative Ct method was applied to calculate gene copy number by analyzing the experimental ΔΔC_{T} values (Rao et al., 2013). The reaction specificity and the amplicon identities were verified by means of melting curve analyses. Gene expression levels were normalized to that of the rpoB gene, which encodes the β-subunit of RNA polymerase and is highly conserved in both E. coli and P. putida (Folloni et al., 2013). The fold-change differences in transcript levels and the associated standard errors were obtained as described by Livak and Schmittgen (2001). Plasmid copy numbers (PCN) were derived from the expression levels of genes encoding antibiotic resistance determinants (Jahn et al., 2016; Lee et al., 2006). The empty pSEVA221 and pSEVA631 vectors were used as a reference; these plasmids were purified using the NucleoSpin™ plasmid EasyPure kit (Macherey-Nagel) according to manufacturer’s instructions and diluted to 1–5 ng µL^{-1} to build calibration curves (Lee et al., 2006). PCN was calculated as the ratio between the concentration of the antibiotic-resistance marker in the plasmid (pDNA), i.e. aac for Gm or neo for Km resistance) and the concentration of the genome-encoded, house-keeping marker (gDNA), i.e. rpoB in each sample, replicate (i.e. pDNA/gDNA) according to the methodology described by Jahn et al. (2016).

Proteomic analysis was used to quantify relative levels of selected proteins in samples from 50-mL shaken-flask cultures of some engineered strains as indicated in the text. Samples were taken at the same data points as per qPCR analysis (5 h and 10 h for early and late exponential growth phase, respectively), and 1-mL aliquots from each culture were pelleted at 10,000 × g for 10 min and flash-frozen with liquid N₂. Pellets were stored at –80 °C until they were processed according to the procedure of Bongers et al. (2020). Cells were lysed in 6 M guanidinium-HCl, 5 mM tris(2-carboxyethyl)phosphine, 10 mM chloroacetamide and 100 mM Tris-HCl (pH = 8.5) while being disrupted in a Mixer Mill (MM 400 Retsch, Haan, Germany) at 25 Hz for 5 min at room temperature, followed by 10 min in a thermomixer at 95 °C at 2000 rpm. A clarified supernatant was obtained by centrifugation at 15, 000 × g for 10 min. The protein concentration in the cell-free lysate was estimated by means of the bicinchoninic acid method, and 100 µg of proteins were tryptically digested for 8 h. After this digestion step, 10 µL of 10% (w/v) trifluoroacetic acid was added and samples were fractionated using a StageTip C18 (Empore, 3M, USA). Next, 1 µg of the purified peptides was injected into an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific). Data acquisition, analysis and statistical treatment were performed as indicated by Kozaeva et al. (2021).

2.5. Cultivation conditions for anthranilate production and general analytical procedures

Batchwise anthranilate production experiments were performed in 250-mL Erlenmeyer flasks filled with 50 mL of M9 minimal medium added with 20 mM glucose (and, in the case of auxotrophic strains, further supplemented with L-Trp at 0.1 mM). These cultures were incubated at 30 °C with agitation at 200 rpm. Two additional glucose pulses (reaching 20 mM) were added after sampling at 20 h and 48 h, unless stated differently. The biomass concentration was estimated by measuring the OD_{600} in a Jenway UV/visible scanning spectrophotometer (model 6705; Cole-Parmer, Vernon Hills, IL, USA). In this device, the OD_{600} correlates to the cell dry weight (CDW) according to 1 OD_{600} = 0.529 g CDW L^{-1}. Samples taken during the cultivation were centrifuged at 13,000 × g for 5 min and the supernatants were stored at –20 °C for analysis by high-performance liquid chromatography (HPLC). To monitor the consumption of glucose and the formation of its oxidized derivatives (i.e. gluconate and 2-ketogluconate), a Dionex Ultimate 3000 system (Thermo Fisher Scientific) with a HPX87H ion exclusion column (125–0140, AminexTM, Bio-Rad Corp.) was used with 5 mM H_{2}SO_{4} as eluent at a flow of 0.6 mL h^{-1} for 30 min at 30 °C (Nikel et al., 2021). Detection was done with both a UV detector at 210 nm and a refractive index detector. Anthranilate production and L-Trp
consumption or secretion were analyzed with a C18 reverse phase column (Agilent ZORBAX Eclipse Plus C18, 959961-902K; Agilent Technologies, Santa Clara, CA, USA) at a flow of 1.0 mL h\(^{-1}\) and 30 °C (gradient of H\(\text{2O} + 0.05\%\) (v/v) acetic acid (pump A) and of acetonitrile (pump B): 0–1.5 min gradient 5–12% B, 1.5–4.5 min gradient 12–30% B, 4.5–8 min gradient 30–70% B, 8–9.5 min gradient 5–70% B and 9.5–11 min 5% B). Product detection was done with an UV detector at 260 nm.

### 2.6. Endo-metabolomics analysis of anthranilate producing strains

Samples for metabolomics were taken before and after adding glucose pulses as indicated in the text, and immediately filtered through a MF-Millipore™ membrane filter (0.45-μm pore size; Sigma-Aldrich Co., St. Louis, MO, USA). The biomass-containing filter was rapidly placed on a mini-Petri dish containing 1 mL of a precooled (−20 °C) quenching solution (composed by 100 mM formic acid in a 40:40:20 (v/v/v) acetonitrile-methanol-water mixture, and the proper internal standards for metabolite quantification) (Kozaeva et al., 2021; McCloskey et al., 2016). The quenching solution was transferred into a 2-mL Eppendorf tube. Solvents were evaporated in a SpeedVac centrifuge concentrator (Thermo Fisher Scientific) for 2 h at −50 °C and bacterial lysis (Balagaddé et al., 2008; Ge et al., 2020; Saeidi et al., 2011; Tsao et al., 2010). The Esa QS system from \(P. \text{stewartii}\) (Beck von Bodman and Farrand, 1995; von Bodman et al., 2003), for instance, has been used to mediate both transcriptional activation and repression of target genes (Dinh et al., 2020; Dinh and Prather, 2019; Gu et al., 2020; Gupta et al., 2017; Shen et al., 2019b; Shong and Collins, 2013; Wu et al., 2020b). The Esa QS system consists of three main components: (i) EsaI, the enzyme N-(3-oxohexanoyl)-l-homoserine lactone synthase that synthesizes the signal molecule (i.e. the AHL), (ii) EsaR, the transcriptional regulator that recognizes and binds AHL, thereby interacting with esa box sequences within (iii) several QS-responsive promoters (including \(P_{\text{esaT}}\) or \(P_{\text{esaR}}\) that control the expression of target gene(s) (Fig. 2A). In the absence of AHLs (or at very limited concentrations of the signal molecule, i.e. at low cell densities), the EsaR transcriptional regulator binds to the \(P_{\text{esaT}}\) or \(P_{\text{esaR}}\) promoters, either activating or repressing, respectively, the transcription of gene(s) placed under control of these promoters (Dinh and Prather, 2019; Shong and Collins, 2013). Accumulation of AHL above a threshold concentration (in the native context, when the bacterial cell density increases) mediates the release of the EsaR regulator from the QS-responsive promoters, leading to the subsequent deactivation of the \(P_{\text{esaT}}\) or \(P_{\text{esaR}}\) promoter or activation of the \(P_{\text{esaT}}\) promoter (Fig. 2A). Thus, the individual Esa QS components can be adopted to construct synthetic genetic circuits for controlling either the transcriptional repression or overexpression of target genes in response to changes in the cell culture density.

In order to implement Esa QS-based genetic circuits in Gram-negative bacteria, we first constructed two families of broad-host-range plasmids by rigorously adopting the rules set by SEVA, the Standard European Vector Architecture (Silva-Rocha et al., 2013). These plasmids for synthetic QS regulation were termed pSensor and pResponse (Fig. 2B and Table 1). While pSensor plasmids are expected to detect and transduce changes in the bacterial cell density, pResponse plasmids should respond to these environmental cues by altering the expression of the target gene(s) cloned under the transcriptional control of the \(P_{\text{esaT}}\) or \(P_{\text{esaR}}\) promoters. The pSensor plasmids pSensor1-3 were constructed by USER assembly as described in the Supplementary Data. These standard vectors contain a synthetic operon formed by codon-optimized versions of \(esaI\) and \(esaR\) under transcriptional control of constitutive promoters displaying different strengths (\(P_{\text{lac}}\), \(P_{\text{Em}}\) and \(P_{\text{lac}}\)) (Fig. 2B and Table S2 in the Supplementary Data). The mutant \(esaR^{\text{L}}\) variant is frequently used as a transcriptional regulator in QS circuits, as it responds to higher threshold AHL concentrations than the wild-type EsaR protein (Shong et al., 2013)—thus increasing the dynamic range of the system. The pResponse plasmids pResponse2 and 4 carry a fluorescent reporter gene (i.e. \(gfp\) or \(gfp\) (LVA)) under the control of the \(P_{\text{esaT}}\) or \(P_{\text{esaR}}\) promoters, respectively (Fig. 2B). The genetic \(gfp\) (LVA) variant encodes a fluorescent GFPmut3 protein with reduced half-life in \(E. \text{coli}\) and \(P. \text{putida}\) compared to that of the wild-type GFPmut3 protein (Andersen et al., 1998). Plasmids pResponse1 and 3 were adopted in control experiments, as they harbor the same fluorescent sensor but pResponse2 were used in control experiments, as they harbor the same fluorescent reporter gene indicated above under transcriptional control of the \(P_{\text{lac}}\) promoter, which is constitutively active in \(P. \text{putida}\) (Fig. 2B). Note that both the pSensor and pResponse plasmid series carry compatible origins of vegetative replication [i.e. oriV(RK2) and oriV(pBBRII)] and antibiotic resistance markers [i.e. Km\(^{\text{R}}\) and Cm\(^{\text{R}}\)], which facilitates testing different plasmid configurations as needed. With this QS toolbox at hand, we set to test the output of the Esa QS system in two Gram-negative bacterial hosts as explained in the next section.

### 3. Results and discussion

#### 3.1. Design of a quorum-sensing circuit for dynamic regulation of gene expression in Gram-negative bacteria

Natural QS systems control various cell density-dependent processes in bacteria. Elements of QS systems have been repurposed to dynamically regulate the production of recombinant proteins, cell population equilibrium and bacterial lysis (Balagaddé et al., 2008; Ge et al., 2020;
transcriptional regulation exerted by the Esa QS system in its native context, these elements should mediate an increase in the fluorescence output as the cell density in the culture rises (i.e. repression → activation). Strains R0 (either E. coli or P. putida) carry the empty pSEVA221 vector and plasmid pResponse2, and were used in control experiments as they lack the sensing components of the Esa QS system. Analogously, the S group of engineered strains harbor both plasmid pResponse4 (carrying P\textsubscript{esas}–gfp(LVA)) and the pSensor plasmids 1 to 3. In this case, the Esa QS device is expected to mediate an activation → repression transition as the cell density increases. Note that, in this case, we resorted to the unstable GFP(LVA) variant derived from gfp\textsubscript{mut3} (Andersen et al., 1998). Wild-type GFP is a very stable protein in its mature, fluorescent form, and its mutant GFP\textsubscript{mut3} derivative has been reported to be approximately 20-fold more fluorescent than wild-type GFP when excited at 488 nm (Tombolini et al., 1997), which allows for efficient spectral separation from other fluorophores. However, we reasoned that the extraordinary stability of GFP could hinder our ability to monitor the dynamic changes brought about by the Esa QS system. Hence, we adopted the short-lived, unstable GFP(LVA) variant that carries a short C-terminal amino acid extension (RPAANDENYALVA) in the experiments involving the P\textsubscript{esas}–gfp(LVA) promoter. The RPAANDENYALVA peptide tag renders otherwise stable proteins susceptible to degradation by intracellular, tail-specific proteases, which results in an in vivo half-life of the mature GFP(LVA) of around 40 min (Andersen et al., 1998). The addition of synthetic Srα degradational tags has been previously adopted in P. putida as a metabolic engineering strategy to modulate protein levels by harnessing the native proteolytic machinery (Volke et al., 2020c). Again, a set of E. coli and P. putida strains was constructed to be used in control experiments. In this case, the S0 strains carry both the empty pSEVA221 vector and plasmid pResponse4, i.e. lacking the sensing components of the Esa QS system. Other combinations of empty vectors and pSensor/pResponse plasmids are listed in Fig. 3A, and E. coli and P. putida strains carrying these plasmids were likewise used in control experiments.

Next, we grew the recombinant bacterial cells in 96-well microtiter plates, and both the OD\textsubscript{600} values and the GFP fluorescence were continuously measured every 5 min during 20 h, which allowed to cover the whole growth curve of both E. coli and P. putida till the stationary phase was reached. To avoid any interference in the fluorescence measurements due to the use of complex culture media, all recombinant strains were grown in M9 minimal medium with 20 mM glucose as the only carbon source. To assess the overall behavior of the synthetic Esa QS system, we evaluated the maximum value of normalized fluorescence in each culture (i.e. GFP fluorescence OD\textsubscript{600}) during the entire growth curve (Fig. 3B). Likewise, the profiles of GFP fluorescence were represented over time for both E. coli and P. putida recombinants to evaluate dynamic changes in the activation or repression of transcriptional activity by the P\textsubscript{esas} and P\textsubscript{esas} promoters (Fig. 3C). By using this information, we also determined the ‘switching’ OD\textsubscript{600} value (Fig. 3D). This parameter was defined either as (i) the cell density (assessed by OD\textsubscript{600} readings) at which the GFP fluorescence was below a threshold concentration (lower panel) triggers the release of EsaR from the promoters and the subsequent transcriptional activation or repression from the P\textsubscript{esas} and P\textsubscript{esas} promoters, respectively. The use of the mutant EsaR\textsuperscript{I70V} variant enables a wider dynamic range in the synthetic QS system. RNAP, RNA polymerase; GOI, gene of interest. (B) Structure of a broad-host-range family of pSensor and pResponse plasmids encoding the Esa QS elements. The pSensor plasmids contain a synthetic operon formed by codon-optimized esas and esas\textsuperscript{I70V} under control of constitutive promoters with different strengths (P\textsubscript{lac}, P\textsubscript{DEP} and P\textsubscript{lacJ}). The pResponse plasmids harbor fluorescent reporter genes [either gfp or gfp(LVA)] under transcriptional control of either the P\textsubscript{esas} and P\textsubscript{esas} promoters or the constitutively-active P\textsubscript{lac} promoter. Both pSensor and pResponse vectors are based on the SEVA standard (Silva-Rocha et al., 2013). Elements in this outline are not drawn to scale; RBS, ribosome binding site (underlined); Km\textsuperscript{R}, kanamycin resistance; Km\textsuperscript{G}, gentamicin resistance.

3.2.1. The P\textsubscript{esas}–gfp-based QS system exhibits a similar dynamic behavior in E. coli and P. putida

Expectedly, the strains used as a negative control (i.e. E. coli or P. putida transformed with the empty pSEVA221 and pSEVA631 vectors, indicated as E in Fig. 3A–C) did not show any significant fluorescence output over the 20-h long experiments. R0 strains, carrying vector pSEVA221 and plasmid pResponse2 (P\textsubscript{esas}–gfp), had a low level of fluorescence, with values comparable for both E. coli and P. putida. The basal level of GFP fluorescence in these experiments, where no sensing Esa component is present, was significantly lower than that in bacteria transformed with vector pSEVA221 and a P\textsubscript{lac}–gfp construct (i.e. C1 strains, used as a positive control). Such a difference was even more
reflect the stronger nature of the P-gfp production. In synthetic Esa QS system brought about changes in the dynamics of GFP fluorescence of strain R0 (Fig. 3 B). These differences probably from 2-fold (strain R2) and 3-fold (strains R1 and R3) as compared to the output was observed in cells carrying plasmid pSensor1-3, which ranged experiment (Fig. 3 B). The addition of the sensing counterpart to the circuits. In all cases, pSensor and pResponse plasmids is given in Table 1. (B–C) Characterization of the P_esa- and P_em7-based QS circuits for dynamic regulation of fluorescent reporter genes—i.e. overexpression of gfp and transcriptional knock-down of gfp(LVA), respectively. Bacterial growth and GFP fluorescence were assessed in experiments performed in 96-well microwell plates in M9 minimal medium containing 20 mM glucose as the carbon source. The maximum value of GFP fluorescence normalized to the cell density reached by the different strains under study is represented in the bar graphs (B). Each bar represents the mean value ± standard deviation from at least three biological replicates. A.U., arbitrary units. (D) Switching optical density at 600 nm (OD_600) for different bacterial strains harboring synthetic Esa QS circuits. For strains R0-R3, this parameter was estimated as the cell density at which the normalized GFP fluorescence increased stably by >5% h^{-1}; whereas for strains S0-S3, the switching OD_600 was calculated as the cell density corresponding to the time when the maximum normalized GFP fluorescence was reached. Each bar represents the mean value ± standard deviation from at least three biological replicates. (E) Characterization of the P_em7-based QS circuit in P. putida ΔppoR. This mutant strain was constructed by eliminating PP_4647 in P. putida KT2440 (indicated with a Δ symbol in the scheme), and transformed with the plasmids listed in panel (A) to obtain the corresponding C2, S0 and S1 derivatives. The experiments were carried out as detailed in panels (B–C).

Fig. 3. Characterization of the synthetic Esa QS system in E. coli and P. putida. (A) Description of bacterial strains constructed to characterize the synthetic Esa QS circuits. In all cases, E. coli DH5α and P. putida KT2440 were used as the bacterial hosts. The expected switch for each type of system is indicated in the diagram, and a full description of pSensor and pResponse plasmids is given in Table 1. (B–C) Characterization of the P_esa- and P_em7-based QS circuits for dynamic regulation of fluorescent reporter genes—i.e. overexpression of gfp and transcriptional knock-down of gfp(LVA), respectively. Bacterial growth and GFP fluorescence were assessed in experiments performed in 96-well microwell plates in M9 minimal medium containing 20 mM glucose as the carbon source. The maximum value of GFP fluorescence normalized to the cell density reached by the different strains under study is represented in the bar graphs (B). Each bar represents the mean value ± standard deviation from at least three biological replicates. A.U., arbitrary units. (D) Switching optical density at 600 nm (OD_600) for different bacterial strains harboring synthetic Esa QS circuits. For strains R0-R3, this parameter was estimated as the cell density at which the normalized GFP fluorescence increased stably by >5% h^{-1}; whereas for strains S0-S3, the switching OD_600 was calculated as the cell density corresponding to the time when the maximum normalized GFP fluorescence was reached. Each bar represents the mean value ± standard deviation from at least three biological replicates. (E) Characterization of the P_em7-based QS circuit in P. putida ΔppoR. This mutant strain was constructed by eliminating PP_4647 in P. putida KT2440 (indicated with a Δ symbol in the scheme), and transformed with the plasmids listed in panel (A) to obtain the corresponding C2, S0 and S1 derivatives. The experiments were carried out as detailed in panels (B–C).

3.2.2. The P_esa promoter exerts a differential transcriptional regulation in E. coli and P. putida

The Esa QS circuit based on the P_esa promoter was tested by constructing a series of E. coli and P. putida strains carrying plasmids pResponse3 [P_esa→ gfp(LVA)], used as a positive control or pResponse4 [P_em7→ gfp(LVA)] in combination with plasmids pSensor1-3 (Fig. 2B). As expected, the control experiments showed that, in general, there was a very low level of GFP(LVA) accumulated in the recombinant strains (Fig. 3B). All C2 strains, carrying both the empty pSEVA221 vector and plasmid pResponse3, exhibited fluorescence levels similar to that of E. coli strains, used as negative controls. These results confirm the unstable nature of the GFP(LVA) protein in both bacterial hosts, as no net accumulation of the fluorophore was detected during the experiment. Furthermore, the fluorescence levels detected in cultures of E. coli 50
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values 22-, 11- and 4-fold higher than that of E. coli S0 in the strains transformed with plasmid pSensor1, 2 and 3, respectively. Unexpectedly, we observed that P. putida strain S0, lacking the sensing components of the Esa QS circuit, exhibited a similar behavior compared to strains S1–S3 in terms of peak GFP output (Fig. 3B). In the control experiment with strain S0, the fluorescence levels were quite high (and almost 5-fold higher than in any of the E. coli strains of the S series)—and the presence of the esal-earl-0 component did not increase the output of the QS system in any of the configurations (i.e. regulation exerted by the P14f or F14f promoters) as it did in E. coli strains. The dynamic response of the synthetic QS system was also markedly different in E. coli and P. putida (Fig. 3C). For all the P. putida strains tested, the normalized fluorescence values peaked at the beginning of the exponential phase and subsequently dropped to almost zero as the transcriptional knock-down of the reporter gene became predominant. This peculiar behavior, observed even in P. putida S0, was not detected in cultures of the analogous E. coli S0 strain (Fig. 3C) nor has it been previously described in the literature for similar systems (Dinh and Prather, 2019; Shong et al., 2013). Transcription from the Pausat promoter should occur in the presence of the EsaR regulator (Gupta et al., 2017) and only at low AHL concentrations (Fig. 2A)—which is not the case in strain S0, where the esal-earl-0 element is absent. When the switching OD600 values were calculated for all the strains under study (Fig. 3D), we found that the qualitative behavior of strains S1–S3 was similar for both bacterial hosts—yet the decrease in the fluorescence signal was triggered at earlier stages in P. putida (OD600 < 0.15 units) than in E. coli (OD600 between 0.2 and 1 units). Therefore, the Pausat promoter can mediate a cell-density-dependent activation repression transcriptional switch that, in P. putida, is independent of the EsaR regulator or the AHL signal.

In an attempt to disentangle the EsaR-independent behavior of the Pausat promoter in P. putida, we scanned the genome of strain KT2440 (Belda et al., 2016) to try and identify genes encoding potential QS-like regulators. The pattern of GFP fluorescence in the recombinant S0 strain (Fig. 3B–D) would indicate that there could be an endogenous QS system that interferes (i.e. cross-talk) with the synthetic Esa QS circuit. It has been previously described that P. putida KT2440 does not produce any of the common signal molecules typically involved in QS systems described in other bacteria (Fernández-Piñar et al., 2011; Ruiz et al., 2021; Subramoni and Venturi, 2009). The genome of this strain appears to display a single open reading frame (PP_4647) that encodes an unpaired transcriptional regulator belonging to the LuxR protein family—i.e. a QS LuxR homolog that occurs without the corresponding luxI AHL-synthese homolog (Fuqua et al., 1996; Xu, 2020). The 705-bp long PP_4647 open reading frame encodes a protein of 235 amino acids termed PpoR (standing for Pseudomonas putida orphan regulator, Fig. 3E). A BLAST search (Chen et al., 2015) revealed high similarity to orthologues in other Pseudomonas strains: PpoR exhibits >75% similarity to solo protein proteins in P. putida F1, P. putida GB-1, P. putida W619 and P. entomophila I48 (Subramoni and Venturi, 2009). We constructed a ΔpooR deletion mutant of strain KT2440 (Table 1), and this strain was transformed with different combinations of plasmids pSEVA221, pResponse1 and pSensor1 to create the corresponding C2, S0 and S1 derivatives of P. putida ΔpooR. When the growth and fluorescence kinetic experiments were repeated with these recombinant bacteria, we observed no differences as compared to the analogue strains in the wild-type KT2440 background (Fig. 3E). These experiments indicate that the PpoR regulator does not interfere with the synthetic Esa QS circuits. Our results are in line with the observations reported by Steindler et al. (2008), linking the lack of conservation of AHL-dependent QS systems in soil-colonizing Pseudomonas species to the very diverse regulatory roles that such regulatory networks play in rhizobacteria. Although we could not identify the native factor(s) that modulate the behavior of the promoter in P. putida KT2440, we further explored the use of this transcriptional device to modulate protein levels as explained in the next section.

3.2.3. Exploring the dependence of dynamic regulation on gene dosage at the protein level

A set of experiments was designed to explore the potential relationship between gene dosage of the synthetic QS system and the content of target proteins under control of the Esa QS circuit (Fig. 4). To this end, the plasmid copy number of vectors carrying the functional QS elements was determined in both E. coli and P. putida by qPCR. We observed that the plasmid copy number of SEVA vectors used in this study was slightly higher in P. putida as compared to E. coli (Fig. 4A), although in all cases the range of plasmid copies per chromosome was 5–15, in accordance to previously reported observations (Jahn et al., 2016). Expectedly, the plasmid copy number of vectors carrying orV (pBBR1) was higher than those bearing an orV(RK2)—but the addition of the functional Esa QS elements to the backboned did not modify these figures. Importantly, the plasmid copy number of pSensor1 and pResponse4 (the combination of plasmids used to construct E. coli S1 and P. putida S1, see Fig. 3A) was similar in each individual host. Next, we assessed the intracellular content of GFP in P. putida strains carrying these plasmids by quantitative target proteomics (Fig. 4B). In a control experiment, where P. putida was transformed with plasmids pSEVA221 and pResponse1 (i.e. strain C1), the actual GFP levels did not change along the growth curve, correlating with the fluorescence measurements shown in Fig. 3. P. putida S0 and S1, in contrast, showed a significant reduction in the GFP content as the cells transitioned from the early (5 h) to the late (10 h) exponential phase. At this point, GFP could no longer be detected by quantitative proteomics—validating the results obtained at the level of fluorescence measurements (Fig. 3). Taken together, these observations illustrate that the system can be used to impose a

Fig. 4. Exploring the interplay between dynamic regulation at the protein level and gene dosage of Esa QS system elements. (A) Plasmid copy number of empty vectors pSEVA221 and pSEVA631, and plasmids pSensor1 (PpooR→esal-earl-0) and pResponse4 (PpooR→gfp[LVA]) in E. coli DH5α and P. putida KT2440. This parameter was calculated as the relative copy number of plasmid DNA (pDNA) with respect to a genome DNA (gDNA) marker as evaluated by qPCR. Samples were taken during exponential growth (ca. 5 h) in shaken-flask cultures under the cultivation conditions indicated in the legend to Fig. 3. (B) Relative GFP abundance in engineered P. putida strains. Samples for quantitative proteomic analysis were taken from shaken-flask cultures grown as indicated above, and the intracellular content of GFP(LVA) and RpoN was determined during early (5 h) and late (10 h) exponential growth. Abundance values, expressed as fold-change, were averaged among three biological replicates and normalized to the initial content of RpoN (i.e. RpoN0), adopted as a housekeeping protein in these experiments. The strain nomenclature is indicated in Fig. 3A, and the grayed area indicates the minimum and maximum levels of RpoN as a reference across all experimental conditions. In all cases, bars (or data points) represent mean values of the corresponding parameter ± standard deviations of triplicate measurements from at least two independent experiments. N.D., not detected.
3.3. Engineering a growth-to-production switch in P. putida for glucose-dependent anthranilate biosynthesis

3.3.1. A simple genetic circuit based on the P_{esa} promoter restores L-Trp prototrophy in a P. putida ΔtrpDC strain

The results reported so far indicated that the P_{esa} promoter is sufficient to effectively knock-down gene expression in batch cultures of P. putida. We reasoned that this feature could be harnessed to engineer metabolic switches that do not require extensive genetic modifications in the host, since no sensing elements of the Esa QS system would be needed. On this background, we focused on using the P_{esa} promoter for the construction of conditionally-auxotrophic strains of P. putida by rewiring the TrpD node in the lower shikimate pathway (Fig. 1). The sharp increase of the GFP signal, followed by an almost complete knock-down of the system output, observed in P. putida strains carrying P_{esa}→gfp (LVA) (Figs. 3 and 4) indicated that this dynamic regulatory layer could be applied to the conversion of anthranilate catalyzed by anthranilate phosphoribosyltransferase. If successful, this manipulation should lead to a P. putida strain conditionally-auxotrophic for L-Trp that does not require supplementation of this amino acid to promote growth.

To investigate whether the P_{esa} promoter can be used to dynamically regulate the L-Trp requirement of P. putida, we first studied if a genetic construct based on this promoter can complement the L-Trp auxotrophy of a ΔtrpDC deletion mutant (Table 1). This mutant strain, lacking both the transcriptionally-coupled trpD (PP_0421) and trpC (PP_0422) genes (Fig. 5A), responsible for L-Trp biosynthesis, has been used for anthranilate production upon further engineering efforts (Kuepper et al., 2015). We constructed two series of pResponse plasmids, termed pRespT4 and pRespT6 (Table 1)—an acronym which stands for pResponse plasmid for regulation of L-Trp levels; the 4 and 6 codes indicate Str^R and Gm^R determinants, respectively, according to the SEVA nomenclature (Silva-Rocha et al., 2013). These standardized plasmids are derivatives of vectors pSEVA441 and pSEVA631 [i.e. high- and medium-copy-number, respectively (Jahn et al., 2016)], and they contain the native trp operon formed by the trpD and trpC genes of P. putida KT2440 under transcriptional control of the P_{esa} promoter (Fig. 5B). In parallel, plasmids pRespT4D1-2 and pRespT6D1-2 were constructed by introducing different SsrA-peptide motifs in the 3′-end of trpD, where D1 and D2 indicate the LVA and ASV degradation tags, respectively (Fig. 5B). As observed for GFP (Fig. 3), engineering these C-terminal extensions in TrpD is expected to render the resulting protein variants susceptible to degradation by the native proteolytic machinery. As such, the addition of degradation tags ensures a short (and

![Diagram](A) Schematic representation of the pResponse plasmids designed to functionally complement the L-tryptophan (L-Trp) auxotrophy of P. putida KT2440 under transcriptional control of the P_{esa} promoter (Fig. 5B). In parallel, plasmids pRespT4D1-2 and pRespT6D1-2 were constructed by introducing different SsrA-peptide motifs in the 3′-end of trpD, where D1 and D2 indicate the LVA and ASV degradation tags, respectively (Fig. 5B). As observed for GFP (Fig. 3), engineering these C-terminal extensions in TrpD is expected to render the resulting protein variants susceptible to degradation by the native proteolytic machinery. As such, the addition of degradation tags ensures a short (and
adjustable) half-life of the cognate proteins and provides an additional control mechanism to modulate enzyme pools and pathway fluxes ( Brockman and Prather, 2015 ).

This set of pResponse plasmids was transformed by electroporation in the L-Trp auxotrophic strain P. putida ΔtrpDC. Next, the growth of the recombinant strains was investigated in M9 minimal medium containing 20 mM glucose in 96-well microtiter plate experiments ( Fig. 5C ). As expected, all strains, including the control, L-Trp auxotrophic strains P. putida ΔtrpDC/pSEVA441 and ΔtrpDC/pSEVA631 (i.e. transformed with the empty vectors), grew similarly to wild-type P. putida KT2440/ pSEVA441 and KT2440/ pSEVA631 when the culture medium was supplemented with 0.1 mM L-Trp ( Fig. 5C )—both in terms of specific growth rates and final biomass density over 16 h of cultivation. In contrast, different growth patterns were observed in the absence of L-Trp—supplemented with 0.1 mM L-Trp ( Fig. 5C ).

We thus tested the ASV tag ( RPAANDENYAASV ), reported to exert a milder effect on proteolysis rates than the LVA motif—the in vivo half-life of GFP(ASV) was calculated to be 110 min, whereas that of GFP (LVA) is just 40 min ( Andersen et al., 1998 ). Interestingly, the ASV-tagged TrpD variant borne by plasmid pRespT6D2 could completely restore the growth of the ΔtrpDC mutant in a L-Trp-free medium similar to those in cultures of P. putida KT2440 ( Fig. 5C ). This recovery effect was less evident in cultures of strain ΔtrpDC/pRespT4D2, at least within the 16 h-long incubation period.

Fig. 6. L-Trp–independent anthranilate production by conditionally-auxotrophic P. putida strains. (A) Establishing a growth-to-anthranilate production metabolic switch in P. putida at the level of TrpD (anthranilate phosphoribosyltransferase) by implementing the cell density-responsive P nas promoter. CCM, central carbon metabolism; other abbreviations in the diagram are as indicated in the legend to Fig. 1. (B) Genetic constructs used to engineer either plasmid- or genome-based dynamic regulation for the TrpD node. Note that, in the case of genome-based modifications, the transcription of the trpDC genes was insulated from any potential upstream regulatory signal by adding a synthetic T1 terminator element. DFRA stands for dynamic flux regulation for anthranilate production; WT, wild-type. (C–D) Growth profiles of wild-type strain KT2440, the L-Trp auxotrophic ΔtrpDC mutant and its derivatives transformed with either plasmid pRespT4 or pRespT6, and the genome-engineered P. putida DFRA1 and DFRA2 strains. In the control experiments indicated in panel C, both P. putida KT2240 and the ΔtrpDC mutants were transformed with the empty pSEVA441 vector. Growth conditions were as indicated in the legend to Fig. 4. (E–H) Growth profiles, anthranilate production and L-Trp secretion by conditionally-auxotrophic P. putida strains. Repeated-batch experiments were performed in M9 minimal medium with an initial glucose concentration of 20 mM. Bacterial growth was estimated as the optical density measured at 600 nm (OD 600 ). The vertical red lines indicate supplementation of glucose at a final concentration of 20 mM in each case. The concentration of anthranilate and L-Trp in culture supernatants was assessed by HPLC. Each data point represents the mean ± standard deviation from at least three biological replicates. (I) Intracellular metabolite analysis of wild-type and engineered P. putida strains grown under the culture conditions explained in panels (E–H). The content of phosphoenolpyruvate (PEP), L-tyrosine (L-Tyr) and L-Trp (normalized to the biomass) was assessed by LC-MS/MS for P. putida DFRA2 and P. putida KT2440, used as a control (Ctrl.). Intracellular metabolite ratios were calculated as the fraction of intracellular metabolite(s) after the first glucose pulse divided by the corresponding metabolite(s) content before substrate addition. Each bar represents the mean ± standard deviation from at least two biological replicates. As a reference, the dashed gray line indicates an intracellular metabolite ratio = 1, i.e. no changes in metabolite content after the glucose pulse.
These results validated the general approach of establishing a conditional L-Trp auxotrophy to control bacterial growth with the genetic circuits based on the P\text{esas} promoter. The next step was the implementation of these dynamic switches for anthranilate biosynthesis.

3.3.2. Construction of plasmid-free P. putida strains bearing a dynamic, self-regulated growth-to-anthranilate production switch

Prompted by the recovery of the L-Trp auxotrophy afforded by the P\text{esas}-dependent trpDC elements, we investigated their application for glucose-dependent production of anthranilate (Fig. 6A). Our reasoning was that rendering the transient expression of trpDC dependent on the cell density via the P\text{esas} promoter should result in a time-limited L-Trp prototrophy, switching to anthranilate production as TrpD wanes away. Moreover, we implemented two types of metabolic switches: a plasmid-based one, based on the pRespT4 plasmid series, and a plasmid-free version of the system, where all the functional elements are engineered into the P. putida chromosome (Fig. 6B). Based on the experiments implemented to characterize the system kinetics in recovering the aromatic amino acid requirement of a ΔtrpDC mutant (Fig. 5), we decided to adopt either the wild-type version of the trpDC operon and the module encoding the ASV-tagged TrpD variant (Fig. 6B). The LVA-tagged TrpD variants were not used in these experiments, since their short life proved insufficient to restore a L-Trp auxotrophy. The plasmid-borne synthetic modules (pRespT4 series) mediated a partial and gradual recovery of the L-Trp dependence of P. putida ΔtrpDC in 16-h growth experiments in microtiter plates (Fig. 5C), and we decided to further explore this growth pattern upon extended cultivation. Moreover, we engineered the chromosome of wild-type strain KT2440 by allelic replacement of the S'-region upstream of trpDC (PP\text{0421}-PP\text{0422}). This strategy, circumventing the use of replicative plasmids, encompassed the construction of pGNW2S trpD and pGNW2S trpD (ASV) C suicide plasmids (Table 1) to exchange the native trpDC genes in the chromosome of P. putida KT2440 by the synthetic cassettes P\text{esas}→trpDC trpC or P\text{esas}→trpDC (ASV) trpC, respectively. Further details on strain construction procedures are provided in the Supplementary Data. We adopted the genome engineering protocol of Wirth et al. (2020) to insert these modules in the bacterial genome, obtaining the P. putida strains DFRA1 [ΔtrpDC; P\text{esas}→trpDC (ASV) trpC] and DFRA2 [ΔtrpDC; P\text{esas}→trpDC trpC], where the acronym DFRA stands for dynamic flux regulation for anthranilate production (Fig. 6B). Since the trpDC genes lie downstream of and collinear with pabA (PP\text{0420}, encoding 4-aminoo-4-deoxychorismate synthase) in the chromosome of P. putida, we decided to insulate the transcription of the trp genes from any potential regulatory elements placed upstream pabA. Hence, a ρ-independent transcriptional terminator, the mmr T1 element from E. coli (Orozzi et al., 1991; Silva-Rocha et al., 2013), was included in the DFRA1 and DFRA2 strains (Fig. 6B). According to this design, the expression of the trpDC gene (and its ASV-tagged derivative) should only respond to the P\text{esas} promoter placed upstream the cognate coding regions.

3.3.3. Bacterial growth profiles and anthranilate production by engineered P. putida strains

The growth of plasmid-based and plasmid-free engineered P. putida strains was firstly tested in M9 minimal medium with 20 mM glucose as the only carbon source (i.e. in the absence of externally-added L-Trp) over 36 h in microtiter plate experiments (Fig. 6C and D). Expectedly, the P. putida ΔtrpDC mutant, included in these experiments as a negative control, could not grow under these culture conditions. In contrast, the wild-type strain, P. putida KT2440, had the fastest growth and achieved the highest final cell densities among all the strains tested—reaching the stationary phase by ca. 10 h of incubation. Extended cultivation of P. putida ΔtrpDC carrying either plasmid pRespT4 or pRespT4D2 revealed two levels of auxotrophy recovery that were not observed in previous, shorter experiments (Fig. 5). Plasmid-based expression of the synthetic P\text{esas}→trpD trpC cassette fully recovered the growth phenotype of the ΔtrpDC mutant—the final cell density of these cultures was similar to that of the wild-type strain, albeit delayed (the stationary phase was reached by 24 h). The proteolizable version of TrpD borne by plasmid pRespT4D2 could not revert the growth defect of the mutant strain, and the stationary-phase OD\text{600} values of these cultures were roughly halved as compared to the wild-type strain control (Fig. 6C). The genome-engineered DFRA1 and DFRA2 strains, where the Esa QS elements drive the expression of trpDC from the bacterial chromosome, had growth phenotypes qualitatively similar to the plasmid-based counterparts—in that the L-Trp auxotrophy of the ΔtrpDC could be reverted more efficiently by the non-tagged TrpD than TrpD(ASV) (Fig. 6D). The final OD\text{600} values in cultures of strains KT2440, DFRA1 and DFRA2 were around 0.9, 0.4 and 0.6, respectively. Taken together, such growth phenotypes reflect differences in gene dosage between plasmid-based and genome-engineered Esa QS elements—as none of the plasmid-free strains grew as much as the wild-type P. putida did under these conditions—and set the basis for anthranilate production experiments.

Next, anthranilate production was investigated in cultures of these bacterial strains. Repeated-batch production was tested in shaken-flask experiments with M9 minimal medium and an initial glucose concentration of 20 mM. Two glucose pulses were implemented at 20 and 48 h to reach, each time, a final concentration of 20 mM (i.e. total amount of fed glucose = 10.1 g L\text{−1}) to boost bacterial growth and anthranilate accumulation (Fig. 6E–H). In all cases, both the cell density and the concentration of L-Trp and anthranilate were assessed over 72 h. Interestingly, no net L-Trp accumulation was detected in the extracellular medium of any of these cultures, which may indicate that the amino acid was rapidly consumed to support biomass formation during the transient suppression of L-Trp auxotrophy mediated by the P\text{esas} circuit. A very similar growth profile was observed for strain ΔtrpDC carrying either pRespT4D2 (Fig. 6E) or pRespT4 (Fig. 6G), although there was a longer lag phase in cultures of the former strain. The pattern of anthranilate production by the two strains was, in contrast, very different. While the concentration of the aromatic increased rapidly in cultures of P. putida ΔtrpDC/pRespT4 over the first 20 h of cultivation, the strain carrying the plasmid-based proteolizable TrpD(ASV) variant showed a delay in anthranilate production. In this case, we could only detect significant amounts of the product upon feeding the first glucose pulse. The anthranilate concentration did not change between the first and second glucose pulse for plasmid-bearing P. putida strains, while the biomass increased by ca. 1.4-fold in both cases (Fig. 6E and G). Interestingly, anthranilate biosynthesis by the auxotrophic ΔtrpDC strain was only detected at low levels once the supplemented L-Trp was fully depleted (Fig. S1A in the Supplementary Data), while P. putida ΔtrpDC/pRespT4D2 did not accumulate any traces of anthranilate in the presence of L-Trp (Fig. S1B). Kuepper et al. (2015) also reported a similar connection between amino acid availability and anthranilate production by derivatives of P. putida KT2440. This pattern was observed even when the engineered strains overexpressed constructs encoding the feedback inhibition-insensitive AroG(D146N) and TrpE(S40F) enzymes—expected to boost product yields. Similarly, we could not detect any anthranilate in cultures of engineered ΔtrpDC mutants carrying plasmids that fully restored L-Trp prototrophy (Fig. 5C)—pointing that a delicate equilibrium between reversion of the amino acid auxotrophy and anthranilate biosynthesis should be met towards efficient aromatic production.

When the physiology and anthranilate production patterns of the genome-engineered strains was studied in repeated batch cultures (Fig. 6F and H), a very similar growth profile was observed for P. putida DFRA1 and DFRA2—with final OD\text{600} values within the range observed in cultures of the plasmid-bearing counterparts. Product titers were, however, significantly higher than in any other anthranilate production experiment thus far. After 72 h, the anthranilate concentration in cultures of strains DFRA1 and DFRA2 reached 3.7 ± 0.2 and 3.8 ± 0.3 mM, respectively (Fig. 6F and H). The aromatic concentration increased almost linearly over time and its production started earlier in cultures of P. putida DFRA2 (carrying the P\text{esas}→trpD trpC module) than for strain DFRA1. Moreover, the later strain, where the trpD(ASV) allele was
engineered as part of the metabolic switch, had a relatively high variability in product titers among individual experiments. This behavior could be related to the endogenous degradation mechanism of SsrA-tagged proteins. Indeed, Wong et al. (2007) described the presence of highly heterogeneous bacterial populations in cultures of *E. coli* engineered to overproduce SsrA-tagged GFP (tagged with either the LVA or ASV motif). The authors also mentioned discrepancies between single-cell and population-wide fluorescence measurements, which could mask this effect. Moreover, the turnover of endogenous SsrA-tagged proteins in *E. coli* appears to vary significantly according to the growth phase, as it is mediated by ATP-dependent proteases (Lies and Maurizi, 2008). These aspects could contribute to the variability observed in anthranilate production by strain DFRA1. As previously observed for plasmid-bearing engineered *P. putida*, strains DFRA1 and DFRA2 did not accumulate anthranilate when the culture medium was supplemented with 0.1 mM L-Trp (data not shown). In summary, *P. putida* DFRA2 was a robust and reliable bacterial host for anthranilate production from glucose in the absence of any plasmid-based expression of genes or L-Trp supplementation (Fig. 6H), thereby achieving the highest product titer reported thus far in the literature. The behavior of strain DFRA2 was further characterized by quantitative metabolomics and compared to the metabolome profile of wild-type *P. putida* KT2440 at the level of PEP, L-Tyr and L-Trp (Fig. 6I). While the content of PEP, a metabolite upstream the shikimate pathway, did not change significantly upon the glucose pulse, the intracellular concentration of the two aromatic amino acids was altered, following opposite trends. The branching of chorismate between L-Tyr and L-Trp biosynthesis (Fig. 1) could be detected upon activating the Esa QS-dependent switch as an increase in the concentration of the former and a significant decrease in the intracellular content of the latter. These results correlate well with the transition to high levels of anthranilate production (at the expense of bacterial growth) observed in these cultures, and validate the concept of establishing a metabolic switch at the TrpD node to stimulate biosynthesis of the target compound.

4. Conclusion

Microbial anthranilate production has the potential to replace current chemical synthesis processes that use non-renewable petroleum derivatives. Anthranilate is toxic for most microorganisms, and selecting a host that is resistant to aromatic compounds is key to satisfy challenging industrial demands in terms of both productivity and production costs. *P. putida* displays high tolerance to a variety of chemicals and has proven to be a promising platform strain for biosynthesis of multiple aromatic compounds (Kuepper et al., 2015, 2020; Nikel et al., 2016; Schwanemann et al., 2020). Strategies implemented for anthranilate bioproduction in bacterial cell factories required deleting or inactivating *trpD*, which naturally leads to L-Trp auxotrophy (Balderas-Hernández et al., 2009; Sun et al., 2013). Extensive genetic manipulations—together with amino acid supplementation—were necessary to optimize aromatic production. Supplementing the fermentation medium with chemical inducers and aromatic amino acids increases production costs and makes the implementation of these bioprocesses on an industrial scale notably difficult. Genetic circuits built with QS elements can help circumventing these issues, as these devices could rebalance metabolism during fermentation in a cell density-dependent manner (Cui et al., 2019; Long et al., 2020; Yang et al., 2021). Our work represents a first-case example of implementing a synthetic QS circuit in *P. putida* for metabolic engineering purposes. We selected the Esa QS system from *P. stewartii*, reported to allow for both gene overexpression and knock-down when using the P_{esa} and P_{esa} promoters, respectively (Dinh et al., 2020; Dinh and Prather, 2019; Gu et al., 2020; Shong et al., 2013). Upon testing the pSensor and pResponse plasmids in both *E. coli* and *P. putida* (Fig. 3), we concluded that the P_{esa} promoter enables transcriptional activation of target genes in a cell density-dependent fashion in both hosts, expanding the repertoire of portable genetic parts for Gram-negative bacteria. The P_{esa} promoter, in contrast, exhibited an EsaR-autonomous regulation pattern in *P. putida*—which motivated its implementation to self-regulate the TrpD node.

Dynamic flux regulation, modulating the abundance of native enzymes, was demonstrated by high-titer production of anthranilate in a series of plasmid-based and genome-engineered *P. putida* strains. Several conditionally-auxotrophic strains were constructed by placing trpDC variants (encoding the enzymes responsible for anthranilate degradation) under transcriptional control of the P_{esa} promoter (Figs. 4 and 5). The key fermentation parameters calculated for cultures of these strains are listed in Table 2, together with the corresponding values for anthranilate-producing *P. putida* strains reported in the literature (Kuepper et al., 2015). We observed an inverse correlation between biomass formation and anthranilate production—since the metabolic switch engineered by using the P_{esa} promoter operates at the level of the proteinogenic L-Trp availability. This observation was further supported by assessing the intracellular metabolome, where the L-Trp content likewise decreased upon activating the switch. The plasmid-free DFRA1 and DFRA2 strains attained the highest anthranilate titer ever reported, >2-fold higher than that observed in cultures of both the performing *P. putida* strain constructed by Kuepper et al. (2015), which required L-Trp supplementation and plasmid-based expression of the *aroG*<sup>Δ14640n</sup> and *trpE*<sup>S40F</sup> alleles (Table 2). Accordingly, the yield of anthranilate on biomass (Y<sub>P/X</sub>) in our plasmid-free strains was 0.25–0.28 g<sub>anthranilate</sub>/g<sub>CDW</sub>, >3-fold higher than in any of the other strains. Likewise, the volumetric productivity of anthranilate was ca. 8 mg L<sup>−1</sup> h<sup>−1</sup>, >2-fold higher than that in other similar systems. To our knowledge, this work represents the first example of biological anthranilate production.

### Table 2

Comparison of key fermentation parameters of anthranilate-producing *P. putida* strains<sup>6,7</sup>.

<table>
<thead>
<tr>
<th><em>P. putida</em> strain</th>
<th>Final OD&lt;sub&gt;600&lt;/sub&gt;</th>
<th>Anthranilate fermentation parameter</th>
<th>Titer (g L&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>Y&lt;sub&gt;P/X&lt;/sub&gt; (g&lt;sub&gt;anthranilate&lt;/sub&gt;/g&lt;sub&gt;CDW&lt;/sub&gt;)</th>
<th>Y&lt;sub&gt;P/S&lt;/sub&gt; (g&lt;sub&gt;anthranilate&lt;/sub&gt;/g&lt;sub&gt;glucose&lt;/sub&gt;)</th>
</tr>
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<tbody>
<tr>
<td>ΔtrpDC&lt;sup&gt;+/p&lt;/sup&gt;SEVAV441&lt;sup&gt;1&lt;/sup&gt;</td>
<td>7.11 ± 1.37</td>
<td>0.09 ± 0.02</td>
<td>0.025 ± 0.008</td>
<td>0.009 ± 0.002</td>
<td></td>
</tr>
<tr>
<td>ΔtrpDC&lt;sup&gt;+/p&lt;/sup&gt;RespT4</td>
<td>4.20 ± 0.23</td>
<td>0.34 ± 0.02</td>
<td>0.15 ± 0.01</td>
<td>0.032 ± 0.002</td>
<td></td>
</tr>
<tr>
<td>ΔtrpDC&lt;sup&gt;+/p&lt;/sup&gt;RespT4D2</td>
<td>3.99 ± 0.04</td>
<td>0.19 ± 0.09</td>
<td>0.09 ± 0.04</td>
<td>0.018 ± 0.008</td>
<td></td>
</tr>
<tr>
<td>DFRA1</td>
<td>4.32 ± 0.09</td>
<td>0.51 ± 0.06</td>
<td>0.28 ± 0.04</td>
<td>0.047 ± 0.006</td>
<td></td>
</tr>
<tr>
<td>DFRA2&lt;sup&gt;7&lt;/sup&gt;</td>
<td>3.91 ± 0.07</td>
<td>0.52 ± 0.04</td>
<td>0.25 ± 0.02</td>
<td>0.048 ± 0.004</td>
<td></td>
</tr>
<tr>
<td>ΔtrpDC + aroG&lt;sup&gt;Δ14640n&lt;/sup&gt; + trpE&lt;sup&gt;S40F&lt;/sup&gt;G&lt;sup&gt;7&lt;/sup&gt;</td>
<td>N.A.</td>
<td>0.25 ± 0.01</td>
<td>0.09 ± 0.02</td>
<td>N.A.</td>
<td></td>
</tr>
</tbody>
</table>

<sup>6</sup> Anthranilate production experiments were performed in shaken-flask cultivations with M9 minimal medium and a total glucose supply of 60 mM (10.1 g L<sup>−1</sup>) over 72 h. Note that anthranilate titers from Figs. 4 and 5 were converted to g L<sup>−1</sup>.<sup>1</sup> Allow for direct comparisons with data from the literature. Data represent average values ± standard deviation from at least two independent experiments. Abbreviations: OD<sub>600</sub>, optical density measured at 600 nm; Y<sub>P/X</sub>, anthranilate yield on biomass; Y<sub>P/S</sub>, anthranilate yield on substrate; CDW, cell dry weight; N.A., not available.

<sup>1</sup> Experiments carried out in the presence of 0.1 mM L-Trp (Fig. S1 in the Supplementary Data).

<sup>7</sup> Data taken from Kuepper et al. (2015). In the best anthranilate-producing *P. putida* strain reported therein, the mutant aroG<sup>Δ14640n</sup> and trpE<sup>S40F</sup>G alleles were expressed from a Lac<sup>IV</sup>/P<sub>ara</sub> element in plasmid pSEVA234 (Silva-Rocha et al., 2013).
through the shikimate route in conditionally-autotrophic strains that do not require expensive additives in the culture medium. These results lay the foundation for further metabolic manipulations towards boosting aromatic bioproduction (Yu et al., 2016), coupled to scaling-up efforts in bioreactors and in situ product removal to alleviate inhibition of key enzymes and toxicity issues (Bentley et al., 2020; Kuepper et al., 2020; Mohamed et al., 2020). These aspects are central to process optimization, and encompass adaptive laboratory evolution efforts (Fernández-Cabezón et al., 2019), together with engineering biosynthesis of compounds that alleviate product toxicity, e.g. ectoine (Ma et al., 2020).

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Declaration of competing interest

The authors declare no financial or commercial conflict of interest.

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Abbreviations

AHL (3-oxooxaxenol)-L-homoserine lactone
Esa QS Quorum-sensing system of P. stewartii
QS Quorum sensing
L-Trp L-Tryptophan

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgen.2022.05.008.

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