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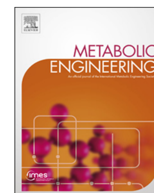
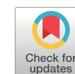


Pseudomonas putida as a functional *chassis* for industrial biocatalysis: From native biochemistry to *trans*-metabolism☆



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ABSTRACT

The itinerary followed by *Pseudomonas putida* from being a soil-dweller and plant colonizer bacterium to become a flexible and engineer-able platform for metabolic engineering stems from its natural lifestyle, which is adapted to harsh environmental conditions and all sorts of physicochemical stresses. Over the years, these properties have been capitalized biotechnologically owing to the expanding wealth of genetic tools designed for deep-editing the *P. putida* genome. A suite of dedicated vectors inspired in the core tenets of synthetic biology have enabled to suppress many of the naturally-occurring undesirable traits native to this species while enhancing its many appealing properties, and also to import catalytic activities and attributes from other biological systems. Much of the biotechnological interest on *P. putida* stems from the distinct architecture of its central carbon metabolism. The native biochemistry is naturally geared to generate reductive currency [i.e., NAD(P)H] that makes this bacterium a phenomenal host for redox-intensive reactions. In some cases, genetic editing of the indigenous biochemical network of *P. putida* (*cis*-metabolism) has sufficed to obtain target compounds of industrial interest. Yet, the main value and promise of this species (in particular, strain KT2440) resides not only in its capacity to host heterologous pathways from other microorganisms, but also altogether artificial routes (*trans*-metabolism) for making complex, new-to-Nature molecules. A number of examples are presented for substantiating the worth of *P. putida* as one of the favorite workhorses for sustainable manufacturing of fine and bulk chemicals in the current times of the 4th Industrial Revolution. The potential of *P. putida* to extend its rich native biochemistry beyond existing boundaries is discussed and research bottlenecks to this end are also identified. These aspects include not just the innovative genetic design of new strains but also the incorporation of novel chemical elements into the extant biochemistry, as well as genomic stability and scaling-up issues.

1. Introduction

Over the last few decades, metabolic engineering approaches, ranging from very simple manipulations to sophisticated designs, have greatly expanded the number of industrially-relevant compounds that can be accessed *via* whole-cell biocatalysis (Nielsen and Keasling, 2016; Park et al., 2018; Woolston et al., 2013). However, the most successful histories of metabolic engineering of microorganisms have been often confined to an assortment of genetic and genomic manipulations that lead to over-production of natively-synthesized metabolites in a handful model microbial cell factories (Smanski et al., 2016), such as *Escherichia coli* and *Saccharomyces cerevisiae*. Along the line, only a very limited number of structurally simple metabolites (e.g., the diols 1,4-butanediol and 1,3-propanediol) and a few natural active compounds (e.g., artemisinin) have found their way towards industrial-scale production (Chubukov et al., 2016; King et al., 2016). Actual commercialization and bioprocesses account today for merely ca. 3.5% of the total production volume of commodity and specialty chemicals (Campbell et al., 2017). A major reason underlying this state of affairs has been the emphasis of contemporary metabolic engineering (and, in particular, approaches aimed at engineering the core metabolism) of individual components in the microbial cell, which resulted into a collection of elegant demonstrations rather than generalizations. In contrast, systematic practices through the adoption of reliable, generalizable tools in standardized microbial *chasses* aimed at developing biosustainable production processes have been rather scarce. During the three decades

☆ This article is dedicated to the memory of the Argentinean microbiologist Norberto J. Palleroni, who passed away at age 96 by the time this review was being written (http://dbm.rutgers.edu/Norberto_Palleroni_obit.html). He was internationally recognized as an authority in taxonomy of the genus *Pseudomonas* and a dedicated researcher for his entire life. May he rest in peace.

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that followed the establishment of metabolic engineering as a discipline on its own merit (including the period that is often referred to as the *post-genomic era*), a staggering volume of *omics* data has been accumulated and more precise analytic techniques for studying cellular metabolism have been established. Empowered by these rapid developments, the focus of metabolic engineers has gradually shifted away from perturbing individual pathways and single metabolic nodes to the manipulation of the entire cell itself, giving rise to the concept of *systems metabolic engineering* (Lee and Kim, 2015; Nielsen, 2017). At the same time, the cost of oligonucleotides and *de novo* DNA synthesis has declined exponentially, and the emergence of a number of tools, afforded by contemporary synthetic biology, has made possible to access the metabolism of non-traditional microbial platforms. In this sense, soil bacteria are a group of microorganisms that have attracted attention as microbial platforms since they are often exposed to extreme environmental conditions, which correlates with a remarkable metabolic and physiological robustness. Among them, the intrinsically high metabolic diversity characteristic of *Pseudomonas* species, has emerged in recent years a solid basis for designing and creating novel pathways for bioproduction.

In this review, we present the most relevant advances in the field of metabolic engineering using the non-pathogenic soil bacterium *Pseudomonas putida* as the functional *chassis*—highlighting a number of synthetic biology approaches that made these manipulations possible. The discussion is focused on what we perceive as the frontiers in the field of engineering *P. putida* and other environmental bacteria beyond the canonical manipulations of the extant biochemistry towards complex designs aimed at re-writing metabolism and the very microbial lifestyle. As indicated in Fig. 1, we argue that the purposeful combination of (i) enabling technologies, such as novel synthetic biology tools, (ii) alternative feedstocks that cannot be processed by other microbial platforms (including man-made waste streams), and (iii) the wealth (if fairly disorganized) of *omics* data will strengthen the value of *P. putida* as the platform of choice for the synthesis of new-to-Nature products compatible with biosustainable industrial production. The establishment of an authentic systems metabolic engineering of *P. putida* is needed to fulfill this purpose, and specific examples are entertained as a step into this direction. The *leitmotiv* of these examples is that the rational exploitation of the native metabolic architecture is

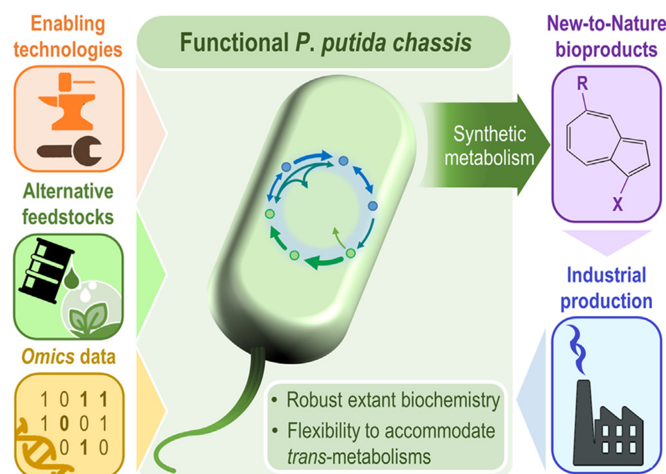

Fig. 1. *P. putida* as a functional *chassis* for developing novel biochemistries leading to new-to-Nature bioproducts. The adoption of specific synthetic biology tools (*enabling technologies*), along with the knowledge brought about by *omics* data, will help re-purposing the rich and flexible central carbon metabolism of *P. putida* to nest *trans*-metabolisms (with reactions and connectivities alien to the extant biochemistry) leading to the synthesis of completely new chemical structures from alternative substrates. Note that the whole process has a cyclic nature (echoing the *design-build-test cycle* of synthetic biology), which benefits from further manipulations to boost bioproduction.

expected to uncover unique biochemistries (which we collectively refer to as «*trans*-metabolisms») that enable the expansion of the biochemical network of *P. putida* towards unusual elements and chemical structures, thereby generating highly valuable compounds that could not be accessed otherwise.

2. *Pseudomonas putida*, *primus inter pares* among soil bacteria

A quick search in the relevant literature reveals that several microbial hosts have been considered and used for applications in metabolic engineering (Beites and Mendes, 2015; Liu and Deutschbauer, 2018). However, to date, no single, naturally-isolated bacterial strain seems to possess all the characteristics that would be desirable in an optimal host. Although the emerging picture indicates that the selection of an adequate bacterial platform will largely depend on the application intended, the adoption of a flexible and robust bacterium as the basis for developing a functional *chassis*, amenable to genetic and metabolic manipulations and endowed with high catalytic performance across a variety of operating conditions is definitely desirable. As hinted in the Introduction, soil bacteria in general, and *P. putida* in particular, could satisfy many of these requirements because of the physicochemical conditions they face naturally in the niches in which they thrive. These conditions include a somewhat continuous exposure to environmental contaminants, often together with all sorts of physicochemical stresses and competing as well as predatory microbial species. Many environmental bacterial species are widely recognized by their versatile, flexible metabolic lifestyles, which allow them to adapt to rapidly changing conditions (e.g., contact with oxidative stressors, temperature challenges, and sudden osmotic perturbations). As indicated in the section below, *P. putida* serves as a prime example of these qualities.

2.1. *The roadmap from soil to the laboratory*

The name «*Pseudomonas*» was coined in 1894 by Migula, who described the genus as a group of cells with polar organs of motility and, in some special cases, the formation of spore-like structures (Migula, 1894). This short, unclear description of the genus prevailed until the publication of the doctoral thesis of den Dooren de Jong in the late 20s (den Dooren de Jong, 1926). This publication revealed one of the features that would define (and classify) the entire genus more accurately: a remarkable capacity to degrade a large variety of organic compounds as part of the process of carbon mineralization. The den Dooren de Jong's thesis was, however, not widely used by contemporary scientists (perhaps because it was written in Dutch), but held out an inspirational vision for Stanier, Palleroni, and Doudoroff, who went on creating the first accurate description of the *Pseudomonas* genus some forty years later (Stanier et al., 1966). A set of differential tables was proposed for the classification of the most relevant *Pseudomonas* species known by then (including *P. putida*), based on nutritional characteristics and morphological features. Upon obtaining the sequence of the 16S rRNA gene of several *Pseudomonas* species in 1973, the bacterial genus was redefined by Palleroni et al. (1973) to encompass the previously described RNA-I group within the subclass of Gram-negative, aerobic γ-Proteobacteria (Palleroni, 2003, 2010). Fig. 2 identifies these milestones in a timeline depicting historical developments in the field of *Pseudomonas* biology.

P. putida is a ubiquitous rhizosphere saprophytic specimen and soil colonizer that belongs to the wide group of fluorescent *Pseudomonas* species. In particular, *P. putida* KT2440 strain is the best-characterized saprophytic member of the group, as it has become with time a model laboratory species which also retains its ability to survive and thrive in natural soil environments (Regenhardt et al., 2002; Timmis, 2002; Wackett, 2003). This *P. putida* strain is a derivative of *P. putida* mt-2, which was isolated from a soil sample in Japan in 1960 as a degrader of 3-methylbenzoic acid (actually, «mt-2» stands for «*meta*-toluate degrader, isolate 2»)) (Nakazawa, 2002). This remarkable metabolic

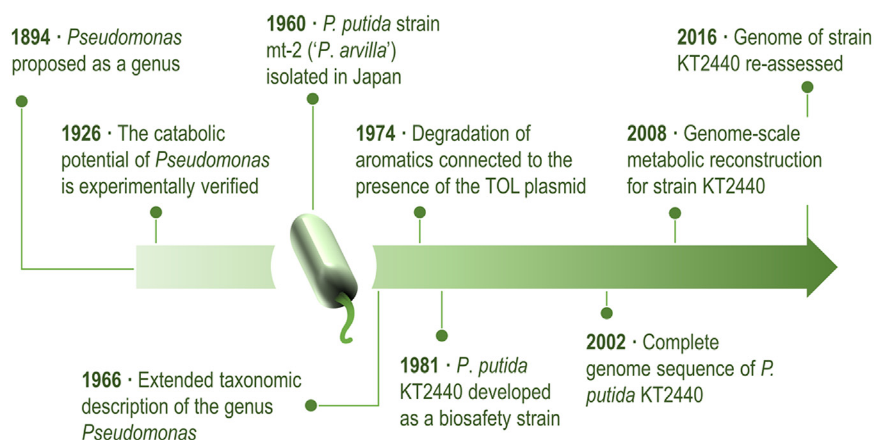

Fig. 2. Schematic timeline of the main findings on the isolation and characterization of *P. putida*. This non-extensive representation of the milestones in the field includes the dates of publication of key articles. Further information on historical developments can be found in the text and references therein.

property was later traced to the presence of the catabolic TOL plasmid pWW0, which encodes dedicated metabolic activities that enable *P. putida* mt-2 to grow on various aromatic substrates as sole carbon and energy sources, e.g., toluene, *m*-xylene, and *p*-xylene (Wong and Dunn, 1974; Worsey and Williams, 1975). Thus, the metabolic potential of *P. putida* and some closely related *Pseudomonas* species was recognized early on for applications in biodegradation of aromatic compounds. Once the catabolic plasmid pWW0 was eliminated from strain mt-2, the plasmid-less variant was designated as strain KT2440 (Bagdasarian et al., 1981) and it soon became the subject of a series of genetic studies in the laboratory, which resulted in the assignment of the *generally recognized as safe* (i.e., GRAS) status as a microbial host for recombinant DNA constructs (Federal Register, 1982; Nikel, 2012; Poblete-Castro et al., 2017; Timmis, 2002). This strain also exhibits remarkable resistance to oxidative stress (Kim and Park, 2014; Lemire et al., 2017; Nikel et al., 2013; Nikel and de Lorenzo, 2014), a remarkable feature closely associated with its capacity to degrade compounds that, on themselves, are sources of stress.

The *in silico* analysis of the 6,181,873-bp long genome sequence of *P. putida* KT2440, which became available first in 2002 (Nelson et al., 2002) and was later revisited in 2016 (Belda et al., 2016), confirmed the lack of any conspicuous virulence factor in the 5592 coding sequences present in the genome. The core and pangenome of *P. putida* has been likewise identified (Udaondo et al., 2016). The updated genome annotation of strain KT2440 generated a list of novel biochemical functions (e.g., the assimilation of alternative carbon sources) that were not previously identified in *P. putida* and resulted in a total of 1256 degradation reactions—including newly-identified catabolic pathways for 32 carbon sources, 28 nitrogen sources, 29 phosphorus sources, and 3 carbon and nitrogen sources. As indicated in the next section, the extremely rich secondary metabolism of *P. putida* KT2440 is wired to a robust core biochemistry characterized by a very distinct architecture.

2.2. The unique core metabolic architecture of P. putida: Reshaping the connectivity of sugar assimilation

In addition to its ability to use a wide variety of carbon and nitrogen sources indicated above, rapid growth and low nutrient demand are some of the added advantages of adopting *P. putida* as a *chassis* (Loeschcke and Thies, 2015; Nikel et al., 2014a, 2014b; Poblete-Castro et al., 2017). Most of these properties arise from (or are intimately connected to) a robust central carbon metabolism (Nikel et al., 2016). How is central carbon metabolism fueled by sugars in *P. putida*? Many Pseudomonads are known to have peripheral pathways for oxidation of sugars (e.g., to gluconate and 2-ketogluconate) in addition to the canonical, phosphorylation-dependent routes for assimilation of hexoses (del Castillo et al., 2007; Lessie and Phibbs, 1984; Sánchez-Pascuala et al., 2017). Interestingly, only one phosphoenolpyruvate-dependent sugar transport system is present in *P. putida*, and it is connected to fructose uptake and phosphorylation (Pflüger-Grau and de Lorenzo, 2014)—an architecture that has been recently shown to conform a complex regulatory device, which senses the availability of fructose *via* the regulatory Cra protein and the intracellular redox status (Chavarría et al., 2016).

Glucose is incorporated either *via* oxidation of the sugar to gluconate or 2-ketogluconate (either oxidized product follows a dedicated catabolic pathway for further processing) or the direct phosphorylation by glucokinase. Irrespective of the first steps in sugar processing (either oxidation or phosphorylation, although the former seems to be preferred over the later in strain KT2440), these pathways converge at the key intermediate 6-phosphogluconate—a metabolite that serves as precursor of the Entner-Doudoroff pathway and the interphase between the oxidative and non-oxidative branches of the pentose phosphate route. As recently reviewed by Udaondo et al. (2018), the regulation of all these catabolic pathways for carbohydrate processing is orchestrated by a versatile set of transcriptional regulators in *Pseudomonas* species. Additionally, the combined activity of enzymes from the Entner-Doudoroff pathway, the pentose phosphate pathway, and the incomplete Embden-Meyerhof-Parnas route form a cyclic metabolic architecture in *P. putida* KT2440 known as *EDEMP cycle* (Fig. 3**A**). When growing on hexose sugars, the operativity of this metabolic architecture endows *P. putida* with high NADPH regeneration rates *via* partial recycling of triose-phosphates (Nikel et al., 2015a)—a metabolic property that is in turn regulated by the presence and extent of oxidative stress conditions. Furthermore, the EDEMP cycle enables different ATP and NADPH formation rates depending on the amount of triose-phosphates recycled, a circumstance that reflects the specific stoichiometry of the Entner-Doudoroff pathway and the Embden-Meyerhof-Parnas pathway. The former yields half the ATP per molecule of glucose than the later, but produces, at the same time, one NADH and one NADPH equivalent. In particular, and assuming that the core metabolic pathways of *P. putida* KT2440 (i) use a NAD^+–dependent glyceraldehyde-3-phosphate dehydrogenase (an activity represented by no less than five isozymes, i.e., GapA, GapB, Epd, PP_0665, and PP_3443) and a $NADP^+$–dependent glucose-6-phosphate dehydrogenase (represented by the three isozymes Zwf, ZwfA, and ZwfB) under homeostatic physiological conditions, (ii) yield pyruvate as the main triose end product (that is further processed to acetyl-coenzyme A), and (iii) the fluxes through the peripheral oxidative loop leading to 2-ketogluconate formation are very low, the net flux of NADPH formation stemming from the activities in the EDEMP cycle (v_{NADPH}) increases linearly with the overall recycling flux from

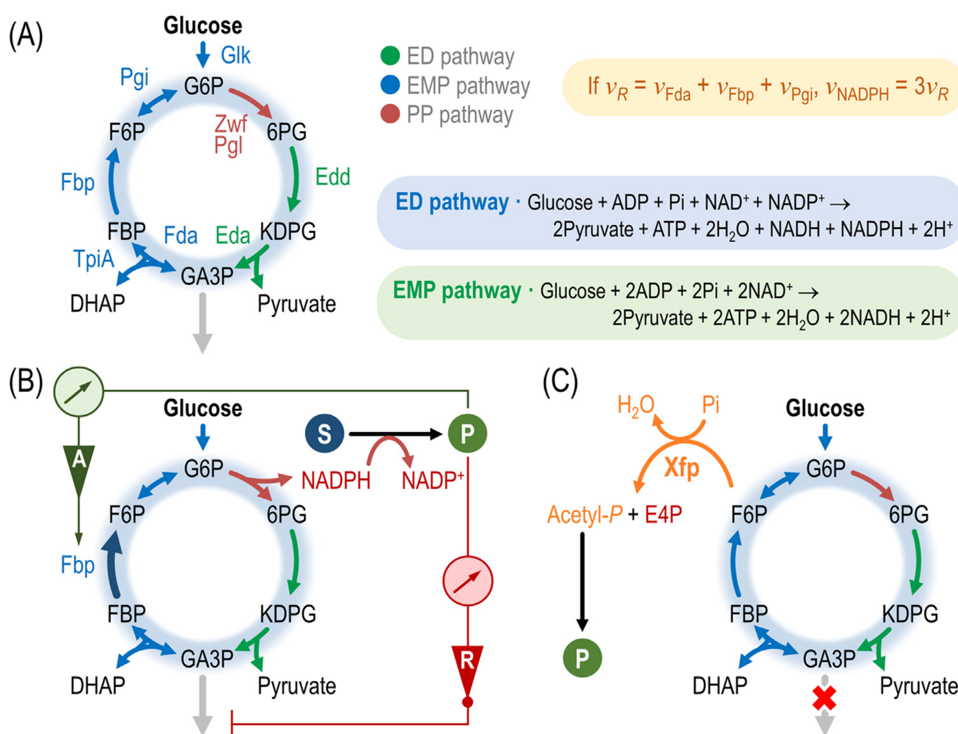

Fig. 3. The EDEMP cycle of *P. putida* KT2440 and two possible uses of this specific metabolic architecture for metabolic engineering purposes. (A) Glucose catabolism occurs mainly through the activity of the Entner-Doudoroff (ED) pathway and part of the trioses-phosphate thereby generated are recycled back to hexoses-phosphate by means of the cycle, that also encompasses activities from the Embden-Meyerhof-Parnas (EMP) and pentose phosphate (PP) pathways. A set of peripheral oxidative reactions, which convert glucose to gluconate and 2-ketogluconate before any phosphorylation of the intermediates occurs, is not shown in the diagram for the sake of simplicity. Each metabolic block is indicated with a different color along with the relevant enzymes catalyzing each step, and the EDEMP cycle is shaded in blue in this illustration. The overall stoichiometry of the ED and the EMP routes from glucose is indicated, along with the rate of NADPH regeneration (v_{NADPH}) as a function of the overall recycling flux of trioses-phosphate (v_R, which depends on the combined activities of Fda, Fbp, and Pgi), assuming that there is no formation of 2-ketogluconate through the peripheral oxidation loop. Further processing of the C3 intermediates is indicated with a gray arrow. The abbreviations used in the figure are as follows: G6P, glucose-6-phosphate; 6PG, 6-phosphogluconate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; GA3P, glyceraldehyde-3-phosphate; FBP, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; F6P, fructose-6-*P*; and Pi, inorganic orthophosphate. (B) Synthetic metabolic circuit for a generic NADPH-dependent reaction that would transform a substrate *S* into a product *P* in a positive feedback loop wired to the EDEMP cycle. The formation of *P* is gauged by two separate sensors connected to individual actuators (e.g., an activator *A* and a repressor *R*) that will, in turn, boost trioses-phosphate recycling and restrict downwards catabolism of C3 units, respectively. (C) Implantation of the *Bifidobacterium* shunt onto the upper metabolic architecture of *P. putida* would allow for the direct C6 to C2 conversion *via* phosphoketolase (Xfp). Note that the spectrum of products *P* that could be potentially obtained by using this strategy encompasses virtually any metabolite derived from acetyl-coenzyme A as the precursor. Acetyl-*P*, acetyl-phosphate; E4P, erythrose-4-phosphate.

glyceraldehyde-3-phosphate upwards (v_R), as indicated in Fig. 3A.

The distinct metabolic architecture in strain KT2440 when the cells grow on sugars has two remarkable sides that offer an interesting starting point for synthetic metabolic designs. Firstly, the connectivity that underlies the cyclic assembly of glycolytic activities in strain KT2440. Among other peculiarities, this architecture is characterized by the functional redundancy of at least four key activities: glucose-6-phosphate dehydrogenase (Zwf, three isozymes), glucose-6-phosphate isomerase (Pgi, two variants), and fructose-1,6-bisphosphate aldolase (Fda, four potential isozymes catalyzing this gluconeogenic reaction). Second, the existence of the EDEMP cycle (Fig. 3A) enables new strategies for engineering entirely novel biochemistries (Aslan et al., 2017). The ability of *P. putida* to sustain high NADPH formation rates is a requisite for implantation of what we could call *redox-expensive metabolic pathways* (Akkaya et al., 2018). In this sense, different engineering strategies can take advantage of the inherent layout of the EDEMP cycle. One attractive possibility for metabolic re-wiring could involve implantation of a metabolic circuit for coupling the activity of orthogonal NADPH-dependent reaction(s), leading to the target product, with two output signals, i.e., (i) increased flux through Fbp (and thus, a higher v_R flux that will further boost NADPH recycling), and (ii) decreased fluxes downwards glyceraldehyde-3-phosphate (e.g., by blocking the Gap activities around this metabolic node), which would prevent further catabolism into acetyl-coenzyme A (Fig. 3B). This synthetic metabolic design would offer the twofold benefit of enhancing the catalytic output of a redox-demanding bioreaction leading to the desired product *via* whole-cell biocatalysis while decoupling bacterial growth from the synthesis of the target molecule.

Besides playing with the fluxes through the native reactions within the core metabolic architecture, the gluconeogenic branch of the EDEMP cycle could be also harnessed as a source of fructose-6-phosphate for directly shunting the natural C6 → C2 conversion *via* the phosphoketolase-catalyzed *Bifidobacterium* shunt or fructose-6-phosphate pathway (Fig. 3C). The reaction executed by phosphoketolase (e.g., Xfp from *B. bifidum* or *B. animalis*) merges ketol cleavage, dehydration, and phosphorolysis to split fructose-6-phosphate into erythrose-4-phosphate (which can be then transformed *via* the non-oxidative branch of the pentose phosphate pathway) and acetyl-phosphate (Henard et al., 2015). Importantly, Xfp also catalyzes the formation of glyceraldehyde-3-phosphate and acetyl-phosphate from xylulose-5-phosphate. If the split of fructose-6-phosphate between the reactions catalyzed by Xfp and Pgi is balanced, the cycle could in principle continue providing NADPH, which has been a limitation on the use of the *Bifidobacterium* shunt in metabolic engineering. The C2-unit acetyl-phosphate can be easily converted into acetyl-coenzyme A (bypassing decarboxylation *via* pyruvate dehydrogenase and thus increasing final products yields)—and from there into a plethora of acetyl-coenzyme A-derived, added-value products.

The two scenarios entertained above represent rather simple manipulations aimed at harnessing the potential of the native biochemistry of *P. putida via* the introduction of a limited number of modifications. Nevertheless, it is possible to further rewire what we could call «*cis*-metabolism», i.e., the enzymatic parts and devices present in the extant metabolic architecture of this bacterium. Predictably, by just reconnecting existing endogenous reactions one could expand the portfolio of products derived from central carbon metabolism. The practical realization of these synthetic metabolic designs is heavily dependent on the access to dedicated synthetic biology tools for genome and genetic engineering—the scope of which is surveyed in the next section.

2.3. *Enabling technologies for metabolic engineering of P. putida: Tools for genome engineering and regulated gene expression*

As indicated above, the entire field of metabolic engineering is experiencing a transition from being a mostly trial-and-error exercise to become an authentic branch of rational engineering thanks to the tools and approaches brought about by contemporary synthetic biology. A clear indication of this transformation is our capacity to manage long DNA molecules, which has evolved from the handling of a few genes at a time up to synthesizing long sequences *de novo* and complete prokaryotic genomes—and soon eukaryotic chromosomes and entire genomes as well. A further example of our capability of handling genetic information includes the use of 3'→2'-phosphonomethyl-threosyl nucleic acid as a xeno-genetic material for *in vivo* applications (Liu et al., 2018). In all, this dynamic situation opens up possibilities for adoption of truly engineering-based approaches for manipulating metabolic pathways of non-traditional microbial platforms. The development and availability of such *enabling technologies* is essential for the design and implantation of veritable synthetic designs in cell factories.

Dedicated tools for gene expression have been developed for *P. putida* over the years and, not surprisingly, this bacterial species has also served as a treasure trove of functional parts (e.g., specific transcriptional regulators and their cognate promoters) that have been harnessed for the construction of novel expression systems. Table 1 presents a recompilation of these gene expression systems and platforms, highlighting those cases in which novel expression platforms have been used for metabolic engineering purposes. Building on the wealth of catabolic pathways in *P. putida*, many transcriptional regulators that respond to complex molecules (e.g., the regulatory proteins that rule aromatic degradation pathways) have been used to design expression systems that could be considered orthogonal when implanted in any surrogate host. In some other cases, expression systems developed for *E. coli* (Dvořák et al., 2015) have been implanted in *P. putida.* For example*,* strain KT2440 lacks the catabolic pathway needed for lactose processing and breakdown, therefore the LacI regulator along with P_{lac} and associated promoters are known to exhibit an orthogonal (if scarcely titrable) response in *P. putida*. The erratic behavior of the LacI/P_{lac} system (and, in reality, that of virtually any other expression platform) when placed in a heterologous host does not come as a surprise: the internal environment of growing cells is highly variable and dynamic (Goñi-Moreno et al., 2017), making it difficult to knock-in and manipulate reliable genetic parts. Additionally, most genetic systems have been thoroughly characterized only in laboratory *E. coli* strains, making extrapolations on their quantitative behavior in alternative hosts almost impossible. An elegant alternative to tackle this issue has been recently proposed by Segall-Shapiro et al. (2018), applying control-theoretic ideas to design promoters that would maintain constant levels of expression at any copy number in *E. coli*. This approach may significantly improve expression systems in *P. putida*, especially considering that the plasmid copy number of well-established origins of replications is different (on average, higher) in *P. putida* and *E. coli* (Cook et al., 2018), which in turns impacts the catalytic capacity of engineered *P. putida* in a strain- and regulatory system-dependent fashion (Jahn et al., 2014; Lindmeyer et al., 2015). Besides plasmid-based gene expression and manipulation, assembly and handling of large DNA segments has been also incorporated to the toolbox recently (Domröse et al., 2017).

The lack of well-established standards, which used to afflict not only engineering efforts using *P. putida* but essentially the entire field of synthetic biology, has only recently been tackled systematically. The hallmark brought about by the *Standard European Vector Architecture* (SEVA; a user-friendly, open-source toolbox platform in constant expansion) has helped to standardize the use of genetic parts that can be applied for metabolic engineering of *P. putida* (Martínez-García et al., 2014a; Silva-Rocha et al., 2013). Besides many of the gene expression systems listed in Table 1, other functionalities have been formatted and added to the SEVA collection, including Tn*5*- and Tn*7*-based transposon vectors (Martínez-García et al., 2011, 2014a, 2014b, 2017; Nikel and de Lorenzo, 2013a)—and the list of novel parts and devices therein continues to expand practically by the month. Needless to say, the inception of technologies based on clustered regularly interspaced short palindromic repeats (CRISPR) and Cas9 (Jakočiūnas et al., 2017) resulted in novel tools for targeted genome manipulations in *P. putida*, including counter-selection strategies for targeted genome mutagenesis (Aparicio et al., 2018; Mougiakos et al., 2017), gene down-regulation (i.e., CRISPR interference) using catalytically deactivated Cas9 (Sun et al., 2018; Tan et al., 2018), and precise plasmid curing (Lauritsen et al., 2017). The impact of CRISPR/Cas-based gene editing techniques is expected to reach genetically intractable microbial species (Shapiro et al., 2018), and would be instrumental to harness the metabolic potential of non-traditional *Pseudomonas* strains. For a recent update on many of the currently available *P. putida* genetic toolbox, the reader is referred to the recent review published by Martínez-García and de Lorenzo (2017). Alas, given the relentless publication of more and more genetic tools for *P. putida* and other non-*E. coli* microbial platforms, chances are that their compilations become obsolete by the time they are published.

Therefore, at the time of writing this article, the challenge is not so much a dearth of synthetic biology tools. Instead, the task is about pushing the boundaries of what *P. putida* can do towards biosynthesis of compounds through importing and nurturing other microorganisms' genes, and the «*biologization*» of otherwise purely chemical bonds and structures. In other words, the frontier in the field is moving from *cis*-metabolism towards *trans*-metabolism. Some avenues to make progress in this direction are discussed below.

3. From extant (*cis*) metabolism towards orthogonal (*trans*) metabolism in the *P. putida chassis*

3.1. *Design and construction of microbial cell factories afresh: Extant biochemistries and synthetic metabolisms*

The textbook definition of metabolism (from the Greek μεταβολή, *transformation* or, more literally, *change*) could be summarized as «*the collection of life-sustaining chemical transformations within individual cells of living organisms*». The canonical definition implies an *enumeration* of components of metabolism from a rather reductionist point of view; something that does not come as a surprise since reductionism has dominated biological research for over a century. Central carbon metabolism provides energy and biomass precursors to the entire cellular network, and its fine regulation is essential to ensure optimal use of substrates and proper allocation of resources to the metabolic pathways that make up for the extant biochemistry of any cell. Overcoming this fine regulation has been the ultimate goal of traditional metabolic engineering. From a broader perspective, a key challenge for biology in the 21st century is to understand the structure and the dynamics of the complex intercellular web of metabolic interactions that contribute to the structure and functioning of the bacterial cell. This has led to the realization that the relational logic of metabolic networks within a cell is shared (to a large degree) by other complex systems, e.g., computer chips and even modern society. This organizational universality indicates that similar laws may govern most complex networks in Nature, which allows the expertise from large and well-mapped non-biological systems to be used in characterizing the intricate interwoven relationships that govern metabolic functions. *Systemic biology* is the view of live systems through the optics of such quantitative perspective, relational logic and analysis tools of *bona fide* engineering being its core tenets by merging systems and synthetic biology with metabolic architectures and multi-scale organization of biological objects.

The process of moving from the study of individual components in central carbon metabolism to re-purposing the whole biochemical network implies that there should be a distinction between the extant

Table 1
Examples of gene expression systems and promoters designed using parts harnessed from *Pseudomonas* species or transplanted into *P. putida* for regulated gene expression, along with some applications of these systems in metabolic engineering.

Expression system (inducer)[a]	Source of regulatory elements	Characterization and examples of uses in metabolic engineering[b]	Reference(s)
XylS/*Pm* (3-methylbenzoic acid)	*P. putida* mt-2	Extensively characterized in *E. coli* and *P. putida*; *p*-coumaric production in engineered *P. putida* strains; heterologous expression of genes for fermentation under low oxygen availability in *P. putida*	de Lorenzo et al. (1993a); Gawin et al. (2017); Calero et al. (2016); Nikel and de Lorenzo (2013b)
XylR/*Pu* (3-methylbenzyl alcohol)	*P. putida* mt-2	Extensively characterized in *E. coli* and *P. putida*; expression of phosphoglucomutase (*celB*) from *Acetobacter xylinum* in recombinant *E. coli* and *Xanthomonas campestris* strains	Ramos et al. (1997); Marqués and Ramos (1993); Blatny et al. (1997)
AlkS/P_{alkB} (short-chain alkanes)	*P. putida* GPo1	Characterized in *E. coli* and *P. putida*; production of (*S*)-styrene oxide and heterologous proteins in recombinant *E. coli* strains	Panke et al. (1999); Makart et al. (2007)
NahR/P_{sal} (salicylic acid)	*P. putida* NCIB 9816-4	Characterized in *E. coli* and *P. putida*; surface reporter of catabolic promoter activity; gene expression cascade with XylS variants	Cebolla et al., (1996, 2001); Becker et al. (2010)
TodST/P_{todX} (4-chloroaniline)	*P. putida* T-57	Characterized in *E. coli*	Vangnai et al. (2012)
CymR/P_{cym} (4-isopropylbenzoic acid)	*P. putida* F1	Characterized in *E. coli*	Choi et al. (2010)
ClcR/P_{clcA} (3- or 4-chlorocatechol)	*P. putida* PRS2015	Characterized in *P. putida*	Guan et al. (2000)
HpdR/P_{hpdH} (3-hydroxypropionic acid)	*P. putida* KT2440	Characterized in *E. coli* and *Cupriavidus necator*	Hanko et al. (2017)
MmsR/P_{mmsA} (3-hydroxypropionic acid)	*P. putida* KT2440	Characterized in *E. coli* and *Cupriavidus necator*	Hanko et al. (2017)
NagR/P_{nagA} (salicylic acid)	*Comamonas testosteroni*	Production of 3-methylcatechol by *P. putida*	Hüsken et al. (2001)
ChnR/P_{chnB} (cyclohexanone)	*Acinetobacter johnsonii*	Characterized in *E. coli*; regulation of biofilm formation in engineered *P. putida* strains	Benedetti et al. (2016a), (2016b)
CprK/P_{DB3} (3-chloro-4-hydroxyphenylacetic acid)	*Desulfitobacterium hafniense*	Characterized in *E. coli* and *P. putida*	Kemp et al. (2013)
MekR/P_{mekA} (methyl-ethyl ketone)	*P. veronii*	Characterized in *E. coli* and *P. putida*; expression of *cre* for recombineering in *P. putida*	Graf and Altenbuchner (2013); Luo et al. (2016)
MtlR/P_{mtlE} (mannitol)	*P. fluorescens*	Characterized in *E. coli* and *P. putida*	Hoffmann and Altenbuchner (2015)
AraC/P_{araB} (arabinose)	*E. coli*	Characterized in *E. coli* and *P. putida*; *p*-coumaric production in engineered *P. putida* strains	Calero et al. (2016)
RhaRS/P_{rhaB} (arabinose)	*E. coli*	Characterized in *E. coli* and *P. putida*; *p*-coumaric production in engineered *P. putida* strains	Calero et al. (2016)
TetR/P_{tetA} (anhydrotetracycline)	*E. coli*	Synthesis of myxochromide S (*mchS* biosynthetic gene cluster from *Stigmatella aurantiaca*), epothilone (*epo* biosynthetic gene cluster from *Sorangium cellulosum*), and tubulysin (*tub* biosynthetic gene cluster from *Cystobacter* sp.) in engineered *P. putida* strains	Fu et al. (2008); Chai et al. (2012)
LacIQ/P_{lac}, P_{tac}, P_{trc} (isopropyl β-D-1-thiogalactopyranoside)	*E. coli*	Extensively characterized in *E. coli* and *P. putida*; biological containment of *P. putida*; production of long-chain rhamnolipids and vanillin in engineered *P. putida* strains	de Lorenzo et al. (1993a,1993b); Pérez-Martín and de Lorenzo (1996); Ronchel et al. (1995); Wittgens et al. (2018); Graf and Altenbuchner (2014)
P_{T5}	Bacteriophage T5	Expression of 6 × His-tagged proteins (*pfrA* siderophore regulatory gene) in *P. putida*	Bertani et al. (1999)
P_{T7}	Bacteriophage T7	Characterized in *P. putida*; containment of *P. putida* strains by conditional expression of the streptavidin gene	Herrero et al. (1993); Szafranski et al. (1997)
P_{EM7}	Synthetic, constitutive	Characterized in *P. putida*; CRISPR-Cas9—based counter-selection for genome manipulations in *P. putida*	Aparicio et al. (2018)
P_{oprL}	Synthetic, constitutive	Characterized in *P. putida*; expression of a bacterial laccase gene from *Shigella dysenteriae* and surface display in *P. putida*	Llamas et al. (2003); Wang et al. (2012)
P_{gro}, P_{tuf}, P_{tufs}	Synthetic, constitutive	Tailor-made for calibrated gene expression in *P. putida*; production of *cis,cis*-muconic acid	Kohlstedt et al. (2018)
P_{JE}	Synthetic, constitutive	Tailor-made for calibrated gene expression in *P. putida*	Elmore et al. (2017)
$P_{SynP\text{-}BG}$	Synthetic, constitutive	Tailor-made for calibrated gene expression in *P. putida*	Zobel et al. (2015)

[a] In some cases, more than one inducer can activate the expression system. Only the typical inducers are listed in the table (usually selected because they are the cheapest and/or the most effective).

[b] Only the first example(s) of uses in practical applications are indicated, and also those studies that have adopted the corresponding expression system for approaches beyond the customary characterization experiments in a surrogate host using fluorescent proteins.

(*cis*) biochemistry and the implanted (*trans*) metabolism. This operational definition indicates that, for some metabolic engineering purposes, re-wiring the components of the *cis*-metabolism would result in the formation of the target product(s), whereas the synthesis of new-to-Nature compounds would require the design and incorporation of *trans*-metabolic reactions into the bacterial *chassis*, e.g., involving novel biochemical transformations. Accordingly, this description of *cis*- and *trans*-metabolism conveys four categories of potential products, as proposed by Lee et al. (2012) and as indicated in Fig. 4: (i) *natural and endogenous* (metabolites that can be produced through native pathways of *P. putida*), (ii) *natural, exogenous* (natural metabolites produced by introducing an heterologous pathway that uses native precursors), (iii) *non-natural* (and therefore, exogenous; metabolites not found in Nature that can be produced by combining metabolic pathways, either native or heterologous), and (iii) *completely new-to-Nature* (for which a completely synthetic pathway is needed, including novel enzymes and reactions). In the next sections, we discuss examples of these two types of engineering operations in *P. putida*.

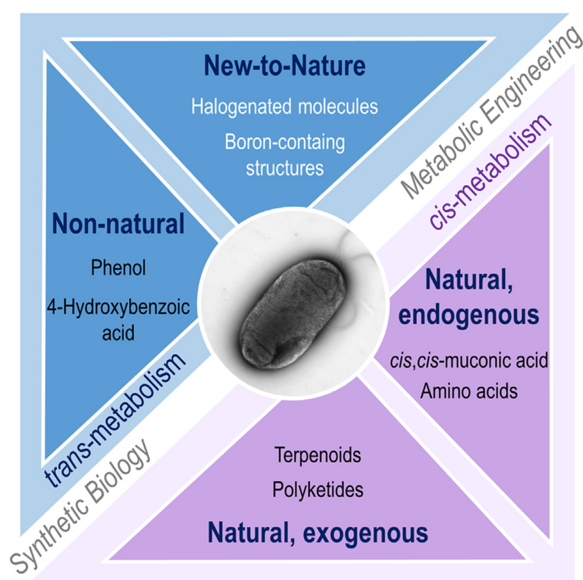

Fig. 4. The *cis*- and *trans*-metabolism realm, highlighting the role of synthetic biology and metabolic engineering in bridging the gap with *P. putida* as the functional *chassis*. Four categories of products are distinguished in either domain, depending on the metabolic pathways needed to synthesize them, and typical examples are provided in each case. Note that entirely new-to-Nature products are indicated in white lettering, as there are no cases reported for their synthesis in *P. putida*.

3.2. Engineering of the cis-metabolism in action: Pseudomonas putida as a host for efficient degradation and production of aromatic compounds

The capacity of *P. putida* strains to degrade aromatic compounds was recognized early on as a key signature of the species. The late 1980s and early 1990s witnessed the golden era of research in the biodegradation of xenobiotic compounds, and the literature reflects the numerous attempts that followed the pioneering work by Chakrabarty while he was working at the Research and Development Center of General Electric Co. At the time, four species of oil-metabolizing bacteria were known to exist, but when introduced into an oil spill, they would compete with each other, thereby limiting the amount of crude oil that they could degrade. Chakrabarty and co-workers described the preparation of recombinant *P. putida* strains (which they called «multi-plasmid hydrocarbon-degrading *Pseudomonas*») by means of plasmid-assisted molecular *breeding*, i.e., propagation of novel catabolic capabilities through directed bacterial conjugation and plasmid transfer (Chakrabarty et al., 1975). The resulting strains were able to break down two-thirds of all the hydrocarbons typically found in a crude oil spill at rates one or two orders of magnitude faster than the previously described individual strains of oil-eating bacteria, a set of results which lead to the first USA patent granted for a genetically modified organism (Chakrabarty, 1981).

3.2.1. Biodegradation of aromatic compounds as a source of added-value metabolites

The huge potential for biodegradation of aromatic compounds written in the *cis*-metabolism of *P. putida* was exploited for transformations beyond the customary bioremediation approaches in the context of which they were discovered (Dvořák et al., 2017). The practical demonstration of this metabolic capability is represented by several engineering strategies developed for the production of *cis*,*cis*-muconic acid [(2*E*,4*E*) 2,4-hexanedioic acid]. This dicarboxylic acid is a relevant platform chemical that is currently recognized for its high industrial value (Xie et al., 2014)—*cis*,*cis*-muconic acid provides synthetic access to terephthalic acid, 3-hexenedioic acid, 2-hexenedioic acid, 1,6-hexanediol, ε-caprolactam, and ε-caprolactone, all of which are important building blocks of commercial plastics, resins, and polymers (e.g., Nylon-6,6 *via* adipic acid). The traditional chemical processes for its production rely on non-renewable, oil-based feedstocks and high concentrations of heavy metal catalysts—and a mixture of the *cis*,*cis*- and *cis*,*trans*-muconic acid isomers is obtained. The synthesis of *cis*,*cis*-muconic acid in *P. putida* mt-2 takes place as an essential part of the upper catabolic pathways dealing with the degradation of aromatic compounds. All of these catabolic segments ultimately converge at the level of catechol (1,2-dihydroxybenzene) as a central metabolic intermediate, which then undergoes intradiol ring *ortho*-cleavage by the action of catechol-1,2-dioxygenase to yield of *cis*,*cis*-muconic acid.

The inactivation of muconate cycloisomerase was one of the first attempts to enhance the production of *cis*,*cis*-muconic acid, given that this enzyme would lactonize the target product and thereby reduce yields. Such strategy enabled the production of *cis*,*cis*-muconic acid *via* the catechol branch of the β-ketoadipate pathway directly from benzoic acid and toluene (Chua and Hsieh, 1990). In addition, the expression of phenol hydroxylating enzymes in *P. putida* KT2440 (i.e., the *dmpKLMNOP*-encoded phenol monooxygenase from *Pseudomonas* sp. strain CF600) enabled efficient *cis*,*cis*-muconic acid synthesis from phenol (Vardon et al., 2015). More recently, the entire protocatechuate branch of the β-ketoadipate pathway was successfully connected to the catechol node, thereby allowing for *cis*,*cis*-muconic acid formation from an even greater number of precursors, including coniferyl alcohol, *p*-coumarate, vanillate, ferulate, and protocatechuate itself (Johnson et al., 2016). As a result, the upper pathways of *P. putida* for aromatic compounds degradation were rendered a *metabolic funnel* to convert heterogeneous mixtures of aromatics with catechol as a central intermediate (Linger et al., 2014)—a circumstance that was adopted to reconstruct efficient modular pathways for *cis*,*cis*-muconic acid synthesis in engineered *E. coli* (Thompson et al., 2018).

3.2.2. From complex alternative feedstocks towards added-value metabolites

Some of the most recent instances on the production of *cis*,*cis*-muconic acid also offer an example of the use of alternative substrates by metabolically engineered *P. putida*, e.g., plant biomass containing lignin. Extensively found in terrestrial plants, lignin is the second most abundant biopolymer on Earth. With the advent of biorefineries that use plant biomass as a source of sugars, lignin has become a common by-product with an enormous potential to be used as raw material in the fuel and chemical industries. However, lignin remains the most under-utilized lignocellulosic biopolymer (Rinaldi et al., 2016). The catalytic potential of *P. putida* in taking part of this endeavor has not passed unnoticed.

As indicated in the previous section, the catechol node plays a central role in the production route leading to *cis*,*cis*-muconic acid production since (i) all the upper segments of the catabolic pathways for degradation of aromatic compounds ultimately yield catechol as the central intermediate, (ii) catechol itself is frequently generated during pre-treatment of lignin, and (iii) catechol is directly converted to the target product by means of catechol-1,2-dioxygenase. However, one of the main problems to overcome in production processes from lignin-based feedstocks is the high toxicity of catechol. First, these raw materials can themselves contain significant fractions of catechol. Second, the conversion of crude mixtures of aromatic compounds requires the engineering of heterologous catabolic pathways into the microbe, all of which additionally contribute to catechol accumulation, potentially leading to forced self-poisoning. These factors could explain why the conversion of complex lignin liquors to *cis*,*cis*-muconic acid by engineered *P. putida* strains has so far resulted in the accumulation of significant amounts of toxic upstream pathway intermediates (which lower final yields, and can altogether hamper the process) and in the production of the target metabolite at only the milligram scale.

In an attempt to overcome this state of affairs, Kohlstedt et al. (2018) designed an entire genealogy of engineered *P. putida* KT2440

strains endowed with enhanced catechol tolerance and high conversion efficiency, and having a wider substrate spectrum than other production platforms. Different metabolic configurations were tested in the engineered strains, and *cis*,*cis*-muconic acid was produced from benzoate, catechol, mixtures of catechol and phenol, and from a lignin hydrolysate liquor (obtained *via* base catalysis and hydrothermal conversion) in bioprocesses tested both at the laboratory- and at the pilot-scale (50 L). When grown under fed-batch conditions using glucose as the main carbon source, some of the engineered strains were able to achieve a *cis*,*cis*-muconic acid titer of ca. 65 g L^{-1} and 13 g L^{-1} from externally added catechol and from the lignin hydrolysate, respectively. More importantly, these engineered *cis*,*cis*-muconic acid producers were successfully applied to provide the first case example of lignin-to-Nylon-6,6 production *via* a cascaded biochemical and chemical integrated process. The bulk of the acid obtained in the process designed by the authors was subsequently hydrogenated to adipic acid *via* a chemical, Pd-assisted hydrogenation process, and the product was directly reacted with hexamethylenediamine to yield Nylon-6,6.

The dependence of these processes on sugars somehow compromises the value of using lignin-derived substrates at the industrial level that, as indicated above, are usually utilized as a co-feed. Novel metabolic engineering strategies are being tested to overcome this problem, and shift to lignin hydrolysates as the only carbon source fueling the process. In a recent example of this sort, Sonoki et al. (2018) engineered a *P. putida* KT2440-based strain to produce *cis*,*cis*-muconic acid from a mixture of vanillic acid (i.e., a guaiacyl-lignin model) and 4-hydroxybenzoic acid (i.e., a *p*-hydroxyphenyl-lignin model) with a yield of ca. 20% (mol mol^{-1}) without the need of glucose supplementation.

3.2.3. Production of aromatic compounds in P. putida

In the same way that *P. putida* is naturally equipped with the metabolic machinery needed for the degradation of aromatic compounds, this bacterium can be used as the functional *chassis* for the synthesis of aromatic compounds—a situation that could lead to sustainable production of some of them (used as building blocks for commodities), since the industrial synthesis of aromatics currently relies almost exclusively on petrochemical-based processes from benzene, toluene, and xylenes (Lee and Wendisch, 2017). Several examples from the literature indicate that *P. putida* has been engineered for the production of aromatic compounds that are often extremely toxic to be handled by other microbial hosts, e.g., cinnamate, *p*-coumarate, *p*-hydroxybenzoate, and phenol (Calero et al., 2018; Molina-Santiago et al., 2016). Although it is not the purpose of this article to survey all these examples, a recent addition to the literature of engineering the *cis*-metabolism of *Pseudomonas* for phenol production deserves attention considering its elegance and simplicity (Wynands et al., 2018). Phenol is an aromatic commodity chemical with a multitude of applications in the chemical industry, and its biological production from tyrosine has been achieved by introducing genes encoding tyrosine-phenol lyase from *Pantoea agglomerans* or *Pasteurella multocida* in *P. putida* or *E. coli* strains (Wierckx et al., 2005). However, the *P. putida* variants capable of producing 1.5 mM phenol from glucose with a yield < 7% C-mol C-mol^{-1} were obtained by non-rational strain engineering techniques, i.e., random mutagenesis followed by extensive high-throughput screening. Building on the knowledge stemming from those early studies, *P. taiwanesis* (a relative of *P. putida*) was forward- and reverse-engineered (22 modifications in total) to finally yield a strain that bears no plasmids (all relevant activities were integrated into the chromosome), and has no auxotrophies (a problem that plagued the engineered *P. putida* strains constructed thus far). The product titer and yield of phenol on glucose reached by the best engineered candidate after 96 h of batchwise shaken-flask cultivation was ca. 3 mM and 16% C-mol C-mol^{-1}, respectively, which are the highest reported in the absence of any additives (e.g., yeast extract) to the culture medium. Considering the high performance of these engineered *P. putida* strains in producing phenol from sugar substrates, it would be interesting to connect this metabolic architecture with the catabolic segments discussed in the preceding section leading to the synthesis of *cis*,*cis*-muconic acid to enable the forward design of a high-yield *cis*,*cis*-muconic acid *P. putida* producer from hexoses.

An entirely new chapter in the synthesis of aromatic compounds is likely to arise from the recent discovery and identification of enzymatic activities for the synthesis of toluene (Beller et al., 2018). Microbial sources of biogenic toluene were first reported more than three decades ago; however, the underlying biochemistry and specific enzymes that catalyze toluene biosynthesis had never been elucidated until now. Phenylacetate decarboxylation is catalyzed by the glycyl radical enzyme PhdB together with its cognate activating enzyme PhdA, a radical *S*-adenosyl-L-methionine enzyme, and the activities were discovered in two distinct anoxic microbial communities known to produce toluene—most likely by a non-cultivated microbial member (strain TolSyn) of the *Acidobacteria* phylum. The unconventional process of enzyme discovery from a complex microbial community (> 300,000 genes) undertaken by the authors involved metagenomics- and metaproteomics-enabled biochemical analysis, as well as *in vitro* confirmation of activities using recombinant enzymes. Yet, the strictly anoxic nature of these glycyl radical enzymes might hinder the design of a *trans*-metabolism for aromatic compounds in *P. putida* KT2440, an obligate aerobe—but there are emerging strategies at hand to overcome this issue, as indicated in the next section.

3.3. Trans-metabolisms engineered in the P. putida chassis: Exploring the untapped metabolic potential of environmental bacteria

The few examples presented in the preceding section are, in a nutshell, the practical demonstration of how flexible the metabolism of *P. putida* is, being able to host complex biochemistries that range from the degradation of complex aromatic substrates down to simple, low-molecular-weight metabolites—and potentially back. This aspect is the subject of the present section, in which we discuss some of the examples available in the literature on the engineering of *trans*-metabolisms in *P. putida*.

3.3.1. Engineering the oxygen-dependent lifestyle of P. putida

As diverse as the natural environmental niches inhabited by *P. putida* and other *Pseudomonas* are, they all share a physicochemical characteristic: oxygen does not usually represent a nutritional limitation. This feature is not surprising, as microbial activity in soil is known to be spatially heterogeneous, and individual species often forming spatial hotspots that differentially contribute to overall biogeochemical processes. Mounting evidence had suggested that spatial organization of bacterial species contributes to the persistence of anoxic hotspots in soils—a hypothesis that has been recently confirmed experimentally with a soil-like system that includes both the obligate aerobe *P. putida* and the facultative anaerobe *P. veronii* (Borer et al., 2018). The strictly aerobic, highly oxidative *cis*-metabolism of *P. putida*, however, hampers its broad application under micro-oxic and anoxic conditions, which in turn excludes the utilization of oxygen-sensitive proteins and metabolites. This situation also leads to complications when it comes to industrial-scale applications. On one hand, the operation of oxic bioprocesses it increases the capital cost, as scaling-up of these processes is significantly limited by the oxygen transfer rate and, due to this, both the maximum and average scales of commercial oxic bioreactors are much smaller in comparison with anoxic bioreactors (Ruiz et al., 2012). On the other hand, oxygen-dependent bioproduction bring about some degree of substrate loss in the form of CO_2, while some anoxic processes can achieve carbon yields close to theoretical yields. Against this background, it is no surprise that engineering an anoxic *P. putida chassis* has been the subject of intense research over the last few years, including the quantitative study of the responses of *P. putida* to different agitation rates and oxygen availability in shaken-flask cultures (Rodríguez et al., 2018).

Three types of engineering approaches can be distinguished, which are based on (i) a *trans*-metabolism for mixed fermentation, (ii) a *trans*-metabolism for nitrate-dependent respiration, and (iii) the adoption of bioelectrochemical systems. In the first type of engineering approach, the genes encoding the acetate kinase from *E. coli* and the ethanol biosynthesis pathway from *Zymomonas mobilis* were introduced in strain KT2440, which resulted in an extended survival in the absence of oxygen (Nikel and de Lorenzo, 2013b). This anoxic-tolerant bacterial *chassis* was further engineered by recruiting two haloalkane dehalogenases from *P. pavonaceae*, which conferred the ability of degrading 1,3-dichloprop-1-ene (a recalcitrant xenobiotic that neither wild-type *P. putida* nor *P. pavonaceae* can degrade under anoxic conditions). In a different study, Steen et al. (2013) constructed two cosmids encoding all the structural, maturation-related, and regulatory genes needed for nitrate reductase and nitrite- and nitric oxide reductase from *P. aeruginosa* in order to establish nitrate-dependent respiration in strain KT2440. The resulting engineered strains efficiently reduced nitrate or nitrite, which in turn sustained an extended anoxic lifespan. Finally, the utilization of bioelectrochemical systems was exploited to develop bioprocesses under conditions with limited oxygen supply. Schmitz et al. (2015) engineered a *P. putida* KT2440 derivative able to synthesize phenazine redox-mediators by expressing the seven-gene cluster *phzA1-G1* together with *phzM* and *phzS* from *P. aeruginosa* PAO1. Formation of the redox-active pyocyanin allowed for partial redox balancing with an electrode under micro-oxic conditions, and the biomass yield on glucose of the engineered *P. putida* was doubled as compared with the wild-type strain. Lai et al. (2016) used $[Fe(CN)_6]^{3-}$ or $[Co(2,2'\text{-bipyridine})_3]^{3+}$ as the high midpoint potential redox mediator when culturing *P. putida* F1 in the anodic compartment of a bioelectrochemical system ran under anoxic conditions in the presence of glucose as the carbon source. Under these conditions, most of the glucose was converted into 2-ketogluconate (with a yield of ca. 0.9 mol mol^{-1}), and overexpression of the endogenous gluconate dehydrogenase in strain KT2440 boosted 2-ketogluconate formation under similarly anoxic culture conditions by > 600% (Yu et al., 2018).

Despite all these valuable efforts, no metabolically engineered *P. putida* strain was able to actually *grow* in the absence of oxygen thus far, which opens up the question of what are the factors missing in the picture that need to be manipulated in order to accomplish the task. It is likely that an authentic genome scale metabolic model-driven design of an anaerobic *trans*-metabolism will help filling the knowledge gap that currently limits the adoption of an oxygen-independent lifestyle by *P. putida*—a situation that will soon be remediated by exploiting the information of the five genome-wide metabolic models currently available for strain KT2440 (Belda et al., 2016; Nogales et al., 2008; Puchałka et al., 2008; Sohn et al., 2010; Yuan et al., 2017), including the latest update by Nogales et al. (2017), in which the *in silico* metabolic potential of *P. putida* has been fully evaluated in the most comprehensive metabolic reconstruction built to date. Meanwhile, and in some cases with the support of such metabolic reconstructions, the already rich *cis*-metabolism of *P. putida* has been used to incorporate new substrates for bioproduction, as disclosed in the section below.

3.3.2. Expansion of the range of carbon substrates

What makes a carbon source into a good substrate for *P. putida*? Hintermayer and Weuster-Botz (2017) attempted to answer this question by estimating the growth parameters of strain KT2440 *in silico* using 57 individual carbon sources, and experimentally validated the results in batch cultures carried out on six of them (i.e., acetate, glycerol, citrate, succinate, malate, and methanol). Glycerol was the carbon source that promoted the highest biomass yield on substrate (0.61 C-mol C-mol^{-1}) with a very good fit between the *in silico* prediction and the experimental validation. The use of glycerol has been explored from a biochemical and genetic point of view in other studies (Nikel et al., 2014a, 2015b), and it has been also used as a substrate to design bioprocesses for the cost-efficient production of polyhydroxyalkanoates by *P. putida* (Beckers et al., 2016; Gomez et al., 2012; Prieto et al., 2016). In fact, biopolymers constitute one of the main products of the *cis*-metabolism of *P. putida* that has been exploited for commercial production, and the reader is referred to the key literature in the domain for further information on polyester production in engineered *P. putida* strains (Arias et al., 2013; Li et al., 2011; Liu et al., 2011; Meng and Chen, 2018; Tripathi et al., 2012).

All these studies indicate that the nutritional landscape of typical *P. putida* niches in Nature (including plant rhizosphere and polluted soils) has pushed its metabolic specialization towards the use of organic acids, amino acids, and aromatic substrates (Martins dos Santos et al., 2004). Considering that glucose and xylose are the two most abundant monosaccharide building blocks of the abundant polysaccharides cellulose and hemicellulose in plant cell walls, both of them are attractive sugars to be used as substrates in bioprocesses. Expansion of the *cis*-metabolism of sugars in *P. putida* to assimilate industrially-relevant hexose and pentose substrates is thus an important topic in the field of metabolic engineering since carbohydrates are not the preferred carbon sources of environmental bacteria.

The carbohydrates on which *P. putida* KT2440 can grow are confined to some hexoses (glucose and fructose), but this strain is unable to metabolize disaccharides or C5 sugars (Köhler et al., 2015), thus limiting the number and nature of sugars that can be used in bioprocesses. Dvořák and de Lorenzo (2018) expanded the *cis*-metabolism of a genome-reduced variant of strain KT2440 to include disaccharides and xylose as the carbon substrates by (i) plugging-in a β-glucosidase from *Thermobifida fusca* (i.e., the enzyme needed for intracellular hydrolysis of cellobiose), (ii) and three separate activities from *E. coli* (i.e., a xylose transporter, a xylose isomerase, and a xylulokinase), and (iii) blocking the oxidative branch of sugar utilization by eliminating the endogenous glucose dehydrogenase. When implemented together, these manipulations enabled co-utilization and total utilization of both cellobiose and xylose in minimal medium by the engineered strain.

Sucrose is another disaccharide alien to the extant catabolic potential of *P. putida*. Being the main sugar component of molasses, thus an interesting feedstock for bioproduction, Löwe et al. (2017a) engineered a reduced-genome variant of strain KT2440 to acquire sucrose permease and invertase activities by delivering the *cscAB* genes from *E. coli* strain W into the chromosome *via* mini-Tn*5* transposons. The resulting *P. putida* variant not only grew on sucrose as the carbon source, but it was also used as a platform strain for photoautotrophic polyhydroxyalkanoate production in a co-culture system along with cyanobacteria. In this setup, a mutant strain of *Synechococcus elongatus* fixes CO_2 and converts it to sucrose, which is in turn used as the main carbon source by the engineered *P. putida* strain to accumulate the intracellular biopolymer with an overall productivity of ca. 24 mg L^{-1} day^{-1} (Löwe et al., 2017b).

Other substrates beyond individual sugars are likewise relevant for industrial production. Lignocellulose, for instance, can be decomposed to cellulose (25–55%), hemicellulose (11–50%), and lignin (10–40%). As most biomass conversion schemes for bioproduction employ a high-temperature processing step, sometimes in the presence of an acid, sugar dehydration products are inevitably formed (Ravindran and Jaiswal, 2016). C5 and C6 sugars can be dehydrated into furfural and 5-(hydroxymethyl)furfural, respectively, and these two compounds are commonly formed during the thermochemical pretreatment, liquefaction, or pyrolysis steps of lignocellulose processing. These aldehydes are considered major inhibitors in microbial conversion processes, but some microorganisms are known to convert these compounds to their less toxic, dead-end alcohol counterparts, furfuryl alcohol and 5-(hydroxymethyl)furfuryl alcohol. *P. putida* KT2440 was engineered to utilize both furfural and 5-(hydroxymethyl)furfural as sole carbon and energy sources *via* genomic integration of the 12-kb *hmf* gene cluster, encoding the eight enzymes previously characterized in *Burkholderia phytofirmans* that transform the two substrates in the common metabolic intermediate 2-furoic acid (Guarnieri et al., 2017).

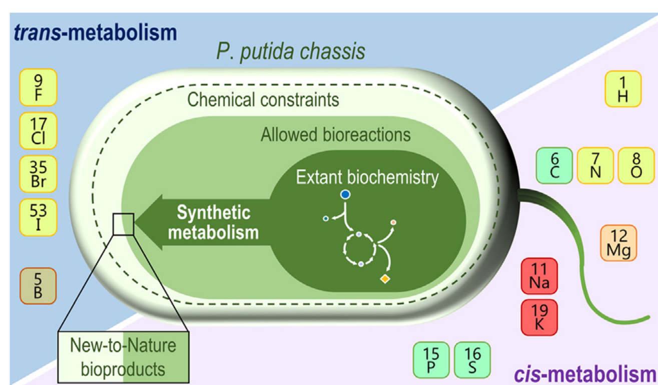

Fig. 5. Towards adoption of novel *trans*-metabolisms in *P. putida*. In order to push the extant biochemistry of the cells into its very boundaries and explore the plethora of potential new-to-Nature products lying there, synthetic pathways (consisting, in some cases, of a purposeful assembly of enzymes specifically evolved for the task) need to be plugged-in in the *chassis*. Note that all the possible biochemical reactions brought about by a synthetic metabolism are constrained by thermodynamics. The typical chemical elements that form part of the *cis*-metabolism, as well as those that would be desirable to bring as part of the core biochemistry of the cell for practical applications, are indicated in either side of the diagram.

4. Conclusions and future prospects: Towards a *bona fide trans*-metabolism in *P. putida*

The examples discussed in the sections above illustrate the potential of *P. putida* in nesting a suite of biochemical pathways from very diverse sources, and in some of these cases, the adoption of harsh reactions demonstrated that only a robust *chassis* can be used to host them. In this concluding section, we summarize the challenges ahead for the design and implementation of entirely new *trans*-metabolisms in the *P. putida chassis*—in particular, considering the strategies that will help the exploration of the interphase between the extant, core biochemistry and all the reactions within the realm of the allowed metabolism (Fig. 5).

4.1. Assimilation of non-biological chemical elements

Most of the examples of metabolic engineering of bioproducts (and *all* of the cases that made their way up to commercialization) are primarily limited to molecules resulting from the combination of only six common elements (i.e., carbon, hydrogen, oxygen, nitrogen, phosphorus, and sulfur). Expanding the chemical palate of microbial cell factories is one of the most sought after objectives in the field nowadays (Reed and Alper, 2018). Enabling novel elemental chemistries within cellular systems will be a major contribution of the systemics biology era to expand the scope of renewable chemistries performed by microorganisms. Among the many uncommon chemical elements that can find their way into biological molecules, halogens are particularly attractive as halogenated organic compounds play a major role in the pharmaceutical, diagnostic, agricultural, and materials industries (O'Hagan and Deng, 2015; Walker and Chang, 2014). While halogenation of organic compounds using synthetic chemistry is well-established (O'Hagan, 2008), these procedures are often characterized by highly toxic chemicals and low yields due to difficulty of separation and purification of enantiomeric substances. Fluorine, the 13th most abundant chemical element on the Earth crust and the most electronegative atom in the periodic table, is such an instance. Due to its unique chemical properties, fluorine has been incorporated into drugs to modify their properties: since the C–F bond is much more hydrophobic than the C–H bond, fluorination affects basicity and enhances lipophilicity, helping cell membrane penetration and increasing bioavailability. Nature, however, has hardly evolved biochemical reactions involving fluorine (O'Hagan et al., 2002). This state of affairs contrasts with the contemporary demand for pharmaceutically-relevant fluorinated biomolecules and building blocks as ca. 25% of the drugs currently licensed contain some type of fluorinated structures. One of the main problems in designing a *trans*-metabolism for targeted fluorination may find a practical solution in *P. putida*: low-molecular-weight organofluorines are known to be very toxic to most cells—but the insight gained from the remarkable chemical feats of *P. putida* can be used to engineer heftier production platforms that can incorporate the extreme biochemistry of fluorine and other halogens. Besides, Pseudomonads species are among the best platforms known for dehalogenation of organohalides (Dvořák et al., 2017; van Pée and Unversucht, 2003), which indicates that the native biochemistry of these species can handle halogenated structures.

Another two elements that seldom form part of the native biochemistry of living cells could soon find an adequate biochemical environment in *P. putida* for the development of *trans*-metabolisms aimed at bioproduction. Directed evolution of proteins has paved the way for bringing boron and silicon into the biochemical agenda of bacteria (Arnold, 2018). Representing a 30% of the total mass of the Earth crust, silicon is chemically very similar to carbon, and it has been hinted that microorganisms and biocatalysts may have a greater capacity for novel silicon-heteroatom bonds than what has been discovered previously. The first instance of an enzyme directly catalyzing the formation of a C–Si bond was demonstrated by using an engineered variant of a cytochrome *c* from the extremophile *Rhodothermus marinus* (Kan et al., 2016). The catalytic scope of this cytochrome was further expanded to include boron, providing access to 16 novel optically-active organoboranes (Kan et al., 2017). These smart strategies for bio-sylylation and bio-borylation open up a novel chapter in *trans*-metabolism that will be surely expanded in the coming years.

4.2. Utilization of alternative carbon substrates

The use of lignocellulosic biomass as an alternative feedstock in bioprocesses is sometimes criticized as it can potentially undermine food security, and also because it is constrained by limited availability and inefficient processing. The replacement of fossil and agricultural feed-stocks with sustainable alternatives for the production of chemicals and fuels is thus a societal and environmental necessity. A promising bioproduction strategy could be the use of reduced inorganic or one-carbon (C1) electron donors to provide reducing power and energy to drive CO_2 fixation, as shown *in vitro* (Erb and Zarzycki, 2016), e.g., CO, methane, methanol, and formic acid (Claassens et al., 2018). Such C1 compounds could be sustainably obtained from waste streams or readily regenerated by the use of unlimited resources (e.g., as light, water, and CO_2). Also, engineering synthesis of sugars *in vivo*, out of CO_2 has been achieved in *E. coli* (Antonovsky et al., 2016). If complex enzymatic systems for both electron transfer and carbon capture were expressed in heterotrophic bacteria, *P. putida* could constitute an interesting alternative to develop a *trans*-metabolic route for CO_2 fixation (Schwander et al., 2016). Production of, e.g., biofuels would then be eased by the innate high solvent tolerance of this bacterium (Blank et al., 2008; Ramos et al., 2015; Smith, 1990).

4.3. Novel biochemistries for structurally complex metabolites

Natural products are widely used as pharmaceuticals and in agrochemistry. Many products traditionally produced using *P. putida* as a microbial cell factory by chemical and biotechnological companies (e.g., Pfizer, Lonza, DSM, DuPont, and BASF) have been mostly focused on this family of compounds (Poblete-Castro et al., 2012). The main interest on these chemical structures currently relies on their extraordinary value as precursors for the pharmaceutical industry, especially when it comes to the synthesis of chiral molecules that are particularly difficult to synthesize using traditional chemical methods (Liu et al., 2017). Complex natural products are often synthesized by

multifunctional mega-synthetases, e.g., multi-domain polyketide synthases (Barajas et al., 2017), the engineering and heterologous expression of which offer huge promise for bioproduction, especially if the natural hosts are genetically intractable, slow growing or unculturable, or even unknown. The assembly of polyketide synthases seems to be a particularly attractive possibility in this respect to access products completely alien to the biochemistry of *P. putida*, especially considering that these modular enzymes can be assembled in different configurations to further expand their catalytic repertoire. Apart from the metabolites brought about by these natural pathways, the structural complexity of novel carbon compounds continues to expand by enzyme engineering and evolution (Erb et al., 2017) to include small carbocycles, e.g., cyclopropane and bicyclobutanes (Chen et al., 2018). In all these cases, a central aspect (unfortunately seldom explored in metabolic engineering designs) when introducing metabolites completely alien to the extant biochemistry is the importance of establishing efficient metabolite repair systems (Sun et al., 2017).

4.4. Dynamic metabolic engineering

Synthetic biology has provided a wealth of gene circuits repurposed as tools for engineering cellular behavior to be dynamically controlled *via* transplantation of feedback loops, diverting feedback loop outputs, or coupling sensory inputs with control devices. However, the potential of such circuits has not been fully exploited for metabolic engineering—let alone in *P. putida*. As recently indicated by Chen and Liu (2018), gene circuits can be divided into three categories based on signal sources: (i) *environmental circuits* that sense environmental signals (e.g., light, temperature, and population density), (ii) *extracellular circuits* that sense extracellular chemical stimuli, and (iii) *intracellular circuits* that sense the accumulation of intracellular chemicals (e.g., endogenous and exogenous metabolites). Dynamically-regulated systems could offer higher catalytic outputs than the customary systems used for metabolic engineering (Tan and Prather, 2017). The regulatory network that rules biodegradation pathways in *P. putida* offer a unique starting point to design such circuits to dynamically control synthetic metabolisms (e.g., as indicated in Fig. 3**B**). The adoption of particular interpretation frameworks for the design of these circuits would be a necessary step towards their implementation, and the formalism of logic gates from electronics (Moon et al., 2012) would be one of the tools to facilitate further engineering of *trans*-metabolisms in *P. putida*.

4.5. From the test tube to the bioreactor

Despite the value of *P. putida* as a metabolic engineering *chassis* argued above, this platform is not alien to the paradox that permeates virtually all microorganisms of industrial interest: the frequent gap between creating a new metabolic property in the laboratory (as discussed above) and developing a large-scale process to fill the breach between design and actual production (Danchin, 2012). One thing is to engineer a quality of interest in the test tube or in a small bioreactor and a quite different one is to implement a complete flowchart for manufacturing bulk amounts of chemicals able to *actually* compete with oil-based counterparts (Sanford et al., 2016). Conspicuous challenges to be addressed include the frequent lack of genomic stability of whole-cell catalysts when bearing complex DNA constructs, the reliance on (large volumes of) sterile liquid media for bacterial growth, and the difficulty of downstream processing for recovering the products of interest. These circumstances make the actual industrial use of engineered *P. putida* strains to be mostly limited to high-added value molecules produced in small-to-medium volume fermentations. Developing strategies for scaling-up the corresponding operations and making them economically viable are thus as important for the biotechnological outcome of this species as the deep genetic engineering attempts discussed in the sections above. In particular, efficient approaches for high-cell-density cultivation of engineered *P. putida* are needed to reach productivities compatible with commercialization of target products (Davis et al., 2015; Diniz et al., 2004; Lee et al., 2000; Sun et al., 2006). The regulatory circuits that are known to affect cell density-dependent physiological traits (e.g., quorum sensing) should be tackled accordingly. Increasing long-term stability of the whole-cell catalysts, achieving high catalytic performance under low-water conditions, and making downstream processing easy are challenges that raise by themselves major scientific questions. Curbing evolvability (Umenhoffer et al., 2010), phenotypic heterogeneity (Nikel and de Lorenzo, 2018), managing water-limitation and saline stress (Yin et al., 2015), and engineering efficient secretion systems (Zhou et al., 2018) will help making the difference between mere academic curiosity and full-fledged (and economically profitable) industrial applications. Even when some efforts have been made in this direction, they are still clearly insufficient to bring about the much pursued shift between an oil-based economy into a biosustainable chemical industry (de Lorenzo et al., 2018; Timmis et al., 2017). While these bottlenecks will hopefully be tackled in the near future, we strongly advocate the worth of *P. putida* for framing the field of metabolic engineering in the wider context of the ongoing 4th Industrial Revolution (Schwab, 2016).

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