Biotechnological domestication of pseudomonads through synthetic biology

by

Pablo I. Nikel, Esteban Martínez-García, and Víctor de Lorenzo*

Systems and Synthetic Biology Program, Centro Nacional de Biotecnología (CNB-CSIC)

Madrid 28049, Spain

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* Corresponding author

V. de Lorenzo

Systems and Synthetic Biology Program

Centro Nacional de Biotecnología (CNB-CSIC)

Darwin 3, Campus de Cantoblanco

Madrid 28049, Spain

Phone: +34 91 585 4536

Fax: +34 91 585 4506

E-mail: vdlorenzo@cnb.csic.es

Preface

Much of contemporary synthetic biology research relies on the use of bacterial chassis for plugging-in and -out genetic circuits and new-to-nature functionalities. However, those microorganisms that are the easiest to manipulate in the laboratory are often suboptimal for downstream industrial applications^[1], which can involve physicochemical stress and harsh operating conditions. Here, we advocate the use of environmental *Pseudomonas* strains as model organisms pre-endowed with the metabolic, physiological and stress-endurance traits that are demanded by current and future synthetic biology and biotechnological needs.

1 The tombstone of Selman Waksman (1888-1973), the discoverer of streptomycin, bears the
2 biblical quote "*The earth will open and bring forth salvation*". The benefits that antibiotics
3 produced by soil microorganisms have brought to mankind have undoubtedly been immense.
4 Yet, Waksman could not imagine during his lifetime the phenomenal wealth of microbial activities
5 concealed in the soil microbiota that could be exploited for human interests. Environmental
6 bacteria not only control global biogeochemical cycling, they also possess an amazing repertoire
7 of biochemical activities. Since the mid-1980s, the development of recombinant DNA technology
8 to manipulate environmental bacterial hosts has enabled the production of new bioactive
9 molecules^{1,2} and the treatment of xenobiotic chemical wastes^{3,4}, among other applications.
10 Attempts to capitalize on microbial soil diversity have recently expanded to include the mining of
11 environmental genes, whether in complete bacterial genomes⁵ or in metagenomes⁶, through the
12 use of functional traps^{7,8} (**BOX 1**) and/or bioinformatic approaches⁹.

13
14 Historically, genetic engineering techniques were used to accomplish these endeavours, although
15 most strategies often lacked an authentic engineering perspective, either technically or
16 conceptually, and largely depended on trial-and-error attempts. By contrast, contemporary
17 synthetic biology addresses the same type of challenges but involves a genuine forward-design
18 engineering approach¹⁰⁻¹². One of the key components of this endeavour is the adoption of
19 specific biological chassis (**Fig. 1**), into which users can plug-in and -out genetic circuits and new-
20 to-nature properties at will. The metaphor of a chassis has become commonplace in synthetic
21 biology¹³; the term evoking the image of an internal framework that supports a man-made object.
22 A bacterial genome, edited where necessary, provides a material frame on which forward-
23 engineered biological components can be placed. The *chassis* metaphor suggests the implanted
24 components (genetic or otherwise) are autonomous with respect to the physiology of the cell in
25 which the chassis is present, and heralds the possibility of conferring predictable properties to
26 biological entities. Unfortunately, unlike the mechanical or electrical parts used in engineering,
27 biological components function in an extremely context-dependent manner because they interact
28 with metabolites and (bio)chemicals; are subjected to Darwinian evolution; can confer emergent
29 properties when combined; and the cells which harbour these components grow and multiply.
30 Overcoming, or at least mitigating, these problems is a fundamental part of the synthetic biology
31 research agenda¹⁴.

1 Selecting a specific bacterium as a chassis for synthetic biology applications can be viewed as
2 being analogous to the selection of animal species for domestication¹⁵ – few species are suitable
3 for this process. One key aspect of the domestication process involves an innate potential (which
4 can be enhanced by breeding) to predictably follow instructions from a master. By the same
5 token, a reliable microbial chassis suitable for synthetic biology should ideally be derived from
6 bacterial species that are naturally pre-endowed with a number of appealing physiological and
7 metabolic properties and are amenable to stable genetic re-programming. The synthetic biology
8 pursuit of biological chassis that can be subjected to deep (and rational) genetic engineering has
9 naturally intersected with the more conventional quest for microbial platform strains and cell
10 factories that are most favourable for consolidated bioprocesses, one of the trademarks of
11 modern industrial biotechnology^{16,17}. Along the line, researchers have been able to domesticate a
12 small number of bacteria for different purposes over time, most notably *Escherichia coli* and
13 *Bacillus subtilis*.

14
15 An ideal bacterial chassis comprises a genome that encodes the basic biological functions
16 required for self-maintenance, growth and stress resistance, but is deleted of other cell structures
17 and signal processing components that divert resources into undesirable destinations. Under
18 natural circumstances, these functions allow microorganisms to interact with their environment,
19 but they may not be wanted in the synthetic biology laboratory or in biotechnological settings.
20 Such strains must also be robust and stable, and easily amenable to genetic manipulations, while
21 simultaneously having low spontaneous variability and physically strong envelopes that can
22 survive operating bioreactor conditions¹⁸. Having efficient transcription/translation systems and a
23 predictable metabolic background is desirable as well. Moreover, easy downstream processing
24 (i.e., recovery and purification of products and recycling or disposal of wastes) is undoubtedly
25 important.

26
27 Although biotechnology has historically dealt with a large variety of platform strains and cell
28 factories, starting with the production of acetone by *Clostridium acetobutylicum*¹⁹ in 1916, *E. coli*
29 has been the microorganism of choice for advanced genetic engineering since the onset of the
30 recombinant DNA era. Not surprisingly, the same bacterium has become the favourite choice for

1 most synthetic biology ventures²⁰. Yet, is this organism really the only bacterium that can be used
2 in both fundamental synthetic biology and applied biotechnology?

3
4 Nature has its own way of shaping bacteria to develop novel traits through which individuals and
5 populations can gain a competitive edge. One archetypal case is that of soil microorganisms that
6 encounter xenobiotic contaminants²¹. In this scenario, bacteria can evolve new metabolic routes
7 to use the new chemical(s) as carbon source(s). The process can be idealized as the encounter
8 of a genetic landscape with a novel chemical landscape^{21,22}. Genotypic and metabolic innovation
9 in such natural settings seems to be elicited by both nutritional and physicochemical
10 environmental stresses²¹⁻²⁵. We argue that the synthetic biology community should take
11 advantage of this natural innovation. Although *E. coli* constitutes an optimal experimental model
12 to test and validate foundational technologies, large-scale deployment of synthetic biology-
13 derived activities calls for a choice of available hosts. The production of complex biofuels and
14 other added-value molecules as well as the biodegradation of recalcitrant chemicals often
15 proceed through the accumulation of intermediates that are toxic for *E. coli*²⁶. In this context, a
16 number of species of the genus *Pseudomonas* seem to fulfill many of the conditions required in
17 an ideal platform strain or microbial cell factory¹⁶⁻¹⁸.

18
19 In this Review we present the case that strains from the genus *Pseudomonas* (and specifically
20 *Pseudomonas putida*) are evolutionarily endowed with many of the properties that current
21 synthetic biology efforts are attempting to develop artificially in other microbial platforms.

22 23 **Why *Pseudomonas*?**

24
25 The genus *Pseudomonas* encompasses a large number (>200 and growing²⁷) of Gram-negative,
26 aerobic γ -proteobacterial species²⁸. The name derives from *pseudes* ($\psi\epsilon\upsilon\delta\epsilon\varsigma$, *false*) and *monas*
27 ($\mu\omicron\nu\acute{\alpha}\varsigma$, *single unit*), and was proposed to broadly designate a genus of non-sporulating rod-
28 shape bacteria with polar flagella²⁹. Although the sequencing of 16S rRNA genes and
29 biochemical tests have helped to distinguish members within the group, the pseudomonad
30 classification criteria are still controversial³⁰. Relevant features shared by many *Pseudomonas*
31 species include their ability to adapt to different physicochemical and nutritional niches, their

1 remarkable capacity to endure both endogenous and exogenous stresses, and their ability to
2 synthesize a large number of bioactive compounds (including antibiotics³¹). These traits are
3 encoded by 1,491 genes³², common to all *Pseudomonas* species sequenced so far.

4
5 That many *Pseudomonas* species are human and plant pathogens can be traced to the
6 production of a suite of virulence factors³³ and the ability of these bacteria to evolve quickly³⁴,
7 which enables the development of resistance to numerous antibiotics. A large number of
8 metabolic genes have been identified as key factors involved in the virulence of some
9 *Pseudomonas* species. In particular, the distinct arrangement of glycolytic pathways in this class
10 of bacteria³⁵ was recognized early for its medical implications. For example, alginate, the viscous
11 exopolysaccharide produced by *P. aeruginosa* and known to be a virulence factor in lung
12 infections of cystic fibrosis patients, has its origins in central metabolic intermediates³⁶. The
13 oxidative stress that *P. aeruginosa* may experience during colonization of host tissues is
14 counteracted by the activity of central pathways *via* the generation of reducing equivalents³⁷. In
15 fact, a genome-wide metabolic model of *P. aeruginosa*³⁸ suggested that the prevalence of the
16 Entner-Doudoroff and pentose phosphate pathways for carbohydrate consumption is an
17 important metabolic asset for infection (**Fig. 3**). This tradeoff, shared by virtually all
18 pseudomonads and involving a high NADPH regeneration rate³⁹, allows the evolution and
19 expression of biochemical pathways in these microorganisms that would be barely tolerated by
20 other bacterial species³⁷. The presence of multiple membrane-bound pumps involved in antibiotic
21 extrusion in virulent strains^{40,41} also has a beneficial effect in non-pathogenic strains, as reflected
22 in the presence of solvent-tolerance efflux pumps⁴².

23
24 Interestingly however, the same metabolic vigor that makes some strains virulent makes others
25 suitable to host and evolve new chemical reactions³⁷. The *Pseudomonas* strains that could
26 potentially be used as platform strains and cell factories in various biotechnological settings are
27 those that retain the inherent beneficial traits (e.g., catalytic vigor) while lacking undesirable traits
28 (e.g., virulence factors). The branch of the *Pseudomonas* family that is most appealing in this
29 regard is centered in the non-pathogenic *P. putida* species. A number of relevant qualities shared
30 by members of this group are discussed below.

General characteristics of *P. putida*

P. putida has been a laboratory model for examining the lifestyle and activities of environmental bacteria^{43,44}. This microorganism made headlines in the late 1970s through the Diamond vs. Chakrabarty patent case⁴⁵ as a bacterium capable of breaking down oil and thus attractive for bioremediation of petroleum spills. Some isolates colonize the rhizosphere⁴⁶ and establish beneficial relationships with plants⁴⁷ by feeding on root exudates and releasing siderophores, antibiotics and biosurfactants^{48,49}. Several environmental strains of *P. putida* can mineralize aromatic hydrocarbons, chloro- and nitro-organic compounds, pesticides, herbicides, and even explosive chemicals⁵⁰. In connection with this trait, this bacterium served as an excellent host to explore inconspicuous reactions in metagenomic libraries. The use of *P. putida* as a host facilitated the implementation of gene traps^{8,51} as a screening method to expose biodegradation processes, by taking advantage of the responsiveness of transcriptional factors to a variety of effectors (**BOX 1**). The extant metabolic diversity of *P. putida*⁵⁰ also includes a metabolic profile, that is likely to change when cells grow in close proximity to other species⁵². This phenomenon, well accredited in *P. aeruginosa*⁵³, suggests the existence of a cryptic metabolic complement, which is expressed under specific circumstances, and enables the use of diffusible metabolites as communication signals to be exploited for engineering inter-cellular communication⁵⁴.

P. putida is often able to withstand exposure to high levels of organic solvents, making it appealing as a biocatalyst for biotransformations in two-phase systems^{39,55} (i.e., fermentations in which the substrates and the products partition between an aqueous and a non-aqueous immiscible phase). In the late 1980s, Horikoshi's group⁵⁶ showed that an isolate of *Pseudomonas* spp. (later classified as *P. putida*) thrived in the presence of toluene at concentrations up to 50% (v/v). Being an aromatic and highly noxious chemical, toluene kills most microorganisms at concentrations as low as 0.1% (v/v). Other *P. putida* strains able to endure high levels of the same chemical in the medium were also reported^{57,58}. How does *P. putida* thrive under such adverse conditions? Much of this tolerance can be traced to the action of efflux pumps that extrude toluene (and possibly other solvents) to the external medium⁵⁹. The effect of pumps is complemented by other physiological changes⁵⁹. Exposure to organic solvents also induces protein unfolding, stimulating a general response mediated by extra-cytoplasmic σ factors that

1 trigger the expression of molecular chaperones and other stress resistance proteins⁶⁰. Again,
2 these features expose the genetic pre-programming of *P. putida* to deal with a hydrophobic
3 milieu, which is desirable for some types of biocatalysis. Ideally, solvent tolerance in a given
4 biotransformation is paralleled by resistance to the toxic effects caused by the products and/or
5 intermediates. In this respect, it is worth mentioning the high butanol tolerance of some
6 pseudomonads⁶¹; not surprisingly, the expression of genes for butanol biosynthesis from *C.*
7 *acetobutylicum* in *P. putida*⁶² resulted in alcohol titers up to 120 mg/L.

8
9 One final feature of *P. putida* that makes it an interesting choice for the downstream applications
10 of synthetic biology is the ability of this species to react to an excess of carbon with respect to
11 other essential elements (e.g., nitrogen or phosphorus) by accumulating intracellular polyester
12 granules as storage material^{63,64}. The biotechnological interest of these compounds stem from
13 their plastic properties, biodegradability, and the broad range of qualities arising from the side
14 chains present in the polymers. In fact, polyhydroxyalkanoates have the potential to replace
15 conventional, oil-derived plastics in several packaging and coating applications⁶⁵. While most
16 polymer-producing microorganisms (including recombinant *E. coli*⁶⁶) accumulate short-chain
17 variants [e.g., poly(3-hydroxybutyrate)], *P. putida* can incorporate linear and branched C6 to C16
18 monomers of 3-hydroxy fatty acids in the chains, thereby producing medium side-chain
19 polyhydroxyalkanoates^{67,68}. Moreover, the coupling of biopolymer accumulation and degradation
20 plays a key role in the overall carbon balance of *P. putida*⁶⁹. Medium side-chain polymers show
21 enhanced elastomeric properties compared to short-side-chain counterparts, also enabling
22 chemical derivatization of the bioplastic. Tailor-made biopolymer production using *P. putida* thus
23 affords a multi-level optimization approach, encompassing not only the engineering of the *chassis*
24 and the key enzymes (e.g., biopolymer synthases), but also the design of fermentation strategies
25 and the introduction of chemical modifications^{70,71}.

26 27 ***P. putida* KT2440**

28
29 The most prominent *P. putida* strain is called KT2440 (KT standing for Kenneth Timmis, who,
30 over the years, characterized this specimen⁷²). This bacterium is a derivative of an environmental
31 isolate named *Pseudomonas arvilla* mt-2⁷³, which was reported to express an enzyme cleaving

the aromatic ring of *m*-toluate⁷⁴. It was later discovered that strain mt-2 harbours the TOL plasmid pWW0, which encodes a pathway for toluene and *m*-/*p*-xylene biodegradation⁷⁵. Strain KT2440 was one of the first bacteria to be certified as a biosafety strain for recombinant DNA experiments and applications, and it is officially classified as GRAS⁷⁶ (*generally regarded as safe*). Inspection of its ca. 6.2 Mb genome⁷⁷ has not only revealed the basis of many of its known characteristics but also exposed some unanticipated properties. One remarkable trait is the presence of >80 genes encoding oxido-reductases⁷⁸, which enable growth on a wide repertoire of aromatic substrates. At least five complete biodegradation pathways encoded in the chromosome have been characterized, which can proceed through β -ketoadipate, phenylacetyl-coenzyme A, homogentisate, gentisate, and homoprotocatechuate as metabolic intermediates⁵⁰. In addition, *P. putida* isolates similar to KT2440 have been found to carry plasmids mediating the breakdown of naphthalene, 4-chloronitrobenzene, 2,4-xyleneol, and phenol⁴⁹.

A closer look at the metabolic properties of this bacterium helps to understand why it can host such variety of biodegradation pathways (**Fig. 4**). *P. putida* KT2440, as do the majority of heterotrophic bacteria, oxidizes organic compounds through peripheral routes that eventually convert these substrates into glycolytic and tricarboxylic acid cycle intermediates. However, glycolysis in *P. putida* has a number of unique features. Several of the oxidative steps are coupled to the reduction of NADP⁺ to NADPH, used to generate anabolic precursors needed for bacterial growth and exploited as antioxidant currency under exogenous and endogenous stressful conditions^{37,79,80}. To help understand these pathways, the first metabolic model of the strain KT2440 was published in 2008⁸¹, and was quickly followed by other two variants^{82,83}. These models have been refined over the years, and have been instrumental in taking steps towards domestication of this microorganism – although expert knowledge is also needed to interpret and implement *in silico* predictions⁷⁹. To date, the most complete metabolic network proposed for KT2440 spans 1,071 reactions and 1,044 metabolites, related to 900 genes. Model-based predictions of *in silico* phenotypes were recently refined by the integration of experimental data on the stoichiometric demands for anabolism and cellular maintenance⁸⁴, enhancing the predictive power of computational design, and data from deep RNA sequencing experiments^{85,86}. Additionally, a comprehensive interaction database of *P. putida* KT2440 has been generated from

three protein-protein interaction methods (*PutidaNET*)⁸⁷, further expanding our understanding of the fundamental biology of this strain.

Getting genes in and out

The roadmap from a naturally occurring bacterium with interesting properties to a reliable and predictable *chassis* that can also deliver on the biotechnological promise of SynBio involves many steps. The challenges include the removal of non-desirable traits, the enhancement of advantageous traits, the insertion of new activities, and the establishment of a language for communicating instructions (receiving, if possible, some feedback in real time). Overcoming these challenges involves genetic manipulations that range in complexity from simple single-gene deletions and/or insertions to the implementation of complex genetic and metabolic circuits.

Deleting genes

The extant, single chromosome of *P. putida* KT2440 encodes 5,481 open reading frames, 76.9% of which have an accredited function, 19.1% of which determine hypothetical proteins, and 4% of which encode products that are not represented in the databases^{77,88}. A genetic toolbox for cutting and pasting both small and large genomic segments as well as executing allelic replacements and stably inserting new DNA segments in the target chromosome is crucial. Although the literature reports a large number of vectors for making site-directed deletions and mutations in Gram-negative microorganisms⁸⁹, many are sub-optimal for making multiple directed changes in the genome of *P. putida* KT2440. Part of the problem stems from the relatively poor frequencies of DNA homologous recombination in this bacterium⁹⁰. Fortunately, adaptation of the chromosome editing tools developed by Pósfai and colleagues to reduce the genomic size of *E. coli*⁹¹ has resulted in a virtually fail-proof method for making seamless deletions in strain KT2440^{92,93}. Using this methodology, a non-motile variant of *P. putida* KT2440 was constructed, in which a ca. 70 kb region (i.e., 1.1% of the genome) spanning the genes needed for flagellar export and assembly had been deleted⁹⁴. This attempt towards the implementation of a reduced-genome *P. putida* KT2440 as microbial cell factory resulted in a number of physiological traits that were enhanced in the non-flagellated mutant, such as increases in the adenylate energy charge

1 and NADPH/NADP⁺ ratio. Separate efforts have been made to reduce the size of the *P. putida*
2 KT2440 genome by random (as opposed to targeted) deletions of large chromosomal regions.
3 While ~7% of the genome has been erased with such procedure⁹⁵, this approach might be limited
4 by the scattering of essential genes through the whole chromosome.

6 *Inserting genes*

8 What is the best procedure to insert new genes into a bacterium? Plasmid vectors have been,
9 and still are, the workhorses for genetic engineering of microorganisms at the laboratory scale,
10 although they are not exempt from problems when used in industrial and environmental settings.
11 Although broad-host-range vectors applicable to *P. putida* have been available since the
12 beginning of the recombinant DNA era⁹⁶, only recently has the architecture of such tools been
13 subject to standardization efforts (**BOX 2**). One example of the power of adopting formatted
14 plasmid vectors in *P. putida* is the recent engineering of a strain to biodegrade the environmental
15 pollutant 1,3-dichloroprop-1-ene under conditions with limited oxygen supply⁹⁷. This example
16 showed the adequacy of using synthetic biology tools to domesticate *P. putida* KT2440, as this
17 bacterium is not naturally endowed neither with the ability to thrive in the absence of oxygen nor
18 the functions needed to mineralize 1,3-dichloroprop-1-ene. In other cases, the objective is not
19 only the acquisition of heterologous DNA fragments but also their stable integration in the
20 chromosome. One possibility is to use the same recombination-based genome editing tools, like
21 the ones mentioned above, but such procedures can be time-consuming. An alternative strategy
22 is the use of Tn5-based and Tn10-based mini-transposon vectors⁹⁸⁻¹⁰⁰, which can deliver multiple
23 DNA segments of unlimited size to the genome of *P. putida* as part of a hybrid mobile element.
24 The downsides of this technique reside in the *a priori* uncertainty of the insertion site, which might
25 affect expression levels because of the uneven distribution of transcriptionally active segments
26 within the bacterial genome¹⁰¹. But this same problem becomes an asset if the user wishes to
27 explore various levels of expression of the same gene(s) through random insertion of the
28 engineered transposon into the target genome¹⁰². Should this not be the case, other genetic
29 systems can be used to direct insertions into specific chromosomal sites. A large number of
30 transposon vectors are available, exploiting the preference of Tn7 to target cognate, single *att*Tn7
31 sequences that are naturally present^{103,104} or can be engineered¹⁰⁵ into the bacterial genome of

choice. A more sophisticated alternative, particularly for very large DNA fragments, involves the exploitation of the integrase activity of an integrative/conjugative element¹⁰⁶, which delivers the DNA of interest into one or more specific sites of the target chromosome. As a whole, the tools listed above allow to conduct any genomic manipulation in *P. putida* KT2440, paving the way towards rational domestication. The next step would be enabling the user to deliver instructions to a biological receiver and get some feedback in return, during or after implanting the desired functionality.

Engineering communication in bacteria

Bacteria can sense physicochemical signals and translate them into specific responses, which most often involve turning on and off gene expression. Promoters controlled by transcription factors (TFs) responsive to more or less natural inducers¹⁰⁷ (e.g., IPTG, arabinose, or tetracycline) have been used to drive the expression of heterologous genes. Such manipulations, which subvert the natural functions of the TF/promoter pairs, signify a primitive yet useful way of programming cells to follow specific orders. The number of such regulatory pairs applicable to *P. putida* is enormous, and many are located within mobile elements (i.e., plasmids and transposons) bearing xenobiotic biodegradation pathways⁵⁰. The promiscuous nature of such itinerant operons forces the encoded enzymes and regulatory elements to evolve to work well in different hosts (i.e., they are to some extent orthogonal). On this basis, environmental genomes and metagenomes are a rich source of TF/promoter pairs that respond to the most diverse repertoire of chemical effectors. Just to name a few, the collection of inducible expression systems operational in *P. putida* KT2440 include those responding to xylenes (XylR^{108,109}), *m*-toluate (XylS¹⁰⁹), salicylate (NahR¹⁰⁹), and short-chain alkanes (AlkS¹¹⁰).

Creating a functional communication system involves combining various regulatory events and types of commands that, at some point, result in a deterministic decision. Bacterial promoters can be faithfully described with Boolean formalisms under which each regulatory event results from the action of binary gates computing up to two inputs into a single output. Such logic networks help explaining the architecture and connectivity of naturally occurring regulatory circuits in *P. putida* (e.g., the above mentioned TOL system¹¹¹). The similarity of transcriptional networks to

1 electronic circuits also stimulated the design of logic gates artificially assembled with bacterial
2 regulatory parts. These devices process specific signals and can be combined with others to
3 implement simple computations (e.g., toggle switches, pulse generators, oscillators, and memory
4 devices). Although they have largely been implemented in *E. coli* thus far, they are also
5 applicable to *P. putida*, and this is a fertile area of research at the present time. Such
6 manipulations are not restricted to TF/promoter pairs, but also allow entering new inputs into TFs
7 and housekeeping proteins (e.g., GroEL¹¹²) by engineering protease-cleaving sites in their
8 structure and thereby modifying their logic behaviour¹¹³.

9
10 Communication is a two-way process: how can researchers get some feedback on the fate of
11 their orders to bacterial cell factories? Visual inspection of cells often provides little information on
12 their physiological or catalytic performance. A useful approach is the adoption of protein-based,
13 TF-based and RNA-based intracellular sensors of specific physiological descriptors coupled to
14 optical reporters. In combination, the simultaneous quantitative monitoring of a number of
15 parameters would allow the user to diagnose the performance of an engineered process. A few *in*
16 *vivo* flux-sensing¹¹⁴ and metabolite-sensing^{115,116} devices are available for *E. coli*. Functional
17 variants of these systems adapted to pseudomonads would be a welcome addition to the
18 repertoire of biosensors already available for these bacteria^{51,115,117}.

19 20 *Programming population behaviour*

21
22 A further step in designing rational *P. putida*-user interactions is engineering chemical signaling.
23 Quorum sensing agents have been used for this purpose¹¹⁸, but the choice of useful molecules is
24 limited. Once more, the biodegradation pathways present in *P. putida* are sources of parts and
25 devices that can be re-factored for programming cell-to-cell communications. An example in this
26 sense is a sender *P. putida* strain, harboring the TOL pathway, which processed environmental
27 toluene as the input cue and generated benzoate as the output signal¹¹⁹. Diffusion of benzoate
28 was sensed by a second, receiver *P. putida* strain that processed benzoate as the input to
29 produce a visual output. Given the large number of possible enzyme/TF/inducer combinations,
30 the opportunities for designing complex population performance are huge. The physical
31 distribution of bacteria in specific physicochemical niches can also be engineered. *P. putida*

KT2440 forms biofilms on abiotic surfaces, the shape of which is determined by the physiological condition of cells, nutrient availability, the surface chemistry of the growth substrate, and nutrient gradients. Catalytic biofilms have been explored with *Pseudomonas* strains by comparing their redox capabilities for *n*-octanol and (S)-styrene oxide production in planktonic growth (stirred tank) and on a solid substrate (membrane reactor)¹²⁰. Biofilm-based systems at an industrial scale could become a realistic alternative to traditional bioprocesses for the bioproduction of fine chemicals.

The challenges ahead

Despite all the potential of *P. putida* discussed above, the reality is that when a promising genomic chassis is combined with a well defined genetic or metabolic implant, the outcome of the combination may not be entirely anticipated. While some genome-edited laboratory strains of *E. coli* are predictable to an extent¹²¹ and current technologies allow the synthesis of DNA sequences the size of a small bacterial genome¹²², the lack of understanding of many basic biological facts (e.g., the interplay between the genetic program and the metabolic network³⁷) is still a considerable hurdle in synthetic biology. In the meantime, how far can we progress in rational design of biological systems? Although individual circuits with a small number of components can be engineered to an acceptable degree of predictability, their connection and interplay within an existing molecular landscape often result in a level of complexity that cannot currently be designed from first-principles. Instead, fine-tuning the inputs to implanted devices with the corresponding outputs needs what we call *biological string-weight engineering*. This term originates in non-mathematical methods used to find optimal parameters for distributing weights in a complex structure by upending a string model of the structure with weights attached at critical heights (this method was used by the Spanish architect Antoni Gaudí¹²³) (**BOX 3**). Nature itself (through gravity in this case) provides the solution to an intricate multi-scale problem that could not be solved using available formalisms. This affords the blending of forward engineering (i.e., the rational assembly of components to the point allowed by current technology) with gravitation towards functional optimality. Within this conceptual framework, the connections among components of a pre-assembled biological device can be allowed to explore a variety of input/output transfer functions¹¹⁷. To this end, the objective is set and the circuitry left to explore

1 *in vivo* the combination of parameters that result in the desired property (e.g., a relevant
2 phenotype). *String-weight engineering* may include *directed evolution* steps to enable exploration
3 of the parameter space by the pre-designed system.

4
5 Once a given optimum is reached, the ultimate challenge lies in protecting the structure from
6 changes and eventual collapse. Evolvability, an inherent property of DNA-based biological
7 systems, is possibly the main obstacle to the engineering of reliable living objects. Some *in vivo*
8 approaches have been proposed to avoid the occurrence of mutations, mostly by increasing
9 genomic stability. For instance, it is possible to counter-select variations in specific DNA
10 sequences by interfacing a conditional lethality circuit with DNA mismatch repair systems¹²⁴.
11 Again, Nature offers remarkable examples of bacteria endowed with lifestyle that oscillate
12 between high- and low-mutation regimes (e.g., *P. aeruginosa*¹²⁵ and *Buchnera aphidicola*¹²⁶),
13 from which we can take inspiration. Chassis programmed to switch between a high and a low-to-
14 none evolutionary regime would constitute excellent frames for designing reliable cell factories.
15 Such objective can be targeted at through the transient expression of a hypermutator
16 phenotype¹²⁷ and the conditional inactivation of the SOS system (therefore implementing the
17 string-weight engineering concept pictured above).

18 19 **Outlook**

20
21 One key endeavour in contemporary synthetic biology is quest of an optimal and realistic
22 genomic chassis for industrial, large-scale applications¹⁸. While synthetic life forms can be
23 entertained that will be altogether programmed *à la carte* in the future for a suite of uses
24 (<http://www.theguardian.com/science/2013/oct/13/craig-ventner-mars>) such all-designed
25 biological objects are unlikely to reach the existing bioindustry anytime soon. In the short term,
26 synthetic biology agents will necessarily merge desirable pre-existing traits (the basis of which
27 might not be fully understood at the time) with rationally engineered implants. It thus makes sense
28 to adopt as the starting point bacteria that Nature has already selected to thrive under many of
29 the demanding conditions that frame industrial operations. Getting to the point where bacteria can
30 be designed with complete reliability still needs clarification of much of their fundamental biology,
31 in particular the interplay between metabolism and genetic stability – as well as a better

understanding of live systems as information-processing chemical machines. Whereas *E. coli*¹²¹ and *Mycoplasma*¹²² are most useful models for addressing such fundamental questions, the industrial performance of these bacteria is limited by their inherent genetic program that reflects their original ecological niches. Other species notwithstanding, we argue that *P. putida* counts among those that hold a superior value in the way towards designer whole-cell catalysts – including both upstream (i.e., biochemical reactions) and downstream (i.e., product recovery) processes. It is likely that, at least for a while, such synthetic biology agents will show more resemblance to bacterial *cyborgs* (i.e., natural properties merged with implanted traits) than *robots* (i.e., systems entirely engineered from first principles).

References

1. McPherson, M.J. & Parish, J.H. Applications of recombinant DNA in biotechnology. *Nat. Prod. Rep.* **4**, 205-224 (1987).
2. Malik, V.S. Biotechnology - the golden age. *Adv. Appl. Microbiol.* **34**, 263-306 (1989).
3. Ramos, J.L. *et al.* The behavior of bacteria designed for biodegradation. *Biotechnology* **12**, 1349-1356 (1994).
4. Pieper, D.H. & Reineke, W. Engineering bacteria for bioremediation. *Curr. Opin. Biotechnol.* **11**, 262-270 (2000).
5. Janssen, D.B., Dinkla, I.J., Poelarends, G.J. & Terpstra, P. Bacterial degradation of xenobiotic compounds: evolution and distribution of novel enzyme activities. *Environ. Microbiol.* **7**, 1868-1882 (2005).
6. Ferrer, M., Beloqui, A., Timmis, K.N. & Golyshin, P.N. Metagenomics for mining new genetic resources of microbial communities. *J. Mol. Microbiol. Biotechnol.* **16**, 109-123 (2009).
7. Dietrich, J.A., McKee, A.E. & Keasling, J.D. High-throughput metabolic engineering: advances in small-molecule screening and selection. *Annu. Rev. Biochem.* **79**, 563-590 (2010).
8. Galvão, T.C., Mohn, W.W. & de Lorenzo, V. Exploring the microbial biodegradation and biotransformation gene pool. *Trends Biotechnol.* **23**, 497-506 (2005).
9. Tuffin, M., Anderson, D., Heath, C. & Cowan, D.A. Metagenomic gene discovery: How far have we moved into novel sequence space? *Biotechnol. J.* **4**, 1671-1683 (2009).

10. Purnick, P.E. & Weiss, R. The second wave of synthetic biology: from modules to systems. *Nat. Rev. Mol. Cell Biol.* **10**, 410-422 (2009).
11. Keasling, J.D. Synthetic biology for synthetic chemistry. *ACS Chem. Biol.* **3**, 64-76 (2008).
12. Endy, D. Foundations for engineering biology. *Nature* **438**, 449-453 (2005).
- A seminal contribution in the synthetic biology field, setting the basis for many of the current tenets within the discipline.**
13. Danchin, A. Scaling up synthetic biology: Do not forget the chassis. *FEBS Lett.* **586**, 2129-2137 (2012).
- An enlightening discussion on the somewhat neglected issue of choosing an appropriate chassis for synthetic biology endeavors.**
14. de Lorenzo, V. Beware of metaphors: chassis and orthogonality in synthetic biology. *Bioeng. Bugs* **2**, 3-7 (2011).
15. Wheeler, M.B. & Campion, D.R. Animal production – A longstanding biotechnological success. *Am. J. Clin. Nutr.* **58**, 276S-281S (1993).
16. Sauer, M. & Mattanovich, D. Construction of microbial cell factories for industrial bioprocesses. *J. Chem. Technol. Biotechnol.* **87**, 445-450 (2012).
17. Schmid, A. *et al.* Industrial biocatalysis today and tomorrow. *Nature* **409**, 258-268 (2001).
18. Foley, P.L. & Shuler, M.L. Considerations for the design and construction of a synthetic platform cell for biotechnological applications. *Biotechnol. Bioeng.* **105**, 26-36 (2010).
19. Dürre, P. New insights and novel developments in clostridial acetone/butanol/isopropanol fermentation. *Appl. Microbiol. Biotechnol.* **49**, 639-648 (1998).
20. Karlsson, E.N., Johansson, L., Holst, O. & Lidén, G. in *The metabolic pathway engineering handbook (Fundamentals)* (ed. Smolke, C.D.) 21.21-21.23 (CRC Press, Boca Raton, FL, 2010).
21. Copley, S.D. Evolution of efficient pathways for degradation of anthropogenic chemicals. *Nat. Chem. Biol.* **5**, 559-566 (2009).
22. de Lorenzo, V. Systems biology approaches to bioremediation. *Curr. Opin. Biotechnol.* **19**, 579-589 (2008).
23. Kivisaar, M. Mechanisms of stationary-phase mutagenesis in bacteria: mutational processes in pseudomonads. *FEMS Microbiol. Lett.* **312**, 1-14 (2010).

24. Pérez-Pantoja, D., Nikel, P.I., Chavarría, M. & de Lorenzo, V. Endogenous stress caused by faulty oxidation reactions fosters evolution of 2,4-dinitrotoluene-degrading bacteria. *PLoS Genet.* **9**, e1003764 (2013).
- This article exposes the role of oxidative stress caused by the biological oxidation of aromatic compounds as the driving force for the evolution of biodegradation pathways.**
25. Nikel, P.I., Pérez-Pantoja, D. & de Lorenzo, V. Why are chlorinated pollutants so difficult to degrade aerobically? Redox stress limits 1,3-dichloroprop-1-ene metabolism by *Pseudomonas pavonaceae*. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **368**, 20120377 (2013).
26. Imlay, J.A. in *EcoSal - Escherichia coli and Salmonella: Cellular and molecular biology* (eds. Böck, A. *et al.*) (ASM Press, Washington, D.C., 2009).
27. Euzéby, J.P. List of bacterial names with standing in nomenclature: a folder available on the internet. *Internatl. J. Syst. Evol. Microbiol.* **47**, 590-592 (1997).
28. Palleroni, N.J. in *Bergey's Manual of Systematic Bacteriology* (ed. Krieg, N.R.) 141-199 (Williams & Wilkins, Baltimore, MD, 1984).
29. Palleroni, N.J. The *Pseudomonas* story. *Environ. Microbiol.* **12**, 1377-1383 (2010).
30. Peix, A., Ramírez-Bahena, M.H. & Velázquez, E. Historical evolution and current status of the taxonomy of genus *Pseudomonas*. *Infect. Genet. Evol.* **9**, 1132-1147 (2009).
31. Gurney, R. & Thomas, C.M. Mupirocin: biosynthesis, special features and applications of an antibiotic from a gram-negative bacterium. *Appl. Microbiol. Biotechnol.* **90**, 11-21 (2011).
32. Loper, J.E. *et al.* Comparative genomics of plant-associated *Pseudomonas* spp.: insights into diversity and inheritance of traits involved in multitrophic interactions. *PLoS Genet.* **8**, e1002784 (2012).
33. Balasubramanian, D., Schneper, L., Kumari, H. & Mathee, K. A dynamic and intricate regulatory network determines *Pseudomonas aeruginosa* virulence. *Nucleic Acids Res.* **41**, 1-20 (2013).
34. Rodríguez-Rojas, A., Oliver, A. & Blázquez, J. Intrinsic and environmental mutagenesis drive diversification and persistence of *Pseudomonas aeruginosa* in chronic lung infections. *J. Infect. Dis.* **205**, 121-127 (2012).
35. Fuhrer, T., Fischer, E. & Sauer, U. Experimental identification and quantification of glucose metabolism in seven bacterial species. *J. Bacteriol.* **187**, 1581-1590 (2005).

- 1 36. May, T.B. & Chakrabarty, A.M. *Pseudomonas aeruginosa*: genes and enzymes of alginate
2 synthesis. *Trends Microbiol.* **2**, 151-157 (1994).
- 3 37. de Lorenzo, V. From the selfish gene to selfish metabolism: Revisiting the central dogma.
4 *BioEssays.* **36**: 226-235 (2014).
- 5 38. Oberhardt, M.A., Puchalka, J., Fryer, K.E., Martins dos Santos, V.A.P. & Papin, J.A.
6 Genome-scale metabolic network analysis of the opportunistic pathogen *Pseudomonas*
7 *aeruginosa* PAO1. *J. Bacteriol.* **190**, 2790-2803 (2008).
- 8 39. Blank, L.M., Ionidis, G., Ebert, B.E., Bühler, B. & Schmid, A. Metabolic response of
9 *Pseudomonas putida* during redox biocatalysis in the presence of a second octanol phase.
10 *FEBS J.* **275**, 5173-5190 (2008).
- 11 40. Aires, J.R., Köhler, T., Nikaido, H. & Plésiat, P. Involvement of an active efflux system in the
12 natural resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicrob. Agents*
13 *Chemother.* **43**, 2624-2628 (1999).
- 14 41. Vettoretti, L. *et al.* Efflux unbalance in *Pseudomonas aeruginosa* isolates from cystic fibrosis
15 patients. *Antimicrob. Agents Chemother.* **53**, 1987-1997 (2009).
- 16 42. Udaondo, Z. *et al.* Analysis of solvent tolerance in *Pseudomonas putida* DOT-T1E based on
17 its genome sequence and a collection of mutants. *FEBS Lett.* **586**, 2932-2938 (2012).
- 18 43. Timmis, K.N. *Pseudomonas putida*: a cosmopolitan opportunist *par excellence*. *Environ.*
19 *Microbiol.* **4**, 779-781 (2002).
- 20 44. Poblete-Castro, I., Becker, J., Dohnt, K., Martins dos Santos, V.A.P. & Wittmann, C.
21 Industrial biotechnology of *Pseudomonas putida* and related species. *Appl. Microbiol.*
22 *Biotechnol.* **93**, 2279-2290 (2012).
- 23 45. Kevles, D.J. Ananda Chakrabarty wins a patent: biotechnology, law, and society. *Hist. Stud.*
24 *Phys. Biol. Sci.* **25**, 111-135 (1994).
- 25 46. Wu, X. *et al.* Comparative genomics and functional analysis of niche-specific adaptation in
26 *Pseudomonas putida*. *FEMS Microbiol Rev* **35**, 299-323 (2011).
- 27 47. Matilla, M.A. *et al.* Complete genome of the plant growth-promoting rhizobacterium
28 *Pseudomonas putida* BIRD-1. *J. Bacteriol.* **193**, 1290 (2011).
- 29 48. Roca, A. *et al.* Analysis of the plant growth-promoting properties encoded by the genome of
30 the rhizobacterium *Pseudomonas putida* BIRD-1. *Environ. Microbiol.* **15**, 780-794 (2013).

49. Silby, M.W., Winstanley, C., Godfrey, S.A., Levy, S.B. & Jackson, R.W. *Pseudomonas* genomes: diverse and adaptable. *FEMS Microbiol. Rev.* **35**, 652-680 (2011).
50. Jiménez, J.I., Miñambres, B., García, J.L. & Díaz, E. Genomic analysis of the aromatic catabolic pathways from *Pseudomonas putida* KT2440. *Environ. Microbiol.* **4**, 824-841 (2002).
- A comprehensive survey on the many biochemical mechanisms that enable *Pseudomonas putida* to grow on diverse carbon substrates.**
51. Mohn, W.W., Garmendia, J., Galvão, T.C. & de Lorenzo, V. Surveying biotransformations with *à la carte* genetic traps: translating dehydrochlorination of lindane (g-hexachlorocyclohexane) into *lacZ*-based phenotypes. *Environ. Microbiol.* **8**, 546-555 (2006).
52. Orphan, V.J. Methods for unveiling cryptic microbial partnerships in nature. *Curr. Opin. Microbiol.* **12**, 231-237 (2009).
53. Watrous, J. *et al.* Mass spectral molecular networking of living microbial colonies. *Proc. Natl. Acad. Sci. USA* **109**, E1743-1752 (2012).
54. Bacchus, W. & Fussenegger, M. Engineering of synthetic intercellular communication systems. *Metab. Eng.* **16**, 33-41 (2013).
55. Ishige, T., Honda, K. & Shimizu, S. Whole organism biocatalysis. *Curr. Opin. Chem. Biol.* **9**, 174-180 (2005).
56. Inoue, A. & Horikoshi, K. A *Pseudomonas* thrives in high concentrations of toluene. *Nature* **227**, 264-265 (1989).
- The very first report on the isolation of a *Pseudomonas* species highly tolerant to solvents.**
57. Ramos, J.L., Duque, E., Huertas, M.J. & Haidour, A. Isolation and expansion of the catabolic potential of a *Pseudomonas putida* strain able to grow in the presence of high concentrations of aromatic hydrocarbons. *J. Bacteriol.* **177**, 3911-3916 (1995).
58. Weber, F.J., Ooijskaas, L.P., Schemen, R.M., Hartmans, S. & de Bont, J.A. Adaptation of *Pseudomonas putida* S12 to high concentrations of styrene and other organic solvents. *Appl. Environ. Microbiol.* **59**, 3502-3504 (1993).
59. Ramos, J.L. *et al.* Mechanisms of solvent tolerance in Gram-negative bacteria. *Annu. Rev. Microbiol.* **56**, 743-768 (2002).

- 1 60. Domínguez-Cuevas, P., González-Pastor, J.E., Marqués, S., Ramos, J.L. & de Lorenzo, V.
2 Transcriptional tradeoff between metabolic and stress-response programs in *Pseudomonas*
3 *putida* KT2440 cells exposed to toluene. *J. Biol. Chem.* **281**, 11981-11991 (2006).
- 4 61. Rühl, J., Schmid, A. & Blank, L.M. Selected *Pseudomonas putida* strains able to grow in the
5 presence of high butanol concentrations. *Appl. Environ. Microbiol.* **75**, 4653-4656 (2009).
- 6 62. Nielsen, D.R. *et al.* Engineering alternative butanol production platforms in heterologous
7 bacteria. *Metab. Eng.* **11**, 262-273 (2009).
- 8 63. Luengo, J.M., García, B., Sandoval, A., Naharro, G. & Olivera, E.R. Bioplastics from
9 microorganisms. *Curr. Opin. Microbiol.* **6**, 251-260 (2003).
- 10 64. Anderson, A.J. & Dawes, E.A. Occurrence, metabolism, metabolic role, and industrial uses
11 of bacterial polyhydroxyalkanoates. *Microbiol. Rev.* **54**, 450-472 (1990).
- 12 65. Keshavarz, T. & Roy, I. Polyhydroxyalkanoates: bioplastics with a green agenda. *Curr. Opin.*
13 *Microbiol.* **13**, 321-326 (2010).
- 14 66. Nikel, P.I., de Almeida, A., Melillo, E.C., Galvagno, M.A. & Pettinari, M.J. New recombinant
15 *Escherichia coli* strain tailored for the production of poly(3-hydroxybutyrate) from
16 agroindustrial by-products. *Appl. Environ. Microbiol.* **72**, 3949-3954 (2006).
- 17 67. Gao, X., Chen, J.C., Wu, Q. & Chen, G.Q. Polyhydroxyalkanoates as a source of chemicals,
18 polymers, and biofuels. *Curr. Opin. Biotechnol.* **22**, 768-774 (2011).
- 19 68. Kim, Y., Kim, H.W., Chung, M.G. & Rhee, Y.H. Biosynthesis, modification, and
20 biodegradation of bacterial medium-chain-length polyhydroxyalkanoates. *J. Microbiol.* **45**,
21 87-97 (2007).
- 22 69. Arias, S., Bassas-Galia, M., Molinari, G. & Timmis, K.N. Tight coupling of polymerization and
23 depolymerization of polyhydroxyalkanoates ensures efficient management of carbon
24 resources in *Pseudomonas putida*. *Microb. Biotechnol.* **6**, 551-563 (2013).
- 25 70. Tripathi, L. *et al.* *Pseudomonas putida* KT2442 as a platform for the biosynthesis of
26 polyhydroxyalkanoates with adjustable monomer contents and compositions. *Bioresour.*
27 *Technol.* **142**, 225-231 (2013).
- 28 71. Escapa, I.F. *et al.* Disruption of β -oxidation pathway in *Pseudomonas putida* KT2442 to
29 produce new functionalized PHAs with thioester groups. *Appl. Microbiol. Biotechnol.* **89**,
30 1583-1598 (2011).

72. Regenhardt, D. *et al.* Pedigree and taxonomic credentials of *Pseudomonas putida* strain KT2440. *Environ. Microbiol.* **4**, 912-915 (2002).
73. Nakazawa, T. Travels of a *Pseudomonas*, from Japan around the world. *Environ. Microbiol.* **4**, 782-786 (2002).
74. Feist, C.F. & Hegeman, G.D. Regulation of the *meta* cleavage pathway for benzoate oxidation by *Pseudomonas putida*. *J. Bacteriol.* **100**, 1121-1123 (1969).
75. Williams, P.A. & Murray, K. Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida* (arvilla) mt-2: evidence for the existence of a TOL plasmid. *J. Bacteriol.* **120**, 416-423 (1974).
76. Federal Register. Appendix E. Certified host-vector systems. **47**, 17197 (1982).
77. Nelson, K.E. *et al.* Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ. Microbiol.* **4**, 799-808 (2002).
The completion of the genome sequencing enabled to understand and rationally explore the metabolic diversity and physiological properties of strain KT2440.
78. Martins dos Santos, V.A.P., Heim, S., Moore, E.R., Strätz, M. & Timmis, K.N. Insights into the genomic basis of niche specificity of *Pseudomonas putida* KT2440. *Environ. Microbiol.* **6**, 1264-1286 (2004).
79. Chavarría, M., Nikel, P.I., Pérez-Pantoja, D. & de Lorenzo, V. The Entner-Doudoroff pathway empowers *Pseudomonas putida* KT2440 with a high tolerance to oxidative stress. *Environ. Microbiol.* **15**, 1772-1785 (2013).
80. Ebert, B.E., Kurth, F., Grund, M., Blank, L.M. & Schmid, A. Response of *Pseudomonas putida* KT2440 to increased NADH and ATP demand. *Appl. Environ. Microbiol.* **77**, 6597-6605 (2011).
81. Nogales, J., Palsson, B.Ø. & Thiele, I. A genome-scale metabolic reconstruction of *Pseudomonas putida* KT2440: iJN746 as a cell factory. *BMC Syst. Biol.* **2**, 79 (2008).
This paper reports on the development of the first genome-wide metabolic model of strain KT2440 that has facilitated its launching as an useful microbial cell factory.
82. Puchałka, J. *et al.* Genome-scale reconstruction and analysis of the *Pseudomonas putida* KT2440 metabolic network facilitates applications in biotechnology. *PLoS Comput. Biol.* **4**, e1000210 (2008).

83. Sohn, S.B., Kim, T.Y., Park, J.M. & Lee, S.Y. *In silico* genome-scale metabolic analysis of *Pseudomonas putida* KT2440 for polyhydroxyalkanoate synthesis, degradation of aromatics and anaerobic survival. *Biotechnol. J.* **5**, 739-750 (2010).
84. van Duuren, J.B. *et al.* Reconciling *in vivo* and *in silico* key biological parameters of *Pseudomonas putida* KT2440 during growth on glucose under carbon-limited condition. *BMC Biotechnol.* **13**, 93 (2013).
85. Nikel, P.I., Kim, J. & de Lorenzo, V. Metabolic and regulatory rearrangements underlying glycerol metabolism in *Pseudomonas putida* KT2440. *Environ. Microbiol.* **16**, 239-254 (2014).
86. Kim, J., Oliveros, J.C., Nikel, P.I., de Lorenzo, V. & Silva-Rocha, R. Transcriptomic fingerprinting of *Pseudomonas putida* under alternative physiological regimes. *Environ. Microbiol. Rep.* **5**, 883-891 (2013).
87. Park, S.J. *et al.* PutidaNET: interactome database service and network analysis of *Pseudomonas putida* KT2440. *BMC Genomics* **10**, S18 (2009).
88. Winsor, G.L. *et al.* *Pseudomonas* Genome Database: improved comparative analysis and population genomics capability for *Pseudomonas* genomes. *Nucleic Acids Res.* **39**, D596-D600 (2011).
89. Schweizer, H.P. Vectors to express foreign genes and techniques to monitor gene expression in Pseudomonads. *Curr. Opin. Biotechnol.* **12**, 439-445 (2001).
90. Tavita, K. *et al.* Homologous recombination is facilitated in starving populations of *Pseudomonas putida* by phenol stress and affected by chromosomal location of the recombination target. *Mutat. Res.* **737**, 12-24 (2012).
91. Kolisnychenko, V. *et al.* Engineering a reduced *Escherichia coli* genome. *Genome Res.* **12**, 640-647 (2002).
92. Martínez-García, E. & de Lorenzo, V. Engineering multiple genomic deletions in Gram-negative bacteria: analysis of the multi-resistant antibiotic profile of *Pseudomonas putida* KT2440. *Environ. Microbiol.* **13**, 2702-2716 (2011).
- A method for the seamless deletion of genetic elements in *Pseudomonas* and other Gram-negative bacteria is presented in this contribution.**
93. Martínez-García, E. & de Lorenzo, V. Transposon-based and plasmid-based genetic tools for editing genomes of Gram-negative bacteria. *Methods Mol. Biol.* **813**, 267-283 (2012).

94. Martínez-García, E., Nikel, P.I., Chavarría, M. & de Lorenzo, V. The metabolic cost of flagellar motion in *Pseudomonas putida* KT2440. *Environ. Microbiol.* **16**, 291-303 (2014).
95. LePrince, A., de Lorenzo, V., Voller, P., van Passel, M.W. & Martins dos Santos, V.A.P. Random and cyclical deletion of large DNA segments in the genome of *Pseudomonas putida*. *Environ. Microbiol.* **14**, 1444-1453 (2012).
96. Bagdasarian, M. *et al.* Specific purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. *Gene* **16**, 237-247 (1981).
97. Nikel, P.I. & de Lorenzo, V. Engineering an anaerobic metabolic regime in *Pseudomonas putida* KT2440 for the anoxic biodegradation of 1,3-dichloroprop-1-ene. *Metab. Eng.* **15**, 98-112 (2013).
- A practical example on how synthetic biology tools can be used to change the entire lifestyle of a bacterium, in this case, moving the metabolism of *P. putida* towards anaerobiosis.**
98. de Lorenzo, V. & Timmis, K.N. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. *Methods Enzymol.* **235**, 386-405 (1994).
99. Martínez-García, E., Calles, B., Arévalo-Rodríguez, M. & de Lorenzo, V. pBAM1: an all-synthetic genetic tool for analysis and construction of complex bacterial phenotypes. *BMC Microbiol.* **11**, 38 (2011).
100. Nikel, P.I. & de Lorenzo, V. Implantation of unmarked regulatory and metabolic modules in Gram-negative bacteria with specialised mini-transposon delivery vectors. *J. Biotechnol.* **163**, 143-154 (2013).
101. Vora, T., Hottes, A.K. & Tavazoie, S. Protein occupancy landscape of a bacterial genome. *Mol. Cell* **35**, 247-253 (2009).
102. Sousa, C., de Lorenzo, V. & Cebolla, A. Modulation of gene expression through chromosomal positioning in *Escherichia coli*. *Microbiology* **143**, 2071-2078 (1997).
103. Choi, K.H. & Schweizer, H.P. mini-Tn7 insertion in bacteria with single *attTn7* sites: example *Pseudomonas aeruginosa*. *Nat. Protocols* **1**, 153-161 (2006).
104. Lambertsen, L., Sternberg, C. & Molin, S. Mini-Tn7 transposons for site-specific tagging of bacteria with fluorescent proteins. *Environ. Microbiol.* **6**, 726-732 (2004).

105. de las Heras, A., Carreño, C.A. & de Lorenzo, V. Stable implantation of orthogonal sensor circuits in Gram-negative bacteria for environmental release. *Environ. Microbiol.* **10**, 3305-3316 (2008).
106. Miyazaki, R. & van der Meer, J.R. A new large-DNA-fragment delivery system based on integrase activity from an integrative and conjugative element. *Appl. Environ. Microbiol.* **79**, 4440-4447 (2013).
107. Cases, I. & de Lorenzo, V. Promoters in the environment: transcriptional regulation in its natural context. *Nat. Rev. Microbiol.* **3**, 105-118 (2005).
108. Blatny, J.M., Brautaset, T., Winther-Larsen, H.C., Haugan, K. & Valla, S. Construction and use of a versatile set of broad-host-range cloning and expression vectors based on the RK2 replicon. *Appl. Environ. Microbiol.* **63**, 370-379 (1997).
109. de Lorenzo, V., Fernández, S., Herrero, M., Jakubzik, U. & Timmis, K.N. Engineering of alkyl- and haloaromatic-responsive gene expression with mini-transposons containing regulated promoters of biodegradative pathways of *Pseudomonas*. *Gene* **130**, 41-46 (1993).
110. Panke, S., de Lorenzo, V., Kaiser, A., Witholt, B. & Wubbolts, M.G. Engineering of a stable whole-cell biocatalyst capable of (S)-styrene oxide formation for continuous two-liquid-phase applications. *Appl. Environ. Microbiol.* **65**, 5619-5623 (1999).
111. Silva-Rocha, R., de Jong, H., Tamames, J. & de Lorenzo, V. The logic layout of the TOL network of *Pseudomonas putida* pWW0 plasmid stems from a metabolic amplifier motif (MAM) that optimizes biodegradation of *m*-xylene. *BMC Syst. Biol.* **5**, 191 (2011).
112. Billerbeck, S., Calles, B., Müller, C.L., de Lorenzo, V. & Panke, S. Towards functional orthogonalization of protein complexes: Individualization of GroEL monomers leads to distinct *quasi*-homogeneous single rings. *ChemBiochem* **14**, 2310-2321 (2013).
113. Calles, B. & de Lorenzo, V. Expanding the Boolean logic of the prokaryotic transcription factor XylR by functionalization of permissive sites with a protease-target sequence. *ACS Synth. Biol.* **2**, 594-603 (2013).
114. Kochanowski, K. *et al.* Functioning of a metabolic flux sensor in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **110**, 1130-1135 (2013).
115. Garmendia, J., de las Heras, A., Galvão, T.C. & de Lorenzo, V. Tracing explosives in soil with transcriptional regulators of *Pseudomonas putida* evolved for responding to nitrotoluenes. *Microb. Biotechnol.* **1**, 236-246 (2008).

- 1 116. Zhang, C., Wei, Z.H. & Ye, B.C. Quantitative monitoring of 2-oxoglutarate in *Escherichia coli*
2 cells by a fluorescence resonance energy transfer-based biosensor. *Appl. Microbiol.*
3 *Biotechnol.* **97**, 8307-8316 (2013).
- 4 117. de las Heras, A., Carreño, C.A., Martínez-García, E. & de Lorenzo, V. Engineering
5 input/output nodes in prokaryotic regulatory circuits. *FEMS Microbiol. Rev.* **34**, 842-865
6 (2010).
- 7 118. Basu, S., Gerchman, Y., Collins, C.H., Arnold, F.H. & Weiss, R. A synthetic multicellular
8 system for programmed pattern formation. *Nature* **434**, 1130-1134 (2005).
- 9 119. Silva-Rocha, R. & de Lorenzo, V. Engineering multicellular logic in bacteria with metabolic
10 wires. *ACS Synth. Biol.* In press, DOI: 10.1021/sb400064y (2014).
- 11 120. Gross, R., Buehler, K. & Schmid, A. Engineered catalytic biofilms for continuous large scale
12 production of *n*-octanol and (S)-styrene oxide. *Biotechnol. Bioeng.* **110**, 424-436 (2013).
- 13 121. Pósfai, G. *et al.* Emergent properties of reduced-genome *Escherichia coli*. *Science* **312**,
14 1044-1046 (2006).
- 15 122. Ma, S., Tang, N. & Tian, J. DNA synthesis, assembly and applications in synthetic biology.
16 *Curr. Opin. Chem. Biol.* **16**, 260-267 (2012).
- 17 123. Huerta, S. Structural design in the work of Gaudí. *Archit. Sci. Rev.* **49**, 324-339 (2006).
- 18 124. Ben Yehezkel, T., Biezuner, T., Linshiz, G., Mazor, Y. & Shapiro, E. Programmable *in vivo*
19 selection of arbitrary DNA sequences. *PLoS One* **7**, e47795 (2012).
- 20 125. Oliver, A., Cantón, R., Campo, P., Baquero, F. & Blázquez, J. High frequency of
21 hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* **288**, 1251-
22 1254 (2000).
- 23 126. van Ham, R.C. *et al.* Reductive genome evolution in *Buchnera aphidicola*. *Proc. Natl. Acad.*
24 *Sci. USA* **100**, 581-586 (2003).
- 25 127. Chou, H.H. & Keasling, J.D. Programming adaptive control to evolve increased metabolite
26 production. *Nature Comm.* **4**, 2595 (2013)
- 27 128. Shetty, R.P., Endy, D. & Knight, T.F. Engineering BioBrick vectors from BioBrick parts. *J.*
28 *Biol. Eng.* **2**, 5 (2008).
- 29 129. Anderson, J.C. *et al.* BglBricks: a flexible standard for biological part assembly. *J. Biol. Eng.*
30 **4**, 1 (2010).

- 1 130. Silva-Rocha, R. *et al.* The Standard European Vector Architecture (SEVA): a coherent
2 platform for the analysis and deployment of complex prokaryotic phenotypes. *Nucleic Acids*
3 *Res.* **41**, D666-D675 (2013).
- 4 **Building on the standardization premise of synthetic biology, in this paper the authors**
5 **propose the adoption of an universal framework for plasmid vectors.**
- 6 131. Pfleger, B.F., Pitera, D.J., Smolke, C.D. & Keasling, J.D. Combinatorial engineering of
7 intergenic regions in operons tunes expression of multiple genes. *Nat. Biotechnol.* **24**, 1027-
8 1032 (2006).
- 9 132. Santos, C.N.S. & Stephanopoulos, G. Combinatorial engineering of microbes for optimizing
10 cellular phenotype. *Curr. Opin. Chem. Biol.* **12**, 168-176 (2008).
- 11 133. Wang, H.H. *et al.* Programming cells by multiplex genome engineering and accelerated
12 evolution. *Nature* **460**, 894-898 (2009).
- 13 134. Agapakis, C.M. & Silver, P.A. Synthetic biology: exploring and exploiting genetic modularity
14 through the design of novel biological networks. *Mol. BioSyst.* **5**, 704-713 (2009).
- 15 135. Jayanthi, S., Nilgiriwala, K.S. & Del Vecchio, D. Retroactivity controls the temporal dynamics
16 of gene transcription. *ACS Synth. Biol.* **2**, 431-441 (2013).
- 17 136. Chavarría, M., Kleijn, R.J., Sauer, U., Pflüger-Grau, K. & de Lorenzo, V. Regulatory tasks of
18 the phosphoenolpyruvate-phosphotransferase system of *Pseudomonas putida* in central
19 carbon metabolism. *mBio* **3**, e00028-00012 (2012).

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Competing interests statement

The authors declare no competing interests.

BOXES

BOX 1. Genetic traps to identify biotransformations

BOX 1

The metagenomic DNA of interest encodes the functions needed for the biotransformation of substrate A into product B. Product B then activates a transcriptional regulator (R) which, upon interaction with product B, switches from an inactive form into a transcriptionally competent factor (R*). R*, in turn, acts on a reporter fusion, modulating its activity. The overall strategy results in the translation of the process $A \rightarrow B$ into a selectable or screenable phenotype in the bacterial host, e.g., a re-factored *Pseudomonas* strain.

BOX 2. The Standard European Vector Architecture.

BOX 2

Compositional and functional standardization of biological parts is one of the trademarks of contemporary SynBio^{12,128,129}. The *Standard European Vector Architecture*¹³⁰ (SEVA) initiative was recently launched as a coherent resource of molecular tools subjected to a concise, minimalist, and standardized format and nomenclature, fully compatible with old and new cloning protocols and DNA assembly methods. Vectors that follow the SEVA format are composed of three variable modules: a cargo, a replication origin, and an antibiotic resistance marker¹³⁰. Target sites for rare restriction enzymes, used to (inter)change the functional modules, are explicitly shown. Segments are separated by three permanent regions, which are shared by all vectors, the T₀ and T₁ transcriptional terminators and the *oriT* conjugation origin for transferring the plasmids into a variety of bacterial recipients. Such simple rules allow for the generation of a huge collection of vectors that cover virtually all genetic and metabolic engineering necessities of *P. putida* KT2440 and other Gram-negative bacteria: genome editing (i.e., deletions and insertions), heterologous gene expression, implementation of logic circuits, biosensors with optical readouts, among many others. The reader is referred to <http://seva.cnb.csic.es> for a complete description of the available vectors and their specific properties. We argue that the SEVA format may become a fundamental reference to speed up biosystems engineering beyond laboratory applications.

BOX 3. String-weight engineering for determining complex parameters.

BOX 3

Before the scientific era, builders of intricate structures often faced the need to estimate parameters that were not amenable to prediction using the analysis and calculation tools available at the time. Architects like Antoni Gaudí (1852-1926), found ways to solve the problem by making string models of the building of interest in which weights were hung at critical places to reveal the effect of local structures on the geometry of the whole object. By turning the model upside down, the optimal arches and angles for maintaining a robust structure could be inferred¹²³. This is an example of how optimal complex designs can be achieved by merging forward engineering with Nature-inspired problem solving. Uncertainties on the best combination of enzymatic steps (1-5) to convert a substrate into a product (Z) include, *inter alia*, the ability to reach a suitable level of transcription (i.e., the function of the promoter P and the regulator R), and the need to identify adequate intergenic regions (IGR) that ensure the necessary stoichiometry in protein production, as well as mRNA stability and termination (T) issues. Current efforts to solve multi-scale design of robust bacterial catalysts through combinatorial and evolutionary approaches^{127,131-133} qualify within the *string-weight engineering* concept.

FIGURES

Fig. 1. The quest for an optimal synthetic biology chassis.

F1

The view of a well-determined genomic frame as a biological chassis for plugging-in and -out genetic circuits and new-to-Nature activities is one of the most powerful metaphors of synthetic biology¹⁴. Such self-replicating chassis should be capable of predictably running a number of background, housekeeping, and pre-defined cellular tasks which must in turn support the functional, long-term operation of engineered traits in a biological system (e.g., a bacterium). Ideally, new functionalities are implanted by means of standardized genetic tools, often based on plasmids or transposon vectors (see main text). The most desirable characteristics of the ideal synthetic biology chassis for biotechnology^{18,134} are often found naturally in microorganisms other than *E. coli* or *Mycoplasma* species. One key aspect of engineering predictable synthetic biology agents is the control of the chassis-implant retroactivity (the biological equivalent to impedance¹³⁵), which most often leads to unexpected changes in the behavior of a circuit, and even to complete collapse. This requires an adequate tuning of the instructions emanating from the inserted DNA to the physiology of the receiving cells³⁷.

Fig. 2. Ecological habitats of *Pseudomonas*.

Bacteria from the genus *Pseudomonas* can thrive in a broad variety of niches that go from natural environments to human associated ecosystems. Members of this genus can be found on the surface of plants (*P. fluorescens*, *P. syringae*), others are common rhizosphere and soil colonizers (*P. putida* KT2440, *P. fluorescens*). Several species have also been isolated from water (*P. fluorescens*, *P. putida* GB-1). Also, other members have been isolated from insects (*P. entomophila*) and from humans (*P. aeruginosa*). Needless to say, several *Pseudomonas* spp. are commonly located in soils with a history of pollution by chemical wastes.

Fig. 3. Catabolism of hexoses in *Pseudomonas*: one substrate, different strategies.

Glycolysis (from *glykys*, sweet and *lysis*, splitting) is the metabolic process converting glucose into pyruvate. The free energy released in these bioreactions is used to form high-energy bonds (ATP) and reducing equivalents (NADH), while converting the substrate in biomass-building blocks. The Embden-Meyerhof-Parnas (EMP) pathway is considered to be the archetypal hexose breakdown route; however, *glycolysis* also refers to other pathways, such as Entner-Doudoroff (ED), pentose phosphate, and various hetero- and homo-fermentative pathways. The ED pathway differs from the linear glycolytic sequence in the nature of the C6 intermediate formed and the aldolase that breaks it down. ED uses 6-phosphogluconate dehydratase (Edd, converting gluconate-6-phosphate into 2-keto-3-deoxyphosphogluconate) and 2-keto-3-deoxyphosphogluconate aldolase (Eda, forming pyruvate and glyceraldehyde-3-phosphate from the C6 intermediate). While *E. coli* has all the enzymes needed to operate functional EMP and ED pathways³⁵, many environmental bacteria use only the ED route due in part to the absence of a 6-phosphofructokinase activity⁸¹. Specifically, pseudomonads exclusively use the ED pathway for sugar catabolism^{35,136}, which seems to enable a high tolerance to oxidative stress⁷⁹.

Fig. 4. The metabolic heart of *P. putida* KT2440.

Lacking a 6-phosphofructokinase, most *Pseudomonas* species metabolize C3- and C6-sugars through the Entner-Doudoroff pathway (instead of the Embden-Meyerhof-Parnas glycolytic route). In the first step of hexose catabolism, glucose is transported through a dedicated ABC uptake system into the cytoplasm, where it is phosphorylated to glucose-6-*P* and gluconate-6-*P*. In the oxidative pathway, in contrast, glucose can diffuse into the periplasm and be converted to gluconate and then to 2-ketogluconate; these intermediates are transported into the cytoplasm to be phosphorylated to gluconate-6-*P* and 2-ketogluconate-6-*P*. These upstream intermediates converge at the gluconate-6-*P* node, entering either the Entner-Doudoroff or the pentose phosphate pathways. In the case of *P. putida*, the pentose phosphate pathway seems to play a most relevant role in NADPH supply, especially through the activity of glucose-6-*P* dehydrogenase, represented by no less than 3 iso-enzymes. All available data thus indicate that the metabolic core of KT2440 is optimally equipped for hosting redox reactions. This not only explains the abundance of strains of *P. putida* among the best aerobic biodegraders of tough substrates, but also advocates their use as a vessel of established (or evolving) new pathways that include intermediate faulty enzyme-substrate couplings.