Biotechnological domestication of pseudomonads through synthetic biology

by

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Running title: Pseudomonads as synthetic biology chassis

Keywords: Synthetic biology; *Pseudomonas putida*; biodegradation; metabolic engineering; stress resistance; energy and redox balance; chassis; biocatalysis

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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.

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

Historically, genetic engineering techniques were used to accomplish these endeavours, although most strategies often lacked an authentic engineering perspective, either technically or conceptually, and largely depended on trial-and-error attempts. By contrast, contemporary synthetic biology addresses the same type of challenges but involves a genuine forward-design engineering approach[10-12]. One of the key components of this endeavour is the adoption of specific biological chassis (**Fig. 1**), into which users can plug-in and -out genetic circuits and new-to-nature properties at will. The metaphor of a chassis has become commonplace in synthetic biology[13]; the term evoking the image of an internal framework that supports a man-made object. A bacterial genome, edited where necessary, provides a material frame on which forward-engineered biological components can be placed. The *chassis* metaphor suggests the implanted components (genetic or otherwise) are autonomous with respect to the physiology of the cell in which the chassis is present, and heralds the possibility of conferring predictable properties to biological entities. Unfortunately, unlike the mechanical or electrical parts used in engineering, biological components function in an extremely context-dependent manner because they interact with metabolites and (bio)chemicals; are subjected to Darwinian evolution; can confer emergent properties when combined; and the cells which harbour these components grow and multiply. Overcoming, or at least mitigating, these problems is a fundamental part of the synthetic biology research agenda[14].

Selecting a specific bacterium as a chassis for synthetic biology applications can be viewed as being analogous to the selection of animal species for domestication[15] – few species are suitable for this process. One key aspect of the domestication process involves an innate potential (which can be enhanced by breeding) to predictably follow instructions from a master. By the same token, a reliable microbial chassis suitable for synthetic biology should ideally be derived from bacterial species that are naturally pre-endowed with a number of appealing physiological and metabolic properties and are amenable to stable genetic re-programming. The synthetic biology pursuit of biological chassis that can be subjected to deep (and rational) genetic engineering has naturally intersected with the more conventional quest for microbial platform strains and cell factories that are most favourable for consolidated bioprocesses, one of the trademarks of modern industrial biotechnology[16,17]. Along the line, researchers have been able to domesticate a small number of bacteria for different purposes over time, most notably *Escherichia coli* and *Bacillus subtilis*.

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

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

most synthetic biology ventures[20]. Yet, is this organism really the only bacterium that can be used in both fundamental synthetic biology and applied biotechnology?

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

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

Why Pseudomonas?

The genus *Pseudomonas* encompasses a large number (>200 and growing[27]) of Gram-negative, aerobic γ-proteobacterial species[28]. The name derives from *pseudes* (ψευδες, *false*) and *monas* (μονάς, *single unit*), and was proposed to broadly designate a genus of non-sporulating rod-shape bacteria with polar flagella[29]. Although the sequencing of 16S rRNA genes and biochemical tests have helped to distinguish members within the group, the pseudomonad classification criteria are still controversial[30]. Relevant features shared by many *Pseudomonas* species include their ability to adapt to different physicochemical and nutritional niches, their

remarkable capacity to endure both endogenous and exogenous stresses, and their ability to synthesize a large number of bioactive compounds (including antibiotics[31]). These traits are encoded by 1,491 genes[32], common to all *Pseudomonas* species sequenced so far.

That many *Pseudomonas* species are human and plant pathogens can be traced to the production of a suite of virulence factors[33] and the ability of these bacteria to evolve quickly[34], which enables the development of resistance to numerous antibiotics. A large number of metabolic genes have been identified as key factors involved in the virulence of some *Pseudomonas* species. In particular, the distinct arrangement of glycolytic pathways in this class of bacteria[35] was recognized early for its medical implications. For example, alginate, the viscous exopolysaccharide produced by *P. aeruginosa* and known to be a virulence factor in lung infections of cystic fibrosis patients, has its origins in central metabolic intermediates[36]. The oxidative stress that *P. aeruginosa* may experience during colonization of host tissues is counteracted by the activity of central pathways *via* the generation of reducing equivalents[37]. In fact, a genome-wide metabolic model of *P. aeruginosa*[38] suggested that the prevalence of the Entner-Doudoroff and pentose phosphate pathways for carbohydrate consumption is an important metabolic asset for infection (**Fig. 3**). This tradeoff, shared by virtually all pseudomonads and involving a high NADPH regeneration rate[39], allows the evolution and expression of biochemical pathways in these microorganisms that would be barely tolerated by other bacterial species[37]. The presence of multiple membrane-bound pumps involved in antibiotic extrusion in virulent strains[40,41] also has a beneficial effect in non-pathogenic strains, as reflected in the presence of solvent-tolerance efflux pumps[42].

Interestingly however, the same metabolic vigor that makes some strains virulent makes others suitable to host and evolve new chemical reactions[37]. The *Pseudomonas* strains that could potentially be used as platform strains and cell factories in various biotechnological settings are those that retain the inherent beneficial traits (e.g., catalytic vigor) while lacking undesirable traits (e.g., virulence factors). The branch of the *Pseudomonas* family that is most appealing in this regard is centered in the non-pathogenic *P. putida* species. A number of relevant qualities shared 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 case[45] as a bacterium capable of breaking down oil and thus attractive for bioremediation of petroleum spills. Some isolates colonize the rhizosphere[46] and establish beneficial relationships with plants[47] 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 chemicals[50]. 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 species[52]. 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 communication[54].

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 group[56] 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 medium[59]. The effect of pumps is complemented by other physiological changes[59]. Exposure to organic solvents also induces protein unfolding, stimulating a general response mediated by extra-cytoplasmic σ factors that

trigger the expression of molecular chaperones and other stress resistance proteins[60]. Again, these features expose the genetic pre-programming of *P. putida* to deal with a hydrophobic milieu, which is desirable for some types of biocatalysis. Ideally, solvent tolerance in a given biotransformation is paralleled by resistance to the toxic effects caused by the products and/or intermediates. In this respect, it is worth mentioning the high butanol tolerance of some pseudomonads[61]; not surprisingly, the expression of genes for butanol biosynthesis from *C. acetobutylicum* in *P. putida*[62] resulted in alcohol titers up to 120 mg/L.

One final feature of *P. putida* that makes it an interesting choice for the downstream applications of synthetic biology is the ability of this species to react to an excess of carbon with respect to other essential elements (e.g., nitrogen or phosphorus) by accumulating intracellular polyester granules as storage material[63,64]. The biotechnological interest of these compounds stem from their plastic properties, biodegradability, and the broad range of qualities arising from the side chains present in the polymers. In fact, polyhydroxyalkanoates have the potential to replace conventional, oil-derived plastics in several packaging and coating applications[65]. While most polymer-producing microorganisms (including recombinant *E. coli*[66]) accumulate short-chain variants [e.g., poly(3-hydroxybutyrate)], *P. putida* can incorporate linear and branched C6 to C16 monomers of 3-hydroxy fatty acids in the chains, thereby producing medium side-chain polyhydroxyalkanoates[67,68]. Moreover, the coupling of biopolymer accumulation and degradation plays a key role in the overall carbon balance of *P. putida*[69]. Medium side-chain polymers show enhanced elastomeric properties compared to short-side-chain counterparts, also enabling chemical derivatization of the bioplastic. Tailor-made biopolymer production using *P. putida* thus affords a multi-level optimization approach, encompassing not only the engineering of the *chassis* and the key enzymes (e.g., biopolymer synthases), but also the design of fermentation strategies and the introduction of chemical modifications[70,71].

***P. putida* KT2440**

The most prominent *P. putida* strain is called KT2440 (KT standing for Kenneth Timmis, who, over the years, characterized this specimen[72]). This bacterium is a derivative of an environmental isolate named *Pseudomonas arvilla* mt-2[73], which was reported to express an enzyme cleaving

the aromatic ring of *m*-toluate[74]. It was later discovered that strain mt-2 harbours the TOL plasmid pWW0, which encodes a pathway for toluene and *m*-/*p*-xylene biodegradation[75]. 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 GRAS[76] (*generally regarded as safe*). Inspection of its ca. 6.2 Mb genome[77] 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-reductases[78], 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 intermediates[50]. In addition, *P. putida* isolates similar to KT2440 have been found to carry plasmids mediating the breakdown of naphthalene, 4-chloronitrobenzene, 2,4-xylenol, and phenol[49].

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 2008[81], 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* predictions[79]. 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 maintenance[84], 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 (*Putida*NET)[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 microorganisms[89], 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 bacterium[90]. 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 deleted[94]. 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

and NADPH/$NADP^+$ ratio. Separate efforts have been made to reduce the size of the *P. putida* KT2440 genome by random (as opposed to targeted) deletions of large chromosomal regions. While ~7% of the genome has been erased with such procedure[95], this approach might be limited by the scattering of essential genes through the whole chromosome.

Inserting genes

What is the best procedure to insert new genes into a bacterium? Plasmid vectors have been, and still are, the workhorses for genetic engineering of microorganisms at the laboratory scale, although they are not exempt from problems when used in industrial and environmental settings. Although broad-host-range vectors applicable to *P. putida* have been available since the beginning of the recombinant DNA era[96], only recently has the architecture of such tools been subject to standardization efforts (**BOX 2**). One example of the power of adopting formatted plasmid vectors in *P. putida* is the recent engineering of a strain to biodegrade the environmental pollutant 1,3-dichloroprop-1-ene under conditions with limited oxygen supply[97]. This example showed the adequacy of using synthetic biology tools to domesticate *P. putida* KT2440, as this bacterium is not naturally endowed neither with the ability to thrive in the absence of oxygen nor the functions needed to mineralize 1,3-dichloroprop-1-ene. In other cases, the objective is not only the acquisition of heterologous DNA fragments but also their stable integration in the chromosome. One possibility is to use the same recombination-based genome editing tools, like the ones mentioned above, but such procedures can be time-consuming. An alternative strategy is the use of Tn*5*-based and Tn*10*-based mini-transposon vectors[98-100], which can deliver multiple DNA segments of unlimited size to the genome of *P. putida* as part of a hybrid mobile element. The downsides of this technique reside in the *a priori* uncertainty of the insertion site, which might affect expression levels because of the uneven distribution of transcriptionally active segments within the bacterial genome[101]. But this same problem becomes an asset if the user wishes to explore various levels of expression of the same gene(s) through random insertion of the engineered transposon into the target genome[102]. Should this not be the case, other genetic systems can be used to direct insertions into specific chromosomal sites. A large number of transposon vectors are available, exploiting the preference of Tn*7* to target cognate, single *att*Tn*7* sequences that are naturally present[103,104] or can be engineered[105] 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 element[106], 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 inducers[107] (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 pathways[50]. 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 (XylS[109]), salicylate (NahR[109]), and short-chain alkanes (AlkS[110]).

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 system[111]). The similarity of transcriptional networks to

electronic circuits also stimulated the design of logic gates artificially assembled with bacterial regulatory parts. These devices process specific signals and can be combined with others to implement simple computations (e.g., toggle switches, pulse generators, oscillators, and memory devices). Although they have largely been implemented in *E. coli* thus far, they are also applicable to *P. putida*, and this is a fertile area of research at the present time. Such manipulations are not restricted to TF/promoter pairs, but also allow entering new inputs into TFs and housekeeping proteins (e.g., GroEL[112]) by engineering protease-cleaving sites in their structure and thereby modifying their logic behaviour[113].

Communication is a two-way process: how can researchers get some feedback on the fate of their orders to bacterial cell factories? Visual inspection of cells often provides little information on their physiological or catalytic performance. A useful approach is the adoption of protein-based, TF-based and RNA-based intracellular sensors of specific physiological descriptors coupled to optical reporters. In combination, the simultaneous quantitative monitoring of a number of parameters would allow the user to diagnose the performance of an engineered process. A few *in vivo* flux-sensing[114] and metabolite-sensing[115,116] devices are available for *E. coli*. Functional variants of these systems adapted to pseudomonads would be a welcome addition to the repertoire of biosensors already available for these bacteria[51,115,117].

Programming population behaviour

A further step in designing rational *P. putida*-user interactions is engineering chemical signaling. Quorum sensing agents have been used for this purpose[118], but the choice of useful molecules is limited. Once more, the biodegradation pathways present in *P. putida* are sources of parts and devices that can be re-factored for programming cell-to-cell communications. An example in this sense is a sender *P. putida* strain, harboring the TOL pathway, which processed environmental toluene as the input cue and generated benzoate as the output signal[119]. Diffusion of benzoate was sensed by a second, receiver *P. putida* strain that processed benzoate as the input to produce a visual output. Given the large number of possible enzyme/TF/inducer combinations, the opportunities for designing complex population performance are huge. The physical 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 extent[121] and current technologies allow the synthesis of DNA sequences the size of a small bacterial genome[122], the lack of understanding of many basic biological facts (e.g., the interplay between the genetic program and the metabolic network[37]) 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 Gaudí[123]) (**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 functions[117]. To this end, the objective is set and the circuitry left to explore

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

Once a given optimum is reached, the ultimate challenge lies in protecting the structure from changes and eventual collapse. Evolvability, an inherent property of DNA-based biological systems, is possibly the main obstacle to the engineering of reliable living objects. Some *in vivo* approaches have been proposed to avoid the occurrence of mutations, mostly by increasing genomic stability. For instance, it is possible to counter-select variations in specific DNA sequences by interfacing a conditional lethality circuit with DNA mismatch repair systems[124]. Again, Nature offers remarkable examples of bacteria endowed with lifestyle that oscillate between high- and low-mutation regimes (e.g., *P. aeruginosa*[125] and *Buchnera aphidicola*[126]), from which we can take inspiration. Chassis programmed to switch between a high and a low-to-none evolutionary regime would constitute excellent frames for designing reliable cell factories. Such objective can be targeted at through the transient expression of a hypermutator phenotype[127] and the conditional inactivation of the SOS system (therefore implementing the string-weight engineering concept pictured above).

Outlook

One key endeavour in contemporary synthetic biology is quest of an optimal and realistic genomic chassis for industrial, large-scale applications[18]. While synthetic life forms can be entertained that will be altogether programmed *à la carte* in the future for a suite of uses (http://www.theguardian.com/science/2013/oct/13/craig-ventner-mars) such all-designed biological objects are unlikely to reach the existing bioindustry anytime soon. In the short term, synthetic biology agents will necessarily merge desirable pre-existing traits (the basis of which might not be fully understood at the time) with rationally engineered implants. It thus makes sense to adopt as the starting point bacteria that Nature has already selected to thrive under many of the demanding conditions that frame industrial operations. Getting to the point where bacteria can be designed with complete reliability still needs clarification of much of their fundamental biology, 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 not withstanding, 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).

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Acknowledgements

This study was supported by the BIO and FEDER CONSOLIDER-INGENIO Program of the Spanish Ministry of Science and Innovation, the ST-FLOW, ARYSIS and EVOPROG Contracts of the EU, the ERANET-IB Program, and the PROMT Project of the CAM. PIN is a researcher from the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina) and holds a Marie Curie Actions Program grant obtained from the EU (ALLEGRO, UE-FP7-PEOPLE-2011-IIF-300508).

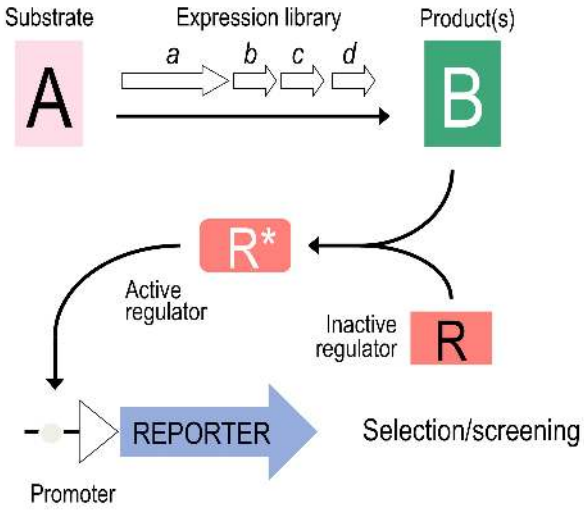
Competing interests statement

The authors declare no competing interests.

BOXES

BOX 1. Genetic traps to identify biotransformations

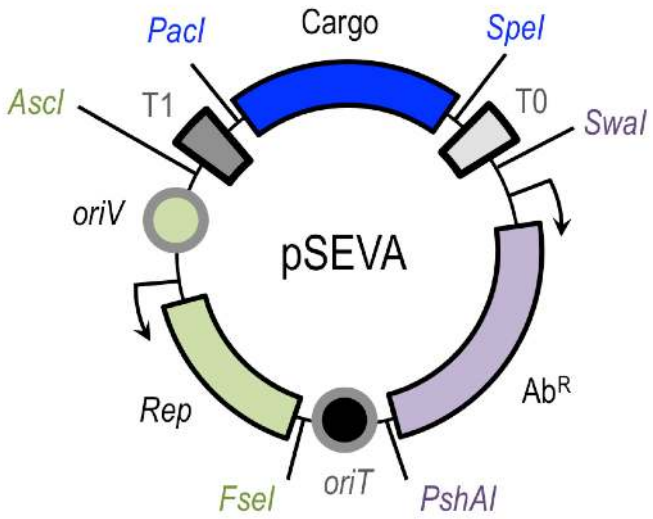
BOX 1



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 ➔ 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.

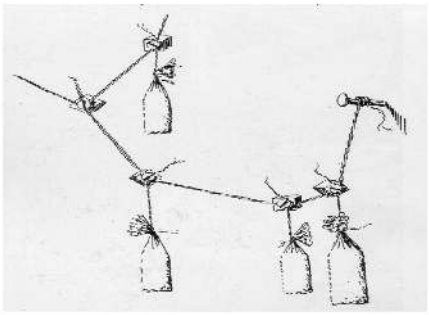
BOX 2

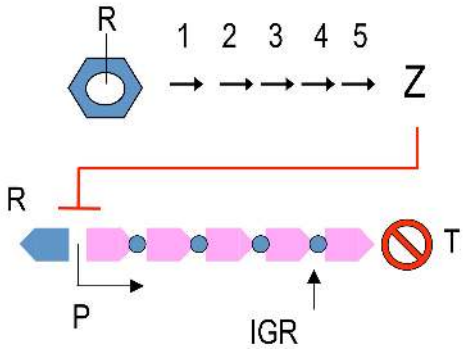


Compositional and functional standardization of biological parts is one of the trademarks of contemporary SynBio[12,128,129]. The *Standard European Vector Architecture*[130] (SEVA) initiative was recently launched as a coherent resource of molecular tools subjected to a concise, minimalist, and standardized 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 marker[130]. 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 T_0 and T_1 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



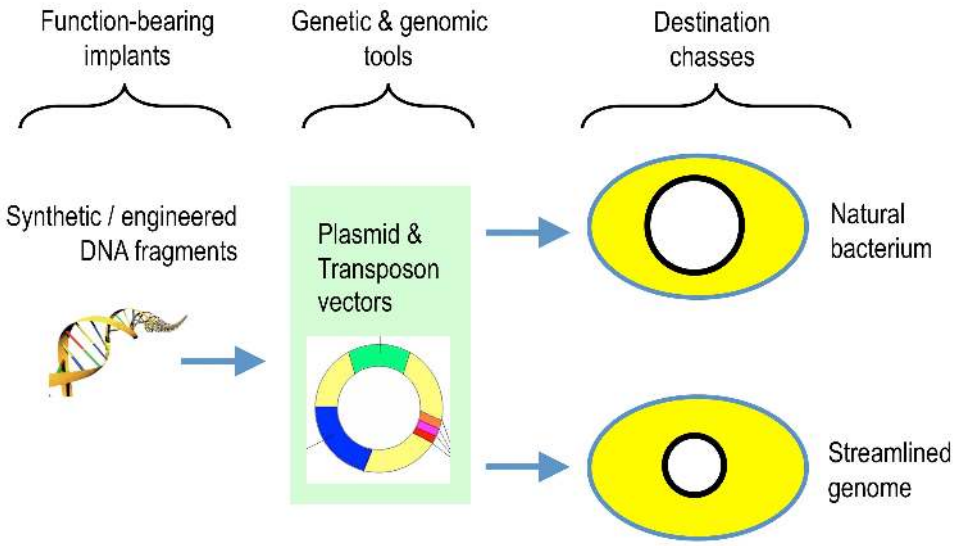


Before the scientific era, builders of intricate structures often faced the need to estimate parameters that were not amenable to prediction using the analysis and calculation tools available at the time. Architects like Antoni Gaudí (1852-1926), found ways to solve the problem by making string models 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 inferred[123]. 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

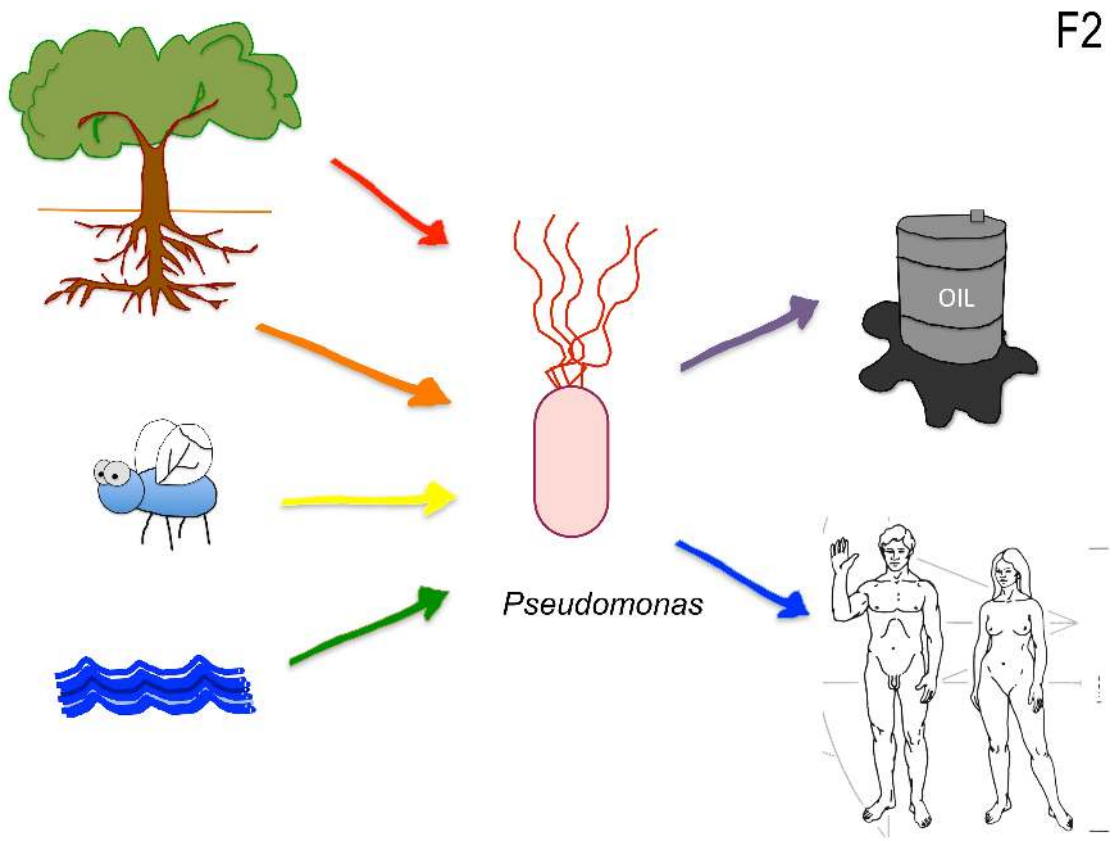
Fig. 1. The quest for an optimal synthetic biology chassis.

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 biology[14]. 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 impedance[135]), 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 cells[37].

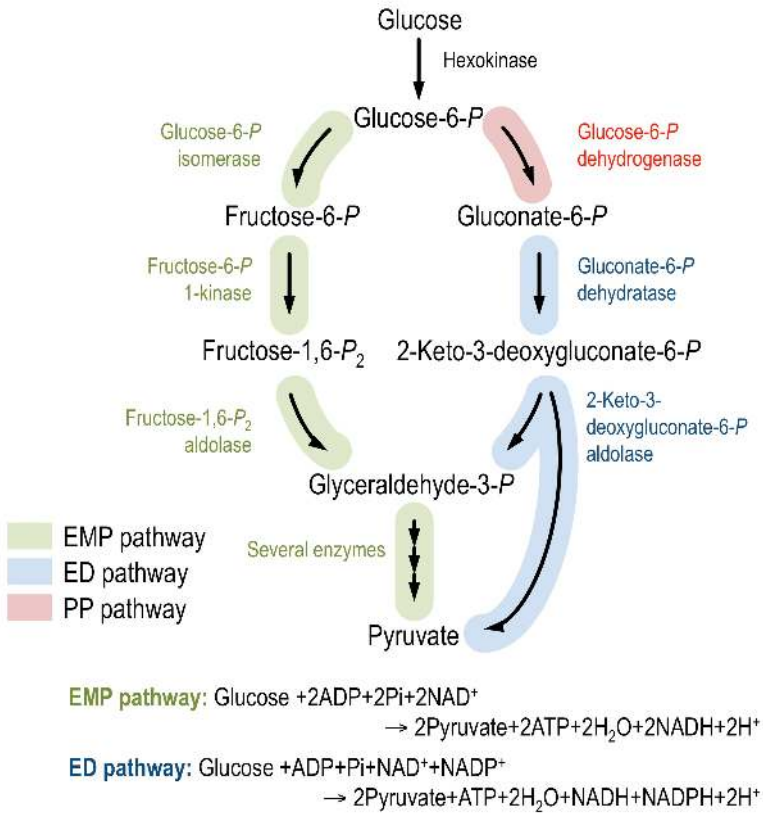
Fig. 2. Ecological habitats of *Pseudomonas*.



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.

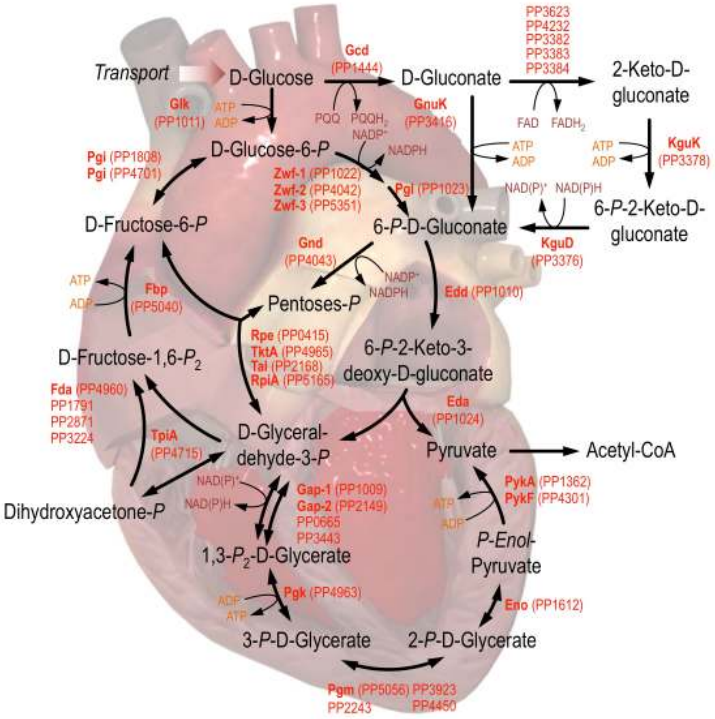
Fig. 3. Catabolism of hexoses in *Pseudomonas*: one substrate, different strategies.

F3



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 pathways[35], many environmental bacteria use only the ED route due in part to the absence of a 6-phosphofructokinase activity[81]. Specifically, pseudomonads exclusively use the ED pathway for sugar catabolism[35,136], which seems to enable a high tolerance to oxidative stress[79].

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.