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# Molecular tools and emerging strategies for deep genetic/genomic refactoring of *Pseudomonas*

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The interest of the genus *Pseudomonas* largely relies on the virulence of some of its species for plants and animals (including humans). Yet, pathogenic features of some isolates coexist with others often present in environmental variants that promote plant growth and degrade chemical pollutants. Many of these traits can be traced to the intrinsic properties of the genomic chassis of this genus along with distinct genetic parts and devices. With the tools of Synthetic Biology these can be enhanced and/or repurposed for the sake of biological control, environmental remediation and whole-cell biocatalysis. In this article we take stock of both conceptual and technological developments that have allowed the virtual domestication of *Pseudomonas* (in particular *P. putida*) as a major biotechnological workhorse with a range of applications of industrial interest. Adoption of a suite of compositional and measurement standards is advocated for bringing *Pseudomonas*-based genetic engineering to a superior level of development.

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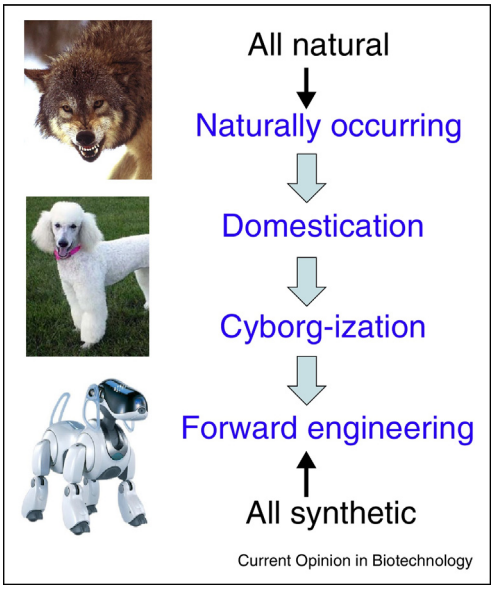
## Introduction

Because of the onset of Systems and Synthetic Biology, contemporary Biotechnology is experiencing a rapid transition from being mostly a trial-and-error exercise to become an authentic branch of engineering. Our capacity to manage DNA has evolved from handling one or few genes at a time to synthesize long sequences and even complete prokaryotic [1] — and soon eukaryotic [2] genomes. The ultimate vision of the bio-industry of the future includes the possibility to rigorously program biological agents *á la carte* for production of given molecules and materials as well as designing autonomous decision-making biological entities able to, for example, act as smart therapeutic agents, get rid of environmental pollution, assist in crop management and even protect and preserve the landscape [3,4]. Alas, all these developments will not happen overnight and a number of intermediate steps will have to be covered along the next decade to move Biotechnology forward towards complete programmability of the biological agents at stake. This state of affairs is not unlike past attempts to domesticate animals, which deals essentially with enhancing their predictability, their productivity and their response to human instructions. As sketched in Figure 1, the way from being wild to being completely predictable includes various stages, one of which (where we seem to be right now) is what we could call cyborg-ization. In this phase, naturally-occurring properties are combined with others entered rationally in the live system (designed) with a predetermined intentionality. Cyborg-ization involves, first, enhancement of innate desirable traits; second, replacement of instrinsic attributes by better ones from other organisms; third, knocking-in entirely new traits; and fourth, elimination of drawbacks. This agenda implies a massive genetic refactoring of the biological entities involved. Ultimately, such systems might be constructed from first principles and programmed with entirely synthetic genomes. But reality is that there is still ample room in the near future for improving biotechnological endeavours with existing biological items owing to the many tools and interpretative frames developed under the aegis of Systems and Synthetic Biology.

In the context above, one major challenge is the pursuit of adequate biological *chassis*, microbial in most instances [5], that provide an optimal biochemical background as well as a genomic frame where engineered modules can be knocked-in and knocked-out with user-friendly genetic technologies. By the same token that there are hundred of thousands of animal species but only about two dozen of them have been domesticated for humans' sake, it is plausible that only a very small collection of microorganisms will eventually succeed as chassis of choice for industrial Biotechnology. What is certain is that the two archetypal bacterial models of reference in Synthetic Biology (*Escherichia coli* and *Mycoplasma*) are unlikely to cover all possible applications. Therefore other species have to be considered for specific purposes. Because of their evolved properties and physiological traits a number of such bacteria has been listed as the most amenable to this end. This review deals with one distinct member of

**Figure 1**




The way towards full predictability of bioengineered systems. The process shows some resemblance to the historical roadmap to domesticate wild animals for the sake of increasing productivity, ease of handling and response to human instructions. Cyborg-ization is the stage where the systems at stake combine naturally-occurring and engineered/synthetic traits.

such a select group of Synthetic Biology chassis-to-be: *Pseudomonas*.

What does *Pseudomonas* stand out as a platform of choice for industrial Biotechnology? Although the genus is mostly known for including many species virulent for humans (*P. aeruginosa*) and plants (*P. syringae*), many other variants also belonging to the group promote plant growth (*P. fluorescens*) by warding off pathogens. Finally, many soil *Pseudomonas* (e.g. *P. putida*) display amazing capacities to degrade toxic environmental pollutants [6,7]. Whether detrimental or beneficial for us, most of these properties stem from a distinct biochemical network that provides metabolic fuel for counteracting oxidative stress, tolerating solvents and hosting strong redox reactions [8•,9••]. This means that *Pseudomonas* is evolutionarily equipped with many of the traits that are desirable in a biological agent expected to perform well under the harsh conditions of a bioreactor in an industrial setting. These circumstances, which were well noticed soon in the development of recombinant DNA, led to an early exploration of *Pseudomonas* genetics along with possible ways to make manipulations to its genomic complement [10]. Since then, a large collection of molecular tools have been reported and thoroughly used both for studying the basic biology of some *Pseudomonas* species and for modifying it with a biotechnological purpose, for example, biological control, bioremediation, biosensing and biotransformations. For these last applications, the species that has prevailed as the most suitable agent for whole-cell catalysis and on which most of Synthetic Biology efforts have focused is *P. putida* and, in particular, strain KT2440 [6,7]. This is not alien to the fact that other species and strains of the genus are often pathogenic and thus more problematic to use in large scale industrial operations.

The sections below take critical stock of some of the major advances in genetic tool development that have either been established specifically for *P. putida* or are applicable to strains of this species [11]. In some instances, the same technologies may be useful for other *Pseudomonas* variants or for other Gram-negative bacteria at large — although experience tells us that both specific and generic tools have to be combined on a case-by-case basis. This means that although many of the approaches discussed below may work in a large variety of Gram-negative bacteria, their parameters and efficiencies could vary dramatically among different target isolates. Still, we argue that having the right tools, preferably standardized, is what makes a difference between a mere trial-and-error genetic *bricolage* and genetic *engineering* in earnest [12].

## The chassis

*P. putida* KT2440 is a derivative of strain *P. putida* mt-2, which was isolated in Japan in the 1970s as a *m*-toluate (i.e. 3-methylbenzoate) degrader [13]. This property was due to the presence of the catabolic TOL plasmid pWW0, which enabled *P. putida* mt-2 to grow on various aromatic substrates, for example, toluene, *m*-xylene and *p*-xylene as sole carbon and energy sources. Once pWW0 was cured (i.e. eliminated from the host strain), the plasmid-less variant was designated KT2440 strain and soon proposed to be a safe host for recombinant DNA constructs. Much later, the complete genome sequence confirmed the lack of conspicuous virulence factors in the 5592 gene/CDS (~6.2 Mb) complement of the strain and its amenability to a suite of manipulations [14,15]. Given the major natural niche of *P. putida* (soil and plant roots, often in sites with a history of chemical pollution), strain KT2440 was first envisioned as an optimal model bacterium for environmental microbiology. This included its use as a vehicle to deliver engineered catabolic activities to sites afflicted by industrial pollution as well as extensive detection of distinct chemicals with whole-cell biosensors based on that strain. While these applications are still feasible, effectively placing engineered strains in the environment for bioremediation or biomonitoring is a considerable scientific and technical challenge and the success stories are very few [16]. However, the metabolic diversity of *P. putida* and its native endurance to physicochemical insults (e.g. solvent tolerance, oxidative stress) kept the interest on strain KT2440 beyond the potential environmental uses that were envisioned earlier. Instead, the emphasis shifted in more recent years