Recombinant bacteria for environmental release: what went wrong and what we have learnt from it

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Abstract

From a biotechnological point of view, bacteria can be seen as either pathogens to target with new drugs or as biocatalysts for large-scale processes in industry, agriculture or the environment. The last includes the exploitation of bacterial activities for bioremediation of toxic waste either in situ or ex situ. The onset of genetic engineering in the late 70s opened the possibility of tailoring recombinant bacteria for environmental release, aimed at biodegradation of otherwise recalcitrant chemicals. However, a few decades later the outcome of this prospect has been quite meager. The literature counts very few cases where the use of genetically engineered bacteria has been proven to be more efficient than natural microorganisms in elimination of recalcitrant compounds under natural (not laboratory) conditions. Fortunately, the emergence of Systems and Synthetic Biology in the last few years is helping to identify what were the caveats of the former approaches and how to correct them. In addition, robust design concepts imported from process engineering provides fresh approaches to the challenge of designing microorganisms à la carte for environmental applications.

Keywords: Biodegradation, Pseudomonas, recombinant antibodies, synthetic biology


Since the early 1980s, genetic engineering of soil bacteria has been claimed to have an extraordinary potential for remediation of environmental pollution, as long as it ultimately produced the design of superior contaminant-breaking live microbial catalysts [1,2]. However, despite intensive efforts in Europe and the USA, the success of such approaches has been very limited thus far [3]. Many problems have been encountered in constructing strains that perform well not only in the laboratory, but also under real environmental conditions (Table 1). It is intriguing that metabolic engineering, which is at the core of any refactored or improved biodegradative pathway, is not the main problem. Rather, bacteria engineered for bioremediation, biocatalysis, or biosensing, require the adoption of hosts, genetic tools and even conceptual frames that diverge from those used for laboratory microorganisms in laboratory-based experiments. In the environment, new information borne by implanted genes and genetic circuits must be stably inherited in the absence of selective pressure, must not be associated with antibiotics, and must not cause the loss of ecological fitness in the carrier.

Because of their genetic promiscuity, the Tn5 and Tn7 transposition systems are optimal sources of biological modules that can be claimed to be authentically context-independent. Thus, they are attractive for developing dedicated molecular tools. We have constructed a large collection of mini-transposon vectors based on Tn5 [4] and Tn7 that allow stable integration of multiple DNA segments into the chromosomes of a whole range of robust Gram-negative soil bacteria such as Pseudomonas putida. These vectors have been instrumental in designing strains that are able to aerobically degrade the otherwise recalcitrant compound 2Cl-toluene in soil [5]. To this end, we inserted catabolic segments with the toluene dioxygenase of the TOD system of P. putida F1 (todC1C2BA) and the entire upper TOL pathway from the pWW0 plasmid of P. putida mt-2 into the chromosome of one 2-chlorobenzoate-degrading Pseudomonas strain. The resulting cells possessed not only the inserted genetic information, but also the functional ability to mineralize 2-chlorotoluene.

However, although these strains did convert the substrate into 2-chlorobenzoate, they failed to grow with 2Cl-toluene as the only carbon source and produced undesirable dead-end hydroxylated products. These results indicate that the real bottlenecks in engineering the degradation of certain pollutants do not rest solely on the enzymology of the process. The rise of systems biology and omics technologies has shed some light on why biotransformations that should work well from an enzymatic point of view happen not to operate properly in the wider context of a live cell. Some revealing pieces of information have been recently published [6,7]...
suggesting that bacteria exposed to aromatic chemicals (toluene and the like) redirect the transcriptional machinery to activate stress response genes. This helps cells to cope with the exposure to organic solvents but thus diverts RNA polymerase from the desired task of expressing catabolic genes for toluene biodegradation.

A separate bottleneck relates to the use of heterologous expression systems for achieving production of the desired enzymes in host cells. Most naturally occurring promoters, even the simplest, have more than two input functions. In engineered biological systems intended to perform in the field, this certainty cannot be overlooked. Let us take, for instance, the Pu promoter encoded in the pWW0 TOL plasmid for biodegradation of this aromatic hydrocarbon in P. putida mt-2 [8]. Pu belongs to the class of promoters that depend on the alternative sigma factor \( \sigma^{54} \) and is activated at a distance by XylR, an \( m \)-xylene-responsive activator (Fig. 1). XylR can be mutated to respond to non-natural aromatic effectors [9,10], making it an ideal basis for developing a large number of aromatic-inducible expression systems ideal for engineering transcriptional circuits. However, the action in vivo of XylR and \( m \)-xylene on Pu depends not only on these two inputs but also on a plethora of additional factors and signals that tune promoter output to the general physiological and metabolic conditions of the cells [8]. To varying degrees, many other natural promoters are affected by the same problem, as prokaryotic regulatory economy tends to compress control elements in increasingly shorter DNA sequences [11]. Fortunately, it is possible to avoid such compressions and come up with promoters and genes relieved of such physiological control [11,12], causing a robust inducer-dependent expression.

A final factor that limits genetic engineering of environmental catalysis is the realization that many, perhaps most, intracellular polypeptides associate into multiprotein structures [13,14] in which many products of orphan genes are likely to scaffold enzymatic complexes [13,15,16]. Furthermore, there are indications that functionally related gene clusters or genomic islands are located in distinct places of the chromosome that target their transcription to given spots of the cytoplasm [17]. This means that each protein needs to be expressed and located in an intracellular physical niche to optimally perform its function. Polypeptides unable to fit within such assemblies might be rendered non-functional and eventually rejected through a simple Darwinian mechanism. Although inserting extra DNA into a cell is straightforward, implantation of the encoded proteins in the molecular ecosystem of the bacterial cytoplasm might be severely counterselected [18].

Table 1. Challenges in constructing genetically engineered bacteria intended for environmental release

<table>
<thead>
<tr>
<th>Problem</th>
<th>Solution</th>
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<tbody>
<tr>
<td>Escherichia coli laboratory strains not robust enough</td>
<td>Use environmental soil bacteria</td>
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<tr>
<td>Antibiotic resistance as selection markers</td>
<td>Non-antibiotic markers and excitable resistance</td>
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<tr>
<td>Plasmids as carriers of engineered traits Expression dependent on chemical inducers Strong selection against implanted genes/circuits</td>
<td>Stable chromosomal integration Expression dependent on environmental inputs Orthogonalization of engineered functions</td>
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![Fig. 1. Factors that affect expression in vivo of the sigma-54-dependent promoter Pu of the TOL plasmid pWW0 of \( m \)-xylene-degrading Pseudomonas putida mt-2. Pu can be transcribed in vitro by combining purified IHF, the sigma factor core RNAP and activated XylR. However, the same promoter is subject in vivo to a plethora of additional factors and inputs, which influence to various degrees the activity of Pu under diverse growth or stress conditions. Mechanistically, such signals enter through the integration host factor (IHF), the IIA\(^{Ntr} \) protein, sigma factor competition, ppGpp levels, temperature, the TurA histone-like proteins, and perhaps other additional inputs.](image)

![Fig. 2. Engineering bacterial consortia with adhesins à la carte. Bacteria A, B and C, which are unable to degrade a given chemical, may, however, bear genes encoding enzymes which, when put together, can give rise to a novel metabolic pathway. However, such bacteria may not naturally have any tendency to associate with each other (right). However, consortia can be forced to form by expressing on the surface of the cells specific adhesins (e.g. single-chain antibodies) anchored to the cell envelope with autotransporter domains (right; see text for explanation).](image)
Do these numerous constraints in designing bacteria intended for environmental release mean the end of this scientific and biotechnological field? Fortunately, the situation is changing rapidly with the advent of synthetic biology and its emphasis on robust design concepts, orthogonality, i.e. context independence, and definition of systems boundaries. In fact, engineering bacteria for bioremediation, biocatalysis or biosensing is receiving renewed attention in view of the possibilities opened by such an emerging discipline [19,20].

Apart from single-strain manipulation, environmental applications of engineered bacteria also encompass the assembly of microbial communities deliberately structured to combine qualities possessed separately by different bacterial strains (Fig. 2). Accordingly, we have devised a general genetic method for surface display of artificial adhesins on the surface of Gram-negative bacteria [21]. This is based on the so-called autotransporter secretion systems, which export and attach both small peptides and completely folded and active proteins to the exterior of the cells [22]. Such a system has been employed for engineering *Ralstonia eutropha* cells coated with a rat metallothionein, aimed at biosorption of heavy metals in soil, which is displayed on the cell surface as a metallothionein–autotransporter hybrid [23]. Finally, the same surface-anchor procedure was employed for targeting expression of Fos/Jun protein dimerization domains to the surface of *Escherichia coli* [21] and for coating live cells with recombinant camel antibodies [22]. All of these new developments herald what can be seen as a rebirth of genetic engineering for environmental applications.

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Transparency Declaration

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