Chapter 7

Designing bacteria for the environment: From trial and error to earnest engineering

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Since the mid-1970s, genetic engineering and the possibility of accidental or deliberate environmental release of modified micro-organisms has been the centre of debates concerning the consequences of altering the ordinary course of nature. For a sound discussion on risks, it is of essence to separate substantive scientific and technical issues from non-informed perceptions of the general public. This chapter advocates this question to be framed on the already extensive history and wealth of data on the design, performance and risk studies made since the early 1980s on genetically modified organisms and more specifically, on available records on genetically engineered micro-organisms (GEMs) designed for non-contained applications as in situ *bioremediation agents. Existing information provides a suitable background for tackling the uncertainties raised by newly engineered agents, including those that may stem from synthetic biology.*

Introduction

There are at least three ways in which genetically modified bacteria can help remove toxic waste. The first is, of course, by the use of environmentally friendly bio-processes and products which are designed *ab initio* precisely to avoid the production of noxious by-products (Schmid et al., 2001). The second case is the recycling or reuse of waste in source for either generation of added value products (e.g. conversion of lignocellulose into biofuels) or for mineralisation into CO_2 and H_2O (Keasling and Chou, 2008; Lee et al., 2008). Finally, there are frequent scenarios in which given chemicals have been released accidentally or chronically to soil or water ecosystems. This pollutes the area with concentrations of the compounds that are high enough to cause a detrimental effect on the biology of the site, but low enough not to warrant an intensive and costly, *ex situ* treatment. These cases are typical candidates for bioremediation interventions (Pieper and Reineke, 2000).

The conceptual frames behind such actions have evolved considerably since 1989, the time of the Exxon Valdez disaster (Harvey et al., 1990), as the deliberate addition of biodegrading bacteria (so-called bio-augmentation) has, in most cases, not been useful (Peterson et al., 2003). For the sake of enumerating biotechnological challenges related to microbial diversity, it should be mentioned that after a long period of stagnation, the field is experiencing a rebirth under the aegis of newly developed insights, for instance in systems and synthetic biology. New bioremediation approaches stem from the growing knowledge on the genomes of soil and marine bacteria and from the analyses of their whole transcriptomes, proteomes and metabolomes (Lovley, 2003; Watanabe and Hamamura, 2003; Pieper and Reineke, 2000; Katsivela et al., 2005; de Lorenzo, 2008). This wealth of data allows the construction of metabolic models that identify bottlenecks in biodegradation reactions. In some cases, these can be overcome through protein design and metabolic engineering aimed at fixing the problems found in natural bacteria. In other instances, the choice is the amendment of the afflicted site with given nutrients that may limit growth or catalysis of the indigenous micro-organisms otherwise (Wenderoth et al., 2003; El Fantroussi and Agathos, 2005). It is also feasible to associate degrading bacteria to plant roots (rhizoremediation), and even the expression of catabolic genes of bacterial origin in transgenic plants (Kuiper et al., 2004; Van Dillewijn et al., 2007).

These approaches are likely to produce successes in the degradation of otherwise recalcitrant pollutants *in situ*, such as chlorinated aliphatics and polychlorinated biphenyls as well as for binding heavy metals. However, bioremediation is not just the encounter of one bacterium with one chemical in a Petri dish. Real environmental cleanup involves various layers of multi-scale complexity involved in removal of toxic waste from polluted sites. Genetics and metabolism are the central, but not the only, aspects of bioremediation. A number of pre-catalysis processes upstream (diffusion in solid matrixes, bioavailability, weathering, abiotic catalysis of pollutants) and downstream post-catalysis (stress, production of toxic intermediates, predation, competition) constrain the outcome of the whole action (de Lorenzo, 2008). To this end, one needs to integrate multi-scale data from the all the biological, chemical and physical actors of the process – a challenging field of action for systems biology.

Genetically modified organisms for the environment: What went wrong?

The concept of using genetically engineered bacteria for environmental release as agents for *in situ* bioremediation of industrial pollution can be traced to the very beginning of the recombinant DNA technology. As early as 1972, Ananda Chakrabarty,

of the University of Illinois in Chicago, made global headlines in his attempt to patent a genetically modified *Pseudomonas* strain able to degrade a suite of petroleum components and thus holding a potential for dissipating oil spills (Cases and de Lorenzo, 2005). After ten years of litigation, the patent of the first man-manipulated live entity was granted, a seminal event that was to trigger a large number of consequences in many different realms e.g. scientific, legal, ethical, biosafey, biosecurity and social acceptance. In the meantime, the first usable tools for facilitating gene cloning were developed by Boyer and Cohen (Cohen et al., 1973) and the arch-famous Asilomar Conference took place (Berg et al., 1975a; 1975b). Although the patented Chakrabarty's strain did not really fulfill its promise, the entire case brought about considerable hype on the potential that genetic engineering could have to endow bacteria with a superior capacity to eliminate pollutants *in situ.* One distinct aspect of such an endeavour is that bacteria tailored for environmental release must be vigorously active rather than attenuated (as was recommended in Asilomar). This posed a fascinating challenge for the genetic engineers of the time, as strains had to be programmed to do their catalytic mission efficaciously while at the same time being safe. The approach proposed by that time was the design of genetic containment and biological containment systems to programme death of the engineered agents once the environmental purpose for which they had been created had been fulfilled (Diaz et al., 1994; Molin et al., 1993; Ramos et al., 1995; Ronchel and Ramos, 2001).

GEMs for *in situ* catalysis, for biological control and for plant protection have been for nearly 20 years the workhorses in which these early concepts have been tested and their success and failures examined (Cases and de Lorenzo, 2005). The balance is extremely good in having expanded the knowledge base on microbial ecology and biodegradation biochemistry – but clearly disappointing in terms of efficacious applications in the field. Despite some early successes in the engineering of sophisticated GEMs able to consume otherwise recalcitrant compounds (Rojo et al., 1987; Ramos et al., 1987) the reality is that bioaugmentation (i.e. increasing removal of pollutants by inoculating the target sites with catalytic bacteria) is not yet a reliable technology. Alas, this applies not only to GEMs, but to virtually all types of micro-organisms, natural or recombinant, the few exceptions being less than five. One is *Dehalococcoides*, an anaerobic bacterium able to cause reductive dechlorination of many chloro-organic compounds when inoculated in polluted aquifers (Lovley, 2003). A second one is *Geobacter* (Amos et al., 2007), which has shown its ability to remediate uranium-contaminated groundwater (Lovley, 2003). The best strains to do the job in both cases occur naturally. Furthermore, many of the toughest recalcitrant molecules (e.g. highly chlorinated aromatics) can be dealt with only by anaerobic bacteria, which are most often not amenable to genetic modification. To finish the less-than-rosy picture for transgenic bacteria, conditional killing circuits were far from achieving a certainty of containment which was hoped for.

On this basis, it is surprising to still see in environmental biotechnology numerous reports that propose engineering this or that bacteria for biodegradation of a target compound for potential use in bioremediation. There is a big gap between the potential and realisation and, for the sake of the field, it is better to accept that basically all early expectations of solving pollution and many other environmental problems through genetic engineering have conspicuously failed (Cases and de Lorenzo, 2005; de Lorenzo, 2009). In contrast, the field has yielded some dividends in the production and application of whole-cell biosensors (Ron, 2007; Vollmer and Van Dyk, 2004; Garmendia et al., 2008; de Las Heras et al., 2008) some of them for *in situ* application for detection of

underground chemicals, as well as bioadsorption an immobilization of heavy ions in engineered bacterial biomass (Valls et al., 2000). These are, however, minor victories in the midst of the debacle that has afflicted the pursuit of superbugs for combating pollution.

Think big: Global challenges

As the world becomes more global, we are becoming more aware that a large number of issues affect entire areas of the planet, with, next to climate change, the issue of global pollution by industrial waste and toxic chemicals. Pollutants produced at a given site are frequently mobilised to the upper layers of atmosphere and then deposited in remote areas, sometimes at high concentrations (Kallenborn, 2006; Daly and Wania, 2005). Unfortunately, it appears that nowhere in the world qualifies properly as a pristine, chemically virgin area. In this respect, it is worth noting that many antibiotics and other pharmaceuticals are eligible as authentic pollutants as well. In reality, there is not a sharp divide between synthetic molecules with antimicrobial activity and the many recalcitrant compounds produced or mobilised by the chemical industry (Alonso et al., 1999, 2001; Martinez et al., 2009). In other cases, xenobiotic compounds or their degradation intermediates become endocrine disrupters with devastating consequences for entire ecosystems. Finally, a set of convergent circumstances, i.e. changes in weather, global dissemination of microbial vectors through expanding transport networks and rapid evolution of antibiotic resistance, have led to the reappearance of epidemic diseases as well as the emergence of new ones. One daunting example of this regards the clear environmental origin of cholera outbreaks, which accounts for the sporadic and erratic occurrence of epidemics of this disease (Colwell, 1996; Colwell et al., 1998).

A better understanding of the connections between man-induced environmental changes and infectious diseases is desperately required. Such information is needed not only for explaining events in retrospect, but also for anticipating outbreaks and informing preventive measures. In summary, climatic change, pollution and infectious processes are at the top of the many issues that must be faced at a global scale. Is there any contribution of the genetic reservoir of microbial diversity for addressing these phenomenal problems?

The history of the planet Earth records a considerable number of changes in the composition of the atmosphere that can be traced to microbial action. One of them occurred 2 to 3 billion years ago, when primitive microbes acquired the ability to generate O_2 out of water using the energy from sunlight. This event altered altogether the ecology of Earth, as organisms were forced to cope with oxidative damage or else faced extinction. This change created new niches and heralded the emergence of the multi-cellular life forms during the Cambrian explosion (approximately 540 million years ago). Since then, the fossil record provides evidence of not less than five mass extinctions. Some of them have been attributed to a sudden change in the global composition of the atmosphere brought about by production of hydrogen sulphide by bacteria that lived in stagnant, deoxygenated water (Grice et al., 2005; Huey and Ward, 2005). Micro-organisms not only sense and reflect global environmental change, but they also contribute actively to bring it about. On this basis, only the global microbiota (which contributes the largest share of the Earth's biomass) has the high-scale catalytic power that would be required to decrease the ramping CO_2 levels, counteract the global warming and neutralise harmful emissions.

Our level of understanding of these processes is not enough yet as to be able to exploit them in our favour, so much more research is still required to this end. One ongoing (and timid) example of the use marine microbes for increasing CO_2 deposition involves the introduction of iron particles in the nutrient-rich, but iron-deficient, ocean waters in order to stimulate the growth of phytoplankton blooms (Pollard et al., 2009). A growing number of marine scientists (as well as businesses) are exploring such fertilisation as a way to foster the onset of plankton populations and sequester large amounts of CO_2 for reducing global warming and preventing ocean acidification. The approach is, however, not devoid of problems (Kintisch, 2008; Tollefson, 2008). When the organic material produced by a plankton bloom sinks to deeper waters, the resulting decomposition may use up oxygen in the medium and cause a destructive effect on marine life. Another concern is the effect of iron fertilisation on nutrients other than iron in the ocean, which may be depleted by phytoplankton growth. Yet, the iron fertilisation concept is not devoid of basis and will surely be applied intensively in the next few years, even at the risk of causing low-oxygen incidents and episodes of local anoxia (Kintisch, 2008; Tollefson, 2008). At the moment, little is known about how these procedures will affect marine food chains, which obviously know no borders. It is likely that the management and even deliberate stimulation of the catalytic capacity of marine microbes and soil bacteria at a planetary scale will be a serious matter of international politics in the not so distant future (Tollefson, 2008).

The onset of systems biology

The applications of systems biology to microbial ecology and environmental biotechnology were booming at the time of writing. The efforts embodied in this conceptual frame to address multi-scale microbiological complexity – from genes to whole communities – is the first step to comprehend more intricate setups where the microbiological constituent is just one of the players of a given system. Phenomena such as microbial pathogenesis, environmental catalysis, let alone climate change, involve a large number of biotic and abiotic components that interact dynamically. Yet, the various disciplines necessary to study these have traditionally been away from each other. Biofilm formation, which is at the core of a large number of microbial functions, is among many conspicuous examples of this sort.

Biofilms can be approached from at least two alternative conceptual frames, each of them using a distinctive descriptive language. Since the pioneering work of Bill Costerton (Costerton et al., 1995), many microbiologists see biofilm formation and evolution, in particular the generation of 3D structures, as the result of a genetically determined developmental programme (Monds and O'Toole, 2009), somewhat reminiscent of those found in animals. On the other hand, the very same phenomena can be described accurately with the only tools of physics and statistical mechanics, with no reference whatsoever to genetically programmed occurrences – a view advocated *inter alia* by Mark van Loosdrecht (van Loosdrecht et al., 2002; Nicolella et al., 2000). This is one of the cases where the divide between descriptive languages becomes more evident. Full understanding of the biofilm phenomena will surely requite the concourse of both approaches (Nadell et al., 2009). Another case involves the bioremediation scenarios mentioned above (de Lorenzo, 2008). The elements that influence the evolution of polluted sites include a combination of biotic and abiotic components, which are to be taken aboard for any useful understanding of each specific case.

Bioremediation could well be a privileged setting for the implementation of a systems science that merges and makes sense out of multi-scale data from all the biological, chemical and physical actors of the process. This endeavour is, however, plagued by the lack of a suitable format to compare and match results arising from different experimental systems and science fields. There is little consensus on the names of the genes, on the conditions of the experiments, on the definition of the parameters, on the activities of the various enzymes, etc. Researchers use *ad libitum* the International Union of Pure and Applied Chemistry's (IUPAC) nomenclature for compounds, together with vulgar names, thus an automated and interactive comparison of the data available is made very difficult to those not inside a given community. Maybe the key for degradation of polyaromatic hydrocarbons (PAHs) relies on a piece of data hidden in a publication on cancer that most microbiologists may never stumble across. Many relevant facts are surely documented, but in a cryptic form and we do not know how to access them and how to benefit from them (Cases and de Lorenzo, 2002). The literature already contains a great deal of information that cannot be properly extracted. The lack of tools to penetrate and process the abundant materials available in specialised publications prevents the translation of such information into useful general principles. Systems biology may provide a remedy to most of these problems because of its insistence on data standards, benchmarking experiments and expressing results in suitable quantitative formats. But we are not there yet. The concourse of computer scientists (including computational linguists) is a must to translate the soft narrative that is so typical of much of the (micro) biological literature into rigorous numerical descriptions of the systems under scrutiny.

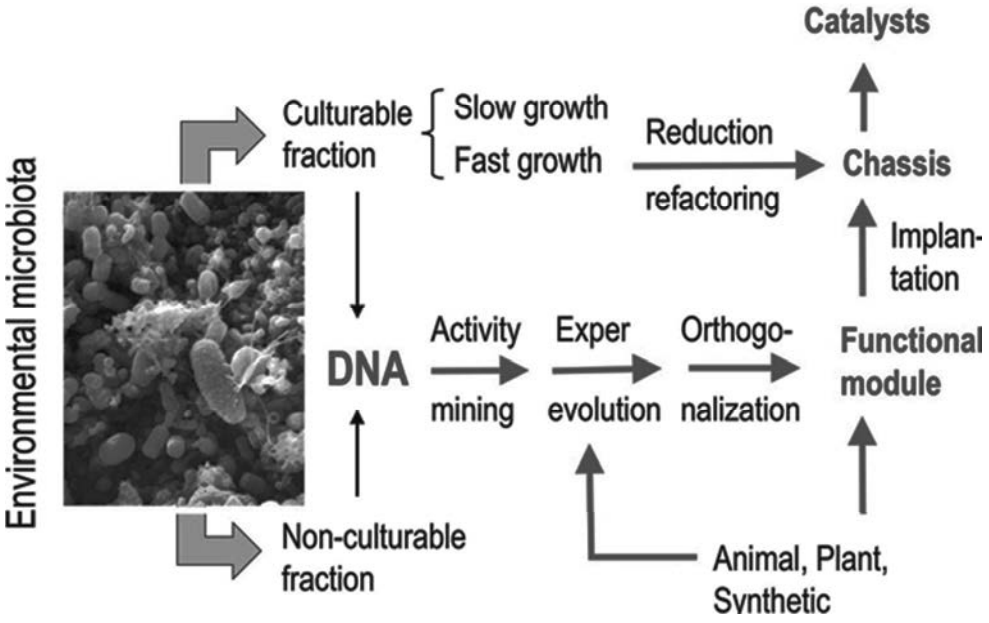
Synthetic biology: The next frontier

The early agenda of recombinant DNA technology in the late 1970s included the notion of genetic engineering as a metaphor of how the new methods would allow us to build new properties in biological systems. All of the activities under the umbrella of synthetic biology convert such an early engineering analogy into a veritable methodology. While traditional genetic engineering uses mostly trial and error approaches to produce new biological designs, synthetic biology attempts to reshape live systems on the basis of a rational blueprint (de Lorenzo and Danchin, 2008). To this end, biological objects are seen as wholes of stand-alone parts hierarchically assembled in modules, devices, subsystems and systems that can be abstracted and completely understood (Endy, 2005; Canton et al., 2008; Arkin, 2008). By the same token, the components of extant biological systems can be de-constructed and rationally re-constructed to build new biological objects with properties *à la carte*. This extreme engineering scene embodies the most extraordinary potential for both understanding the functioning of live systems and for constructing biological materials with a large variety of applications. Yet, implementation of this desirable scenario still needs to fill a large number of gaps in our knowledge of existing biological systems, including the definition of the biological building blocks that can be used for robust engineering; the adoption of a descriptive, quantitative language for biological transactions; and the identification and management of the physical, chemical and evolutionary constraints that frame the functioning of any autonomous biological system (de Lorenzo and Danchin, 2008).

The performance of virtually all biological objects – from proteins to communities – is context-dependent. Furthermore, live entities are perpetually changing under the inexorable laws of Darwinian evolution. Yet, existing biological systems are very robust so it should be possible to design them as well. To this end, a better conceptual frame is needed to understand what minimal biological building blocks are and how they can be

formatted and engineered. The nature of such biological parts is essentially different from e.g. components of electric circuits or mechanic engines. In addition, the nature and description of biological building blocks depends on the scale of the engineering objective. While genetic circuits may rely only on defined promoters and reporters, designing a whole cell will require complete functional modules as building blocks. Similarly, whole cells will be the parts for microbial community design and tissue engineering, and so on. There is a considerable list of research items associated with these issues. Fortunately, the growing ease of synthesising long DNA segments, even complete genomes, should make the field progress at a very fast rate.

Figure 7.1. **Flowchart for the generation of genetically engineered catalysts in the era of systems and synthetic biology**



Notes: The largest reservoir of biological activities is the non-culturable environmental microbiota, including the viral component. Various activity mining strategies employing wet or computational procedures can be used to identify pools of enzymatic activities of interest (pan-enzymes; de Lorenzo, 2008) in the corresponding metagenomic DNA. These can be evolved experimentally for an optimal performance and further orthogonalised (i.e. their functioning made autonomous from the final host). This gives rise to functional modules composed of one or more genes endowed with their cognate regulatory circuit – again, engineered for an optimal performance. On the other hand, the genomes of culturable fast-growing members of the microbial community can be minimised for deletion of undesirable features and optimised as the chassis for implantation of modules of either microbial origin or imported from other kingdoms, including non-natural biological objects (proteins, ribozymes, etc). The outcome of the flowchart is the production of robust and predictable whole-cell catalysts for *in situ* or *ex situ* environmental remediation. It is likely that the genomic chasses for these procedures will soon be altogether synthetic (Gibson et al., 2008).

Source: de Lorenzo, V. (2010), "Exploiting microbial diversity: The challenges and the means", in K. Timmis (ed.), *Handbook of Hydrocarbon and Lipid Microbiology*, Springer, Berlin-Heidelberg, pp. 2 438-2 458.

One possibility in this context is the creation of altogether artificial cells in which the whole genome is synthetic (Gibson et al., 2008) and can be programmed for a given application, an operation reminiscent of writing instructions in a computer programme (Danchin, 2009). Production of synthetic or semi-synthetic bacterial cells of this sort is now at hand, and the ultimate agenda of the genetic engineering that Cohen and Boyer started in the 1970s appears to be within reach. To avoid the re-enactment of the

controversy on GMOs that such synthetic cells could bring about, others see it more feasible to engineer DNA-free vesicles endowed with all basic features of live cells but without any ability to proliferate (Noireaux and Libchaber, 2004; Kuruma et al., 2008). Generation of synthetic cells is not only a biotechnological challenge, but also a serious scientific endeavour which touches upon very fundamental questions, e.g. the origin of life and the emergence of self-maintaining biological systems (Luisi, 2006).

New risks in sight?

The safety concerning accidental or deliberate release of semi-synthetic or entirely synthetic agents is the subject of much ongoing discussion (de Lorenzo, 2010a; de Lorenzo and Danchin, 2008; Schmidt and de Lorenzo, 2012). The large body of literature on GMOs and GEMs for environmental release shows that the more engineered one bacterium is, the less fit it is also to survive once released. However, even heavily engineered organisms function thus far on the basis of what one could call familiar biology, i.e. live systems based on DNA as information-bearing molecules, L-amino acids, D-sugars and a generally very conserved protein translation machinery. Despite the diversity of existing biological systems, they all share these basic building blocks and genetic software. Synthetic biology ultimately ambitions to emancipate biology from such constraints and create in the laboratory live objects based on other principles (Marliere et al., 2011; Marliere, 2009). While this is not yet at hand – and may not be in the near future – it is just a question of time that both organisms and properties new-to-nature (NTN) will be assembled. When the time comes, it will be necessary to anticipate new safety and risk scenarios associated to these new agents on the background of the benefits that they can bring about as well (Schmidt and de Lorenzo, 2012). But, as long as we remain in the realm of such a familiar biology, we may well handle virtually any possible scenario involving the release of GEMs for the next 10-15 years. The problem in most cases is that of its proper and efficacious performance of the engineered agents and not any risk of ecosystem takeover.

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