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Genetically modified organisms for the environment: stories of success and failure and what we have learned from them

Summary. The expectations raised in the mid-1980s on the potential of genetic engineering for in situ remediation of environmental pollution have not been entirely fulfilled. Yet, we have learned a good deal about the expression of catabolic pathways by bacteria in their natural habitats, and how environmental conditions dictate the expression of desired catalytic activities. The many different choices between nutrients and responses to stresses form a network of transcriptional switches which, given the redundance and robustness of the regulatory circuits involved, can be neither unraveled through standard genetic analysis nor artificially programmed in a simple manner. Available data suggest that population dynamics and physiological control of catabolic gene expression prevail over any artificial attempt to engineer an optimal performance of the wanted catalytic activities. In this review, several valuable spin-offs of past research into genetically modified organisms with environmental applications are discussed, along with the impact of Systems Biology and Synthetic Biology in the future of environmental biotechnology. [**Int Microbiol** 2005; 8(3):213-222]

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Introduction: the quest for the superbug

Bioaugmentation (addition of specific microorganisms) and biostimulation (addition of specific compounds to enhance microbial metabolism) are methods that can be applied to accelerate the recovery of polluted sites. In the late 1970s and early 1980s, bacterial genes encoding catabolic enzymes for recalcitrant compounds started to be cloned and characterized. Soon, many microbiologists and molecular biologists realized the potential of genetic engineering for addressing biodegradation. The pioneering work of Gunsalus and Chakrabarty on the genetic basis of degradation of a suite of recalcitrant compounds by *Pseudomonas* strains culminated in 1981 with the granting of a patent for a strain developed by conjugation that could degrade camphor, octane, salicylate, and naphthalene [US Patent #425944], the first living being to be the subject of an intellectual property case. At that point, it seemed that molecular techniques, either through plasmid breeding or sheer genetic engineering, could rapidly produce microbes with higher catalytic abilities, able to basically degrade any environmental pollutant.

This promising picture had a solid basis, as pathways for degradation of novel carbon sources arise through what one could call "natural genetic engineering" processes. For the most part, these involve mutations that broaden the substrate range of pre-existing enzymes, shuffle sequences, horizontally transfer DNA pieces between members of a microbial community, and cut and paste larger DNA segments to form new hybrid genes and metabolic operons [70]. Most such biodegradative gene clusters end up under the control of substrate-responsive promoters [10,68]. While natural selection can accomplish all of these processes, it may take a long time to obtain bacteria that can be deliberately employed as biocatalysts for release in polluted sites. Recreating these natural events in the test tube would accelerate the development of bioremediating strains of microorganisms. Following the seminal work of Chakrabarty and his colleagues on the degradation of petroleum components [26] and chloroaromatic compounds [27], the possibilities of applying the tools of genetic engineering to the biodegradation of recalcitrant pollutants had a sensational boost with the series of papers published by the laboratory of Timmis in the mid- and late 1980s [53,57]. Their work

showed that, by judiciously cutting and pasting DNA from different origins, and placing the resulting assembly of genes under the control of sufficiently strong promoters, it was possible to produce *Pseudomonas* strains bearing hybrid metabolic pathways able to eliminate very recalcitrant compounds (for instance, mixtures of chloro-benzoates and alkyl-benzoates). This influential work raised great expectations about designing microorganisms for in situ bioremediation with the tools of genetics. But it also ignited a scientific and public debate on the possible ecological risks of such applications, and awakened an enormous interest in microbial ecology among many molecular biologists [35].

Imaginary risks *vs.* real bottlenecks

As soon as the prospect of releasing genetically modified microorganisms (GMOs) for bioremediation became a reality, much of the research effort in the field was aimed at biosafety and risk assessment. New and exciting questions were asked, and novel technologies developed to address public concerns. The use of recombinant organisms for environmental applications was bound to differ in important respects from that for contained manipulations [61]. Instead of being propagated as a monoculture in an optimized, controlled environment with nutrients in excess, the recombinant organism is expected to be introduced into a diverse biological community where it must establish itself, interact with other bacteria in unknown ways, and face a multitude of poorly controllable external factors, some of which place it under considerable stress. Moreover, microorganisms generally live in biofilms attached to surfaces, rather than having a planktonic lifestyle. In summary, many environmental conditions encountered in bioremediation are patently unfavorable for the GMOs. Therefore, the specific characteristics of open biotechnological applications clearly required novel genetic tools and concepts to engineer new properties and to meet the demands of eventual applications in the field [66]. Such demands included stability without selection, minimal physiological burden, small-size non-antibiotic selection markers, minimal lateral transfer of cloned genes to indigenous organisms, and traceability of specific genes and strains in complex ecosystems. In particular, the biological and genetic containment of recombinant genes became a challenge, as many schemes were proposed to program cell death once the biocatalyst had fulfilled its mission, or in the event that genetically modified genes could be accidentally transferred [19,50].

The attempts to meet all of these expectations were somewhat suboptimal, as absolute containment of GMOs cannot be achieved [16,67] and inoculated recombinant bacteria often compete poorly with indigenous communities in the long run [61]. With the exception of cases of extreme selection (for instance, activated sludge for the treatment of more or less concentrated waste), only in very few cases has the use of a GMO turned out to be much better than the performance of its natural, non-manipulated counterpart [65,72]. In reality, it can be argued that the bottleneck for the efficacious use of GMOs in the environment has not been the body of legal regulations that limit their release. Instead, it is the lack of knowledge on microbial physiology and ecology, which is a prerequisite for any application of this sort. It is difficult to set up realistic clean-up interventions in the environment unless we comprehend the general rules (let alone the mechanisms) of the processes involved. If the only bottleneck for degradation of a given compound by a community were the lack of one enzyme or one regulatory protein, the massive selective conditions imposed by the presence of the target chemical as a potential nutrient in a polluted niche would likely result in the emergence of a new variant to overcome such a limitation. If that does not happen, it may well mean that the bottleneck lies somewhere else.

From the test tube to the environment

While genetic engineering has produced numerous strains able to degrade otherwise intractable pollutants in a Petri dish [43,56] or in a bioreactor, the practical translation of this research into actual in situ bioremediation practices has been quite scanty [18,61]. One major issue in this respect is the growing realization that the strains and bacterial species that most frequently appear in traditional enrichment procedures are not the ones performing the bulk of biodegradation in natural niches—and may not even be any good as bioremediation mediators. The use of stable isotope probing (SIP) and equivalent methods in microbial ecology [73] have revealed that *Pseudomonas*, *Rhodococcus*, and the typical aerobic fast-growers that are widely favored as hosts of biodegradation-related recombinant genes are far less significant under natural conditions. Actually, fast-growers optimize production of biomass versus consumption of the available C-source. A few calculations make it readily apparent that the toll for using fast-growers as agents for biodegradation is the inevitable buildup of unwelcome biomass. Instead, the optimal clean-up agent would be the one that displays a maximum catalytic ability with a minimum of cell mass, a quality that can hardly be enriched for with traditional methods. The expression of biodegradation genes can be artificially uncoupled from growth with the use of stationary-phase promoters or starvation promoters [40,41], but then cells have to be maintained active at the expense of a second C-source (such as the exudates of plant roots, see below).

Whether fast-growers or slow-growers, the introduction of laboratory strains in complex microbial communities faces not only resistance-to-colonization effects, but also subopti-

mal niche specificity and poor adaptation of the transcriptional machinery of the GMO existing at the start point. In particular, the stationary-phase sigma factor RpoS (and perhaps other sigma factors) seems to undergo a niche-specific selection of certain alleles that ensures optimal fitness in defined physico-chemical conditions [22]. Furthermore, work on *Escherichia coli* and *Pseudomonas* has revealed that the non-homogeneous texture of natural niches selects distinct populations that quickly diverge genetically from the initial inoculum [49]. In view of all this, what are the determinants for the correct expression of a desired catabolic activity in time and space? Can we really engineer and release GMOs with a high degree of predictability and biotechnological performance?

The efficacy of a desired in-situ catalytic activity (biodegradation or otherwise) depends first on its very presence in the target site. One key enzyme may not be there, or it may pre-exist in the site but not be manifested. Alternatively, it can be hosted by just a very minor part of the whole microbial population, so that its factual expression in the site might not be significant. In these cases, is inoculation of GMOs a choice? Note that the use of microorganisms for bioremediation does diverge from the dispersion of bacteria for plant protection (for instance, ice-minus *Pseudomonas* or insecticidal *Bacillus thuringiensis*), as the catalyst must reach pollutants well below the surface of the afflicted site. In reality, at this stage, whether the introduced bacterium is recombinant or not makes little difference, because the problem is that of implantation of foreign microbes in an unfamiliar territory. The introduction of bacterial biomass in an existing niche may create a palatable niche for protozoa [29] that prevents the bacterial population to grow beyond a certain level. Ingenious approaches have been developed to circumvent this problem, including encapsulation of the inoculum in a polymeric matrix or protection in plastic tubing [5], but these may not be lasting solutions. In this respect, there is a great need to develop a sort of "Environmental Galenics" which—like Galenic medicine—is concerned with the formulation, dosage, and delivery of biological remedies to an environment in poor condition.

One promising strategy includes increasing the population of the bacterial catalysts in the rhizosphere of adequate plants that can be employed as a vehicle for inoculating and spreading the microorganisms in polluted soil [33,34,69]. This approach—generically called *rhizoremediation*—does hold a promise for pollution clean-up, as the system benefits also from water and water-soluble compounds flowing through the bacterial community on their way to the root surface. Moreover, the basic metabolism of plant root colonizers does not depend on degradation of the target compounds, but on consumption of root exudates. This makes the thermodynamic balance between degradation and physiological burden much more favorable, as biomass production is uncoupled from elimination or detoxification of the contaminant in soil [6]. A variant of this concept is the use of endophytic bacteria to the same end, as microorganisms—although never reaching high numbers—are then protected from predators and other stresses [3]. This allows suitable genes to be placed and expressed in heterologous hosts that are optimum for thriving in a particular environment. In another approach, publication of the complete sequence of one strain of *Deinococcus radiodurans* [7] raised great expectations because this species endures harsh environmental conditions, including high radiation doses, and is also amenable to a whole suite of genetic manipulations. A more sophisticated—and surely promising—approach to augment the presence of biodegradative genes in a community is promoting the dissemination of DNA encoding the products of interest among indigenous populations by means of promiscuous plasmids or other mobile genetic elements [64]. It is a revealing paradox that so many research efforts have dealt in the past with containing gene transfer while, in the future, the challenge might be to achieve exactly the contrary, i.e., to stimulate propagation of a given DNA segment through a target microbial community.

Whether recombinant or not, the directed delivery of bacteria or their genes into contaminated sites remains a phenomenal technological challenge. A growing trend is the generation of transgenic plants able to express biodegradation or detoxification genes recruited from bacteria. The examples of the Hg-volatilizing poplar [59] or the trinitrotoluene (TNT)-removing *Arabidopsis* [25] illustrate well this point. The use of natural or genetically modified plants as tools of bioremediation (phytoremediation) has several advantages. One of them is that the geotropism of the roots delivers spontaneously the catalysts well below the soil surface. A second advantage is that inoculation strategies benefit from the methods already optimized for extensive agricultural dispersion of plant seeds. And third, horizontal gene transfer is far less probable. In view of the difficulties for delivering microbial GMOs to polluted sites, it is plausible that the ectopic expression of bacterial genes in plant carriers will open a new perspective to interventions aimed at environmental clean-up—as well as to the very value of transgenic plants for non-food purposes.

From genetic engineering to eco-engineering

On the basis of the comments above, does in situ intervention with microorganisms as biocatalysts still have a future? The answer is one unequivocal yes. Microbial processes are at the very basis of the cycles of nutrients in the biosphere, and bacteria will still be our main assets to tackle environmental pol-

lution. Yet, future research may no longer focus on the design and delivery of super-bugs. Instead, researchers will probably attempt to grasp what are the conditions that either stimulate or limit the emergence and expression of the desired in situ degradation activities: N and P sources, electron acceptors, oxygen tension, temperature, osmotic pressure, etc.—as has been clearly substantiated by the experience of the *Exxon Valdez* oil spill [47]. Microbial degradation of environmental, (often) xenobiotic pollutants is generally limited by a series of physiological and regulatory blockages that include: (i) the lack of induction of the correct catabolic operons when microorganisms are exposed to the target compounds because of low concentration or failure to be identified as regulatory effectors; (ii) the lack of recognition of the chemicals of interest as substrates for the catabolic enzymes present in the bacteria; (iii) the stress caused to the cell by the same substrates or other environmental conditions; (iv) the metabolic chaos caused by the generation of toxic and dead-end products brought about by the coexistence in a given site of multiple contaminants along with a diversity of microbes bearing manifold metabolic pathways; (v) the lack of solubility of the contaminants; and, most importantly, (vi) the thermodynamic balance of the degradation process, which is framed by the redox potential of the contaminated site and the availability of suitable terminal electron acceptors. In fact, the composition of the community growing at a given site and the ability of their members to express distinct sets of genes depend on the prevailing environmental physico-chemical circumstances. The consequence of these notions is that we could promote expression in situ of specific catalytic activities by altering the conditions of the affected site—provided that we understand how population dynamics and gene expression are connected to multiple environmental inputs. There is thus an ongoing shift from genetic engineering of the biocatalyst for in situ delivery towards eco-engineering of the site for stimulating the onset of the pursued metabolic activities within the indigenous community.

While an understanding of all of the factors that influence the interplay between environmental conditions and community-wide gene expression capacities is not yet at hand, we can at least consider what happens to the individual microorganisms involved. In their habitually polluted niches, bacteria face two concurrent physiological choices (Fig. 1). First, there is a choice between nutrients (A and B), so that those with higher energetic returns and/or that are more available are preferred over those more difficult to metabolize. However, bacteria are subjected to various types of environmental stresses (including temperature, solvents, UV radiation, desiccation, starvation, and predation), and their endurance may divert much of the metabolic energy that would be otherwise employed in the buildup of catabolic pathways for given C sources. A side aspect of such choices is that environmental pollutants, including aromatic hydrocarbons and xenobiotics, can be both nutrients and stressors due to their toxic effects on cells that grow in contaminated sites. Under these circumstances, bacteria must either activate the genetic program for consumption of the pollutants as nutrients, or those for enduring simultaneously various kinds of stresses [10]. The outcome of such an impasse, which each cell of a given population has to undergo (Fig. 1), is then projected into both the gross composition of the community and the level of expression of the genes involved in the degradation of the available C sources.

Regardless of the specific activation mechanism, every bacterial promoter can be described rigorously through a limited number of parameters (Fig. 2). Various techniques reveal distinct parameters. For instance, transcriptome analysis with DNA chips reveals the induction or inducibility of given genes, but provide little information on promoter capacity. The factual level of expression of any given gene in a single cell or in an entire population of cells is then subject to population effects. For instance, genes encoded in plasmids or located closer to the replication origin in the chromosome may have a factually higher gene dose and thus be expressed at higher levels [63]. Even in homogeneous bacterial populations, the distribution of promoter strengths may not be (and in fact is not) identical (Fig. 2).

The microbiological questions behind eco-engineering are therefore about: (i) understanding the control of expression of catabolic genes in their natural environment; (ii) learning how bacteria evolve the ability to respond transcriptionally and post-transcriptionally to novel environmental signals (for instance, xenobiotic compounds); (iii) how such

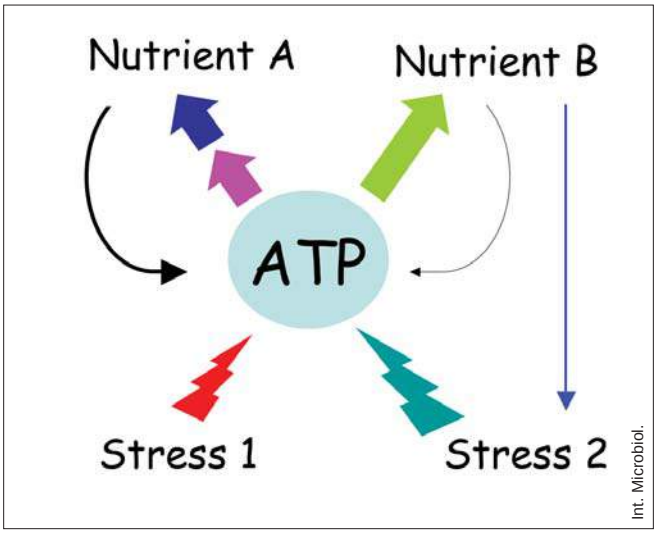

Fig. 1. Regulatory choices in environmental bacteria. The accessible energy resources for single cells is operatively represented here as ATP. Bacteria must spend a considerable share of such energy for the buildup of catabolic pathways for the dedicated metabolism of available C-sources (and to a lesser extent N, P sources and oligonutrients as well).

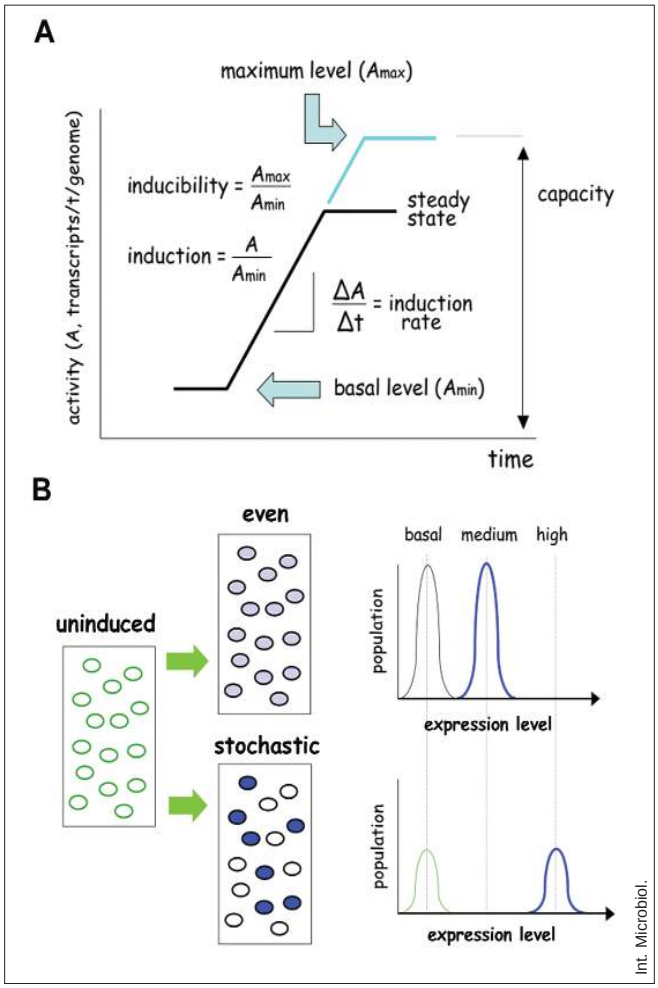

Fig. 2. Intrinsic qualities of bacterial promoters and population effects. The most important property of a promoter is its strength (**A**), which is defined as the number of productive transcripts initiated per second and per genome unit. Capacity is the maximum absolute activity level. (**B**) Some promoters exhibit a wide range of activities that increase proportionally to the extent of the inducing signal (rheostatic response [32]. Others, by contrast, display an on/off switch (typically, the *lac* promoter of *Escherichia coli*) that is activated by a larger portion of the population in response to the corresponding stimulus [4,32]. From a population point of view, the operative output of gene expression might be similar in both cases, but mechanistically they are quite different [21,58].

a response is integrated in the global regulatory network of single cells and communities [11]; and (iv) how physiological competence of each of the partners is translated into community composition.

Evolving regulation: acquisition of new specificities for environmental inputs

The above-discussed scenario of complex communities undergoing a considerable stress seems to be perfect for the rapid evolution of transcriptional control systems to respond to novel environmental signals, such as new chemical structures released into the environment [15,68]. This control generally involves an effector-specific transcriptional regulator, the activity of which on its cognate promoter is directly or indirectly controlled by physiological signals (such as metabolites and small molecules) that report the physiological status and check promoter output [10]. Probably, many transcriptional regulators that are affected by small molecules start out by having a crevice in their structure, which evolves as a molecular pocket for a given compound in a more or less specific fashion. For instance, the LysR-type ClcR protein, which activates a pathway for the degradation of 3-Cl catechol in *Pseudomonas putida*, is directly inhibited by fumarate [42], an intermediate of the tricarboxylic acid (TCA) cycle, thereby entering a degree of metabolic control in the system. In other cases, entire protein folds seem to be recruited by transcriptional activators as effector-binding sites. The toluene-binding domain of the transcriptional regulator XylR of the TOL plasmid of *Pseudomonas* mt-2 seems to have the same overall structure as the substrate-binding site of the enzyme catechol O-methyltransferase [17].

As discussed elsewhere [15], the acquisition of specificity for a new inducer in a transcriptional regulator requires a pre-existing regulator with a certain escape (i.e. responsiveness to non-legitimate effectors), upon which new specificity can be built by natural (or artificial) rounds of mutagenesis and selection. Yet, the move from specificity for compound A to specificity for B rarely takes place in a single step even if A and B are related structurally. Changes in effector specificity in bacterial regulators have been attempted experimentally in various cases, not only for designing new expression systems [12,51] but also as biological components of biosensors [13,75]. Genetic screenings for new specificities systematically result in regulator variants, which broaden the range of effectors towards the new one—rather than exchanging specificities altogether [12,23]. By computer-aided manipulation of effector-binding sites in periplasmic sugar-binding proteins of *E. coli*, Hellinga et al. have calculated that the number of combinatorial possibilities to alter the native sugar-binding pocket of such proteins to recognize specifically a different effector is in the range of 10^{76} (same magnitude than the number of atoms in the universe) [36]. Since the best technologies for generation of molecular diversity in the laboratory hardly go beyond 10^{12}–10^{14}, it is clear that evolution of the specificities of transcriptional factors rarely occur in a single step. On the contrary, it is probable that moving between peaks of effector specificity is bound to occur through valleys of unspecificity. In fact, regulators of microbial pathways for recent compounds (e.g. many environmental pollutants) are not too specific for their substrates [15], which may reflect an ongoing evolution not entirely optimized yet for the unusual, sometimes xenobiotic, nutrients [68,70].

The modularity of business domains in transcriptional factors

While most bacterial transcription factors (90% in *E. coli*) are multi-domain proteins, the actual number of different domains for DNA binding and signal-reception and response functions is comparatively small. There are approximately 300 transcription factors in *E. coli*, but only 11 different DNA-binding domains and 46 input domains [37,38]. Given this limited repertoire of modules, the diversity required to respond to a wide range of signals and to deploy a number of regulatory strategies can be achieved by combining available domains. There are 74 distinct domain architectures in *E. coli* [37], which still suggest a significant degree of gene duplication. One possibility to respond to similar signals in different ways is to combine similar input modules with sets of different DNA-binding domains, as the location in the overall protein structure affects the regulatory activity of the transcription factor. Repressors tend to have their DNA-binding domains at their C-terminus while activators place the same activity in their N-terminus [46]. Moreover, similar domain architectures can have different effects depending on the locations of their binding site relative to the transcription start site. In this way, the same regulator can activate some genes when it binds to upstream binding sites, while repressing others when it binds downstream to RNA-polymerase-binding sites, thus providing another level of variability [37].

Evolving pockets and developing regulatory networks

One critical realization for understanding the buildup of a given transcriptional response in the environment is that such a response is not limited to the emergence of a single regulator or regulatory domain with a given effector specificity. In fact, it looks as if the evolutionary drive of regulators were at odds with conventional engineering wisdom. For instance, there are multiple regulatory systems for operons encoding the biodegradation of toluene in *Pseudomonas*. In the archetypical case of the TOL system of *Pseudomonas putida* mt-2, the regulator XylR interacts directly with the pathway substrate and triggers transcription of the corresponding metabolic operon [52]. Other toluene-degrading *Pseudomonas* strains activate their catabolic operons for the compound by means of the 2-component system *todST* [55], in which the sensor protein interacts with toluene. Yet a third toluene-degrading gene cluster born by *P. stutzeri* OX1 [60] is activated not by toluene, but by methyl-catechol (a downstream intermediate of the metabolic pathway), so that a certain basal level of activity of the route is required prior to the buildup of enough inducer for the regulator TouR [1]. In other words, it seems that one regulatory problem (having an operon induced in the presence of toluene) can be solved through at least three different outcomes (and possibly many more).

A second feature of the same issue is how very similar substrates end up associated with different types of regulators [20]. Benzoate and salicylate are similar molecules that can be degraded, respectively, by *P. putida* mt-2 and *P. putida* NAH7 through very similar catabolic pathways [15]. However, the activator of the benzoate-degradation operon is the AraC-like factor XylS, while that of the salicylate operon is the LysR-type protein NahR. The surprising aspect of such a difference is that XylS mutants that respond to salicylate [54], as well as NahR mutants that respond to benzoate [12], can be easily isolated in the laboratory. Why did the two proteins evolve so divergently despite regulating very similar pathways for very similar substrates? As in the case of other regulatory paradoxes, perhaps the answer can be found in the selection of whole or partial regulatory networks that merge specific responses (e.g. induction by a substrate) with more general environmental inputs—instead of the selection of just one component of the network. In this respect, note that multiple regulatory schemes can be generated artificially by random combination of just a few control elements [24].

Although there are few experimental data, it is plausible that the evolutionary unit of transcriptional control is not the sole regulator or the promoter, but the network or network motif to which they belong (Fig. 3). The practical consequence of these notions is that heterologous expression systems can hardly be grafted into environmental bacteria while disregarding the wider regulatory network in which every cell promoter is embroiled. Small chemical species (nutrients and signal molecules) can interact with proteins and with RNAs in riboswitches, but not with other small molecules or with DNA (Fig. 3). In addition, RNAs can interact with proteins and other RNAs [30], and even be the direct sensors of environmental inputs such as temperature [31], whereas DNA interacts only with proteins. In a homogeneous population, small molecules can diffuse out and regulate the transcription of genes in neighboring cells (typically, in quorum sensing), so that regulatory networks of individual cells can become coordinated at a whole-population level. But in the typical heterogeneous environmental scenarios, with multiple nutrients and a diverse microbial community, diffusion can include metabolic intermediates as well, which allow a degree of metabolic integration [45]. Bacteria do exchange signal molecules and nutrients with other microorganisms, plants, and animals, so that regulatory networks involving various populations of diverse species can be selected for optimal fitness of the entire biological community [10]. Horizontal gene transfer may help in some cases to evolve an optimal topology and density of regulatory networks held by a microbial consortium.

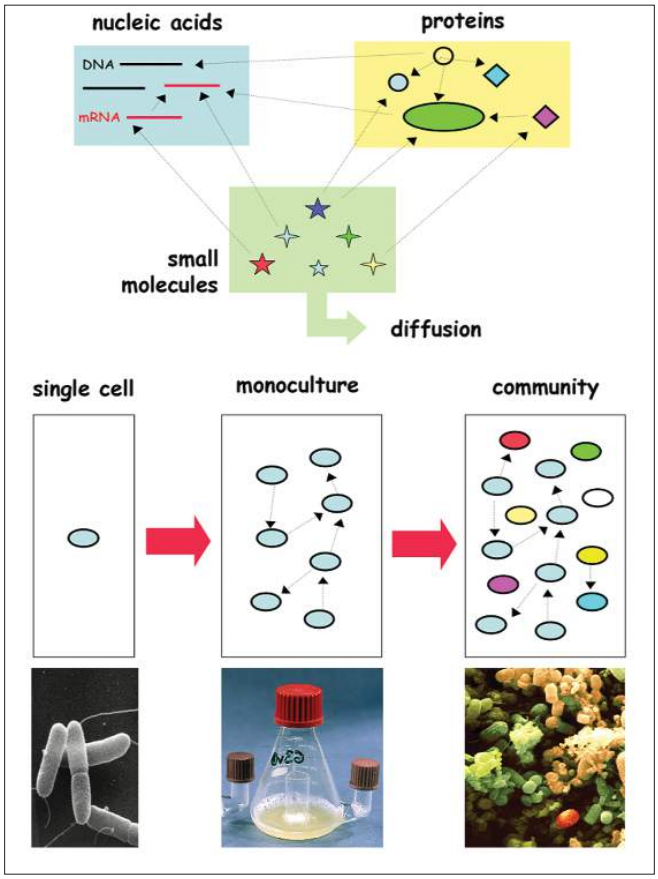

Fig. 3. Iterative networks in the regulation of gene expression in environmental bacteria. At the level of a single cell, transcriptional networks involve proteins, nucleic acids, and small molecules. Although all of them can be nodes of scale-free networks [2] or components of small worlds and contribute to their topology and density, only proteins can interact physically with all of the other components.

Engineering in situ expression of heterologous genes

One of the reasons for the early optimism about GMOs in the environment [35] was the notion that strong promoters could simply be engineered upstream of the required genes and the microorganisms would thus express the desired activities at high levels upon their release. This can indeed be done with little difficulty in a Petri dish. However, now it is clear that the regulatory intricacies that determine the behavior of bacteria in the environment flaws any attempt to design heterologous expression systems in GMOs destined for liberation. Besides the necessity to subordinate transcription levels to the overall metabolic status and stress conditions, a number of post-transcriptional checks further ensure the linkage of any protein within the wider context of the cell proteome. Growth conditions influence in many ways mRNA stability, so it cannot be taken for granted that a strong promoter ensures the expression of a gene or gene cluster at high levels. Instead, the strong expression of cloned genes might be the main cause for the loss of fitness. Moreover, enzymes do not float loose in the cell cytoplasm, but form highly structured and functionally related multiprotein complexes [8]. The expression of new proteins in subrogate environmental hosts may not automatically translate into an efficient performance of the desired activity if the corresponding polypeptide is not accompanied by the correct constellation of interacting proteins. However, we do not have to give up the possibility of expressing recombinant genes in the environment, as we can still approach the problem from an engineering point of view. Instead of forcing well-known strong promoters to perform in a very different context [50], it is more sensible to search for promoters that are actually optimized for delivering in a given site, and exploit such promoters as expression assets. In this respect, the last few years have witnessed the emergence of a collection of techniques under the denomination of IVET (*in vivo expression technologies*) which attempt to recognize promoters that become active exclusively in very specific niches—regardless of the promoter type or regulatory mechanism [48]. Once such promoters are identified, they can be exploited for constructing niche-specific expression systems à la carte, thereby offering invaluable opportunities for introducing desired genes into predetermined sites. The success of this approach, however, has been mostly limited to the expression of reporter genes in strains destined for biological control of plant diseases [74], and not so much for bioremediation.

Outlook: molecular biology for a green environment and a sustainable society

In a 1992 article [14], one of us argued that "[E]nvironmental applications of genetically engineered microorganisms are currently hampered not only by legal regulations restricting their release, but also by the frequent dearth of adequate genetic tools for their construction in the laboratory […]." After 15 years, regulations have relaxed and we have plenty of genetic tools—yet we are still far from dominating the construction of GMOs for in situ bioremediation. What then appeared to be a simple DNA cut and paste exercise appears now framed within a complex network of intracellular and intercellular metabolic and regulatory interactions that cannot be tackled with traditional genetic approaches. Yet, the recent rise of the "omics" technologies (genomics, proteomics, metabolomics) and the expected generalization of Systems Biology approaches [45] open new avenues for taking a fresh look at such a complex problem. Systems Biology is about complexity as such, without breaking it down into smaller parts. This is quite a dramatic methodological shift, as Systems Biology attempts to overcome the traditional reductionist meth-

ods of molecular biology and instead provide computational and conceptual tools for tackling intricate biological questions from a holistic perspective. In this context, the integration of data on the catalytic performance of microbial communities with information on the chemical fate of pollutants will offer a sound scientific basis to eco-engineering of interventions, which are still dominated by trial-and-error, experience-based approaches.

While the mutagenesis/phenotypic analysis approach is of great value to decipher regulatory phenomena with a limited number of variables, it may fail to reveal major aspects of regulatory networks. Like a three-legged table, the response to a given environmental input might include unrelated but equally essential genes or groups of genes, and the removal (i.e. mutation) of one of them may make the whole response collapse, thereby disguising the equally important roles of the others. In other cases, as in a four-legged table, a redundant and highly connected regulatory network might lose altogether one of its components and the loss would not be detected phenotypically. Current approaches, such as DNA chips and proteomic analysis of nucleoprotein complexes, are helping to sort out networks of this type that are otherwise opaque to traditional genetic scrutiny.

In the meantime, many of the early efforts for the construction of GMOs for the environment have been exploited by the biocatalysis/biotransformations sector, so the wealth of unusual reactions that form parts of many degradative pathways now serves as one of the bases of modern green chemistry [62]. While delivering predictable GMOs for in situ bioremediation is still an enormous challenge, making them work as catalysts under the controlled conditions of a reactor is a comparatively easy operation. In this respect, genetic engineering is helping not only to optimize many biotransformations that give rise to high-added-value substances, but also to simplify downstream processing of the reaction products [39]. The large reservoirs of biocatalytic activities that remain unexplored in the realms of the anaerobic and the non-culturable bacteria can now be accessed with meta-genomic technologies [9]. In this way, key genes can be scavenged from nature for practical purposes without caring for the original host. In 2005, the interface between genetic engineering and chemical engineering looks a lot more promising than that between genetic engineering and in situ bioremediation. A clear example of this shift is the growing attention to transgenic plants not just as vehicles for delivering bacterial biodegradation activities to soil (see above), but as genuine cell factories for the production of precious materials, including antibodies and plastics [76].

Further bits and pieces of the earlier body of research on environmental GMOs have found stimulating spin-offs in the field of whole-cell biosensors for detecting chemicals. This is based on the exploitation of the promoters of bacterial biodegradative or detoxification operons that naturally respond to pathway substrates in order to express instead a reporter gene that can be detected and quantified [13,75]. The pioneering work of Gary Sayler and his *lux* fusions to the salicylate-responsive promoter of the pNAH7 plasmid of a soil *Pseudomonas* [28] has now expanded into other contexts and other bacteria for recognizing a suite of chemicals and heavy metals in the environment—an issue that is not devoid of problems [71].

Finally, we argue that the logical (and radical) follow up of genetic engineering for environmental or industrial catalysis is the brand new field of Synthetic Biology. This emerging discipline attempts to recreate rationally from scratch phenomena and qualities that are characteristic of life, including the design of artificial cells à la carte with predetermined metabolic and catalytic properties [44]. We are thus about to witness a new era in environmental biotechnology, in which the most fruitful interfaces will not focus (as has happened so far) on microbiology and chemistry. Instead, frontline research in the field will involve engineering complex systems and redesigning biological components inspired by electric and electronic circuitry. Whether or not such new approaches are ultimately successful may make a difference in our ability to reduce wastes, eliminate industrial pollution, and enjoy a more sustainable future.

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Organismos modificados para el medio ambiente: historias de éxito y fracaso, y qué hemos aprendido de ellas

Resumen. Las expectativas surgidas a mediados de la década de 1980 sobre las posibilidades de la ingeniería genética para remediar in situ problemas de contaminación ambiental no se han cumplido totalmente. A pesar de ello, se ha aprendido mucho sobre la expresión de las vías catabólicas por parte de las bacterias en sus hábitat naturales y sobre la influencia que ejercen las condiciones ambientales en la expresión de las actividades catalíticas deseadas. Las numerosas opciones entre los nutrientes y las respuestas al estrés forman una red compleja de regulación transcripcional que, debido a la redundancia y la robustez de los circuitos que intervienen, no se puede ni descifrar mediante un análisis genético estándar ni programar artificialmente de manera simple. Los datos disponibles sugieren que la dinámica de poblaciones y el control fisiológico de la expresión catabólica de los genes prevalece sobre cualquier intento de buscar artificialmente el funcionamiento óptimo de las actividades catalíticas deseadas. En esta revisión se comentan varios efectos valiosos de la investigación en organismos modificados genéticamente para su aplicación ambiental, así como el impacto de la Biología de Sistemas y de la Biología Sintética en el futuro de la biotecnología ambiental. [**Int Microbiol** 2005; 8(3):213 -222]

Palabras claves: *Pseudomonas* · biodegradación · eco-ingeniería · Biología Sintética

Organismos modificados para o meio ambiente: histórias de sucesso e fracasso, e daí aprendimos delas

Resumo. As expectativas surgidas em meados da década de 1980 sobre as possibilidades da engenharia genética para remediar in situ problemas de contaminação ambiental não se cumpriram totalmente. Apesar disso, se aprendeu muito sobre a expressão das vias catabólicas por parte das bactérias em seus hábitats naturais e sobre a influência que exercem as condições ambientais na expressão das atividades catalíticas desejadas. As numerosas opções entre os nutrientes e as respostas ao stress formam uma rede complexa de regulamento transcripcional que, devido à redundância e a robustez dos circuitos que intervêm não se pode nem decifrar mediante uma análise genético standard nem programar artificialmente de maneira simples. Os dados disponíveis sugerem que a dinâmica de povoações e o controle fisiológico da expressão catabólica dos genes prevalece sobre qualquer tentativa de buscar artificialmente o funcionamento ótimo das atividades catalíticas desejadas. Nesta revisão se comentam vários efeitos valiosos da investigação em organismos modificados geneticamente para sua aplicação ambiental, assim como o impacto da Biologia de Sistemas e da Biologia Sintética no futuro da biotecnologia ambiental [**Int Microbiol** 2005; 8(3):213 -222]

Palavras chave: *Pseudomonas* · biodegradação · eco-engenharia · Biologia Sintética