The Behavior of Bacteria Designed for Biodegradation

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Mineralization of organic molecules by microbes is essential for the carbon cycle to operate. The massive mobilization of compounds stored in natural resources, or the introduction of xenobiotics into the biosphere, leads to unidirectional fluxes, which result in the persistence of a number of chemicals in the biosphere, and thus constitute a source of pollution. Molecular biology offers the tools to optimize the biodegradative capacities of microorganisms, accelerate the evolution of “new” activities, and construct totally “new” pathways through the assemblage of catabolic segments from different microbes. Although the number of genetically engineered microbes (GEMs) for potential use in biodegradation is not large, these recombinant microbes function in microcosms according to their design. The survival and fate of recombinant microbes in different ecological niches under laboratory conditions is similar to what has been observed for the unmodified parental strains. rDNA, both on plasmids and on the host chromosome, is usually stably inherited by GEMs. The potential lateral transfer of rDNA from the GEMs to other microbes is significantly diminished, though not totally inhibited, when rDNA is incorporated on the host chromosome. The behavior and fate of GEMs can be predicted more accurately through the coupling of regulatory circuits that control the expression of catabolic pathways to killing genes, so that the GEMs survive in polluted environments, but die when the target chemical is eliminated.

A large proportion of organic compounds of biological and chemical origin are ultimately mineralized (ie degraded to carbon dioxide, water and other inorganic compounds), predominantly by microorganisms, as part of the continuous cycling of carbon between inorganic and organic states. Mineralization of a compound involves its structural alteration and the formation of metabolic intermediates that serve either as carbon skeletons for cellular constituents or as fuels for energy generation (Fig. 1).

However, not all organic molecules are readily mineralized, and these may persist in the biosphere. When the structural elements of such compounds are chemically very stable, have novel features not generally found in organic molecules of biological origin, are toxic to microorganisms, or inhibit degradative enzymatic attack, then the compound itself is said to be recalcitrant to biological degradation (Fig. 1).

Mineralization of organic compounds is a central feature of the carbon cycle and is a process critical to the maintenance of life on this planet. However, biotransformation or partial degradation of an organic compound does not necessarily have a beneficial outcome: microbes may not gain energy for growth, even more recalcitrant compounds or highly reactive products which undergo thereafter chemical changes such as polymerization may be formed (Fig. 1). The resulting products may be even more toxic than their precursors.

Most naturally occurring molecules are easily mineralized, as are industrial chemicals with structure similar to organic compounds of biological origin. However, many xenobiotics (compounds that exhibit structural elements not found in natural ones) may be recalcitrant, or may be transformed to recalcitrant products. Industrial chemicals have been present in the environment for only an instant in evolutionary time, a period that in some cases has not been long enough for the appropriate functional groups to evolve in catalytic sites of enzymes. In other cases accumulation of chemicals in the biosphere is the result of the mobilization of large amounts of compounds as a consequence of industrial activities. The introduction of xenobiotics or the massive mobilization of compounds present in natural resources cause a problem regarding the integration of these compounds into the natural nutrient cycles. As a consequence of the progressive accumulation of pollutants in the biosphere, environments hostile to biological systems, ie those polluted by aliphatic and aromatic hydrocarbons, chlorinated compounds, nitrotoluenes, dyes, explosives, organic solvents, etc are increasing1.

Effective steps should be taken to protect the environment from pollution. With regard to chemical pollutants, it is evident that the manufacture of toxic and recalcitrant compounds should diminish and eventually cease. Such chemicals must be replaced with nontoxic alternatives that can be mineralized. However, these changes will inevitably take a number of years, as the possible negative effects of many compounds have not yet been investigated. This makes active measures necessary to eliminate or detoxify pollutants. One of the most promising approaches is to optimize the biodegradative capacities of microorganisms, accelerate the evolution of new activities, and exploit them to eliminate these pollutants2.

The introduction of some chemicals into the environment selects for the evolution of the corresponding catabolic pathways, ie, the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) and insecticides such as DDT and parathion, can be mineralized by single microbes or communities of microbes2–5. However, other chemicals are highly recalcitrant, ie, organic solvents, polychlorinated aromatics, dioxins, dibenzofurans, etc. In such cases the evolution of new activities in the laboratory may be helpful because the frequency and types of genetic events needed (e.g., mutation, alteration of gene expression, gene dosage, gene transfer, etc.) can be carefully controlled under selective conditions5–6. The experimental evolution of catabolic
Recombinant Bacteria

Bacteria belonging to the genus *Pseudomonas* exhibit a wide range of metabolic activities against natural and xenobiotic compounds. The so-called fluorescent *Pseudomonas* group includes strains whose biochemical, physiological and genetic characteristics have been well characterized. A number of genetic tools—wide host range vectors, transposons and mini-transposons, reverse genetics etc.—have made it possible to design recombinant derivatives of this group of bacteria with increased biodegradative properties. Several strains with natural biodegradative properties have been intensively investigated to expand the range of xenobiotics they can degrade. Some of these constructions are summarized below.

1. *Pseudomonas putida* KT2440 harboring the self-transmissible TOL plasmid pWW0 grows on a variety of aromatic hydrocarbons used as solvents, including toluene, m- and p-xylene, and m-ethyltoluene. The lateral alkyl chain of these aromatics is oxidized to yield alkylbenzoates, which are further metabolized to Krebs cycle intermediates via benzoates and alkylbenzoates. The bacterium exhibits several blocks to the metabolism of p-ethylbenzoate (Fig. 2). Through the introduction of a series of mutations in the TOL plasmid, we constructed pWW0-EB62, a recombinant TOL plasmid that allows the host microbe to grow on p-ethylbenzoate while maintaining its ability to grow on benzoate and m- and p-methylbenzoate. The strategy involved the isolation of *xylS4*, a *meta*-cleavage pathway regulator mutant that recognizes *p*-ethylbenzoate as an effector. The mutant regulator exhibited a single amino acid change with respect to the wild-type protein, arginine 45 being replaced by threonine. When the mutant regulator was introduced in *Pseudomonas* bearing the TOL plasmid, p-ethylbenzoate was oxidized to p-ethylcatechol, which inactivated catechol 2,3-dioxygenase (Fig. 2). We then isolated a mutant gene *xylE106* encoding an enzyme resistant to inactivation by its substrate: p-ethylcatechol. The mutant catechol 2,3-dioxygenase exhibits a single amino acid substitution (threonine 253→isoleucine) (Wassermann, personal communication). The *xylS4* and *xylE106* mutations were recombined in the TOL plasmid pWW0-EB62 so that the bacteria grew on 4-ethylbenzoate as the sole C-source (Fig. 2). This microbe has been extensively studied in microcosms (see below).

2. *Pseudomonas* sp. B13 exhibits two ortho-cleavage pathways, one each for the metabolism of benzoate and the other for catalysis of 3-chlorobenzoate. These pathways do not allow the mineralization of other chlorobenzoates or alkylbenzoates. The chromosomal ortho-cleavage pathway for benzoate seems to be similar to other catabolic pathways for the metabolism of benzoate by *Pseudomonas* sp. The 3-chlorobenzoate pathway is partially chromosome encoded and partially plasmid encoded. In this pathway, 3-chlorobenzoate offers considerable potential for accelerating the evolution of bacteria able to degrade toxic industrial chemicals, and this may be useful for reducing environmental pollution. Several strategies have been successfully applied to construct bacteria able to eliminate a wide range of organic solvents, such as toluene derivatives, a number of chlorinated aromatics arising from human industrial activities, for example chlorobenzoates and chlorophenols, and the more recalcitrant polychlorinated biphenyls and explosives such as 2,4,6-trinitrotoluene.
zolate is first oxidized to 3-chlorocatechol by chromosome encoded genes (Fig. 3); then 3-chlorocatechol undergoes ring cleavage and chlorine elimination, a process in which three plasmid-encoded enzymes—catechol 1,2-dioxygenase, chloromuconate cycloisomerase and dienelactone hydrolase—are involved. Growth of the bacteria on 3-chlorobenzoate resulted in amplification of the plasmid encoded genes involved in the process, in order to increase protein dosage. Pseudomonas sp. B13 cannot grow on 4-chlorobenzoate or mixtures of alkyl- and halobenzoates. In contrast, Pseudomonas sp. FR1 (pFRC20P), a derivative of B13 that was constructed through the recruitment of genes from different microorganisms, is able to grow on 4-chlorobenzoate (Fig. 3) and mixtures of alkyl- and chloroaromatics. Its construction involved chromosomal integration of the genes for the metabolism of 4-chlorobenzoate and alkylbenzoates to 4-chlorocatechol and alkylcatechols respectively. The construct was completed by the introduction of a mobilizable plasmid (pFRC20P) carrying a key gene for the conversion of alkyl-lactones resulting from the metabolism of alkylcatechols. The genes encoding the TOL plasmid toluate dioxygenase (xy/XYZ) and the next enzyme in the pathway (a dehydrogenase encoded by xy/L) plus the positive regulator xylS, were cloned into Tn5 and delivered into the host chromosome. This allowed the recombinant bacteria to grow on 4-chlorobenzoate, and allowed the metabolism of 4-methylbenzoate to γ-methyl-lactone. The resulting bacterium was called Pseudomonas sp. FR1. Cloning of an isomerase from Alcaligenes that allowed the conversion of the γ-methyl-lactone into β-methyl-lactone in plasmid pFRC20P allowed mineralization of the alkylbenzoate. Further, this isolate grew, and simultaneously assimilated, mixtures of alkyl- and halobenzoates. Both Pseudomonas sp. B13 and FR1 (pFRC20P) have been studied in soil and aquatic microcosms.

The bioremediation of sites polluted by compounds at very low concentrations may be favored by microbes able to grow in plant rhizospheres, where the nutrient supplied by the plant may help to colonize soil sites poor in available substrates and ultimately improve the elimination of pollutants. Pseudomonas fluorescens sp. F13, an isolate from the sugarcane rhizosphere, is an excellent root colonizer and shows potential for serving as an instrument of biological control. This microbe has been subjected to pathway expansion to include biphenyl. Improving the aerobic degradation of polychlorinated biphenyls is of current interest, although the aerobic process is limited to some congeners, which in most cases yield chlorobenzoates. The bph gene cluster seems to be designed to deal with the unsubstituted substrate. Pseudomonas sp. F13-PCB is a derivative that carries the bph A1,2,3,4BCD genes for the conversion of biphenyl into benzoate. The bph genes were cloned into a mini-Tn5 operon encoding resistance to biolaphos in the suicide plasmid pDDPCB, and then delivered into the host chromosome through this vector system. Pseudomonas sp. clone A is a natural isolate that uses 2,4,6-trinitrotoluene (TNT) as the sole N-source. The microbe removes all three nitro groups successively from the nitroarene ring to yield Toluene, which cannot be used as a C-source by this strain since it lacks the enzymatic machinery to oxidize this hydrocarbon. Because Pseudomonas sp. clone A belongs to rRNA group I, it was reasoned that the transfer of a Km-TOL plasmid should allow this isolate to mineralize TNT. Matings between Pseudomonas sp. clone A and P. putida (pWW0-Km) yielded Km transconjugants of clone A, which used TNT as the sole C- and N-source. This strain has been shown to eliminate TNT in waste waters from a factory manufacturing this compound (E. Duque, personal communication).

5. Mercury (Hg) is a toxic metal that has been released into the environment in substantial quantities. Its toxicity results from the capacity of Hg(II) to bind sulfhydryl, thioether and imidazole groups and thereby inactivate enzymes. Organic species of mercury, both alkyl and aromatic derivatives, accumulate in tissues of higher organisms generating serious health problems. The merTPABD genes of transposon Tn5, which encodes the enzymes involved in organomercury resistance, were cloned into a mini-Tn5 that was used to insert the genes into the genome of P. putida. Transconjugants expressing constitutively the mer genes on basis to high resistance to phenylmercury were selected. This allowed the engineered bacteria to cleave Hg from an organic moiety and reduce the liberated Hg(II) to Hg(0). These properties were also combined with benzene and toluene catabolism of several Pseudomonas strains, allowing the degradation of its aromatic moiety as well as detoxification of the metal component.

Tracking GEMs in Microcosms
Most of the recombinant bacteria constructed for biodegra-
Potential of the New Genetic Material to Transfer Later-duce New Epitopes on a Bacterial Surface Protein

The ability of the bacterium to survive and multiply in the environment, this mAb specifically recognized the strain when grown in different culture media and at different growth stages. The mAb, which is highly specific, was used to track the strain in situ.

Features to be Analyzed with Recombinant Bacteria

A series of features that are crucial for the safe and effective functioning of some of the above recombinant and wild-type bacteria have been examined in soil microcosms with and without plants, and in sewage microcosms:

(i) The ability of the bacterium to survive and multiply in the microcosms into which it is introduced;
(ii) the stability of the introduced genetic material;
(iii) effects, if any, of the GEMs on the structure and function of the microcosms into which it is introduced;
(iv) the ability of the recombinant microbe to effectively carry out the activity for which they were designed under the conditions prevailing in the microcosm;
(v) the potential of the new genetic material to transfer laterally to other microorganisms;
(vi) coupling of a biological containment system to the catabolic pathways, so that programmed cell death can be designed;
(vii) design of barriers to gene dispersal.

Survival and Propagation of GEMs in Their Target Ecosystem

Microcosms offer a suitable approach to evaluate the survival and functioning of GEMs, however, it should not be ignored that the samples are taken from nature and introduced into the laboratory. This imposes some limitations, since a number of parameters are closely controlled, i.e. incubation temperature, humidity, light/dark cycles, etc. The survival of some of the recombinant microbes described above was assayed by introducing both parental and recombinant strains in soil, rhizosphere and aquatic microcosms. The microcosms used were: (i) sandy-loam agricultural soils, whose pH were in the range of 7.0 to 7.5 and in which sugarbeet or maize seeds were introduced as required, and (ii) sewage water from a city water treatment plant. These recombinant bacteria had been designed to eliminate pollutants and they would be added to polluted soils and aquatic microcosms in relatively high numbers, e.g. 10^9-10^10 colony forming units (CFU) per gram of soil or milliliter of sewage water.

Most of the studies have focused on the behavior of these microbes during the initial period after introduction into the microcosms, usually between 4 and 8 weeks. It was generally observed that both the recombinant microbes and their parental strains were able to establish in soil, rhizosphere and aquatic microcosms. In each ecosystem the number of bacteria tended to reach the microcosm’s carrying capacity, so that in microcosms rich in organic matter the number of microbes was usually higher than in microcosms poor in nutrients.

Recombinant bacteria survived better than the parental strains only when they were introduced in soil or aquatic microcosms in which up to 0.1% (wt/wt or wt/vol) pollutant had been added, i.e., conditions strongly favorable for the GEM. For instance, introduction of P. putida EEZ15 (pWW0-EB62) or P. putida sp. FR1 (pFRC20) in soils supplemented with 0.1% (wt/wt) p-ethylbenzoate and p-methylbenzoate, respectively, allowed the strain to become established at at least one order of magnitude higher than in soils without the supplement. A similar observation was made when these bacteria were introduced in microcosms consisting of water from a sewage treatment plant. Although survival of parental and recombinant bacteria approached 100% in short term experiments, in the long term a steady decline was observed in CPU per gram of soil, until numbers in some cases were below our detection limits.

Both recombinant and parental bacteria not only survived in soils but also multiplied. In fact, following introduction of a low number, ie 10^7 CFU per gram of soil of P. putida (pWW0-EB62) in non-sterile soils it multiplied to reach a density of 10^10 CFU per gram of soil.
after prolonged incubation in soil retained the ability to use the aromatic compounds for which they were designed to deal. The effects of the introduction of wild-type or recombinant microbes on the indigenous microbiota have been studied either by estimating the "total" number of culturable microbes in relatively rich medium, ie, peptone agar, or by using the indigenous population able to use a compound as the sole C-source, ie p-hydroxyphenylacetic acid degraders, the denitrifying bacteria or the "heterotrophic" population13-15 (M.C. Ronchel et al., unpublished). In general, it has been observed that neither the wild-type nor the recombinant microbe affected the parameter studied, which suggests that natural environments have a certain buffering capacity to deal with the introduced microbes.

The above cases suggest that the possible risk derived from the use of recombinant microbes in bioremediation are similar to those posed by the parental non-modified strains. It also seems that the physico-chemical and biological parameters of the microcosms affect equally the parental and recombinant microbes.

**Functionality of Recombinant Microbes in Target Microcosms**

The introduction of bacterial strains into the environment for *in situ* bioremediation will usually require that microorganisms be able to survive in high numbers and express the desired catabolic phenotype.

For pollutants to be efficiently mineralized by natural and genetically modified microbes, the degrading microbe not only must become established in the polluted sites, but also express catabolic genes in response to the pollutant, even in the presence of other compounds. Soils, river sediments and sewage treatment plants are complex environments in which gene expression can be inhibited or stimulated, *Pseudomonas putida* harboring either the wild-type pWW0 or the recombinant TOL plasmid were able to mineralize ¹⁴C-labeled substrates, eg, p-methyl-¹⁴C- benzoate, for at least a month46. In soils, mineralization was monitored as evolution of ¹⁴CO₂, whereas in aquatic microcosms, metabolism of alkyl- and chloroaromatics was chromatographically monitored by measuring the disappearance of the target chemical from the polluted site43. It was shown that *Pseudomonas* sp. FR1 (pFRC20P) enhanced the rate of degradation of a mixture of 3-chlorobenzoate and 4-methylbenzoate which had been added to the water column of sediment cores made of intact layered sediments from the Plussee Lake and the Rhine River41. *Pseudomonas* sp. FR1 bearing the bph genes inserted on the chromosome was able to remove up to 100 ppm of 4-chlorobiphenyl per g of sediment slurry in 5 days. In these assays, recombinant bacteria were established at close to 10⁶ CFU per g of sediment.

**Transfer of Recombinant DNA from *Pseudomonas* to Other Microorganisms**

Gene transfer from recombinant microbes has been analyzed under optimal laboratory conditions and in soil and aquatic microcosms. The genetic information introduced into *Pseudomonas* FI13-PCB was inserted into the host chromosome via a mini-Tn5 lacking the transposase gene. In the case of *Pseudomonas* sp. FR1 (pFRC20P) the information was partially located on the bacterial chromosome (Tn5.;ϕXYXZ.;ϕYS) and partially on a mobilizable broad-host-range plasmid (pFRC20P). In *P. putida* (pWW0-EB62) the recombinant information was on a self-transmissible TOL plasmid. The recombinant DNA in these three strains was stably maintained under laboratory growth conditions, as well as in bacteria introduced into soil and aquatic microcosms. In the laboratory there was no transfer of the recombinant DNA from the chromosome of *Pseudomonas* sp. FI13-PCB or *Pseudomonas* sp. FR1 (pFRC20P) to other *Pseudomonas*, or to indigenous bacteria in soil and aquatic microcosms19,40,42. In contrast, plasmid pFRC20P was transferred to other microbes, but only if a helper plasmid was supplied in the mating mixture42. In these cases, transfer of the plasmid led to about 10⁴ transconjugants per recipient. Transfer of the wild-type TOL plasmid or the recombinant pWW0-EB62 was restricted to microorganisms belonging to members of the pseudomonad rRNA group I (P. putida, P. fluorescens, P. stutzeri, etc.) and some enterobacteriaceae. In matings on plates the rate of TOL transfer was of the order of 1 to 10⁴ transconjugants per recipient. In soils, the TOL plasmid was transferred from *P. putida* to other strains of *Pseudomonas* only at high cell densities (higher than 10⁶ CFU/g soil). Transfer was influenced by the type of soil used, the incubation temperature, the initial inoculum size and the presence of chemicals that affected the survival of donor or recipient bacteria. Maximal transfer was observed in soils incubated at 15 to 17°C, when the donor and recipient loads were about 10⁶ CFU per g of soil47.

In conclusion, recombinant DNA can be transferred between microorganisms. Transfer is limited by the vector used to introduce the recombinant DNA into the host microbe and by the
nature of the recipient microorganism. Chromosomal information is less likely to be transferred than is information on mobilizable plasmids. The latter is less likely to be transferred than recombinant DNA on self-transmissible plasmids.

Not all locations on the chromosome are similar in terms of expression, physical structure or mobilization. This is particularly true in *Pseudomonas* where most auxotrophic markers are located on only 50% of the chromosome. Ramos-González et al. labeled the chromosome of *P. putida* randomly at 34 independent positions with the same marker, a mini-Tn5-Km. It was shown that the TOL plasmid was able to mobilize these insertions on the host chromosome at a rate between $10^{-4}$ and $10^{-5}$ transconjugants/recipient. Furthermore, the mobilization of the host chromosome by TOL occurred when the TOL plasmid and the marked chromosome were located in the two independent microbes, a phenomenon called retrotransfer. Retrotransfer involves the movement of a self-transmissible replicon to a recipient organism, the capture of DNA, and its return to the original donor. It then follows that, although chromosomal insertions are less likely to be mobilized than rDNA on plasmids, they can nonetheless potentially undergo transfer.

**Biological Containment Based on Substrate Availability**

The full exploitation of recombinant microbes for bioremediation will inevitably involve their release in the environment, where non-controllable conditions prevail. This issue raises a series of scientific and public concerns due to the lack of knowledge on the behavior and fate of microbes in the environment. This also raises an inevitable question, can we extrapolate the results obtained in microcosms to the “real” scenario, the open environment?

The goal behind “biological containment” is that of increased predictability in the behavior and fate of recombinant microbes. Biological containment can be passive, i.e., through the impairment of strains by the introduction of mutations (auxotrophies, recA, etc.), or active, this is based on genes that induce cell killing. The active system imposes a series of criteria, namely, (i) the killing efficiency should be high, (ii) the killing gene should perform effectively in a wide range of microbes, (iii) the suicide system should not interfere with the “modifications” introduced in the microbes and (iv) the mutation rate of the killing system should be as low as possible.

One model case of chemical induction of suicide as a biological containment principle (for release purposes) was developed for the biodegradation of xenobiotics. The strategy was to combine the killing function with the regulatory system of a degradative pathway in such a way, that the cells survive in the presence of the xenobiotic (repression of the killing gene), but after completion of degradation of the compound the killing gene is turned on, and the cells die. This approach has been tested in relation to the TOL pathway responsible for the mineralization of different benzoates. With the detailed knowledge about the genetic control of the genes involved in this degradative pathway, it was possible to design engineered combinations of the expression system of the TOL genes and a suicide gene. One of the promoters of the TOL genes was fused with the E. coli lacI gene whose product represses a promoter, *P*, which in the present context was fused with the killing gene. In the presence of *m*-methylenbenzoate the TOL promoter is activated, which implies that the repressor protein is made, and consequently the killing function is not expressed; the cells survive, and degradation of *m*-methylenbenzoate is allowed. After removal of the aromatic inducer the repressor is no longer expressed from the TOL promoter, and therefore no inhibition is exerted on the promoter responsible for expression of the killing function. After completion of the task of the designed organism (degradation of *m*-methylenbenzoate), it destroys itself through substrate regulated induction of the suicide system.

It should be emphasized that effective containment systems can only be constructed if the performance of the organism in the proper environmental context is at least partially characterized. In fact, this biological containment system was shown to function in soils (Fig. 4).

**Universal Barriers to Gene Dispersal**

To address concerns due to the potential transfer and spread of rDNA into the native population of microorganisms, a genetic circuit to suppress lateral spread of cloned genes from recombinant microbes to indigenous microorganisms was conceived. The system involves two genes, the *colE3* gene, encoding the RNAse colicin E3, and the immunity *immE3* gene, which codes for a specific inhibitor of the lethal function, the immunity E3 protein. The target of colicin E3 is located at the 3' end of the 16S rRNA which is conserved virtually in all prokaryotic and some eukaryotic genera. Cleavage of this sequence separates the mRNA binding sites from the remainder of the 16S rRNA, thereby inhibiting protein synthesis and leading to cell death. In the GEM, the gene specifying the lethal function is closely linked to the determinant of the novel phenotype, whereas the immunity gene is located far from the recombinant genes, so that co-transfer of immunity gene with the *kill* gene is highly unlikely. Transfer of the determinant of the novel phenotype to a new organism will be accompanied by transfer of the lethal function, but not of the immunity function, and as a consequence the recipient organism will be killed. The genes *colE3* and *immE3* have been cloned and constitutively expressed separately. The *immE3* gene was integrated into the
chromosome of different E. coli and Pseudomonas putida strains. On the other hand, the colE3 gene was cloned in a promiscuous plasmid able to replicate both in gram-positive and gram-negative bacteria. Effectiveness of the containment system was tested with different DNA transfer mechanisms (transformation and conjugation), and different recipient microorganisms of environmental importance. There were representatives of the main subgroups of Proteobacteria: P. putida and P. fluorescens (γ subgroup), Alcaligenes eutrophus and Com­ mononas acidovorans (β subgroup), Rhizobium meliloti and Agrobacterium tumefaciens (α subgroup). In all cases the coli­E3 lethal function decreased the frequency of plasmid trans­fer by four to five orders of magnitude. In combination with transposon cloning vectors, the circuit is predicted to reduce the lateral spread of specific genes to extremely low levels and to increase substantially the ecological predictability of novel recombinant microorganisms.

Future Prospects

To bring about in vitro evolution of the catabolic pathways usually requires a thorough knowledge on the biochemistry of the reactions involved in the degradation of a compound and of the genetics of the pathway, i.e. the organization of the genes encoding for the enzymes and the operation of the regulatory circuits governing the activation of gene expression. In recent years a number of new catabolic pathways dealing with toxic and recalc­itrant compounds have been elucidated. These include path­ways for the metabolism of dibenzoxinoids, new pathways for chlorocatearins, polar and apolar nitroaromatics such as nitro­benzoxides and mono- and poly-substituted nitro­arenes. Equally important is that many enzymes are extremely relaxed in their substrate specificity, which makes them able to deal with a wide range of benzene derivatives, which makes them able to deal with a wide range of aromatic hydrocarbons and expansion of the substrate range of the degradation pathway. J. Bacteriol. 171:6782–6790.

Duque, E., Haidour, A., Godoy, F. and Ramos, J. L. 1993. Construction of a Pseudomonas hybrid strain that mineralizes 2,4-trinitro­benzene. J. Bacteriol. 175:2278–2283.

Dowling, D. N., Piipe, R. and Dwyer, D. F. 1993. A DNA module encoding traits capable of the degradation of polychlorinated biphenyls (PCBs). FEMS Microbiol. Lett. 123:149–154.

Lehrbach, P. R., Zeyer, J., Reineke, W., Knackmuss, H.-J. and Timmis, K. N. 1984. Enzyme responsible for in vitro use of cloned genes to extend the range of haloaromatics degraded by Pseudomonas sp. strain B13. J. Bacteriol. 158:1025–1032.

Ramos, J. L., Stok, A., Reineke, W. and Timmis, K. N. 1986. Altered effector specificities in regulators of gene expression: TOL plasmid α2 mutants and their use to engineer expansion of the range of aromatics degraded by bacteria. Proc. Natl. Acad. Sci. USA 83:8467–8471.

Ramos, J. L., Wasserfallen, A., Rose, K. and Timmis, K. N. 1987. Redesigning metabolic routes: manipulation of TOL plasmid pathways for catabolism of alkylbenzoxides. Science 238:593–596.

Reineke, W. and Knackmuss, H.-J. 1979. Construction of halogen-utilizing bacteria. Nature 277:385–386.

Rojo, F., Piiper, D., Engesser, K. H., Knackmuss, H.-J. and Timmis, K. N. 1987. Assembly of catabolic routes for degradation of chloro­ and bromoaromatics. Science 238:1395–1398.

Sokatch, J. (Ed.). 1986. The Bacteria, vol X. The Biology of Pseudomonas. Academic Press, N.Y.

Gallar, E., Silver, S. and Witholt, B. (Eds.). 1992. In: Pseudomonas: Molecular Biology and Biotechnology. American Society for Microbiology. Washington DC.

Tiedje, J. M. and Williams, P. J. 1974. Metabolism of toluidine and toluidine sulphones by Pseudomonas putida (avila) mt-2: evidence for a new function of the TOL plasmid. J. Bacteriol. 124:7–13.

Dorn, E., Heilig, M., Reineke, W. and Knackmuss, H.-J. 1974. Isolation and characterization of a 3-chlorobenzoate degrading pseudomonic. Arch. Microbiol. 99:61–70.

Rangnekar, V. 1988. Variation in the ability of Pseudomonas sp. strain B13 to utilize m-chloro-benzoate is associated with tandem amplification and desmutilication of DNA. J. Bacteriol. 170:1907–1912.

Piiper, D. H., Engesser, K. H., Don, R. H., Timmis, K. N. and Knackmuss, H.-J. 1985. Modified ortho-cleavage pathway in Alcaligenes eutrophus JMP134 for the degradation of 4-methylcyclohexanol. FEMS Microbiol. Lett. 29:63–67.

Dowling, D. N. and O’Gara, P. F. 1994. Genetic manipulation of ecologically adapted Pseudomonas putida for PCB degradation. In: Current Topics in Molecular Genetics. (Life Sci. Adv.) 21:1–8.

Bopp, L. H. 1986. Degradation of highly chlorinated PCBs by Pseudomonas strain LB400. J. Ind. Microbiol. 1:233–239.

Niehar, E. and Richardson, D. H. S. 1980. The replacement of the nondescript term “beaty metals” by a biologically and chemically significant classification of metal ions. Environ. Pollut. Ser. B 1:13–26.

Lloyd, R. A. and Brown, N. P. 1989. Regulation of transcription in Escherichia coli from the mer and mrr promoters in the transposon Tn501. J. Mol. Biol. 205:343–355.

Ramos, J. L., Brunke, M., Deckwer, W.-D. and Timmis, K. N. 1994. Pseudomonas putida strains which constitutively express mercury resistance for biotransformation of organomercurial pollutants. Appl. Environ. Microbiol. 60:357–362.

Tiedje, J. M., Colwell, R. K., Grossman, Y., Hodson, R. E., Lennik, R. E., Mack, R. N. and Regal, P. J. 1989. The planned introduction of genetically engineered organisms: ecological considerations and recommendations. Ecol. Appl. 1:298–315.

Ramos, J. L., Duque, E. and Ramos-González M.J. 1991. Survival in soils of an herbicide-resistant Pseudomonas putida strain bearing a recombinant TOL plasmid. Appl. Environ. Microbiol. 57:1683–1687.

Herrero, M., de Lorenzo, V. and Timmis, K. N. 1990. Transposon vectors containing non-antibiotic resistance selection markers for the cloning and stable chromosomal insertion of foreign genes in gram negative bacteria. J. Bacteriol. 172:6557–6567.

De Lorenzo, V., Herrero, M., Jacobizk, U. and Timmis, K. N. 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing and chromosomal insertion of cloned DNA in gram-negative bacteria. J. Bacteriol. 172:6568–6572.

Drahos, D. J., Barry, G. F., Hemmung, B. C., Brandt, B. E., Skipper, H. F., Kline, M. L., Kluepfel, D. A., Hughes, T. A. and Gooden, D. T. 1988. Pre­test release testing procedures: U.S. field test of a LacZΔY-engineered soil bacterium, P. lih. 181–191. In: The release of genetically engineered microorganisms, Sussman, M., Attwood, C. H., Skinner, F. A. and Stewart-Tull, D. E. (Eds.). Academic Press, London.

Kristensen, C. S., Ebel, L., Sánchez-Romero, J. M., Givkov M., Molin, S. and de Lorenzo, V. 1994. Site-specific insertion of cloned DNA into chromosomally located DNA sequences with the multiutter system encoded by the barCBA operon the broad-host-range plasmid PR4. J. Bacteriol. in press.

Ebel, L., Kristensen, C. S., Givkov, M., Grohmann, E., Gerlitz, M. and Sahl, H. W. 1994. Analysis of the multiutter system encoded by the barCBA operon the broad-host-range plasmid PR4. Mol. Microbiol. 12:131–141.
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---

34. Prosser, J. I. 1994. Molecular marker systems for detection of genetically engineered microorganisms in the environment. Microbiology 140:5-17.
35. Ramos-González, M. I., Ruiz-Cabello, F., Brettar, I., Garrido, F. and Ramos, J. L. 1992. Tracking genetically engineered bacteria: monoclonal antibodies against surface determinants of the soil bacterium *Pseudomonas putida* 2440. J. Bacteriol. 174:2978-2985.
36. Gebauer, F., Posthumus, W. P. A., Correa, I., Lennon, J. A., Meloen, R. H. and Enjuanes, L. 1991. Residues involved in the antigenic sites of transmissible gastroenteritis coronavirus S glycoprotein. Virol. 183:225-238.
37. Erb, R. W. and Wagner-Döbler, I. 1993. Detection of polychlorinated biphenyl degradation genes in polluted sediments by direct DNA extraction and polymerase chain reaction. Appl. Environ. Microbiol. 59:4065-4073.
38. Atlas, R. M. 1992. Molecular methods for environmental monitoring and containment of genetically engineered microorganisms. Biodegradation 3:137-146.
39. Brazil, G. M., Kenefick, L., Callanan, M., Haro, A., de Lorenzo, V., O’Gara, F. and Dowling, D. N. 1994. Expanding the metabolic functions of a rhizosphere competent pseudomonad to degrade haloaromatics. Microb. Releases 1:23-28.
40. Delgado, A., Duque, E. and Ramos, J. L. 1992. Behavior in agricultural soils of a recombinant *Pseudomonas* bacterium that simultaneously degrades alkyl- and haloaromatics. Microb. Releases 1:23-28.
41. McClure, N. C., Weightman, A. J. and Fry, J. C. 1989. Survival of *Pseudomonas putida* UWCl containing cloned catabolic genes in a model activated-sludge unit. Appl. Environ. Microbiol. 55:2627-2634.
42. Nusslein, K., Maris, D., Timmis, K. N. and Dwyer, D. F. 1992. Expression and transfer of engineered catabolic pathways harbored by *Pseudomonas* spp. introduced into activated sludge microcosms. Appl. Environ. Microbiol. 58:1259-1265.
43. Pipke, R., Wagner-Döbler, I., Timmis, K. N. and Dwyer, D. F. 1992. Survival and function of a genetically engineered *Pseudomonas* in aquatic sediment microcosms. Appl. Environ. Microbiol. 58:1259-1265.
44. Wagner-Döbler, I., Pipke, R., Timmis, K. N. and Dwyer, D. F. 1992. Evaluation of aquatic sediment microcosms and their use in assessing possible effects of introduced microorganisms on ecosystem parameters. Appl. Environ. Microbiol. 58:1249-1258.
45. Duque, E., Ramos-González, M. I., Delgado, A., Contreras, A., Molin, S. and Ramos, J. L. 1992. Genetically engineered *Pseudomonas* strains for mineralization of aromatics: survival, performance, gene transfer, and biological containment. p. 429-437. In: *Molecular Biology and Biotechnology.* American Society for Microbiology. Washington DC.
46. Duque, E., Marqués, S. and Ramos, J. L. 1993. Mineralization of p-methyl-14C-benzoate in soils by *Pseudomonas putida* (PW00). Microb. Releases 2:175-177.
47. Ramos-González, M. I., Duque, E. and Ramos, J. L. 1991. Conjugal transfer of recombinant DNA in cultures and in soils: host range of *Pseudomonas putida* TOL plasmids. Appl. Environ. Microbiol. 57:3020-3027.
48. Holloway, B. N. 1986. Chromosome mobilization and genomic organization in *Pseudomonas,* p. 217-250. In: *The Bacteria,* vol. X, J. Sokatch (Ed.) Academic Press, N.Y.
49. Ramos-González, M. I., Ramos-Díaz, M. A. and Ramos, J. L. 1994. Chromosomal gene capture mediated by the *Pseudomonas putida* TOL catabolic plasmid J. Bacteriol. 176:4635-4641.
50. Mergrey, M., Lejeune, P., Sadouk, A., Gerits, A. J. and Fabry, L. 1987. Shuttle transfer (or retrotransfer) of chromosomal markers mediated by plasmid pULB113. Mol. Gen. Genet. 209:61-70.
51. Molin, S., Boe, L., Jensen, L. B., Kristensen, C. S., Givskov, M., Ramos, J. L. and Bej, A. K. 1993. Suicidal genetic elements and their use in biological containment of bacteria. Ann. Rev. Microbiol. 47:139-166.
52. Molin, S. 1992. Designing microbes for release into the environment. Sci. Progress Oxford 76:139-148.
53. Contreras, A., Molin, S. and Ramos, J. L. 1991. Conditional-suicide containment system for bacteria which mineralize aromatics. Appl. Environ. Microbiol. 57:1904-1908.
54. Marqués, S. and Ramos, J. L. 1993. Transcriptional control of the *Pseudomonas putida* TOL plasmid catabolic pathways. Mol. Microbiol. 9:923-929.
55. Jensen, L. B., Ramos, J. L., Kaneva, Z. and Molin, S. 1993. A substrate-dependent biological containment system for *Pseudomonas putida* based on the *Escherichia coli* gpt gene. Appl. Environ. Microbiol. 59:3713-3717.
56. Díaz, E., Munthali M., de Lorenzo, V. and Timmis, K. N. 1994. Universal barriers to lateral spread of specific genes among microorganisms. Mol. Microbiol. 13:855-861.
57. Jakes, K. S. 1982. The mechanism of action of colicin E2, colicin E3 and cloacin DF13, p. 131-167. In: *Molecular Action of Toxin and Viruses.* Cohen, P. and van Heyningen, S. (Eds.). Elsevier, Amsterdam. Biomedical Press.
58. Lasater, L. S., Cann, P. A. and Glitz, D. G. 1989. Localization of the site of cleavage of ribosomal RNA by colicin E3. J. Biol. Chem. 264:21798-21805.
59. Blasco, R., Wittich, R.-M., Timmis, K. N. and Pieper, D. H. 1994. From xenobiotic to antibiotic: Ecotoxicity of some chloroaromatic pollutants is due to microbial formation of protoanemonin. Submitted.
60. Groesewegen, P. E. J., Breeuwer, P., van Heilvoet, J. M. L. M., Langenhoff, A. M. M., de Vries, F. P. and de Bont, J. A. M. 1992. Novel degradative pathway of 4-nitrobenzoate in *Comamonas acidovorans* NBA-I0. J. Gen. Microbiol. 138:1599-1605.
61. Spanggord, R. J., Spain, J. C., Nishino, S. F. and Mortelmans, K. E. 1991. Biodegradation of 2,4-dinitrotoluene by a *Pseudomonas* sp. Appl. Environ. Microbiol. 57:1320-1325.
62. Robertson, J. B., Spain, J. C., Haddock, J. D. and Gibson, D. J. 1992. Oxidation of nitrotoluene by toluene dioxygenase: evidence for a monooxygenase reaction. Appl. Environ. Microbiol. 58:2643-2648.