Catabolic Plasmids

Secondary article

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Catabolic plasmids are circular, accessory DNA elements present in the cytoplasm of many soil bacteria. They confer on their host the ability to degrade and recycle complex, naturally occurring and synthetic molecules, including a vast array of environmental pollutants.

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Introduction

For most of this century agriculture and industry have used a wide range of synthetic chemicals which appear to have no counterparts in nature. Many of these compounds, by their very nature and complexity, are resistant to degradation when released into soil, water and air. Unfortunately, the repeated use and release of such synthetics has become an everyday occurrence, resulting in widespread environmental pollution.

Where degradation does occur it is carried out almost exclusively by microorganisms, predominantly bacteria. The complexity of these molecules is such that to degrade them to simple and harmless end products, many enzymatic steps are required. For many soil bacteria these degradative (catabolic) activities are encoded by large groups of genes clustered on the main chromosome or on catabolic plasmids. Such plasmids are usually large (80 to > 200 kb) with one or more clusters of multicistronic transcriptional units, possessing up to 10–15 degradative genes. In many instances catabolic plasmids are self-transmissible by cell-to-cell contact (conjugation) and are transferred freely throughout diverse soil bacterial populations (broad host range), leading to novel combinations of degradative genes capable of degrading the most complex, the most recalcitrant and the most persistent of synthetic molecules.

In some instances these catabolic gene clusters occur within transposable elements and therefore they move unfettered between plasmids and the main chromosome. Rearrangements of existing genes, the activities of transposable elements and the broad host range of catabolic plasmids provide powerful mechanisms for the evolution of bacteria capable of rapidly degrading and recycling mutagenic, carcinogenic and/or teratogenic chemicals such as dioxins, trichloroethylene (TCE), polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), dichlorodiphenyltrichloroethane (DDT), γ-1,2,3,4,5,6-hexachlorocyclohexane and phenoxyherbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). The evolution and spread of bacterial genes involved in the degradation of xenobiotic molecules is a powerful and positive development in the fight against environmental pollution.

Bacterial degraders

In the 1960s, Stanier, Palleroni, Doudoroff and their co-workers carried out much of the pioneering research on the degradative functions of bacteria. Their work demonstrated that species of the genus *Pseudomonas* possessed considerable catabolic versatility. The epitome was '*Pseudomonas multivorans*', loosely translated as 'the pseudomonad that ate everything'. It has gone through several phylogenetic reincarnations first to '*Pseudomonas cepacia*' and is now known as *Burkholderia cepacia*. With the success of molecular genetic analyses the variety of bacterial degraders under study has also grown. From Gram-negative bacteria such as *Pseudomonas putida*, *P. oleovorans*, *P. convexa*, *P. arvilla* and a wide array of other pseudomonads, the field has expanded to include *Ralstonia eutropha* (formerly '*Alcaligenes eutrophus*'), *Alcaligenes paradoxus*, *Sphingomonas paucimobilis* (formerly '*Pseudomonas paucimobilis*'), to various species in the genera *Aerobacter*, *Agrobacterium*, *Klebsiella*, *Streptomyces*, *Erwinia*, *Kurthia*, *Rhizobium*, *Xanthomonas*, to name but a few. Among the Gram-positive bacteria are members of the genus *Rhodococcus*, which are common in soil and the guts of insects. The association between insecticide-degrading bacteria and insects may indicate that insecticide resistance may not be due solely to changes in the insects' genetic make-up, but could result from the evolution and/or spread of resistance (detoxification genes) among bacteria associated with the insect itself. With the expansion in the numbers of species and genera of bacteria under study, novel or alternative dissimilatory routes for normally recalcitrant organic molecules are emerging. Dechlorination is also carried out by obligate anaerobes, providing an additional source of metabolic versatility (Loffler *et al*., 1997).

Selective forces

In many instances the level of the synthetic molecule in a particular environment is critical. While the level present may pose a substantial hazard to humans and other living

organisms, it may not be broken down and recycled simply because the levels are insufficient to induce the production of specific enzymes which allow its use as a source of carbon and energy for growth. For the organochlorine herbicide 2,4-D, which is applied at high concentrations, degradation takes 8–12 weeks. However, after three or four serial applications of 2,4-D to the same soil, the herbicide disappears in 1–3 weeks. This increase in the rate of degradation reflects a selection for an increased population of pre-existing 2,4-D degraders and their persistence between applications. Where the 2,4-D-degradative genes are located on broad host range catabolic plasmids, the numbers of new 2,4-D degraders can be increased significantly by plasmid transfer.

For the closely related but far less biodegradable organochlorine herbicide 2,4,5-T the problem does not lie with the level of the substrate but rather with the inability of existing enzymes to degrade the molecule to any appreciable extent. In these circumstances it has been possible to select a 2,4,5-T-degrading strain by exposure of a pre-existing strain of *B. cepacia* to high levels of 2,4,5-T over a long period of time. In this instance the 2,4,5-T degrader which was eventually isolated differed from the original parental strain by the accumulation of mutations in various organochlorine catabolic genes. These altered genes encoded altered enzymes which allowed the rapid and complete degradation of what was a virtually nonbiodegradable molecule.

Plasmid location of catabolic determinants

The very first catabolic plasmid to be isolated was the CAM plasmid of *Pseudomonas putida*; this plasmid controlled the oxidation of the naturally occurring terpene, camphor (Rheinwald *et al*., 1973). More surprising was the subsequent discovery that plasmids also encoded the degradation of synthetic molecules. The first such plasmid, pJP1, encoded the degradation of the notorious organochlorine herbicide 2,4-D (Pemberton and Fisher, 1977). The intensively studied 2,4-D plasmid pJP4 (carried by *Ralstonia eutropha* JMP134) is typical of many of these plasmids in that it is a large, conjugative plasmid, with a very broad host range and one or more clusters of degradative genes which encode the degradation of the organochlorines 2,4-D and 3-chlorobenzoate (3-CBA).

Catabolic phenotypes specified

In the years since the CAM and 2,4-D plasmids were isolated a range of plasmids, transposons and gene clusters have been identified which encode the degradation of a range of naturally occurring and synthetic molecules. Some examples of catabolic plasmids are given in **Table 1**.

Other phenotypes specified

Plasmids can and do encode a wide variety of characteristics of evolutionary advantage to themselves and their host. In addition to their degradative genes many catabolic plasmids are self-transmissible via conjugation and possess clusters of transfer (*tra*) genes. When the *tra* genes are expressed the plasmid-bearing cell produces long, thin, hair-like, surface appendages (sex pili) which allow it to attach to a potential recipient strain, to which it will transfer a copy of its plasmid. Sex pili are the site of attachment of plasmid-specific bacterial viruses which enter the cell and go through their life cycle, leading to cell lysis and the release of many viral progeny. While some plasmid-bearing strains promote the attachment and growth of certain viruses, the growth of other viruses can be inhibited by the plasmid's presence. Other characteristics frequently encoded by catabolic plasmids are resistance to mercury, tellurite, ultraviolet (UV) irradiation and, less frequently, antibiotics. Catabolic plasmids are characteristically large, and relatively few of their genes have been assigned functions. Extensive DNA sequencing may provide some answers.

Compatibility types

A characteristic of closely related plasmids is that they are unable to stably coexist in the same cell – they are incompatible with one another. Use has been made of this characteristic to group most plasmids of the Enterobacteriaceae into 20 or so incompatibility (Inc) groups; the various groups have been denoted by letters of the alphabet, IncA, B, C etc. Since this classification has been based primarily on plasmids derived from clinical isolates it does not reflect plasmids from a wide range of environmental isolates. Indeed incompatibility groupings have been developed for *Pseudomonas* (IncP1, P2...P14) and other organisms whose plasmids do not fit into this incompatibility grouping.

Most plasmids have a narrow host range, being only transferable between members of the same species. However there is a small group of plasmids which can be transferred to and replicated in a wide range of bacteria – the so-called broad host range plasmids. These appear to be confined to the IncP, Q, W, N and X groups in the enterobacterial classification; all are conjugative with the exception of the small IncQ plasmids, which are nonconjugative but can be mobilized at high frequency by a coresident IncP plasmid. While some broad host range catabolic plasmids such pJP4 (IncP) can be classified by the enterobacterial system, and others such as pTOL by the *Pseudomonas* system, many fall into neither system, indicating that the evolutionary diversity of broad host range plasmids may be greater than originally thought.

Table 1 Catabolic plasmids

Bacterium	Phenotype (substrate degraded)	Plasmid
Pseudomonas putida	Camphor (moth repellent; used in the manufacture of plastics, varnishes and explosives)	pCAM
Alcaligenes eutrophus	2,4-D (organochlorine herbicide)	pJP4
Burkholderia cepacia	2,4,5-T (organochlorine herbicide)	Mega-plasmids
Rhodococcus erythropolis	PCBs and PBBs (widespread use in insulation and many household items)	Plasmids
Sphingomonas sp.	Chlorinated and unchlorinated dibenzofurans and dibenzo-*p*-dioxins	Gene clusters on main chromosome
Pseudomonas sp.	Nitrotoluene (explosive)	Plasmid
Sphingomonas paucimobilis	γ-Hexachlorocyclohexane (organochlorine insecticide)	Plasmid
Pseudomonas sp.	Styrene (used in manufacture of plastics and rubber)	Plasmid
Flavobacterium sp.	Nylon (used in manufacture of synthetic fibres for textile and domestic uses)	pOAD2
Rhodococcus erythropolis	Trichloroethylene (solvent for paints, fats, oils, rubbers, etc.)	pBD2 (linear plasmid)
Pseudomonas putida	Naphthalene (insecticide, substrate for manufacture of indigo blue dye)	pNAH
Pseudomonas putida	Salicylate (substrate for manufacture of aspirin)	pSAL
Pseudomonas convexa	Nicotine/nicotinate	pNIC
Pseudomonas oleovorans	Octane (component of crude petroleum oil)	pOCT
Pseudomonas arvilla	Toluene (solvent, substrate for manufacture of dyes and explosives)	pTOL

Transmissibility

Central to the rapid evolution of novel catabolic activities is the transmission of clusters of catabolic genes between bacteria, most frequently on broad host range plasmids. By combining defined catabolic genes from two or more different bacteria it is possible to produce novel catabolic activities not present in either parent strain. For example the TOL plasmid was transferred to a soil pseudomonad capable of degrading 3-chlorobenzoate. The resultant strain could now degrade both toluate and 3-CBA. Using continuous culture selection techniques mutants were isolated which could degrade the recalcitrant organochlorines 4-chlorocatechol and 3,5-dichlorocatechol, neither of which is degraded to any appreciable extent by either parental strain (Reineke and Knackmuss, 1979). Subsequently, the same techniques were used to isolate AC1100, a strain of *Burkholderia cepacia* capable of the complete degradation of the recalcitrant organochlorine herbicide 2,4,5-T.

Structure, Transposons and Insertion Sequences

It is now known that many novel catabolic activities arise by rearrangements of DNA sequences promoted by the activities of transposable elements. Recent research indicates that insertion sequences and other transposable elements are prevalent in the main chromosomes and plasmids of a wide range of organisms involved in the breakdown and recycling of naturally occurring and synthetic molecules. Indeed many clusters of catabolic genes are located on transposable elements. A few examples of catabolic transposons are listed in **Table 2**.

Stability and the Interactions with Chromosomes

Many bacterial degraders are isolated by enrichment techniques which select a single strain capable of using a complex organic molecule as a sole source of carbon and energy for growth. Where 2,4-D is used as the carbon source, a variety of strains, predominantly species of *Pseudomonas* and *Alcaligenes*, can be isolated. Virtually all carry 2,4-D plasmids. Removal of the selection pressure, e.g. growth on 2,4-D, results in irreversible loss of the 2,4-D plasmids and their 2,4-D-degradative ability in at least 50% of the isolates. While 2,4-D plasmids such as pJP4 are stable in the absence of 2,4-D, prolonged growth on this substrate does select for a subpopulation of pJP4 plasmids which have extensive duplications of the 2,4-D genes. In other strains the catabolic plasmid relinquishes its separate existence in the cytoplasm and enters the main chromosome; the plasmid is present but not in a form detectable by plasmid extraction techniques.

Table 2 Catabolic transposons

Bacterium	Phenotype (substrate degraded)	Transposon
Ralstonia eutropha (formerly *Alcaligenes eutrophus*)	Biphenyl 4-chlorobiphenyl	Tn*4371* (55 kb)
Burkholderia cepacia	2,4-D	Tn*5530* carried by pIJB1
Pseudomonas putida	Toluene	Tn*4654*
Alcaligenes sp.	3-Chlorobenzoate	Tn*5271* (Class I transposon with class II insertion sequences on either end)
Pseudomonas putida	Naphthalene	Tn*4655* carried by pNAH7
Sphingomonas sp.	Carbofuran (insecticide)	IS1412, 1487, 1488

Such a Jekyll and Hyde existence occurs with a conjugative, 2,4-D plasmid of *Alcaligenes paradoxus* which can integrate and excise from the main chromosome (Ka and Tiedje, 1994). This integration and excision is typical of many plasmids. It can occur where the plasmid is normally unstable in its host but selection pressure to maintain plasmid-borne characteristics, such as catabolic activity, selects for a subpopulation of cells where the integrated plasmid is stably replicated and maintained as part of the main chromosome. Where a plasmid has DNA sequences in common with the main chromosome, entry (integration) and exit (excision) from the main chromosome can occur by the host cell-encoded RecA dependent homologous recombination. Where the plasmid carries a class II transposon, homology is not required and the plasmid can integrate into virtually any part of the main chromosome by replicative transposition.

Catabolic Pathways, Operon Structure and Regulation

While any catabolic operon is potentially naturally mobilizable and certainly can be cloned, the degradative operons occurring on wild-type plasmids are useful but nonessential for the survival of the host cell in the absence of their substrate. They confer an advantage on their host when their specific substrate is abundant; indeed they may be essential when the substrate is toxic, but the host bacteria preferably utilize other carbon and energy sources. This is reflected in the structure and control of the catabolic operons.

There is an emphasis on host cell-supplied global negative regulation (catabolite suppression) systems, such as the cyclic adenosine monophosphate (cAMP)-dependent C-reactive protein (CRP) and the cAMP-independent catabolite repression systems of *Escherichia coli*, or the cAMP-independent systems of *Bacillus*, acting in opposition to specific, plasmid-borne positive regulation. This is based generally on a two-component sensor/regulator system which detects the substrates of the degradative pathway. The regulatory genes, and sometimes the degradative genes, are expressed constitutively at a low level, but in the presence of substrate they activate the transcription of the degradative genes as well as enhancing their own expression. In the most simple cases there is an operon which contains all the enzymes necessary for the complete degradation of a particular catabolite. In more complex systems, where the catabolite can be encountered in several forms of varying complexity, two or more semi-independent operons with a more complex regulatory structure may be encountered.

In the case of the TOL plasmid pWWO of *Pseudomonas putida* there are two operons: the upper pathway, which degrades toluene and xylene to catechol, and the lower pathway, which degrades catechol to central metabolites. When the upper pathway is activated the lower pathway is activated as well, but if the lower pathway is activated in the presence of catechol the upper pathway remains repressed. This is achieved by two divergent regulator genes, *xylR* and *xylS*. *xylS* activates the lower pathway, consisting of the *meta* pathway (*xylTEGFJQKIH*) and the three-component aromatic ring dioxygenase and the dehydrodiol dehydrogenase (*xylXYZL*), while *xylR* activates *xylS* and the upper pathway (*xylCMABN*) (Marques *et al*., 1998). This system allows the host cell to produce only those enzymes necessary for the degradation of catechol.

Differences in codon usage between the two operons suggest that they evolved independently, most likely in different organisms. Comparable arrangement is common, particularly in pathways for the degradation of aromatic hydrocarbons, and serves to streamline the degradation process. Different enzymes are employed to convert a vast array of substrates to a limited number of key intermediates such as catechol and protocatechuate. These intermediates are then channelled into *meta* or *ortho* cleavage pathways which supply central metabolic routes such as the tricarboxylic acid cycle (van der Meer *et al*., 1992). The aromatic ring fission catalysed by the dioxygenases of these pathways takes place either between the hydroxyl groups

(intradiol or *ortho* cleavage), or adjacent to one of the hydroxyls (extradiol or *meta* cleavage). *Meta* cleavage pathways such as the lower pathway on pWWO, as well as pathways on pNAH7 and plasmid VI150 (from a *Pseudomonas* strain that uses phenol, cresols and 3,4-dimethylphenol as sole carbon and energy sources) are plasmid borne and show homology. Unlike the *ortho* cleavage pathways, which are generally present on the chromosome, the *meta* cleavage pathways are able to degrade methylated catecholic substrates.

Metabolism of chlorobenzoates, chlorobenzene and chlorophenoxyacetate produces chlorinated catechol intermediates which are dealt with by plasmid-borne, modified *ortho* cleavage pathways (Harwood and Parales, 1996) like the *tfd* pathway for the degradation of 2,4-D carried by pJP4 (**Figure 1**). The genes of this pathway are arranged in five operons (**Figure 1**, **Table 3**) the largest of which (*tfdCDEF* and $tfdC_{II}D_{II}E_{II}F_{II}$) contain genes of a modified *ortho* cleavage pathway. *tfdA*, *tfdB* and $tfdB_{II}$ also encode enzymes, while *tfdK* encodes a transporter protein involved in the uptake of 2,4-D. Two identical regulatory genes, *tfdR* and *tfdS*, are associated with the functional operons. *tfdT* is a remnant of a regulator which had been inactivated by the insertion of ISJP4.

Both pilus and opacity genes are central to the pathogenicity of their respective hosts, and phase variation allows the pathogen to evade the host's defence mechanisms.

Clusters of catabolic genes also appear to be subject to DNA slippage. Certain deletion mutations in the structural genes of the protocatechuate 3,4-dioxygenase of *Acinetobacter calceoaceticus* are associated with DNA sequence repetitions, suggesting the existence of DNA slippage structures (Gerischer and Ornston, 1995). DNA slippage is potentially a significant source of genetic diversity for catabolic genes, which provides the variations in substrate specificities necessary to degrade mixtures of closely related molecules. For example, enzymes involved in the degradation of PCBs will encounter PCB molecules with varying numbers of chlorine atoms. To degrade molecules with slightly different structures generally requires enzymes with slightly different specificities. For any given gene and the enzyme it encodes, such variations can arise by changes in the DNA sequence due to DNA slippage.

Generation of Enzyme Diversity – DNA Slippage

Tandemly repeated DNA sequences occur throughout the genomes of prokaryotes. These small DNA repeats (3–9 nucleotides) can consist of simple homopolymers of poly(A), poly(T), poly(G) or poly(C) or mixtures of nucleotides, e.g. CTA CTA CTA, etc. The numbers of repeats are variable and in many instances arise by slipped-strand mispairing due to DNA polymerase slippage during chromosome replication. If the template strand bulges during replication the DNA polymerase may fail to replicate one or more of the tandem repeats, leading to a reduction in the numbers. Conversely, if the nascent strand bulges the number of repeats may increase. Depending on the genomic location of these hypervariable regions the consequences can be quite dramatic.

If variation in the numbers of these repeats alters the space between the -10 to -35 region upstream of a gene, then the level of expression of the gene can increase or decrease dramatically. Phase variation in fimbrial genes of *Haemophilus influenzae* occurs by this mechanism.

If this variation occurs within the coding region it can result in variations to the reading frame, leading to alterations in the primary amino acid sequence. Shifting of the translational reading frame for any of the opacity (Opa) proteins of *Neisseria gonorrhoeae* results in the simultaneous production of different Opa proteins.

Modular Evolution and Sequence Relationships

The substrates degraded by catabolic plasmids often require complex pathways for complete degradation. The genes involved are generally clustered together in groups of operons containing the enzymes responsible for the sequential steps in a pathway and their regulatory genes (see **Figure 1**). DNA rearrangement, deletion and transposition can lead to new genes or parts of genes being brought into existing modules where they can establish novel degradative capabilities. Such events may occur on all scales, from a simple joining by proximity of the genes of two degradative pathways, the end product of one of which can be used as the initial substrate by the other, through the introduction of new enzymes to complement an existing pathway, to the fusion of parts of genes from different phylogenetic sources to form multicomponent enzymes. Examples of the above include the toluene degradation pathway on pWWO, the codon usage patterns of which suggest the upper and lower pathways came together from separate origins, the 2,4-D metabolism of pJP4 – *tfd*A and *tfd*B appear to have been recruited during the evolution of the *tfd* pathway, and enzymes like the 2-oxo-1,2-dihydroquinoline 8-monooxygenase of *P. putida* 86, which consists of class IB-like reductase and class ZA-like oxygenase.

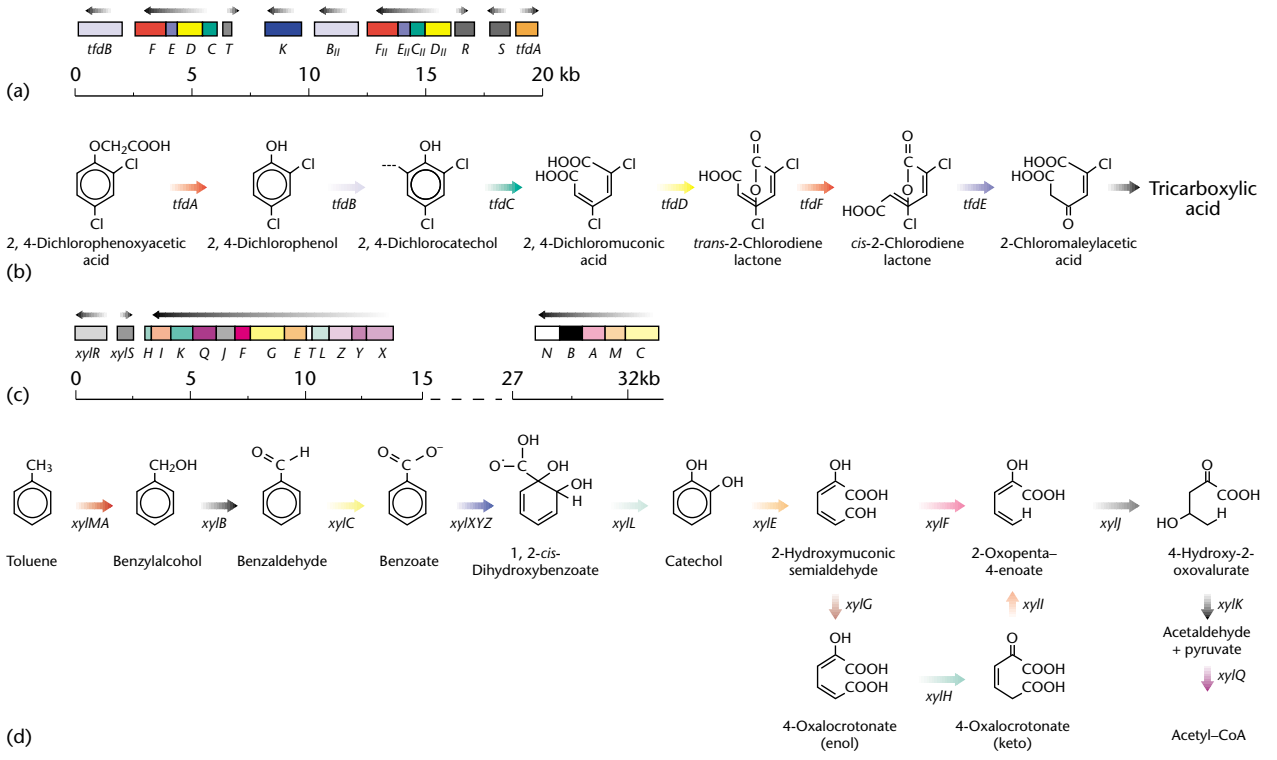

Figure 1 Plasmid-encoded pathways for the degradation of natural and synthetic molecules. (a) The *tfd* regulon on pJP4. (b) The proposed pathway for 2,4-dichlorophenoxyacetic acid degradation facilitated by the *tfd* regulon. (c) The *xyl* regulon on pWWD. (d) The proposed pathway for toluene degradation facilitated by the *xyl* regulon.

Ecology, Distribution among Genera and Clonality

Recycling of naturally occurring aromatic molecules, derived primarily from plant-based materials, forms a central and essential component of the earth's carbon cycle. It is to be expected that bacteria which degrade aromatic compounds occur predominantly in soil, on the surfaces of plants, in the guts of organisms that feed on plants and, to a lesser extent, in water. The bacterial genera represented among the aromatic degraders reflect these ecological origins – *Pseudomonas*, *Bacillus*, *Streptomyces*, *Rhodococcus*, *Alcaligenes*, *Burkholderia*, *Ralstonia*, *Xanthomonas*, etc. The distribution of catabolic plasmids also reflects soil microorganism-borne origins, with the greatest contribution to date from the genus *Pseudomonas*, whose members occur in very large numbers in soil and on plant surfaces.

In addition to recycling naturally occurring aromatic molecules, many soil microorganisms have specifically evolved enzymes to degrade chlorinated substrates such as 2,4-D, 2,4,5-T, PCBs, 3-CBA, etc. This degradation of organochlorines can occur via modified *ortho* cleavage pathway enzymes encoded by catabolic plasmids.

For clusters of catabolic genes that occur on broad host range plasmids and/or transposable elements it is anticipated that they will be widely distributed, particularly among Gram-negative bacteria. Under these circumstances evolutionary divergence of individual genes will be subtle, while gene arrangements and regulation will show more pronounced variations from one bacterium to another. The limited numbers of pathways studied and the initial concentration on Gram-negative bacteria may produce a superficial impression that a particular class of catabolic genes arose from a single ancestral gene. That this might not be the case is indicated by studies of chlorocatechol catabolism in *Rhodococcus opacus*, which suggest that a functionally similar pathway evolved quite separately from that which occurs in the majority of bacteria so far studied (Eulberg *et al*., 1998).

Bacterial genes involved in the degradation of chlorinated compounds could have arisen by the evolution of one or a small number of bacterial genes in one place in response to the use of chlorinated compounds and then spread worldwide. This appears to be the case with the atrazine catabolism genes *atzABC*, where geographically distinct atrazine-degrading bacteria contain *atzABC* genes which are 99% homologous at the DNA level (de Souza *et al*., 1998). In contrast, degradative genes such as those

Table 3 Catabolic genes of the *tfd* and the *xyl* pathways

Gene	Protein product
tfd pathway	
tfdA, A_{II}	2,4-Dichlorophenoxyacetate monooxygenase
tfdB, B_{II}	2,4-Dichlorophenol hydroxylase
tfdC, C_{II}	Chlorocatechol-1,2-dioxygenase
tfdD, D_{II}	Chloromuconate cycloisomerase
tfdE, E_{II}	Chlorodiene lactone isomerase
tfdF, F_{II}	Chlorodienelactone hydrolase
tfdK	2,4-D transporter protein
tfdR	Transcriptional activator
tfdS	Transcriptional activator
tfdT	Inactivated regulator
xyl pathway	
xylA	Toluene side-chain monooxygenase
xylB	Benzyl alcohol dehydrogenase
xylC	Benzaldehyde dehydrogenase
xylE	Catechol 2,3-oxygenase
xylF	2-Hydroxymuconic semialdehyde hydrolase
xylG	2-Hydroxymuconic semialdehyde dehydrogenase
xylH	4-Oxalocrotonate tautomerase
xylI	4-Oxalocrotonate decarboxylase
xylJ	2-Oxopent-4-enoate hydratase
xylK	4-Hydroxy-2-oxovalerate aldolase
xylL	Benzoate *cis*-diol dehydrogenase
xylM	Toluene side-chain monooxygenase
xylN	Probable toluene-specific porin
xylQ	Acetaldehyde dehydrogenase
xylR	Transcriptional activator
xylS	Transcriptional activator
xylT	Ferredoxin
xylX	Benzoate dehydrogenase
xylY	Benzoate dehydrogenase
xylZ	Benzoate dehydrogenase

involved in the degradation of 3-CBA also occur in geographically distinct populations of bacteria but show little DNA homology (Fulthorpe *et al*., 1998).

Applications in Biotechnology

The decision in the case of Diamond versus Chakrabarty (1980), which stated that it is possible to patent a living organism, allowed research into the applications of organisms in industry to be profitable and led directly to a major expansion of biotechnology. Catabolic plasmids were central to that case and they continue to hold an essential position today. The advantages of catabolic genes carried by plasmids are numerous. The number of copies of a particular catabolic pathway per host cell can be manipulated, the degradative genes and operons can be transferred to different, better suited strains or species provided compatible replicative/transcription apparatus are present, and, perhaps most importantly, the rearrangement, addition and deletion of genes within the operons, as well as the compilation of a number of operons on a single plasmid is a relatively simple, straightforward procedure.

Catabolic plasmids have uses in bioremediation, particularly the degradation of highly recalcitrant compounds (e.g. dioxins, PCBs), pesticides (e.g. 2,4-D, atrazine) and explosives (e.g. trinitrotoluene (TNT)), in the breakdown of chemicals to provide compounds necessary in industrial processes, and in the monitoring of environmental pollution. Naturally occurring catabolic plasmids can be selected and used, but often it is necessary to modify the control and/or the structure of the catabolic operons, and the copy number of the plasmids carrying them, in order to achieve the optimum conditions for the expression of the catabolic genes and maximize their industrial usefulness. Furthermore, genomic sequencing of various bacteria and archaea has revealed the location of many new chromosomally located catabolic genes, operons and their regulatory apparatus (Armengaud *et al*., 1998). This has allowed the selection and expression on plasmids of new degradative capabilities previously only encountered on the chromosomes. At the same time, selective mutations have resulted in the production of degradative pathways for novel synthetic compounds.

Bioremediation is one of the main areas to benefit from the selection and development of catabolic plasmids. Bacteria involved in degradation of pollutants may not grow very fast, nor can they often carry out all the steps necessary in the conversion of a xenobiotic to a harmless end product. This is usually a community effort with a number of different species involved. Bacterial survival in a polluted zone is also most often limited to areas of relatively low concentration of the pollutant.

Catabolic plasmids can be used in a number of ways in bioremediation. Pathways already expressed in the on-site community can be selected and expressed on plasmids to increase their copy number and modify their control within individual bacteria, and to provide them to other members of the microbial community. Novel pathways not present in the local population can be introduced or pathways specifically engineered to produce a particular end product can be provided. Since contamination is usually a combination of a number of different chemicals, a number of operons can be expressed on a single plasmid to provide members of the microbial community with greater catabolic diversity. Of course, the actual breakdown of the pollutants is only part of the problem. The survival of bacteria in the presence of toxic chemicals is attributed in large part to specific efflux systems capable of pumping the chemical out of the cell. It may therefore be useful to

introduce these mechanisms into the natural community on plasmids alongside the degradative pathways or, in some cases, only introduce the genes necessary for survival and allow the bacteria present to degrade the pollutants by the pathways they already possess. Such genes would allow the degradative bacteria to survive greater concentration of toxic chemicals, allowing them to penetrate deeper into the polluted zone and therefore remediate it at a faster rate.

Of course using catabolic plasmids in bioremediation is not quite that simple. Questions of the ethics of release of novel replicons into the environment aside, the catabolic plasmids must not only be stably maintained but the catabolic genes they carry must be expressed. Furthermore, in order to be maintained in the community they must provide an advantage for their host which outweighs the metabolic cost of maintaining the plasmid and expressing its genes. The spread of transmissible catabolic plasmids within soil and water can be enhanced by the activities of other organisms. For example the spread of the 2,4-D organochlorine catabolic plasmid pJP4 throughout the soil profile is greatly enhanced by the burrowing activities of earthworms. Those worms, which limit their activities to the top few centimetres of soil, promote shallow soil transmission. In contrast deeper burrowing species of worms distribute the plasmid much deeper into the soil profile, providing a greater level of soil bioremediation (Daane *et al*., 1997). The spread of conjugative plasmids in a severely polluted site may therefore be drastically slowed if the chemical present is toxic to earthworms.

Detailed knowledge of the regulation of catabolic pathways has led to the development of catabolic plasmids as biosensors for environmental pollution. Biosensors have the potential to be developed in all cases where the degradative genes are turned on in response to the presence of the particular organic contaminant which they degrade. A reporter gene such as luciferase, green fluorescent protein or β-galactosidase is placed under the control of a promoter regulated by a transcriptional activator which responds to the presence of the contaminant. In the presence of the contaminant the activator is switched on, transcription of the reporter gene is enhanced and the reporter protein is produced (**Figure 2**). Biosensors of this type have been constructed to detect toluene, naphthalene, and benzene, benzoate and their derivatives (Willardson *et al*., 1998). Not all of these are plasmid borne. There is no absolute requirement for the biosensor to be a plasmid construct, but the advantages of plasmid over chromosomal DNA are the same here as in other applications: ease of manipulation and greater copy number. Higher copy numbers in turn potentially translate into greater sensitivity. The advantages of biosensors of this type over conventional chemical tests are their low cost, their adaptability to field analysis and their suitability to *in situ* monitoring.

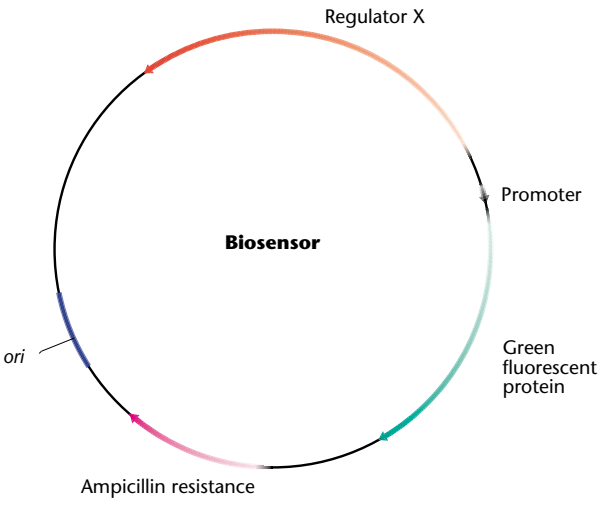

Figure 2 Simplified plasmid map of a hypothetical biosensor construct. Regulator X is a transcriptional activator that responds to an environmental pollutant. Green fluorescent protein is a reporter gene activated by regulator X. *ori* is the origin of plasmid regulation. Ampicillin resistance is a selectable gene. In this model, regulator X responds to an environmental signal (e.g. polychlorinated biphenyl) and attaches to the promoter. Transcription from the promoter is activated and green fluorescent protein is produced. The level of fluorescence detected is proportional to the size of the signal and therefore to the concentration of the pollutant.

Apart from their obvious use in pollution control, catabolic plasmids and the genes they encode can be used for a variety of additional purposes. One of the earliest herbicide-resistance genes to be cloned into plants was *tfdA* from pJP4. This gene encodes an enzyme which detoxifies the herbicide 2,4-D; plants genetically engineered with *tfdA* show high-level resistance to 2,4-D. Such crops allow easy weed control with minimal crop damage. In the future the selective use of individual catabolic genes could lead to cheaper production of certain industrial dyes and solvents, to inbuilt biodegradability in plastics, to new synthetic fibres and to the development of novel pharmaceuticals.

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