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Evolution of novel metabolic pathways for the degradation of chloroaromatic compounds

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Abstract

Chlorobenzenes are substrates not easily metabolized by existing bacteria in the environment. Specific strains, however, have been isolated from polluted environments or in laboratory selection procedures that use chlorobenzenes as their sole carbon and energy source. Genetic analysis indicated that these bacteria have acquired a novel combination of previously existing genes. One of these gene clusters contains the genes for an aromatic ring dioxygenase and a dihydrodiol dehydrogenase. The other contains the genes for a chlorocatechol oxidative pathway. Comparison of such gene clusters with those from other aromatics degrading bacteria reveals that this process of recombining or assembly of existing genetic material must have occurred in many of them. Similarities of gene functions between pathways suggest that incorporation of existing genetic material has been the most important mechanism of expanding a metabolic pathway. Only in a few cases a horizontal expansion, that is acquisition of gene functions to accomodate a wider range of substrates which are then all transformed in one central pathway, is observed on the genetic level. Evidence is presented indicating that the assembly process may trigger a faster divergence of nearby gene sequences. Further 'fine-tuning', for example by developing a proper regulation, is then the next step in the adaptation.

Introduction

The potential of microorganisms to degrade environmental pollutants, many of which have only recently been introduced into nature, is very large. It is intriguing to find out how so many different metabolic pathways have developed in bacteria and if new capabilities are still arising which enable microorganisms to use compounds which they have not encountered before.

We have been studying the possibilities for bacteria to degrade chlorobenzenes, which can be considered as novel substrates not generally degradable. We, and others, could isolate bacteria which use these compounds as sole carbon and energy source, from environments exposed to chlorinated benzenes for extended periods. From unexposed material, however, these bacteria could not be isolated, although the genetic material seems to be present, and we propose that they have evolved this new metabolic pathway under selective constraints.

Here I will give a brief overview of the different genetic mechanisms that can lead to changes in the DNA of microorganisms, of which it is believed they were and still are involved in creating the pool of variants from which present day's metabolic pathways were selected. I will further shortly summarize the available data on the genetic organization of (chloro-) aromatic pathways in aerobic bacteria as it will give an idea of the similarities between many of these genes. Understanding the different genetic processes that account for the development of the chlorobenzene pathway in particular, will certainly be of value for understanding the evolution of metabolic pathways in general.

Mechanisms of genetic adaptation

Metabolic versatility in the bacterial world is obvious to all microbiologists. We take it for granted that some bacteria grow by digesting human tissue, others by metabolizing toluene or by using CO_2 and H_2 as their sole carbon and energy source. But how could so many varieties arise? And did the formation of bacterial metabolic pathways more or less stabilize during evolutionary time or is there still an ongoing process of creating more variants? We will see below that this process has not stopped, and that new variants of metabolic pathways such as that for chlorobenzene degradation keep emerging.

One of the main ideas of the last decades was that many man-made compounds, which were introduced in large quantities into the environment, have led to a selection of bacteria with novel characteristics capable of degrading these materials. Especially compounds like chlorinated benzenes, PCBs or other chlorinated aromatics, various herbicides and pesticides, were considered to be substances that bacteria had never encountered before and thus - at best - metabolized through existing enzyme systems. For some substances or for combinations of substances, like chlorobenzoates and methylbenzoates, clear indications of the limitations of existing pathways in specific laboratory organisms were obtained (Ramos & Timmis 1987). Enzymatic incapabilities were discovered (e.g., for catechol 2,3-dioxygenase) which in some cases could be resolved by selecting induced mutants (Ramos 1987). Through genetic engineering some of the limitations could be raised and the concept of metabolic patchwork assembly is still important in the work of many laboratories (Rojo 1987; Mokross 1990; Timmis 1990).

In the mean time, many groups worked on the enrichment and isolation of bacterial strains from (polluted) environments, with the hope that natural selection had done some of the pathway assembly. This was successful for some substances, for example for 2,4-dichlorophenoxy acetic acid (Ka 1994a; Ka 1994b) or chlorobenzenes (van der Meer 1987), for others it remained only partially an improvement. There is still no one single microorganism that can use higher chlorinated biphenyls as sole carbon and energy source and break the substance down completely to CO_2, H_2O and Cl^- (Brenner 1994). From some of these 'newly' enriched microorganisms the genes for the degradation pathways and a great deal of the enzymology have been described. A comparison of those with genes for aromatic degradation pathways in other microorganisms shows us something about these natural adaptation processes and their underlying mechanisms.

Nowadays we believe that various cellular processes create changes in the genetic material of (micro-) organisms either spontaneously or induced, and as such are the driving forces for creating the 'pool' of variants from which novel characteristics can be selected. A summary of such changes in the DNA and cellular processes that may be causing these changes, is presented in Table 1. These mechanisms can be particularly well observed in the metabolic adaptation of microorganisms (Clarke 1984; van der Meer 1992), or for instance in the acquisition of resistances to antibiotics (Terzaghi & O'Hara 1990; Amabile-Cuevas & Chicurel 1992). At present, it is too early to add clear rates or frequencies to each of the mechanisms. Many processes can in principle take place within a short timescale (i.e., one generation of a bacterium), although at relatively low frequencies. For instance, the rate of 'spontaneous' change of any nucleotide into another in a growing (micro-) organism, is estimated to be 0.003 per genome per replicative round (Drake 1991). 'Spontaneous' transfer of a plasmid from one bacterium to another can have rates between 0.01 (for plasmid RP4) and 10^{-7} (for a *Pseudomonas* sp. strain B13 conjugable element) per cell to cell contact (R. Ravatn, unpubl.). Higher incidences may be found during specific phases of the lifetime of (micro-) organisms or as a response to specific environmental signals or conditions. For example, a substantially higher 'spontaneous' mutation rate was found in non-growing bacterial cultures in the presence of a potentially useful substrate (so-called adaptive mutations) (Cairns 1988; Foster 1993; Harris 1994). The mechanistic details of these higher rates or specific responses is still subject of debate, but various cellular processes, such as the SOS response or the recBCD pathways are presumed to be involved (Cairns 1988; Drake 1991; Foster 1993; Harris 1994).

DNA replication and DNA repair systems are supposed to account for the smallest-sized changes, such as base-pair transitions, transversions, small lesions, short repetitions or duplications (Schaaper 1986; Drake 1991). Despite their proofreading capacity, replication and repair pathways are error-prone to a certain level and are thus raising a detectable rate of mutations (van Houten 1990; Echols & Goodman 1991; Modrich 1991; Hoeijmakers 1993). The local primary structure of the DNA is apparently an important factor for the occurrence and site of DNA change (Schaaper 1986). Short sequence repetitions or palindromic sequences

Table 1. Mechanisms causing alterations in DNA sequence and examples of the effects of such alterations in aromatic degradation

Type of change	Cellular processes	Size	Effects	Examples
Transition	DNA replication	1 nt	altered protein specificities	XyIS mutants (Ramos 1986), AmiE mutants (Clarke 1984)
Transversion		1 nt	disturbed reading frame	Mutations that inactivate *pcaHG* (Gerischer & Ornston 1995)
Inversion	DNA repair	1-several nt	altered promoter	Spontaneous mutation in *lac1* (Schaaper 1987)
Duplication		1-several nt		cap-domain mutation in haloalkane dehalogenase (Pries 1994)
Deletion		1-several nt		
Inversion	DNA recombination	several nt up	protein domain swapping	Modular development of BphC (Han 1995
Duplication	(RecA dependent and	to several kb	gene organization	Figs 1, 2, 3 and 4
Deletion	illegitimate)		gene dosage	Mutations that inactivate *pcaHG* (Gerischer & Ornston 1995)
Strand exchange				Duplications of *xylS* (Assinder 1993)
				Duplications on pOAD nylon-degradation plasmids (Negoro 1994)
			gene repair	*tfdR/S* duplication (You & Chosal 1995; Matrubutham & Harker 1994)
				catJ/pcaJ recombinatorial repair (Gregg-Jolly & Ornston 1994)
Duplication	Site specific recombination	several nt up to several kb	silencing of gene expression	Mutations that inactivate *pcaHG* (Gerischer & Ornston 1995)
Excision	(IS-elements, Transposons)		activation of gene expression	RS1100 (Haughland 1990)
Integration			disturbance of reading frames	*tfdT* inactivation by ISJP4 (J. H. J. Leveau, in prep.)
Inversion			gene mobilization	Tn52*80* chlorobenzene dioxygenase transposon (van der Meer 1991)
				Overview of catabolic transposons in (Wyndham 1994)
DNA uptake	Transformation	several kb	increase of total genetic information content	Transformation in *A. calcoaceticus* (Gregg-Jolly & Ornston 1990)
Gene transfer	Plasmid-mediated conjungation	several kb up to hundreds of kb	increase of total genetic information content	catabolic plasmids, see (Frantz & Chakrabarty 1986; Sayler, 1990)
	Phage-mediated transfection		disturbance of reading frames	

may serve as sites for false hybridization or template shift during DNA replication, thus creating DNA changes. These type of alterations may occur more frequently than single base- pair substitutions due to misincorporation (Schaaper 1986; Drake 1991; Harayama & Rekik 1993; Gerischer & Ornston 1995), and they create larger changes in the DNA sequence in a single 'mutational' event. This may make calculations of phylogenetic relationships, which are now based on estimating neutral base-pair substitutions, considerably more complicated (Drake 1991; Harayama & Rekik 1993). It should also be noted that various compounds in the environment are directly acting as mutagenic agents, triggering DNA changes by the activity of DNA repair systems (Walker 1984; Woodgate & Sedgwick 1992; Hoeijmakers 1993; Levine 1994). The effects of these smallest sized DNA changes are trivially clear and well-documented. For example, a single

base-pair change can effect the catalytic activity of an enzyme or disturb the genetic reading frame altogether (Table 1).

The second group of mechanisms involves the recombination systems of the microbial cell. Regions of the DNA can be exchanged or rearranged to a new relative position whenever sequence homology exists between them. RecA-dependent recombination requires a homologous region of at least 15–25 base-pairs (Summers 1994). Stunningly, also smaller homologous regions (as small as 4 base-pairs) can be sufficient for recombination (Gerischer & Ornston 1995), although at lower frequencies than when larger overlaps exist (Summers 1994). These recombinations are generally independent of RecA-activity and may be mediated by other mechanisms, such as by the activity of DNA gyrase (Summers 1994). As the DNA sequence is non-random, it is obvious that the potential for DNA rearrangements through recombinational processes is high. The sizes of these rearrangements range from several nucleotides to fragments containing parts of or complete genes. It has even been proposed that large scale rearrangements may take place on chromosome level through recombination between multiple copies of homologous DNA sequences (Stern 1984). These repetitive sequences occur in non-coding regions of the chromosome and have sizes between 35 and 154-base pairs (Stern 1984; Dimri 1992; Louws 1994; Houghton 1995). Probably they are in some form conserved in all eubacteria (Louws 1994).

Recombinations between DNA fragments can also be mediated by the enzymes encoded on mobile elements, such as insertion sequences or transposons (Galas & Chandler 1989). During the process of moving of mobile elements, DNA rearrangements are created. These elements can replicate and integrate themselves into a target sequence on the DNA or create a deletion when transposing (Grindley & Reed 1985). In certain cases they will move into a genetic reading frame and thereby disturb gene expression (Schaaper 1986; Gerischer & Ornston 1995). They can also influence gene expression by providing transcription signals as their ends often contain sequences resembling promoters (Galas & Chandler 1989). Movement of IS-elements and transposons occurs in response to environmental signals or simply in a stochastic manner (Terzaghi & O'Hara 1990). Their movement can even lead to capture and mobilization of other genes. In many cases IS- elements and transposons are associated with genes for degradation of environmental pollutants and it is believed that they have been important in the formation and distribution of new catabolic pathways (Nakatsu 1991; van der Meer 1992; Wyndham 1994).

Probably the largest changes in the genetic content of the bacterial cell occur through plasmid-mediated gene transfer, uptake of naked DNA molecules or transfection by phages. In all of these processes, the 'accepting' bacterial cell faces an instantaneous increase in total genetic information content. The localization of many catabolic gene clusters on plasmids is well recognized (see also below) and thus their potential to be distributed in a mixed population of bacteria (Frantz & Chakrabarty 1986; Sayler 1990; van der Meer 1992). These transfer processes by which DNA fragments move from one bacterium to another is probably one of the most important ways by which existing gene functions are acquired or captured by other microorganisms.

The blueprints for metabolism of aromatic compounds

A survey of the available genetic information for degradation pathways of aromatic compounds in aerobic bacteria (Figures 1, 2, 3 and 4) reflects the outcome of all these changes in the DNA during time. Selective constraints on protein functions may have forced slow divergence in some cases, whereas in others the local DNA structure may have been a site for continuous changes (see for instance) (Hartnett & Ornston 1994; Kowalchuk 1995). This overview shows that the operons and gene clusters encoding the different pathways have many genes in common, yet often in new combinations.

It has been often proposed that the main strategy of microorganisms to degrade aromatic pollutants is to use a number of so-called peripheral enzymes which convert the substances to a common intermediate, usually a dihydroxy- (catechol-like) compound (Dagley 1986; Ramos & Timmis 1987; Schlömann 1994). In this way, a larger variety of initial substrates could be channeled into one central pathway. These intermediates are then converted in a number of enzymatic steps to intermediates of central metabolic pathways, such as acetate, acetaldehyde, succinate or pyruvate. Examples of lower pathways are the *ortho* cleavage pathway, the gentisate pathway, the *meta* cleavage pathway (Dagley 1986), or the modified *ortho* cleavage (or chlorocatechol oxidative) pathway. Although it is certainly possible to expand the use of a lower pathway

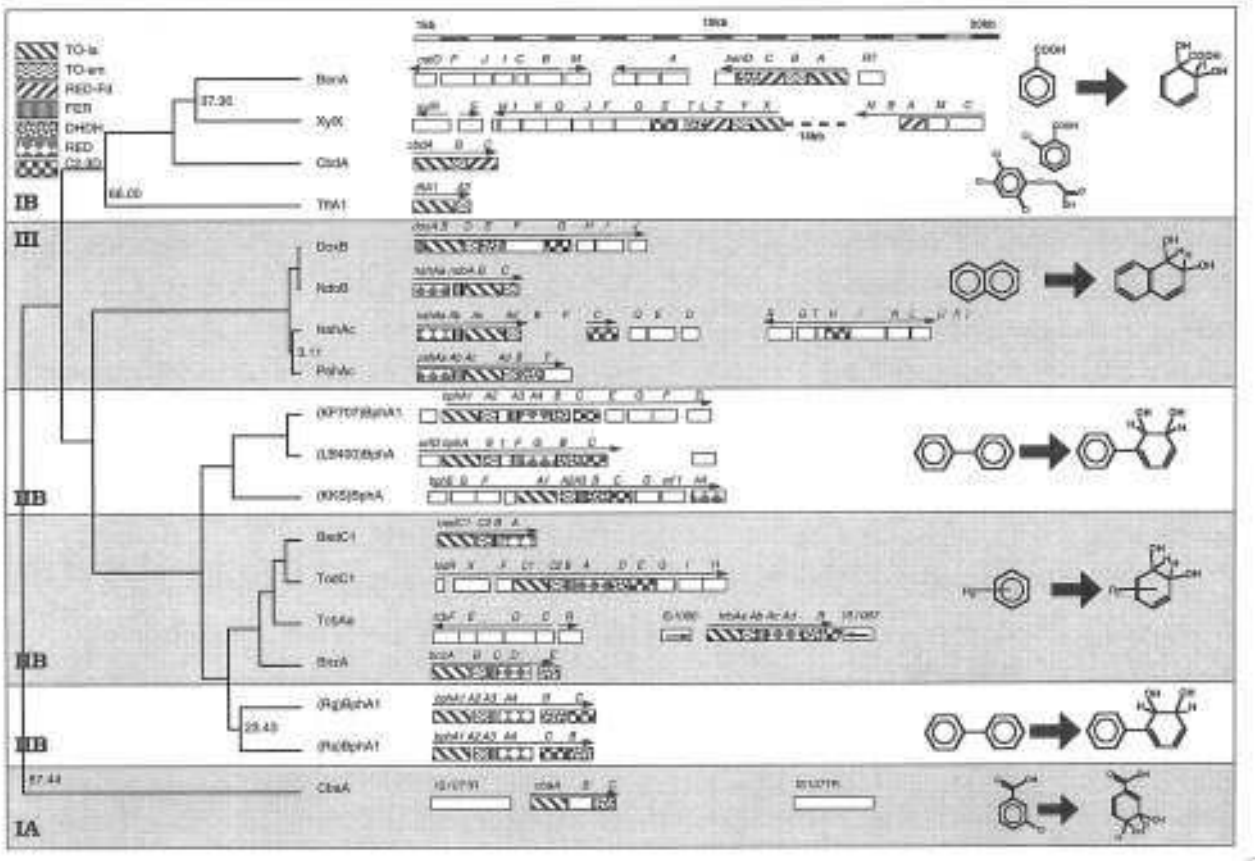

Figure 1. Blueprints of degradation pathways for aromatic compounds in which aromatic ring dioxygenases (ARD) play a role. The central part of the figure depicts the organization of genes for ARD and their neighbours (as far as known). Shown are the ARD genes for benzoate degradation (class IB dioxygenases) (Mason and Cammack, 1992), naphthalene (class III), biphenyl (class IIB), benzene and toluene (class IIB) and 3-chlorobenzoate (class IA). Gene names are given above each cluster and arrows indicate the direction of transcription. Genes are drawn according to their size and relative position, and the scale bar on top of the figure indicates the absolute gene sizes in kilobase-pairs (kb). Accentuated are the individual genes for the aromatic ring dioxygenase, the dihydrodiol dehydrogenase and the extradiol dioxygenase in each cluster. Similar shadings or patterns represent DNA and amino acid similarity of that particular gene. The left part of the figure shows a PILEUP clustering (Devereux 1984; Pearson & Lipman 1988) derived from a pairwise comparison of the amino acid sequences of the large subunits of the terminal oxygenase. Different groups obtained in the Pileup-program are clustered graphically in the figure by alternating grey and white background color. To get an idea of the degree of similarity in the clustering, several values of pair-wise comparisons are indicated (values obtained in the program DISTANCES as uncorrected number of amino acid substitutions per 100). The value of 66.00 belongs to the BenA:TftA1 comparison; 87.44 to a BenA:CbaA comparison. The right part of the figure depicts the principle reactions catalyzed by ARDs of that group. Pileup clustering for any other gene of ARD would more or less give the same picture (Werlen 1996). Abbreviations and references: TO-la, large subunit of the terminal oxygenase; TO-sm, small subunit of the terminal oxygenase; Red-Fd, 'combined' ferredoxin-NADH-ferredoxin reductase; Fer, Ferredoxin; DHDH, dihydrodiol dehydrogenase; RED, ferredoxin-NADH-reductase; C2,3D, extradiol dioxygenase. *cat* and *ben* genes code for benzoate degradation in *Acinetobacter calcoaceticus* (Neidle 1988; Neidle 1989; Neidle 1991; Neidle 1992; Hartnett 1994; Shanley 1994; Romero-Arroyo 1995); *xyl* genes for xylene degradation of *P. putida* mt–2 (TOL plasmid) (Inouye 1983; Nakai 1983; Harayama & Rekik 1990; Harayama 1991; Horn 1991; Suzuki 1991; Neidle 1992; Harayama & Rekik 1993; Harayama 1994), *cbd* genes code for 2-halobenzoate 1,2-dioxygenase in *Pseudomonas cepacia* (plasmid pBAH1) (Haak 1995), *tft* for 2,4,5-trichlorophenoxyacetic acid dioxygenase in *B. cepacia* AC1100 (Danganan 1994), *dox* for dibenzothiophene and naphthalene in *Pseudomonas* (Denome 1993), *ndo* for naphthalene 1,2-dioxygenase (Kurkela 1988; Simon 1993), *nah* for naphthalene degradation in *P. putida* (NAH7 plasmid) (Schell 1985; You 1988; Harayama 1987; Harayama & Rekik 1989; You 1991; Eaton & Chapman 1992; Simon 1993; Eaton 1994), *pah* for naphthalene and phenanthrene dioxygenase and DHDH in *P. putida* (Takizawa 1994), *bph* for biphenyl degradation in *P. pseudoalcaligenes* KF707 (Taira 1992; Furukawa 1994), in *Pseudomonas* sp. LB400 (Erickson & Mondello 1992; Hofer 1993), and in *Pseudomonas* sp. KKS102 (Kimbara 1989; Fukuda 1994; Kikuchi 1994a; Kikuchi 1994b), *bed* genes for plasmid-encoded benzene dioxygenase in *P. putida* ML2 (Tan & Fong 1993), *tod* for toluene degradation in *P. putida* F1 (Zylstra & Gibson 1989; Menn 1991; Lau 1994; Wang 1995), *tcb* for plasmid- encoded chlorobenzene degradation in *Pseudomonas* sp. P51 (van der Meer 1991a; van der Meer 1991b; van der Meer 1991d; Werlen 1996), *bnz* for benzene degradation (Irie 1987), *bph* for biphenyl degradation in *Rhodococcus globerulus* (Asturias & Timmis 1993; Asturias 1995), and in *Rhodococcus* sp. (Masai 1995), and *cba* genes for plasmid-encoded 3-chlorobenzoate 4,5-dioxygenase of *Alcaligenes* sp. BR60 (Nakatsu & Wyndham 1993; Nakatsu 1995).

with another peripheral enzyme (as in *Pseudomonas* sp. strain B13) (Rojo 1987), most gene clusters for aromatic degradation pathways seem to be complete and do not show such a horizontal expansion. For instance, the toluene degradation pathway of *P. putida* mt2 (Williams & Sayers 1994) allows degradation of toluene, xylenes, (methyl-)benzoates, but not of phenol or salicylate, although part of the genetic information could be properly used for this (see below). Aromatic ring dioxygenases, such as toluene (TOD) (Gibson 1990) or chlorobenzene (TCB) dioxygenase (Werlen 1996) catalyze the conversion of many different aromatic substrates, yet this is not always productive, since enzymes encoded in a 'later' stage, such as catechol 1,2-dioxygenase or catechol 2,3-dioxygenase are not able to convert the intermediates arising in the first steps. Interestingly, the gene organization and the similarities in the different aromatic degradation pathways suggest a much greater deal of variety in the 'central' metabolic pathways, and a great conservation in the 'peripheral' enzymes. I propose that this indicates that pathways have evolved in a vertical manner by acquisition of genes which would just encode the transformation reactions necessary for a particular step. Alternatively, as will be seen for the different *meta* cleavage pathways, non-necessary gene functions may have disappeared or have been counterselected for. Expansion in a horizontal manner, which would lead to metabolism of a wider variety of initial substrates in a single organism, that are all channeled in the same 'lower' or 'central' pathway, seems to be less frequent.

It is almost impossible by now to indicate all the relations between all genes in every cluster. Therefore, I will focus on a number of clear examples. Most of the relationships have been recognized by other authors, yet mostly for genes of individual interest.

Aromatic ring dioxygenases

Aromatic ring dioxygenases are found in many aerobic degradation pathways and catalyze the incorporation of dioxygen on the aromatic substrate at the expense of NADH-oxidation. The aromatic compound is hereby transformed to a dihydrodiol (Figure 1). Aromatic ring dioxygenase enzyme complexes can be categorized into different classes on the basis of the number of individual subunits, cofactor requirements and type of electron transfer centre (e.g, Iron sulfur clusters) (Harayama 1992; Mason & Cammack 1992; Powlowski & Shingler 1994). Examples of class IA are dioxygenases with two subunits, such as phthalate dioxygenase (Nomura 1992), 3-chlorobenzoate 4,5- dioxygenase (Nakatsu & Wyndham 1993) (Figure 1) or the dioxygenase catalyzing 3- or 4-carboxydiphenyl ether oxidation (Dehmel 1995). Class IB aromatic ring dioxygenases have three subunits, two of which form the terminal oxygenase and one is a flavin-containing protein with ferredoxin-NADH-reductase activity. Examples are benzoate 1,2- dioxygenase or toluate 1,2-dioxygenase (Figure 1). The largest aromatic ring dioxygenases (class II and III) are formed by the four subunit complexes, such as toluene, biphenyl or naphthalene dioxygenase. In the class II and III complexes, two separate proteins exist for the ferredoxin- and for the NADH-ferredoxin-reductase-function (Harayama 1992; Mason & Cammack, 1992). The reductase component of naphthalene dioxygenase (class III) is substantially shorter and biochemically different from those in class II systems (Mason & Cammack 1992). Clustering analysis of a large number of genes for aromatic ring dioxygenase indicates that all classes are related through the large subunit of the terminal oxygenase, although class IA enzymes are clearly the most distant ones when compared with classes IIB and III (Figure 1). Except for class IA enzymes, all others have a gene for the small subunit of the terminal oxygenase. Class IB then differs from the others (II and III) because in this group no separate genes for a ferredoxin and a NADH-reductase are found (Neidle 1991). Interestingly, the clustering obtained for the terminal oxygenase large subunit genes not only reflects the biochemical subclasses of the enzymes, but also correlates well with a particular gene order. The gene order for the class II enzymes is that for large subunit, small subunit, ferredoxin and reductase, whereas for class III the gene for reductase and ferredoxin are upstream of those for the large and small subunit. Small changes in gene order also exist within the group of the biphenyl dioxygenases. In *Pseudomonas* sp. strain KKS102 the gene for the reductase (*bphA4*) is not directly present near those for the other dioxygenase subunits (Kikuchi 1994a). The biphenyl genes in this group further contain two extra open reading frames when compared with their closest relatives, the toluene and benzene dioxygenase genes or with biphenyl dioxygenases of *Rhodococcus* strains (Figure 1). Interestingly, in almost all dioxygenase gene clusters the gene for the dihydrodiol dehydrogenase (the enzyme catalyzing the next step in the transformation process) is present directly downstream of the dioxygenase genes. It is interesting that the degree of relatedness and clustering correlates so well with a

particular gene order. Perhaps the rearrangements or recombinations which caused gene order differences, somehow accelerated subsequent changes in the DNA sequence of the genes itself.

Sometimes complete 'blocks' of genes ('modules') have acquired a totally different relative position in another microbe and encode part of another metabolic pathway. The genes for the class IB benzoate and toluate dioxygenase and for the dihydrodiol dehydrogenase show this clearly (Figures 1 and 3). The *benABCD*-genes are part of a chromosomally located regulon for benzoate degradation in *Acinetobacter calcoaceticus* (Neidle 1991). In this strain they lay in close proximity to the genes for an *ortho* cleavage pathway (the *cat*-genes). The related *xylXYZL*-genes of *P. putida* mt2 (Harayama 1991; Neidle 1991) occur on the TOL-plasmid but form here one transcriptional unit with the genes for a *meta* cleavage pathway (*xylT* through *H*). This movement of modules can similarly be seen in the clusters for biphenyl and toluene dioxygenases (Figures 1 and 3). Here a block of five genes for the aromatic ring dioxygenase and dihydrodiol dehydrogenase is rather conserved but form part of a transcriptional unit with the genes for a shorter *meta* cleavage pathway (e.g., *bphCEGFD* or *todFEGIH*). A similar *tcbAaAbAcAdB* gene block of *Pseudomonas* sp. strain P51, however, is no longer integrated with the genes for the short *meta* pathway, but cut out by the action of two IS-elements and moved to a new position nearby the genes for a chlorocatechol pathway (van der Meer 1991d; Werlen 1996) (see below). Except for the *tcb*-encoded pathway, in which case the dioxygenase genes were recombined by the activity of IS-elements, the underlying mechanisms for such rearrangements of gene blocks are not clear. Perhaps in some cases the general recombination pathways mediated rearrangements when DNA sequences of sufficient homology were present. Such 'DNA migratory elements' were postulated in a rearrangement observed in the relative position of the *catA* gene in two strains of *P. putida* (Houghton 1995). If these mechanisms involve illegitimate recombination pathways, however, it may not be an easy task to find (remnants of the) DNA regions which triggered the rearrangement.

Single component hydroxylases

Single component (flavin containing) hydroxylases catalyze transformation reactions in a wide variety of aromatic degradation pathways (Figure 2). Some of them are particularly useful in transformation of halogenated compounds. These enzymes are mostly involved in the initial stages of transforming aromatic substances with a hydroxy-group on the ring into dihydroxy-derivatives. These are then subject to degradation in one of the lower pathways. Unlike the aromatic ring dioxygenase the genes encoding single component hydroxylases are distantly related to one another (Figure 2), although this may be biased by the lack of information on other bacteria. Furthermore, it appears as if these genes occur mostly as loners, without much conservation in neighbouring genes between different pathways, suggesting that they were 'captured' and are useful for many different reactions. The two highest related ones, although occurring in two totally different clusters, are *pheA* for phenol monooxygenase in *P. putida* (Nurk 1991) and *tfdB* for 2,4-dichlorophenol hydroxylase in *Alcaligenes eutrophus* (Perkins 1990). Both of these genes are plasmid-located. Phenol monooxygenase converts phenol to catechol, which is then cleaved by the catechol 1,2- dioxygenase encoded by *pheA* (see Figure 4). *PheA* and *pheB* are flanked by IS-elements and it seems that this is an example of an 'incomplete' cluster, since no genes for the subsequent degradation of muconic acid are present nearby. The gene *tfdB* is located in the vicinity of a number of gene clusters on plasmid pJP4 which encode the complete degradation of 2,4-dichlorophenoxy acetic acid. It looks as if the *tfdB* gene was one of the genes captured in this pathways, since it is not part of the transcriptional unit for *tfdCDEF*, encoding the chlorocatechol oxidative pathway, which lays directly upstream of *tfdB*. A third member of the cluster with *pheA* and *tfdB* is a gene for 2-hydroxybiphenyl monooxygenase of *P. azelaica* sp.strain HBP1 (A. Schmidt, in prep.). In this case, however, the gene for the hydroxylase is part of a cluster for a short *meta* pathway, containing the genes for a *meta*-cleavage enzyme and a hydrolase similar to those for biphenyl degradation. Two other members of the single component hydroxylase family, i.e. *pcpB* for pentachlorophenol-4-monooxygenase of *Flavobacterium* (Orser 1993) and *tbuD* for phenol/cresol hydroxylase of *P. pickettii* PKO1 (Kukor & Olsen 1992) have been sequenced (Figure 2). The *pcpB* gene seems to be part of a short cluster with genes for a XylZ-type reductase and a regulatory gene (Orser & Lange 1994). The *tbuD* gene on its hand occurs nearby the genes for a *meta* cleavage pathway (Kukor & Olsen 1991). Very distantly related to the other members of this family are *p*-hydroxybenzoate hydroxylase

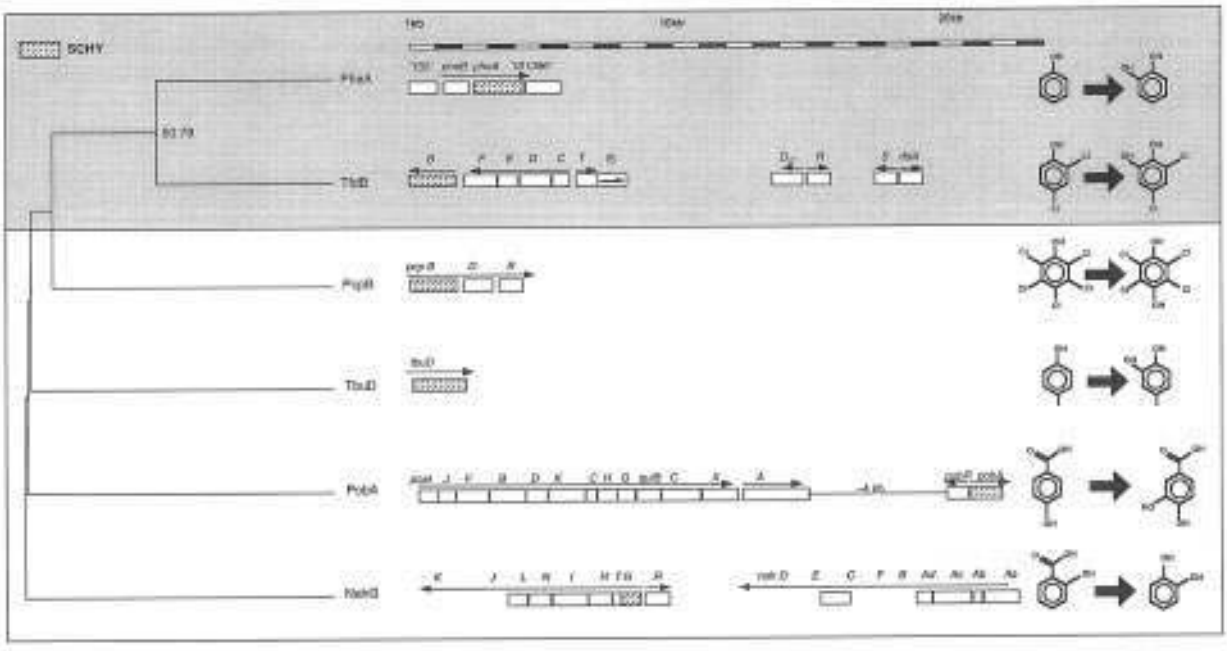

Figure 2. Overview of the gene organization of pathways in which a gene for a flavin-containing single component hydroxylase (SCHY) occurs. For structure and explanation of the figure, see Figure 1. PILEUP clustering was performed for the primary sequence of the SCHYs. Gene assignments and abbreviations: *phe* genes code for plasmid-located phenol monooxygenase (*pheA*) and catechol 1,2-dioxygenase (*pheB*) of *Pseudomonas* sp. EST 1001 (Kivisaar 1991; Nurk 1991), *tfd* for 2,4-dichlorophenoxyacetic acid degradation in *Alcaligenes eutrophus* JMP134 (on plasmid pJP4) (Streber 1987; Perkins 1990; Matrubutham & Harker 1994; You & Ghosal 1995), *pcp* code for pentachlorophenol–4-monooxygenase of *Flavobacterium* (Orser 1993; Orser & Lange 1994), *tbuD* for phenol/cresol hydroxylase of *P. pickettii* PKO1 (Kukor & Olsen 1992), *pobA* for *p*-hydroxybenzoate hydroxylase of *A. calcoaceticus* (DiMarco 1993b) and *nahG* for salicylate hydroxylase of *P. putida* PpG7 (NAH plasmid) (see Figure 1).

(encoded by *pobA*) (DiMarco 1993b) and salicylate hydroxylase (encoded by *nahG*) (You 1991). The gene *nahG* is also substantially shorter than the others (see further below). These two genes may be examples of DNA rearrangements which have had time to smoothen out (see below). The *nahG* gene is transcriptionally fused with the genes for a *meta* cleavage pathway and is properly regulated by the product of the divergently located *nahR* gene (Schell 1985). Also *pobA* is regulated in such a sense by *pobR* (DiMarco 1993a). The other members of the single component hydroxylase family appear to be more lose in their neighbouring associations and (as far as understood) in their regulatory control (see below).

Extradiol dioxygenases

The family of the extradiol dioxygenases represents a group of related genes (and enzymes) from one of the more 'central' transformation pathways. We will see, however, that this is a rather arbitrary view. Extradiol dioxygenases catalyze *meta* cleavage of aromatic compounds which carry two adjacent hydroxy-groups. The aromatic ring structure is broken up, although for binuclear compounds only on one of the rings (Figure 3). In these cases first one of the rings is cleaved and partially removed, which leaves an intermediate such as benzoate or salicylate behind. This aromatic ring structure then goes through a second round of cleavage, although catalyzed by related but not the same enzymes. The similarities between the primary structures of these enzymes were recognized by many authors and have been well described (e.g.) (Harayama & Rekik 1989; Williams & Sayers 1994). A clustering analysis reveals different discrete subgroups, which again correlate well with a particular gene order (Figure 3). The enzymes of relatively homogenous subgroups, such as that with DmpB, PhlH, NahH and XylE, show nevertheless important differences in their substrate range (Carrington 1994; Cerdan 1995). Between subgroups differences in substrate prefer-

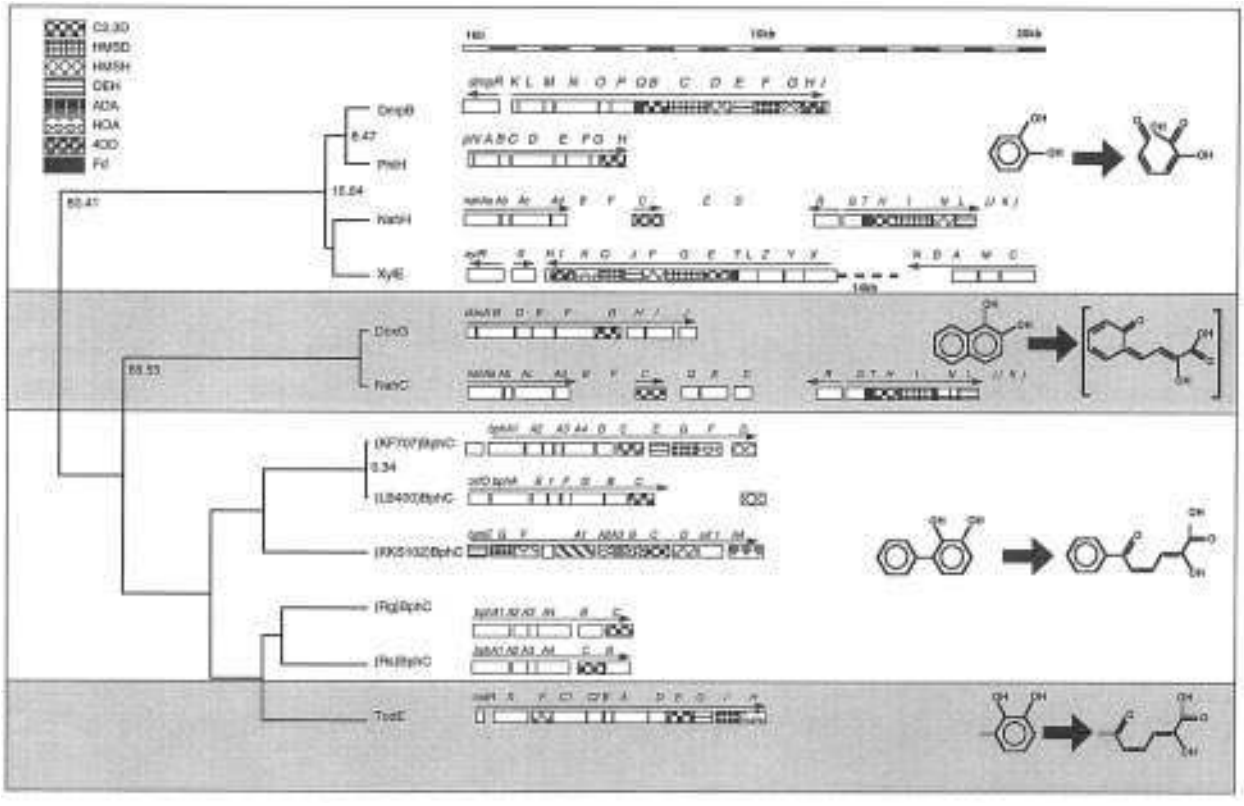

Figure 3. Similar overview as in Figures 1 and 2, now for the genes for extradiol dioxygenase and other *meta* cleavage pathway enzymes. PILEUP clustering was performed for the primary sequence of the extradiol dioxygenases. Non-trivial uncorrected distances: DmpB:NahH, 15.64; DmpB:NahC, 80.41; DoxG:(KF707)BphC, 66.55.Abbreviations and gene assignment (as far as not explained in Figures 1 and 2): C2,3D, extradiol dioxygenase (*meta* cleavage enzyme); HMSD, 2- hydroxymuconic semialdehyde dehydrogenase; HMSH, 2- hydroxymuconic semialdehyde hydrolase; OEH, 2-oxopent–4-dienoate hydratase; ADA, aldehyde dehydrogenase (acylating); HOA, 4-hydroxy- -2-oxovalerate aldolase; 4OD, 4-oxalocrotonate decarboxylase; Fd, ferredoxin-like protein. *dmp* Genes code for phenol metabolism in *Pseudomonas* sp. CF600 (pVI150) (Nordlund 1990; Shingler 1992; Shingler 1993; Powlowski & Shingler 1994), and *phl* genes for phenol hydroxylase and catechol 2,3-dioxygenase in *P. putida* (pPGH1) (Herrmann 1995).

ences are more pronounced (Hirose 1994; Kim & Zylstra 1995). What is interesting to notice is that one subgroup of the extradiol dioxygenase genes, the catechol 2,3-dioxygenases in stricter sense (e.g., XylE, DmpB, NahH), occur in a very clear conserved gene cluster (Figure 3). These clusters, e.g. *dmpQBCDEFGHI* (Shingler 1992) or *xylTEGFJQKIH* (Harayama & Rekik 1990), encode the complete so called *meta* cleavage pathway (Harayama & Rekik 1990; Powlowski & Shingler 1994). The cluster appears as another gene module which is found in different combinations with other upstream located genes (Figures 1, 2 and 3). For example, transcriptionally fused combinations occur with genes for phenol hydroxylase (*dmpLMNOP*) (Nordlund 1990) or *phlBCDEF* (Herrmann 1995), for salicylate hydroxylase (*nahG*) or for toluate and benzoate 1,2-dioxygenase and the dihydrodiol dehydrogenase (*xylXYZL* or *bphXYZL*) (Carrington 1994). A non-transcriptionally fused combination seems to be present with the *tbuD* -encoded phenol/cresol hydroxylase (Kukor & Olsen 1991).

Two major variations on this *meta* cleavage gene cluster have been found. One of these, a short *meta* pathway, occurs in microorganisms degrading biphenyl or toluene (Figure 3). Remarkably, the transformation steps of this short *meta* pathway are not a 'central' route, but occur in the initial stages of transformation. The genes encoding this short *meta* pathway have no particular conserved gene order, although in most cases the gene for the extradiol dioxygenase (e.g., *bphC*) is followed by that for hydratase (*bphE*), aldehyde dehydrogenase (*bphG*) and aldolase (*bphF*). The gene for the hydrolase (*bphD* or its equivalent), can be at a different location. This shows that exten-

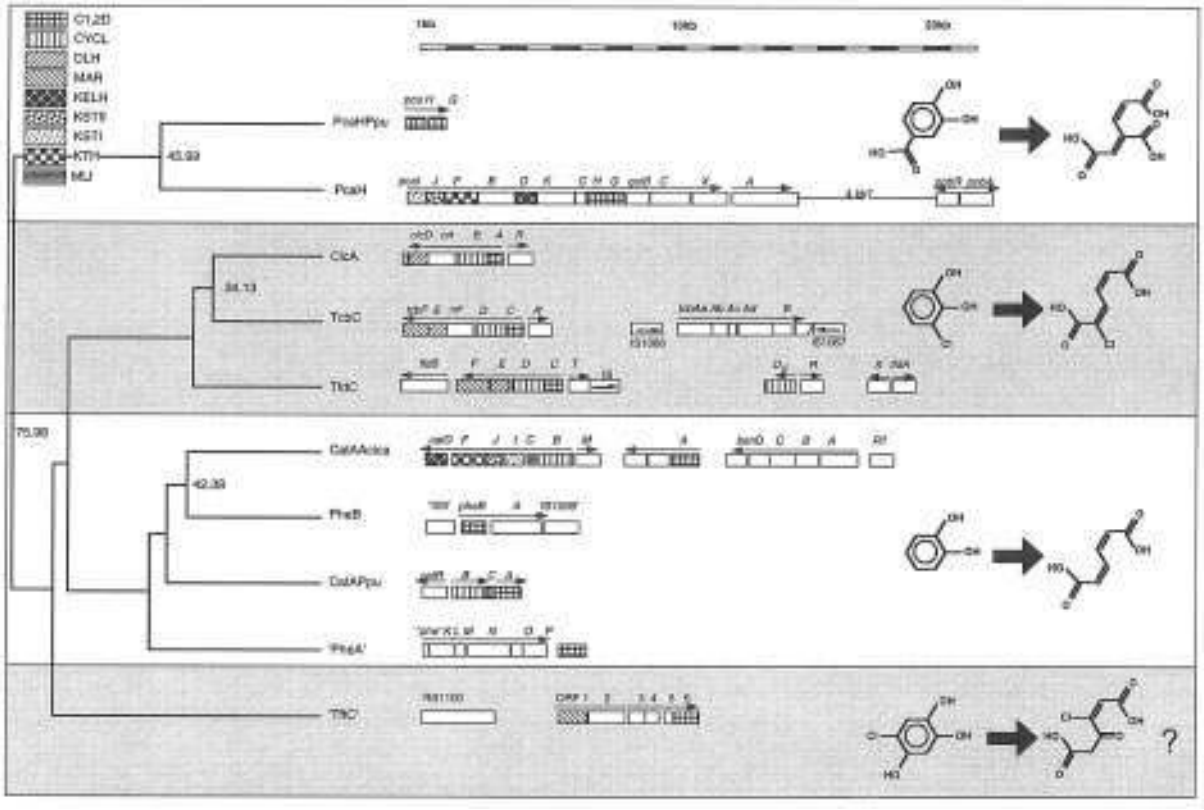

Figure 4. Overview for the genes for *ortho* cleavage pathway enzymes. PILEUP clustering was performed for the predicted primary sequences of the intradiol dioxygenases. Not self-explanatory uncorrected distances: PcaHPpu:ClcA, 75.98. Abbreviations and gene assignment (as far as not explained in Figures 1,2 or 3): C1,2D, intradiol dioxygenase (*ortho* cleavage enzyme); CYCL, (chloro-)muconate cycloisomerase; DLH, dienelactone hydrolase; MAR, maleylacetate reductase; KELH, β-ketoadipate enol lactone hydrolase; KSTII, component II of β-ketoadipate:succinyl CoA transferase; KSTI, component I of β-ketoadipate:succinyl CoA transferase; KTH, β-ketoadipyl CoA thiolase; MLI, muconolactone isomerase. *pcaHG* Encode protocatechuate 3,4-dioxygenase of *P. putida* (Frazee 1993), *pca* genes encode protocatechuate pathway in *A. calcoaceticus* (Hartnett 1990; Hartnett & Ornston 1994; Elsemore & Ornston 1995), *clc* genes encode the chlorocatechol oxidative pathway of *P. putida* (pAC27) (Frantz & Chakrabarty 1986; Coco 1993), *tcb* that of *Pseudomonas* sp. strain P51 (see Figure1) and *tfd* that of *A. eutrophus* JMP134 (see Figure 2), *cat* genes encode catechol metabolism in *A. calcoaceticus* (see Figure 1) and in *P. putida* (Houghton 1995), *pheB* codes for catechol 1,2-dioxygenase in *Pseudomonas* sp. EST1001 (see Figure 2), '*pheA*' for catechol 1,2-dioxygenase in a phenol catabolic pathway of *A. calcoaceticus* (Ehrt 1995), and '*tftC*' for an intradiol dioxygenase involved in 2,4,5-trichlorophenoxy acetic acid metabolism in *B. cepacia* (Daubaras 1995) (see Figure1).

sive rearrangements between the genes for the different *meta* pathways have taken place. If the extended *meta* pathway genes evolved from the shorter one, several genes were acquired. Alternatively, several genes may have become deleted if the shorter *meta*-pathway cluster originated in the longer one. These observations indicate that gene clusters, and consequently, metabolic pathways develop in a modular sense. A third type of *meta* pathway occurs in the naphthalene degradation pathways (e.g., *dox*) (Denome 1993) or *nah* (Harayama & Rekik 1989; Eaton & Chapman 1992), although only the gene for the extradiol dioxygenase (*doxG* or *nahC*) is related to that of the other *meta* pathway genes.

Intradiol dioxygenases

The intradiol dioxygenases catalyze the first reaction in a series of transformation steps which lead to an alternative way of breaking up the aromatic ring of catecholic compounds (Figure 4). The best characterized intradiol cleaving enzymes are protocatechuate 3,4-dioxygenase, catechol 1,2-dioxygenase and chlorocatechol 1,2-dioxygenase. The complete series of trans-

formation reactions are collectively named the *ortho* cleavage pathways. Catechol, methyl- or chlorocatechols, or protocatechuate are processed in these pathways to end with 3-oxoadipate. In contrast to the *meta* cleavage pathway gene clusters, the genes for the *ortho* cleavage pathways are often not located in one transcriptional unit. This is clearly seen in the position of the gene for catechol 1,2-dioxygenase (*catA* or *pheB*) (Figure 4). In *A. calcoaceticus* the *catA* gene is separated from the other structural *cat* genes (*catBCIJFD*) (Neidle 1988). In two strains of *P. putida* the *catA* gene is not part of the transcriptional unit *catBC* and not located in their vicinity (Aldrich 1987; Houghton 1995). Yet in one case the *catA* gene appears directly downstream of *catBC*, although apparently still independently transcribed (Houghton 1995). Likewise as for the genes for extradiol dioxygenase, the genes for intradiol dioxygenase seem to have become incorporated in different clusters. For example, the catechol 1,2-dioxygenase gene *pheB* in *Pseudomonas* sp. strain EST1001 (Kivisaar 1991) is situated in front of a gene for the phenol monooxygenase and is not associated with those for the muconate cycloisomerase or lactonizing enzyme (e.g., *catB* and *catC*). In a phenol degradation pathway in *A. calcoaceticus* the gene for a catechol 1,2-dioxygenase is located downstream from the genes for the multicomponent phenol hydroxylase (Ehrt 1995) (Figure 4). The *cat* or *phe* encoded catechol 1,2-dioxygenases are only moderately related to the protocatechuate 3,4-dioxygenase genes (Figure 4). Protocatechuate 3,4-dioxygenase has two different subunits which have similar three-dimensional structure, encoded by the genes *pcaH* and *pcaG*. The two subunits share 18% identical amino acids which suggests a common ancestry, perhaps through gene duplication (Hartnett 1990). The *pcaHG* genes are clustered in one transcriptional unit with the other genes for the *ortho* cleavage pathway in *A. calcoaceticus* (overview in reference) (Elsemore & Ornston 1995) and with a part of those (*pcaDC* and *pcaB*) in *Agrobacterium tumefaciens* (Parke 1995). All these variations make it questionable to consider the *ortho* cleavage pathway as a central, conserved pathway from a genetic viewpoint.

An intermediate position in the clustering analysis is taken by a putative intradiol dioxygenase (hydroxyquinol 1,2-dioxygenase) encoded by ORF6 of the *tft* pathway (Daubaras 1995). The gene for this intradiol dioxygenase is part of a six gene cluster in *B. cepacia* AC1100. Not all functions encoded by these genes are known, but supposedly only two gene products (that of ORF1 and ORF6) are be needed for cleavage and transformation of the substituted catechol to give chloromaleylacetate (Daubaras 1995). As such, this *ortho* cleavage pathway would be substantially shorter than the others.

A relatively strong conservation in gene order is found for a separate group of intradiol cleaving enzymes, the chlorocatechol 1,2-dioxygenases. The chlorocatechol 1,2-dioxygenases are approximately 40% identical in amino acid sequence to the catechol 1,2-dioxygenases (Neidle 1988; van der Meer 1991a). The genes for chlorocatechol 1,2-dioxygenase form one cluster with the others involved in chlorocatechol breakdown, such as that for chloromuconate cycloisomerase, dienelactone hydrolase and maleylacetate reductase (Kasberg 1995). The pathway for chlorocatechol breakdown has received considerable attention because it was viewed as a typical novel pathway evolved for chloroaromatics degradation (see the historic overview in Schlömann 1994). All of these clusters described so far, e.g., *clcABD* (Frantz & Chakrabarty 1987), *tcbCDEF* (van der Meer 1991c), and *tfdCDEF* (Perkins 1990) are located on plasmid molecules (see below). Remarkably, only the gene for the intradiol dioxygenase and that for chloromuconate cycloisomerase are related to counterparts in the 'normal' catechol pathways. The other two downstream genes (and an ORF of unknown function) do not share homology with any of the *cat*, *phe*, or *pca* genes. It has been speculated that these two were separately 're-cruited' from another origin (Kukor 1989; Schlömann 1990).

Origins of the chlorobenzene pathways

Several bacteria were isolated on the basis of enrichment techniques from polluted environments or in laboratory matings which use chlorobenzenes as sole carbon and energy substrate (Reineke & Knackmuss 1984; Schraa 1986; Kröckel & Focht 1987; Spain & Nishino 1987; Oltmanns 1988; Sander 1991; van der Meer 1991c; Nishino 1992). It seems that for most of them, the range of metabolizable substrates expands from monochlorobenzene up to 1,2,4-trichlorobenzene. One strain was described which can also use 1,2,4,5-tetrachlorobenzene (Sander 1991). The different strains seem to degrade either monochlorobenzene, 1,2-dichloro-, 1,4-dichloro- and 1,2,4-trichlorobenzene (Sander 1991; van der Meer 1991c; Nishino 1992), or 1,4-dichloro- and 1,3-dichlorobenzene (but not 1,2-dichloro- and 1,2,4-

trichloro-) (Schraa 1986; Spain & Nishino 1987). All biochemical evidence indicates that the first transformation steps are performed by a multicomponent dioxygenase which catalyzes the NADH-dependent conversion of chlorobenzenes to *cis*-chlorobenzene dihydrodiols, and a dihydrodiol dehydrogenase which catalyzes the (formal) oxidation of the chlorobenzene dihydrodiol to chlorocatechol with concomittant reduction of NAD^+ (Schraa 1986; Spain & Nishino 1987; Werlen 1996). We have characterized the genes for these two enzymes and some aspects of their biochemistry from a chlorobenzene degrader, *Pseudomonas* sp. strain P51 (Werlen 1996). It turned out that the chlorobenzene dioxygenase is a four-subunit class IIB aromatic ring dioxygenase which is very similar to the toluene or benzene dioxygenase (Figure 1). These enzymes have a relatively broad substrate range and are capable of converting benzene rings with various substituents (not carboxyl-), but also naphthalene (with ring substituents) and biphenyls (Gibson 1990; Werlen 1996). It seems as if this multi-usage was successfully applied by bacteria degrading chlorobenzenes. In strain P51 the dioxygenase genes are part of a composite transposable element, Tn*5280*, and may thus have been moved from another origin by the mobilizing activity of two copies of an IS-element (van der Meer 1991d). We have no real evidence from which organism the 'ancestor' *tcb* dioxygenase genes originated, but it seems not unlikely that it was a microorganism carrying *tod* or *bnz* genes (Werlen 1996) (Figure 1).

The chlorocatechols formed from chlorobenzenes are subsequentely converted to 3-oxoadipate by the activity of (probably) four enzymes: Chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase, dienelactone hydrolase and (chloro-)maleylacetate reductase (van der Meer 1991a; Schlömann 1994; Kasberg 1995). At least two chlorine atoms can be removed during these steps, either spontaneously or mediated by the activity of chloromuconate cycloisomerase (Vollmer 1994) and maleylacetate reductase (Kaschabek & Reineke 1995). In the chlorobenzene pathway of strain P51, these enzymes are encoded by the *tcbCDEF* genes (van der Meer 1991a). As mentioned above, the *tcbCDEF* genes are related to the genes for other chlorocatechol pathways, such as *clcABD* of *P. putida* (pAC27) (Frantz & Chakrabarty 1987) and *tfdCDEF* of *Alcaligenes eutrophus* JMP134 (pJP4) (Perkins 1990). Detailed biochemical work, which was carried out to quite an extent in the group of Schlömann, has indicated that the enzymes of the pathway have a preference for catalyzing chloro- (and methyl-) substituted compounds in comparison with non-chlorinated ones (reviewed in reference) (Schlömann 1994). Does this mean that these enzymes were specifically selected for usage in the metabolism of chlorinated compounds? Schlömann calculated the time period for the divergence of the genes for catechol 1,2-dioxygenase (e.g., *catA*) and those for chlorocatechol 1,2-dioxygenase (e.g., *clcA*) from a common ancestor, considering neutral substitutions only and a particular absolute evolutionary rate of 7×10^{-9} mutations per site and year (Harayama & Rekik 1993; Schlömann 1994). With these propositions the calculated divergence time between *tfdC* and *clcA* was around 140 million years, which is much longer than the time span during which mankind has introduced its chlorinated chemicals. It could be that the divergence time is overestimated when under certain circumstances mutation rates would raise considerably or other divergence mechanisms would lead to multiple subtitutions at a time (see above). On the other hand, there may have been chlorinated chemicals around in nature (Gribble 1992; De Jong 1994), which selected for enzymes specific in degradation of chlorinated compounds a long time ago. The novelty of the chlorobenzene pathway lays in the combination of two existing gene clusters. This was a relatively fast process, expanding the capacities of microorganisms easily, which was selected for upon pollution of the environment with chlorobenzenes.

Studies on another chlorobenzene degrader, tentatively named *Alcaligenes* sp. strain JS705 (J. R. van der Meer, in prep.), have given more insight in where the genes for the chlorobenzene pathway may have originated and if the novel combination was selected for by chlorobenzene pollution. This strain was isolated from contaminated groundwaters in Florida, both by enrichment techniques (Nishino 1992) and by colony hybridization with chlorobenzene pathway genes (J. R. van der Meer, in prep.). Gene cloning, hybridizations and DNA sequencing showed that this strain carries the genes for a chlorobenzene dioxygenase similar to those of strain P51 (Figure 5), and that the chlorocatechol pathway is encoded by a set of genes almost identical to the *clcABD* genes. The presence of *clcABD* genes in this microorganism suggests the remarkable distribution of this gene cluster among various bacterial species across different continents. A small variation in this strain is that another identical copy of the *clcR-A* genes is present. Selection for chlorobenzene degradation apparently led to another type of combina-

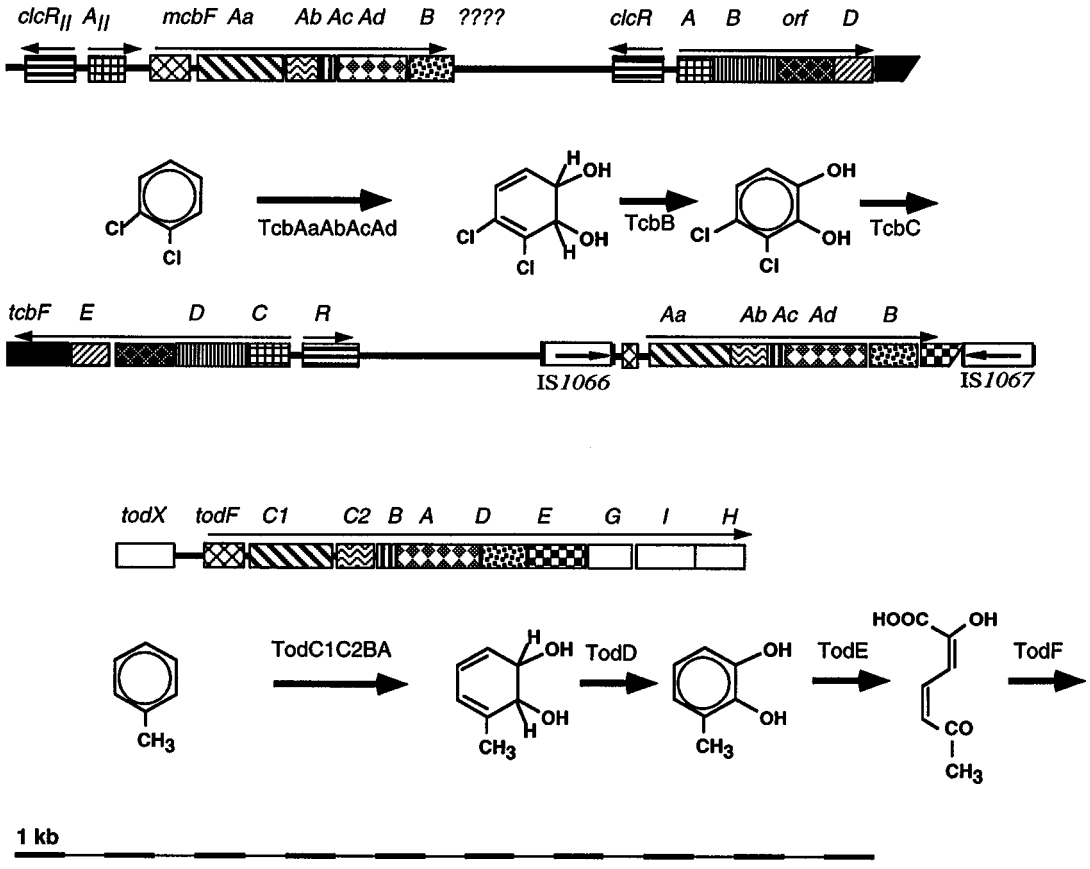

Figure 5. Comparison of the genes for chlorobenzene degradation of *Pseudomonas* sp. strain P51 (*tcb*), *Alcaligenes* sp. strain JS705 (*clc* and *mcb*) and those for toluene degradation in *P. putida* F1 (*tod*). Similar genes are depicted with similar shading or hatching. Question marks in the *mcb* cluster indicate a region which has not been analyzed by DNA sequencing. Parts of degradation pathways are presented. For gene explanations, see figures 1,2,3 or 4.

tion in this strain of genes encoding benzene dioxygenase and dihydrodiol dehydrogenase, and those encoding a chlorocatechol pathway. We have screened the contaminated groundwater and groundwater from the same aquifer which comes from an upstream located well without chlorobenzene pollution, for presence of the genetic material for chlorobenzene degradation of strain JS705. PCR-amplification indicated clearly that fragments from the *clcA* gene are present in the uncontaminated site (Figure 6). However, we have not been able yet to isolate a strain from the uncontaminated groundwater, which contained either one of both gene clusters, solely on the basis of its genotype.

Regulatory concepts for a novel pathway

What does it mean for a cell when a new gene cluster is acquired and inserted on a certain position in its DNA? Are these genes immediately properly expressed, do primitive promoters exist (Janssen 1994) or do new regulatory functions have to develop in the course of time? Many features on the DNA and quite a number of different proteins may determine a proper regulation. Several aromatic degradation pathways appear to be regulated by a class of proteins named the LysR-type transcriptional activators (Figure 1–4), such as NahR (Schell & Sukordhaman 1989), TcbR (van der Meer 1991b), ClcR (Coco 1993), CatR (Parsek 1992), CatM (Romero-Arroyo 1995), TfdR (Matrubutham & Harker 1994), TfdS (You & Ghosal 1995), or PcpR (Orser & Lange 1994). All details of the mechanism of transcription activation are not known for these reg-

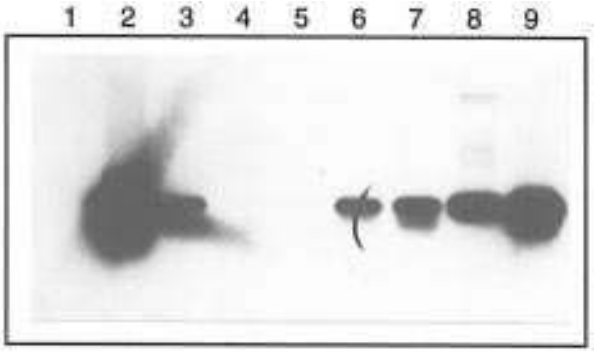

Figure 6. Autoradiogram of Southern hybridizations with DNA fragments obtained by amplification in the polymerase chain reaction of a 425- base pair region of the *clcA* gene. Template DNAs were isolated from chlorobenzene polluted (S1) and unpolluted groundwater (S2). As probe in the hybridizations the *clcA* gene of *Alcaligenes* sp. strain JS705 was used. For further explanation, see text. Lanes: 1, control (no DNA), 2, plasmid containing *clcA* gene, 3, DNA marker, 4, control (no DNA), 5, 6 and 7, DNA prepared from bacteria filtered from 0.5 l of S2 groundwater, diluted 1/100, 1/10 and 1/1, 8 and 9, DNA prepared from S1 filtered groundwater, diluted 1/10 and 1/1.

ulators, but there seems to be no involvement of other proteins than the regulator, a σ^{70} factor and RNA polymerase (Schell 1993). It is thought that upon binding with an effector molecule, the regulator is able to induce a conformational change, which may favour start of RNA synthesis (Wang 1992; Parsek 1995). A tightly controlled regulatory system, which is (almost) not induced when no effectors are present and efficiently induced as soon as an effector is signalled, would be useful. Furthermore, the system would have to be protected from cross- interaction by other DNA-binding proteins which may aspecifically interact with the promoter region (Fernandez 1994; Perez-Martin & de Lorenzo, 1995a). The intricacies of such optimized regulatory systems are indicated by studies on the regulator protein XylR, DmpR and XylS. XylR and XylS are the major transcriptional activators of *xyl* gene expression in *P. putida* mt2 (Inouye 1983; Mermod 1987; Nakazawa 1990; Marques & Ramos 1993). Specific binding sites in the P_u, P_s (for XylR) and P_m (for XylS)-promoter regions ensure proper binding (Marques & Ramos 1993). Expression from P_u and P_s promoters requires in addition to XylR the sigma factor σ^{54}. False binding by other proteins seems to be inhibited by bending the DNA in the promoter region. Hereto, the P_u-promoter region contains an IHF-binding site at which a bend is induced in the DNA by an IHF-like protein (Perez- Martin & de Lorenzo 1995b). The P_s-promoter, however, does not require IHF, although it contains potential IHF-binding sites. The DNA sequence in this promoter region forms a static bend, assisted by protein HU . New systems, arising after the event of for instance DNA rearrangements, may not be so optimized, as we will see below. Here, cross-activation by other existing DNA binding proteins in the cell may become important for regulation.

For the expression of the *pheB* catechol 1,2-dioxygenase gene such a cross-activation was clearly observed. The *pheB* gene is found on plasmid pEST1226 (Kivisaar 1989) in a small gene cluster which contains first an IS-element, and downstream of *pheB* the gene *pheA* for phenol monooxygenase (Figures 2, 4) and another IS- element. Upstream of the first IS-element there appears to be a binding site for the regulatory protein CatR, which is involved in the transcriptional activation of *catBC* in *P. putida* (Parsek 1992). When a plasmid containing the *phe* gene cluster is introduced in *P. putida,* expression of the *pheBA* genes is activated by CatR (Kasak 1993) through binding to this region upstream of the first IS-element (Parsek 1995). Another example of potential cross-activation in a catabolic pathway for aromatic compounds is found on plasmid pJP4 of *A. eutrophus*. Three gene clusters (*tfdA*, *tfdB*, and *tfdCDEF*) are located on this plasmid which encode the enzymes for conversion of 2,4-dichlorophenoxyacetic acid to 3-oxoadipate. Several copies of potential regulatory elements have been found. Two of these, *tfdR* and *tfdS*, are identical (Matrubutham & Harker 1994; You & Ghosal 1995). It is not clear if both of them are transcribed in *A. eutrophus* or if one is preferentially expressed. The TfdR or TfdS regulatory protein is capable of binding to the *tfdA* promoter and to a region upstream of a duplicated *tfdD* gene (Matrubutham & Harker 1994). From DNA sequence analysis and comparison with other clusters for chlorocatechol degradation (Figure 4) it became clear that another putative regulatory gene was located upstream of *tfdC* and oriented in opposite direction. We discovered that this gene, *tfdT*, is actually interrupted by an IS-element, causing a premature ending of the open reading frame (J. H. J. Leveau, in prep.). The shortened gene product is not capable of binding to the *tfdC* promoter region, nor can it activate transcription from this promoter. By replacing *tfdT* for *tfdR*, however, we were able to show that the gene product of *tfdR* can activate the *tfdC* promoter (J. H. J. Leveau, in prep.). The TfdR (TfdS) protein may thus have taken over the role of activating the *tfdC* promoter when the 'original' TfdT protein became inactivated,

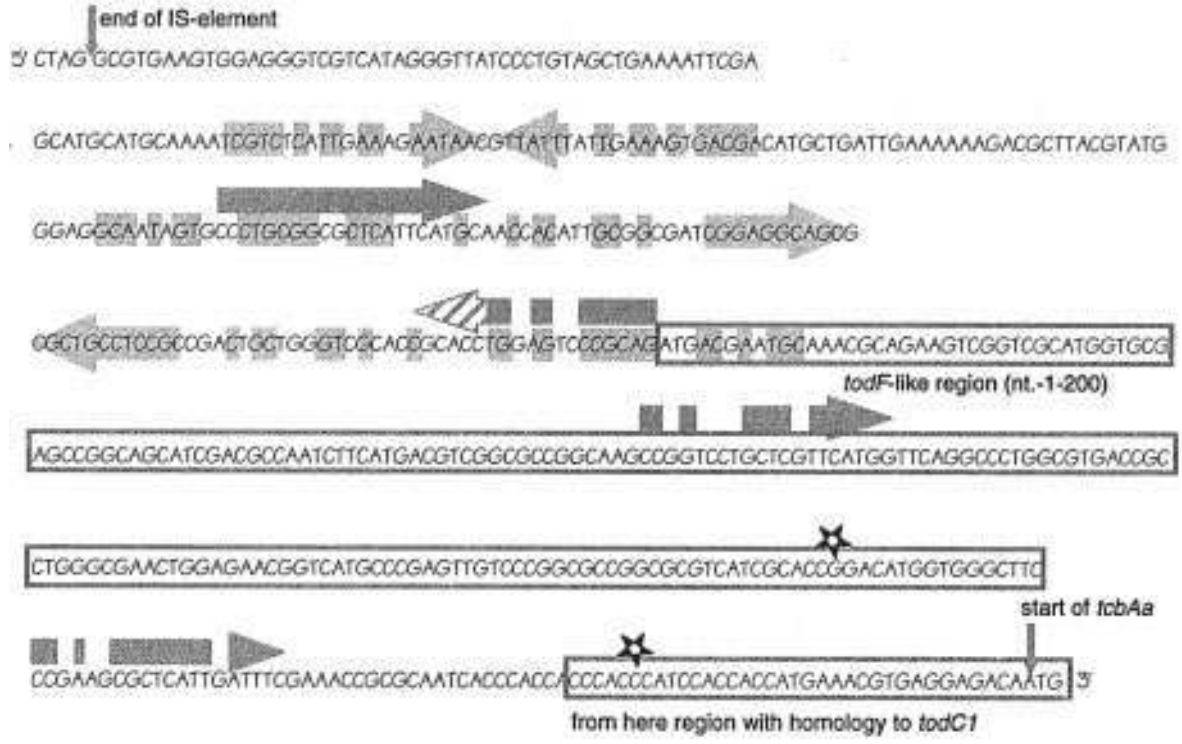

Figure 7. Upstream region of the *tcbAa* gene of *Pseudomonas* sp. strain P51. The picture shows the various features explained in the next: Region homologous to *todF*, the most obvious direct and inverted repeats and the mRNA start sites observed so far (indicated with an asterix).

because it was capable of cross-binding to this DNA region.

We do only partially understand how the *tcb* genes for chlorobenzene degradation are regulated. The *tcbCDEF* cluster is transcribed from a promoter upstream of *tcbC* and transcriptional activation is mediated by the LysR-type regulator TcbR. From induction experiments with the TcbR-*tcbCDEF* system we observed that the system without inducer is not 'tightly' shut off (there is a background chlorocatechol 1,2-dioxygenase activity level of about 5% of the maximum level observed during growth on 1,2,4-trichlorobenzene) (van der Meer 1991c). Maximal induction appears to be around a factor of 20. If we compare this with the factor of 15,000 induction observed for the XylR-Pu system (Perez-Martin & de Lorenzo 1995b), this could mean that the TcbR-*tcbC* system is not evolutionarily optimized. How and if the *tcbAB* genes are regulated, remains unclear at this moment. This gene cluster may have been integrated on the plasmid pP51 nearby the *tcbCDEF* genes by the activity of the IS- elements. The DNA fragment with the five genes of the *tcbAB* loci (Werlen 1996), however, were not transposed without disturbing their surroundings. The upstream region of the *tcbAa* genes is a mixture of different sequences of which we could trace some of the origins. We could identify a region of 200 nucleotides with high identity to *todF*, which is the gene upstream of *todC1* (Menn 1991; Werlen 1996) (Figure 7). Outside this region, we find some direct and inverted repeat structures with no homology to one of the other dioxygenase gene clusters described so far. Did the activity of the IS- elements mix this region, as it occurs in the *todFC1* and *mcbFAa* gene regions (Figure 6), completely, and does a new regulatory system now have to develop? As far as we have analyzed by now, the TcbR protein does not bind at this DNA region. Primer extension mappings have so far led to several mRNA starting points in this region (Figure 7). Oxygen uptake experiments and initial Northern studies seem to indicate that the *tcbAB* genes are transcribed to almost a similar extent when the cells are grown on acetate as when they are grown on chlorobenzenes (C. Werlen, unpubl.). Further analysis will have to show that perhaps an existing DNA binding protein in strain

P51 will bind here and that this can be the beginning of a new regulatory system.

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