Genetic adaptation of bacteria to chlorinated aromatic compounds

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Abstract: Genetic mechanisms in bacteria provide a continuous source of alterations in DNA sequences that may lead to favourable adaptations. Bacteria that use chlorinated aromatics as sole carbon and energy sources show evidence of these different genetic alterations. The distinct effects of single base-pair mutations on adaptation of bacterial strains (e.g. by changing the substrate specificity of a key metabolic enzyme or regulator protein) have been demonstrated in various studies. In addition to these small sequence modifications, intermolecular or intercellular gene exchange mechanisms can result in new strains with altered metabolic capabilities. The details of these evolutionary processes with respect to the metabolism of chlorobenzenes and chlorocatechols are reviewed in this manuscript.

Key words: Adaptation; Pseudomonads; Chlorinated aromatic compounds; Evolution

Introduction

Over the past few decades, the large scale industrial production and extensive use of synthetic chemicals in all levels of society have led to a wide distribution of harmful compounds in the environment. In almost any compartment of our environment (e.g. soil, air, water, and biota), traces of synthetic organic compounds can be detected. At many waste disposal sites or other point sources of contamination, concentrations of these chemicals may be so great that direct toxicological risks are encountered.

Microorganisms play a central role in the degradation of organic compounds and therefore are very important for the mineralization or detoxification of toxic organic chemicals. Unfortunately, not all organic compounds are equally well biodegradable, and, in some cases, environmental conditions may be unfavourable for microbial activity. If the molecular structure of a compound is not commonly encountered in nature, microorganisms may not have the necessary degradative enzyme systems. The presence of substituents, such as halogen atoms, nitro- or sulfate groups, on otherwise easily degradable compounds may make the compound less accessible for biodegradation and thus more persistent in the environment [1,2].

It has been shown that microbial populations may adapt to use previously persistent compounds as novel carbon and energy sources [3–5]. Such an adaptation may be caused by the selection of mutant strains which have acquired novel metabolic activities or altered enzymatic specificities. Here I discuss some of the genetic mecha-
nisms which may have resulted in the formation of the metabolic pathway of chlorinated benzene degradation in pseudomonads.

Genetic mechanisms for adaptation

A wide variety of processes exist that cause changes in existing genetic material and result in altered metabolic functions. The details of the different genetic mechanisms in adaptation to xenobiotic compounds have recently been reviewed elsewhere [6,7]. When considering the different genetic mechanisms involved in evolution and alterations of DNA sequences, a distinction should be made between vertical and horizontal evolutionary processes [8]. Vertical processes are those which lead to a divergence in DNA sequence in daughter cells, due to the accumulation of mutations. These mutations can be single base-pair changes or those leading to larger sequence changes, e.g. deletions and duplications. The effects of point mutations on enzyme specificity or on effector recognition by regulator proteins have been well established [9–12], and it has become clear that these processes can have direct consequences for the adaptation of strains to xenobiotic compounds. Other possible processes of sequence divergence, e.g. slipped strand mispairing, can lead to more extensive sequence changes and may be a general driving force for the divergence of gene sequences [13–15]. The effect of such a process in microorganisms, however, has not yet been well investigated.

Horizontal processes are those which cause an exchange of DNA sequences between the genome of two different organisms (intercellular movement), or between different DNA molecules, e.g. within the chromosome and extrachromosomal elements inside one organism (intermolecular movement) [8]. Horizontal movement of genetic information may be caused by recombination or other mechanisms of gene exchange, e.g. conjugation, transduction, or transformation. It has become very clear that horizontal gene exchange plays an important role in the adaptation of microorganisms to xenobiotic compounds [6]. The list of bacteria with self-transmissible plasmids encoding the degradation of aromatic or toxic organic compounds is extensive [16]. Although many of these plasmids have a similar backbone structure encoding probably replication and transfer functions, they carry different catabolic genes [17]. This strongly suggests the existence of processes by which plasmids can acquire genes or gene clusters and subsequently disseminate them in a microbial population [18,19]. Such a recent example of horizontal gene exchange has been the discovery of transposable elements of different families containing catabolic genes. The xyl and nah genes are located on Tn3-type transposons, Tn4561 (56 kb) [20] and Tn4655 (55 kb) [21], respectively. Other elements which have been described include Tn5271, encoding 4-chlorobenzoate metabolism [22], Tn4371, for 4-chlorobiphenyl metabolism [23], IS931, associated with the 2,4,5-trichlorophenoxyacetic acid genes [24], the DEH element, which carries the dehalogenase genes of Pseudomonas putida PP3 [25], and Tn5280 of the chlorobenzene plasmid pP51 [26] (see below). Some of these elements reside on the chromosome (such as IS931, Tn4371, and DEH), whereas the others were originally isolated from plasmids.

Evolution of the metabolic pathway for chlorobenzene degradation

Since very few bacteria had been described which were able to degrade chlorobenzenes completely, we considered these compounds suitable substrates for evolutionary studies of bacterial adaptation. Characterization of Pseudomonas sp. strain P51, capable of degrading 1,2,4-trichlorobenzene, 1,2-dichloro- and 1,4-dichlorobenzene, along with strains isolated by others [5,27–29], led to a reasonably good picture of the metabolic pathway for chlorobenzene degradation and of the different genes which are involved in this pathway [26,29–31]. The first enzymes of the pathway, a three-component aromatic ring dioxygenase and a benzene glycol dehydrogenase, catalyze the incorporation of a dioxygen molecule into the aromatic ring and the subsequent dehydrogenation which gives rise to a chlorinated
catechol. The chlorinated catechol is then cleaved in a second dioxygenation reaction, catalyzed by chlorocatechol 1,2-dioxygenase, and further degraded, via a number of different chlorinated intermediates, probably to (chloro-)3-oxoadipate. In strain P51 the different genes encoding these metabolic steps were found on a 110-kb plasmid [29]. Three different transcriptional units were found: (i) the ‘upper pathway’ cluster, containing the genes encoding the chlorobenzene dioxygenase and the chlorobenzene glycol dehydrogenase; (ii) the ‘chlorocatechol oxidative’ cluster, encoding the genes for the conversion of chlorocatechols (see below); and (iii) the regulatory gene tcbR, which encodes a transcriptional activator for the chlorocatechol oxidative operon.

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Fig. 1. Schematic representation of metabolic channeling in the aerobic degradation of aromatic compounds by bacteria. (A) A number of different aromatic compounds and the positions at which initial enzymatic attack can take place (indicated by numbered arrows). Dotted arrows between different structures indicate that this particular compound can occur as intermediate in the degradation of the previous one. Numbers: 1, biphenyl dioxygenase [82]; 2, dibenzofuran dioxygenase [83]; 3, naphthalenesulfonic acid dioxygenase [84]; 4, naphthalene dioxygenase [85,86]; 5, dibenzo-p-dioxin dioxygenase [83]; 6, ‘2,4-dichlorophenoxyacetate monooxygenase’ [69]; 2,4-D/α-ketoglutarate dioxygenase [49]; 7, γ-HCH dehydrochlorinase [87]; 8, 1,2-dihydroxynaphthalene dioxygenase [33,84]; 9, 2,2',3-trihydroxybiphenyl dioxygenase [56]; 10, pentachlorophenol 4-monoxygenase [88,89]; 11, 2,3-dihydroxybiphenyl dioxygenase [90]; 12, benzoate 1,2-dioxygenase [44]; 13, 4-chlorobenzoate dehalogenase [58]; 14, phenol hydroxylase [50]; 15, xylene monoxygenase [91]; 16, toluene dioxygenase [32]; 17, toluene 4-monoxygenase [51]; 18, dinitrotoluene dioxygenase [92]; 19, salicylate hydroxylase [47]; 20, benzene sulfonate dioxygenase [93]; 21, chlorobenzene dioxygenase [29]; 22, 2,4-dichlorophenyl hydroxylase [38]. Different shadings indicate enzyme families with sequence homologies or with comparable activities: (black), extradiol dioxygenases; (stippled), aromatic ring dioxygenase; (grey dotted), monoxygenases and hydroxylases; (striped), dehalogenase activities. (B) The different ortho dihydroxylated central intermediates. Ring cleavage of these intermediates takes place as indicated by the arrows. Open arrows represent intradiol dioxygenases, filled arrows the extradiol dioxygenases. Numbers: 1, catechol 2,3-dioxygenase; 2, catechol 1,2-dioxygenase; 3, protocatechuate 4,5-dioxygenase; 4, protocatechuate 3,4-dioxygenase; 5, chlorocatechol 1,2-dioxygenase. Below these are shown the ring cleavage products of the intradiol- (ortho-) and extradiol- (meta) cleavage reactions. No complete overview of all possible reactions is intended.
The upper pathway gene cluster in strain P51 is flanked by two iso-insertion elements, ISI060 and IS1007. This complete element, Tn5280, was shown to be a functional transposon, able to insert in single copy and at random into the genome [26].

As a result of these analyses, it appears that the metabolic pathway for chlorobenzene degradation in strain P51 developed from two different genetic elements. The transposable element containing the genes for the aromatic ring dioxygenase and the benzene glycol dehydrogenase, i.e. Tn5280, may have originated in bacteria degrading toluene by direct dioxygenation, such as P. putida F1 [32]. The dioxygenase in this strain was shown to have a broad substrate range, and could oxidize chlorinated benzenes to the corresponding dihydrodiols [32]. If such a dioxygenase gene cluster became captured by two copies of an insertion element, i.e. ISI060 and IS1007, successful transfer of the dioxygenase transposon to a catabolic plasmid containing a chlorocatechol oxidative operon, could provide the resulting transconjugant strain with the necessary genetic information to carry out complete chlorobenzene degradation. Strain P51, then, provides a nice example of different evolutionary mechanisms which may be required for the generation of a new catabolic pathway, i.e. (i) recombination events involving horizontal gene transfer, and (ii) vertical evolution of specialized enzyme systems for new (chlorinated) substrates (see below).

Convergence and variations in metabolic pathways for (chloro-) aromatic compounds

It has been established that metabolic pathways of aromatic compounds in bacteria generally follow similar strategies and involve a limited number of central steps. Pathways for aerobic degradation of aromatics apparently converge to form a relatively small number of intermediates (Fig. 1). These intermediates carry at least two hydroxyl groups (in ortho or para positions) and can contain other substituent groups. They are then cleaved either by intradiol dioxygenase enzymes (ortho cleavage) or by extradiol dioxygenases (meta cleavage). The intradiol and extradiol dioxygenases appear to have no significant similarities on the amino acid level, and therefore are not evolutionary closely related [33]. Within the extradiol dioxygenases and intradiol dioxygenases, different enzyme groups also exist with relatively little sequence similarity. The archetype extradiol dioxygenase, catechol 2,3-dioxygenase (encoded by the xylE, nahH or dmpB genes) (Fig. 1B), does not show significant sequence similarity with the extradiol enzyme protocatechuate 4,5-dioxygenase [34]. Of the intradiol dioxygenases, three subgroups have been described: catechol 1,2-dioxygenase (encoded by catA of Acinetobacter calcoaceticus or P. putida) [14], protocatechuate 3,4-dioxygenase (encoded by the pcaHG genes of e.g. P. putida, P. cepacia, A. calcoaceticus) [35–37], and chlorocatechol 1,2-dioxygenase (encoded by tcbC of Pseudomonas sp. strain P51 [30], tdcC of Alcaligenes eutrophus [38,39], or clcA of P. putida pAC27 [40], and described for Pseudomonas sp. B13 [41]).

Among the enzymes which catalyze the initial steps in aromatic degradation pathways, different classes are found with extensive homology to each other and to other enzymes of the central pathways (Fig. 1), suggesting similar evolutionary strategies. The most important class of these enzymes is probably that of the aromatic ring dioxygenases, which catalyze insertion into the aromatic ring of two hydroxyl groups derived from molecular oxygen and cofactors such as NADH (recently reviewed in references [42,43]). It currently appears that all of these dioxygenases are multi-component enzymes with three or four different protein subunits. These proteins comprise a short electron transfer chain, by which electrons are transferred from NADH, via a reductase and a ferredoxin, to the terminal oxidase. The reductase and the ferredoxin may be combined as two domains of one protein molecule, as is the case for the toluate and benzoate dioxygenases [13,44]. In some cases, the different protein subunits of the aromatic ring dioxygenases share significant amino acid sequence homology, e.g. the two subunits which make up the terminal oxidase of the toluate dioxygenase and those of the naphthalene, toluene or biphenyl dioxygenase [44,45].
Many other aromatic ring dioxygenases have not yet been characterized on the DNA sequence level, and comparison can only be made on the basis of biochemical information [43].

Another class of enzyme activities catalyzing initial steps in aromatic metabolism are the monooxygenases or hydroxylases. These catalyze the incorporation of a single hydroxyl group on the aromatic ring or oxidize alkyl side chains. Several different enzyme groups are found which are not significantly related on amino acid sequence level. Single component aromatic ring flavoprotein hydroxylases are exemplified by p-hydroxybenzoate hydroxylase, with a monomer size of 45 kDa [46]. An overall amino acid sequence identity of 25% was found between p-hydroxybenzoate hydroxylase and salicylate hydroxylase (encoded by the NAH plasmid gene nahG) [47], whereas 2,4-dichlorophenol hydroxylase (encoded by the gene tfdB of plasmid pJP4) [38] and phenol hydroxylase (encoded by pheA) [48] are substantially larger, and only share significant sequence similarity in two regions, one of which may be involved in FAD binding. The 2,4-dichlorophenoxyacetic acid monooxygenase (encoded by tfdA on plasmid pJP4) is not related to these single component monooxygenases [49].

Multicomponent aromatic ring monooxygenases are also found, e.g. phenol hydroxylase from Pseudomonas CF600 (encoded by the dmp-KLMNOP genes) [50], and toluene 4-monooxygenase from P. mendocina KR1 (encoded by the tmoABCDE genes) [51]. These two enzyme complexes have three protein subunits in common [51]. Furthermore, the TmoC ferredoxin and TodB ferredoxin of the aromatic ring dioxygenases share 32% amino acid sequence identity and therefore may be of similar evolutionary origin [51]. Another class of monooxygenase activities is found in enzymes which oxidize alkyl side groups on aromatic rings, such as xylene monooxygenase [52] and toluene sulfonate methyl monooxygenase [53]. These two enzymes have biochemically distinct properties, however, and do not appear strongly related [53].

Several other enzymatic steps are required to convert aromatic substrates to the hydroxylated intermediates. Very interesting from an evolutionary point of view are the extradiol cleavage enzymes, 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) [54] and 1,2-dihydroxynaphthalene dioxygenase (NahC) [33], which share significant overall amino acid sequence similarity with the catechol 2,3-dioxygenases XylE, NahH [33] and DmpB [55]. Other extradiol cleavage enzymes such as 2,2',3-trihydroxybiphenyl dioxygenase [56] may also belong to this large protein family. Dehydrogenases catalyze the reduction of the dihydrodiol compounds formed by the activity of the aromatic ring dioxygenases to form catechols. These dehydrogenases were shown to be related to one another and to belong to the family of short-chain alcohol dehydrogenases [57]. Unique enzyme activities found in the first steps of aromatic metabolism may have been recruited into these pathways from yet unknown evolutionary origin, e.g. dehalogenating enzymes (4-chlorobenzoate dehalogenase of Pseudomonas sp. CBS3 [58] or dehydrochlorinase of P. paucimobilis UT6 [59]).

In conclusion, several classes of enzymes catalyzing the early stages of transformation of aromatic compounds are found (Fig. 1). Enzymes within these classes, such as the multi-component dioxygenases, share significant sequence similarities with one another. Some of them, e.g. the extradiol dioxygenases appear to form evolutionary related protein families with enzymes from 'deeper' metabolic branches, such as catechol 2,3-dioxygenase. This evolutionary relatedness and the genetic organization of catabolic gene clusters [6] suggest genetic processes by which DNA fragments containing several genes (sometimes referred to as 'gene modules' or 'gene cassettes') or gene fragments are combined to form new metabolic pathways or protein activities. An example of the existence of such gene modules may be a DNA fragment containing the genes for the aromatic ring dioxygenase and the benzene glycol dehydrogenase (Fig. 2). These genes can be found at totally different positions in the genomes of different bacteria [6]. Other examples of some putative gene modules within aromatic degradation pathways, are the meta
cleavage pathway genes (in stricter sense) and the genes for a modified ortho cleavage pathway. The meta cleavage pathway genes have been found in almost identical genetic organization as part of the operons for salicylate degradation (nah genes), toluate and metatoluate degradation (xyl genes), phenol degradation (dmp genes), and toluene degradation (tod genes) [6]. The reactions catalyzed by these gene modules are shown in Fig. 2.

**Evolution of the chlorocatechol oxidative pathway**

An important central pathway for the aromatic degradation of chlorinated compounds in bacteria is the chlorocatechol oxidative pathway or modified ortho cleavage pathway [2,41,60]. Chlorinated catechols are converted by a specific set of enzymes to finally 3-oxoadipate [61,62], which may carry one chlorine atom, depending on the amount of chlorine substituents on the catechol. Chlorocatechol 1,2-dioxygenase, the first enzyme of the pathway, is an enzyme that is approximately 40% identical to the normal catechol 1,2-dioxygenase, but that has a much wider substrate range with respect to conversion of chlorocatechols [30,63]. Chloromuconate cycloisomerase, the next enzyme in the pathway, can have very different substrate specificities depending on the strain from which it was isolated [64]. Chloromuconate cycloisomerases have a high sequence similarity to the muconate cycloisomerase (approximately 40% overall amino acid sequence identity) [30]. Some chloromuconate cycloisomerases are thought to have an active dechlorination mechanism, as opposed to the spontaneous dechlorination in the conversion of 3-chloromuconate by muconate cycloisomerase. The chlorodienelactones which are formed by the activity of chloromuconate cycloisomerase are then further transformed by dienelactone hydrolase and by maleylacetate reductase. The latter enzyme may also have a dechlorinating activity [62].

The genetic organization of the chlorocatechol oxidative pathway differs substantially from both that of the normal ortho cleavage pathway, such as that characterized from *Acinetobacter calcoaceticus*, and that of the protocatechuate pathway [14,35,36,44]. In the normal ortho cleavage pathway, *catA* encoding catechol 1,2-dioxygenase, is separated from the other genes of the pathway (Fig. 3A). The only genes in the protocatechuate pathway with significant similarity to those of the modified ortho pathway genes, are the *pcaHG* genes encoding the protocatechuate 3,4-dioxygenase (Fig. 3B). However, these show even less conservation in their localization within the pathway gene clusters (Fig. 3B). In the chlorocatechol oxidative pathway, the gene encoding the chlorocatechol 1,2-dioxygenase is directly coupled to the chloromuconate cycloisomerase gene [30]. Interestingly, only these two genes are common to the ortho and modified ortho pathways. After the stage of the cycloisomerase, the pathways diverge. In the operons for the chlorocatechol oxidative pathway, we find evidence for DNA rearrangements after the chloromuconate cycloisomerase gene [30]. Two of the three different characterized operons have an extra DNA fragment between the genes encoding chloromuconate cycloisomerase and dienelactone hydrolyase, whereas the other has only remnants of this.

![Fig. 2. Metabolic reactions catalyzed by enzymes encoded on three putative gene modules. Open arrows between different intermediates indicate that more than one enzymatic step is required, closed arrows depict single enzymatic steps. Black arrows indicate the cleavage site for the catechol dioxygenase.](https://academic.oup.com/femsre/article-abstract/15/2-3/239/614288)
DNA fragment. It could be that the first part of the operon, i.e. the chlorocatechol 1,2-dioxygenase and chloromuconate cycloisomerase genes, became fused with a different set of genes from another origin, since the latter genes have no apparent sequence homology with genes from the normal \textit{ortho} cleavage pathway.

A special case is required for the different regulatory genes which are involved in the regulation of the (chloro)catechol oxidative pathways. Regulatory genes for the \textit{ortho} cleavage pathways include \textit{catM} (\textit{A. calcoaceticus}) \cite{65}, \textit{catR} (\textit{P. putida}) \cite{66,67}, \textit{tcbR} (\textit{Pseudomonas} sp. strain P51) \cite{31}, \textit{clcR} (\textit{P. putida} pAC27) \cite{68}, and \textit{tfdS} (\textit{Alcaligenes eutrophus} JMP134) \cite{69,70}. All encode proteins which belong to the group of LysR transcriptional activators and are located at a similar position with respect to the rest of the pathway genes which they regulate (Fig. 3), suggesting that they form an ancient type of regulation. Although CatR and ClcR respond to the inducer muconate, it is not known to what extent the various regulators differ in their inducer specificity or in recognition of DNA-binding sites at the operator. It will be interesting to further analyze changes that may have occurred in the different modified \textit{ortho} cleavage pathways before they obtained their final form and to determine the possible ‘original’ substrates for this pathway in bacteria.

\textbf{Concluding remarks}

Comparative studies in bacteria have revealed evidence of evolutionary processes that created or modified different metabolic pathways, e.g.
those for degradation of xenobiotic compounds. It has become clear that some specialized functions, e.g. enzymes for conversion of chlorinated aromatics, probably evolved prior to the introduction of xenobiotics into the environment. On the other hand, new evolutionary events such as horizontal gene transfer processes or point-mutations are still taking place and can have an important impact on the adaptability of strains. The question of whether the occurrence of large quantities of synthetic, toxic compounds has led to a rapid evolution of new bacterial genotypes, however, is still open. Several studies have recently indicated that mutations would be possible in bacteria which are ‘environmentally induced’ [71–76], although the issue is subject to debate [77,78]. It will be very interesting to find more evidence of regulatory circuits in bacteria that, sensing changing environments [79] or the presence of toxic compounds [80,81], switch on mechanisms leading to favorable genetic alterations.

Acknowledgement

I would like to thank Flynn Picardal for critical review of the manuscript.

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