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Evolution of chlorocatechol catabolic pathways

Conclusions to be drawn from comparisons of lactone hydrolases

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Abstract

The aerobic bacterial degradation of chloroaromatic compounds often involves chlorosubstituted catechols as central intermediates. They are converted to 3-oxoadipate in a series of reactions similar to that for catechol catabolism and therefore designated as modified *ortho*-cleavage pathway. Among the enzymes of this catabolic route, the chlorocatechol 1,2-dioxygenases are known to have a relaxed substrate specificity. In contrast, several chloromuconate cycloisomerases are more specific, and the dienelactone hydrolases of chlorocatechol catabolic pathways do not even convert the corresponding intermediate of catechol degradation, 3-oxoadipate enol-lactone. While the sequences of chlorocatechol 1,2-dioxygenases and chloromuconate cycloisomerases are very similar to those of catechol 1,2-dioxygenases and muconate cycloisomerases, respectively, the relationship between dienelactone hydrolases and 3-oxoadipate enol-lactone hydrolases is more distant. They seem to share an α/β hydrolase fold, but the sequences comprising the fold are quite dissimilar. Therefore, for chlorocatechol catabolism, dienelactone hydrolases might have been recruited from some other, preexisting pathway. Their relationship to dienelactone (hydrolases identified in 4-fluorobenzoate utilizing strains of *Alcaligenes* and *Burkholderia (Pseudomonas) cepacia* is investigated). Sequence evidence suggests that the chlorocatechol catabolic operons of the plasmids pJP4, pAC27, and pP51 have been derived from a common precursor. The latter seems to have evolved for the purpose of halocatechol catabolism, and may be considerably older than the chemical industry.

Introduction

As a prerequisite for ring cleavage, aerobic bacterial degradation of non-halogenated aromatic compounds often demands an initial transformation to dihydroxybenzene derivatives such as catechol, protocatechuate, homoprotocatechuate, gentisate, homogentisate or hydroquinone (Dagley 1978, 1986; Chapman 1979). In a few cases, the introduction of a third hydroxyl group prior to ring opening is necessary, giving rise, for example, to 1,2,4-trihydroxybenzene (Bayly & Barbour 1984). The peripheral pathways bring about a convergence of many different aromatic compounds to a limited number of ring cleavage substrates. This metabolic funnel is especially impressive in the case of catechol, which has long been known to be a metabolite in the catabolic pathways of benzoate, phenol, anthranilate, tryptophan, benzene, naphthalene, salicylate, toluene, mandelate and others (Stanier & Ornston 1973). Like protocatechuate, catechol can be further converted to intermediates of the tricarboxylic acid cycle either following *meta*-cleavage adjacent to the hydroxyl groups or following *ortho*-cleavage between them.

The aerobic mineralization of chloroaromatic compounds appears to be governed by similar principles. However, an additional aspect to be considered is the way in which dehalogenation is achieved. Thus, some substrates are dehalogenated prior to ring cleavage and are then converted via one of the usual central intermediates. Well established variations on this theme are the hydrolytic dechlorination of 4-chlorobenzoate

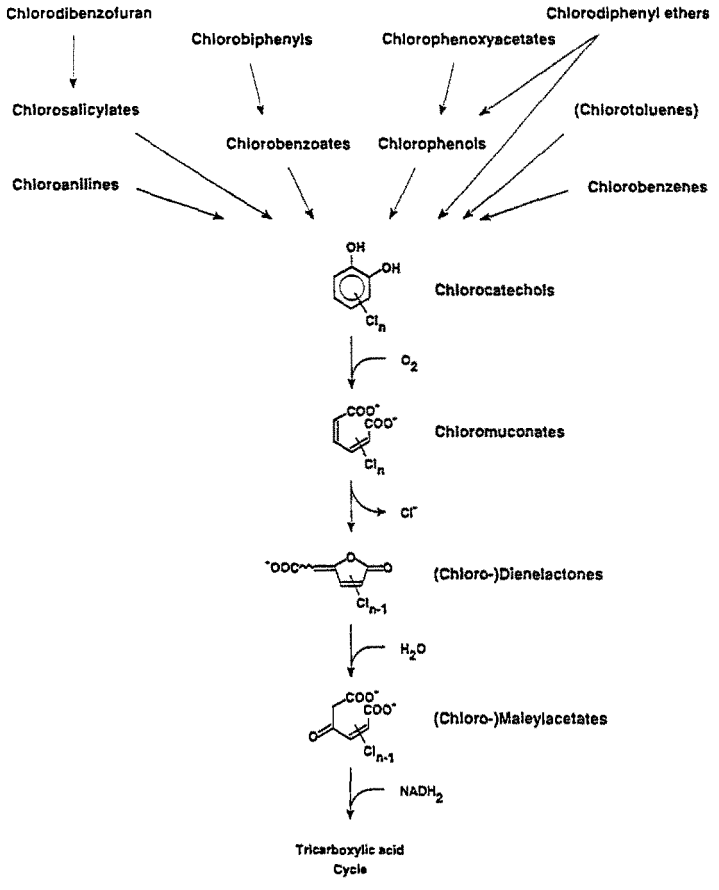

Fig. 1. Convergence of degradative pathways at chlorosubstituted catechols. For references, see text.

(after activation by CoA) and its further conversion via protocatechuate, the oxygenolytic dehalogenation of 4-chlorophenylacetate yielding homoprotocatechuate, the dehalogenation of 2-chlorobenzoate by dioxygenases giving rise to catechol as the ring cleavage substrate and the oxidative, as well as reductive dechlorination steps in pentachlorophenol degradation via the intermediate 1,2,4-trihydroxybenzene (see recent reviews, for example, by: Engesser & Fischer 1991; Häggblom 1992). For many other chloroaromatic compounds, the degradative pathways converge at chlorosubstituted catechols as central intermediates (Fig. 1). In such cases, dehalogenation takes place after ring cleavage has been accomplished. Corresponding catabolic routes were originally described for chlorosubstituted phenoxyacetates (Bollag et al. 1968; Evans et al. 1971a,b; Gaunt & Evans 1971a), for chlorobenzoates (Dorn et al. 1974; Hartmann et al. 1979, 1989; Miguez et al. 1990), and for chlorophenols (Knackmuss & Hellwig 1978; Gorlatov et al. 1989; Pieper et al. 1989). More recently, degradation via chlorocatechols has also been reported for different chlorobenzenes (Reineke & Knackmuss 1984; De Bont et al. 1986; Schraa et al. 1986; Van der Meer et al. 1987; Haigler et al. 1988; Sander et al. 1991), chloroanilines (Latorre et al. 1984; Zeyer et al. 1985; Surovtseva et al. 1986), chlorosalicylates (Rubio et al. 1986; Schindowski et al. 1991), chlorotoluenes (Haigler & Spain 1989; Rey et al. 1992), chlorobiphenyls (Mokross et al. 1990; Havel & Reineke 1991), chlorodiphenyl ethers (Schmidt et al. 1992, 1993), and chlorodibenzofuran (Wittich 1992).

The reactions yielding chlorocatechols from the respective chloroaromatic precursors can, at least in some cases, be catalyzed by ordinary peripheral enzymes which may be unspecific enough to convert chlorosubstituted compounds in addition to their normal non-chlorinated substrates (Knackmuss & Hell-

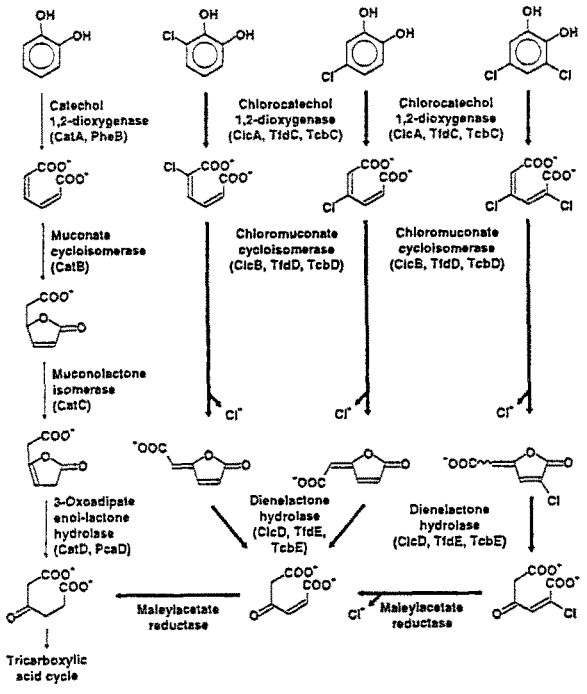

Fig. 2. Parallel pathways for the degradation of catechol (light arrows) and some chlorocatechols (heavy arrows). Enzyme names are given, as are their designations as gene products.

wig 1978; Reineke & Knackmuss 1978a,b; Alexander 1979; Spain & Gibson 1988). In non-adapted strains such a transformation may result in the accumulation of chlorocatechols followed by their oxidation and polymerization (Horvath 1972; DiGeronimo et al. 1979; Haller & Finn 1979; Knackmuss 1983; Fava et al. 1993). In other cases, 2-chloro-*cis,cis*-muconate or *cis*-dienelactone (*cis*-4-carboxymethylenebut-2-en-4-olide) were observed as products of incomplete degradation of chloroaromatic compounds (Janke et al. 1989). Thus, the enzymes of catechol catabolic pathways seem, in general, to be unable to catalyze an efficient conversion of chlorocatechols or their metabolites. This can be due to steric or electron-withdrawing effects of the chlorine-substituent or to differences in metabolite structure resulting from chloride elimination.

Those bacteria which utilize chloroaromatic compounds as sole source of carbon and energy, and which mineralize them via chlorocatechols as intermediates, have developed mechanisms to overcome these problems. They thus provide a basis for the investigation of the biochemical and genetic nature of this adaptation.

Altered, but not necessarily low substrate specificity

Chlorocatechols are generally degraded via the so-called modified *ortho*-cleavage pathway. The first reactions of this catabolic route are analogous to those of the catechol branch of the 3-oxoadipate pathway (Fig. 2). *ortho*-Cleavage of catechol or chlorocatechols yields *cis,cis*-muconate or its chlorosubstituted derivatives, respectively. *cis,cis*-Muconate is cycloisomerized to muconolactone, an intramolecular ester which is relatively stable. It therefore has to be isomerized to 3-oxoadipate enol-lactone before hydrolysis of the lactone ring yields 3-oxoadipate (Stanier & Ornston 1973). Chloro-*cis,cis*-muconates are also subject to cycloisomerization, but this reaction can be coupled to chloride elimination, thus giving rise to dienelactones (4-carboxymethylenebut-2-en-4-olides) which, depending on their respective precursors, might still carry chlorine or other substituents (Tiedje et al. 1969; Evans et al. 1971a,b; Gaunt & Evans 1971a; Schmidt & Knackmuss 1980). The dienelactones have an additional double bond as compared to muconolactone, endowing them with an enol-lactone structure. They can therefore be hydrolyzed without prior isomerization. Maleylacetate and 2-chloromaleylacetate, the products of dienelactone and 2-chlorodienelactone hydrolysis, are reduced to 3-oxoadipate (Duxbury et al. 1970; Chapman 1979; Kaschabek & Reineke 1992; Vollmer et al. 1993) and thus can enter the lower part of the 3-oxoadipate pathway which by CoA-transfer and thiolytic cleavage results in the formation of succinate and acetyl CoA. Unlike the initial reactions of chlorocatechol degradation, the reduction of maleylacetate has no equivalent in catechol catabolism.

Bacteria degrading chloroaromatic compounds via *ortho*-cleavage of chlorocatechols seem, in general, to have two sets of enzymes; one set for catechol and a separate set for chlorocatechol catabolism. This was originally observed in the 3-chlorobenzoate utilizing strain *Pseudomonas* sp. B13 which, during growth with this substrate, induces a chlorocatechol 1,2-dioxygenase (EC 1.13.11.–) that differs from the usual catechol 1,2-dioxygenase (EC 1.13.11.1) in having higher affinities and activities with substituted catechols (Dorn & Knackmuss 1978a,b). By investigating dioxygenase activities with several substituted catechols, usually during growth on various aromatic compounds and their chlorosubstituted analogues, enzymes differing in specificity have meanwhile been shown to be inducible in quite a number of other strains (Kilpi

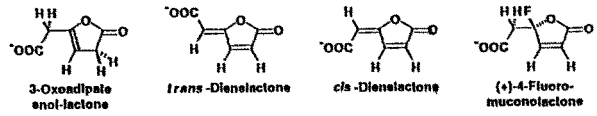

Fig. 3. Substrates for dienelactone hydrolases or 3-oxoadipate enol-lactone hydrolases: comparison of structures.

et al. 1983; Zeyer et al. 1985; Spain & Nishino 1987; Pieper et al. 1988; Gorlatov et al. 1989; Hickey & Focht 1990; Loidl et al. 1990; Miguez et al. 1990; Sander et al. 1991; Schindowski et al. 1991; Van der Meer et al. 1991a,b). Some of the chlorocatechol dioxygenases have been purified (partially or to homogeneity) and were investigated in detail (Ngai & Ornston 1988; Pieper et al. 1988; Broderick & O'Halloran 1991; Maltseva et al. 1991; Hinteregger et al. 1992; Solyanikova et al. 1992; Bhat et al. 1993; Miguez et al. 1993). Despite considerable variation in their relative specificities, the overall picture that emerges from the investigation of the chlorocatechol dioxygenases is one of enzymes with relaxed substrate specificity.

A relatively low specificity has also been reported to distinguish the chloromuconate cycloisomerase (EC 5.5.1.7) of *Pseudomonas* sp. B13 from its counterpart muconate cycloisomerase (EC 5.5.1.1; Schmidt & Knackmuss 1980). Similar kinetic parameters were found for the cycloisomerases of the chloroaniline utilizing strain *Comamonas acidovorans* CA28 (Hinteregger et al. 1993). In contrast to the enzymes from these strains, however, several other chloromuconate cycloisomerases convert *cis,cis*-muconate very slowly or with low affinity (Surovtseva et al. 1986; Kuhm et al. 1990; Solyanikova et al. 1993; Vollmer & Schlömann 1994). Thus, the cycloisomerases of chlorocatechol degradation tend to be more specific than the dioxygenases. In addition, the chloromuconate cycloisomerases of *Pseudomonas* sp. B13 and of the 2,4-dichlorophenoxyacetate utilizing strain *Alcaligenes eutrophus* JMP134 (pJP4) have the capability to dehalogenate 2-chloro-*cis,cis*-muconate after cycloisomerization, a feature which is lacking in normal muconate cycloisomerases as recently observed (Vollmer et al. 1994). Thus, altered specificity is not the only difference between muconate and chloromuconate cycloisomerases.

After some early reports on the separation of dienelactone hydrolases (EC 3.1.1.45) and chloromuconate cycloisomerases (Gaunt & Evans 1971b; Sharpee et al. 1973), Schmidt & Knackmuss (1980) were able to differentiate the dienelactone hydrolase of *Pseudomonas* sp. B13 from the analogous enzyme of catechol catabolism, 3-oxoadipate enol-lactone hydrolase (EC 3.1.1.24). The dienelactone hydrolase did not convert 3-oxoadipate enol-lactone, nor did the enol-lactone hydrolase turn over *cis*- or *trans*-dienelactone. This is remarkable because of the similar overall structure of these lactones (Fig. 3). More recently, dienelactone hydrolases from two other degraders of chloroaromatic compounds have been purified and characterized (Schlömann 1988; Maltseva et al. submitted). Both the dienelactone hydrolase encoded by pJP4 of *A. eutrophus* JMP134 and the corresponding enzyme from the chlorophenols utilizing strain *Rhodococcus erythropolis* 1CP, resemble the B13 hydrolase in not converting 3-oxoadipate enol-lactone. With respect to turnover of the two dienelactone isomers the three enzymes showed significant differences (Table 3). The dienelactone hydrolase of *Pseudomonas* sp. B13 has some preference for *trans*-dienelactone, as evident from an almost thirty-fold lower K_m value for this than for the *cis*-isomer, the V_{max} values being of similar magnitude. For the pJP4-encoded enzyme similar kinetic parameters were found with both dienelactones. In contrast, the dienelactone hydrolase of *R. erythropolis* 1CP converts only the *cis*-isomer and not *trans*-dienelactone, a specificity corresponding to that of the dioxygenase and cycloisomerase of this strain (Maltseva et al. 1991; Solyanikova et al. 1993).

The examples mentioned above show that conclusions about enzymes of chlorocatechol degradation drawn solely from dioxygenases are not necessarily valid. In fact, one lesson to be learnt from cycloisomerases and hydrolases is that the chlorocatechol degradation pathway is not just a low specificity variant of catechol catabolism. Some of the enzymes rather have a relatively high, albeit altered, specificity.

Distant relatedness of 3-oxoadipate enol-lactone hydrolases and plasmid-encoded dienelactone hydrolases

Except for a maleylacetate reductase (EC 1.3.1.32; Don et al. 1985; Kukor et al. 1989; Vollmer et al. 1993), the enzymes of chlorocatechol degradation are generally encoded by degradative plasmids (Fisher et al. 1978; Pemberton et al. 1979; Chatterjee et al. 1981; Pierce et al. 1981; Vandenbergh et al. 1981; Grishchenkov et al. 1983; Amy et al. 1985; Chaudhry & Huang 1988;

Hickey & Focht 1990; Van der Meer et al. 1991a; Mäe et al. 1993; Bhat et al. 1994). Those which have most thoroughly been investigated are pJP4, encoding the 2,4-dichlorophenoxyacetate catabolic enzymes in *A. eutrophus* JMP134 (Don et al. 1985), pAC27, encoding the 3-chlorobenzoate degradative enzymes in *Pseudomonas putida* AC866 (Chatterjee & Chakrabarty 1984), and pP51, encoding the enzymes for 1,2,4-trichlorobenzene catabolism in *Pseudomonas* sp. P51 (Van der Meer et al. 1991a). From *Pseudomonas* sp. B13, the genes for chlorocatechol catabolism have been shown to be transmissible to various recipients (Reineke et al. 1982). However, the plasmid, named pB13 or pWR1, was difficult to isolate and gave low yields (Chatterjee & Chakrabarty 1983; Weisshaar et al. 1987). It was found to be very similar to pAC25, the precursor of pAC27 (Chatterjee & Chakrabarty 1982, 1983). Furthermore, the dienelactone hydrolases purified from *Pseudomonas* sp. B13 and from a clone with pAC27 genes were found to be identical in every discernible respect (Frantz et al. 1987; Ngai et al. 1987). The enzymes for chlorocatechol degradation of *Pseudomonas* sp. B13 and pAC27 are therefore not differentiated in this review.

The similarity of the pathways suggests that the modified *ortho*-cleavage pathway for chlorocatechol degradation evolved from the catechol branch of the ubiquitous 3-oxoadipate pathway. Sequencing of the pAC27- , pJP4- , and pP51-encoded chlorocatechol 1,2-dioxygenases and chloromuconate cycloisomerases (Frantz & Chakrabarty 1987; Ghosal & You 1988, 1989; Perkins et al. 1990; Van der Meer et al. 1991b) has indeed shown them to be homologous to the usual catechol 1,2-dioxygenases and muconate cycloisomerases (Aldrich et al. 1987; Neidle et al. 1988; Kivisaar et al. 1991; Eck & Belter 1993; Shanley et al. 1994; Houghton et al. personal communication). In the sequence alignment of the 1,2-dioxygenases, as reported by Eck & Belter (1993), catechol and chlorocatechol 1,2-dioxygenases share between 25 and 35% amino acid sequence identity (while among the catechol 1,2-dioxygenases the percentage of identical positions varies from 27 to 58%, and among the chlorocatechol 1,2-dioxygenases from 55% to 66%). The similarity between muconate and chloromuconate cycloisomerases is even greater (Table 1).

The dienelactone hydrolase of *Pseudomonas* sp. B13 and the 3-oxoadipate enol-lactone hydrolases of *P. putida* and *A. calcoaceticus* have also been assumed to share a common ancestor. This hypothesis was originally based on similar molecular masses, inhibition by *p*-chloromercuribenzoate, and similarities of the NH_2-termini and amino acid compositions (Ngai et al. 1987). In addition, an identical pentapeptide neighbouring a cystein residue in the *P. putida* 3-oxoadipate enol-lactone hydrolase and the pAC27-encoded dienelactone hydrolase was interpreted to be conserved because of its proximity to the active site (Frantz et al. 1987). However, the elucidation of the three dimensional structure of the pAC27-encoded dienelactone hydrolase showed that Cys-123, rather than Cys-60 which neighbours the pentapeptide, is part of the active site (Pathak et al. 1988, 1991; Cheah et al. 1993a).

Sequencing of the 3-oxoadipate enol-lactone hydrolases of *A. calcoaceticus* and *P. putida* (Hartnett & Ornston 1994; Shanley et al. 1994; Houghton et al. personal communication) has now revealed a surprisingly low sequence similarity to the dienelactone hydrolases encoded by pAC27, pJP4, and pP51 (Table 2; Frantz & Chakrabarty 1987; Perkins et al. 1990; Van der Meer et al. 1991b). Multiple sequence alignments of the six lactone hydrolases by using CLUSTAL (Higgins & Sharp 1988, 1989), cluster the enol-lactone hydrolases into one group and the dienelactone hydrolases into another. Indeed, the alignments of these two groups yield differing results depending on the chosen parameter values (i.e. penalties for opening and extending gaps), suggesting that the alignments are not reliable. Thus, simple sequence comparisons do not allow the conclusion that 3-oxoadipate enol-lactone hydrolases and the plasmid-encoded dienelactone hydrolases are homologous, and consequently it is problematic to calculate the ratios of identical positions (see below).

The determination of the three-dimensional structure of the pAC27-encoded dienelactone hydrolase (Pathak et al. 1988, 1991; Pathak & Ollis 1990) allowed the identification of a structural core, the α/β hydrolase fold (Ollis et al. 1992), which this enzyme shares with others. The core is an α/β sheet consisting of eight β-strands connected by α-helices, and has been found in a diverse group of enzymes with different functions, sizes and oligomer structures, and with no obvious sequence similarity. Besides dienelactone hydrolase, this group initially included a fish acetylcholine esterase, wheat carboxypeptidase II, a fungal lipase, and haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10. Despite additional structural components in some of these enzymes, the catalytic triad residues, comprising a nucleophile (Cys, Ser, or Asp), a histidine, and an acid (Asp or Glu), occupy

Table 1. Pairwise comparisons of cycloisomerase sequences.

catB P. putida	–	54.5	52.1	52.0	54.5
catB A. calcoaceticus	52.4	–	48.3	49.1	48.3
tfdD pJP4	41.6	40.3	–	64.7	66.7
clcB pAC27	41.1	40.5	63.2	–	72.0
tcbD pP51	44.1	40.8	65.9	75.7	–
	catB	*catB*	*tfdD*	*clcB*	*tcbD*
	P.putida	*A.calcoac.*	pJP4	pAC27	pP51

The ratio (%) of identical positions in pairwise comparisons of DNA sequences is shown above the diagonal, the ratio (%) of identical positions in pairwise comparisons of predicted amino acid sequences below it. The latter were aligned pairwise by using the algorithm of Myers & Miller (1988) as implemented in the Intelligenetics PC/GENE program package (open gap cost 7, unit gap cost 2). DNA sequences were aligned so that gap positions corresponded to those in protein sequences. The *catB*-encoded enzymes are muconate cycloisomerases, the others chloromuconate cycloisomerases. References for the sequences: Frantz & Chakrabarty 1987; Perkins et al. 1990; Van der Meer et al. 1991b; Shanley et al. 1994; Houghton et al. personal communication (the latter includes some differences when compared to the sequence of Aldrich et al. 1987). For some of the comparisons the same or similar values are found in the cited papers as well as in Ghosal & You (1989).

Table 2. Pairwise comparisons of dienelactone and 3-oxoadipate enol-lactone hydrolase sequences.

pcaD P. putida	–	46.7	49.2	n.d.	n.d.	n.d.
pcaD A. calcoacet.	43.6	–	51.6	n.d.	n.d.	n.d.
catD A. calcoacet.	40.8	44.0	–	n.d.	n.d.	n.d.
tfdE pJP4	(9.8)	(9.4)	(7.3)	–	54.7	57.5
clcD pAC27	(11.0)	(11.0)	(9.3)	53.8	–	60.3
tcbE pP51	(8.8)	(8.0)	(6.7)	52.1	50.0	–
	pcaD	*pcaD*	*catD*	*tfdE*	*clcD*	*tcbE*
	P.putida	*A.calcoac.*	*A.calcoac.*	pJP4	pAC27	pP51

The ratio (%) of identical positions in pairwise comparisons of DNA sequences is shown above the diagonal, the ratio (%) of identical positions in pairwise comparisons of predicted amino acid sequences below it. The sequences of the dienelactone hydrolases encoded by pJP4 (*tfdE*), pAC27 (*clcD*), and pP51 (*tcbE*) were taken from Perkins et al. (1990), Frantz & Chakrabarty (1987), and Van der Meer et al. (1991b), respectively. The sequences of 3-oxoadipate enol-lactone hydrolases from *A. calcoaceticus* (*catD*, *pcaD*) and *P. putida* (*pcaD*) were obtained from L.N. Ornston's laboratory (Hartnett & Ornston 1994; Shanley et al. 1994; Houghton et al. personal communication). The alignment shown in Fig. 4 was used for the calculations. DNA sequences were aligned so that gap positions corresponded to those in protein sequences. For comparisons between dienelactone hydrolases on the one hand and 3-oxoadipate enol-lactone hydrolases on the other, the ratios of protein are sequence identities given in brackets, because they were dependent on a manual alignment. For these comparisons identical positions in DNA sequences were not determined (n.d.). For some of the comparisons, similar values are given in papers cited above.

the same positions within the three dimensional structure. This similarity in structural detail, the similar overall topology, the same order of the catalytic triad residues in the sequence, and conserved loops at the active site, allowed Ollis et al. (1992) to conclude that the α/β hydrolase fold enzymes diverged from a common ancestor. The same authors identified other members of this protein family based on their three dimensional structures (other lipases, carboxypeptidase A) or because of previously reported sequence similarities to enzymes with an α/β hydrolase fold (thyroglobulin, an epoxide hydrolase, hydrolases of *meta*-cleavage pathways). Other authors (e.g. Kawasaki et al. 1992; Derewenda & Sharp 1993) reported on additional members of the family. Enzymes possessing an α/β hydrolase fold differ from other groups of enzymes with catalytic triads (e.g. the serine proteases) with respect to the fold and the order of the triad

residues in the sequence, implying that catalytic triads have developed several times by convergent evolution (Ollis et al. 1992).

An especially useful feature for the identification of other α/β hydrolase fold enzymes is a conserved sequence pattern at the 'nucleophile elbow': Sm-X-Nu-X-Sm-Sm (where X represents any amino acid, Sm is a small residue such as Gly, Val or Ala, and Nu is the nucleophile; Ollis et al. 1992). This pattern is also found in the three 3-oxoadipate enol-lactone hydrolases around a serine residue at position 101 (Fig. 4). Furthermore, this region has high sequence similarity to part of a hydrolase involved in biphenyl catabolism which had been connected to enzymes with an α/β hydrolase fold (Ollis et al. 1992; Hartnett & Ornston 1994). Consequently, serine rather than the previously assumed cysteine (Yeh & Ornston 1984) appears to be the nucleophile which attacks the ester carbonyl group (Hartnett & Ornston 1994). As required for enzymes with α/β hydrolase fold, the three 3-oxoadipate enol-lactone hydrolases also have a conserved acidic residue (Asp-201) which precedes a conserved histidine (His-248). In Fig. 4 a sequence comparison between three dienelactone hydrolases and three 3-oxoadipate enol-lactone hydrolases is shown. Since the CLUSTAL program did not give a clear-cut result, the dienelactone hydrolases on one hand and the 3-oxoadipate enol-lactone hydrolases on the other were aligned manually, so that the presumed catalytic triad positions coincided. The gaps which necessarily had to be introduced because of the different sizes of the enzymes (dienelactone hydrolases ca. 25–26 kDa, 3-oxoadipate enol-lactone hydrolases ca. 29–34 kDa) were placed so that they would not interrupt known or predicted α-helices or β-strands. The manual alignment was performed mainly to obtain a reasonable basis for comparison between the two groups of hydrolases, not to predict the structure of the 3-oxoadipate enol-lactone hydrolases. However, the following two points might lend some credibility to the manual alignment. First, 5 gaps covering a total of 89 positions had to be introduced into either all three dienelactone hydrolases or all three 3-oxoadipate enol-lactone hydrolases, resulting in 9 positions identical in all six sequences. During several attempts with the CLUSTAL program, using various penalties for opening and extending gaps in the final alignment, a maximum of 7 positions identical in all six sequences was obtained, and this required 12 gaps through all dienelactone hydrolases covering a total of 73 positions. Thus, the manual alignment was not worse than the computer calculated ones. Second, the two large gaps inserted into all three dienelactone hydrolase sequences occur at positions in which other enzymes with an α/β hydrolase fold are known to carry additional helices (Ollis et al. 1992). Thus, if 3-oxoadipate enol-lactone hydrolases should in fact turn out to belong to this family of proteins, they might be expected to have additional structural elements in the regions corresponding to these two gaps.

It should be noted that the above mentioned pentapeptide found close to a cysteine residue in the *P. putida* 3-oxoadipate enol-lactone hydrolase and in the pAC27-encoded dienelactone hydrolase occurs at completely different positions in the proteins and is not conserved in the other lactone hydrolases (Fig. 4).

Future investigations might show whether or not 3-oxoadipate enol-lactone hydrolases have a structure similar to that implicated in the alignment of Fig. 4. Efforts directed towards crystallization of these enzymes are being made in our laboratory. For now it might be concluded from the above that the plasmid-encoded dienelactone hydrolases and the 3-oxoadipate enol-lactone hydrolases are related, in that they share a common ancestor with an α/β hydrolase fold. However, it is a much more distant relationship than observed for the 1,2-dioxygenases and the cycloisomerases, and it includes major insertions or deletions as well as a change in the active site nucleophile.

Other types of dienelactone hydrolases: Candidates for the missing link?

The large differences between the plasmid-encoded dienelactone hydrolases and the 3-oxoadipate enol-lactone hydrolases suggest that the former might not have evolved directly from the latter for the purpose of playing a role in the degradation of chloroaromatic compounds. Therefore, the question has to be raised whether a different hydrolase from some other pathway might have been recruited for dienelactone hydrolysis in chlorocatechol catabolism. This presumed missing link could have shared more similarity with the plasmid-encoded dienelactone hydrolases than do the 3-oxoadipate enol-lactone hydrolases. A comparison of the sequences of the pJP4- , pAC27- , and pP51-encoded dienelactone hydrolases to the SwissProt and PIR databanks (as of August 93) by using FASTA (Pearson & Lipman 1988) detected the similarities among the three dienelactone hydrolases, but no other hydrolase with convincing similarity could be found. Thus, using this approach another enzyme

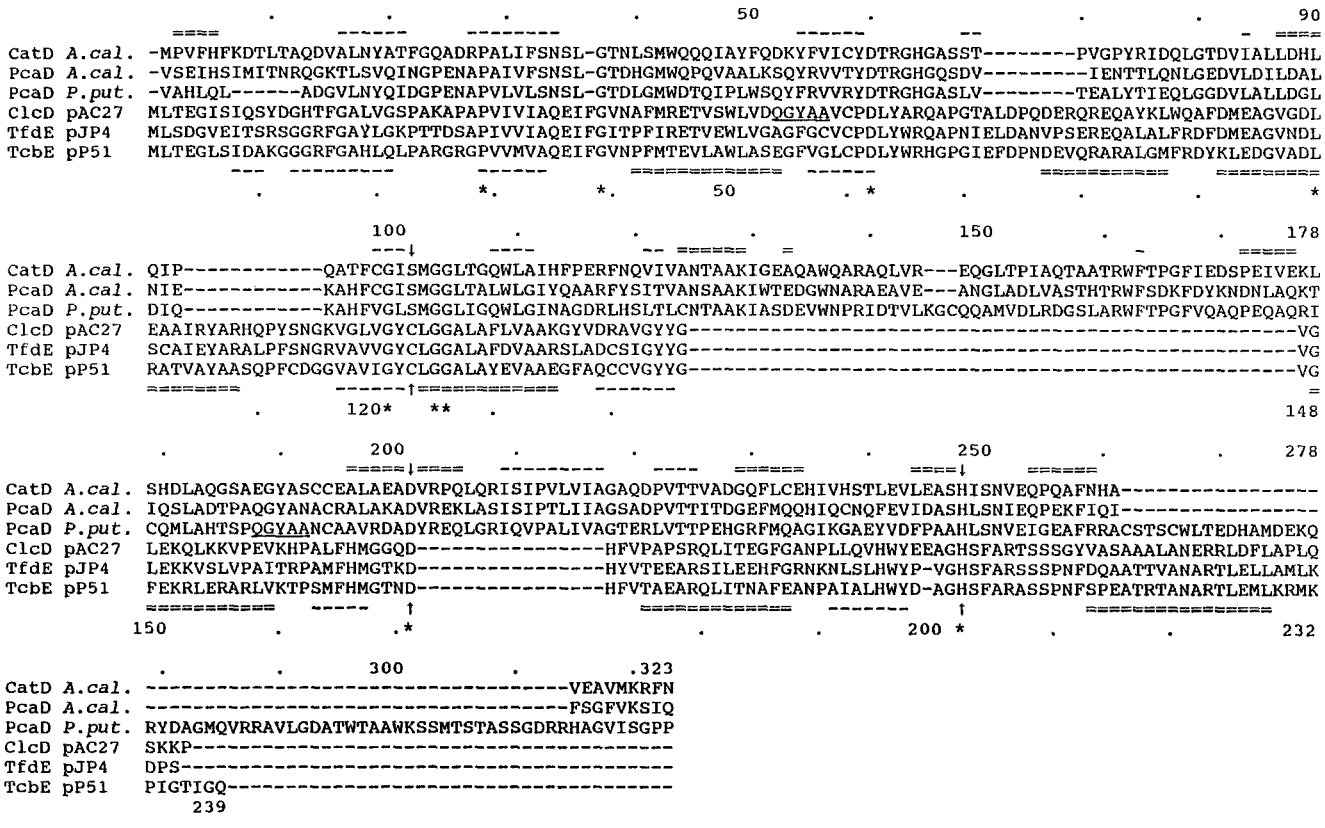

Fig. 4. Comparison of the sequences of 3-oxoadipate enol-lactone hydrolases (CatD, PcaD) from *A. calcoaceticus* and *P. putida* and dienelactone hydrolases (ClcD, TfdE, TcbE) of plasmids pAC27, pJP4, and pP51. For references on the sequences, see the legend to Table 2. Initially, a multiple sequence alignment was performed using the program CLUSTAL (Higgins & Sharp 1988, 1989) as implemented in the PC/GENE program package, by using the default parameters excepting higher open and unit gap costs (30 instead of 10). The 3-oxoadipate enol-lactone hydrolase cluster on the one hand, and the dienelactone hydrolase cluster on the other, were then aligned manually, so that the respective (presumed) catalytic triad residues coincided (marked by arrows). Gaps were introduced somewhat arbitrarily, but so that possibly corresponding secondary structure elements aligned and were not interrupted by gaps. The positions of α-helices (= = = =) and β-strands (- - - -) of the pAC27-encoded dienelactone hydrolase as given by Pathak & Ollis (1990) are shown below the alignment. For the 3-oxoadipate enol-lactone hydrolases they were predicted from the sequences according to the algorithm of Garnier et al. (1978) as implemented in PC/GENE (default parameters). In a control run the program detected almost all secondary structure elements of the pAC27-encoded dienelactone hydrolase correctly, with the exception of strand 7 and helix F, and with inaccuracies at the beginnings and ends of the respective helices and strands. α-Helices and β-strands are shown above the sequences only in those cases where the prediction for all three enol-lactone hydrolases gave the same result. Stars denote residues identical in all sequences. A pentapeptide occurring in ClcD of pAC27 as well as in PcaD of *P. putida* is underlined. Numbers above the sequences count 3-oxoadipate enol-lactone hydrolase positions, including those where one or two of the sequences have gaps. Positions of dienelactone hydrolases are numbered below the sequences.

from which the plasmid-encoded dienelactone hydrolases may have been derived, could not yet be identified.

Obvious candidates for the presumed precursors of dienelactone hydrolases for chlorocatechol degradation are dienelactone hydrolases identified in 4-fluorobenzoate degrading strains of *Alcaligenes* and *Burkholderia (Pseudomonas) cepacia* (Schlömann et al. 1990a). With the exception of *A. eutrophus* JMP134, these strains had not previously been reported to utilize haloaromatic compounds, and they do not grow with 3-chlorobenzoate, 4-chlorobenzoate, or 2,4-dichlorophenoxyacetate. During growth with 4-fluorobenzoate they induce dienelactone hydrolase and maleylacetate reductase activities, but apparently no chlorocatechol 1,2-dioxygenase or chloromuconate cycloisomerase (Schlömann et al. 1990a). These observations show that some bacteria possess dienelactone hydrolases without employing them for chlorocatechol catabolism. The natural function of these enzymes is presently not known.

Table 3. Comparison of 3-oxoadipate enol-lactone hydrolases and different types of dienelactone hydrolases.[a]

Enzymes (Representative strains or encoding plasmids)	Substrates Converted				Molecular mass (kDa)	Inactivation		pH optimum
	3-oxoadipate enol-lactone	*trans*-diene-lactone	*cis*-diene-lactone	4-Fluoro-mucono-lactone		*p*CMB	EDTA	
3-Oxoadipate enol-lactone hydrolase *A. calcoaceticus*, *P. putida*	+[b]	–	–	+	29.1–34.5[c]	+	n.d.[d]	7.5–9
Dienelactone hydrolase type I *A. eutrophus* 335 and JMP222	+	+	–	+	58[e]	+	+	7.5
Dienelactone hydrolase type II *B. cepacia* ATCC17759	–	–	+	–	32.4[f]	–	–	5.5
Dienelactone hydrolase type III pAC27, *Pseudomonas* sp. B13	–	+ K_m = 15 μM	+ K_m = 400 μM	+	25.4–25.8[c]	+	–	7.5
pJP4		K_m = 110 μM[g]	K_m = 140 μM[g]					
Dienelactone hydrolase from *R. erythropolis* 1CP	–	–	+ K_m = 28 μM	–	30[h]	+	–	7.8–8

[a] The properties compiled here have been shown for at least one member of each group; they are not necessarily valid for all of them. The data were taken from the following references: Ornston 1966; Schmidt & Knackmuss 1980; Schlömann 1982, 1988; Yeh & Ornston 1984; Frantz & Chakrabarty 1987; Ngai et al. 1987; Perkins et al. 1990; Schlömann et al. 1990a, 1990b, 1993; Van der Meer et al. 1991b; Hartnett & Ornston 1994; Shanley et al. 1994; Maltseva et al. submitted; Houghton et al. personal communication.

[b] + = high turnover rate; – = activity marginal or zero.

[c] Monomeric enzymes, molecular masses predicted from DNA sequences.

[d] n.d. = no information available.

[e] Determined by gel filtration.

[f] Determined by SDS polyacrylamide gel electrophoresis. Gel filtration indicated 39 kDa.

[g] Determined in cell-free extract, not with purified enzyme.

[h] Determined by SDS polyacrylamide gel electrophoresis and gel filtration.

Dienelactone hydrolases were originally differentiated based on their substrate specificities (Table 3; Schlömann et al. 1990a). Those detected, for example, in *A. eutrophus* 335 (the type strain), *A. eutrophus* H16, and *A. eutrophus* JMP222 (a cured derivative of JMP134) convert *trans*-, but not *cis*-dienelactone and were designated as type I. The dienelactone hydrolase of *B. cepacia* has the opposite preference, hydrolyzing only the *cis*-isomer, and was termed type II. The plasmid-encoded dienelactone hydrolases for chlorocatechol catabolism showed turnover of both isomers, although with some variation in the kinetic parameters (see above). Their designation as type III resulted from the former hypothesis that they might represent a relatively recent development.

The type II dienelactone hydrolase of *B. cepacia* was purified to apparent homogeneity, and was found to differ from the enzymes encoded by pAC27 and pJP4 (type III) in basic properties, such as pH optimum of activity, inhibition by *p*-chloromercuribenzoate, and amino acid composition (Table 3; Schlömann et al. 1993). Thus, dienelactone hydrolases of types II and III seem not to be closely related, implying the possibility that the capability to hydrolyze dienelactones might have arisen several times by convergent evolution. A recently described dienelactone hydrolase of chlorophenol degrading *R. erythropolis* 1CP resembled the *B. cepacia* hydrolase with respect to substrate specificity (Maltseva et al. submitted). Other properties, however, suggested that it might be more closely related to the plasmid-encoded type III dienelactone hydrolases (Table 3), implying that substrate specificity is not necessarily a valid property for differentiation of the dienelactone hydrolases.

Characterization of the dienelactone hydrolase type I of *A. eutrophus* 335 revealed that this enzyme, in addition to *trans*-dienelactone, also converts 3-oxoadipate enol-lactone (Schlömann 1988). The type I enzyme thus has a specificity intermediate between that of the plasmid-encoded dienelactone hydrolases and that of the 3-oxoadipate enol-lactone hydrolases (Table 3), and it might therefore appear to be an evolutionary intermediate. However, from both these groups of lactone hydrolases, the dienelactone hydrolase type I dif-

fers in being Mn^{2+} dependent and in having a larger molecular mass under non-denaturing conditions (Schlömann 1988).

In order to obtain a basis for a more detailed analysis of the evolutionary relationships among the lactone hydrolases, the dienelactone hydrolase type I gene was cloned into *E. coli* (Schlömann et al. 1991). For this purpose the efficiency of the transformation system of *A. calcoaceticus* BD413 derivatives was found to be a valuable tool, because it allowed the rapid screening of an *E. coli* library of *A. eutrophus* DNA for those clones that were able to complement a 3-oxoadipate enol-lactone hydrolase deficient mutant of *A. calcoaceticus*. One class of clones obtained expresses dienelactone hydrolase activity with the *trans*-isomer (Hinner et al. 1994), while two other groups of clones apparently contain the genes for the two 3-oxoadipate enol-lactone hydrolases of *A. eutrophus* (Johnson & Stanier 1971). Further analysis of these clones is expected to shed some light on the relatedness of the type I dienelactone hydrolase to other hydrolases.

Single versus multiple origin of chlorocatechol degradative pathways

A major question in the assessment of environmental pollution is whether bacteria have the potential to evolve largely novel pathways when exposed to a xenobiotic compound, or whether their capability is more or less limited to combining preexisting pathways and improving their expression. With respect to chlorocatechol degradation, the first possibility above predicts the pathways found in various chloroaromatics utilizing bacteria would have developed from, for example, catechol catabolic routes several times independently. In contrast, the second option envisages a single origin of the modified *ortho*-cleavage pathway and a subsequent worldwide distribution, involving also plasmid transfer to various genera.

As noted previously (Schlömann 1992), the sequence similarities of the chloromuconate cycloisomerases encoded by pAC27, pJP4 and pP51 can most easily be understood when the assumption is made that these enzymes share a common ancestor that is not shared by the muconate cycloisomerases sequenced to date (Fig. 5A). It seems highly unlikely that convergent evolution due to the steric or electronic necessities of chloromuconate turnover could have resulted in such a degree of sequence similarity as now found among the chloromuconate cycloisomerases.

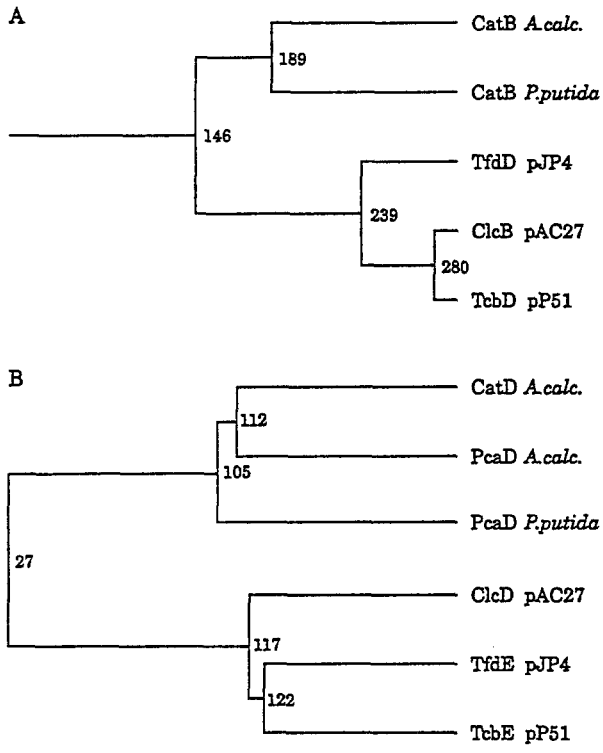

Fig. 5. (A) Dendrogram showing the relatedness of muconate cycloisomerases (CatB) and chloromuconate cycloisomerases (TfdD, ClcB, TcbD) as calculated by the CLUSTAL program (Higgins & Sharp 1988, 1989). The numbers are the scores at which the respective sequences are clustered. For references on the sequences, see the legend to Table 1. (B) Dendrogram showing the relatedness of 3-oxoadipate enol-lactone hydrolases (CatD, PcaD) and dienelactone hydrolases (TfdE, ClcD, TcbE), calculated in the same way. For references, see the legend to Table 2. The graphic output of CLUSTAL was adjusted manually with respect to the horizontal axis, to allow a comparison of both dendrograms. Note that the cycloisomerase sequences are ca. 50% longer than the hydrolase sequences and therefore potentially yield higher scores.

However, theoretically the sequence similarity might still have been inherited from a relatively recent common ancestor which was a muconate cycloisomerase, and not a chloromuconate cycloisomerase. In this case, the development of the new substrate specificity would still be an example of functionally convergent evolution. An analogous argument applies to the evolution of the chlorocatechol 1,2-dioxygenases.

The amino acid sequences of the 3-oxoadipate enol-lactone hydrolases on the one hand, and of the pAC27-, pJP4-, or pP51-encoded dienelactone hydrolases on the other, are so different that a multiple evolution of the former to the latter for the purpose of chlorocatechol degradation can certainly be excluded. Correspondingly in Fig. 5B, the branching between the two

clusters for dienelactone hydrolases and 3-oxoadipate enol-lactone hydrolases is considerably deeper than that between muconate and chloromuconate cycloisomerases (Fig. 5A). If one assumes a recruitment of preexisting dienelactone hydrolases into chlorocatechol catabolic operons, then multiple occurrences of this process would not be compatible with the structural similarity of the operons found on pAC27, pJP4 and pP51 (Fig. 6). Thus, the hydrolases, even more convincingly than the dioxygenases and cycloisomerases, suggest that the chlorocatechol degradation operons of these plasmids diverged from an ancestral operon of the same function (that is, an operon encoding at least chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase, and dienelactone hydrolase).

This conclusion gains additional support from two other groups of proteins involved in chlorocatechol degradation. (1) The *tfdCDEF* operon of pJP4 and the *tcbCDEF* operon of pP51 contain genes, *tfdF* and *tcbF* (Fig. 6), whose products appear to function as maleylacetate reductases (Seibert et al. 1993; Schell et al. 1994). These genes have apparently been recruited from some other than the catechol catabolic pathway, and as discussed for the hydrolases, their similar position in the operons contradicts a multiple occurrence of the recruitment. (2) It has recently been pointed out by Coco et al. (1993) that the regulatory proteins ClcR and TcbR are more similar to each other than to the corresponding and homologous regulatory proteins of the catechol pathway, CatR of *P. putida* and CatM of *A. calcoaceticus*. This fits to the previous assumption that the chlorocatechol degradation genes evolved as clusters including a regulatory gene (Coco et al. 1990).

The dendrogram in Fig. 5B shows the pJP4- and the pP51-encoded hydrolases to be more similar to each other than to the pAC27-encoded hydrolase. This branching pattern differs from that observed for the chloromuconate cycloisomerases, where TcbD and ClcB are most similar to each other (Fig. 5A), as well as from that for the chlorocatechol 1,2-dioxygenases (see Van der Meer et al. 1991b). Since the pattern of divergence among the cycloisomerases and dioxygenases is also shared by the dienelactone hydrolase genes (Tables 2 and 4), it must be assumed that the *clcABD* and the *tcbCDEF* operons share a common ancestor, not shared by the *tfdCDEF* operon. This conclusion is also consistent with the operon structures (Fig. 6). The differing branching patterns of the hydrolases at the protein level point to the existence of steric or electronic necessities of substrate turnover, which might soon be understood based on the present knowledge about dienelactone hydrolase mechanism (Cheah et al. 1993a,b).

Since pAC25 (the precursor of pAC27), pJP4, and pP51 were obtained from strains that were isolated on different continents (Pemberton et al. 1979; Chatterjee et al. 1981; Van der Meer et al. 1987), a worldwide distribution of a basic module for chloroaromatics degradation seems indeed to have taken place. And because the corresponding regions of other plasmids for chlorocatechol degradation were found to be similar to the chlorocatechol catabolic genes of pJP4 (Amy et al. 1985; Mäe et al. 1993; Bhat et al. 1994), the postulated ancestral chlorocatechol degradation operon seems to have been evolutionary successful, being the precursor of several of the known modified *ortho*-cleavage pathways.

However, Holben et al. (1992) recently reported on the occurrence of 2,4-dichlorophenoxyacetate degrading bacteria, whose *tfdCDEF* genes did not hybridize to probes of the corresponding pJP4 genes, while their *tfdA* and *tfdB* genes did. Furthermore, the properties of the chlorocatechol 1,2-dioxygenase and especially of the chloromuconate cycloisomerase of *R. erythropolis* 1CP suggest that chlorocatechol catabolism in this organism might have evolved independently from the pJP4- or pAC27-encoded pathways (Solyanikova et al. 1994; Maltseva et al. in preparation). The hypothesis that functional convergence might have occurred in the evolution of chlorocatechol degradative pathways is presently being investigated.

Evolution for the purpose of halocatechol degradation

Cometabolism is known to play a major role in the degradation of xenobiotic compounds (Horvath 1972; Alexander 1979; Slater & Lovatt 1984; Janke & Fritsche 1985; Neilson et al. 1985; Gottschalk & Knackmuss 1993). In addition, the genetic information for halocatechol assimilation has been assumed to preexist in nature without being related to any natural function (Knackmuss 1984). The question thus arises whether the original chlorocatechol catabolic operon postulated above evolved in order to function in the degradation of halogenated catechols, whether it primarily played a role in the degradation of other compounds, or whether it was devoid of a discernible function. The following suggests that the first possibility is indeed correct:

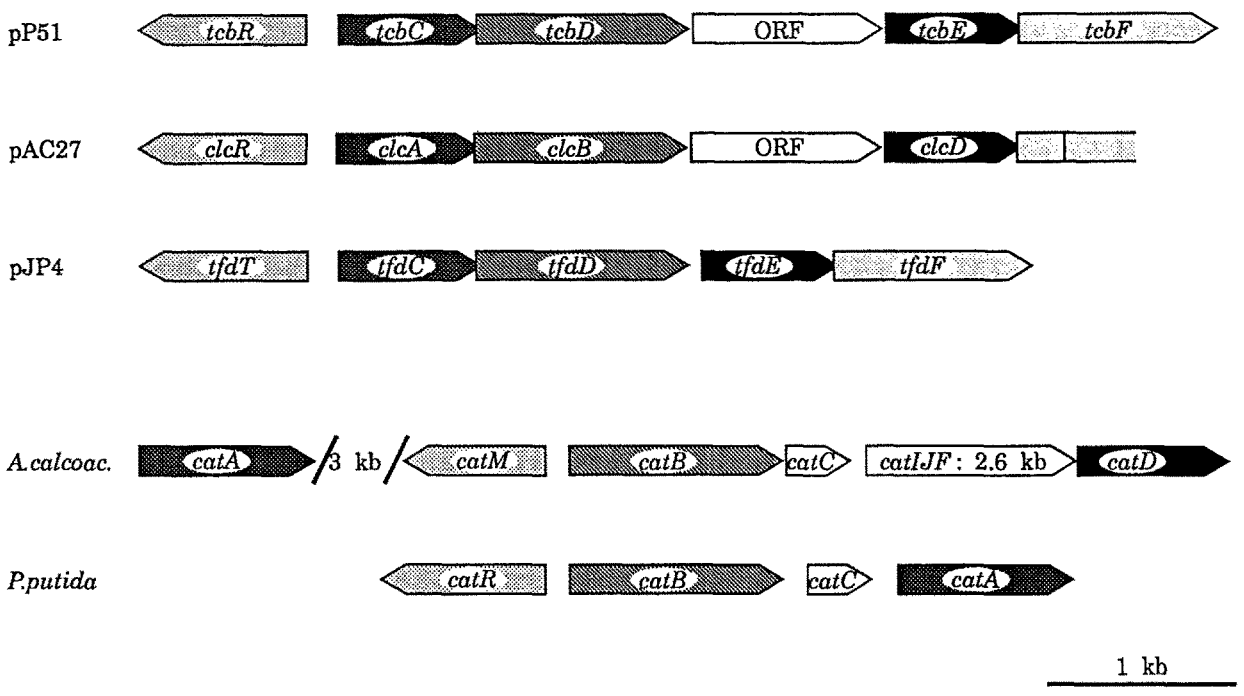

Fig. 6. Operons of catechol and chlorocatechol degradation with adjacent regulatory genes. Each arrow corresponds to an open reading frame. Homologous genes are shaded in the same way. Arrows without tips denote an overlap of the reading frames. Information compiled from the following sources: Frantz & Chakrabarty 1987; Aldrich & Chakrabarty 1988; Neidle et al. 1989; Perkins et al. 1990; Rothmel et al. 1990; Van der Meer et al. 1991b,c; Brown et al. 1992; You 1992; Coco et al. 1993; Shanley et al. 1994. Instead of, or in addition to *tfdT* (also referred to as *tfdX*), another regulatory gene, *tfdR*, appears to be involved in the regulation of the *tfdCDEF* operon (Kaphammer et al. 1990). In *P. putida*, the 3-oxoadipate enol-lactone hydrolase gene belongs to the *pcaBDC* operon (Ornston et al. 1990), rather than to the *cat* operon (with respect to the latter possibly differences between the two strains under investigation, possibly not drawn to scale).

1. The substrate specificities of the 1,2-dioxygenases and cycloisomerases indicate that the enzymes evolved to convert substituted catechols and muconates, respectively. This criterion for the natural substrate, however, is not very restrictive, since in addition to chlorine it also allows substituents such as methyl groups.
2. The chloromuconate cycloisomerases encoded by pAC27 and pJP4, and probably those encoded by related plasmids, evolved the capability to catalyze chloride elimination following 2-chloro-*cis,cis*-muconate cycloisomerization, a characteristic not shared by usual muconate cycloisomerases (Vollmer et al. 1994).
3. Each of the chlorocatechol catabolic operons found on pAC27, pJP4, and pP51 includes a dienelactone hydrolase. Such an enzyme is necessary for degradation only if, compared to muconolactone, an additional double bond is formed during or after cycloisomerization. Thus it should be conceivable that the substituent referred to above (point 1) is removed from the molecule in an elimination reaction, thereby giving rise to the other double bond.
4. TfdF of plasmid pJP4 has recently been shown to share similarity in the NH_2-terminal sequence with the maleylacetate reductase of *A. eutrophus* JMP134 (Seibert et al. 1993). The hypothesis that TfdF might be a reductase for maleylacetate or substituted maleylacetates has recently been corroborated (Schell et al. 1994). Thus, TfdF, TcbF, and their Clc equivalent, like the dienelactone hydrolases, have a potential catabolic function only if an additional double bond is formed in an elimination reaction.
5. The chlorocatechol degradation enzymes are inducible in response to the presence of chloroaromatic compounds (Harker et al. 1989; Pieper et al. 1989; Kaphammer et al. 1990; Van der Meer et al. 1991c; Coco et al. 1993; see also references given for substrate specificity). Since the structural and the regulatory genes for chlorocatechol degradation seem to have coevolved (Coco et al. 1990), the

Table 4. Synonymous differences between various chlorocatechol catabolic genes and some catechol catabolic genes for comparison.

Enzymes	Compared sequences[a]		Ratio of identical bases in third triplet position[b] (%)	Ratio of synonymous substitutions (K_s value)[c]	Corrected ratio of synonymous substitutions (K_s^{corr})[d]
Dienelactone hydrolases	*tfdE*	*clcD*	27.9	0.721	2.44
	tfdE	*tcbE*	30.8	0.692	1.92
	clcD	*tcbE*	46.0	0.540	0.95
3-Oxoadipate enol-lactone hydrolases	$pcaD_{P.put.}$	$pcaD_{A.cal.}$	17.2	0.828	–[e]
	$pcaD_{P.put.}$	$catD_{A.cal.}$	38.3	0.617	1.30
	$pcaD_{A.cal.}$	$catD_{A.cal.}$	41.8	0.582	1.12
Cycloisomerases	*tfdD*	*clcB*	35.2	0.648	1.50
	tfdD	*tcbD*	35.7	0.643	1.46
	clcB	*tcbD*	44.4	0.556	1.01
	tfdD	$catB_{P.put.}$	32.2	0.678	1.76
	tfdD	$catB_{A.cal.}$	27.2	0.728	2.65
	clcB	$catB_{P.put.}$	48.7	0.513	0.86
	clcB	$catB_{A.cal.}$	35.1	0.649	1.50
	tcbD	$catB_{P.put.}$	38.9	0.611	1.26
	tcbD	$catB_{A.cal.}$	37.0	0.630	1.37
	$catB_{P.put.}$	$catB_{A.cal.}$	38.2	0.618	1.30
1,2-Dioxygenases	*tfdC*	*clcA*	34.5	0.655	1.55
	tfdC	*tcbC*	33.3	0.667	1.65
	clcA	*tcbC*	41.5	0.585	1.14
TfdF, TcbF	*tfdF*	*tcbF*	25.9	0.741	3.32
Regulatory proteins	*clcR*	*tcbR*	27.3	0.727	2.61

[a] The *tfd* genes are encoded by pJP4, the *clc* genes by pAC27, and the *tcb* genes by pP51. For references and the alignment of the hydrolase and the cycloisomerase sequences, see the legends to Tables 1 and 2. The chlorocatechol 1,2-dioxygenase sequences (Frantz & Chakrabarty 1987; Perkins et al. 1990; Van der Meer et al. 1991b) were compared when aligned as shown by Eck & Belter (1993). TfdF and TcbF (same references) as well as TcbR and ClcR (Van der Meer et al. 1991c; Coco et al. 1993) could be aligned without the introduction of gaps. In all cases, DNA sequences were aligned to fit the corresponding amino acid sequences.
[b] Ratio of identical bases (%) in the third triplet position of the four codon families TCN-serine, CTN-leucine, CCN-proline, CGN-arginine, ACN-threonine, GTN-valine, GCN-alanine, and GGN-glycine (determination according to Harayama et al. 1987).
[c] Calculated by subtracting the ratio of identical positions from 100%.
[d] Correction for multiple substitutions at the same site: $K_s^{corr} = -(3/4)\ln[1-(4/3)K_s]$ (Kimura & Ohta 1972). Note that for many of the K_s values ($K_s > 0.55$) this formula underestimates the frequency of substitutions that have taken place (Takahata & Kimura 1981; Miyata et al. 1982).
[e] The formula does not allow to correct K_s values ≥ 0.75.

induction also points to the natural function of this gene cluster.

Considering all this evidence it is difficult to escape the conclusion that the postulated ancestral operon evolved for the purpose of chlorocatechol or possibly bromocatechol degradation.

Chlorocatechol catabolism: An old pathway for the degradation of natural compounds or a recent answer to man-made pollution?

If the chlorocatechol degradative pathway were just a low specificity variant of catechol degradation, then it would be reasonable to consider its evolution as a relatively recent event (Coco et al. 1990), an answer to anthropogenic environmental pollution (Ghosal et al. 1985). Such a view was, for example, the basis for the experimental strategy which resulted in the isolation of *Pseudomonas* sp. B13 (Dorn et al. 1974); it

was assumed that a 3-chlorobenzoate degradative pathway could evolve de novo from benzoate catabolism within a few months of increasing selection pressure. However, as detailed above, the chlorocatechol pathway is more elaborate than previously acknowledged (substrate specificity, capability for dehalogenation, recruitment of additional genes/enzymes) and an ancient chlorocatechol degradation operon which gave rise to the *clc*, *tfd*, and *tcb* versions must be postulated. This forces us to reconsider the question already raised by Chapman (1976) and Reineke (1984), whether the pathway evolved recently, implying that its function from the beginning was destruction of man-made pollutants, or whether chlorocatechol degradation evolved long ago, its original function being the catabolism of naturally occurring halogenated compounds. Chlorosubstituted and bromosubstituted aromatics have been shown to exist in nature (Faulkner 1980; Fenical 1981; Hager 1982; Strunz 1984; Neidleman & Geigert 1986; Gribble 1992; Naumann 1993), and they may be present in considerable concentrations (Asplund & Grimvall 1991; De Jong et al. 1994) thus providing the selection pressure necessary to account for the evolution of an ancient chlorocatechol degradation operon.

Harayama & Rekik (1993) have recently estimated that the enzymes for *meta*-cleavage pathways of the TOL plasmid pWW0, of NAH7, and of pVI150 have diverged 20–50 million years ago (see also: Harayama et al. 1987). Based on the neutral theory of evolution (Kimura 1968, 1991; King & Jukes 1969), they assumed that those base substitutions which do not result in an altered amino acid sequence are evolutionary almost neutral, because they are subject to no or only a weak selection. Such substitutions can therefore accumulate, an observation made on various genes (Kimura 1977; Jukes 1980; Miyata et al. 1980). The observed frequency of synonymous substitutions (K_s) thus provides a basis to calculate the time of divergence (T) for two homologous sequences, provided that an absolute evolutionary rate (V_s), the substitution frequency per time interval, is known. The relationship is given by the equation: $V_s = -(3/4)\ln[1-(4/3)K_s]/2T$ (Kimura 1977; Miyata et al. 1980). Since the term in front of K_s is meant to compensate for multiple substitutions, the equation can be simplified to $V_s = K_s^{corr}/2T$ or $T = K_s^{corr}/2V_s$, with $K_s^{corr} = -(3/4)\ln[1-(4/3)K_s]$ being the estimated frequency of synonymous substitutions that occurred during divergence. The absolute evolutionary rate V_s of the *Escherichia coli – Salmonella typhimurium* divergence has been reported to be 0.7–0.8%/10^6 years (Ochman & Wilson 1987). This value was estimated by first relating branching points of the phylogenetic tree of bacteria, chloroplasts, and mitochondria to major ecological events in the past, and by then comparing the observed synonymous substitutions of various genes to the time scale thus derived. The absolute evolutionary rate (V_s) can be calculated from generation time and mutation rate, the latter having been estimated to be 0.5×10^{-9} per site per generation for *E. coli* (Drake 1991a). As pointed out by Harayama & Rekik (1993), using this mutation rate, a V_s value of 7×10^{-9} mutations per site per year (Ochman & Wilson 1987) corresponds to a generation time which is in the same range as that obtained from ecological studies for natural environments (Babiuk & Paul 1970).

For genes involved in chlorocatechol catabolism, the ratio of synonymous substitutions was calculated using identities in protein sequences for those amino acids which are coded for by at least four different triplets (Harayama et al. 1987). The data are shown in Table 4. Also included are some comparisons between chlorocatechol genes and their counterparts in catechol catabolism, as well as among catechol catabolic genes from different organisms. Because of the pattern of divergence (see above), for the estimation of the age of the chlorocatechol pathway, the differences between *tfd* genes on the one hand and the corresponding *clc* or *tcb* genes on the other are most relevant. In these comparisons the corrected ratio of synonymous substitutions (K_s^{corr}) averages 2.0 (calculated from data of Table 4). Using $V_s = 7 \times 10^{-9}$ mutations per site and year as the absolute evolutionary rate, this value corresponds to a divergence time of ca. 140 million years. Under the same assumptions the structural *clc* and *tcb* genes diverged ca. 70 million years ago, while for the divergence of corresponding *P. putida* and *A. calcoaceticus* genes ca. 90 million years is obtained (the highest K_s value had to be omitted, because the correction for multiple hits could not be performed). For the latter genera Ochman & Wilson (1987) based on 16S rRNA sequences calculated a divergence time of 600 million years.

Against such calculations the contentions can be raised that (1) synonymous substitutions are not necessarily neutral, and that (2) mutation rates are not constant. The first problem can, for example, be due to preferences in codon usage (Miyata & Hayashida 1981; Ikemura 1981; Sueoka 1988; Sharp et al. 1989), or to effects on mRNA secondary structure (Lawrence et al. 1991). As shown in Table 5 between the *tfd* genes

Table 5. Codon usage and GC content of chlorocatechol and catechol catabolic genes.

Amino acid	Triplet	Average Frequency (%) of Triplets Coding for the Respective Amino Acid[a]				
		pAC27: *clcA, clcB clcD*	pP51: *tcbC, tcbD tcbE*	pJP4: *tfdC, tfdD tfdE*	*P.putida: catB, pcaD*	*A. calcoac: catA, catB, catD, pcaD*
Leu	TTA	3	1	5	0	25
	TTG	21	14	16	8	25
	CTT	8	14	23	8	13
	CTC	16	19	31	9	5
	CTA	3	4	0	0	12
	CTG	50	47	24	75	21
Val	GTT	10	7	19	5	27
	GTC	29	38	35	32	16
	GTA	9	4	8	5	22
	GTG	52	51	37	59	35
Ala	GCT	15	7	15	7	20
	GCC	32	32	18	62	23
	GCA	12	12	20	9	40
	GCG	40	50	47	22	18
Gly	GGT	20	6	35	20	47
	GGC	49	60	26	73	31
	GGA	8	15	22	3	16
	GGG	23	19	16	3	5
GC content[b] (%)		60.9	63.4	56.5	63.5	44.7

[a] The codon usage for the four most frequent amino acids with at least four synonymous codons is illustrated by the frequency with which the triplets code for the respective amino acid (adding up to ca. 100% for each amino acid). For each plasmid or organism an average has been calculated from two, three, or four of those genes which have also been used for the calculation of synonymous differences. For references on the sequences, see the legends to Tables 1 and 2.

[b] The average GC content is shown for the same genes for which the codon usage is presented.

and the corresponding *clc* or *tcb* genes there are some changes in codon usage, which relate to altered GC content of the genes. And while the different codon usage of *P. putida* and *A. calcoaceticus* genes is obvious, there does not seem to be a significant difference in codon preferences between the *clc* and the *tcb* genes. This suggests that a selection pressure on codon usage should not have had a major influence on the frequency of synonymous substitutions among the chlorocatechol catabolic genes compared here.

The second contention mentioned above is certainly more difficult to deal with. The ongoing controversy on the possible existence of directed mutations points to the fact that the mutation rate is not a constant, but that it is dependent on the physiological conditions of the organism (Cairns et al. 1988; Hall 1990; Mittler & Lenski 1990; Drake 1991b; Echols & Goodman 1991; Foster 1993; Koch 1993; MacPhee 1993). Generally, mutation rates appear to be highly regulated so that, despite short term changes, in the long run the mutation rate per genome is nearly invariant among DNA based microorganisms (Drake 1991a), and so that the absolute evolutionary rate of synonymous substitutions (per year) in enteric bacteria is similar to that in animals and plants (Ochman & Wilson 1987). Therefore, bacterial genes should be expected to have a long term mutation rate within certain limits. In the study of Ochman and Wilson the synonymous substitution rate for 22 protein encoding regions from *E. coli* and *S. typhimurium* varied by a factor of 40.

With respect to the chlorocatechol catabolic genes the question then is: Are there specific reasons to

assume a higher than normal mutation rate? The chlorocatechol degradation pathway appears to be usually encoded by plasmids, and these could be expected to evolve more rapidly than chromosomal genes (Eberhard 1990). In addition, the pathway may be expected to have been evolved and maintained in the bacterial population at locations where chloroaromatic compounds were present. These or their metabolites might have had a mutagenic effect, possibly because they acted as chemical stress (Blom et al. 1992) and thus might have increased error-prone replication (Van der Meer et al. 1992). Thus, there certainly is a likelihood that the mutation rate by which the chlorocatechol pathways of pAC27, pJP4, and pP51 diverged, was higher than assumed in the calculation of the divergence time.

However, in order to be relevant for the initial question of whether the original chlorocatechol degradation pathway evolved before or as a result of man-made environmental pollution, arguments for an increased mutation rate would have to account for a factor of 10^6 (to bring the calculated divergence time of 140×10^6 years down to the age of large scale anthropogenic production of chemicals). In the absence of good evidence to the contrary, it must be doubted that this can, in fact, occur. It thus seems, the chlorocatechol degradation pathway may be considerably older than the chemical industry.

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