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MICROBIAL DEGRADATION OF SYNTHETIC RECALCITRANT COMPOUNDS

Prepared for publication by

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Microbial degradation of synthetic recalcitrant compounds

ABSTRACT

Man-made chemicals can often be degraded to varying extents by the natural microflora. Except for simple compounds such as chlorobenzoates, the biochemical and genetic basis of biodegradation is poorly understood. Highly chlorinated compounds, which are not generally subject to rapid biodegradation, may be used as substrates for strains developed under strong selective pressure. Determining the catabolic pathways and defining the regulation of genes involved in degradation is critical for constructing improved bacterial strains capable of enhanced degradation of recalcitrant compounds.

INTRODUCTION

Over the past 30-40 years the microbial consortia have been challenged to evolve metabolic pathways to dissimilate toxic chemicals released into the environment. Numerous synthetic chemicals, including herbicides, pesticides, and polychlorinated biphenyls (PCB's), can be degraded to varying extents by microorganisms isolated from soil and water (1). Although degradation of man-made toxic chemicals does not always lead to detoxification, in many cases the products are less hazardous and are susceptible to further degradation. Factors that adversely affect the biodegradability of compounds have been defined (2). The general rules are: (1) oligomerization increases the recalcitrant nature of a compound; (2) the presence of halogen atoms, nitro and sulfo groups or branched carbon chains decreases biodegradability; and (3) the more side chains on a molecule, the more recalcitrant the compound.

The biochemical processes induced by microorganisms have been categorized into four groups based on the chemical activity and toxic nature of the products (3). These processes are mineralization, the conversion of an organic compound to its constituents and inorganic product(s); detoxification, the conversion of toxicants to innocuous metabolites; cometabolism, the metabolism of compounds without utilization of the substrate as a source of energy; and activation, the conversion of a nontoxic molecule to a toxic one. These chemical conversions have been shown to occur in both aerobic and anaerobic conditions (1). However, due to limited space, only the processes leading to the partial or complete dissimilation of toxic compounds by isolated pure aerobic cultures will be discussed in this review. An overview of our current understanding of the biochemical and genetic basis of biodegradation of synthetic compounds is presented.

METABOLISM OF SYNTHETIC COMPOUNDS

A number of organisms including *Pseudomonas aeruginosa*, *P. putida*, *P. cepacia*, *Alcaligenes eutrophus* etc. are able to metabolize naturally occurring organic, as well as a number of synthetic compounds (4,5). The genes responsible for dissimilation of these compounds are often located on plasmids as indicated in Table 1. The transmissible nature of plasmids may lead to a rapid spread, to the microbial consortia, of genes encoding metabolic enzymes

TABLE 1. Representative Naturally Occurring Degradative Plasmids in *Pseudomonas*

Plasmid	Degradative pathway	Size (kilobase pairs)	Reference ^a
CAM	Camphor	500	(9)
OCT	Octane, decane	500	(10)
SAL1	Salicylate	85	(11,12)
NAH	Naphthalene	83	(12)
TOL	Xylene, toluene	117	(13)
NIC	Nicotine, nicotinate	Unknown	(14)
pRA500	3,5-Xylenol	500	(15,16)
pCITI	Aniline	100	(17)
PEG	Styrene	37	(18)
pBS271	ϵ -Caprolactam, ϵ -aminocaproic acid	500	(19)
pCS1	Parathion hydrolysis	68	(20)
pJP2	2,4-Dichlorophenoxyacetic acid	58	(21)
pJP4	2,4-Dichlorophenoxyacetic acid	83	(21,22)
pWR1	3-Chlorobenzoic acid	111	(23,24)
pAC25	3-Chlorobenzoic acid	117	(24)
No designation	2,6-Dichlorotoluene	96	(25)

^aAlthough a number of plasmids encoding degradation of compounds such as xylene/toluene and naphthalene are known, only those most extensively studied are named.

required to degrade man-made toxic compounds (6). Evidence supporting this proposal includes the high degree of homology seen between a number of degradative plasmids (7,8), just as many antibiotic resistant plasmids are homologous to one another. There are a number of metabolic pathways that can be utilized for the biological transformation of synthetic compounds (3). The enzymes involved in the catalysis of many of these herbicides and pesticides can generally be grouped into two classes. There are hydrolases, such as esterases, amidases, and halohydrolases, which mediate the transfer of chemical groups to water; and oxidoreductases, consisting of dehydrogenases and oxygenases, which require NAD⁺ or NADP⁺ as hydrogen acceptors. This section will discuss the enzymatic reactions involved in dissimilating a number of synthetic compounds, by a variety of microorganisms.

Hydrolytic cleavage of chlorinated aliphatic acids (Fig. 1), such as trichloroacetic acid, can be utilized by pure cultures of *Moraxella* sp. strain B and *Pseudomonas putida* PP3 (26,27) as their sole carbon source. Trichloroacetic acid (TCA) and dalapon (2,2-dichloropropionic acid) are degraded via hydrolytic dechlorination to yield saturated aliphatic acids, which

Group	Example	Structure
A. Chlorinated Aliphatic Acids	Dalapon	$\text{CH}_2\text{CCl}_2\text{COOH}$
B. N-Substituted Carbamates	Carbofuran	
C. Thiocarbamates	EPTC	$\text{CH}_3\text{CH}_2\text{S}=\text{C}(\text{N}(\text{C}_2\text{H}_5)_2)$
D. Urea Herbicides	Diuron	
E. S-Triazines	Atrazine	
F. Organophosphates	Parathion	
G. Phosphonate Herbicides	Glyphosate	
H. Halogenated Aromatics		
phenols	PCP	
benzoic acids	3-Chlorobenzoate	
phenoxyalkanoic acids	245 T	
biphenyls	PCB 2, 4, 5, 2', 3' pentachlorobiphenyl	

Fig. 1. Representative groups and examples of synthetic compounds used as herbicides and pesticides, as well as industrially useful compounds.

are readily degraded. Several 2-haloaliphatic acid dehalogenases have been purified and characterized (28,29) from *Pseudomonas* and at least one appears to be encoded on a transposable element within the chromosome (30). Other enzymes, such as the haloacetate halide-hydrolase, from *Moraxella*, however are plasmid-encoded (26,31). Not all chlorinated aliphatics are degraded by hydrolysis as described for the dissimilation of trichloroethylene (32). Degradation of TCE, by the bacterium strain G4, can be activated by intermediates of aromatic biodegradation, which utilizes oxygenases. Indeed, the toluene dioxygenase from *P. putida* F1 catalyzes the degradation of TCE (33).

The hydrolytic cleavage of the carbamate linkage of N-substituted carbamates (Fig. 1), used as herbicides and pesticides, at the amide or ester linkage completely neutralizes these compounds. Several researchers (34,35,36) have reported on the degradation of carbamate herbicides and pesticides by pure cultures. Hydrolytic enzymes, purified from *Pseudomonas striata* (34) act on a wide range of N-phenylcarbamate herbicides including propham and chloropham. N-methyl carbamates, such as carbofuran may be utilized as a nitrogen source, as shown by a culture of *Achromobacter* that encodes a microbial hydrolase (35,36). Related to the N-substituted carbamates are the thiocarbamates (Fig. 1), which have a sulfur atom at the ester oxygen. EPTC, a herbicide, and a member of this group can be metabolized as a sole carbon source by an *Arthrobacter* sp. (37). A plasmid within this strain was shown to be required for degradation. Other related compounds include the substituted urea herbicides (Fig. 1), which have an amide bond that is easily hydrolyzed by enzymes, such as the arylacylamidase isolated from *Bacillus sphaericus* (38).

One of the most important group of herbicides are the s-triazines (Fig. 1). Microbial degradation of atrazine, a commonly used herbicide, has been described (39,40,41,42). A recent article reports on the isolation of several *Pseudomonas* species capable of dealkylating and dechlorinating atrazine, while utilizing it as a carbon source (43). A partially purified dechlorinating hydrolase from *Rhodococcus corallinus* was shown to dechlorinate several s-triazines (44).

A fairly well characterized hydrolase is parathion hydrolase, which is involved in the metabolism of the insecticide parathion (0,0-diethyl 0-nitrophenyl phosphate) (Fig. 1) and related organophosphates (45). A *Flavobacterium* sp. (46) and *Pseudomonas diminuta* MG (47) carry degradative plasmids (47,48) that encode the genes responsible for metabolism of this insecticide. The phosphotriesterase isolated from the *Flavobacterium* also hydrolyzes the pesticide coumaphos (0-0-diethyl-0-3-chloro-4-methyl-2-oxo-2H-1-benzopyran-7-yl phosphate, ref. 49). The *Flavobacterium*, however, cannot open the benzene ring as it can with diazinon and parathion.

There are at least two degradative pathways for the dissimilation of the widely used broad-spectrum phosphonate herbicide glyphosate (Fig. 1). One pathway, as exemplified by the isolated *Pseudomonas* sp. PG2982 (50), proceeds by the cleavage of the C-P bond, giving rise to the formation of sarcosine (51). The second pathway metabolizes glyphosate by hydrolysis to aminomethylphosphonic acid (AMPA), which is then subsequently mineralized. A *Flavobacterium* sp. (52) as well as several *Arthrobacter* sp. (53) are capable of utilizing this pathway.

Very little information is available on the mechanism of penta chlorinated phenol (PCP) aerobic metabolism (Fig. 1). Recently, a *Flavobacterium* strain (54) and a *Rhodococcus* strain (55) have been isolated, which can use PCP as a sole carbon source. *Rhodococcus chloro-phenolicus* PCP-1 attacks pentachlorophenol producing tetra- or trichlorohydroquinone intermediates via *para*-hydroxylation (55). It was shown that the oxygen molecule for the *para*-hydroxylation was derived from H₂O, however this reaction only proceeded in the presence of molecular oxygen.

Generally, methylated and halogenated aromatics (Fig. 1), including polychlorinated biphenyls, are dissimilated via oxygenases (56). However, nucleophilic attack catalyzed by dehalogenases has also been documented. Lingens and colleagues (57,58) have isolated a 4-chlorobenzoate dehalogenase, from *Pseudomonas* strain CBS3, that not only dehalogenates 4-chlorobenzoate, but also converts nitrochlorobenzoates to nitrophenols. In the more typical electrophilic reaction, bacteria transform aromatics into dihydroxyderivatives, such as substituted catechols, which serve as substrates for oxygenolytic cleavage of the aromatic ring (59). Chlorocatechols are generally metabolized by *ortho* cleavage pathways (as described in the following sections), whereas methylcatechols follow a *meta* cleavage pathway. Substrates may be misrouted down a cleavage pathway resulting in the accumulation of toxic intermediates as described by Rojo *et al.* (59).

Chlorobenzoic acids (Fig. 1) have been shown to be degraded by several *Pseudomonas* strains as well as cometabolized by *Acinetobacter calcoaceticus* strain BS5 (60). The genes for the metabolism of the chlorocatechol intermediate are plasmid-encoded (Fig. 2). The genetics for chlorobenzoate degradation are discussed in a following section. The strain WR1306 (61) isolated from a chemostat is capable of metabolizing chlorobenzene, as its sole carbon source, through a pyrocatechol (catechol) intermediate. Chlorophenoxy herbicides (Fig. 1),

such as 2,4-D (2,4-dichlorophenoxyacetic acid) and MCPA (4-chloro-2-methylphenoxyacetic acid) are degraded by a number of organisms. Bacteria including *Pseudomonas* sp., *Arthrobacter* sp., *Mycoplana* sp. and *Flavobacterium peregillum* are capable of cleaving the ether linkage between the oxygen and the aliphatic side chain of 2,4-D to form glyoxylate and 2,4-dichlorophenol (61). The dichlorophenol is then metabolized through the β -ketoacid pathway (3-oxoadipate) pathway (Fig. 3). The genes responsible for this degradation have been found to be encoded on several different plasmids, such as pJP2 and pJP4 (21). Similarly, MCPA is metabolized by *Pseudomonas* sp. NCLB9340 and *Arthrobacter* sp. (60), through an oxidative cleavage. The phenoxyacetic herbicide 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), however is metabolized through a different pathway as described in a subsequent section. Other aromatic compounds that are metabolized through catechol intermediates include the chlorinated anilines. Herbicides such as monuron, diuron, lonuron, and propanil are members of this group. Depending on the structure of the chlorocatechol intermediate, dissimilation proceeds via the *meta* or *ortho* pathway (60).

Polychlorinated biphenyls (PCB'S) (Fig. 1) have been used widely in industrial applications because of their thermal stability and excellent dielectric properties. There are 209 possible congeners of PCB'S containing 1 to 10 chlorine atoms per biphenyl molecule. Aroclors (Monsanto) and Kanechlors (Kanehafuchi) contain 60 to 80 congeners, therefore posing a difficult challenge for microbial degradation. Nonetheless, recent studies have indicated that aerobic and anaerobic transformations do occur (62) and several studies have led to isolation of microorganisms capable of degrading a number of PCB derivatives (reviewed in reference 63). Furukawa et al. (64) demonstrated that *Acinetobacter* sp. strain P6 selectively degrades congeners containing one to four chlorines. Bedard et al. (65) have isolated a diverse group of PCB degrading bacteria including a *Corynebacterium* sp., various pseudomonads, an *Alcaligenes faecalis*, and *Alcaligenes eutrophus*. There appears to be two pathways for the aerobic dissimilation of PCB'S. The principle route involves a 2,3-dioxygenase attack at an unsubstituted 2,3 (or 5,6) position (63,66), exemplified by the *Corynebacterium* strain MBL (67). An alternative route is used by *A. eutrophus* H850, which utilizes a 3,4-dioxygenase (67). These organisms differ in their substrate specificity. For example, MBL is able to partially degrade 4,4'-dichlorobiphenyl, whereas H850 shows only poor activity on this congener. Another organism *P. putida* LB400 is capable of degrading congeners containing up to six chlorines (68). LB400 apparently has both dioxygenase activities. The genes for the first four enzymes of the 2,3-dioxygenase pathway, which allows LB400 to utilize biphenyl as a sole carbon source, have recently been cloned (69). It is presumed that these genes are responsible for the metabolism of a variety of PCB congeners. 2,3-Dihydroxybiphenyl dioxygenases from biphenyl-degrading *Pseudomonas pseudoalcaligenes* and *Pseudomonas aeruginosa* have recently been purified and shown to require a iron (II) as a prosthetic group (70). This enzyme catalyzes the third metabolic step of biphenyl degradation, the ring opening of 2,3-dihydroxybiphenyl. The chromosomal gene encoding the estradiol dioxygenase has been cloned (71) and sequenced (72) from *Pseudomonas pseudoalcaligenes*.

GENETICS AND MOLECULAR BIOLOGY OF THE DEGRADATION OF CHLORINATED COMPOUNDS

As discussed previously, a large number of synthetic chemicals are degraded by the natural microflora leading to their partial or complete utilization. While in most cases, the biochemical or genetic basis of such degradation is not well understood, in a few cases the biodegradative pathways have been well delineated and the organization and regulation of the respective genes carefully studied. For non-chlorinated synthetic compounds such as parathion, it has been mentioned before that the hydrolytic enzyme is encoded by a plasmid in *P. diminuta* MG as well as by a different plasmid in a *Flavobacterium* species, although the *opd* gene specifying the hydrolase enzyme appeared to be highly homologous, and presumably identical in both of the plasmids (47,48). Many chlorinated aliphatic compounds, such as the chlorinated alkanic acids, which are toxic, are also known to be biodegradable (30,31). In some cases the dehalogenase genes involved in the metabolism of the chlorinated alkanic acids have been shown to be plasmid-borne (26), while in others they have been inferred to be located on chromosomal transposable elements (30).

The most detailed understanding concerning the organization and regulation of the genes specifying degradation of chlorinated compounds has come from studies that involve the degradation of chlorinated aromatic acids. Many chlorinated aromatics have been released into the environment in massive amounts as herbicides and pesticides over a long period of time and it is possible to isolate pure cultures from nature that are capable of utilizing simple chlorinated compounds as their sole source of carbon and energy (1). In at least two cases, *viz.* in the degradation of 3-chlorobenzoic acid (3Cba) and 2,4-dichlorophenoxyacetic acid (2,4-D), the genes specifying the degradative pathways have been shown to be plasmid-borne (1,22), while in the case of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), at least part of the genes appear to be located on the chromosome (73). In addition, there is a plasmid present in the 2,4,5-T degrading *P. cepacia* AC1100, which exhibits homology to the plasmids encoding degradation of 3Cba and 2,4-D, indicating that some plasmid genes may have a role to play in the degradation of 2,4,5-T.

GENETICS OF THE DEGRADATION OF 3-CHLOROBENZOIC ACID

The genes encoding degradation of 3-chlorobenzoate have been characterized to be plasmid encoded in two different *Pseudomonas* species, viz. plasmid pAC27 in *P. putida* (74) and plasmid pWR1 in *Pseudomonas* sp. (75). The two enzymes that are involved in the conversion of 3-chlorobenzoate to 3-chlorocatechol (3-Clc) are chromosomally encoded in *P. putida* and are assumed to be the same needed for growth on benzoate. The plasmid pAC27, on the other hand encodes a chlorocatechol degradative pathway consisting of three enzymes (Fig. 2 and Fig. 3) that allow conversion of 3-Clc to maleylacetate. Presumable reduction of maleylacetate to β -keto adipate (Fig. 2) is mediated by a chromosomally-encoded enzyme and β -keto adipate itself is metabolized by a set of chromosomally-encoded enzymes (4,76).

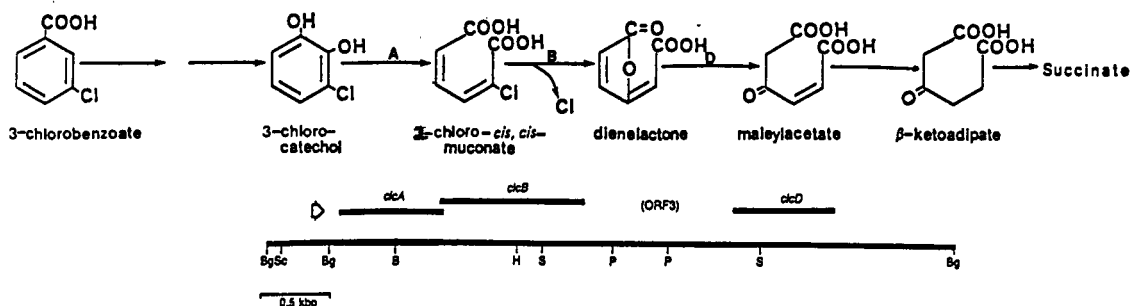


Fig. 2. The biodegradative pathway for 3-chlorobenzoate, showing the three plasmid-encoded genes involved in dissimilation of 3-chlorocatechol. Steps A, B, and D are mediated by chlorocatechol degradative (*clc*) genes *clcA*, *clcB*, and *clcD*, respectively encoding pyrocatechase II, muconate lactonizing enzyme II, and hydrolase II. The location of the promoter is indicated by the arrowhead. The initiation codon of *clcB* overlaps with the stop codon of *clcA*. The restriction sites are as follows Bg, *Bgl*III; Sc, *Sac*II; B, *Bam*HI; H, *Hind*III; S, *Sal*I; P, *Pst*I.

Very little is known about the physical properties of the plasmid pWR1, since it has never been isolated in significant yields. Chatterjee and Chakrabarty (24) demonstrated that the 111 kb pWR1 is highly homologous with the 117 kb pAC25 plasmid, from which pAC27 has been derived due to spontaneous deletion of a 6 kb region. It has subsequently been demonstrated that all the chlorocatechol degradative (*clc*) genes are clustered on a 4.2 kb *Bgl*III fragment of the plasmid pAC27 (Fig. 2). There is an adjoining 385 bp *Bgl*III segment that contains the promoter for the structural genes carried on the 4.2 kb segment (Fig. 2, ref. 77). The complete nucleotide sequence of these two fragments has been determined and the organization of the three genes encoding the three critical enzymes - catechol oxygenase II (Chlorocatechol dioxygenase), cycloisomerase II (chloromuconate cycloisomerase) and dienelactone hydrolase has been delineated (Fig. 2). It should be emphasized that similar enzymes, differing in substrate specificity, are involved in the degradation of the natural, nonchlorinated parental molecule, pyrocatechol (catechol). The catechol degradative enzymes are chromosomally-coded and have very high specificity towards catechol or its metabolites. For example, the relative specificity of catechol oxygenase I (pyrocatechase I), or cycloisomerase I, the chromosomally encoded enzymes involved in catechol degradation, have less than 1% activity to 3-chlorocatechol or 2-chloromuconate (2-chlorolexa-2,4-diendioate (78)). The chromosomally-encoded enol- lactone hydrolase has very little activity towards dienelactone. In contrast, the pAC27-specific pyrocatechase II and cycloisomerase II have broader substrate specificities, and can act on both chlorinated and nonchlorinated catechol and muconate, respectively. Dienelactone hydrolase is, however, quite specific for dienelactone and has no activity towards enol-lactone. The homology detected between benzoate and 3-chlorobenzoate degradative enzymes illustrates one mode of evolution where chromosomal genes are recruited onto a plasmid and subsequently evolve to alter the enzymes' substrate specificity to varying degrees (4). This is in contrast to the type of evolution that occurs under strong selective pressure as seen in the development of the 2,4,5-T degradative enzymes (see below).

The complete nucleotide sequence of the 385 bp and the 4.2 kb *Bgl*III fragments of the plasmid pAC27 revealed four major open reading frames (ORF's) the first initiating 23 bp downstream from the 5' *Bgl*III end of the 4.2 kb segment. N-terminal amino acid sequence analysis of purified catechol oxygenase II, from *E. coli* cells harboring the cloned 4.2 kb segment under the *tac* promoter, agreed with the predicted amino acid sequence from the DNA, and the total amino acid composition of the purified protein agreed with that predicated from the DNA (77). Thus this ORF was designated as the *clcA* gene. The termination codon for *clcA* at base 1182 overlaps the initiation codon of a second major ORF, which by agreement with N-terminal amino acid sequence analysis and the total amino acid composition of the purified protein has been designated as the *clcB* gene, encoding muconate cycloisomerase II. A third major ORF follows *clcB*, with possible initiation codons at bases 2320 and 2464, but no polypeptide for this ORF has been detected in *E. coli* maxicells, nor has any enzyme function

been identified. The N-terminal coding sequence of ORF 4 matched the N-terminal sequence of purified dienelactone hydrolase from *Pseudomonas* sp. B13, and this ORF has therefore been designated as the *clcD* gene (4).

The *clcABD* gene cluster behaves typically as an operon. There is a single promoter for all three genes and no known transcription termination signals in between the genes. In addition, when the operon is placed downstream of the *tac* promoter, in the broad host range plasmid pMMB22, the *clcABD* gene cluster directs the synthesis of all three enzymes in both *E. coli* and *P. putida* only on induction with isopropyl- β -D-thiogalactoside. This suggests that the cluster is regulated as a single unit under the control of a single promoter. The promoter appears to be under positive control, since the 4.2 kb BglIII fragment allows slow growth on 3-chlorobenzoate only on amplification in absence of the activator gene (79). Similar amplification of the corresponding pWR1 plasmid DNA fragment when *Pseudomonas* sp. B13 cell were grown on chlorobenzoate has been reported (80). Preliminary results indicate that the regulatory gene is divergently transcribed upstream of the *clcABD* operon and its protein product induces transcription of the *clcABD* operon in the presence of chlorobenzoate.

GENETICS OF THE DEGRADATION OF 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D)

It has been previously mentioned that a number of plasmids have been reported to specify the degradation of 2,4-D (21). One typical plasmid that has been studied extensively in this regard is pJP4. pJP4 is a P1-incompatibility group plasmid, which encodes resistance to mercury, dissimilation of 2,4-D, and a slow dissimilation of 3-chlorobenzoate. Weightman *et al.* (22) have demonstrated converging pathways for 3-chlorobenzoate and 2,4-D degradation via chlorocatechol, as indicated in Fig. 3. They further demonstrated that the chlorocatechol

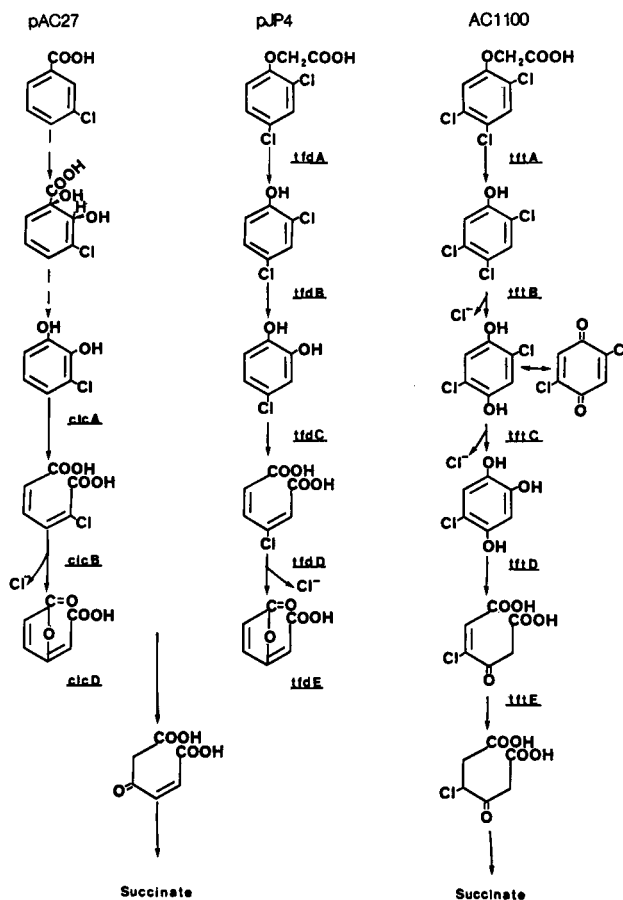


Fig. 3. Pathways for degradation of chlorobenzoate, 2,4-dichlorophenoxyacetic acid and a tentative pathway for 2,4,5-trichlorophenoxyacetic acid. The first two enzymatic steps in chlorobenzoate degradation are chromosomally-encoded, whereas the genes converting 3-chlorocatechol to maleylacetate are plasmid-borne. All 2,4-D degrading genes are plasmid-encoded. It is likely that the genes for 2,4,5-T degradation are located on both plasmids and the chromosomal DNA of AC1100. However, *ttd* has definitely been shown to be chromosomally-encoded (73).

genes in the pJP4 plasmid were clustered in a 3 kb region within the pJP4 *EcoRI* B fragment. Since the chlorocatechol genes are also present in the chlorobenzoate degradative plasmid pAC27, Ghosal et al. (79) hybridized various fragments of plasmid pJP4 with various fragments of plasmid pAC27 and demonstrated that a 10 kb DNA segment, containing three structural genes for chlorocatechol metabolism, present on plasmid pJP4 showed appreciable homology with the 4.2 kb *BglIII* fragment of plasmid pAC27, containing the chlorocatechol genes. In spite of the detectable sequence homology in the structural genes present on both plasmids, the regulation of their expression was quite different. The chlorocatechol genes of pJP4 are normally not expressed efficiently when the cells are grown with chlorobenzoate. Isolation of variants having high rate of growth demonstrated extensive rearrangements in the chlorocatechol gene region, suggesting that regulation of the two pathways is quite different. The location of individual 2,4-D degradative genes (*tfd*) present on the plasmid pJP4 has been reported by Don et al. (81). Transposon mutagenesis has allowed the localization of five genes for enzymes involved in 2,4-D and chlorocatechol pathways. Four of the genes, *tfdB*, *tfdC*, *tfdD*, and *tfdE*, encoded 2,4-dichlorophenol hydroxylase, dichlorocatechol 1,2-dioxygenase, dichloromuconate cycloisomerase, and chlorodienelactone hydrolase, respectively. No function has been assigned so far to *tfdF*. Inactivation of genes *tfdC*, *tfdD*, and *tfdE*, which encode the transformation of dichlorocatechol to chloromaleylacetic acid, prevented the host strain from degrading both 3-chlorobenzoate and 2,4-D, suggesting that the pathways for these two substrates utilize common enzymes for the dissimilation of chlorocatechols. All the 2,4-D degradative genes were found to be present on a 14 kb *EcoRI* fragment. The cloning and nucleotide sequence of the first gene of the 2,4-D pathway viz. *tfdA* encoding 2,4-D monooxygenase has also been accomplished (82). Regarding the location of 2,4-D degradative genes on plasmid pJP2, Ghosal et al. (83) reported the isolation of several 2,4-D+ clones of *P. cepacia* from an *EcoRI* library of plasmid pJP2. Restriction analysis of the recombinant plasmids demonstrated the presence of *EcoRI* fragment A and F of pJP2 in common among all 2,4-D clones, suggesting some essential roles played by these two fragments in 2,4-D degradation. The 16 kb *EcoRI*-A fragment of pJP2 also demonstrated strong homology with pJP4 *EcoRI* fragments B, E, G, and F, which are known to harbor chlorocatechol degradative genes including the regulatory genes. It is thus likely that the *EcoRI*-A fragment of pJP2 harbors the genes encoding the late part of the 2,4-D degradative pathway.

GENETICS OF THE DEGRADATION OF 2,4,5-TRICHLOROPHENOXYACETIC ACID (2,4,5-T)

Pseudomonas cepacia AC1100, developed under strong selective pressure in a chemostat (7), is capable of growth and complete mineralization of the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). AC1100 harbors at least two plasmids, but their role in degradation is not understood. Ghosal et al. (83) showed that one plasmid has a considerable amount of homology to region of the plasmid pJP4, from *Alcaligenes eutrophus* (21), responsible for 2,4-D degradation. This region may contain a gene that encodes the monooxygenase activity required for the conversion of 2,4,5-T to 2,4,5-trichlorophenol (2,4,5-TCP, see Fig. 3). The metabolic pathway for the dissimilation of 2,4,5-T is in the process of being delineated (Fig. 2) by Dr. U. Sangodkar of our laboratory and Dr. P.J. Chapman of the U.S. EPA laboratory at Gulf Breeze. It is interesting to note that the 2,4,5-TCP derived from 2,4,5-T is metabolized through formation of a chlorohydroxy hydroquinone intermediate, rather than a chlorocatechol. In general pseudomonads metabolize chlorinated aromatics via chlorocatechols, as described previously. Degradation through chlorohydroquinone intermediates have only recently been shown in the degradation of PCP by *Flavobacterium* and *Rhodococcus* species (54,55).

A 2,4,5-T degradative gene has been cloned by complementation of a *Tn5* insertional mutation of AC1100, pT88 (73), from a cosmid library carrying DNA fragments of the wild-type AC1100 genome. The *P. cepacia* 2,4,5-T mutant, pT88, accumulates the intermediate 5-chloro-1,2,4-trihydroxybenzene (73). A simple assay was developed for detecting the expression of the enzyme, necessary for metabolism of this intermediate, permitting the cloning of the required gene. Subcloning experiments showed that a 4kb *BamHI*-*PstI* fragment and a 258 bp *PstI*-*EcoRI* fragment, separated by a 1.3 kb distance were required for complementation. The 258 bp fragment is needed for transcription and presumably contains the promoter region. Hybridization studies revealed that both fragments are chromosomal in origin and that the original mutant pT88 has a large deletion in this region. The ancestry of the cloned 2,4,5-T gene has not been identified. This gene shows no homology to any degradative plasmids or to chromosomal DNA from any other *Pseudomonas* strains tested. Another interesting feature of the AC1100 genome is the presence of a repeated sequence, RS1100, which has been sequenced and shown to resemble an insertion sequence (84). RS1100 has recently been demonstrated to be a transposable element, capable of mobilizing intervening DNA between one replicon to another, and having strong outwardly-directed promoter activity for genes at the site of transposition (85). This sequence, although present in *P. cepacia* AC1100, does not show any homology to the total DNA of a large number of pseudomonads examined to date. As with the 2,4,5-T cloned gene the origin of this sequence is unknown. It is interesting to speculate that this repeated sequence was involved in the recruitment of 2,4,5-T degradative genes by a transpositional event from an organism unrelated to pseudomonads. Thus under selective pressure the demand to quickly evolve a new catabolic pathway enables an organism to utilize the gene pool from unrelated organisms.

CONCLUDING REMARKS

The use of synthetic chemicals, while essential in modern industrial society, has created severe problems of environmental pollution that must be recognized and remedied. Microorganisms have evolved the capacity to dissimilate a number of herbicides and pesticides by altering substrate specificity of enzymes, already encoded in the genome, as seen in the evolution of the 3-chlorobenzoate pathway. However, many synthetics, especially those that are highly halogenated, remain recalcitrant to biodegradation. It is possible, nonetheless, to use selective evolution (through use of a chemostat) to develop strains capable of degrading recalcitrant compounds, such as 2,4,5-T. This type of evolution occurs at a much accelerated rate compared to that in nature and is facilitated by the presence of a large gene pool, so organisms can readily take up foreign DNA. It is interesting to note that under selective pressure for 2,4,5-T degradation, a *Pseudomonas* strain evolved. It is possible that pseudomonads in nature are able to evolve rapidly through the uptake of foreign DNA, which is the likely mode for evolution in *P. cepacia* AC1100. This ability can be utilized in a laboratory setting for further development of strains capable of enhanced degradation of toxic, synthetic pollutants in the environment.

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