Ann. Rev. Microbiol. 1988. 42:263–87

MICROBIAL DEGRADATION OF HALOAROMATICS

Walter Reineke

Bergische Universität—Gesamthochschule Wuppertal, Fachbereich 9, Gauss-Strasse 20, D-5600 Wuppertal 1, Federal Republic of Germany

Hans-Joachim Knackmuss

Institut für Mikrobiologie der Universität Stuttgart, Azenbergstrasse 18, D-7000 Stuttgart 1, Federal Republic of Germany

---

CONTENTS

INTRODUCTION

Public concern over the possible effects of chemicals on humans and their environment has largely focused on a few classes of compounds. Of these compounds chlorinated aromatics are the most spectacular. Polychlorinated biphenyls (PCBs) and chlorinated benzenes show the entry points of those chemicals into the environment. PCBs were first produced in 1929 in the United States. Total cumulative world production of PCB has been estimated

0066-4227/88/1001-0263$02.00

at about 750,000 tonnes. Of this total, approximately 60% have gone into closed electric uses (e.g. transformers, capacitors), 15% to nominally closed uses (hydraulic and heat-transfer fluids), and 25% to dispersive uses (plasticizers, paint and printing ink components, adhesives, and additives for cutting oils, textile auxiliaries, and pesticides). It may be deduced therefore that 300,000 t have entered the environment since 1929 in widely disseminated form and that 450,000 t are either still in service or in landfills.

Most chlorinated benzenes are used mainly as intermediates in the synthesis of fine chemicals, and enter the environment as losses and wastes from production sites. As losses are normally 1–2% of raw materials, their total quantities do not exceed a few hundred tonnes per annum. The exceptionable aspects are the dispersive uses of mono- and dichlorobenzenes and waste disposal.

Two types of pollution may result from releases of chemicals into the environment: In *point source pollution* the concentration of the chemical is high; this may occur in landfills, waste dumps, and industrial effluents or at sites of accidents associated with transportation and application of chemicals. In *dispersed pollution* the concentrations are low and result from losses from production sites via volatilization or from agricultural uses. Different strategies are necessary to stem the impact of low concentrations of dispersed chemicals occurring worldwide and the high concentrations of point source pollutants that occur in smaller areas. If a dispersed chemical such as an agrochemical tends to persist, its use and introduction into the environment can only be regulated legislatively. Technological solutions, however, can be applied for more highly concentrated pollutants, e.g. in industrial wastewater and dumps.

In this paper we discuss metabolic pathways elaborated in single microorganisms that are able to grow with haloaromatics as carbon and energy source. These pathways include the major catabolic routes to amphibolic intermediates. Cometabolism, i.e. the widespread ability of microorganisms to catalyze partial transformation to products that do not support growth, is discussed as a minor aspect. The central role of some simple chlorinated benzene derivatives in the degradation of certain chloroaromatic pollutants is schematized in Figure 1, to show the importance of the pathways in the degradation of some pollutants summarized here. The paper closes with some aspects of the application of chlorinated aromatic-degrading bacteria.

BIODEGRADATION MECHANISMS

The biodegradation of a halogenated arene can be considered complete only when its carbon skeleton is converted into intermediary metabolites and its organic halogen is returned to the mineral state. The crucial point is the

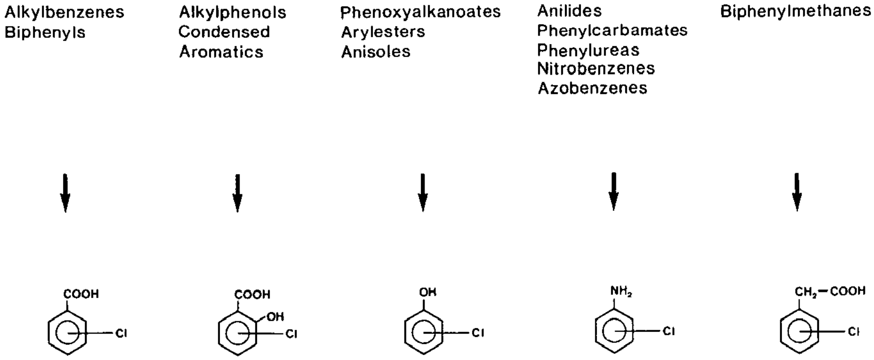

Figure 1 Chlorinated aromatic compounds metabolized to chlorinated aromatic carbonic acids, phenols, and anilines.

removal of halogen substituents from the organic compound. This may occur at an early stage of the degradative pathway with reductive, hydrolytic, or oxygenolytic elimination of the halosubstituent. Alternatively, nonaromatic structures may be generated, which spontaneously lose halide by hydrolysis or hydrogen halide by β-elimination.

Displacement of Halogen Through Hydrogen

Molecular oxygen is required not only as the terminal electron acceptor during respiration, but also for insertion into the aromatic compound during ring-activating hydroxylation and ring cleavage. Microorganisms have of necessity evolved different mechanisms for degradation in the absence of oxygen, including ring fission of aromatic compounds. Although details of the pathways and the enzymes involved are still missing, metabolism of the aromatic ring in the absence of molecular oxygen is now known to proceed in at least five different situations (90): (*a*) through anaerobic photometabolism; (*b*) under nitrate-reducing conditions in mixed cultures and by single strains of *Bacillus* sp., *Pseudomonas* sp., and *Moraxella* sp.; (*c*) with sulfate as electron acceptor; (*d*) in consortia through fermentation coupled to methanogenesis, and (*e*) through fermentation. Anaerobic degradation of aromatic compounds is reviewed by Evans & Fuchs (27a) in this volume.

The first lines of evidence for anaerobic degradation of halogenated aromatics were presented by Horowitz et al (42) and Suflita et al (94). They found that an anaerobic microbial consortium isolated from sewage sludge could degrade a number of *meta*-substituted chlorinated benzoates. The most interesting degradative reaction was the loss of the chloride without the alteration of the aromatic ring. When all the chlorine atoms were successively removed, ring fission led to methane and carbon dioxide. Dechlorination occurred only under methanogenic conditions. Analysis of the kinetics of the dechlorination steps suggested that the dichlorinated parent compound was the preferred substrate and inhibited the dechlorination of the monochlorinated intermediate (95). Shelton & Tiedje (89) recently isolated and characterized organisms in an anerobic consortium that mineralized 3-chlorobenzoate. Based on the organisms isolated there appears to be a three-tiered food chain (Figure 2). The methanogenic consortium consisted of one dechlorinating bacterium, one benzoate-oxidizing bacterium, two butyrate-oxidizing bacteria, two H_2-consuming methanogens (*Methanospirillum hungatei, Methanobacterium* sp.), and a sulfate-reducing bacterium (*Desulfovibrio* sp.). The dechlorinating bacterium converted 3-chlorobenzoate stoichiometrically to benzoate, which accumulated in the medium. The presence of butyrate-oxidizing bacteria and the sulfidogen in the enrichment is unexplained, since they do not appear to be in the main path of carbon flow. The growth substrate of the dechlorinating bacterium in the enrichment is not clear. Presumably, one or more of the organisms in the enrichment were cross-feeding the dechlorinating bacterium. This may offer an explanation for the presence of the butyrate-oxidizing and sulfate-reducing bacteria.

The dechlorination reaction appeared to be enzymatic, since it occurred after induction and because of the low substrate K_m of 67 μM, loss of activity at temperatures above 39°C, and high degree of substrate specificity (*o*- and *p*-chlorobenzoate were not dechlorinated) (43, 95).

Recently, Dolfing & Tiedje (20) established a defined 3-chlorobenzoate-degrading consortium consisting of the key organisms from the above-

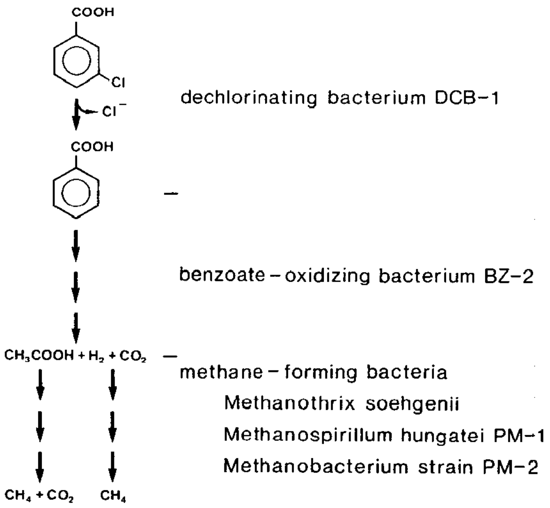

Figure 2 3-Chlorobenzoate-degrading food chain.

mentioned consortium, i.e. the dechlorinating organism (DCB-1), the benzoate degrader (BZ-1), and the lithotrophic methanogen (*Methanospirillum* strain PM-1). The chlorine released from the aromatic ring was recovered in stoichiometric amounts as chloride ion. The reducing power required for reductive dechlorination was obtained from the hydrogen produced in the acetogenic oxidation of benzoate. One third of this hydrogen was consumed via the reductive dechlorination, while two thirds was left to the methanogen.

Reductive dechlorination has also been shown for 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), chlorophenols, and 1,2,4-trichlorobenzene (1,2,4-TCB). A methanogenic consortium grown on 3-chlorobenzoate dechlorinated 2,4,5-T at the *para* position to form 2,5-dichlorophenoxyacetic acid (2,5-D) (96). These microorganisms did not metabolize 2,5-D or several other chlorinated phenoxyacetic acids. However, when 2,4,5-T was incubated in sludge samples the first conversion was to 2,4,5-trichlorophenol (2,4,5-TCP), whereas the initial event observed in pond sediment or methanogenic aquifer samples was dehalogenation (38). The cleavage of the ether bond is consistent with the results of Mikesell & Boyd (63), who found trichlorophenol as the initial metabolic product when 2,4,5-T was incubated in sewage sludge.

Boyd & Shelton and their colleagues (7, 8) investigated the anaerobic degradation of mono- and dichlorophenol isomers by fresh sludge and by sludge acclimated to either 2-, 3-, or 4-chlorophenol. In unacclimated sludge, each of the monochlorophenol isomers was degraded. The rates of disappearance were in the order: *ortho* > *meta* > *para*. For the dichlorophenols, reductive dechlorination of the chlorine-group *ortho* to phenolic OH was observed. The respective monochlorophenol compounds released were subsequently degraded. 3,4-Dichlorophenol (3,4-DCP) and 3,5-DCP were persistent. Specific cross-acclimation patterns were observed for monochlorophenol degradation. Sludge acclimated to 2-chlorophenol (2-CP) cross-acclimated to 4-CP but did not utilize 3-CP. This sludge also degraded 2,4-DCP. Sludge acclimated to 3-CP cross-acclimated to 4-CP but not to 2-CP. This sludge degraded 3,4-DCP and 3,5-DCP but not 2,3-DCP or 2,5-DCP. The data indicated that two unique microbial activities exist that are not present in fresh sludge. The active microbial population in the 4-CP-acclimated sludge appeared to be a mixture of both populations present in the 2-CP- and 3-CP-acclimated sludges, because it was able to degrade all three monochlorophenol isomers and 2,4-DCP and 3,4-DCP. ^{14}C-Labeled 4-CP, 2-CP, and 2,4-DCP were converted to $^{14}CH_4$ and $^{14}CO_2$. Even pentachlorophenol (PCP) was completely dechlorinated by a mixture of the 2-CP-, 3-CP-, and 4-CP-acclimated sludges (64). With repeated PCP additions, 3,4,5-trichlorophenol (3,4,5-TCP), 3,5-DCP, and 3-CP accumulated. All chlorinated compounds disappeared after the PCP additions were stopped.

When sludge was incubated with [^{14}C]PCP, 66% of the added ^{14}C was mineralized to $^{14}CO_2$ and $^{14}CH_4$.

The proposed PCP degradation pathway, based on the sequential appearance and disappearance of 3,4,5-TCP, 3,5-DCP, and 3-CP (Figure 3), appeared to result from the relatively higher rate of PCP dechlorination by the 2-CP-acclimated sludge. This sludge rapidly removed chlorine from positions 2 and 6 of PCP to give 3,4,5-TCP. The *para*-chlorine was then removed by populations present in the 2-CP- or the 4-CP-acclimated sludge or both, which have been shown to dechlorinate this position (8). 3,5-DCP and 3-CP were probably dechlorinated by the 3-CP-acclimated sludge.

Tsuchiya & Yamaha (102, 103) isolated *Staphylococcus epidermidis* from the intestinal contents of rats. Whole cells converted 1,2,4-TCB to *o*-dichlorobenzene, which was further converted to monochlorobenzene. These conversions, which resulted also from *m*- and *p*-dichlorobenzene, proceeded only in a hydrogen atmosphere. Dried and broken cells also maintained the dechlorinating activity, which was stimulated by the addition of NADPH.

Displacement of Halogen by Hydroxyl

The first lines of evidence implicating replacement of chlorine from the aromatic ring through hydroxyl were presented by Johnston et al (48). They isolated a *Pseudomonas* species capable of utilizing 3-chlorobenzoate as the sole carbon source. Manometric studies showed that cells grown on either benzoate or 3-chlorobenzoate oxidized 3-chloro-, 3-hydroxy-, and 2,5-dihydroxybenzoate without a lag period. During growth with 3-chlorobenzoate, 3-hydroxy- and 2,5-dihydroxybenzoate were excreted into the culture medium. From these data the authors proposed a degradative sequence with chloride elimination in the first step and 3-hydroxybenzoate as the reaction product.

In 1975 Chapman (14) isolated *Micrococcus* spp. able to grow with 4-chlorobenzoate. Degradation apparently proceeded via 4-hydroxybenzoate and protocatechuate (Figure 4). The same degradative sequence was also reported for an *Arthrobacter* sp. (80), a *Nocardia* sp. (52), a *Pseudomonas* sp. (53), and *Arthrobacter globiformis* (106, 107). Chlorinated benzoates other than 4-chlorobenzoate did not support growth. The dechlorinating enzymes of the *Pseudomonas* sp. and *A. globiformis* were inducible by 4-chlorobenzoate but not by 4-hydroxybenzoate. The enzymes from the

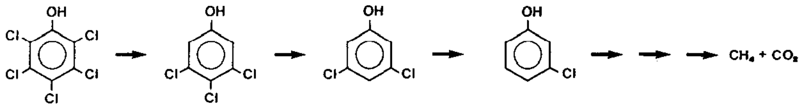

Figure 3 Proposed pentachlorophenol pathway.

Micrococcus sp. and *Pseudomonas* sp. strain CBS3 converted 4-bromobenzoate, whereas 4-fluorobenzoate was not accepted as a substrate. In contrast, an *Arthrobacter* sp. isolated by Marks et al (59) was able to dehalogenate 4-chloro-, 4-fluoro-, and 4-bromobenzoate, although fluorobenzoate did not support growth. Marks et al (59) were able to prepare an active cell extract from this organism, and it converted 4-chlorobenzoate at approximately 5% of the rate observed for whole cells. The dehalogenase activity had an optimum pH of 6.8 and an optimum termperature of 20°C. It was inhibited by dissolved oxygen and stimulated by Mn^{2+}. Unlike the other cell-free aromatic dehalogenase system reported by Klages & Lingens (53), this system was not stimulated by the addition of Fe^{2+}. The apparent Michaelis constant of the cell-free extract for 4-chlorobenzoate was 30 μM. The enzyme also appeared to be highly specific for *para*-substituted monohalobenzoates.

Additional data were recently reported for the dechlorinating enzyme from *Pseudomonas* sp. strain CBS3 (98) partially purified by ammonium sulfate fractionation. The pH optimum was between 7.0 and 7.5. The K_m values for 4-chloro-, 4-bromo-, and 4-iodobenzoate were found to be 0.15, 0.068, and 0.12 mM, respectively. In contrast to the activity reported for the cell-free extract of Klages & Lingens (53), this activity could not be increased through the addition of Fe^{2+}.

The mechanism of the dehalogenation process has recently been clarified by labeling experiments using $^{18}O_2$ and $H_2{}^{18}O$ (60, 67). The data indicate that the dechlorination reaction utilizes water as the hydroxyl donor and not molecular oxygen. The results showed that the enzymatic conversion of 4-chlorobenzoate to 4-hydroxybenzoate proceeds via a hydrolytic cleavage of the carbon-chlorine bond.

Replacement of a chlorine substituent by a hydroxy group has recently also been shown for the degradation of pentachlorophenol by use of mutants of an aerobic, chlorophenol-utilizing *Flavobacterium* sp. (93). The pathway for PCP degradation was initiated by the conversion of PCP to tetrachloro-*p*-hydroquinone. Labeling experiments using $H_2{}^{18}O$ and $^{18}O_2$ demonstrated that the initial dechlorination of PCP proceeded by a hydrolytic displacement of chlorine, rather than by an oxygenase-catalyzed mechanism. Two reductive dechlorinations of tetrachloro-*p*-hydroquinone followed, yielding first trichlorohydroquinone and then 2,6-dichlorohydroquinone. These results are

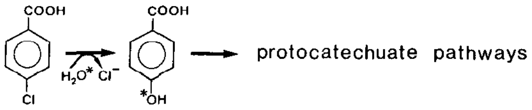

Figure 4 Hydrolytic dechlorination of 4-chlorobenzoate.

in agreement with some pathway intermediates proposed earlier by Suzuki (97) and Reiner et al (79). Chlorine is also hydrolytically displaced from PCP by *Rhodococcus chlorophenolicus* PCP-I (2). However, the novel hydroxy group occurred in position 4 whether or not a substrate had chlorine substituents in this position. This indicates that the enzymes only fortuitously function as a dehalogenating enzyme. The *para*-hydroxylation, although considered a hydrolase reaction, required the presence of molecular oxygen. Further metabolism of the reaction product tetrachloro-*p*-hydroquinone was claimed to proceed under anaerobiosis.

Oxygenolytic Halogen-Carbon Bond Cleavage

Fortuitous dehalogenation by dioxygenases is another mechanism to remove halogen substituents from haloaromatic compounds. Goldman et al (39) reported such oxygenolytic dehalogenation of an arylhalide in investigating 2-fluorobenzoate metabolism by a pseudomonad. This organism metabolized 2-fluorobenzoate by two pathways (65) because nonselective dioxygenation by the benzoate 1,2-dioxygenase generated a mixture of 2- and 6-fluoro-1,2-dihydro-1,2-dihydroxybenzoate (Figure 5). Some 85% of the 2-fluorobenzoate underwent defluorination to produce catechol, which was degraded through the 3-oxoadipate pathway. The other pathway, which accounted for the remaining 15% of the 2-fluorobenzoate utilized by the organism, proceeded through 3-fluorocatechol to 2-fluoro-*cis,cis*-muconate, both of which were isolated from the growth medium. 2-Fluoro-*cis,cis*-muconate was not further metabolized.

The nonenzymatic nature of the elimination of fluorine from 2-fluoro-1,2-dihydro-1,2-dihydroxybenzoate was clearly demonstrated with a mutant of *Alcaligenes eutrophus* that was defective in the dihydrodihydroxybenzoate dehydrogenase (78), but able to use 2-fluorobenzoate as the growth substrate (27). Of the originally covalently bound fluorine, 80% was released as fluoride. The remaining 20% of the substrate utilized was identified as 6-fluoro-1,2-dihydro-1,2-dihydroxybenzoate. As fluorine is a leaving group, it is eliminated spontaneously from 2-fluoro-1,2-dihydro-1,2-dihydroxybenzoate as an anion. Decarboxylation of the resulting β-keto acid gives cate-

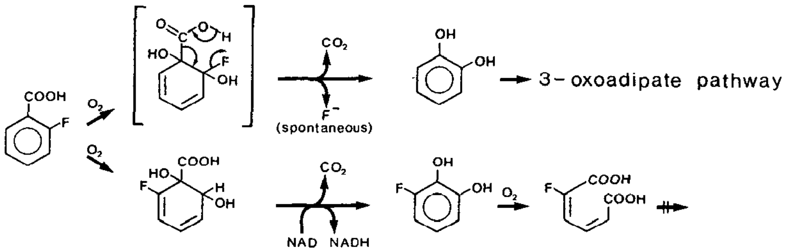

Figure 5 Degradation pathway for 2-fluorobenzoate.

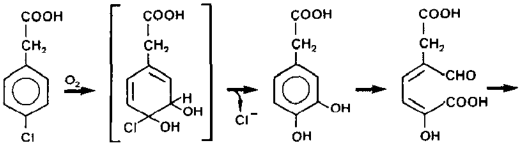

Figure 6 Reaction sequence in the degradation of 4-chlorophenylacetate.

chol. The bacterial defluorination of 2-fluorobenzoate is therefore a fortuitous event. The reaction product of the rearomatization, catechol, can then be degraded by the usual catabolic enzymes of the 3-oxoadipate pathway.

An analogous type of degradation sequence was suggested in the mineralization of 2-chlorobenzoate by *Pseudomonas cepacia* (108).

Oxygenolytic elimination from a *cis*-dihydrodiol produced by dioxygenation probably accounts for the initial dehalogenation of 4-halophenylacetates by *Pseudomonas* sp. strain CBS3 (54). This explanation is in accord with the organism's ability to use phenylacetate, 4-fluoro-, 4-chloro-, and 4-bromophenylacetate as the sole carbon source. Degradation apparently proceeded via homoprotocatechuate, which was further metabolized by a *meta*-cleavage pathway (Figure 6). On fractionation of a crude extract on Sephacryl S-200 the dehalogenating enzyme activity could be separated into two components, which were both necessary for the reaction. The highest activity was obtained in the presence of cofactors Fe^{2+} and NADH (61). One component of this system was purified and characterized (62).

A novel mechanism for expelling halogen by a dioxygenase has been shown recently with a bacterial strain isolated for growth on 5-chlorovanillate (49). Cell suspensions of this strain grown on 5-chlorovanillate released chloride quantitatively and readily oxidized 5-chloroprotocatechuate, a proposed metabolite of 5-chlorovanillate. An inducible protocatechuate 4,5-dioxygenase further converted 5-chloroprotocatechuate. Ring opening is followed by a nucleophilic displacement of chloride from the acylchloride (Figure 7). This reaction does not require an additional cyclizing enzyme to give 2-pyrone-4,6-dicarboxylate. In contrast, this last metabolite arises from the natural compound protocatechuate by the action of the 4,5-dioxygenase

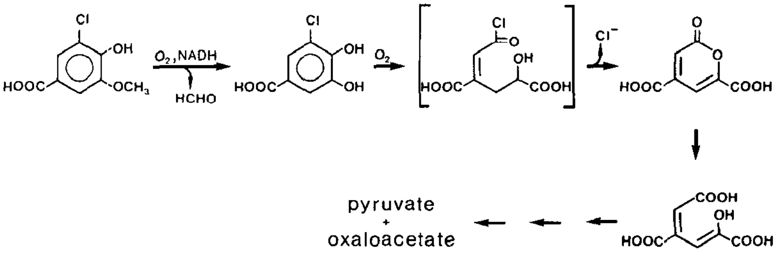

Figure 7 Reaction sequence proposed for the degradation of 5-chlorovanillate.

and further oxidation of the resulting aldehyde by a specific dehydrogenase. Partially purified protocatechuate 4,5-dioxygenase and pure protocatechuate 4,5-dioxygenase from *Pseudomonas testosteroni* showed similar substrate specificities. 5-Chloro-, 5-bromo-, and 5-fluoroprotocatechuate were mainly converted to 2-pyrone-4,6-dicarboxylate.

Chloride Eliminations From Nonaromatic Intermediates

The biochemistry of the biodegradation of chlorinated benzoates, benzenes, anilines, and phenoxyacetates, involving chlorinated intermediates prior to the dechlorination step, has recently been investigated. A common feature of these pathways is the elimination of chloride after *ortho* cleavage of chlorocatechols. Chloride appears to be eliminated spontaneously after the carbon halogen bond has been labilized through isomerases or reductases.

PERIPHERAL ENZYME SEQUENCES GENERATING CHLOROCATECHOLS

Reaction sequences that describe the formation of chlorocatechols from chlorinated aromatics are shown in Figure 8. Various soil bacteria have been reported to cleave the ether linkage of phenoxyacetate and to produce 2,4-dichlorophenol from 2,4-D, 2-methyl-4-chlorophenol from 2-methyl-4-chlorophenoxyacetate (MCPA), and 4-chlorophenol from 4-chlorophenoxyacetate (5, 37, 58). Experiments with resting cells and cell-free extracts from bacteria grown on phenoxyacetate showed that ether bond cleavage requires oxygen (41, 99). Oxygen is incorporated into the side chain so that glyoxylate is released. The enzymatic production of 4-chlorocatechol from 4-chlorophenol and of 3,5-dichlorocatechol from 2,4-dichlorophenol required both oxygen and NADPH (6). 3-Methyl-5-chlorocatechol is the product of hydroxylation of 2-methyl-4-chlorophenol.

Recently, phenol hydroxylases have been purified from 2,4-D–degrading bacteria such as an *Acinetobacter* sp., a *Pseudomonas putida* strain, and *Alcaligenes eutrophus* 335. The 2,4-D plasmid pJP4–encoded hydroxylase is a true 2,4-dichlorophenol hydroxylase because it readily converted 2,4-dichlorophenol, 2,4-dibromophenol, and 2-methyl-4-chlorophenol to the corresponding catechols. 4-Chlorophenol and 2-chlorophenol were hydroxylated at lower rates, and phenol was not hydroxylated (57). The substrate specificity of the pJP4–encoded hydroxylase differed from that of the *Acinetobacter* sp., for which only 2,4-dichlorophenol, 4-chloro-2-methylphenol, and 4-chlorophenol were found to be true substrates. Other substituted phenols evoke the oxidation of NAD(P)H and oxygen consumption without undergoing hydroxylation. Instead, the product is hydrogen peroxide, which suggests that electron flow is uncoupled from hydroxylation in the presence of these compounds (4).

The productive degradation of chloroanilines also proceeds via chlor-

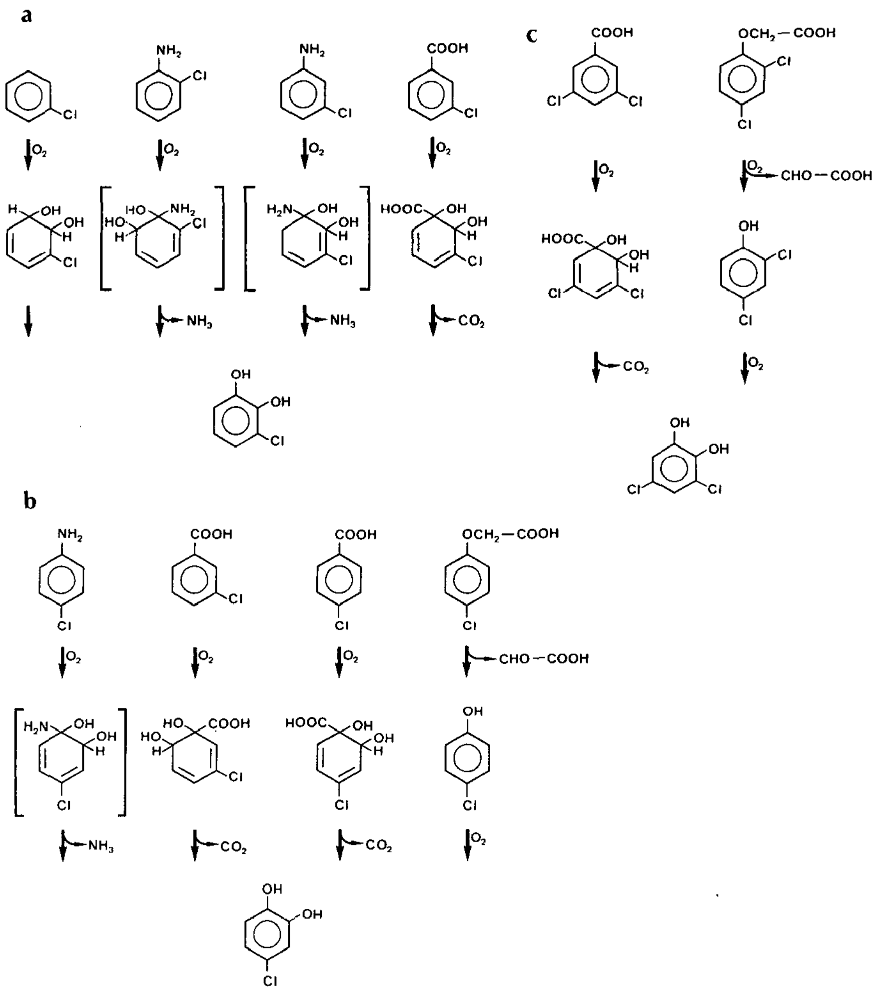

Figure 8 Peripheral sequences generating chlorocatechols; (*a*) 3-chlorocatechol, (*b*) 4-chlorocatechol, (*c*) 3,5-dichlorocatechol.

ocatechols (55, 109, 110). In the catabolism of aniline (3) ammonia is liberated through a dioxygenase reaction. In *Pseudomonas* sp. strain JL4, which uses 2-, 3-, or 4-chloroaniline as the growth substrate, 2-chloroaniline is subject to 1,6-dioxygenation, which yields 3-chlorocatechol (55). This compound is also generated from 3-chloroaniline. Dioxygenation of 4-chloroaniline results in the formation of 4-chlorocatechol. Aniline-utilizing cells of *Rhodococcus* sp. strain An 117, however, hydroxylated 3-chloroaniline in the 1,6 position to give 4-chlorocatechol (85).

Furukawa et al (30–34) have studied the conversion of various isomers of

PCB by *Alcaligenes* sp. and *Acinetobacter* sp. grown on biphenyl. A probable pathway for the degradation is shown in Figure 9. This pathway was proposed based on the detected accumulated metabolites and in analogy to the known pathway for biphenyl (11–13, 101). A *cis*-dihydrodiol is formed and is dehydrogenated to yield a 2',3'-dihydroxybiphenyl; *meta*-cleavage of this intermediate generates chlorinated derivatives of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoates, which are further metabolized, yielding chlorinated benzoates and 2-hydroxypenta-2,4-dienoate.

The reaction sequences involved in the conversion of chlorinated benzoates into the respective catechols proceed via chlorosubstituted 1,2-dihydro-1,2-dihydroxybenzoates as intermediates (72, 73). This conversion has mostly been studied with the 3-chlorobenzoate-utilizing *Pseudomonas* strain B13 and its derivative strains. Nonselective dioxygenation generated a mixture of 3- and 5-chloro-1,2-dihydro-1,2-dihydroxybenzoate; 67% of the 3-chlorobenzoate was degraded through 3-chlorocatechol and the remaining 33% through 4-chlorocatechol (72). The benzoate 1,2-dioxygenase in *Pseudomonas* strain B13 is rather specific; only benzoate and 3-chlorobenzoate were converted at a considerable rate. This enzyme is unable to oxidize benzoates containing a chlorine substituent in the *ortho* or *para* position. In this respect the B13 dioxygenase is similar to that in benzoate-utilizing bacteria of many different genera (1, 10, 18, 44–46, 92, 104, 105). However, other benzoate 1,2-dioxygenases with different regioselectivities have been described. Dioxygenation in the 1,6 position was found with an enzyme from an *Arthrobacter* sp. (44), so that only 4-chlorocatechol resulted from 3-chlorobenzoate. In contrast, a *Pseudomonas aeruginosa* strain accumulated solely 3-chlorocatechol from 3-chlorobenzoate (46). Benzoate 1,2-dioxygenases with considerably relaxed substrate specificity function in derivatives of *Pseudomonas* strain B13 containing the TOL plasmid and in *Pseudomonas* sp. strain WR912 (40, 71, 74, 75). Both 3- and 4-chlorobenzoate and even 3,5-dichlorobenzoate were turned over at a considerable rate, yielding 4-chloro- and 3,5-dichloro-1,2-dihydro-1,2-dihydroxybenzoate and finally 4-chloro- and 3,5-dichlorocatechol, respectively.

The metabolism of chlorobenzene to 3-chlorocatechol was described for

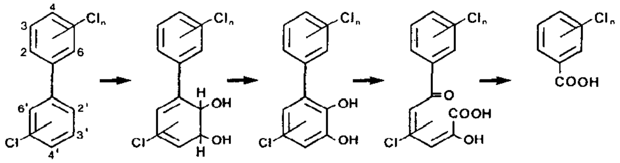

Figure 9 Proposed major metabolic sequences for the degradation of polychlorinated biphenyl (n = 1–4).

strain WR1306 (76). It proceeds via 3-chloro-1,2-dihydro-1,2-dihydroxybenzene as an intermediate. The benzene 1,2-dioxygenase was found to be rather specific, since only benzene, chlorobenzene, and bromobenzene were substrates. Fluorobenzene and dichlorobenzenes were not oxidized. Analogous sequences with a dioxygenation *ortho* and *para* to a chloro-substituent were recently observed for the degradation of 1,3- and 1,4-dichlorobenzene, yielding 3,5- and 3,6-dichlorocatechol, respectively (19, 68, 84, 91).

ASSIMILATION OF CHLOROCATECHOLS In 2,4-D- and 3-chlorobenzoate-degrading bacteria catechol 1,2-dioxygenases were found, which exhibit high activities with mono- and dichlorocatechols (21–23). The products from *ortho*-cleavage of 3- and 4-chlorocatechol were 2- and 3-chloro-*cis,cis*-muconates, respectively (83). For the cycloisomerization of 2-chloro-*cis,cis*-muconate by enzyme preparations from *Pseudomonas* strain B13 grown on 3-chlorobenzoate, Schmidt & Knackmuss (82) proposed 4-carboxychloromethylbut-2-en-4-olide as an intermediate; this intermediate spontaneously generated *trans*-4-carboxymethylenebut-2-en-4-olide by *anti* elimination of hydrogen chloride (Figure 10). Identification of *cis*-4-carboxymethylenebut-2-en-4-olide as the enzymatic cycloisomerization product of 3-chloro-*cis,cis*-muconate suggested 4-chloro-4-carboxymethylbut-2-en-4-olide as an intermediate, from which hydrogen chloride could be released spontaneously by *anti* elimination. Both *cis* and *trans* isomers of 4-carboxymethylenebut-2-en-4-olide were converted into maleylacetate by a 4-carboxymethylenebut-2-en-4-olide hydrolase. Cell-free extracts of B13 grown on 3-chlorobenzoate catalyzed the total degradation of 3,5-dichlorocatechol (86). On the basis of separated enzyme activities, the catabolic pathway of 3,5-dichlorocatechol was proposed to proceed via 2,4-dichloromuconate, *trans*-2-chloro-4-carboxymethylenebut-2-en-4-olide, *cis*-2-chloro-4-carboxymethylenebut-2-en-4-olide, *β*-chloromaleylacetate, and maleylacetate.

Previously Bollag et al (6) used an enzyme preparation that liberated stoichiometric amounts of chloride from 4-chlorocatechol and showed that 4-carboxymethylenebut-2-en-4-olide was the reaction product. Enzymes isolated from an *Arthrobacter* sp. catalyzed the conversion of 4-chloro- and 3,5-dichlorocatechol to 3-chloro- and 2,4-dichloro-*cis,cis*-muconate, respectively (100). Sharpee et al (88) prepared 2-chloro-4-carboxymethylenebut-2-en-4-olide from 2,4-dichloro-*cis,cis*-muconate with a partially purified lactonizing enzyme from an *Arthrobacter* sp. grown on 2,4-D. The 2-chloro-4-carboxymethylenebut-2-en-4-olide and the unsubstituted 4-carboxymethylenebut-2-en-4-olide, the corresponding chlorinated *cis,cis*-muconates, and the chlorocatechols were converted enzymatically and yielded identical products, which were tentatively identified as *β*-chloromaleylacetate and maleylacetate, respectively (100). On the basis of isolated metabolites and induction experiments, Evans et al (29) reported the same maleylacetate pathway for the degradation of 4-chlorocatechol in a pseudomonad capable of utilizing 4-chlorophenoxyacetate. An *ortho*-fission enzyme that converted 3,5-dichlorocatechol into 2,4-dichloro-*cis,cis*-muconate (28) was reported in cell-free extracts of *Pseudomonas* sp. strain NCIB 9340 grown on 2,4-D. Enzymatic lactonization yielded 2-chloro-4-carboxymethylenebut-2-en-4-olide with release of chloride. Further degradation due to a hydrolyzing enzyme gave β-chloromaleylacetate. Degradation of 3-methyl-5-chlorocatechol, the intermediate in MCPA metabolism, was found to proceed through 2-methyl-4-chloro-*cis,cis*-muconate. Further metabolism to 2-methyl-4-carboxymethylenebut-2-en-4-olide followed by hydrolysis gave β-methylmaleylacetate (Figure 10). Sequences involved in the degradation of 3-chloro-, 4-chloro-, 3,5-dichloro-, and 3-methyl-5-chlorocatechol are summarized in Figure 10.

A maleylacetate reductase and a 3-oxoadipate succinyl-CoA transferase from *Pseudomonas* strain B13 grown on 3-chlorobenzoate and strain WR1306 grown on chlorobenzene were found to metabolize maleylacetate to 3-oxoadipate and 3-oxoadipyl-CoA (Figure 11). Enzymes isolated from the *Arthrobacter* sp. converted maleylacetate and β-chloromaleylacetate to succinate (24) by consumption of two equivalents of NADH or NADPH. Be-

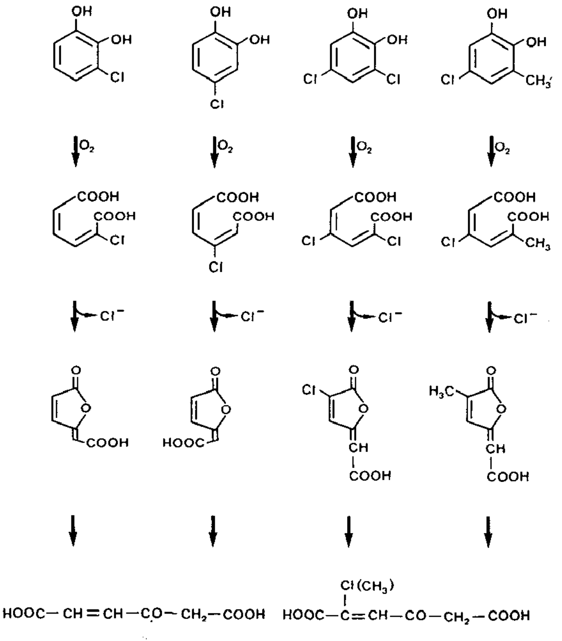

Figure 10 Degradation of chlorocatechols to maleylacetates.

cause succinate was also formed enzymatically from chlorosuccinate, Duxbury et al (24) hypothesized that β-chloromaleylacetate is degraded via 2-chloro-4-oxoadipate and chlorosuccinate to produce succinate (Figure 11). Succinate was also found as a product of 2,4-D when ring-labeled 2,4-D was metabolized by a soluble enzyme preparation (100).

Chapman (15) proposed an alternative mechanism (Figure 11) for the degradation of β-chloromaleylacetate. He observed that a maleylacetate reductase that normally funtions in the degradation of resorcinol (16) was able to convert β-chloromaleylacetate to 3-oxoadipate and chloride. In accordance with this mechanism, two moles of reduced nicotinamide nucleotide were consumed per mole of substrate.

In *Pseudomonas* strain B13 and derivative strains, catechol and chlorocatechols were assimilated via two separate *ortho*-cleavage pathways. Correspondingly, two types of isofunctional enzymes for ring fission were found. Pyrocatechase type I, highly specific for catechol, was present in cells grown on benzoate. This enzyme, together with the isofunctional enzyme pyrocatechase type II, which exhibited relaxed specificities and high activities for the chlorosubstituted substrates, was induced when 3-chlorobenzoate was the growth substrate (22, 23). As has been shown with derivatives of strain B13 harboring the TOL plasmid, the relative amounts of these two isoenzymes induced are dependent on the mode of chlorine substitution of the aromatic growth substrate (75). Cell-free extracts from cells grown on 4-chloroben-

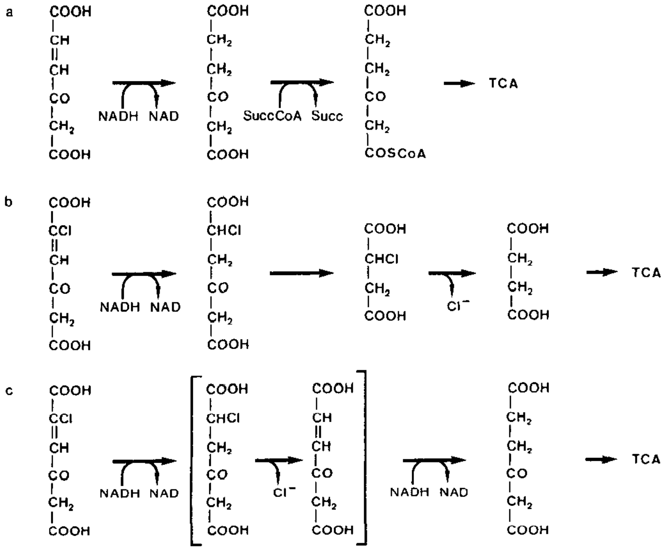

Figure 11 Proposed pathways for the degradation of maleylacetate (*a*) and β-chloromaleylacetate (*b* and *c*).

zoate contained considerably higher amounts of pyrocatechase type II than extracts from cells grown on 3-chloro- or 3,5-dichlorobenzoate. Both types of *ortho*-cleaving enzymes were also induced in chloroaniline-degrading derivatives of *Pseudomonas* strain B13 (55) and the chlorophenol-degrading *Alcaligenes* strain A7-2, which had acquired the genes encoding the maleylacetate pathway from strain B13 (87).

Two types of isofunctional enzymes were also found for cycloisomerization of *cis,cis*-muconate and *cis,cis*-chloromuconates. Cycloisomerase type I, functioning in the 3-oxoadipate pathway, is highly specific for *cis,cis*-muconate, whereas cycloisomerase type II has high activity for *cis,cis*-muconate, 2-chloromuconate, and 3-chloromuconate (82). In contrast to the ring-fission enzymes, cycloisomerase type I is only induced in *Pseudomonas* strain B13 grown on benzoate and cycloisomerase type II is only induced in B13 grown on 3-chlorobenzoate.

Cycloisomerization of *cis,cis*-muconate gave 4-carboxymethylbut-2-en-4-olide, while 4-carboxymethylenebut-2-en-4-olides were formed from chloro-substituted *cis,cis*-muconates. The latter butenolides resulted from chloride elimination that occurred during or after cycloisomerization. The subsequent enzymes in the maleylacetate pathway cannot function in the 3-oxoadipate pathway because of the different reduction states of the respective metabolites (82). The 4-carboxymethylbut-2-en-4-olide was further transformed by an isomerase to 4-carboxymethylbut-3-en-4-olide prior to delactonization. The delactonizing enzymes in both pathways forming the dicarboxylic acids are not isoenzymes because they exhibit no metabolic cross-activities (82). 4-Carboxymethylenebut-2-en-4-olide hydrolase, which was only induced during growth on 3-chlorobenzoate, was highly specific for its substrate and exhibited no activity for 4-carboxymethylbut-3-en-4-olide, the corresponding metabolite of the 3-oxoadipate pathway. Similarly, 4-carboxymethylbut-3-en-4-olide hydrolase did not accept 4-carboxymethylenebut-2-en-4-olide as a substrate.

3-Oxoadipate is a common metabolite of the normal *ortho* pathway, the resorcinol pathway (35, 36), and the modified *ortho* pathway of chlorocatechol and 3,5-dichlorocatechol assimilation. Maleylacetate reductase functions in the convergence of these pathways by introducing reduction equivalents into metabolites of the more highly oxidized substrates, resorcinol and chlorocatechols.

ISOLATION AND APPLICATION OF HALOAROMATIC-DEGRADING BACTERIA

Isolation of Haloaromatic-Degrading Strains

In four billion years microorganisms have evolved an extensive range of enzymes, pathways, and control mechanisms in order to be able to degrade a wide array of naturally occurring aromatic compounds. In contrast, the list of pure, biochemically well characterized cultures able to grow at the expense of haloaromatics is short. Haloaromatic-assimilating microbial strains have been obtained by (*a*) enrichment from nature, (*b*) in vivo genetic manipulation, and (*c*) in vitro genetic engineering.

The theory of enrichment culture is simple. The haloaromatic compound to be degraded is supplied as the growth-limiting and usually sole source of an essential nutrient in a culture medium. Of the many organisms added at the start of the experiment, only those with the necessary degradative ability will grow significantly under these conditions. Enrichment cultures grown on chloroaromatics often require several months for isolation. This indicates that besides selection genetic events might be involved. For the degradation of some chlorinated aromatics natural gene exchange has to occur.

An important prerequisite for the construction of bacterial strains capable of degrading novel chlorinated aromatic compounds is the recognition and acquisition of genes coding an enzyme sequence able to convert chlorinated aromatics to the respective chlorocatechols. Genes allowing total degradation of chlorocatechols are borne on some transmissible plasmids (summarized in 70).

The first report of in vivo construction of a catabolic pathway for the mineralization of chlorinated aromatics using external genetic information for the acquisition of a novel phenotype described work with *Pseudomonas* strain B13 and *Pseudomonas putida* mt-2 and the novel growth substrate 4-chlorobenzoate. Strain B13 was isolated by enrichment culture with 3-chlorobenzoate. It oxidizes 3-chlorobenzoate to 3- and 4-chlorocatechol (see above) and uses the maleylacetate pathway for further breakdown. Strain B13 is unable to utilize 4-chlorobenzoate, since the benzoate 1,2-dioxygenase has a very narrow specificity and will not accept 4-chlorobenzoate as a substrate. However, strain B13 can oxidize 4-chlorocatechol, the expected metabolite in the degradation of 4-chlorobenzoate. The benzoate 1,2-dioxygenase in *Pseudomonas putida* mt-2 determined by the TOL plasmid has a broader specificity than the B13 enzyme and can accept 4-chlorobenzoate as a substrate (72).

The primary transconjugant from the mating between strains mt-2 and B13 grew on 3-chlorobenzoate but not on 4-chlorobenzoate, although it was able to use *m*-toluate, a substrate for the enzyme sequence determined by the TOL plasmid. Transfer of the TOL plasmid from *Pseudomonas putida* mt-2 to *Pseudomonas* sp. strain B13 was not sufficient to produce a strain that could utilize 4-chlorobenzoate for growth, even though this compound is oxidized by the TOL-encoded benzoate 1,2-dioxygenase. However, derivatives of *Pseudomonas* strain WR211 such as WR216 were obtained by spontaneous mutation on plates containing 4-chlorobenzoate. These strains had lost the ability to grow on *m*-toluate because of an insertion found in the catechol 2,3-dioxygenase gene, *xylE* (47).

Chatterjee & Chakrabarty (17) followed the same procedure using the TOL plasmid and the plasmid pAC25, which codes the chlorocatechol-degrading enzyme sequence, to isolate strains that could grow on 4-chlorobenzoate and 3,5-dichlorobenzoate. In addition to the transfer of external genetic information, mutational events have to occur before a novel compound can be used as a growth substrate and misrouting of 4-chlorocatechol can be avoided. Several authors have described similar experiments in which the range of substrates utilized by bacterial isolates has been extended.

Schwien & Schmidt (87) transferred the genes coding the maleylacetate pathway from *Pseudomonas* strain B13 to *Alcaligenes* strain A7, which is able to grow on phenol. The transconjugant *Alcaligenes* strain A7-2 could utilize all three isomeric chlorophenols, which are not attacked by any of the parent strains. A similar transfer to *Pseudomonas* strain WR401, which is capable of growing on methylsalicylate but not on chlorosalicylates, created transconjugants that could use the latter compounds for growth (77). Transfer of the genes coding the maleylacetate pathway to an aniline-degrading *Pseudomonas* strain, JL1, allowed the isolation of chloroaniline-degrading bacteria (55). The manifestation of the same catabolic sequence of strain B13 in *Pseudomonas putida* F1 (degrading benzene) led to strains that could use chlorobenzene as the sole source of carbon and energy (68).

The strategy of combining genes of separate pathways to produce hybrid pathways could also be applied to the use of cloned catabolic genes. This approach has some advantages over conventional crosses for the construction of strains with novel catabolic properties. (*a*) Specific and well-characterized cloned DNA fragments can be used. (*b*) The use of DNA fragments carrying only essential genes avoids the introduction of unproductive enzymes and the consequent need for inactivation of the corresponding genes by mutation. However, this approach has only been shown for the degradation of chlorosalicylate and chlorobenzoate. Lehrbach et al (56) cloned the *xylD* gene alone and the *xylD* and *xylL* genes together (encoding the toluate 1,2-dioxygenase and the dihydrodihydroxytoluate dehydrogenase) into *Pseudomonas* strain B13 to extend the chlorinated benzoates utilized. The introduction of the cloned *xylD* gene alone allowed strain B13 to degrade 4-chlorobenzoate, while the introduction of both the cloned *xylD* and *xylL* genes, followed by spontaneous mutational divergence, resulted in emergence of B13 variants that could also degrade 3,5-dichlorobenzoate. Lehrbach et al (56) also cloned the naphthalene-degradative gene *nahG*, which encodes a broad-specificity salicylate hydroxylase (66), from the plasmid NAH7 and introduced the cloned gene into strain B13. Expression of *nahG* enabled B13 to completely mineralize novel substrates such as chlorosalicylates.

Application of Degradative Strains

Certain xenobiotics, particularly those with polychlorinated aromatic rings, are not known to be growth substrates but are nevertheless subject to cometabolism. Recent work (9) demonstrated that degradation of PCB in soil was not enhanced by addition of a PCB-cometabolizing, biphenyl-utilizing *Acinetobacter* strain. Enhancement of both substrate disappearance and mineralization was only brought about by the addition of the substrate analog, biphenyl. This substrate-analog enrichment selectively increased the number of biphenyl degraders and thereby the cometabolic activity within the indigenous microflora. Upon depletion of the (co)substrate the number of biphenyl oxidizers declined exponentially.

Xenobiotics present in soil at rather high concentrations may be mineralized by laboratory strains that grow at the expense of the contaminant. Edgehill & Finn (26) have shown that soil was more rapidily cleared of the wood-preserving chemical PCP if inoculated with specialized *Arthrobacter* cells.

In another study, soil contaminated with a high concentration of 2,4,5-T was repeatly inoculated with *Pseudomonas cepacia* strain AC1100, which is capable of utilizing 2,4,5-T as the sole source of carbon and energy (50, 51). The contaminated soil samples showed more than 90% degradation after six treatments with strain AC1100, while the concentration of the contaminant remained unchanged in the uninoculated soil. Although strain AC1100 was effective in removing most of the 2,4,5-T from soil, the residual concentration of the herbicide or its metabolites was still high enough (2–10 $\mu g\ g^{-1}$ soil) to impair plant growth. Apparently, some of the 2,4,5-T or its breakdown products became bound to soil particles and was unavailable for microbial degradation. Assessment of the long-term survival of AC1100 in uncontaminated soil indicated that its initial cell titer of 10^7 cells g^{-1} soil fell to an undetectable level within 12 wk of incubation. When 2,4,5-T was added AC1100 became detectable again after an initial lag of 2 wk. By use of a Nal^r mutant it was demonstrated that the 2,4,5-T-degrading capability was not transferred within the indigenous soil population.

Pertsova et al (69) have shown that in soil columns of chernozem soil inoculated with a *Pseudomonas putida* strain able to grow with 3-chlorobenzoate, continuously added 3-chlorobenzoate was totally degraded. The native microflora of the soil, however, was unable to degrade 3-chlorobenzoate, as shown by a breakthrough of 3-chlorobenzoate in the effluent. Taxonomic characteristics of 3-chlorobenzoate-utilizing bacteria isolated from the inoculated soil columns indicated that the 3-chlorobenzoate-degrading plasmid from *Pseudomonas putida* had been transferred in the course of incubation.

Many potentially hazardous pollutants are recalcitrant, highly toxic, or insoluble and thus escape degradation or disturb conventional treatment systems. Conventional biological treatment procedures are often inadequate for the removal of such pollutants from industrial wastewaters. A wide variety of halogenated aromatic compounds can be degraded by adapted microbial strains or defined mixed cultures. The use of such microorganisms for biotreatment of industrial wastewaters containing chlorinated aromatic pollutants requires extrapolation of laboratory studies to real-world treatment systems. Extrapolation is imprecise because only some of the biological and chemical real-world parameters that influence the biodegradation potential of laboratory strains can be assessed under laboratory conditions in well-controlled experiments. A major problem is the retention of a biomass with a specific haloaromatic-degradative capability or the establishment of a specific trait within the autochthonous microflora of a treatment-plant sludge.

The degradation of chlorophenols has been studied in laboratory and scaled-up systems by the addition of laboratory strains. By using a fill-and-draw reactor, domestic activated sludge was adapted to utilize 40 mg liter $^{-1}$ of pentachlorophenol in a synthetic industrial waste. This procedure routinely required 6–7 days. To reduce the lag period another procedure was used, whereby PCP-degrading bacteria were inoculated directly into an operating activated sludge unit in the laboratory. Part of the mixed liquor was removed and replaced by an equivalent volume of a batch culture of *Arthrobacter* strain ATCC 33790 grown with PCP. The level of PCP was reduced from 40 mg liter $^{-1}$ to less than 1 mg liter^{-1} in 1–2 day instead of 6–7. Once acclimated, the activated sludge was stable under laboratory conditions (25).

That degradative sequences for chloroaromatics can be established in activated sludge has been demonstrated with a mixture of the isomeric chlorophenols as model compounds and *Pseudomonas* strain B13 as an organism harboring the capability to assimilate the halosubstituted aromatic ring and to transfer this trait to other bacteria (81). A synthetic sewage containing alkanols, acetone, and phenol was readily degraded by two stably coexisting organisms, a methanol-degrading *Pseudomonas extorquens* strain and *Alcaligenes* strain A7, which can degrade phenol at high concentration in addition to ethanol, isopropanol, and acetone. Unsteady-state transient conditions were observed by increasing loads of chlorophenols. However, a culture augmented with an inoculum of *Pseudomonas* strain B13 grown on 3-chlorobenzoate developed a stable community that totally degraded high loads of chlorophenols. Proof of mineralization was provided by low dissolved organic carbon (DOC) in the cell-free effluent and by the elimination of equimolar amounts of chloride. Population analysis revealed that the establishment of the chlorocatechol-degrading capacity is more important than the number of viable cells of strain B13 for obtaining a functioning mixed culture. Hybrid organisms that emerged in the population, such as *Alcaligenes* strain A7-2, which had acquired the halocatechol-degrading genes from strain B13, were much more competitive with respect to phenol and chlorophenol utilization than strain B13.

To establish the potential for biodegrading chlorinated aromatic compounds as a stable property of the indigineous microflora of a biological treatment system, several requirements must be met. (*a*) The system must productively break down the chemical so that by increasing biomass the system can transiently respond to increasing loads. (*b*) Catabolic traits such as the assimilation of chlorocatechols within the microbial population must be transmissible, to ensure that through the recruitment of existing catabolic enzymes and gene regulators with appropriate effector/substrate specificities new hybrid pathways for haloaromatics can be generated. Thus in addition to physiological flexibility, the adaptability of the system to shifts of substrates such as congenerous compounds would be guaranteed.

Literature Cited

1. Alexander, M., Lustigman, B. K. 1966. Effect of chemical structure on microbial degradation of substituted benzenes. *J. Agric. Food Chem.* 14:410–13
2. Apajalahti, J. H. A., Salkinoja-Salonen, M. S. 1987. Dechlorination and *para*-hydroxylation of polychlorinated phenols by *Rhodococcus chlorophenolicus. J. Bacteriol.* 169:675–81
3. Bachofer, R., Lingens, F., Schäfer, W. 1975. Conversion of aniline into pyrocatechol by a *Nocardia* sp. Incorporation of oxygen-18. *FEBS Lett.* 50:288–90
4. Beadle, C. A., Smith, A. R. W. 1982. The purification and properties of 2,4-dichlorophenol hydroxylase from a strain of *Acinetobacter* species. *Eur. J. Biochem.* 123:323–32
5. Bollag, J.-M., Helling, C. S., Alexander, M. 1967. Metabolism of 4-chloro-2-methylphenoxyacetic acid by soil bacteria. *Appl. Microbiol.* 15:1393–98
6. Bollag, J.-M., Helling, C. S., Alexander, M. 1968. 2,4-D metabolism. Enzymatic hydroxylation of chlorinated phenols. *J. Agric. Food Chem.* 16:826–28
7. Boyd, S. A., Shelton, D. R. 1984. Anaerobic biodegradation of chlorophenols in fresh and acclimated sludge. *Appl. Environ. Microbiol.* 47:272–77
8. Boyd, S. A., Shelton, D. R., Berry, D., Tiedje, J. M. 1983. Anaerobic biodegradation of phenolic compounds in digested sludge. *Appl. Environ. Microbiol.* 46:50–54
9. Brunner, W., Sutherland, F. H., Focht, D. D. 1985. Enhanced biodegradation of polychlorinated biphenyls in soil by analog enrichment and bacterial inoculation. *J. Environ. Qual.* 14:324–28
10. Cain, R. B., Tranter, E. K., Darrah, J. A. 1968. The utilization of some halogenated aromatic acids by *Nocardia. Biochem. J.* 106:211–27
11. Catelani, D., Colombi, A. 1974. Metabolism of biphenyl. Structure and physicochemical properties of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid, the *meta*-cleavage product from 2,3-dihydroxybiphenyl by *Pseudomonas putida. Biochem. J.* 143:431–34
12. Catelani, D., Colombi, A., Sorlini, C., Trecanni, V. 1973. Metabolism of biphenyl. 2-Hydroxy-6-oxo-6-phenylhexa-2,4-dienoate: the *meta*-cleavage product from 2,3-dihydroxybiphenyl by *Pseudomonas putida. Biochem. J.* 134:1063–66
13. Catelani, D., Sorlini, S., Treccani, V. 1971. The metabolism of biphenyl by *Pseudomonas putida. Experientia* 27: 1173–74
14. Chapman, P. J. 1975. Bacterial metabolism of 4-chlorobenzoic acid. *Abstr. Annu. Meet. Am. Soc. Microbiol.* 1975:192
15. Chapman, P. J. 1979. Degradation mechanisms. In *Microbial Degradation of Pollutants in Marine Environments. EPA-600/9-79-012,* ed. A. W. Bourquin, P. H. Pritchard, pp. 28–66. Gulf Breeze, Fla: US Environ. Prot. Agency. 552 pp.
16. Chapman, P. J., Ribbons, D. W. 1976. Metabolism of resorcinylic compounds

by bacteria: alternative pathways of resorcinol catabolism in *Pseudomonas putida*. *J. Bacteriol.* 125:985–98

17. Chatterjee, D. K., Chakrabarty, A. M. 1982. Genetic rearrangements in plasmids specifying total degradation of chlorinated benzoic acids. *Mol. Gen. Genet.* 188:279–85
18. Clarke, K. F., Callely, A. G., Livingstone, A., Fewson, C. A. 1975. Metabolism of monofluorobenzoates by *Acinetobacter calcoaceticus* N.C.I.B. 8250. Formation of monofluorocatechols. *Biochim. Biophys. Acta* 404: 169–79
19. De Bont, J. A. M., Vorage, M. J. A. W., Hartmans, S., van den Tweel, W. J. J. 1986. Microbial degradation of 1,3-dichlorobenzene. *Appl. Environ. Microbiol.* 52:677–80
20. Dolfing, J., Tiedje, J. M. 1986. Hydrogen cycling in a three-tiered food web growing on the methanogenic conversion of 3-chlorobenzoate. *FEMS Microbiol. Ecol.* 38:293–98
21. Dorn, E., Hellwig, M., Reineke, W., Knackmuss, H.-J. 1974. Isolation and characterization of a 3-chlorobenzoate degrading pseudomonad. *Arch. Microbiol.* 99:61–70
22. Dorn, E., Knackmuss, H.-J. 1978. Chemical structure and biodegradability of halogenated compounds. Two catechol 1,2-dioxygenases from a 3-chlorobenzoate–grown pseudomonad. *Biochem. J.* 174:73–84
23. Dorn, E., Knackmuss, H.-J. 1978. Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of catechol. *Biochem. J.* 174:85–94
24. Duxbury, J. M., Tiedje, J. M., Alexander, M., Dawson, J. E. 1970. 2,4-D metabolism: enzymatic conversion of chloromaleylacetic acid to succinic acid. *J. Agric. Food Chem.* 18:199–201
25. Edgehill, R. U., Finn, R. K. 1983. Activated sludge treatment of synthetic wastewater containing pentachlorophenol. *Biotechnol. Bioeng.* 25:2165–76
26. Edgehill, R. U., Finn, R. K. 1983. Microbial treatment of soil to remove pentachlorophenol. *Appl. Environ. Microbiol.* 45:1122–25
27. Engesser, K.-H., Schmidt, E., Knackmuss, H.-J. 1980. Adaptation of *Alcaligenes eutrophus* B9 and *Pseudomonas* sp. B13 to 2-fluorobenzoate as growth substrate. *Appl. Environ. Microbiol.* 39: 68–73

27a. Evans, W. C., Fuchs, G. 1988. Anaerobic degradation of aromatic compounds. *Ann. Rev. Microbiol.* 42:289–317

28. Evans, W. C., Smith, B. S. W., Fernley, H. N., Davis, J. I. 1971. Bacterial metabolism of 2,4-dichlorophenoxyacetate. *Biochem. J.* 122:543–51
29. Evans, W. C., Smith, B. S. W., Moss, P., Fernley, H. N. 1971. Bacterial metabolism of 4-chlorophenoxyacetate. *Biochem. J.* 122:509–17
30. Furukawa, K., Matsumura, F., Tonomura, K. 1978. *Alcaligenes* and *Acinetobacter* strains capable of degrading polychlorinated biphenyls. *Agric. Biol. Chem.* 42:543–48
31. Furukawa, K., Tomizuka, N., Kamibayashi, A. 1979. Effect of chlorine substitution on the bacterial metabolism of various polychlorinated biphenyls. *Appl. Environ. Microbiol.* 38:301–10
32. Furukawa, K., Tomizuka, N., Kamibayashi, A. 1983. Metabolic breakdown of Kaneclors (polychlorobiphenyls) and their products by *Acinetobacter* sp. *Appl. Environ. Microbiol.* 46:140–45
33. Furukawa, K., Tonomura, K., Kamibayashi, A. 1978. Effect of chlorine substitution on the biodegradability of polychlorinated biphenyls. *Appl. Environ. Microbiol.* 35:223–27
34. Furukawa, K., Tonomura, K., Kamibayashi, A. 1979. Metabolism of 2,4,4'-trichlorobiphenyl by *Acinetobacter* sp. P6. *Agric. Biol. Chem.* 43:1577–83
35. Gaal, A., Neujahr, H. Y. 1979. Metabolism of phenol and resorcinol in *Trichosporon cutaneum*. *J. Bacteriol.* 137:13–21
36. Gaal, A. B., Neujahr, H. Y. 1980. Maleylacetate reductase from *Trichosporon cutaneum*. *Biochem. J.* 185:783–86
37. Gaunt, J. K., Evans, W. C. 1961. Metabolism of 4-chloro-2-methylphenoxyacetic acid by a soil microorganism. *Biochem. J.* 79:25p–26p
38. Gibson, S. A., Suflita, J. M. 1986. Extrapolation of biodegradation results to groundwater aquifers: reductive dehalogenation of aromatic compounds. *Appl. Environ. Microbiol.* 52:681–88
39. Goldman, P., Milne, G. W. A., Pignataro, M. T. 1967. Fluorine containing metabolites formed from 2-fluorobenzoic acid by *Pseudomonas* species. *Arch. Biochem. Biophys.* 118:178–84
40. Hartmann, J., Reineke, W., Knackmuss, H.-J. 1979. Metabolism of 3-chloro-, 4-chloro-, and 3,5-dichlorobenzoate by a pseudomonad. *Appl. Environ. Microbiol.* 37:421–28

41. Helling, C. S., Bollag, J.-M., Dawson, J. E. 1968. Cleavage of ether-oxygen bond in phenoxyacetic acid by an *Arthrobacter* sp. *J. Agric. Food Chem.* 16:538–39
42. Horowitz, A., Shelton, D. R., Cornell, C. P., Tiedje, J. M. 1982. Anaerobic degradation of aromatic compounds in sediments and digest sludge. *Dev. Ind. Microbiol.* 23:435–44
43. Horowitz, A., Suflita, J. M., Tiedje, J. M. 1983. Reductive dehalogenation of halobenzoates by anaerobic lake sediment microorganisms. *Appl. Environ. Microbiol.* 45:1459–65
44. Horvath, R. S., Alexander, M. 1970. Cometabolism of *m*-chlorobenzoate by an *Arthrobacter*. *Appl. Microbiol.* 20: 254–58
45. Hughes, D. E. 1965. The metabolism of halogen-substituted benzoic acids by *Pseudomonas fluorescens*. *Biochem. J.* 96:181–88
46. Ichihara, A., Adachi, K., Hosokawa, K., Takeda, Y. 1962. The enzymatic hydroxylation of aromatic carboxylic acids; substrate specificities of anthranilate and benzoate oxidases. *J. Biol. Chem.* 237:2296–302
47. Jeenes, D. J., Reineke, W., Knackmuss, H.-J., Williams, P. A. 1982. TOL plasmid pWWO in constructed halobenzoate-degrading *Pseudomonas* strains: enzyme regulation and DNA structure. *J. Bacteriol.* 150:180–87
48. Johnston, H. W., Briggs, G. G., Alexander, M. 1972. Metabolism of 3-chlorobenzoic acid by a pseudomonad. *Soil Biol. Biochem.* 4:187–90
49. Kersten, P. J., Chapman, P. J., Dagley, S. 1985. Enzymatic release of halogens or methanol from substituted protocatechuic acids. *J. Bacteriol.* 162: 693–97
50. Kilbane, J. J., Chatterjee, D. K., Chakrabarty, A. M. 1983. Detoxification of 2,4,5-trichlorophenoxyacetic acid from contaminated soil by *Pseudomonas cepacia*. *Appl. Environ. Microbiol.* 45: 1697–700
51. Kilbane, J. J., Chatterjee, D. K., Karns, J. S., Kellog, S. T., Chakrabarty, A. M. 1982. Biodegradation of 2,4,5-trichlorophenoxyacetic acid by a pure culture of *Pseudomonas cepacia*. *Appl. Environ. Microbiol.* 44:72–78
52. Klages, U., Lingens, F. 1979. Degradation of 4-chlorobenzoic acid by a *Nocardia* species. *FEMS Microbiol. Lett.* 6:201–3
53. Klages, U., Lingens, F. 1980. Degradation of 4-chlorobenzoic acid by a *Pseudomonas* sp. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Reihe* C 1:215–23
54. Klages, U., Markus, A., Lingens, F. 1981. Degradation of 4-chlorophenylacetic acid by a *Pseudomonas* species. *J. Bacteriol.* 146:64–68
55. Latorre, J., Reineke, W., Knackmuss, H.-J. 1984. Microbial metabolism of chloroanilines: Enhanced evolution by natural genetic exchange. *Arch. Microbiol.* 140:159–65
56. Lehrbach, P. R., Zeyer, J., Reineke, W., Knackmuss, H.-J., Timmis, K. N. 1984. Enzyme recruitment in vitro. Use of cloned genes to extend the range of haloaromatics degraded by *Pseudomonas* sp. strain B13. *J. Bacteriol.* 158: 1025–32
57. Liu, T., Chapman, P. J. 1984. Purification and properties of a plasmid-encoded 2,4-dichlorophenol hydroxylase. *FEBS Lett.* 173:314–18
58. Loos, M. A., Roberts, R. N., Alexander, M. 1967. Phenols as intermediates in the decomposition of phenoxyacetates by an *Arthrobacter* species. *Can. J. Microbiol.* 13:679–90
59. Marks, T. S., Smith, A. R. W., Quirk, A. V. 1984. Degradation of 4-chlorobenzoic acid by *Arthrobacter* sp. *Appl. Environ. Microbiol.* 48:1020–25
60. Marks, T. S., Wait, R., Smith, A. R. W., Quirk, A. V. 1984. The origin of oxygen incorporated during the dehalogenation/hydroxylation of 4-chlorobenzoate by an *Arthrobacter* sp. *Biochem. Biophys. Res. Commun.* 124:669–74
61. Markus, A., Klages, U., Krauss, S., Lingens, F. 1984. Oxidation and dehalogenation of 4-chlorophenylacetate by a two-component enzyme system from *Pseudomonas* sp. strain CBS3. *J. Bacteriol.* 160:618–21
62. Markus, A., Krekel, D., Lingens, F. 1986. Purification and some properties of component A of the 4-chlorophenylacetate 3,4-dioxygenase from *Pseudomonas* species strain CBS. *J. Biol. Chem.* 261:12883–88
63. Mikesell, M. D., Boyd, S. A. 1985. Reductive dechlorination of pesticides 2,4-D, 2,4,5-T, and pentachlorophenol in anaerobic sludge. *J. Environ. Qual.* 14:337–40
64. Mikesell, M. D., Boyd, S. A. 1986. Complete reductive dechlorination and mineralization of pentachlorophenol by anaerobic microorganisms. *Appl. Environ. Microbiol.* 52:861–65
65. Milne, G. W. A., Goldman, P., Holz-

man, J. L. 1968. The metabolism of 2-fluorobenzoic acid. II. Studies with $^{18}O_2$. *J. Biol. Chem.* 243:5374–76

66. Morris, C. M., Barnsley, E. A. 1982. The cometabolism of 1- and 2-chloronaphthalene by pseudomonads. *Can. J. Microbiol.* 28:73–79
67. Müller, R., Thiele, J., Klages, U., Lingens, F. 1984. Incorporation of [^{18}O] water into 4-hydroxybenzoic acid in the reaction of 4-chlorobenzoate dehalogenase from *Pseudomonas* spec. CBS3. *Biochem. Biophys. Res. Commun.* 124:178–82
68. Oltmanns, R. H., Rast, H. G., Reineke, W. 1988. Degradation of 1,4-dichlorobenzene by enriched and constructed bacteria. *Appl. Microbiol. Biotechnol.* 28:609–16
69. Pertsova, R. N., Kunc, F., Golovleva, L. A. 1984. Degradation of 3-chlorobenzoate in soil by pseudomonads carrying biodegradative plasmids. *Folia Microbiol. Prague* 29:242–47
70. Reineke, W. 1986. Construction of bacterial strains with novel degradative capabilities for chloroaromatics. *J. Basic Microbiol.* 26:551–67
71. Reineke, W., Jeenes, D. J., Williams, P. A., Knackmuss, H.-J. 1982. TOL plasmid pWWO in constructed halobenzoate-degrading *Pseudomonas* strains: prevention of *meta* pathway. *J. Bacteriol.* 150:195–201
72. Reineke, W., Knackmuss, H.-J. 1978. Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of benzoic acid. *Biochim. Biophys. Acta* 542:412–23
73. Reineke, W., Knackmuss, H.-J. 1978. Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on dehydrogenation of 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid. *Biochim. Biophys. Acta* 542:424–29
74. Reineke, W., Knackmuss, H.-J. 1979. Construction of haloaromatics utilising bacteria. *Nature* 277:385–86
75. Reineke, W., Knackmuss, H.-J. 1980. Hybrid pathway for chlorobenzoate metabolism in *Pseudomonas* sp. B13 derivatives. *J. Bacteriol.* 142:467–73
76. Reineke, W., Knackmuss, H.-J. 1984. Microbial metabolism of haloaromatics: Isolation and properties of a chlorobenzene-degrading bacterium. *Appl. Environ. Microbiol.* 47:395–402
77. Reineke, W., Wessels, S. W., Rubio, M. A., Latorre, J., Schwien, U., et al. 1982. Degradation of monochlorinated aromatics following transfer of genes encoding chlorocatechol catabolism. *FEMS Microbiol. Lett.* 14:291–94
78. Reiner, A. M., Hegeman, G. D. 1971. Metabolism of benzoic acid by bacteria. Accumulation of (-)-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid by a mutant strain of *Alcaligenes eutrophus*. *Biochemistry* 10:2530–36
79. Reiner, E. A., Chu, J., Kirsch, E. J. 1978. Microbial metabolism of pentachlorophenol. In *Pentachlorophenol: Chemistry, Pharmacology and Toxicity*, ed. K. R. Rao, pp. 67–81. New York: Plenum
80. Ruisinger, S., Klages, U., Lingens, F. 1976. Abbau der 4-Chlorbenzoesäure durch eine *Arthrobacter*-Species. *Arch. Microbiol.* 10:253–56
81. Schmidt, E., Hellwig, M., Knackmuss, H.-J. 1983. Degradation of chlorophenols by a defined mixed microbial community. *Appl. Environ. Microbiol.* 46: 1038–44
82. Schmidt, E., Knackmuss, H.-J. 1980. Chemical structure and biodegradability of halogenated aromatic compounds. Conversion of chlorinated muconic acids into maleoylacetic acid. *Biochem. J.* 192:339–47
83. Schmidt, E., Remberg, G., Knackmuss, H.-J. 1980. Chemical structure and biodegradability of halogenated aromatic compounds. Halogenated muconic acids as intermediates. *Biochem. J.* 192: 331–37
84. Schraa, G., Boone, M. L., Jetten, M. S. M., van Neerven, A. R. W., Colberg, P. J., Zehnder, A. J. B. 1986. Degradation of 1,4-dichlorobenzene by *Alcaligenes* sp. strain A175. *Appl. Environ. Microbiol.* 52:1374–81
85. Schukat, B., Janke, D., Krebs, D., Fritsche, W. 1983. Cometabolic degradation of 2- and 3-chloroaniline because of glucose metabolism by *Rhodococcus* sp. An 117. *Curr. Microbiol.* 9:58–67
86. Schwien, U. 1984. *Bakterieller Abbau von Dichloraromaten: Metabolismus von 3,5-Dichlorbrenzcatechin*. PhD thesis. Univ. Göttingen, Fed. Rep. Germany. 101 pp.
87. Schwien, U., Schmidt, E. 1982. Improved degradation of monochlorophenols by a constructed strain. *Appl. Environ. Microbiol.* 44:33–39
88. Sharpee, K. W., Duxbury, J. M., Alexander, M. 1973. 2,4-Dichlorophenoxyacetate metabolism by *Arthrobacter* sp.: Accumulation of a chlorobutenolide. *Appl. Microbiol.* 26:445–47

89. Shelton, D. R., Tiedje, J. M. 1984. Isolation and partial characterization of bacteria in an anaerobic consortium that mineralizes 3-chlorobenzoic acid. *Appl. Environ. Microbiol.* 48:840–48

90. Sleat, R., Robinson, J. P. 1984. The bacteriology of anaerobic degradation of aromatic compounds. *J. Appl. Bacteriol.* 57:381–94

91. Spain, J. C., Nishino, S. F. 1987. Degradation of 1,4-dichlorobenzene by a *Pseudomonas* sp. *Appl. Environ. Microbiol.* 53:1010–19

92. Spokes, J. R., Walker, N. 1974. Chlorophenol and chlorobenzoic acid cometabolism by different genera of soil bacteria. *Arch. Microbiol.* 96:125–34

93. Steiert, J. G., Crawford, R. L. 1986. Catabolism of pentachlorophenol by a *Flavobacterium* sp. *Biochem. Biophys. Res. Commun.* 141:825–30

94. Suflita, J. M., Horowitz, A., Shelton, D. R., Tiedje, J. M. 1982. Dehalogenation: a novel pathway for the anaerobic biodegradation of haloaromatic compounds. *Science* 218:1115–17

95. Suflita, J. M., Robinson, J. A., Tiedje, J. M. 1983. Kinetics of microbial dehalogenation of haloaromatic substrates in methanogenic environments. *Appl. Environ. Microbiol.* 45:1466–73

96. Suflita, J. M., Stout, J., Tiedje, J. M. 1984. Dechlorination of (2,4,5-trichlorophenoxy)acetic acid by anaerobic microorganisms. *J. Agric. Food Chem.* 32:218–21

97. Suzuki, T. 1977. Metabolism of pentachlorophenol by a soil microbe. *J. Environ. Sci. Health Part B* 12:113–27

98. Thiele, J., Müller, R., Lingens, F. 1987. Initial characterisation of 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. CBS3. *FEMS Microbiol. Lett.* 41:115–19

99. Tiedje, J. M., Alexander, M. 1969. Enzymatic cleavage of the ether bond of 2,4-dichlorophenoxyacetate. *J. Agric. Food Chem.* 17:1080–84

100. Tiedje, J. M., Duxbury, J. M., Alexander, M., Dawson, J. E. 1969. 2,4-D metabolism: Pathway of degradation of chlorocatechols by *Arthrobacter* sp. *J. Agric. Food Chem.* 17:1021–26

101. Tittmann, U., Lingens, F. 1980. Degradation of biphenyl by *Arthrobacter simplex* strain BPA. *FEMS Microbiol. Lett.* 8:225–58

102. Tsuchiya, T., Yamaha, T. 1983. Reductive dechlorination of 1,2,4-trichlorobenzene on incubation with intestinal contents of rats. *Agric. Biol. Chem.* 47:1163–65

103. Tsuchiya, T., Yamaha, T. 1984. Reductive dechlorination of 1,2,4-trichlorobenzene by *Staphylococcus epidermidis* isolated from intestinal contents of rats. *Agric. Biol. Chem.* 48:1545–50

104. Walker, N., Harris, D. 1970. Metabolism of 3-chlorobenzoic acid by *Azotobacter* species. *Soil Biol. Biochem.* 2:27–32

105. Yamaguchi, M., Fujisawa, H. 1980. Purification and characterization of an oxygenase component in benzoate 1,2-dioxygenase system from *Pseudomonas arvilla* C-1. *J. Biol. Chem.* 255:5058–63

106. Zaitsev, G. M., Karasevich, Y. N. 1980. Utilization of 4-chlorobenzoic acid by *Arthrobacter globiformis. Mikrobiologiya* 50:35–40

107. Zaitsev, G. M., Karasevich, Y. N. 1980. Preparative metabolism of 4-chlorobenzoic acid in *Arthrobacter globiformis. Mikrobiologiya* 50:423–28

108. Zaitsev, G. M., Karasevich, Y. N. 1982. Utilization of 2-chlorobenzoic acid by *Pseudomonas cepacia. Mikrobiologiya* 53:75–80

109. Zeyer, J., Kearney, P. C. 1982. Microbial degradation of *para*-chloroaniline as sole carbon and nitrogen source. *Pestic. Biochem. Physiol.* 17:215–23

110. Zeyer, J., Wasserfallen, A., Timmis, K. N. 1985. Microbial mineralization of ring-substituted anilines through an *ortho*-cleavage pathway.. *Appl. Environ. Microbiol.* 50:447–53

Annual Review of Microbiology
Volume 42, 1988

CONTENTS

(*continued*)

Indexes