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Microbial Degradation of Halogenated Compounds

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Summary. The mode of degradation of various halogenated compounds in isolated pure cultures and the disposition of the degradative genes have been studied. In many cases the degradative genes are found to be clustered on plasmids and appear to be under positive control. Genetic selection in vivo and genetic manipulations in vitro have allowed construction of strains having wider biodegradative potentials than their natural counterparts. Molecular cloning of the degradative gene clusters for halogenated compounds in vectors with a broad host range also allows the transfer of such genes to a large number of Gram-negative bacteria. The application of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T)–degrading microorganisms has demonstrated the effectiveness of this strain in removing large amounts of 2,4,5-T from contaminated soil within a short period, and such soil has been shown to support the growth of plants normally sensitive to low concentrations of 2,4,5-T. The two major challenges that must be addressed in the near future are the development of appropriate microbial technology for the decontamination of soil containing hazardous halogenated compounds, and the promulgation of appropriate regulations to ensure the safety and well-being of the public during the application of genetically improved strains in an open environment.

In the industrialized world halogenated compounds play a major part in day-to-day life. Large amounts of these compounds used as refrigerants, fire retardants, paints and varnishes, solvents, herbicides, pesticides, and so on have been released into the environment. Not only are most of these compounds toxic to man and animals, some of them, such as insecticides and rodenticides, are used widely because of their toxicity. Massive pollution problems resulting from large-scale use of such chemicals have confronted most of the countries in the world (*1*). In response to such incidences of pollution, the U.S. Congress in 1976 passed the Toxic Substances Control Act (TSCA), requiring that all new chemical substances intended for large-scale use be approved by and registered with the Environmental Protection Agency. Specifically, TSCA, the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), the Clean Water Act, and several others have called for assessment of the environmental hazard and impact on human health of synthetic halogenated compounds and other toxic environmental pollutants.

The assessment of risks and hazards is usually associated with the determination of two important parameters of the chemicals: their toxicological properties and their fate in the environment. While the long-term toxicological properties of halogenated compounds present interesting challenges to toxicologists, regulators, and environmentalists, the recalcitrance of many halogenated compounds to microbial degradation (*2*) presents equal challenges to microbiologists and geneticists. Many halogenated compounds are extremely persistent in nature, while others are biodegradable (*3*). An understanding of the biochemical and genetic mode of microbial dissimilation of biodegradable halogenated compounds may be a logical step toward the construction of genetically improved strains that might be used in the future for enhanced removal of the more persistent compounds.

This article is an overview of our current understanding of the physiological and genetic basis of biodegradation of some halogenated compounds, specifically chlorinated aromatics, by isolated pure aerobic cultures. The role of genetic manipulations in the construction and molecular analysis of such strains is stressed. Excellent reviews are available on the degradation of halogenated compounds by natural microbial consortia, both aerobic and anaerobic (*4*), and this will not be dealt with in this article.

Significance of Studies on the Microbial Degradation of Halogenated Compounds

Even after three decades of uninterrupted growth of information in the field of microbial genetics, the genetic, biochemical, and molecular basis of transformation and mineralization of halogenated compounds in the biosphere by microbial populations still offers a unique niche for the study of evolution. Structural plasticity of the genetic machinery of the microbes endows them with a degradative capability that can be quickly adapted in response to substrate variability. Such expanding catabolic versatility may reveal principles not yet encountered in the intensively studied metabolic pathways of *Escherichia coli, Salmonella*, and other genera and species. Unlike enterobacteria, which are confined in the intestinal milieu, saprophytic microbes inhabit widely divergent ecosystems, both aerobic and anaerobic,

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of soil and aquatic environments.

Most of the halogenated compounds are man-made and do not have counterparts in nature. Rapid evolution of catabolic pathways in microorganisms exposed to such a substrate may be achieved in two ways. First, the existing enzyme machinery may be employed, provided the new substrate is to some extent chemically analogous to a natural substrate. The kinetic limitation to new substrate utilization can frequently be overcome by enzyme overproduction through a gene dosage effect, by inactivating or changing the stringent control of the regulatory genes involved, or by producing enzymes with altered specificity by mutational divergence. Second, novel enzyme activities may also be derived from heterologous or preexisting genes for related enzymes or proteins by a variety of genetic mechanisms, such as gene recruitment, genetic rearrangement by legitimate and illegitimate recombination, and so on.

Even though the halogenated compounds have been synthesized and released into the environment only during the past few decades, degradative capabilities against many such compounds have already been observed in the natural microflora. The two mechanistic modes of evolution of a new catabolic phenotype mentioned above are easily conceivable when the existing genes or enzymes are not subject to allosteric control. In such cases the microevolutionary processes could be mimicked in the laboratory by continuous culture in a chemostat or by using techniques of genetic manipulation including recombinant DNA techniques.

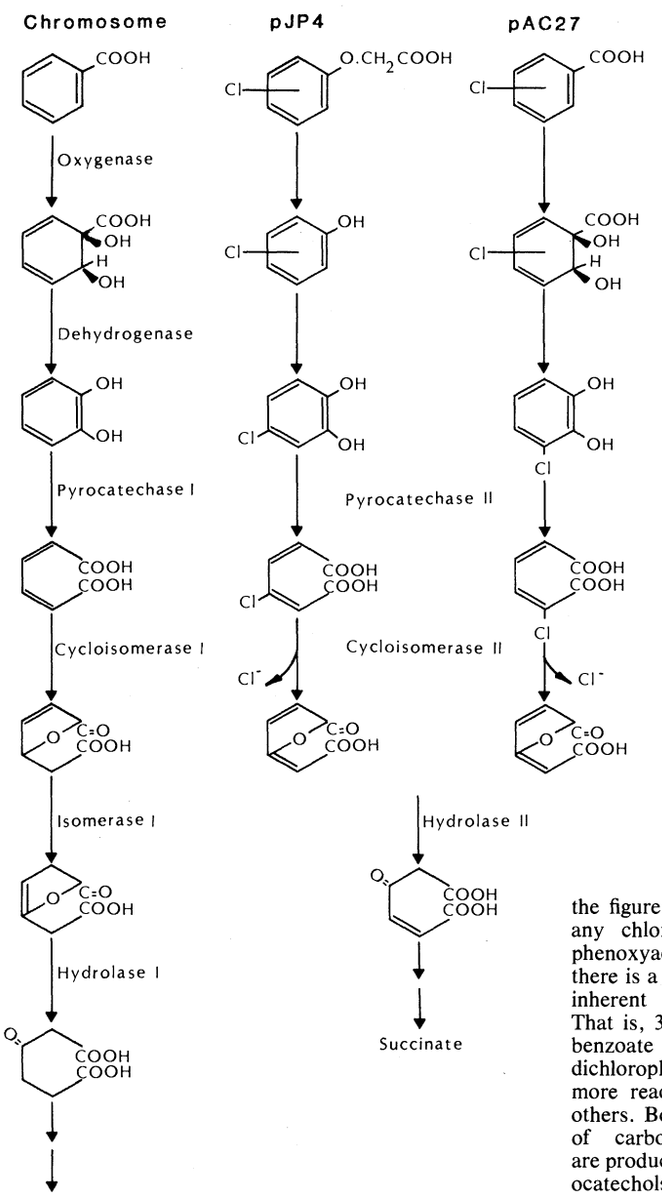

Fig. 1. Pathways for the degradation of benzoate, chlorophenoxyacetates, and chlorobenzoates. The benzoate degradative pathway is known to be chromosomally coded (indicated by "chromosome" at the top), while the 2,4-dichlorophenoxyacetic acid degradative and 3-chlorobenzoate degradative genes are known to be borne on plasmids pJP4 and pAC27, respectively. The plasmid-encoded pyrocatechase II and cycloisomerase II differ from the chromosomally encoded pyrocatechase I and cycloisomerase I in their substrate specificities, while hydrolase II may be entirely different from hydrolase I in this property. In the conversion of the chlorinated catechols encoded by plasmids pJP4 and pAC27, the involvement of β-ketoadipate as an intermediate is not firmly established. Although the figure seems to indicate that any chlorobenzoate or chlorophenoxyacetate can be degraded, there is a great deal of specificity inherent in such degradations. That is, 3-, 4-, and 3,5-dichlorobenzoate and 4- and 2,4-dichlorophenoxyacetate are much more readily degraded than the others. Both *cis* and *trans* forms of carboxymethylenebutenolide are produced from different chlorocatechols, although only one form is shown in the figure.

Among the saprophytic organisms, bacteria and fungi are of major importance in the dissimilation of halogenated compounds. Although it is apparent that the fungi and bacteria employ different strategies in degrading halogenated substrates, very little is known about the genetics and biochemistry of the fungal degradation of halogenated compounds. Similarly, most of the information available on bacterial degradation of halogenated compounds concerns oxidative degradations. Recently, it has been reported that anaerobic bacterial consortia from lake and river sediments transform and mineralize several halogenated compounds; however, these bacteria have not yet been characterized and genetically analyzed (*5*).

For convenience, the haloorganic compounds degraded by microorganisms are classified in three groups: (i) haloaliphatic, (ii) haloaromatic, and (iii) haloheterocyclic. In order to study the genetics of the microbial degradation of various halogenated compounds, isolation of a pure culture is essential. This has been accomplished in the case of a number of such compounds, and these compounds and the microorganisms capable of degrading them are listed in Table 1. The list is by no means complete; it covers only some of the compounds degradable by naturally occurring microbial populations. Nevertheless, such studies are useful, since an understanding of the biochemistry and genetics of the degradation of simple halogenated compounds will make it possible to characterize the appropriate genes and ultimately to transfer them in order to construct improved strains. An understanding of the cellular regulatory processes will also present opportunities to manipulate the genes or change the cellular environment so as to remove the rate-limiting steps and enhance degradation of the compounds. In the following sections we discuss the genetic and molecular mechanisms controlling the bacterial degradation of some halogenated compounds and indicate how an understanding of these mechanisms can be useful in the construction of genetically improved strains for the rapid degradation of more recalcitrant environmental pollutants.

Biochemical and Genetic Basis of Degradation

There have been many studies of the ability of soil and aquatic microorganisms to dissimilate halogenated compounds (*2–4*); however, detailed studies of the biochemical and genetic basis of

such biodegradations are lacking. Two exceptions are the biodegradation of chlorobenzoic acids, notably 3-chlorobenzoic acid (3Cba), and the biodegradation of chlorinated phenoxyacetic acids, notably 4-chlorophenoxyacetic acid and 2,4-dichlorophenoxyacetic acid (2,4-D).

Microbial Metabolism of 3-Chlorobenzoic Acid

The biochemical and enzymological basis of the degradation of 3Cba by *Pseudomonas* sp. strain B13 has been described by Knackmuss and co-workers (*6, 7*). These workers demonstrated the conversion of 3Cba to the corresponding chlorocatechol by the same enzymes probably involved in the conversion of benzoate to catechol, that is, benzoate oxygenase and the dihydrodiol benzoate dehydrogenase (Fig. 1). The chlorocatechol is further oxidized by a modified *ortho* pathway to chloro-*cis, cis*-muconic acid by a ring-cleaving pyrocatechase II, which specifically acts on the chlorocatechol rather than the nonchlorinated parent. The chloromuconic acid is believed to be dehalogenated by a fortuitous reaction catalyzed by *cis,cis*-chloromuconate lactonizing enzyme (cycloisomerase II), for which both nonchlorinated and chlorinated *cis, cis*-muconates are substrates. The product of the cycloisomerase II reaction is believed to be the chlorolactone, which undergoes spontaneous conversion to *trans*-4-carboxymethylene but-2-en-4-olide. This is then converted by the hydrolase II, which has strict specificity toward the diene-lactone generated from chloromuconate and very little activity toward lactones generated from nonchlorinated muconates (*6*), to maleylacetic acid. Maleylacetic acid is further converted to succinate, presumably through formation of β-ketoadipate (*6*), although β-ketoadipate has not been shown to accumulate during growth of another $3Cba^+$ *Pseudomonas putida* on 3Cba (*8*). It is interesting to note that resting cells of a *P. putida* strain capable of dechlorinating 3Cba can also completely dehalogenate 3-bromo- and 3-fluorobenzoic acid, while the iodinated analog is only slowly dehalogenated (*9*).

It is not clear whether all the genes involved in 3Cba degradation are present on plasmids. It is known that some of the key enzymes are plasmid-specified. For example, pyrocatechase II, cycloisomerase II, hydrolase II, and presumably the enzyme or enzymes acting on maleylacetic acid (Fig. 1) appear to be coded by genes borne on plasmid pWR1 in *Pseudomonas* sp. strain B13 (*6, 10*) or plasmid pAC25 present in a $3Cba^+$ strain of *P. putida* (*8*). Chatterjee and Chakrabarty (*11*) demonstrated that pWR1 and pAC25 are highly homologous and are presumably identical, although pWR1 has a short deletion of about 6 kb. A deletion in pAC25 (117 kb) resulted in the formation of plasmid pAC27 (110 kb), which retains the 3Cba degradative and transfer genes of pAC25 and which has been the subject of intensive study in our laboratory because of the ease with which the plasmid DNA can be isolated. As in pWR1, the pAC25 DNA is difficult to isolate; the 7-kb deletion in pAC27, however, allows isolation of this plasmid DNA in much higher yields. A physical map of this plasmid is shown in Fig. 2A.

Molecular cloning studies demonstrated that the 20-kb Eco RI B fragment (EB) of pAC27 (pDC10) has all the structural and regulatory genes (*12*), since introduction of this fragment alone to $3Cba^-$ *P. putida* strains enables them to grow rapidly with 3Cba. Introduction of a much shorter 4.2-kb Bgl II E (BE) fragment (pDC25, Fig. 2A) also allows $3Cba^-$ *P. putida* cells to utilize 3Cba, but at a much lower rate. Continued streaking on a minimal 3Cba plate allows better growth; however, isolation of plasmid DNA from such cells usually shows amplification of the Bgl II E fragment with a copy number of about 8, which suggests that expression of the structural genes present on the 4.2-kb Bgl II E fragment is under positive control by a regulatory gene which is not present on this fragment (*12*). Amplification is necessary to compensate for the presence of a positively acting activator protein, similar to those proposed for the degradation of naphthalene and toluene encoded by the NAH and TOL plasmids (*13, 14*).

Plasmid pAC27 (which is homologous with pWR1 studied by Knackmuss and co-workers) (*11*) is not the only plasmid that specifies 3Cba degradation. Don and Pemberton (*15*) described the characterization of a broad-host-range *inc*Pl plasmid pJP4 in *Alcaligenes eutrophus* that encodes degradation of 2,4-D in addition to 3Cba (Fig. 1). Weightman *et al.* (*16*) showed that common genes involved in chlorocatechol degradation are involved in both 2,4-D and 3Cba degradation. Ghosal *et al.* (*12*) demonstrated that the pAC27 Bgl II E fragment harboring the 3Cba degradative genes shows extensive homology with the pJP4 Eco RI fragments EB and EE (Fig. 2B). Cloning

Table 1. Bacterial degradation of some halogenated compounds. References are cited only for those where the degradative genes are known to be plasmid-borne.

Compounds	Bacteria	Plasmid	References
Fluoro-, chloro-, and bromoacetate; propionate and butanoate; chloroethane, chloroethanol, and chloromethane	*Pseudomonas* sp., *Moraxella* sp., *Hyphomicrobium* sp.	pUO1	(*50*)
3-, 4-, and 3,5-dichlorobenzoates	*Pseudomonas* sp.	pWR1, pAC25, pAC27, pAC29	(*8, 10, 39*)
Chlorosalicylate	*Bacillus* sp., *Pseudomonas* sp.	pWR1 + cloned *nah*G	(*44*)
2,6-Dichlorotoluene	*Pseudomonas* sp.	No designation	(*51*)
Chlorobenzene	Uncharacterized strains		
Chlorophenols	*Alcaligenes* sp., *Pseudomonas* sp., *Flavobacterium* sp.		
Mono- and dichlorobiphenyls	*Alcaligenes* sp. *Acinetobacter* sp.	No designation pKF1	(*52*) (*42*)
Chlorophenoxyacetic acids: 4-chloro-, 2,4-dichloro-, and 2,4,5-trichlorophenoxyacetic acid	*Alcaligenes* sp., *Pseudomonas* sp.	pJP2, pJP4	(*15*)
Deethylsimazine (6-chloro-*N*-ethyl-1,3,5-triazine 2,4-diamine)	*Rhodococcus corallinus*, *Pseudomonas* sp.		
Chloropicrin	*Pseudomonas* sp.		

experiments demonstrated that Eco RI fragments EB, EE, and EF of pJP4 (pYG1943, Fig. 2B) are enough to give the 3Cba$^-$ *P. putida* strain a 3Cba$^+$ phenotype and that cloning of the EB fragment alone (pYG419, Fig. 2B) allows only slow growth on 3Cba. As with the pAC27 Bgl II E fragment, cells harboring the pJP4 Eco RI B (pYG419) fragment show amplification of this fragment when enriched by continued growth on 3Cba (*12*). Whether the same kinds of sequences are involved in the amplification of the 3Cba degradative genes present on pAC27 and pJP4 is not known.

Biochemistry of 2,4-D and 2,4,5-T Degradation

Phenoxy alkanoic acids such as 2,4-D and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) are among the most widely used herbicides. Although there have been a number of reports on the degradation of 2,4-D, information on the microbial degradation of 2,4,5-T is rather sparse. For instance, the complete pathway for 2,4-D degradation by *Arthrobacter* sp. and the enzymes involved in the sequential steps have long been established (*17, 18*). The pathway of 2,4-D degradation by *Pseudomonas* sp. appears to be similar to the *Arthrobacter* sp. pathway except for the presence of a few additional metabolic products (*19*). Recently, it was reported that degradation of 2,4-D occurs through dechlorination prior to ring cleavage in *Aspergillus niger* (*20*). Herbicides 2,4-D and 2,4,5-T are structurally related, the latter having an extra chlorine atom at position 5. Unlike 2,4-D, 2,4,5-T is poorly biodegradable and persists for long periods, hence constituting a pollution problem (*21*). Cometabolism of 2,4,5-T by *Brevibacterium* sp. resulted in the formation of product tentatively identified as 3,5-dichlorocatechol (*22*). Bacterial cometabolism of 2,4,5-T was also described by Rosenberg and Alexander (*23*), who proposed a degradation pathway of 2,4,5-T in soil (*24*). Reductive dechlorination of 2,4,5-T by anaerobic microorganisms was described by Suflita *et al.* (*5*).

Recently, *Pseudomonas cepacia* strain AC1100 isolated from a chemostat enrichment was found to use 2,4,5-T or 2,4,5-trichlorophenol (2,4,5-TCP) as its sole source of carbon and energy (*25, 26*). Resting cells of AC1100 oxidize or dehalogenate pentachlorophenol; pentafluorophenol; tetra-, tri-, and dichlorophenols; 2,4-D; and so on (*26*). Like 3Cba, the bromine and fluorine derivatives are dehalogenated much more readily than the iodine derivatives (*26*). When AC1100 is grown with 2,4,5-T or 2,4,5-TCP, several metabolic products are detected in the culture medium by gas chromatography. Gas chromatographic–mass spectrometric (GC-MS) analysis of the 2,4,5-T metabolites after methylation revealed the presence of the following compounds: a product tentatively identified as hydroxy-2,4,5-T; three isomers of dichlorohydroxyphenoxyacetic acids, one of them tentatively identified as 2,4-dichloro-6-hydroxyphenoxyacetic acid; 2,4,5-TCP; and three isomers of dichlorocatechols including 3,5-dichlorocatechol, 4-chlorophenol, and succinic acid. The following metabolites formed during the growth of AC1100 on 2,4,5-TCP have been identified: two isomers of dichlorocatechols (essentially the same as those formed during the 2,4,5-T degradation except for 3,5-dichlorocatechol) and 4-chlorophenol. Dechlorination of 2,4,5-T by an anaerobic methanogenic consortium grown on 3Cba occurs at the *para* position to form 2,5-dichlorophenoxyacetic acid, as described by Suflita *et al.* (*5*). Three types of dehalogenation mechanisms are employed in the microbial metabolism of 2,4,5-T: simultaneous hydroxylation and dehalogenation, reductive dehalogenation, and elimination of the halogen as the hydrogen halide after ring fission. Various enzymatic mechanisms have been postulated for the dechlorination of various chlorinated compounds (*5*).

Relationships Between the 2,4-D and 2,4,5-T Pathways

Some similarities can be found between the 2,4-D and 2,4,5-T pathways: side chain removal at an initial stage resulting in the formation of the corresponding chlorophenol, presence of common metabolic intermediates such as 3,5-dichlorocatechol and succinate, and ring hydroxylation or ring dechlorination prior to side chain cleavage. Although some common intermediates are formed during the degradation of 2,4-D and 2,4,5-T, AC1100 is unable to grow on 2,4-D, suggesting high specificity of the initial enzyme or enzymes or induction specificity in 2,4,5-T metabolism. Enzymes that degrade 2,4,5-TCP are inducible by 2,4,5-TCP, whereas enzyme (or enzymes) for the conversion of 2,4,5-T to 2,4,5-TCP is constitutive (*27*). The 2,4,5-TCP degradative enzymes are also known to be subject to catabolite repression by organic acids such as succinate (*27*).

Genetics of 2,4-D and 2,4,5-T Degradation

Plasmid involvement in the degradation of chlorinated phenoxy herbicides such as 2,4-D was first described by Pemberton and Fisher (*28*). Subsequently, 2,4-D–degrading plasmids were found among many naturally occurring soil microorganisms, and they can be readily transferred from one host to another (*15*). Two of these plasmids, isolated from *A. eutrophus*, were designated pJP2 and pJP4 (*15*). Plasmids pJP2 and pJP4 belong to the *inc*P3 and *inc*P1 incompatibility groups, respectively, and both plasmids are self-transmissible. Although little is known about the physical and genetic organization of plasmid pJP2 encoding the degradation of phenoxyacetic acid (PAA) and 2,4-D, plasmid pJP4 is rather well characterized. It has a molecular size of 83 kb (Fig. 2B) and encodes resistance to merbromin and mercuric chloride and degradation of 3Cba as well as 2,4-D (*15*). A physical and functional map of pJP4 was recently established by transposon mutagenesis, and some of the catabolic genes have been mapped on the plasmid genome (*16*).

On the other hand, however, very little is known about the genetics of 2,4,5-T degradation. Although 2,4,5-T–degrading *P. cepacia* AC1100 harbors at least two plasmids and there is circumstantial evidence for the involvement of plasmids in 2,4,5-T degradation (*12*), the precise role of plasmids in the degradation has yet to be established. Several different bacteria capable of utilizing 2,4,5-T as the sole carbon source were reportedly isolated by conventional enrichment techniques and two of them were found to contain plasmids (*16*). However, analysis of 2,4,5-T$^-$ transposon-generated mutants of one of these isolates showed the presence of transposons on the chromosome, indicating the involvement of chromosomal genes in 2,4,5-T degradation by this isolate (*16*). Ghosal *et al.* (*12*) demonstrated considerable homology of the pJP4 EB fragment harboring chlorocatechol degradative genes with a 4-kb fragment of plasmid DNA isolated from the 2,4,5-T$^+$ *P. cepacia* AC1100 strain. On the other hand, hybridization of the chromosomal and plasmid DNA isolated from two different Tn5-generated 2,4,5-T$^-$ mutants of AC1100 with Tn5 as a probe showed the presence of Tn5 on the chromosome (*29*). The precise role of plasmid and chromosomal genes in the degradation of 2,4,5-T by strain AC1100 remains to be elucidated.

Laboratory Construction of 2,4-D and 2,4,5-T–Degrading Strains

Segregants of *Alcaligenes* sp. lacking plasmids still retain the ability to degrade 2,4-dichlorophenol (*15, 28*). To determine whether 2,4-D plasmids encode a complete pathway of 2,4-D degradation, plasmids pJP2 and pJP4 were transferred by conjugation to strain 383 of *P. cepacia*. Analysis of *P. cepacia* exconjugants showed that they could grow on 2,4-D as the sole source of carbon and energy and that they all contained either pJP2 or pJP4 in unmodified form. Similarly, conjugal transfer of pJP2 and pJP4 to 2,4,5-T^+ Nal^r *P. cepacia* DC102 resulted in the construction of new strains capable of utilizing both 2,4,5-T and 2,4-D as growth substrates (*12*). Analysis of plasmids isolated from DC102 exconjugants harboring pJP2 showed the presence of intact pJP2 as well as unmodified resident plasmids of DC102. However, in all exconjugants harboring pJP4 analyzed so far, an approximately 10-kb fragment of pJP4 has apparently been lost. This might be due to interaction between incoming plasmid pJP4 and the resident plasmids in the recipient, since no such change was observed in wild-type strain 383 of *P. cepacia*. The restriction profiles of the plasmids in all exconjugants remained unchanged whether the growth substrate was 2,4-D or 2,4,5-T. All efforts to grow *P. putida* strains harboring pJP4 in minimal medium containing 2,4-D have failed, although the transfer of the plasmid could be achieved by selecting for the Hg^r character. Since 2,4-D plasmids pJP2 and pJP4 are expressed in *P. cepacia* upon their transfer, a plasmid-less *P. cepacia* strain 383 was used as a host for subsequent cloning experiments.

Cloning of the 2,4-D Degradative Genes in *Pseudomonas*

To determine the minimum fragment size of pJP2 and pJP4 required to confer the 2,4-D^+ phenotype, cloning experiments were carried out (*12*) with broad-host-range vectors pLAFRI and pCP13 (*30, 31*), using *P. cepacia* 383 as the host. Selections were directly made on minimal agar containing 2,4-D. Several 2,4-D^+ clones were obtained from the Eco RI library of pJP2. Although none was obtained from the Eco RI library of pJP4, one 2,4-D^+ clone was obtained from the Hind III library of pJP4 (*12*). Restriction analysis of plasmid DNA isolated from various 2,4-D^+ clones revealed that all 2,4-D^+ clones obtained from the pJP2 Eco RI library contained Eco RI fragments A and F (EA and EF) of pJP2 in common, indicating some essential role of these two fragments in 2,4-D degradation. It was mentioned previously that the structural and regulatory genes for chlorocatechol degradation are present on Eco RI fragments EB, EE, and EF of pJP4 and the Eco RI B fragment of pAC27. Hybridization of a number of pJP4 Eco RI fragments (EA, EB, EC/ED, EE, EF, EG, and EH) with various pJP2 fragments showed strong homology of pJP4 EB, EE, EF, and EG fragments with the 16-kb Eco RI A (EA) fragment of pJP2. Since this fragment, along with EF of pJP2, is present in all the 2,4-D^+ clones, it is reasonable to conclude that many of the chlorocatechol and other 2,4-D degradative genes are present on the EA fragment, and presumably also on the EF fragment of pJP2. Whether this fragment (pJP2 EF) contains any regulatory or structural genes for 2,4-D degradation is not known.

Genetic Rearrangements in Haloaromatic-Degrading Bacteria

It is generally assumed that deletion and amplification are the recombination mechanisms that play a significant role in the evolution of new genetic machinery. Detailed molecular analysis of plasmid evolution and DNA sequence analysis in model systems such as the *lac* operon to study the genetic and molecular basis of deletion formation have yielded new insights into the mechanisms underlying these two processes (*32, 33*). Intramolecular amplification of tetracycline resistance determinants or a whole set of resistance determinants in plasmid NR1 (*34*) due to selection pressure using higher antibiotic concentration in growth medium is well documented. Amplification of the pAC27 Bgl II E fragment or the pJP4 Eco RI B fragment to provide seven to ten copies of the cloned fragment in association with a single copy of the vector to enable the host cells to utilize 3Cba (*12*) has been mentioned.

An example of genetic rearrangement (deletion-fusion followed by genetic duplication) is the modification of plasmid pJP4 (83 kb) to generate plasmid pYG2 (93 kb), which allows ready growth of *P. putida* cells on 3Cba (*12*). This modification occurs spontaneously when pJP4 is transferred to *P. putida*, selecting for $3Cba^+$. The transfer frequency under these circumstances is about 10^{-7}, and the $3Cba^+$ *P. putida* exconjugants grow well on 3Cba. If selection is made for Hg^r (encoded by pJP4), the transfer frequency is 10^{-2} and the Hg^r *P. putida* exconjugants harbor unmodified pJP4 and are phenotypically $3Cba^-$. Ghosal *et al.* (*12*) showed that expression of the pJP4 3Cba genes in *P. putida* requires deletion of a segment of about 15 kb from

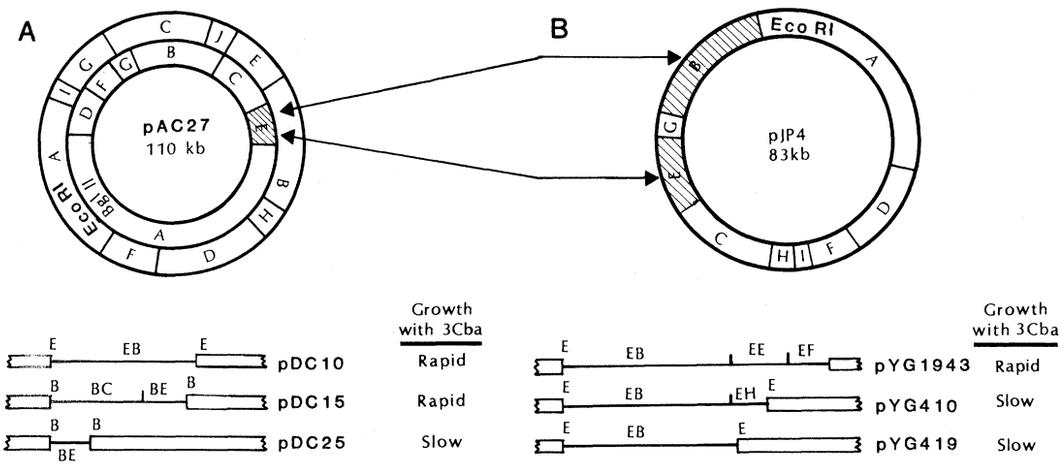

Fig. 2. Physical maps of the plasmid pAC27 (A) with Eco RI and Bgl II and plasmid pJP4 (B) with Eco RI. Shaded areas represent regions of homology, as indicated by the arrows. The cloning of various restriction fragments of each plasmid (single line) to generate recombinant plasmids and the ability of such recombinant plasmids to allow growth of the host cells with 3-chlorobenzoic acid are shown below the maps.

pJP4, followed by duplication of a 25-kb segment as an inverted repeat, as shown in Fig. 3.

Duplication-amplification as well as deletion-fusion events are often thought to involve partially or completely homologous sequences located at distinct points on the chromosome (*35*). These sequences lead to intramolecular recombinational events such as DNA-slippage replication (*36*) or intermolecular unequal recombination between daughter chromosomes or between two replicons (*37*). Such partially or completely homologous sequences may be present on the chromosome in a direct or inverted orientation. Several plasmids from haloaromatic-degrading bacteria, such as pJP4, pAC25, and pAC1100, have been examined for direct or inverted repeats. In all these cases the presence of homologous sequences as direct repeats was revealed by Southern hybridization or inverted repeats were detected as "snap-back" stem-loop structures by electron microscopy. Eco RI–digested pJP4 DNA, when denatured and rapidly reannealed, shows many stem-loop structures (Fig. 4). Preliminary studies indicate that the end points for concomitant deletion and duplication lie near or at these repeated sequences, suggesting a potential role of the repeated sequences in genetic rearrangements in these degradative plasmids.

Laboratory Construction of Strains with Broader Biodegradative Potential

Genetic selection in vivo. As mentioned previously, transfer of pJP2 or pJP4 to the 2,4,5-T–degrading strain of *P. cepacia* DC102 allows it to utilize both 2,4-D and 2,4,5-T. An important factor in the construction of bacterial strains capable of degrading novel chlorinated aromatic compounds is the recognition that a complete set of genes allowing degradation of chlorocatechols is borne on some transmissible plasmids (*8, 38*). Thus *Pseudomonas* sp. strain B13 can transfer the chlorocatechol degradative genes to many other *Pseudomonas* strains. In addition, introduction of other degradative genes greatly broadens the substrate specificity of bacterial strains. For example, strain B13 is incapable of utilizing 4-chlorobenzoic acid (4Cba) or 3,5-dichlorobenzoate (3,5-Dcb). Reineke and Knackmuss (*10*) and Hartman *et al.* (*39*) showed that it was possible to transfer the TOL plasmid to strain B13 and select under continuous cultivation variants of strain B13 that could use not only 4Cba but also 3,5-Dcb as the sole source of carbon and energy. They delineated the role of the TOL plasmid in providing a broad substrate-specific toluate oxidase that allows conversion of 4Cba and 3,5-Dcb to the corresponding chlorocatechols. Such chlorocatechols could then be utilized by strain B13 through the plasmid pWR1 (after some mutational divergence for 3,5-Dcb utilization).

Chatterjee and Chakrabarty (*40*) followed the same procedure and isolated similar variants from their $3Cba^+$ *P. putida* strain AC858 harboring the plasmid pAC25. Growth of AC858 in a chemostat in the presence of cells harboring the TOL plasmid allowed emergence of cells that could also utilize 4Cba. Such $4Cba^+$ cells harboring pAC27 showed transposition of a segment of the TOL plasmid on their chromosome to provide the broad substrate-specific benzoate oxygenase. Further enrichment of the $4Cba^+$ cells in the presence of 3,5-Dcb allowed emergence of cells that could slowly utilize this compound as the sole source of carbon and energy. Isolation of plasmid DNA from the slowly growing $3,5\text{-}Dcb^+$ cells demonstrated the appearance of a second plasmid, pAC29, which was shown by hybridization studies to be derived primarily by duplication of a segment of pAC27 with further mutational divergence and recruitment of the replication-incompatibility genes of TOL (*40*). Continued selection on 3,5-Dcb gave rise to faster growing variants that had a single plasmid (pAC31) with a restriction profile identical to that of the original pAC27 plasmid. Plasmid pAC31 allowed ready growth on 3,5-Dcb but not on 3Cba or 4Cba, although mutants could be isolated that allowed growth on all three compounds. It was concluded that pAC31 was generated by a homologous recombination of the segment containing mutated genes allowing degradation of 3,5-Dcb with exact substitution of the original segment from pAC27.

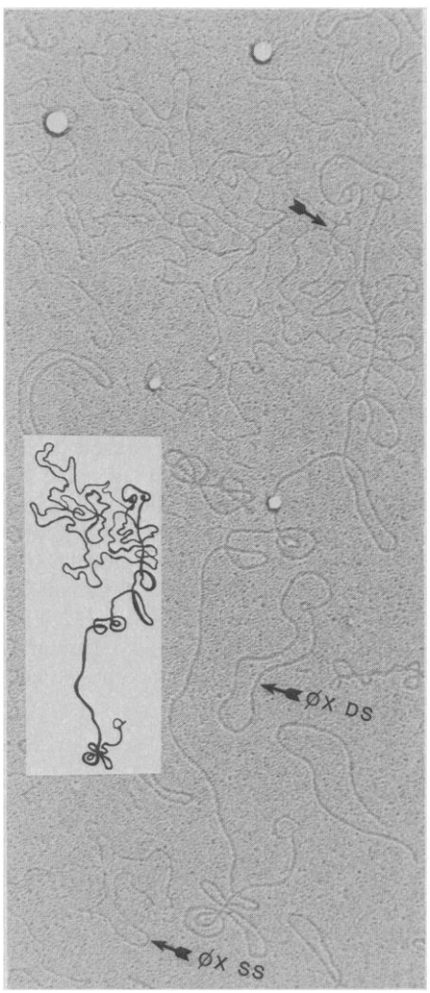

Fig. 3. Presence of a 25-kb duplication in an inverted orientation in plasmid pYG2 DNA, as revealed by electron microscopic detection of snap-back structures of the denatured and renatured whole DNA. The plasmid pYG2 (93 kb) is derived from pJP4 during selection on 3Cba and demonstrates deletion of 15 kb of pJP4 DNA and duplication of 25 kb of pJP4 DNA containing Eco RI fragments B, G, E, and F. A diagram of this molecule and the double-stranded (DS) and single-stranded (SS) ϕX-174 DNA (5.386 kb) molecules is also shown.

The use of TOL and pAC27 or pAC31 to allow degradation of various mono- and dichlorobenzoates is not the only example of in vivo genetic manipulations for the degradation of halogenated compounds. Schwien and Schmidt (*41*) showed that transfer of the chlorocatechol degradative genes from *Pseudomonas* sp. B13 to *Alcaligenes* strain A7, which is capable of growing on phenol, allows the exconjugant *Alcaligenes* strain A7-2 to utilize all three isomeric chlorophenols, which are not attacked by any of the parents. Similar plasmid transfer to *Pseudomonas* sp. WR401, which is capable of growing on salicylate but unable to attack chlorosalicylate, allows the exconjugant cells to utilize chlorosalicylate (*38*).

The role of transmissible plasmids in the total mineralization of environmental pollutants such as mono- or dichlorobiphenyls was demonstrated by Furukawa and Chakrabarty (*42*). They characterized a plasmid, pKF1, that allowed conversion of various chlorinated biphenyls to the corresponding chlorinated benzoic acids; however, no further conversion could take place. The role of pAC27 and pAC31 in specifying complete degradation of 3Cba, 4Cba, and 3,5-Dcb has been mentioned. The combined growth of bacterial strains harboring pKF1 and pAC27 or pAC31 allowed total mineralization of 4-chloro- or 3,5-dichlorobiphenyls, which could not be accom-

plished by any of the parents. Another example of the putative role of plasmid genes in the emergence of new degradative capability is that of 2,4,5-T degradation by *P. cepacia* strain AC1100 (*43*). Strong selection under continuous cultivation for 8 to 10 months in the presence of a pool of plasmid genes led to a strain of *P. cepacia* AC1100 that could not only utilize 2,4,5-T but also completely dechlorinate chlorophenols such as 2,4,5-TCP and pentachlorophenol (*26*). What little is known of the biochemistry of 2,4,5-T degradation by this strain has already been discussed.

Gene cloning. The role of the TOL plasmid in extending the substrate specificity of *Pseudomonas* sp. strain B13 from 3Cba to 4Cba or 3,5-Dcb has been mentioned. Under such circumstances, the TOL plasmid undergoes a mutation at the *xyl*E locus to prevent the formation of toxic intermediates from 3-chlorocatechol via the TOL-specified *meta* pathway. An alternative strategy was used by Lehrbach *et al.* (*44*), who cloned the *xyl*D gene and the *xyl*D and *xyl*L genes into *Pseudomonas* B13. Normally strain B13 can grow on 3Cba but not on 4Cba, 3,5-Dcb, salicylate, or chlorosalicylate. Introduction of the cloned *xyl*D gene allowed *Pseudomonas* B13 to degrade 4Cba, while introduction of the cloned *xyl*D and *xyl*L genes, followed by spontaneous mutational divergence, allowed emergence of *Pseudomonas* B13 variants that could also degrade 3,5-Dcb. Similar mutational divergence was previously shown to be necessary in pAC29 to allow growth with 3,5-Dcb (*40*). Lehrbach *et al.* (*44*) also cloned the naphthalene degradative gene *nah*G encoding salicylate hydroxylase from the plasmid NAH7 (*45*) and introduced the cloned gene into *Pseudomonas* B13. Expression of *nah*G enabled *Pseudomonas* B13 to convert salicylate and chlorosalicylate to catechol and chlorocatechol, which could be used as carbon sources by *Pseudomonas* B13 through pWR1-encoded enzymes. The cloned gene therefore enabled this organism to completely mineralize a new substrate such as chlorosalicylate.

Effectiveness of Genetically Manipulated Microorganisms in the Environment

The development of strains capable of degrading halogenated compounds such as 2,4,5-T, penta- and tetrachlorophenol, 2,4,5-trichlorophenol, and chlorosalicylate raises the interesting question of whether such strains can be used in toxic waste dump sites for removal of such compounds, many of which are not only toxic themselves, but are potential sources of dioxins including the highly toxic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Dump sites usually contain many chemicals in high concentrations, and even sites contaminated with a single toxic chemical will have many other easily assimilable carbon sources that may be utilized in preference to the toxic chemical. In addition, the indigenous microflora in contaminated soil may outcompete any exogenously added genetically improved strains.

It is, however, known that chemicals such as parathion and pentachlorophenol are rapidly degraded in nature by naturally adapted microorganisms (*46*). Crawford and co-workers (*46*) also demonstrated the effectiveness of a pentachlorophenol-degrading *Flavobacterium* species in removing pentachlorophenol from river, lake, and ground waters. Decontamination was most effective between 15° and 30°C, and pentachlorophenol concentrations (10 parts per billion to 100 parts per million) were reduced to undetectable levels within 2 or 3 days, suggesting the usefulness of a microbiological wastewater treatment process.

Using the 2,4,5-T–degrading *P. cepacia* AC1100, Chatterjee *et al.* (*47*) showed removal of more than 98 percent of the 2,4,5-T present at 1000 ppm from soil over a period of 1 week. Kilbane *et al.* (*48*) showed that weekly applications of this strain in soil contaminated with 10,000 to 20,000 ppm 2,4,5-T allowed removal of more than 90 percent of the chemical within 6 weeks. Removal of 2,4,5-T by AC1100 enabled the soil to support growth of broadleaf plants that

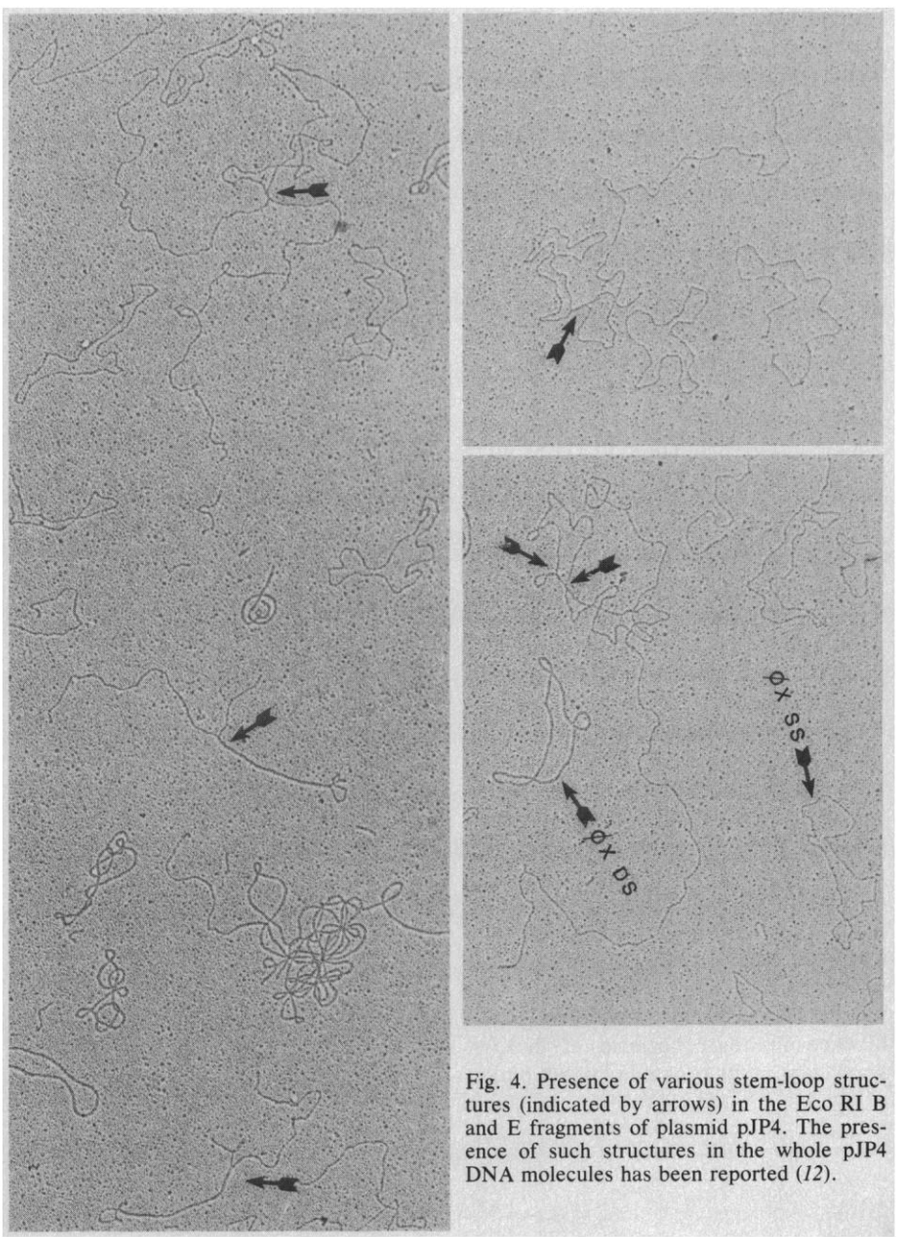

Fig. 4. Presence of various stem-loop structures (indicated by arrows) in the Eco RI B and E fragments of plasmid pJP4. The presence of such structures in the whole pJP4 DNA molecules has been reported (*12*).

are normally sensitive to low concentrations (10 to 15 ppm) of this herbicide. Kilbane *et al.* (*48*) also found that once the bulk of the 2,4,5-T was gone, the AC1100 cells could not compete with the indigenous microorganisms and died off rapidly, becoming undetectable in a few weeks. Availability of 2,4,5-T, however, allowed rapid proliferation of a few remaining cells. It was concluded by Kilbane *et al.* (*48*) that treatment of contaminated soil with a laboratory-developed microorganism such as AC1100 allows almost complete restoration of the soil with little threat of potential ecological disaster, since such strains are unable to survive and compete with indigenous microorganisms in the absence of the target chemical.

Concluding Remarks

Microorganisms in nature have apparently evolved the ability to degrade many halogenated compounds, particularly those with relatively few halogen atoms. The genes for the degradation of halogenated compounds have often been found to be plasmid-associated. Like the degradative genes for natural hydrocarbons such as toluene or naphthalene, encoded by TOL and NAH plasmids (*13, 14*), the degradative genes for halogenated compounds also appear to be clustered (*12*); this allows cloning of many such gene clusters in broad-host-range vectors for dissemination to a large number of Gram-negative bacteria. If genetically improved bacteria prove to be effective for the in situ degradation of other toxic chemicals besides 2,4,5-T, then the broad host range of such cloned gene clusters would lead to highly useful applications in contaminated areas.

An issue that must be addressed in this regard is that of regulating the application of genetically improved strains in an open environment. Such strains may prove to be useful not only for the removal of toxic chemicals from the environment but also for other purposes such as oil recovery, prevention of ice nucleation in agricultural applications, plant growth promotion, and so on. In a highly publicized case, the field application of a strain of *Pseudomonas syringae* to prevent frost nucleation has been blocked in court because of lack of knowledge of its probable impact on the environment (*49*). Although Congress is considering the development of appropriate guidelines or legislation and the Environmental Protection Agency is attempting to formulate the steps that might be needed for safe and hazard-free construction and release of genetically improved microorganisms, no definitive plans for regulation are in place. An understanding of the genetic and molecular basis of biodegradation of halogenated compounds by microorganisms, effective use of such understanding in constructing appropriate strains for the enhanced degradation of a number of high-priority toxic chemicals, and the development of guidelines to govern the application of such genetically improved strains will be key ingredients in solving our toxic chemical pollution problems.

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