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Degradation and Bioremediation of Herbicides by Microorganisms

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ABSTRACT: Herbicides are in dispensable tools in modern agriculture as they allow effective weed control, thereby increasing the efficiency of crop production. The indiscriminate use of chemical herbicides has caused an irreparable damage to every aspect of the environment and human welfare. There has also been destruction of the natural enemies of pests and non-target organisms like earthworms and nitrogen-fixing microbes. Nodulation in legumes has either been reduced or not even formed due to these toxic compounds. There has also been extensive damage to flora and fauna due to wide spread herbicide contamination of water bodies. Thus, there is a need to devise methods to (i) get rid of excessive use of herbicides without affecting the overall production and (ii) adopt microbiological and bioremediation measures to decontaminate the environment from residues of these persistent herbicides. In this article we have focussed on the second objective. Although metabolic pathways are characterized, genes encoding herbicide degrading enzymes have not been cloned. Therefore, further work involving characterization of the genes encoding newer herbicide degrading enzymes is required that will offer considerable promise for engineering herbicide resistance into crop plants so that high residues do not pose phytotoxic problem.

Keywords: Microorganisms, Degradation, Bioremediation, Herbicides, Herbicide degrading genes, enzymes.

INTRODUCTION

Herbicides are indispensable tools in modern agriculture as they allow effective weed control, thereby increasing the efficiency of crop production. Herbicides reach the soil through pre-planting and pre-emergence application or as foliage run off from post-emergence applications and later through the return of crop residues to the soil. Finally, residues of herbicides reach water bodies through surface runoff and percolates deep into the water table. Thus, both soil and water have been contaminated. Moreover, most of these herbicides are resistant to degradation resulting in their accumulation in soil and water.

The indiscriminate use of chemical herbicides has caused an irreparable damage to every aspect of the environment and human welfare. There is an endless list of health problems caused by herbicide poisoning, such as genetic damage, birth defects, psychological problems, immune deficiencies, kidney and liver damage, skin allergies, various forms of cancer etc. There has also been destruction of the natural enemies of pests and non-target organisms like earthworms and nitrogen-fixing microbes. Nodulation in legumes has either been reduced or not even formed due to these toxic compounds. There has also been extensive damage to flora and fauna due to widespread herbicide contamination of water bodies.

Thus, there is a need to devise methods to get rid of excessive use of herbicides without affecting the overall production and adopt microbiological and bioremediation measures to decontaminate the environment from residues of these persistent herbicides. In this article we have focussed on Microbial degradation and bioremediation of commonly used herbicides.

As we know biodegradation by microbes offers considerable promise as a strategy for bioremediation of contaminated sites and only a few herbicides have received the attention namely: phenols, phenoxy acids, nitriles, organophosphates, thiocarbamates and triazines. The phenols and triazines represent the most widely used persistent classes of xenobiotics. Among these groups, herbicides which require an immediate attention because of their hazardous effects on non-target organisms and their residual problems are represented in Table 1.

Table 1: Some Important herbicides used in agriculture and forestry.

Abbreviation	Chemical name
Atrazine	2-chloro-4- (ethylamino)- 6- (isopropylamino) – 1,3,5 – triazine
Dicamba	3,6-dichloro salicylic acid (DCSA)
EPTC	S-ethyl dipropyl thio-carbamate
2,4-D	2,4 -dichloro phenoxy acetic acid
2,4,5-T	2,4,5-trichloro acetic acid
TCA	Trichloro acetic acid
PCP	Penta chloro phenol
DCMU	3,(3,4-Dichloro phenyl)-1,1-dimethyl urea
DSMA	Disodium methyl arsenate
MCPA	2-methyl-4-chloro- phenoxy acetic acid
PMG	N-(phosphono methyl) glycine

Voluminous information is available on the diversity of microbes capable of degrading large number of herbicides. Progress has also been made with respect to the purification and characterization of enzymes involved and establishment of the metabolic pathways. Although considerable progress has been made on genetic aspects of microbial degradation of common herbicides like 2,4-D, 2,4,5-T much has not been done with respect to the several other herbicides which are also the common contaminants of the environment. This article also focuses on the use of catabolic genes in the degradation of herbicides, their characterization and finally transformation into suitable hosts for bioremediation.

II. MICROBIAL DEGRADATION OF CURRENTLY USED HERBICIDES

Herbicides like many other aromatic hydrocarbons are degraded both by biological and non-biological processes. However, in natural environment, it is difficult to distinguish between these two modes, though some reactions are clearly non-biological, such as photolysis, others: such as hydrolysis can be either non-biological or biological. Nonbiological processes are never complete whereas microbial activities lead to complete mineralization. Microbes, over billions of years have evolved an extensive range of enzymes, pathways and control mechanisms in order to degrade a wide array of aromatic compounds. Many species of algae, fungi, bacteria and actinomycete are known to degrade a large number of xenobiotics including herbicides. These microbes having degrading ability are obtained from soil by enrichment culture or non-selective techniques. Soil enrichment involves the increase in the microbial population possessing degrading activities, on continued exposure to any specific xenobiotic/s (1). Some microbes eventually adapt themselves to degrade other related compounds in the enrichment process called cross adaptation. Although many microbial species are known to degrade pesticides, molecular biology of only a few bacterial species have been worked out. This has partly been attributed to rapid growth rate, easy manipulation though these herbicides are quite persistent and extremely toxic. Diverse microorganisms are known to degrade phenols as given in the Table 2.

Table 2.

Herbicides	Microorganisms	References
Phenols and PCP	*Pseudomonas stutzeri* SPC2	Ahamad and Kuhni [2]
	Pseudomonas sp.	Raidehaus and Schmidt [3]
	Pseudomonas aeruginosa	Premlatha and Rajkumar [4]
	Pseudomonas sp. strain CP4	Babu *et al.,* [5]
2,4-D	*Arthrobacter*	Tiedje and Aiexander [6]
	Pseudomonas sp. *Pseudomonas* sp.	Lillies *et al.,* [7] Musarrat *et al.,* [8]
	Alcaligenes eutrophus	Don *et al.*, [9]
	Flavobacterium sp.	Chaudhry and Huang [10]
Alachlor	*Streptomyces capoamus, Streptomyces galbus, Streptomyces bikiniensis*	Sette *et al.,* [11]
2,4,5-T	*Nocardiodes simplex*	Kozyreuva *et al.,* [12]
Mecoprop	*Alcaligenes denitrificans*	Zipper *et al.,* [13]
Paraquat	*Agrobacterium tumefacians* *Pseudomonas fluorescens* *Bacillus cereus*	Tu and Bollen [14]
Thiocarbamates: EPTC	*Rhodococcus* sp. TE-1	Behki and Khan [15]
Triazines		
Atrazine	*Phanerochaete chrysosporium*	Mougin *et al*., [16]
	Pleurotus pulmonarius	Masaphy *et al*., 1994 [17]
	Pseuodmonas	Mandelbaum *et al*., 1995 [18]
	Agrobacterium tumefaciens	Devers *et al.,* 2005 [19]
	Rhodococcus erythropolis	Andersen *et al.,* 2001 [20]
s-triazines	*Pseuodmonas* spp.	Behki and Khan [15]
	Rhodococcus corallinus	Cook and Hutter,1986 [21]
Glyphosate	*Pseuodmonas*	Papkeand Amrhein [22]
	Flavobacterium	Papkeand Amrhein [22]
	Arthrobactor atrocyaneus ATCC 13752	Papkeand Amrhein [22]
	Pseudomonas sp GLC 11	Selvapandian and Bhatnagar [23]

A. Phenols

Phenol and its derivatives are common environmental pollutants originating, mainly, from coal gasification plants, coke ovens, petroleum refineries, plastic and pharmaceutical industries, wood preservation and air craft maintenance. Babu *et al*., [5] also isolated several strains of *Pseudomonas* sp. which utilized as much as 1.5 g phenol/l as sole source of carbon. Similarly, Ahamad and Kunhi, [2] reported a strain of *Pseudomonas stutzeri* SPC2, from municipal sewage, which utilized phenols up to 1200 ppm as a sole source of carbon and energy, however, the degradation followed an ortho pathway though majority of bacterial species degrade phenol through the meta pathway. Among phenols, PCP (pentachlorophenol) is one of the most widely used broad spectrum biocide used as fungicide, insecticide, herbicide and algicide [24]. Various microorganisms are known to degrade these xenobiotics are briefly mentioned in Table 2.

An enzyme that catalyses the dehalogenation of PCP has been isolated and partially purified from *Arthrobacter* (Schenk *et al*., [25]. The involvement of two genes pcp B and pcp-C in the metabolism of PCP along with their respective enzymes have been reported. Various bacterial species completely utilized PCP in batch cultures as a sole source of carbon and energy. Complete degradation of PCP to CO, with the release of chloride ion by *P. aeruginosa* has also been reported [4]. However, in certain cases degradation of PCP was found to be through co-metabolism using glucose as its co-substrate.

B. Phenoxy Acids

Since the release of the herbicide 2,4-D in the 1940's and the understanding that it was readily decomposed by a variety of microorganisms, microbial degradation was considered to be a major factor in the dissipation of the phenoxy herbicides in the environment. The most widely used members of this group are: MCPA, 2,4-D and 2,4,5-T. Apart from several effects on non-target organisms, they have been reported to cause multiple chromosomal aberrations and has been implicated in birth malformations and neurological problems in affected populations. As these chemicals are manufactured and used in very large quantities, effective handling of their production, wastes and the contaminated environment is urgently required [26]. 2, 4-D does not persist for long in the environment (half-life in soil is 1-6 weeks) as it is susceptible microbial degradation. However, adverse conditions such as low pH and low temperature are known to promote its longevity. 2, 4,5-T on the other hand is degraded relatively slowly by soil bacterial populations [26]. It has been suggested that its persistence is determined by the number and position of chloride atoms as substances containing a chloride ion in the meta position are more stable and persistent to degradation.

Biodegradation of 2,4-D has received considerable attention lately, not only due to its extensive use but also because it serves as a model compound for understanding the mechanism of biodegradation of other, structurally related and environmentally significant halo-aromatic compounds. Metabolism of 2,4-D by various bacteria have been reported such as *Arthrobacter* sp., *Pseudomonas* sp., *Alcaligenes eutrophus* and *Flavobacterium* sp. (Table 2). Information on its metabolism, pathways involved and the catabolic genes responsible have been discussed elsewhere Kumar *et al*., [27,28]

An haloalkaliphilic bacteria, belonging to the family Halomonadaceae have been found to degrade 2,4-D [29]. Probably this bacterium utilizes the samebiochemical pathways as most of the 2,4-D degrading bacteria from nonextreme environments. Hybridization of the DNA of this isolate with pJP_4 genes tfd A, B, C and D encoding the first four steps of the 2,4-D degradation pathways in A. eutrophus JMP134 was found to be at medium or low stringency. Further, comparison of the partial sequences of the tfd A gene from the alkali Lake isolates with those of bacteria from non-extreme environments suggested a common genetic origin of the 2,4-D degradation pathway in the two groups of microorganisms. In extreme environment, this strong compatibility of degradative enzyme can be exploited further to develop a suitable bioremediation system.

In addition to bacterial species, actinomycetes are also capable of metabolizing 2,4-D and related herbicides. For instance, Kozyreuva *et al.*, [12] reported actinomycete Nocardiodes simplex SE capable of degrading different chloroaromatic compounds including 2,4-D and 2,4,5-T by the dehalogenation pathways. 2,4-D is dehalogenated at the fourth position on the aromatic ring and is replaced by an OH group. Evidently, microbial degradation pathway of 2,4-D, 2,4,5-T etc. have been investigated in detail, however, the microbial metabolism of mecoprop (RS)-2-(4-chloro-2-methylphenoxy) propionic acid) remains to be elucidated. Two more bacterial cultures with the ability to utilize mecoprop as a growth substrate have been isolated [30, 31]. Both strains *Sphingomonas herbicidovarans* (previously designated *Flavobacterium* sp. Strain MH [30] and *Alcaligenes denitrificans* were able to use mecoprop as the sole carbon and energy source [13]. When strain MH was offered racemic mecoprop as the growth substrate it could completely mineralized both its (R) and (S) racemic enantiomers as is shown by biomass formation, substrate consumption, and stoichiometric chloride release. However, the (S) enantiomer disappeared much faster from the culture medium than the (R) enantiomer. These results suggest the involvement of specific enzymes for the degradation of each enantiomer and it also emphasizes on the importance of using enantiomer specific analytical tools and the necessity of treating enantiomers as distinct molecules.

C. Thiocarbamates

Soil microbes contribute significantly in the bioremediation of thiocarbamate when incorporated into the soil. Microbial degradation of EPTC (S-ethyl dipropyl thiocarbamate) involves hydrolysis at the ester linkage with the formation of a mercaptan, CO_2, and a secondary amine. The mercaptan is converted into an alcohol by trans-thiolation and further oxidized to an acid before entering into the metabolic pool. The amines are oxidized to ammonia, CO_2, and water. Bacterial degradation of EPTC appears to be largely confined to *Rhodococcus* sp. Strain TE 1 [32] and *Rhodococcus* sp. Strain JE1 [33]. The latter authors proposed hydroxylation of the α-propyl carbon of the N, N-dialkyl moiety as the major route of EPTC metabolism by strain JE1. The unstable α-hydroxypropyl EPTC thus produced would decompose into propionaldehyde and N-dipropyl EPTC. Another important metabolite identified was EPTC-sulfoxide [33]. Dipropylamine but not N-dipropyl EPTC was generated during degradation of EPTC by *Rhodococcus* sp. Strain TE 1, suggesting the existence of another pathway in this strain [32].

D. Triazines

Herbicides containing an s-triazine (symmetrical triazine) ring are relatively persistent in the environment. They include atrazine, cynazine, melamine, propazine and simazine. Of these, atrazine is the most widely used herbicides in the world for the control of annual grasses and broad leaf weeds in corn and sorghum fields. It is microbially degraded by hydrolysis at the second carbon or by N-dealkylation of side chains or by deamination. Despite that apparent biodegradability, atrazine has led to the contamination of terrestrial ecosystems and ground waters. It causes ecological and public health problems and can even cause damage at concentrations above 3 µg/L. Atrazine [2-chloro-4- (ethylamino)- 6- (isopropylamino) – 1,3,5 – triazine], a derivative of s-triazines is the most extensively used herbicides in agriculture and forestry sectors. Many workers reported the biodegradation of atrazine [34, 35]. This strain has a potential to be used for the remediation of atrazine under in situ conditions. Further the degradation process can be maximized by optimizing the degradation parameters like pH and temperature. Neutral pH was the most suitable for the biodegradations as the increase in pH decreases the rate of breakdown. Mixed bacterial consortium of bacterial species like *Rhizobium, Pseudomonas alcaligenes, Rhodococcus, P. aeruginosa, Klebsiella* sp. *Comomonas* sp. etc. was capable of eliminating atrazine from contaminated soil [36-38].

A number of soil microorganisms which metabolize atrazine have been reported Table 1. However, the enzymatic systems involved in their metabolism have not yet been characterized. The biostability of the N-alkyl side chains of the herbicide atrazine has been a major difficulty in obtaining the degradation of this compound by microorganisms. The streptomyces sp. PS1/5 was capable of removing the N-alkyl groups of atrazine. Degradation of atrazine occurred during the growth phase and increasing aeration resulted in complete disappearance of low levels of atrazine [39]. Atrazine was found to be converted into N, N-didealkyl atrazine (CAAT) in addition to traces of CIAT only.

The major degradation products generated by white rot fungi such as *Phanerochaete chrysosporium* (Mougin *et al*., [16] and *Pleurotus pulmonarius* (Masaphy *et al*., [17] are N-dealkylated products. Certain mycorrhizal fungi such as *Hymenoschyphus ericaeare* able to metabolize, triazine but their metabolic products have not yet been characterized. Bacterial isolates capable of s-triazine dealkylation have been identified as *Pseudomonas* spp. [32] or as nocardioform actinomycetes namely Nocardia sp. and *Rhodococcus corallinus* NRRLB 15444 [40, 21]. Behki *et al*., [41] reported that *Rhodococcus* sp. strain TE 1 can degrade atrazine efficiently to produce the dealkylated metabolites deisopropylatrazine (2-chloro-4-amino-6-isopropyl amino-s-triazine [CIAT] and Deethylatrazine (2-chloro-4-amino-6-isopropyl amino-s-triazine [CIAT] and that this catabolic function was associated with an indigenous 77-Kb plasmid in this bacterium. There are two more isolates of *Pseudomonas* [18] where the degradation is more efficient but by different pathways. The dechlorinating enzyme, s-triazine hydrolase, encoded by the trz A gene was purified from R. corallines [42]. By combining the catabolic activities of both *Rhodococcus* sp. StrainTEI and *R. corallinus* would result in dealkylation and dechlorination of atrazine. Cook and Hutter [40] also reported similar results where a mixed culture of *R. corallinus* and *Pseudomonas* sp. strain NRRLB-12227 can use the dechlorinated and (partially) dealkylated s-triazine ring as a nitrogen source [40]. The degradative pathway of these triazine ring has been elucidated for these strains by Cook and Hutter, [40].The DNA fragments carrying the genes required for ring cleavage were also cloned [43,44]. The cloning, characterization and expression of the dichlorination gene involved in atrazine degradation has been reported by de Souza *et al*., [45]. A 22-Kb Eco RI genomic DNA fragment designated as pMDI, was shown to encode atrazine dichlorination activity in *Escherichiacoli* DHS. Atrazine degradation was demonstrated by a zone-clearing assay on agar medium containing crystalline atrazine and by chromatographic methods. A gene conferring the atrazine- clearing phenotype was subsequently subcloned as a 1.9Kb AvaI fragment in pACYC184, designated as pMD4, and was expressed in *E. coli.* This result and random Tn-5 mutagenesis established that the 1.9 Kb AvaI fragment was essential for atrazine dichlorination [45]. *E. coli* containing pMD4 degraded atrazine to hydroxy-atrazine which was detected only transiently. Thus, hydroxy-atrazine appears to be the first metabolite in atrazine degradation *by Pseudomonas* sp. strain ADP and the genes encoding such degradations are widespread in nature and contribute to the formation of hydroxy-atrazine in soil. The widespread contamination by this herbicide has provided a selective pressure to different genes and biochemical mechanisms for the mineralization of atrazine in the environment.

Evidently, dealkylation is a major pathway for atrazine biodegradation in soils however, there is no bacterial or fungal enzymes involved in this degradation process. It was shown that the 77-Kb plasmid of *Rhodococcus* sp. Strain TEI, which is responsible for the degradation of thiocarbamate herbicides such as EPTC [46], also enables N dealkylation of atrazine and related s-triazine (Behki *et al*., [41, 47]. N-dealkylation of atrazine also constitutes a crucial step in the bacterial catabolism of EPTC (Dick *et al.,* [33]. Metabolic pathway for the degradation of s-triazine has been proposed by Cook [48] using *Rhodococcus corallinus* NRRLB 15444R and three genes encoding the enzymes have also been characterized (Table 3).

The results of Cook and Hutter [21] were further confirmed by Eaton and Karns [43] where they observed that genes trz-B, tra-C and trz-D are closely linked on a DNA segment of 6.1 Kb. This segment is bordered by apparently identical DNA segments of size between 2.2-2.6 Kb which seem to cause the bordered DNA to undergo transpositions. The presence of these genes on an apparently mobilizable DNA segment flanked by insertion sequence elements may be important in bringing about rapid evolutionary changes by causing genetic re-arrangements that result in altered gene regulation, gene duplication and the recombination of genes into novel units.

This supports the idea that genetic exchange is important in the evolution of novel pathways for the degradation of xenobiotics by naturally occurring bacteria.

E. Glyphosate

Currently, glyphosate is the most extensively used non-selective herbicide. There have been reports of microbial degradation of this phosphonate herbicide by *Pseudomonas, Flavobacterium* and *Arthrobacter atrocyaneus* ATCC 13752 which utilize it as a phosphate source [22]. Selvapandian and Bhatnagar [23] have also isolated *Pseudomonas* sp. GLC11 capable of growth in the presence of upto 125 µg glyphosate. Unlike other bacterial strains, this isolate grows equally well in commercial as well as analytical grade glyphosate. These workers have partially purified the carbon phosphorus (c-p) lyase, the 220KD enzyme responsible for c-p cleavage in this herbicide. This enzyme is located in the periplasmic space of *Pseudomonas* sp. GLC 11 [23]. Since glyphosate is utilized as a phosphate source, extra phosphate inhibits the glyphosate transport, or degradation and/or represses these processes at the level of gene expression. Studies on phosphonate metabolism in *Escherichia coli* and *Enterobacter aerogenes* have shown that genes encoding both the transport and degradatory functions in phosphonate metabolism are subject to repression by inorganic phosphate as part of the PHO regulon [49]. Although metabolic pathways are characterized, genes encoding glyphosate degrading enzymes have not been cloned. Therefore, further work involving characterization of the genes encoding glyphosate degrading enzymes is required that will offer considerable promise for engineering glyphosate resistance into crop plants so that high residues do not pose phytotoxic problem.

III. MOLECULAR GENETICS OF HERBICIDE DEGRADATION

Although many species of fungi and other microbes are known to degrade herbicides, genetic simplicity of bacterial genomes is particularly appealing. The degradative genes in bacteria are located either on the main chromosome, plasmids or on transposons. There is also a possibility that they are distributed in parts between these two components of bacterial genome. Degradative plasmids carry genes that code for enzymes responsible for herbicide metabolism. If a degradative plasmid is conjugative, it can be transferred to other hosts thereby transferring a non-degradative to a degradative phenotype. This forms the basis for plasmid assisted molecular breeding (PAMB) technology. A large proportion of degradative plasmids isolated from various species of Alcaligenes and Pseudomonas possess a broad host range [50] whereas, majority of the plasmids have narrow range. Such a broad host range specificity provides an important route for the evolution and spread of degradative functions through soil microbes. The first report of plasmid borne degradative genes among soil bacteria came from P. putida capable of degrading camphor, an insect repellent [51]. Now several bacterial species with plasmids borne degradative character are available and the use of a few of them in the degradation of selected herbicides is discussed (Table 3).

Table 3: Genes encoding various pesticide degrading enzymes.

Genes	Enzymes	Substrate
tfdA	2,4-D monooxygenase	2,4-D
tfdB	2,4-hydroxylase	2,4-dichlorophenol
tfdC	3,5-chlorocatechol-1,2-dioxygenase	3,5-dichlorocatechol
tfdD	2,4-dichloromuconate	2,4-dichlorocyclo isomerase muconate
tfdF	chlorodiene lactone hydrolase	trans-2-chlorodienelactone
tfdE	chlorodiene lactone isomerase	cis-2-chlorodienelactone
PCP-B	PCP-4-monoxygenase	pentachlorophenol
PCP-C	TeCH-reductive dehalogenase	Tetrachlorohydroquinone
tftA+tftB	2,4,5-trichlorophenol oxygenase	2,4,5-trichloro phenoxy acetic acid
tftC+tftD	2,4,5-trichlorophenol hydrolase	2,4,5-trichloro phenoxy acetic acid
tftH	hydroxy quinol 1,2 dioxygenase	ortho-hydroxyquinol 1,2,4-trihydroxybenzene
tftE	maleyl acetate reductase	maleyl acetate
trzA	s-triazine hydrolase	melamine and deethylsimazine
trzB	ammeline amino liydrolase	triazine herbicides
trzC	ammelide amino hydrolase	triazine herbicides
trzD	cyanuric acid aminohydrolase	triazine herbicides
atzA	atrazine chlorohydrolase	hydroxyatrazine

Adapted from Kumar *et al.,* [27].

A. Nitriles

The benzonitrile herbicide, bromoxynil is extremely effective inhibitor of nitrification in soil. However, soil microbes have acquired the ability of degrading this herbicide to its respective benzamide and benzoic acid derivatives. A strain of *Klebsiella ozanae* has been isolated that uses bromoxynil as a source of nitrogen. This strain harbours 82 Kb plasmid containing then itrilase gene (bxn) which is constitutively expressed. AN itrilase enzyme is highly specific for bromoxynil and acts on the cyano-group converting it to a non-phytotoxic compound.

The bxn gene has been constitutively expressed in E. coli by direct transfer [52]. Irreversible loss of the ability to metabolize bromoxynil, both in *K. ozanae* and *E. coli*, has been found to be associated with the reduction in size of the plasmid from 83 Kb to 68 Kb. In E. coli this conversion is the result of a host recA' dependent recombination. Even the polarity and location of bxn gene have been determined by assaying hybrid constructs of the nitrilase gene fused with the heterologous lac promoter. Success has also been achieved in obtaining transgenic plants resistant to the nitrile herbicide, bromoxynil [53]. This has been a result of expressing the bxn gene encoding bromoxynil detoxification enzyme, nitrilase from *Klebsiella ozanae* into crop plants.

B. Phenoxy Acids

The first report on plasmid mediated degradation of synthetic pesticides, came for the herbicides 2,4-D and MCPA [54]. Infect, 2, 4-D was the first xenobiotic compound to be biodegraded to innocuous products of the tricarboxylic acid cycle and one of the first to provide the molecular basis for the organization of catabolic genes [55]. Fisher *et al.*, [54] isolated and characterized the plasmid pJP1 D from *Alcaligenes paradoxus* responsible for 2,4-D degradation. The genes responsible for the metabolism of 2,4-D are located on this conjugal plasmid and mapped by transposon mutagenesis [56]. The pJP4 genes (tfd-A to tfd-F) encoding various enzymes required for the different steps in 2,4-D degradation pathway are outlined are as follows. The tfd-A gene codes for an α-ketoglutarate dependent dioxygenase that convert 2,4-D to 2,4 dichlorophenol and is located upstream of and transcribed separately from the other tfd genes [57, 58]. The tfd B genes codes for phenol hydroxylase that converts 2,4-dichlorophenol to dichlorocatechol and it is downstream of the other tfd genes and is also transcribed separately. The tfd-C gene encodes a 1,2-dichlorocatechol dioxygenase and is found in a single operon with the tfd DEF genes. The latter produce enzymes that oxidize the ring cleavage product to β-ketoadipate which is then metabolized by enzymes encoded on genome [59].

These plasmid borne tfd genes are highly homologous and fairly ubiquitous, for instance, these have been found in *P. putida* isolated in Estonia pEST4011 [60], *Pseudomonas cepacia* isolated in India [pMABI [61], *Flavobacterium* species isolated in Halmenas Florida PRC10 [10] and even in an alkalophile [29]. In spite of these reports, all 2,4-D degraders do not necessarily carry plasmid encoded catabolic genes identical to those on pJP4. Genes less homologous or not at all homologous to the tfd genes are commonly encountered but poorly characterized. Amy *et al.,* [62] described several strains that show no homology to pJP4. It appears that the genes of bacterial populations responding to 2,4-D in a Michigan soil are not homologous to the tfd genes. Two studies have demonstrated that 2,4-D catabolic determinants are chromosomally encoded in some strains [63]. The tfd genes block cannot be used as universal gene probe for 2,4-D degradative bacteria [64] here genetic recombination would play a major role in the evolution of these catabolic genes.

Thus, most of the genes responsible for 2,4-D degradation are plasmid borne, however, one of the key gene associated with the enzyme chloromethylacetate reductase is chromosomally encoded. The significance of this particular gene can be visualized from the fact that although pJP4 is transmissible to other bacterial strains, and its genes may be expressed in these hosts, only those strains that possess a chromosomally encoded reductase are able to utilize 2,4-D as a sole source of carbon [65]. In addition, two regulatory genes tfd-R and a monooxygenase gene tfd-S have also been identified which plays a significant role in the degradation of herbicides [58, 66].

During the 80's, Amy *et al.,* [62] have also reported plasmid mediated degradation of 2,4-D in a number of aquatic bacterial isolates. These workers constructed a hybrid plasmid by cloning 2,4-D degradative genes from pEML159 into the vector plasmid pBR325. This hybrid plasmid has been expressed in E. coli HB 101. The resultant E. coli pSA122 has been found to contain a 14.8 Mb fragment from pEML159 which confers the ability to release CO□ from 2,4-D leveled in the acetate portion.

Chaudhry and Huang [10] isolated a new plasmid pRC10 from *Flavobacterium* sp. 50001 which harbours genes essential for degradation of 2,4-D and MCPA. Comparison of pRC10 with pJP4 from *A. eutrophus* has shown regions of homology between the two plasmids. Restriction fragment of pRC10, which shares homology with the tfd genes of pJP4, have been cloned into a broad host range plasmid and studied in *P. putida*. The cloned DNA fragment has been found to express the genes tfd A and tfd B. In spite of the similarity in function, the size and restriction pattern of pRC10 (45 Kb) are considerably different from those of pJP4 (80 Kb).

An actively growing pure culture of *Pseudomonas cepacia* AC 1100 can degrade more than 97% of 2,4,5-T (1 ppm) within six days and utilize it as the sole source of carbon and energy [67]. Of pure culture capable of growing on 2,4,5-T, obtained initially as a mixed culture is an indication of the various gene transfers and recombinational mechanisms operative in the selection of a faster growing variant in the utilization of xenobiotics. Since plasmids play a critical role in supplying gene pools, which the mixed bacterial population can use to evolve 2,4,5-T degradative capability [67]. It is anticipated that the degradative genes would be part of one or more plasmids in the final strain AC 1100. The instability of 2,4,5-T degradation genes in AC 1100 points to this possibility.

C. Thiocarbamates

The involvement of plasmid specified enzymes in thiocarbamate degradation has been hypothesized on the basis of the rapid irreversible loss of degradation activity in some isolates. Tam *et al.,* [46] reported the isolation of *Rhodococcus* sp. TE1., initially identified as an *Arthrobacter* sp., which can metabolize EPTC.

The degradation of EPTC in this strain has been associated with a 77 Kb plasmid possessed by this organism. Another bacterium, *Flavobacterium* sp. VI 15 is also capable of utilizing EPTC as a sole source of carbon [68].

Derivative of strain VI 15 that are unable to utilize EPTC have been found to lack a 100 Kb plasmid pSMB2. The correlation of these two findings indicates a possibility of a common origin of pSMB2 and the 77 kb plasmid of strain TE1. Although the outstanding metabolic versatility of *Rhodococcus* species is well documented [69], understanding at the molecular and genetic levels of the enzymatic processes involved is generally poor. Further research is required in this area in order to characterize more of the thiocarbamate metabolizing genes and establish the exact role of the cytochrome P-450 system.

D. Chloroaromatic Herbicides

A well-known example of this class of herbicides is the Dicamba, used as a model for the in-situ bioremediation of many recalcitrant compounds. This herbicide is degraded and used as the sole carbon source by *Pseudomonas* sp. strain *Pseudomonas Xanthomonas maltophilla* (PXM). The dicamba degrading ability is attributed to the presence of a large plasmid pDKI [70], approximately 250 Kb in size. Cork and co-workers purified this megaplasmid and described it as the largest prokaryotic plasmid encoding for chloroaromatic degrading enzymes. This plasmid could be stabilized by vigorous environmental control and genetic manipulation [71].

Studies with whole cells [71, 72] have shown that the first degradation product of dicamba is 3,6-dichloro salicylic acid (DCSA). Wang *et al.,* [73] reported the presence of an O-demethylase in *P. maltophilla* DI-6 due to which dicamba was rapidly converted to DCSA. This enzyme can be separated into three components classified as a reductase, a ferredoxin, and an oxygenase [73].

E. Paraquat

Paraquat herbicide has been widely used over five decades to control annual and perennial weeds of cotton, rice, soybean etc. However, due to high toxicity and environmental pollution many countries have banned its active use [74, 75]. Several bacterial and fungal species belonging to different genera have been isolated from paraquat -contaminated soils by enrichment culture techniques and characterized based on microbial and molecular tools. As illustrated in Table 2. many paraquat degrading microorganisms, like, *Aerobactor aerogenes, Agrobacterium tumefaciens, Pseudomonas fluorescens,* and *Bacillus cereus* have been isolated from contaminated soils. These bacterial species can efficiently mineralize and utilize paraquat as a sole source of carbon and nitrogen [76]. A few other bacteria like *Oscillospira* sp. BCk-1, *Clostridium prazmowski* BCK-2 and *Sporobactor orenetal* BCK-3 efficiently degraded paraquet approximately upto 80% after 3 days of treatment [77]. Microbes have been found to convert paraquat to monoquat by demethylation. Further, oxidative cleavage of monoquat led to the formation of 4-carboxy-1-methylpyridinium ion [78]. Pyridinium ring carbons are released into the soil as CO_2 and methylamine by microbial activity [79]. Methylamine can further be utilized as sole source of nitrogen and carbon. However, detailed pathway and the enzymology involved in degrading various metabolites is not yet properly understood. Bacteria isolated from paraquat-contaminated environments with high degradation capacity and potential for bioremediation holds promise for decontamination strategy. In this regard, microbial consortia instead of single strain can produce better degradation results. In order to enhance, degradation efficiency, the relationship between members of such a consortium and their adaptability to adverse environments should be studied. Further, more bacterial species should be screened with better adaptability to wide range of soil factors such as pH, temperature, salinity, metal ions and nutrient availability.

F. Triazines

Catabolic genes responsible for the degradation of the thiocarbamate seems to have evolved through cross adaptation with either atrazine or vice-versa. For example, only those strains of *Rhodococcus* that could degrade EPTC have been found to metabolize atrazine under aerobic conditions to CIAT and CEAT as dead-end products [41]. This bacterium can also metabolize other s-triazines (Cyanazine, propazine and simazine) and these reactions have been associated with a 77 Kb plasmid, previously known to be required for EPTC degradation [46]. Thus, all the atrazine degrading strains are also capable of degrading EPTC, whereas strains unable to degrade atrazine likewise cannot degrade EPTC. This may be due to the fact that microbial degradation of thiocarbamates also involves hydrolysis, as is the case for s-triazines. It should, however, be noted that triazines and thiocarbamates have very different chemical structures.

Therefore, it is evident that metabolism of both atrazine and EPTC is associated with the 77 Kb plasmid of strain TE1. However, strain TE1 does not use atrazine as a sole nitrogen source, suggesting that the molecule is not deaminated nor dealkylated by an oxidative mechanism as the absence of oxygen inhibits the metabolism. Further expression of atrazine degrading activity does not require induction by this substrate. Apart from s-triazines, thiocarbamates and organophosphates insecticides, the 77 Kb plasmid of strain TEI has also been reported to be associated with the degradation of aryl N-methylcarbamate insecticides by a co-metabolic process [80]. The association of this 77 Kb plasmid with the biodegradation of such a wide array of xenobiotics could be exploited while constructing a suitablemicrobe for bio-reclamation purpose. There is a strong possibility that this cytochrome P-450 system is also required for atrazine degradation. Apparently, this strain is capable of degrading structurally different herbicides by the use of a cytochrome P-450 with relaxed substrate specificity.

Infect, it was the same plasmid of *Rhodococcus* sp. strain TEI that is required for degradation of both s-triazines and thiocarbamates and is also essential for ring hydroxylation of N-methyl carbamate insecticide [80]. By using the cloned genes of *Rhodococcus* sp. strain NI 86/21, it should be possible to elucidate whether the cytochrome P450 system or other plasmid encoded enzymes are responsible for the degradation of variety of pesticides viz; thiocarbamates, s-triazine N-methylcarbamate. There is another possibility of having a system similar to the nonspecific oxidative lignin decomposing system of white rot fungi which enables them to degrade a variety of environmental pollutants, including halogenated aromatic compounds [81, 82]. The involvement of this system in atrazine degradation cannot be ruled out [16, 17]. This knowledge of dechlorinating enzymes and the corresponding genes involved in the further breakdown to dealkylated triazines by *R. corallinus* together with the availability of triazine ring-cleaving *Pseudomonas* isolate, creates a new perspective for the engineering of strains with a potential for accelerated degradation of triazine herbicides. Further, manipulation of the cloned genes may also produce enzymes with higher activities. The cloned genes could be further engineered for introduction into the plant protoplasts such that atrazine resistant or tolerant plants are generated for use when atrazine residues in soil may pose a phytotoxicity problem.

CONCLUSIONS

Microbial degradation offers considerable promise as a strategy for bioremediation of contaminated sites and the herbicides that have received the most attention include phenols, phenoxy acids, nitriles, organophosphates, thiocarbamates and triazines. Bioremediation methods have to be devised to get rid of excessive amount of herbicide residues left in the soil and to make the environment free from their contaminants. However, the potential of biodegradation processes to control pollution from herbicide waste must acknowledge the obstacles that confront the practical applications of this technology. Compared with the list of widely used herbicides, only a few characterized microbial strains transform many of these recalcitrant herbicides into less toxic or more labile products to environmentally safer limits. For many other compounds which have been introduced a few years back, no microbial strain with biodegradation potential have been identified. There is a need to isolate organisms which have the abilities to degrade such herbicides. In this regard, microbial consortia instead of single strain can produce better degradation results. In order to enhance, degradation efficiency, the relationship between members of such a consortium and their adaptability to adverse environments should be studied. Further, more bacterial species should be screened with better adaptability to wide range of soil factors such as pH, temperature, salinity, metal ions and nutrient availability.

In addition, gene cloning in microbes offers a great challenge in channelizing their potentials to solve the present crises of environmental decontamination. The development of biochemical and genetic techniques has expanded the potential for experimental advances in biodegradation leading to the isolation and further manipulation of potentially useful biodegradative genes from diverse microbial strains.

Biodegradation strategies using engineered strains although offer great promise, it has also generated a lot of controversy as to their fate in the environment. It is also not clear that to what extent genetically engineered microbes (GEMS) will replace the native strains and whether these would be suitable for pilot scale studies on pesticide biodegradation. The use of suicidal genes upstream the promoter sequence if generated, will help in recalling such organisms. All this can be achieved only if we have a clear understanding of genes involved, biochemical pathways of degradation, their control and finally expression in other organisms including higher plants. Gene transfer from native, microbial strains to industrially useful bacterial strains will undoubtedly lower the cost of production of important biodegradation enzymes for bioreactors. A new genetic engineering techniques have gained broad applicability in biodegradation field.

Till now lot of laboratory data has been generated on isolation, characterization of enzymes and genes involved, however, little progress has been made to successfully transfer the bio remediation technology to the field. This is primarily due to two reasons (i) transfer of indigenous microbes degrading pesticides or other xenobiotics into the field is not always smooth as their survival, multiplication and degrading capability is hindered and (ii) use of GEMs is still a controversial issue and probably would take more time to be finally released into the environment. Moreover, monitoring of their ecological impact is essential and this data has to be made available before their release into the environment. In spite of these problems, continued attempt should be made to utilize pesticide degrading microbes either by improvising the indigenous organisms or by using GEMS carrying suicidal genes so that these can be recalled once the site is bioremediated. The development of bioremediation system or consortia also require more knowledge about microbes as regard to their catabolic activities. This will need an untiring effort in monitoring clean and polluted environments and the response of indigenous and applied microbial flora to changing circumstances. Some of the tools for doing so already exist including polymerase chain reaction (PCR) based assays and the use of fluorescent gene markers.

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