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Genetic Engineering and Problems of Environmental Pollution

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I. Environmental Pollution: a perspective

We live in an era where chemicals play a very important role in our every day life. The production and use of synthetic chemicals in the United States has doubled every eight years during the last two decades and the usage of pesticides alone has increased from 1.1 billion pounds a year in 1971 to 1.4 billion pounds in 1977 and has approached more than 1.5 billion pounds by the end of the seventies. Uncontrolled production and environmental release of large amounts of synthetic chemicals has produced massive pollution problems, that led the United States Congress to promulgate in October, 1976 the Toxic Substances Control Act (TSCA), stipulating that no persons may manufacture or process a chemical substance for a new use without obtaining specific approval from the U.S. Environmental Protection Agency. Specifically TSCA, FIFRA (Federal Insecticide, Fungicide and Rodenticide Act), the clean Water Act and several others have sought to assess the environmental hazard and impact on human health of a bewildering number of synthetic chemicals manufactured and released by the chemical industry. The assessment of environmental hazard and human health can best be done by following two key parameters, viz. the toxicological properties of the chemicals and their fate in the

environment. The environmental fate of chemicals, specifically the biodegradation of natural and synthetic compounds, by natural microflora has been the subject of a number of books and reviews (Bourquin and Pritchard, 1979; Chakrabarty, 1982; Leisinger et al, 1981; Omenn and Hollaender, 1984) and will not be dealt with in this article. Other articles in this volume specifically deal with principles, methods and techniques in the biodegradation and disposal of a variety of environmental pollutants. The primary objective of this article is to draw the readers'attention to the techniques and the potential of the emerging microbial genetic engineering technology that may in future provide a means of disposing of many of the persistent, toxic chemicals from the environment through use of genetically-manipulated microorganisms or their products.

IIa. Microbial degradation of chlorinated compounds

A major cause of environmental pollution in the industralized world is the manufacture and usage of highly halogenated compounds, chlorinated aromatics in particular. Incidences of toxicity problems with chemicals such as DDT (Dichloro-diphenyl-trichloroethane), PCBs (Polychlorinated biphenyls), 2,4,5-T (2,4,5-Trichlo-

rophenoxyacetic acid), TCDD (2,3,7,8-tetrachloro-dibenzo-p-dioxin) etc., are rampant and have raised important questions about the fate of such chemicals in the environment and the ability of microorganisms to dissimilate many of these synthetic compounds in soil and water (Kaufman and Kearney, 1976). Based on such concerns, various symposia have been organized to address the problem of microbial inability to attack highly chlorinated compounds and how to enhance such ability (Bourquin and Pritchard, 1979; Leisinger et al, 1981; Omenn and Hollaender, 1984). In general it has been observed that microorganisms can decompose many chlorinated compounds, some at an appreciable rate and others more slowly. A list of representative compounds that can be attacked by isolated pure cultures is shown in Table 1. Many more are known to be degraded in nature, particularly by microbial community under aerobic or anaerobic conditions (Kaufman and Kearney, 1976; Suflita, Stout and Tiedje, 1984; Slater and Bull, 1982).

While soil and isolated microbial community studies provide important insights into the nature and mode of degradation of many of the toxic chemicals and are critical for determining the persistence and associated toxicological characteristics of the chemicals and their biodegradation products, such studies offer little hope of enhancing the process of micro-

bial biodegradation where such a process is shown to be extremely slow or non-existent. Mixed cultures are difficult to study genetically as well as toxicologically, since it might not be possible to cultivate in the laboratory all the individuals cultures that may contribute to the process of biodegradation. Until the rate limiting steps are studied carefully in individual strains during the degradative process, no significant genetic improvement can be accomplished. It is thus customary to study pure cultures where effective genetic improvements can be made by studying the biochemistry and the genetics of biodegradation of various synthetic compounds.

Studies of biodegradation of chlorinated compounds by pure cultures have provided some interesting clues regarding the disposition of the biodegradative genes. Many of the genes involved in the biodegradation of chlorinated compounds are found to be clustered and borne on plasmids (Ghosal et al, 1984; Weightman et al, 1984). A list of representative plasmids encoding degradation of some of these compounds is given in Table ?. There are several interesting characteristics of the degradative genes. As mentioned previously, the genes have been shown to be clustered, where studied. Even when the same catabolic genes (degradation of 3-chlorobenzoic acid, for example) are borne on

two different plasmids, they may demonstrate considerable amount of homology. This is exemplified by the homology of the 3-chloro-benozoate (3Cba) degradative structural genes present on plasmids pAC27 and pJP4 (Fig. 1). In case of 3Cba genes present on pJP4, the regulatory sequences are separated from structural genes by a segment for which no counterpart exists in pAC27. Some structural rearrangements are necessary for rapid 3Cba growth when plasmid pJP4-harboring cells are selected by continued growth on 3Cba (Ghosal et al, 1984). Similar to the degradation of naphthalene and toluene encoded by NAH and TOL plasmids (Yen and Gunsalus, 1982; Inouye et al, 1984), the expression of the 3Cba genes in both plasmids pAC27 and pJP4 appears to be under control by positive regulatory elements. In absence of such positively acting elements, the structural gene clusters in both pAC27 and pJP4 undergo rapid amplification in order to increase the gene copy number to compensate for the absence of the activator protein encoded by the regulatory gene(s).

IIb. Microbial degradation of hydrocarbons and oily wastes

Similar to chlorinated compounds, microorganisms are well known for their ability to degrade a variety of hydrocarbons present in

crude oil. The use of microorganisms in the natural decomposition of oil spills as well as residues in oil tankers and storage drums has been reported (Atlas, 1981; Gutnick and Rosenberg, 1977). The degradative genes for a number of hydrocarbons are known to be borne on plasmids, and construction of multiplasmid strains capable of enhanced growth on crude oil has also been reported (Friello et al, 1976). The clustering of the hydrocarbon degradative genes to form operon type of control units and the positive nature of such regulation has previously been mentioned (Franklin et al, 1981; Inouye et al, 1984; Yen and Gunsalus, 1982; Shapiro et al, 1984). An interesting property of many of the hydrocarbon degrading bacteria is the production of surface active agents (Cooper and Zajic, 1980). Such surface active agents are believed to bring the fine oil droplets in phase with the aqueous media, thereby emulsifying the oil in water for facilitated transport to the intracellular enzymes.

An example of a hydrocarbon utilizing bacterium producing a potent emulsifier is that of P. aeruginosa strain SB1 producing the emulsifier EM (Banerjee et al, 1984). Strain SB1 can utilize a large number of normal straight chain alkanes (C_{10} to C_{36}) as its sole source of carbon and energy and produces EM during growth with liquid alkanes (C_{10} to C_{20}). An emulsifying

non-producing mutant SB3 cannot grow with any of the liquid alkanes unless EM is added exogenously. Thus EM production is critical for strain SB1 to utilize liquid alkanes. Interestingly, mutant SB3 cells can grow on solid alkanes such as tetracosane (C_{24} H_{50}) or candle wax, essentially as well as the wild type cells SB1, suggesting that EM production is essential only for growth with liquid hydrocarbons. Since mutants such as SB3 cannot utilize liquid alkanes but can grow rapidly with wax, it is clear that such mutants can be quite useful for dewaxing, since the liquid alkanes with high fuel value are not affected. Another role of genetic manipulations in hydrocarbon microbiology is the isolation of derepressed mutants that can produce large amounts of the emulsifier during growth with any carbon source. For example, emulsifiers are normally produced during growth with the hydrocarbons. Thus SB1 produces EM maximally only when grown with liquid alkanes such as hexadecane. A derepressed mutant SB30, however, produces five fold more EM even when growing with a cheap carbon source such as corn oil or chicken fat. The ability of such EM preparation, when isolated from corn oil grown cells of SB30, to disperse an oil slick and remove oily residues from solid surfaces is seen from Fig. 2. These experiments were conducted by Dr. Keith Pugh and Jennifer Williamson of Emultech Limited, Stock-

ton, U.K. By using a 10% solution of SB30 EM on a 'rocking beaker' experiment, they determined the oil slick dispersion activity of SB30 EM and assigned a rating of 1.8 out of 0 to 2, 2 being the maximum possible efficiency. The cleanliness of the side of the beaker, as compared to the control, should also be noted. Thus removal of oil slick and oily wastes through application of surfactants and other surface active agents produced by genetically manipulated strains might in the future be a valuable tool in the fight against environmental pollution.

III. Tools of genetic manipulation

(a) Transposon mutagenesis

Transposon mutagenesis is accomplished by the insertion of a transposable element on a gene, leading to the inactivation of the gene. Transposable elements are DNA sequences that can move from one replicon to another in absence of the host recombination functions or extensive homology between the element itself and the site of integration of the second replicon. Many transposons are now known in both prokaryotes and eukaryotes. In general they are diverse in size and functional organization, although characterized by certain common features such as encoding of the enzyme transposes (Berg and Berg,

1983). Use of transposon mutagenesis has several advantages over the use of chemical mutagens. Firstly, the insertion of a transposon usually provides a stable mutant phenotype that can be studied indefinitely without problems of high rates of reversion in most cases. Secondly, since insertion of a transposon is usually polar and interrupts transcription, the order of genes in an operon can be determined by measurement of enzymatic activity and accumulation of intermediates. Thirdly, the insertion of a transposon usually is accompanied by antibiotic (or mercury) resistance, so that it is easy to select for mutants by simply treating the recipient cells with donors harboring the transposon under conditions where transfer of the transposon-containing replicon cannot lead to replication of the replicon so that the transposon is inserted either into the chromosome or into plasmids of the recipient cells. Fourthly, transposons provide portable region of homology for chromosomal mobilization. The presence of a transposon in the chromosome and in a conjugative plasmid permits insertion of that plasmid into the chromosome by a single reciprocal exchange between the homologous transposon sequences, occasionally with oriented chromosome transfer from the site of transposon insertion (Krishnapillai et al, 1981). Fifthly, transposon insertions provide valuable markers for the study of

genes that have no readily selectable markers, as exemplified by genes whose products mediate cell-cell interactions during differentiation but make no contribution to the phenotype of colonies derived from single cells (Kuner and Kaiser, 1981). Insertion of transposons allows the cloning of neighboring genes which can then be used as hybridization probes to fish out the wild type gene from a genomic library. Furthermore, most transposable elements have unique restriction sites so that they are good markers for isolating defined deletion derivatives or locating the precise position of the gene by heteroduplex mapping.

There are a number of methods for conducting transposon mutagenesis. When transposition onto a plasmid is desired, the plasmid is usuallty introduced into the host cells harboring a transposon in the chromosome or on a non-conjugative plasmid. The transposon which is transposed from the chromosome or the resident plasmid to the plasmid to be mutated is then selected for by its transfer to a second strain by selection on a medium where only the second strain harboring the transposon can grow (Yen and Gunsalus, 1982; Franklin et al, 1981). A second method is to introduce the transposon via bacteriophages that are either defective for productive life cycle or are capable of injecting the DNA but the DNA cannot replicate any further. This is best

exemplified by the introduction of Tn5 to a myxobacterium by coliphage P1 (Kaiser and Dworkin, 1975). An essentially identical method is to introduce the transposon via plasmids that are rendered transmissible to the recipient cells but are either incapable of replicating naturally (such as ColE1 type of plasmids in many non-enteric Gram negative bacteria) or because of conditional replication defect (i.e., inability to replicate at 42 C). When a transposon is introduced as part of such replicon into a recipient cell which is selected for the retention of the transposon carried antibiotic phenotype, transposition will occur before the vehicle plasmid gets eliminated. If the insertion of the transposon is needed in a gene present on a high copy plasmid, a simple procedure is to introduce the high copy plasmid in a host carrying a copy of the transposon, such as Tn5, on the chromosome. Plating of such cells in presence of high concentrations of kanamycin (which do not allow growth of cells with a single copy of the Tn5 on the chromosome) will select for Tn5 transposition on the high copy plasmid. Further analysis can then be conducted to find out which clone contains the Tn5 on the desired gene.

(b) Gene cloning

A most powerful method for constructing microorganisms for application in environmental pollution clean up is gene cloning or formation of *in vitro* recombinant DNA. In this method, specific genes are either isolated, usually by digestion of a genetic segment with various restriction enzymes, or by making a complementary DNA copy of a messenger RNA molecule and then coupling such a DNA segment with the vector DNA by means of poly A and poly T complementary single stranded ends. If the vector or the foreign DNA fragments have been generated by digestion with TypeII restriction endonucleases, which produce staggered cuts, they can be joined initially through hydrogen bonds by annealing at a low temperature and then by joining the fragments permanently through covalent bonds generated by ligase. This method is very useful since the hybrids can be dissected to regenerate the foreign DNA fragment for further analysis. A drawback of the method is that if the foreign gene itself has a site for the endonuclease or there are no restriction endonuclease sites on the foreign DNA segment, such genes cannot be cloned.

There are several advantages for the complementary DNA (c-DNA) cloning by the homopolymer tailing method (Salser, 1978).

Because no endonuclease cutting is involved, rather large DNA segments, having no or large number of restriction endonuclease sites or complete c-DNA copies of eukaryotic mRNA, can be conveniently tailed and cloned with bacterial plasmids. Since individual homopolymer tailed preparations cannot undergo ring closure, this method ensures hybrid formation between poly dA-tailed gene copy and poly dT-tailed plasmid DNA and facilitates recovery of such hybrids.

Various vectors have been developed for facilitated gene cloning (Kahn et al, 1979; Bagdasarian and Timmis, 1982). The most useful are the ones that have broad host range properties so that they can be transferred among most of the Gram negative microorganisms. This is useful, since transformation systems for many of such microorganisms is not known, so that cloning of a gene segment in E. coli and its subsequent transfer to a variety of Gram negative microorganisms will allow such gene clusters to be replicated, and depending upon individual strains, to be expressed (Franklin et al, 1981). A list of commonly used plasmid and cosmid cloning vectors is given in Table 3.

In general, three types of broad host range plasmids have been used for gene cloning. The Inc P plasmid RK2 and its derivatives have been widely used because of the small size, the availability of a number of antibiotic esistance

properties and the presence of unique restriction sites allowing insertional inactivation (Kahn et al, 1979). The IncQ plasmid RSF1010 has also most of the above features and in addition it replicates with a higher copy number (usually about 50, as compared to about 5 for RK2). Thus cloning of a gene with a RSF1010 derivative such as pKT230 (Bagdasarian and Timmis, 1982) and its expression allows synthesis of a much higher quantity of the gene product which might be useful under certain conditions. It should be stressed, however, that cloning in a high copy number plasmid is not always desirable. Thus cloning of genes in high copy vectors encoding membrane proteins or ribosomal RNA can often lead to loss of viability for the cell.

A third broad host range plasmid that has been used for gene cloning belongs to IncW plasmid S-a (Leemans, et al, 1982). This vector plasmid is non-conjugative but is mobilizable by a number of other plasmids. A major disadvantage with the use of plasmids as cloning vectors is the fact that large gene segments cannot be conveniently cloned since this increases the size of the recombinant plasmid with a drastic reduction in the transformation frequency. Thus larger plasmids are transformed at a much lower frequency and consequently difficult to detect. A class of plasmids used for cloning large DNA segments is the cosmids (Hohn, 1979). Such plasmids

contain the phage lambda cos site that allows them to be packaged in the phage heads, provided the size of the total DNA segment is of the order of 45 to 50 kilobase pairs (kb). Since most of the cosmids range from 7 to 25kb (Hohn, 1979), it is clear that they must have anywhere from 20 to 40kb additional DNA for packaging in E. coli cells. For cloning, the cosmid DNA is treated with restriction enzymes, ligated with foreign DNA similarly treated, and packaged in phage lambda heads. Such phages can be used for transfection, selecting for the antibiotic resistance specified by the cosmid. The recombinant cosmids can then be mobilized by other conjugative replicons (Darzins and Chakrabarty, 1984).

(c) Nucleotide sequencing and promoter specificity

Once a gene (or a set of genes) has been cloned, it is very useful to sequence the gene as well as its upstream and downstream regions to look for regulatory sequences that may control the expression of the genes. This is particularly critical for the Pseudomonas degradative genes which determine biodegradation of various hydrocarbons and toxic chlorinated aromatic compounds. For example, it is known that many Pseudomonas degradative plasmids are not ex-

pressed in E. coli and other soil bacteria (Sakaguchi, 1981). In a detailed study on the nucleotide sequences surrounding transcription initiation sites of the TOL plasmid borne xylABC operon in P. putida, Inouye et al, (1984) could not detect the consensus sequences of E. coli promoters in either the -10 region (Pribnow box) or the -35 region (RNA polymerase recognition site). They could detect a sequence complementary to the 3' end of the 16S rRNA of P. aeruginosa and E. coli in front of the predicted start codon of the xylA gene. Thus it appears that Pseudomonas promoter sequences may be different from those found in E. coli.

Detection and study of promoter sequences are of prime importance to maximize the expression of cloned genes (Bagdasarian et al, 1983). Promoters are the DNA segments that direct RNA polymerase binding and initiation of transcription. No clear correlation can be established between the strength of a promoter and its primary structure even though the sequences of a large number of promoters have been established (Rosenberg and Court, 1979). It should be emphasized that Pseudomonas promoters are not the only promoters that are different from E. coli. Streptomyces genes are also poorly expressed in E. coli (Hopwood et al, 1983) because of different promoter sequency specificity. It is also known that B. subtilis produces different forms of RNA

polymerases that recognize different promoters, and a set of genes, either vegetative or sporulation specific, may be turned on because of this altered recognition of specific promoters (Moran et al, 1982). In order to increase the yeild of degradative enzymes for enhanced degradation of environmental pollutants, expression vectors containing promoter seqeunces capable of allowing high level expression of adjacent cloned genes are deemed extremely useful. Bagdasarian et al (1983) have constructed such an expression vector contained in a broad host range plasmid with the hybrid trp-lac (tac) promoter and the promoter of the lacI gene of E. coli which allows efficient expression of Pseudomonas genes such as that for catechol 2,3- dioxygenase.

(d) Gene amplification

It was pointed out in the previous section that one way to greatly increase the amount of enzyme synthesis is by cloning the gene(s) under the control of a strong promoter such as tac. Occasionally, however, genes are either found on the genome without a promoter or are cloned without a strong promoter or positive regulatory element so that the level of expression is extremely low. For example, the bgl operon of E. coli is normally found in a cryptic state in the genome because of the lack of a functional pro-

moter. The operon specifies a transport protein (bgl-c) and a hydrolase (bgl-B), and can be activated either by IS elements or by gyrase mutations. When activated the operon is inducible by B-glucoside sugars and is under positive control by the bglS gene product (DiNardo et al, 1982). Such activation does not, however, lead to enhanced enzyme production.

Gene amplification is one way by which microorganisms can greatly increase the amount of enzyme synthesis in response to some environmental or other selection pressure. The clear cut example is the amplification of antibiotic resistance genes in response to high concentrations of antibiotics present in the growth media. Under such conditions, some of the resistance genes undergo amplifictions to greatly increase the gene copy numbers so that more enzymes can be produced for the modification of the antibiotics (Perlman and Rownd, 1975; Ptashne and Cohen, 1975).

A relevant example of such gene amplification process in the area of biodegradation of chlorinated aromatics is the amplification of 3-chlorobenzoate (3Cba) degradative genes in the 2,4-dichlorophenoxyacetic acid (2,4-D) degradative plasmid pJP4. pJP4 is a 83kb plasmid which on EcoR1-digestion produces 9 fragments (Fig. 1). Cloning of various fragments for rapid 3Cba growth has produced $3Cba^+$ clones which demon-

strate in their plasmids the presence of EcoR1 fragments B,E and F. Both fragments B and E demonstrate appreciable homology with a single BglII-E fragment (4.2kb) of another 3Cba degradative plasmid pAC27 (Ghosal et al, 1984), suggesting that both the pJP4 EcoR1 fragments B and E contain the 3Cba degradative structural and regulatory genes. It has also been possible to clone the EcoR1-B fragment (15kb) of plasmid pJP4. The presence of this fragment alone allows only slow growth with 3Cba with production of brown coloration. Agarose gel electrophoresis of the EcoR1-digested plasmid (pYG419) isolated from slow-growing $3Cba^+$ cells demonstrate the presence of the EcoR1-B fragment in highly amplified form. Electron microscopic contour length determination of plasmid pYG419 demonstrates its size at 120kb. Since the vector is 20kb, and the EcoR1 fragment B is about 15kb, and EcoR1 digestion of this plasmid produces only these two fragments, it is clear that the EcoR1 fragment B is present in about 7 copies on this plasmid. Growth in absence of any selective pressure (i.e., without 3Cba) produces phenotypically $3Cba^-$ cells. Isolation of plasmid DNA from such $3Cba^-$ cells demonstrates the presence of a single plasmid species of about 35kb which on EcoR1 digestion produces a single copy EcoR1-B fragment along with the vector. Since no such amplification occurs in plasmid pYG1943 which contains

fragments B, E and F and which allows rapid growth with 3Cba, it is clear that the EcoR1 fragment B contains all the 3Cba degradative genes but presumably lacks a putative positive control regulator gene. In absence of this gene product, efficient transcription of the structural genes cannot take place. Under strong selective conditions of 3Cba growth, this gene cluster (EcoR1 fragment B) undergoes amplification to compensate for the low level of transcription so that inefficient transcription at a number of sites will allow appreciable enzyme synthesis for 3Cba growth (Ghosal et al, 1984). In presence of the E and F fragments (plasmid pYG1943) which encode the putative positive regulatory element, efficient transcription occurs so that the cells do not have any need for the gene dosage effect.

(e) Gene duplication and divergence

Gene duplication and subsequent mutational divergence play important roles in the evolution of new degradative functions. There are numerous examples of such processes in bacterial evolution which have been dealt with in some excellent reviews (Clarke, 1984; Mortlock, 1982). There are examples of gene duplication and divergence occuring in extending the substrate range of a strain of P. aeruginosa AC867, capable of growing

with 3- and 4-chlorobenzoate but not with 3,5-dichlorobenzoate (3,5-Dcb), to utilize 3,5-Dcb (Chatterjee and Chakrabarty, 1982). For example, during chemostatic selection of this strain in presence of strains harboring the TOL plasmid and with 3,5-Dcb as a major source of carbon, it was possible to obtain variants of AC867 that could grow slowly with 3,5-Dcb as a sole source of carbon and energy. Isolation of plasmid and chromosomal DNA and subsequent hybridization of restriction digests of such DNA with TOL and the chlorobenzoate plasmid DNAs as probes demonstrated the emergence of a new plasmid pAC29 which harbored duplicate copies of gene segments from the chlorobenzoate degradative plasmid and the replication/incompatibility genes of the TOL plasmid. The duplicated chlorobenzoate gene segment was inferred to have undergone further mutational divergence so as to encode a set of new enzymes that allowed utilization of 3,5-Dcb and its metabolites as substrates (Chatterjee and Chakrabarty, 1982). Thus gene duplication and mutational alterations for change in the substrate specificity of degradative gene product are important parameters in the development of new bacterial strains capable of utilizing chlorinated and non-chlorinated environmental pollutants. Presence of a duplicated segment in an inverted orientation in plasmid pYG2, which is derived from plasmid pJP4 on 3Cba enrichment, has

also been shown by Ghosal et al (1984).

IV. Construction of strains with broad biodegradative potential

(a) in vivo genetic selection

A major step 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 (Reineke and Knackmuss, 1979; Chatterjee et al, 1981). Thus Pseudomonas sp. strain B13 can transfer its degradative potential for 3-chlorocatechol utilization to other cells. Strain B13 is, however, incapable of utilizing 4-chlorobenzoate (4Cba) or 3,5-Dcb. Hartmann et al, (1979) demonstrated that it was possible to select under continuous cultivation variants of strain B13 that could not only utilize 4Cba but also 3,5-Dcb as a sole source of carbon and energy. They delineated the role of the TOL plasmid in providing a broad substrate-specific benzoate oxygenase which allows conversion of 4Cba or 3,5-Dcb to the corresponding chlorocatechols. Such chlorocatechols could then be utilized by strain B13 (after some mutational divergence for 3,5-Dcb utilization) through the plasmid pWR1 present in strain B13. Following

the demonstration by Hartmann et al, (1979), Chatterjee and Chakrabarty (1982) isolated similar variants from their 3Cba$^+$ P. putida strain AC858 harboring the plasmid pAC25. Growth of AC858 in a chemostat in presence of cells harboring the TOL plasmid allowed emergence of cells that could also utilize 4Cba. Such 4Cba$^+$ cells harboring the plasmid pAC27 demonstrated the presence of a portion of the TOL plasmid on their chromosome to provide the broad substrate specific benzoate oxygenase. Enrichment of 4Cba$^+$ P. aeruginosa AC867 cells in presence of 3,5-Dcb similarly produced cells that could slowly utilize this compound as a sole source of carbon and energy. Isolation of plasmid DNA from such 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 the pAC27 plasmid and a fragment from TOL, with further mutational divergence. Continued selection on 3,5-Dcb gave rise to faster growing colonies that demonstrated the presence of a single plasmid (pAC31) with identical restriction profile as the original plasmid pAC27, with the difference that this plasmid allowed ready growth on 3,5-Dcb but not with 3Cba or 4Cba. Mutants, however, could be isolated that would allow 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.

The role of TOL and pAC25 or pAC27 plasmids to allow degradation of various mono- and dichlorobenzoates is not the only example of in vivo genetic manipulations for the degradation of novel compounds. Schwien and Schmidt (1982) have also demonstrated that transfer of the chlorocatechol degradative potential from Pseudomonas sp. B13 to an Alcaligenes strain A7 capable of growing with phenol will allow the exconjugant Alcaligenes strain A7-2 to utilize all three isomeric chlorophenols, which were not attacked by any of the parents. Similar gene transfer to Pseudomonas sp. WR401 capable of growing with salicylate but unable to attack chlorosalicylate allows the exconjugant cells to utilize chlorosalicylate (Reineke et al, 1982). An interesting example of the role of a chromosomal dehalogenase gene to extend the substrate range of a bacterium is the formation of R-prime derivative with plasmid R68.44 (Slater and Bull, 1982). Thus P. putida PP3 dehalogenates 2-chlorobutanoate to 2-hydroxybutanoate, which cannot be utilized by PP3 but can be used by another strain HB2001. Strain HB2001 cannot utilize 2-chlorobutanoate. A community of P putida PP3 harboring R68.44 and HB2001 in a chemostat allowed emergence of recombinants able to grow on both 2-chlorobutanoate

and 2-hydroxybutanoate.

The role of transmissible plasmids in the total mineralization of environmental pollutants such as mono- or dichlorobiphenyls has also been emphasized by Furukawa and Chakrabarty (1982). These workers characterized a plasmid pKF1 that allowed conversion of various chlorinated biphenyls to their corresponding chlorinated benzoic acids, but could not specify any further degradation. However, Chatterjee and Chakrabarty (1982) described the characterization of plasmids pAC27 and pAC31 that allowed complete degradation of 3Cba, 4Cba and 3,5-Dcb. Thus combined growth of bacterial strains harboring pKF1 and pAC27 or pAC31 allowed total mineralization of 4-chloro or 3,5-dichlorobiphenyls (Furukawa and Chakrabarty, 1982). A combination of strong selective pressure and the introduction of plasmid gene pools for the evolution of a new degradative pathway has allowed the isolation of a strain of P. cepacia that can utilize 2,4,5-T as its sole source of carbon and energy (Karns et al, 1984). Normally, 2,4,5-T is known to be degraded slowly in nature by co-metabolic activities of various microorganisms. Selection in a continuous culture where both microorganisms from waste dump sites and microorganisms that harbored various degradative plasmids were present and where 2,4,5-T was gradually introduced to replace easily assimilable plasmid substrates over a

period of 8 to 10 months led to the emergence of a strain of P. cepacia AC1100 that could not only utilize 2,4,5-T but could completely dechlorinate various chlorophenols such as 2,4,5-trichlorophenol, pentachlorophenol etc. (Chakrabarty et al, 1984; Karns et al, 1984). Details regarding the nature of regulation of 2,4,5-T and chlorophenol degradation have been published (Karns et al, 1984).

(b) in vitro gene cloning

It has been mentioned previously that transfer of the halocatechol degradative genes from Pseudomonas sp. strain B13 to other strains possessing phenol hydroxylase or salicylate hydroxylase (which allow conversion of phenol and salicylate to catechol) results in the emergence of strains that can utilize chlorophenols and chlorosalicylates (Reineke et al, 1982; Schwien and Schmidt, 1982). The hydroxylase enzymes allow conversion of the chlorinated phenol and chlorinated salicylate to the corresponding chlorinated catechol, which can then be utilized by the chlorocatechol degradative genes transferred from Pseudomonas sp. strain B13. Another way of accomplishing the same results is to transfer the phenol or salicylate hydroxylase genes to Pseudomonas sp. strain B13 so that this strain will have all the genes for the complete

degradation of chlorophenol or chlorosalicylate. It has also been mentioned that the benzoate oxygenase present in Pseudomonas B13 can use 3Cba as a substrate, but not 4Cba. The TOL encoded benzoate oxygenase, on the other hand, has a broad substrate specificity so that it can use both 3Cba and 4Cba as substrates. Instead of introducing the entire TOL plasmid into Pseudomonas B13 which neccesitates a mutation in the xylE gene for keeping the chlorocatechol to be channeled into the toxic intermediate formation by the meta pathway, Lehrbach et al, (1984) cloned the xylD and xylD and L genes into Pseudomonas B13 which could grow on 3Cba but not 4Cba, 3,5-Dcb, salicylate or chlorosalicylate. Introduction of cloned xylD gene allowed Pseudomonas B13 to degrade 4Cba whereas the cloned xylD-xylL genes, followed by spontaneous mutational divergence, allowed emergence of Pseudomonas B13 variants that could also degrade 3,5-Dcb. Similar mutational divergence has previously been shown to be necessary in the plasmid pAC29 to allow growth with 3,5-Dcb (Chatterjee and Chakrabarty, 1982). Lehrbach et al, (1984) also cloned the naphthalene degradative gene nahG encoding salicylate hydroxylase from the plasmid NAH7 (Yen and Gunsalus, 1982), and introduced the cloned gene into Pseudomonas B13. Expression of nahG gene in Pseudomonas B13 enabled it to convert salicylate and chlorosali-

cylate to catechol and chlorocatechol, which could be used as carbon sources by Pseudomonas B13. The cloned gene therefore enabled this organism to utilize these compounds.

There are several advantages and some disadvantages of the in vitro technique over the in vivo selection mechanism. The major advantage is the fact that introduction of well defined cloned genetic segments allows detailed study of the conversion processes that occur in the cell. This is particularly useful since it is possible to make useful mutations in the cloned derivative by site-directed or oligonucleotide-directed mutagenesis which also avoids complications due to operation of non-productive pathways or pathways leading to accumulation of toxic intermediates. Gene cloning also allows introduction of completely foreign genes into the cell which can be expressed at high levels because of its location under a strong promoter; such foreign genes cannot normally be introduced by natural plasmid transfer processes. A major drawback of the in vitro technique is that it necessitates a thorough understanding of the location of the gene(s) and its regulation. Also, only a single gene or genes that are clustered together can be conveniently cloned. Quite frequently, such recombinant plasmids are unstable in the new cellular environment and tend to get lost or undergo deletions spontaneously at a high

frequency. Thus a combination of both *in vivo* and *in vitro* selective techniques is expected to provide a rational approach for the construction of improved strains for enhanced degradation of toxic, persistent compounds.

V. Survival and effectiveness of laboratory developed strains in an open environment

A major reason for the construction of genetically-engineered strains capable of utilizing toxic, persistent pollutants is to examine their effectiveness for degrading such chemicals in toxic waste dump and other contaminated sites. Although microorganisms have been used for decades for sewage sludge digestion, and have occasionally been used for cleaning up oil polluted areas (Atlas, 1981; Gutnick and Rosenberg, 1977), no definitive information exists regarding the ability of microorganisms to decompose toxic chemicals in an open environment. Various arguments have been put forward as to why genetically-engineered microorganisms may not produce desired effects in terms of removing recalcitrant pollutants from contaminated environment (Karns *et al*, 1984). The major two arguments state that (i) most genetically-engineered microorganisms would be unable to compete with indigenous microorganisms and would not therefore survive for too long to utilize the

toxic chemicals and that (ii) the genetically-engineered microorganisms would have no incentive to attack the toxic chemicals because of the presence of large amounts of other easily assimilable carbon sources. A clue to this intrigue has recently been obtained from experiments where a laboratory developed strain of P cepacia AC1100 capable of utililizing 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) as a sole source of carbon and energy was used in soil contaminated with various concentrations of 2,4,5-T (100 ppm to 20,000 ppm) to test its effectiveness to remove 2,4,5-T. This strain was demonstrated to remove more than 98% of 2,4,5-T in a week when the 2,4,5-T concentration in soil was 1000 ppm (Chatterjee et al, 1982). Kilbane et al. (1983) further demonstrated that AC1100 could remove more than 90% of 2,4,5-T from the contaminated soil in about 6 weeks when the concentration of 2,4,5-T in soil was of the order of 10,000 to 20,000 ppm.

Since 2,4,5-T is a herbicide highly effective for broad leaf plants, usually its presence in soil at a concentration of 25 ppm inhibits germination of plants such as lettuce. It was demonstrated by Kilbane et al (1983) that whereas soil contaminated with high concentrations (1000ppm) of 2,4,5-T did not allow any germination of lettuce seeds, the same soil when treated with AC1100 for a week allowed germination and growth of lettuce seeds, although at reduced

level. The reduced plant growth was ascribed to the presence of small amounts (5 to 7 ppm) of 2,4,5-T in the soil, indicating removal of more than 98% of 2,4,5-T as is normally obtained in laboratory culture. It was concluded by Kilbane et al (1983) that treatment of 2,4,5-T contaminated soil with a laboratory developed microorganism such as AC1100 allows almost a total restoration of the soil.

The question as to what happens to the microorganisms after the toxic chemical is gone was also addressed by Kilbane et al (1983). It was demonstrated that AC1100 could not compete with the indigeneous microflora in absence of 2,4,5-T and died off rapidly, becoming undetectable in about 6 weeks from an initial titer of $5X10^7$ cells/gm soil. However, a very diluted population was maintained in the soil, since addition of 2,4,5-T after 8 weeks when AC1100 titer in soil was virtually undetectable allowed rapid proliferation of AC1100 after a 2 week lag period with consequent removal of the chemical from the soil. Thus the strain remained viable only when there was an adequate supply of 2,4,5-T. The conclusion was drawn by Kilbane et al (1983) that there was little possibility of any ecological disaster due to the use of AC1100, since the strain could not survive in absence of 2,4,5-T. The major outcome was the fact that only the target chemical was completely removed.

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VI. Guidelines for environmental applications of genetically-manipulated microorganisms

As mentioned previously, genetically-engineered microorganisms for degradation of toxic, environmental pollutants can be developed both by *in vivo* and *in vitro* gene manipulation techniques. Since in most countries *in vitro* gene manipulation techniques (recombinant DNA) are under some form of control, and since the after effect of any large scale use of genetically-engineered microorganisms in an open environment is at present unknown, some forms of guidelines are deemed necessary to ensure protection of the public health and environment. Unfortunately, in absence of any meaningful field experiments, it is difficul to predict as to what sort of precautions one should take. In the United States, large scale field experiments approved by the National Institutes of Health Recombinant DNA Advisory Committee have been blocked by the courts until the environmental impact of such usage can be determined. The U.S. Congress is actively considering the form of guidelines or legislation that should be in place, and the mode of enforcement of such guidelines is also under active consideration by the U.S. Environmental Protection Agency. It is presumably a matter of time before such guidelines take shape. Hopefully, once such guide-

lines are drawn and enough field tests are conducted to determine the effectiveness of microorganisms and the safety of public health, more active interest will ensue in the development of microbial technology for the removal of toxic chemicals from the environment.

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LEGENDS TO FIGURES

Fig. 1: Restriction maps of the two degradative plasmids pAC27 (encoding degradation of 3-chlorobenzoic acid) and pJP4 (encoding degradation of 2,4-D and 3-chlorobenzoic acid). The extent of homology between the two plasmids is demonstrated by the hatched areas as indicated by the arrows. The structural genes for chlorocatechol degradation are clustered in the BglII-E fragment of pAC27 while they are clustered in the two EcoR1 fragments B and E of plasmid pJP4.

Fig. 2: Oil slick dispersion by EM preparations of corn oil grown cells of SB30. The left beaker is the control without EM, while the right beaker shows the dispersion of the oil slick during slow shaking with the EM preparations. Note also the cleanliness of the sides of the beaker as a result of EM treatment.

Table 1: List of some bacterial genera capable of degrading halogenated organic compounds.

Bacteria	Type of compounds
Pseudomonas, Flavobacterium, Arthrobacter, Alcaligenes Moraxella	Monofluoro- and Monochloroacetate, Monochloro- and Monobromopropionate, Monochlorobutanoate, Dichloropropionate, Chloroethane, Chloromethane, Chloroethanol
Pseudomonas, Arthrobacter, Flavobacterium	Chloropicrin, 2,3-Dichloroallyl mercaptan
Pseudomonas, Alcaligenes, Flavobacterium, Bacillus	3- or 4-Chlorobenzoate, 3,5- or 2,6-Dichlorobenzoate, Mono-, Di-, Tri- or Pentachlorophenol, Chlorosalicylate, Chlorotoluene
Pseudomonas, Acinetobacter, Arthrobacter, Klebsiella	Chlorinated biphenyls such as mono- or dichlorobiphenyl
Pseudomonas, Alcaligenes	Chlorophenoxyacetates such as 2,4-Dichlorophenoxyacetic acid, 2,4,5-Trichlorophenoxyacetic acid

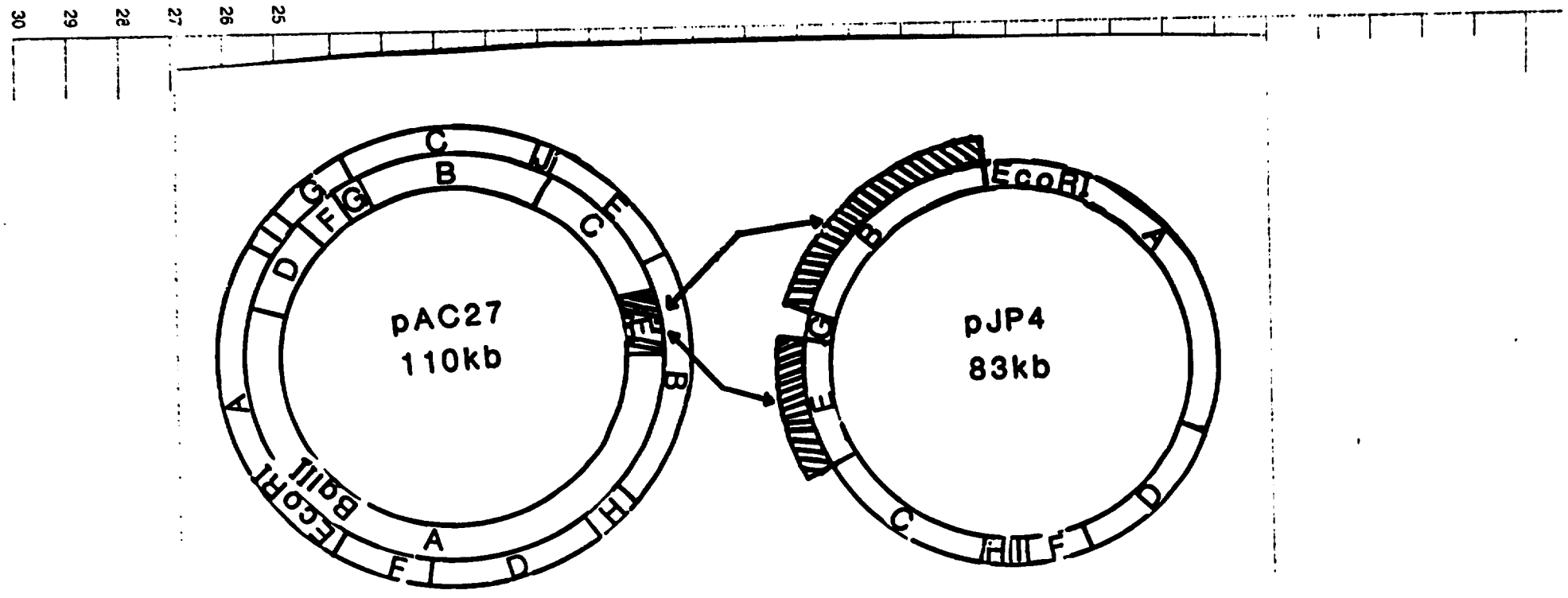
Table 2: List of plasmids encoding degradation of some halogenated compounds.

Plasmid	Molecular size (Mdal)	Halogenated Substrate	Reference
pUO1	43	Haloacetate	Kawasaki, et al (1981)
pAC21	65	p-Chlorobiphenyl	Kamp and Chakrabarty (1979).
pKF1	53	p-Chlorobiphenyl	Furukawa and Chakrabarty (1982).
No designation	35, 50	p-Chlorobiphenyl	Sayler et al (1984)
pJP2	36	2,4-D	Don and Pemberton (1981)
pJP4	52	2,4-D, 3-Chlorobenzoate	Don and Pemberton (1981)
pWR1	72	3-Chlorobenzoate	Reineke and Knackmuss (1979)
pAC25	76	3-Chlorobenzoate	Chatterjee and Chakrabarty (1982)
pAC31	72	3,5-Dichlorobenzoate	Chatterjee and Chabarty (1982)
No designation	63	2,6-Dichlorotoluene	Vandenberg et al (1981)

Table 3: List of some widely used plasmid cloning vectors

Plasmid	Size kb	Selective markers	Cleavage sites for cloning
pBR322	4.6	Tc , Ap	EcoR1, HindIII, BamHI, SalI, PstI, PvuI, AvaI, PvuII
pACYC184	4.3	Cm , Tc	EcoR1, HindIII, BamHI, SalI
pRK248	9.6	Tc	EcoR1, BglII, SalI
pRK2501	11.1	Tc , Km	EcoR1, BglII, SalI, HindIII, XhoI
pMW79	12.6	Tc , Ap	BamHI, SalI, HindIII
pKT230	11.9	Sm , Km	EcoR1, BamHI, HindIII, XhoI, SstI, XmaI
pLAFR1	21.6	Tc	EcoR1, SalI
pVK100	23	Tc, Km	EcoR1, HindIII, SalI, XhoI
pMMB34	13.7	Km	EcoR1, BamHI, SstI

Abbreviations used under 'selective markers': Tc, tetracycline; Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Sm, streptomycin.



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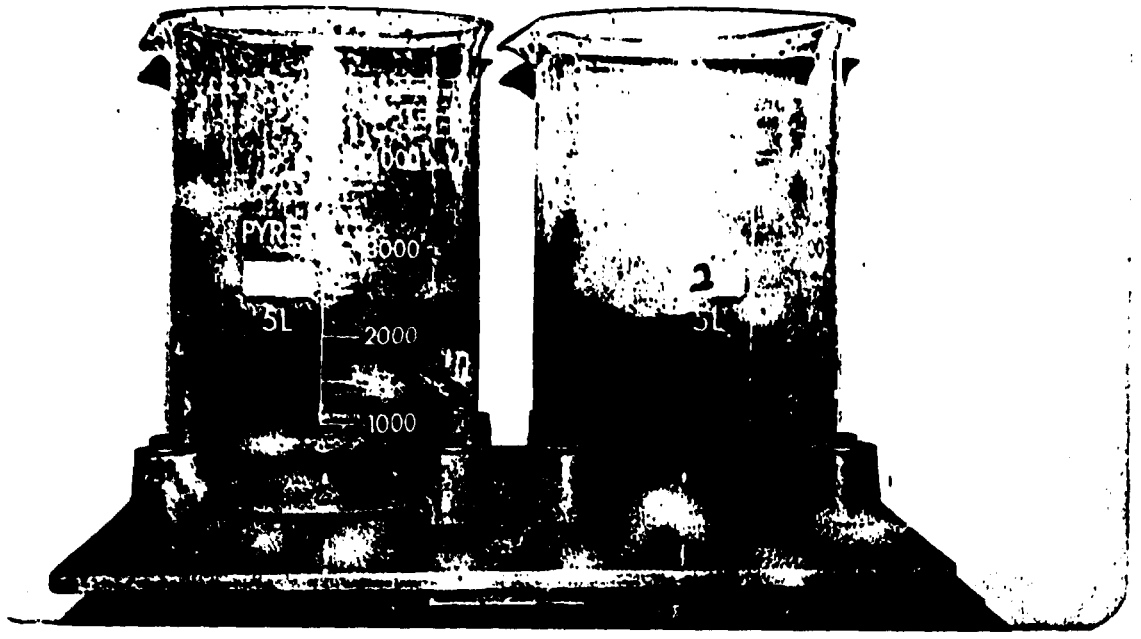

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