REVIEW ARTICLES

MICROBIAL DEGRADATION OF SYNTHETIC TOXIC CHEMICALS

A.M. CHAKRABARTY AND S. MONGKOLSUK[1, 2]

Department of Microbiology & Immunology, University of Illinois College of Medicine, P.O. Box 6998, M/C 790 Chicago, IL 60680, U.S.A., and Laboratory of Biotechnology, Chulabhorn Research Institute[1], Department of Biotechnology, Mahidol University[2], Bangkok, Thailand.

(Received 3 January, 1992)

ABSTRACT

Environmental release of synthetic compounds in the form of herbicides/pesticides, solvents, refrigerants etc. has created major concerns with regard to their effects on human health because of the persistence of many such compounds. The persistence of these compounds is a reflection of the inability of natural microorganisms to utilize them as a sole source of carbon and energy. Many microorganisms can utilize simple chlorinated compounds such as 3-chlorobenzoate (3Cba) or 2,4-dichlorophenoxyacetate (2, 4-D) as their sole carbon source but cannot utilize higher chlorinated forms such as 2, 4, 5-trichlorophenoxyacetic acid (2, 4, 5-T) and others. Under strong selection in a chemostat with 2, 4, 5-T as the only major source of carbon (directed evolution), it has been possible to isolate a strain of Pseudomonas cepacia *AC1100 that can utilize 2, 4, 5-T as its sole source of carbon and energy Molecular cloning of the genes both for 3Cba/2, 4-D as well as 2, 4, 5-T degradation has shown that while the chlorocatechol* (clc) *genes for 3Cba and 2, 4,-D dissimilation are highly homologous, the 2, 4, 5-T degradative* (tft) *genes show no homology with any of the genomic DNA of a large number of pseudomonads and other bacteria. Thus, while natural evolution encompasses recruitment of analogous genes and their subsequent divergence to provide new substrate specificity to their gene products, directed evolution such as what occurred in the evolution of* tft *genes in* P. cepacia *AC1100 in the chemostat may involve recruitment of totally non-homologous genes from different species or genera in response to short-term, strong selection. The presence of IS931 type of sequences upstream of the* chq or tmo *gene cluster may additionally suggest that directed evolution may also necessitate the presence of IS elements with strong outwardly-directed promoter activity so as to ensure the expression of newly recruited non-homologous genes in a different cellular background. It would be important to examine other cases of directed evolution to see how widespread such gene recruitment and gene expression mechanisms are in nature.*

INTRODUCTION

The primary uses of synthetic compounds, particularly chlorinated ones, relate to their release in the environment as herbicides/pesticides, refrigerants, solvents or as industrially useful compounds such as PCBs (polychlorinated biphenyls) which have been used as dielectric fluids in electrical transformers. The extent of chlorination varies for individual compounds, with comparatively lower level of chlorination for chlorinated aliphatic compounds used as solvents and higher level of chlorination for aromatic compounds usually used as pesticides or in the form of PCBs. In general, the higher is the extent of chlorination, the higher is the level of recalcitrance of the compounds to microbial attack.[1,2] Thus, while aromatic compounds with 1 or 2 chlorine atoms are usually biodegradable, compounds with 3 or more chlorines generally are recalcitrant and are biodegraded in nature extremely slowly by cooxidative metabolism.[2,3,4] The positions of the chlorines on the aromatic ring also dictate to a large extent as to how biodegradable the compound is likely going to be.

For natural microorganisms to utilize a chlorinated compound, they must have an array of enzymes that should convert the target compound through a series of intermediates to a product, which can then enter the general metabolic pool. The rate of conversion in each of these steps would dictate that final efficiency of the dissimilation of the compound. The rate of conversion for each step in turn would depend upon the nature of the enzyme. If the enzyme has high turn over rate for the substrate, the conversion would be rapid. For many chlorinated compounds, the enzymes are extremely inefficient, since in many cases, they are designed to attack a structurally analogous non-chlorinated natural compound rather than a synthetic chlorinated compound as a substrate. As a result, the chlorinated compound is degraded rather slowly, which explains the relative persistence of many synthetic chlorinated compounds in nature. Such persistence, particularly when the chemicals are toxic, invariably leads to deposition in human and animal tissues of significant concentrations of the chemicals, giving rise to toxicological symptoms.[5]

Many simple chlorinated compounds have been manufactured for a long period of time and released into the environment in appreciable amounts so that they have been exposed to the natural microflora for a significant length of time. As a result, many natural microorganisms demonstrate an ability to relatively rapidly utilize a number of chlorinated compounds as their sole source of carbon and energy. Such ability to utilize a chlorinated compound is primarily due to evolution in the microorganisms of a new set of genes that encode new enzymes with high affinity for the chlorinated compound and its intermediates as substrates. Understanding how microorganisms evolve new genes for the complete utilization of a synthetic chlorinated compound may provide us with some insight not only into the mechanism of evolution of genes, but may allow us to accelerate the process of evolution so that specific microorganisms can be developed in the laboratory which would evolve the capability to utilize a known recalcitrant compound as its sole source of carbon and energy. Understanding the molecular basis of the evolution of structural and regulatory genes and their interactions with natural and synthetic inducer compounds to allow degradation of such compounds should provide the basis for future genetic manipulations of microorganisms for enhance degradation of toxic synthetic chemicals.

METABOLISM OF SYNTHETIC COMPOUNDS

A number of organisms including *Pseudomonas aeruginosa, P. putida, P. cepacia, Alcaligenes eutrophus* etc. are able to metabolize naturally occurring organic, as well as a number of synthetic compounds.[3, 4] The genes responsible for dissimilation of these compounds are often located on plasmids as indicated in Table 1. The transmissible nature of plasmids may lead to a rapid spread to the microbial consortia of genes encoding metabolic enzymes required to degrade man-made toxic compounds.[6]

TABLE 1. Representative Naturally Occurring Degradative Plasmids in Pseudomonas (taken from Ref. 7)

Plasmid[a]	Degradative pathway	Size (kilobase pairs)
CAM	Camphor	500
OCT	Octane, decane	500
SAL1	Salicylate	85
NAH	Nephthalene	83
TOL	Xylene, toluene	117
NIC	Nicotine, nicotinate	Unknown
pRA500	3, 5-Xylenol	500
pCITI	Aniline	100
pEG	Styrene	37
pBS271	ϵ-Caprolactam, ϵ-aminocaproic acid	500
pCS1	Parathion hydrolysis	68
pJP2	2, 4-Dichlorophenoxyacetic acid	58
pJP4	2, 4-Dichlorophenoxyacetic acid	83
pWR1	3-Chlorobenzoic acid	111
pAC25	3-Chlorobenzoic acid	117
No designation	2, 6-Dichlorotoluene	96

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

Evidence supporting this proposal includes the high degree of homology seen between a number of degradative plasmids,[8] just as many antibiotic resistant plasmids are homologous to one another. There are a number of metabolic pathways that can be utilized for the biological transformation of synthetic compounds.[8] The enzymes involved in the catalysis of many of these compounds can generally be grouped into two classes. There are hydrolases, such as esterases, amidases, and halidohydrolases, which mediate the transfer of chemical groups to water; and oxidoreductases, consisting of dehydrogenases and oxygenases, which

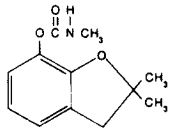
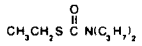
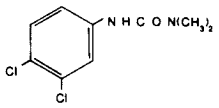
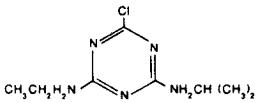
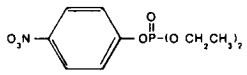
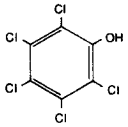
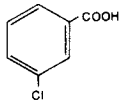
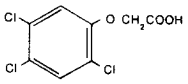
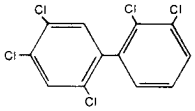
Group	Example	Structure
A. Chlorinated Aliphatic Acids	Dalapon	CH_3CCl_2COOH
B. N-Substituted Carbamates	Carbofuran	
C. Thiocarbamates	EPTC	
D. Urea Herbicides	Diuron	
E. S-Triazines	Atrazine	
F. Organophosphates	Parathion	
G. Phosphonate Herbicides	Glyphosate	
H. Halogenated Aromatics		
phenols	PCP	
benzoic acids	3-Chlorobenzoate	
phenoxyalkanoic acids	245-T	
biphenyls	PCB 2, 4, 5, 2', 3'-pentachlorobiphenyl	

Fig. 1 Representative groups and examples of synthetic compounds used for agricultural or industrial purposes.

require NAD^+ or $NADP^+$ as hydrogen acceptors. This section will discuss the enzymatic reactions involved in dissimilating a number of synthetic compounds by a variety of microorganisms.

Hydrolytic cleavage of chorinated aliphatic acids (Fig. 1), such as trichloroacetic acid, can be utilized by pure cultures of *Moraxella* sp. strain B and *Pseudomonas putida* PP3 as their sole carbon source.[9] Trichloroacetic acid (TCA) and dalapon (2, 2-dichloropropionic acid) are degraded via hydrolytic dechlorination to yield saturated aliphatic acids, which are readily degraded. Several 2-haloaliphatic acid dehalogenases have been purified and characterized from *Pseudomonas* and at least one appears to be encoded on a transposable element within the chromosome.[10] Other enzymes, such as the haloacetate halido-hydrolase from *Moraxella,* however, are plasmid-encoded. Not all chlorinated aliphatics are degraded by hydrolysis as described for the dissimilation of trichloroethylene.[11] Degradation of TCE, by the becterium strain G4, can be activated by intermediates of aromatic biodegradation, which utilizes oxygenases. Indeed, the toluene dioxygenase from *P.putida* F1 catalyzes the degradation of TCE.[12]

The hydrolytic cleavage of the carbamate linkage of N-substituted carbamates (Fig. 1), used as herbicides and pesticides, at the amide or ester linkage completely neutralizes these compounds. Derbyshire *et al*[13] have reported on the degradation of carbamate herbicides and pesticides by pure cultures. Hydrolytic enzymes, purified from *Pseudomonas striata,* act on a wide range of N-phenylcarbamate herbicides including propham and chloropham. N-methyl carbamates, such as carbofuran, may be utilized as a nitrogen source, as shown by a culture of *Achromobacter* that encodes a microbial hydrolase.[13] Related to the N-substituted carbamates are the thiocarbamates (Fig. 1), which have a sulfur atom at the ester oxygen. EPTC, a herbicide, and a member of this group can be metabolized as a sole carbon source by an *Arthrobacter* sp.[14] A plasmid within this strain was shown to be required for degradation. Other related compounds include the substituted urea herbicides (Fig. 1), which have an amide bond that is easily hydrolyzed by enzymes, such as the arylacylamidase isolated from *Bacillus sphaericus.*[15]

One of the most important groups of herbicides are the s-triazines (Fig. 1). Microbial degradation of atrazine, a commonly used herbicide, has been described.[16] A recent article reports on the isolation of several *Pseudomonas* species capable of dealkylating and dechlorinating atrazine, while utilizing it as a carbon source.[17] A partially purified dechlorinating hydrolase from Rhodococcus corallinus was shown to dechlorinate several s-triazines.

A fairly well characterized hydrolase is parathion hydrolase, which is involved in the metabolism of the insecticide parathion (*O, O*-diethyl-*O*-nitrophenyl phosphate) (Fig. 1) and related organophosphates. A *Flavobacterium* sp.[18] and *Pseudomonas diminuta* MG[19] carry degradative plasmids that encode the genes responsible for metabolism of this insecticide. The phosphotriesterase isolated from the *Flavobacterium* also hydrolyzes the pesticide coumaphos (*O-O*-diethyl-*O*-3-chloro-4-methyl-2-oxo-2*H*-1-benzopyran-7-yl phosphate). The *Flavobacterium* however, cannot open the benzene ring as it can with diaxinon and parathion.

There are at least two degradative pathways for the dissimilation of the widely used broad-spectrum phosphonate herbicide glyphosate (Fig. 1). One pathway, as exemplified by the isolated *Pseudomonas* sp. PG2982, proceeds by the cleavage of the C-P bond, giving rise to the formation of sarcosine.[20] The second pathway metabolizes glyphosate by hydrolysis to aminomethylphosphonic acid (AMPA), which is subsequently mineralized. A *Flavobacterium* sp. as well as several *Arthrobacter* sp. are capable of utilizing this pathway.[21]

Very little information is available on the mechanism of penta chlorinated phenol (PCP) aerobic metabolism (Fig. 1). Recently, a *Flavobacterium* strain[22] and a *Rhodococcus* strain[23] have been isolated, which can use PCP as a sole carbon source. *Rhodococcus chlorophenolicus* PCP-1 attacks pentachlorophenol producing tetra-or trichlorohydroquinone intermediates via para-hydroxylation.[23] It was shown that the oxygen molecule for the *para*-hydroxylation was derived from H_2O, however this reaction only proceeded in the presense of molecular oxygen.

Generally, methylated and halogenated aromatics (Fig. 1), including polychlorinated biphenyls, are dissimilated via oxygenases. However, nucleophilic attack catalyzed by dehalogenases has also been documented. Lingens and colleagues[24] have isolated a 4-chlorobenzoate dehalogenase from *Pseudomonas* strain CBS3 that not only dehalogenates 4-chlorobenzoate, but also converts nitrochlorobenzoates to nitrophenols. In the more typical electrophilic reaction, bacteria transform aromatics into dihydroxy derivatives, such as substituted catechols, which serve as substrates for oxygenolytic cleavage of the aromatic ring. Chlorocatechols are generally metabolized by *ortho* cleavage pathways (as described in the following sections), whereas methylcatechols follow a *meta* cleavage pathway. Substrates may be misrouted down a cleavage pathway resulting in the accumulation of toxic intermediates as described by Rojo *et al.*[25]

Chlorobenzoic acids (Fig. 1) have been shown to be degraded by several *Pseudomonas* strains as well as cometabolized by *Acinetobacter calcoaceticus* strain BS5. The genes for the metabolism of the chlorocatechol intermediate are plasmidencoded (Fig. 2). The genetics for chlorobenzoate degradation are discussed in the following section. The strain WR1306 isolated from a chemostat is capable of metabolizing chlorobenzene, as its sole carbon source, through a pyrocatechol (catechol) intermediate, and many such chlorobenzene degrading microorganisms are known.[26] Chlorophenoxy herbicides (Fig. 1) such as 2, 4-D (2, 4-dichlorophenoxyacetic acid) and MCPA (4-chloro-2-methylphenoxyacetic acid) are degraded by a number of organisms. Bacteria including *Pseudomonas* sp., *Arthrobacter* sp., *Mycoplana* sp. and *Flavobacterium peregilum* are capable of cleaving the ether linkage between the oxygen and the aliphatic side chain of 2, 4-D to form glyoxylate and 2, 4-dichlorophenol. The dichlorophenol is then metabolized through the β-ketoadipate (3-oxoadipate) pathway (Fig. 3). The genes responsible for this degradation have been found to be encoded on several different plasmids, such as pJP2 and pLP4. Similarly, MCPA is metabolized by *Pseudomonas* sp. NCLB9340 and *Arthrobacter* sp. through an oxidative cleavage. The phenoxyacetic herbicide 2, 4,5-T (2, 4, 5-trichlorophenoxyacetic acid), however is metabolized through a different pathway as described in a subsequent section. Other aromatic compounds that are metabolized

through catechol intermediates include the chlorinated anilines. Herbicides such as monuron, diuron, lonuron, and propanil are members of this group. Depending on the structure of the chlorocatechol intermediate, dissimilation proceeds via the *meta* or *ortho* pathway.[7]

Polychlorinated biphenyls PCB'S) (Fig. 1) have been used widely in industrial applications because of their thermal stability and excellent dielectric properties. There are 209 possible congeners of PCB'S containing 1 to 10 chlorine atoms per biphenyl molecule. Aroclors (Monsanto) and Kanechlors (Kanegafuchi) contain 60 to 80 congeners, posing a difficult challenge for microbial degradation. Nonetheless, recent studies have indicated that aerobic and anaerobic transformations do occur[27] and several studies have led to isolation of microorganisms capable of degrading a number of PCB derivatives.[28] Furukawa *et al.*[29] demonstrated that *Acinetobactor* sp. strain P6 selectively degrades congeners containing one to four chlorines. Bedard *et al.*[30] have isolated a diverse group of PCB degrading bacteria including a *Corynebacterium* sp., various pseudomonads, an *Alcaligenes faecalis*, and Alcaligenes eutrophus. There appear to be two pathways for the aerobic dissimilation of PCB's. The principle route involves a 2, 3-dioxygenase attack at an unsubstituted 2, 3 (or 5, 6) position, exemplified by the *Corynecbacterium* strain MB1.[30] An alternative route is used by *A. eutrophus* H850, which utilizes a 3, 4-dioxygenase. These organisms differ in their substrate specificity. For example MB1 is able to partially degrade 4, 4′ -dichlorobiphenyl, whereas H850 shows only poor activity on this congener. Another organism *P. putida* LB400 is capable of degrading congeners containing up to six chlorines.[31] LB400 apparently has both dioxygenase activities. The genes for the first four enzymes of the 2, 3-dioxygenase pathway, which allows LB400 to utilize biphenyl as a sole carbon source, have recently been cloned. It is presumed that these genes are responsible for the metabolism of a variety of PCB congeners. 2, 3-Dihydroxybiphenyl dioxygenases from biphenyl-degrading *Pseudomonas pseudoalcaligenes* and *Pseudomonas aeruginosa* have recently been purified and shown to require iron (II) as a prosthetic group. This enzyme catalyzes the third metabolic step of biphenyl degradation, the rind opening of 2, 3-dihydroxybiphenyl. The chromosomal gene encoding the estradiol dioxygenase has been cloned and sequenced from *Pseudomonas pseudoalcaligenes*, and a great deal is known regarding the organization of the (chloro) biphenyl genes.[32]

GENETICS OF CHLOROBENZOATE DEGRADATION

Genetic organization of the *cat* and *clc* genes

The isolation and characterization of a plasmid pAC27 from a strain of *Pseudomonas putida* that allowed the host cells to utilize 3-chlorobenzoate (3Cba) has previously been reported.[6] While many microorganisms can utilize the nonchlorinated natural compound benzoate,[33] introduction of a single chlorine atom in this molecule makes it quite recalcitrant to microbial attack. It was thus of interest to us to determine the biochemical and genetic basis of the biodegradation of 3Cba by such a natural isolate. Previously, Knackmuss, Reineke and their colleagues in Germany[34] isolated a *Pseudomonas* species strain B13 which was also capable of utilizing 3 3Cba as its sole source of carbon and energy. These researchers delineated the

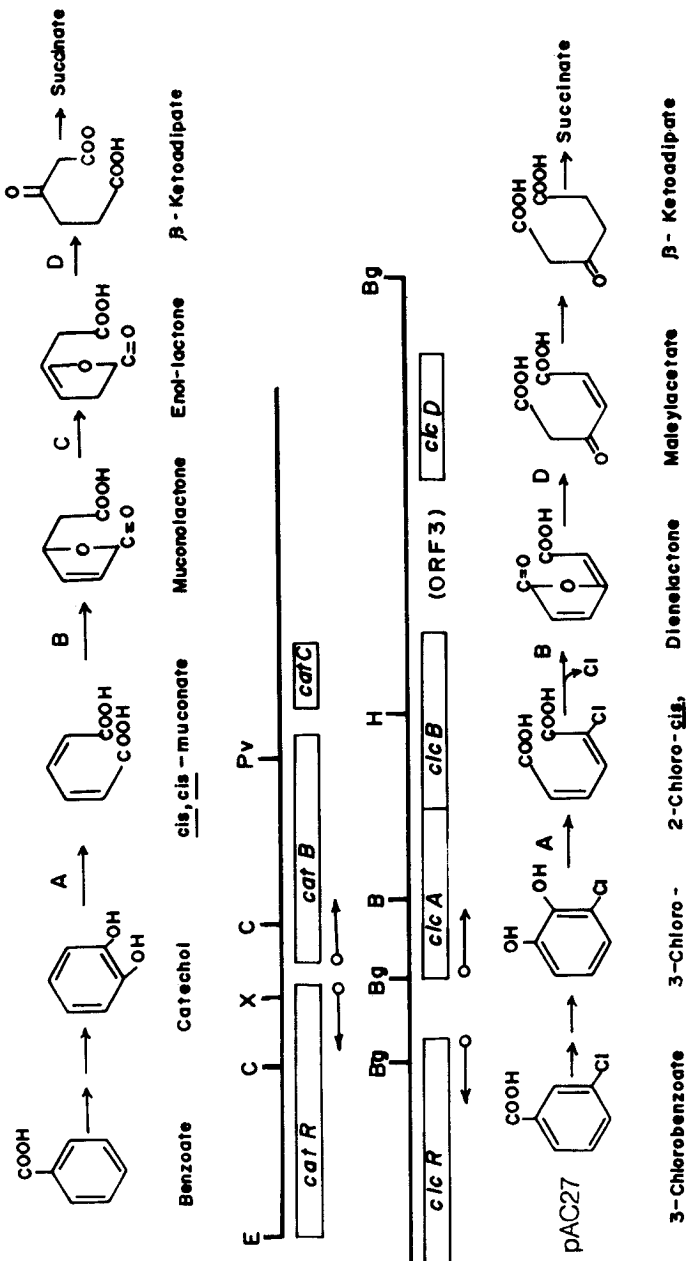


Fig. 2 Pathways of the degradation of benzoate and 3-chlorobenzoate and the organization of *cat*R-*cat*BC and *clc*ABD operons. The arrows in opposite directions indicate divergent transcriptions of the regulatory genes from the operons they control. The nature of ORF3 in the *clc*ABD operon is unknown.

pathway of the degradation of 3Cba, where 3Cba was shown to be converted to 3-Chlorocatechol (3Clc) by the chromosomally-coded benzoate oxygenase system; 3Clc in turn was shown to be converted to β-ketoadipate through four enzymes, chlorocatechol-1,2-dioxygenase (pyrocatechase II), chloromuconate cycloisomerase (cycloisomerase II), dienelactone hydrolase (hydrolase II) and maleylacetate reductase.[35]It appears that in pseudomonads such as *P. cepacia* (but not in *P. aeruginosa PAO)* and other bacteria, maleylacetate reductase is chromosomally encoded,[36] so that the conversion of 3Clc to β-ketoadipate involves participation of 3 key enzymes, *viz.* pyrocatechase II, cycloisomerase II and hydrolase II. The cloning of a 4.2 kb segment from the plasmid pAC27 enabled 3 Cba-negative *P. putida* host cells to utilize 3Cba extremely slowly[37] demonstrating that structral genes for all the chlorocatechol enzymes were borne on this fragment, but the promoter of the gene cluster and a positive regulatory gene were missing.[38]

The slow growth of *P. putida* cells harboring the cloned 4.2 kb segment of the plasmid pAC27 on a broad host range vector implied that some critical regulatory gene (s) needed for rapid growth was missing. Indeed, Ghosal *et al.*[37] demonstrated that cloning of a 3.2 kb *Bgl* II-*Eco*RI upstream region *in trans* will allow rapid utilization of 3Cba by host cells harboring the 4.2 kb *Bgl* II fragment. Thus, a regulatory gene (s) was postulated to be present on this fragment. In absence of this 3.2 kb fragment, mutant cells harboring the 4.2 kb *Bal* II fragment and capable of rapid growth with 3 Cba could also be obtained at a low frequency. Such rapid-growing cells showed amplification of the 4.2 kb *Bgl* II fragment, amounting to about 8 copies, suggesting that in absence of a positive regulatory gene, the structural genes needed to be amplified to produce enough enzymes to allow appreciable growth on 3Cba.[37]

In order to understand the organization of the chlorocatechol genes on the 4.2 kb *Bgl* II fragment and the mode of regulation of the structural genes by the putative regulatory gene on the upstream 3.2 kb *Bgl* II-*Eco*RI fragment, the sequence of the 4.2 kb *Bgl* II fragment and the fragment harboring the regulatory gene was determined. The sequence of the 4.2 kb *Bgl* II fragment and an adjoining 385 bp *Bgl* II fragment was determinde by Frantz and Chakrabarty[38] who delineated the organization of the genes *clc*A, *clc*B and *clc*D encoding pyrocatechase II, cycloisomerase II and hydrolase II respectively (Fig. 2). The *clc*A, *clc*B and *clc*D genes were demonstrated to be under the control of a single promoter present on the 385 bp adjacent *Bgl* II fragment, suggesting that the 3 genes are regulated as an operon. The promoter of this operon shows considerable homology with other promoters such as the *nah, sal* and *xyl* promoters.[38]

Figure 2 also demonstrates the pathway of the degradation of the nonchlorinated parent benzoate. Benzoate is utilized by a large number of pseudomobads and other bacteria by the β-Ketoadipate pathway through formation of catechol, *cis, cis*-muconate and the enol-lactone.[33] Since the chlorocatechol genes could conceivably evolve from the catechol *(cat)* genes, we decided to clone and sequence the *cat* genes. Aldrich *et al.*[39, 40] cloned the *cat*B and the adjoining *cat*C gene and sequenced the gene cluster. In *p. putida,* the *cat*B and *cat*C genes encoding cycloisomerase I and muconolactone isomerase respectively, are clustered together on the chromosome and regulated as an operon under a single promoter.[40] Aldrich and Chakrabarty[40] not only sequenced the *cat*BC operon but Aldrich et al also delineated some of the critical nucleotides of the promoter

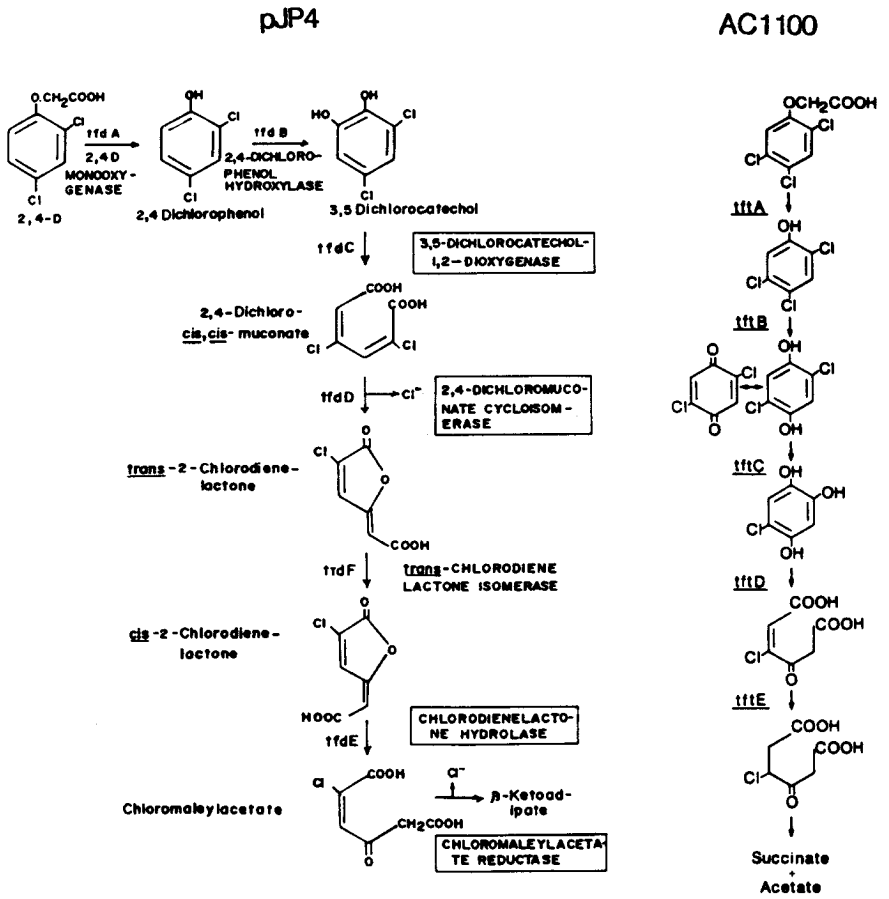


Fig. 3 Pathway of the degradation of 2, 4-D (left), and a tentative pathway for 2, 4, 5-T degradation (right).

by site directed mutagenesis.[41] Two unique base alterations within the-10 region of the *cat*BC promoter greatly affected its activity. Substitution of an A at-11 inactivated the promoter, while introducing a T at-12 caused the promoter to become constitutive in *P. putida* and abolished the requirement of *cat*R for its activation. Altering a nucleotide at the 35 region at a site highly conserved in *E. coli* had little effect on the wild type promoter activity. Thus, site-directed mutagenesis of the *cat*BC promoter provided a good deal of information about some of the critical nucleotides of this promoter.

In contrast to the *cat*BC operon which is chromosmal, the *clc* genes are regulated as an operon not only on the plasmid pAC27, but also on the plasmid pJP4 which specifies a complete degradative pathway for the herbicide 2,4-Dichlorophenoxyacetic acid (2,4-D). 2,4-D is metabolized through formation of 2,4-dichlorophenol (2,4-DCP) and 3,5-dichlorocatechol via 2,4-dichloromuconic acid and chloromaleylacetic acid (Fig. 3). The conversion of 3,5-dichlorocatechol to chloromaleylacetate is mediated by 4 genes *tfd*C, *tfd*D, *tfd*E and *tfd*F which encode chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase, 2-chlorodiene lactone hydrolase, and presumably *trans*-chlorodiene lactone isomerase respectively (Fig. 3). Thus, *tfd*C, *tfd*D and *tfd*E genes closely resemble *clc*A, *clc*B and *clc*D genes with regard to their functions, even though such genes are parts of two entirely different plasmids (pJP4 and pAC27) which belong to two different incompatibility groups and isolated in two different cellular backgrounds *(Alcaligenes eutrophus* and *P. putida)* from two different countries (Australia and U.S.A.). Similar to the *clc*ABD operon, the *tfd*C, *tfd*D and *tfd*E genes are clustered together along with *tfd*F,[42] and regulated as an operon under control of a regulatory gene *tfd*R.[43]

There is another gene *tdf*O (also known as *tfd*S) which is divergently transcribed from *tfdA* and which is assumed to be involved in the positive regulation of *tfd* genes.[44, 45] The positive expression of the *tfd*CDEF operon by the regulatory gene *tfd*R occurs only in presence off inducers such as 2,4-D, 2,4-DCP or 4-chlorocatechol. In absence of such effectors, *tfd*R acts as a negative controlling element.[43] Unlike the *cat*R/*cat*BC or *clc*R/*clc*ABD operons, however, the *tfd*R gene maps several kb upstream of the *tfd*CDEF operon and exerts dual control on both the operon as well as the *tfd*A gene which maps as a separate gene several kb away similar to *cat*A gene mapping several kb awat from the *cat*BC operon in *Pseudomonas.*[46] Kaphammer and olsen[47] have recently shown that TfdS acts as a repressor of *tfd*B in the absence of an effector and is an activator of *tfd*B when the effector, presumably chloromaleylacetate, is present. The presumptive role of TfdS in the activation of *tfd*A is not demonstrated as yet.

The *cat*BC operon is regulated positively by a regulatory gene *cat*R which is transcribed divergently (Fig.2) from the *cat*BC operon.[48] We have reported (48) the characterization of a regulatory gen *cle*R (Fig. 2) which, similar to *cat*R, is divergently transcribed from the operon it controls (*clc*ABD). Molecular details of these genes and operons will be described later, but it is clear that both the *cat*BC and the *clc*ABD operons, along with their regulatory genes, exhibit striking similarity in their organization.

Characteristics and homologies of the *cat* and *clc* genes and enzymes

An important consideration with regard to the *cat*BC, *clc*ABD and *tfd*CDEF operons is the specificity of the enzymes they encode. The *cat*BC operon is involved in the utilization of the non-chlorinated catechol intermediates, while *clc*ABD operon is involved in the utilization of monochlorocatechol and *tfd*CDEF operon in the utilization of dichlorocatechol. In conformity with such substrate diversity, the enzymes show interesting differences with regard to their V_{max} and K_m for various substrates.[49] Frantz and Chakrabarty[38] described the insertion of the 4.2 kb *Bgl* II fragment harboring the *clc*ABD operon under the *tac* promoter which led to the hyperproduction of pyrocatechase II, cycloisomerase II and hydrolase II. These enzymes were purified for N-terminal amino acid sequence determination and other properties.[50] Pyrocatechase I, which is induced during growth with benzoate has a high relative V_{max} (given a value of 100) for catechol as a substrate but a low value for 3-chlorocatchol or 3,5-dichlorocatechol (< 1.0). In contrast, pyrocatechase II encoded by pWRI in *Pseudomonas* sp. strain B13, a plasmid analogous to pAC27,[51] has a V_{max} of 164 for 3-chlorocatechol (relative to a value of 100 for catechol) and 148 for 3,5-dichlorocatechol, suggesting that the evolved enzyme is more efficient with cholorocatechols as substrates. The pJP4-encoded pyrocatechase II shows higher V_{max} value for 3,5-dichlorocatechol (181) than 3-chlorocatechol (124). This specificity is also apparent for cycloisomerase I and cycloisomerase II. Aldrich *et al.*[39] hyperproduced cycloisomerase I by placing the *cat*B gene under the *tac* promoter and verified the DNA sequence from the N-terminal amino acid sequence of purified *cat*B gene product. The chromosomally coded cycloisomerase I has a relative V_{max} of 100 for *cis, cis*-muconate, but only 0.8 for 2-chloro-*cis, cis*-muconate. In contrast, the plasmid coded cycloisomerase II has a V_{max} value of 180 for 2-chloromuconate relative to 100 for muconate.[49] Interestingly, the dichloromuconate cycloisomerase encoded by the pJP4 plasmid has a relative V_{max} of 125 for 2,4-dichloro-*cis, cis*-muconate, suggesting that the pJP4-encoded enzymes appear to have a higher turn over rate with dichlorinated catechols or their intermediates than the monochlorinated catechols.[49] However, acquisition of broad substrate specificity may in some cases lead to formation of inefficient enzymes with lower catalytic activity, as demonstrated by the pAC27 encoded pyrocatechase II and cycloisomerase II.[50]

Regarding the difference between hydrolase I and hydrolase II, the chromosomally encoded hydrolase I is quite specific for enol-lactone while the plasmid-encoded hydrolase II is quite specific for dienelactones.[52, 53] However, among the plasmid-encoded hydrolase II, the enzyme encoded by pWRI (pAC27) has a higher affinity ($K_m = 15\mu M$) for *trans*-dienelactone (*trans*-4-carboxymethylenebut-2-en-4-olide) which is an intermediate of 3-chlorocatechol degradation, than the corresponding *cis*-isomer ($K_m = 400\mu m$). On the other hand, plasmid pJP4 allows conversion of 3,5-dichlorocatechol through formation of *cis*-2-chloro-4-carboxymethyenebut -2-en-4-olide, and the hydrolase II encoded by this plasmid appears to be equally active both for the *trans*-and the *cis*-isomer ($K_m = 190\ \mu m$ for *trans*, 180 *v*m for *cis*, ref. 49). Thus, the plasmid encoded enzymes show interesting differences with respect to their chlorinated substrate specificities, while the chromosomally encoded enzyme has an entirely different substrate profile. It is also important to point out that there is remnant of a gene downstream of the *clc*D

gene in pAC27 plasmid that shows a 57% DNA sequence homology with *tfd*F.[54] *tfd*F is essential for growth with dichlorocatechols and the absence of a complete functional *trans*-chlorodienelactone isomerase encoded by the pAC27 plasmid may explain the inability of pAC27 plasmid to allow host cells to utilize dichlorocatechols.

The sequencing of the *cat*BC operon and the *clc*ABD operon (40, 38), and that of the *tfd*CDE genes by Ghosal and You[55, 56] and *tfd*CDEF by Perkins *et al.*[54] allowed a sequence homology comparison among these genes. It is interesting to note that the order of genes in the *cat*BC, *clc*ABD and *tfd*CDE operons is the same as the order of the degradative steps in the pathway, and the enzymes pyrocatechase I and pyrocatechase II show a good deal of homology with regard to their amino acid sequence.[57] Similarities in the nucleotide sequences of gene *clc*A and *tfd*C encoding pyrocatechase II type of enzymes, have now been reported;[55] *tfd*C gene is somewhat smaller than the *clc*A gene and shows 63% nucleotide sequence homology with it.[55, 56] The lengths of the *cat*B and *clc*B genes are 1128 bp and 1113 bp respectively and the two genes are 52% homologous at the nucleotide level. The homology is scattered throughout the genes, but there are several highly conserved regions, suggesting that the two genes may be products of divergent evolution from a common ancestral gene. Similar comparison of the nucleotide sequences of *tfd*D gene,which is 1107 bp and is therefore slightly smaller than the 1113 bp *clc*B, with that of *clc*B shows a sequence homology of 63% between the genes.[56] The presence of an incomplete non-functional *tfd*F like gene downstream of the *clc*D gene has also been reported.[54]

In contrast to the pyrocatechases and the cycloisomerases, the hydrolases appear to have diverged widely.[53] The N-terminal amino acid sequences between hydrolases I and II are dissimilar, although the amino acid sequence surrounding one of the cysteine residues in hydrolase I closely resembles the amino acid sequence neighboring Cys-60 in hydrolase II, suggesting that the catalytic region alone might have been constrained against divergence because of its critical role in the enzymatic catalysis.[52] It should, however, be emphasized that hydrolase I has very little activity with the dienelactone as a substrate while the hydrolase II has very little activity with the enol-lactone as substrate and it is therefore not surprising that the primary structures of the two hydrolases have diverged so widely. The two hydrolases encoded by plasmids pAC27 and PJP4 are, however, quite homologous since the genes *tfd*E and *clc*D demonstrate approximately 50% homology at the nucleotide sequence level.[56] It is interesting to note that there is a 4 bp overlap between the stop codon of *clc*A and the start codon of *clc*B, and a similar 4 bp sequence overlap exists between the stop codon of *tfd*C and start codon of *tfd*D. Thus, the organization of the *clc* genes in pAC27 and pJP4 is remarkably similar in fine details, even though the *clc*B and *clc*D genes in plasmid pAC27 are separated by approximately I kb of an open reading frame of unknown function,[38] while the *tfd*D and *tfd*E genes are contiguous on plasmid pJP4.[54, 56]

The nature of the regulatory genes involved in the *cat* and *clc* pathways has recently been delineated. The presence of the regulatory genes *cat*R and *clc*R was predicted from the upstream sequence of the *cat*BC and *clc*ABD operons by Henikoff *et al.*[45] We showed that both the *cat*BC and the *clc*ABD operons are positively regulated by two regulatory proteins *Cat*R and *Clc*R (Fig. 2). The *tfd*CDEF operon has been shown to be regulated by a gene *tfd*R

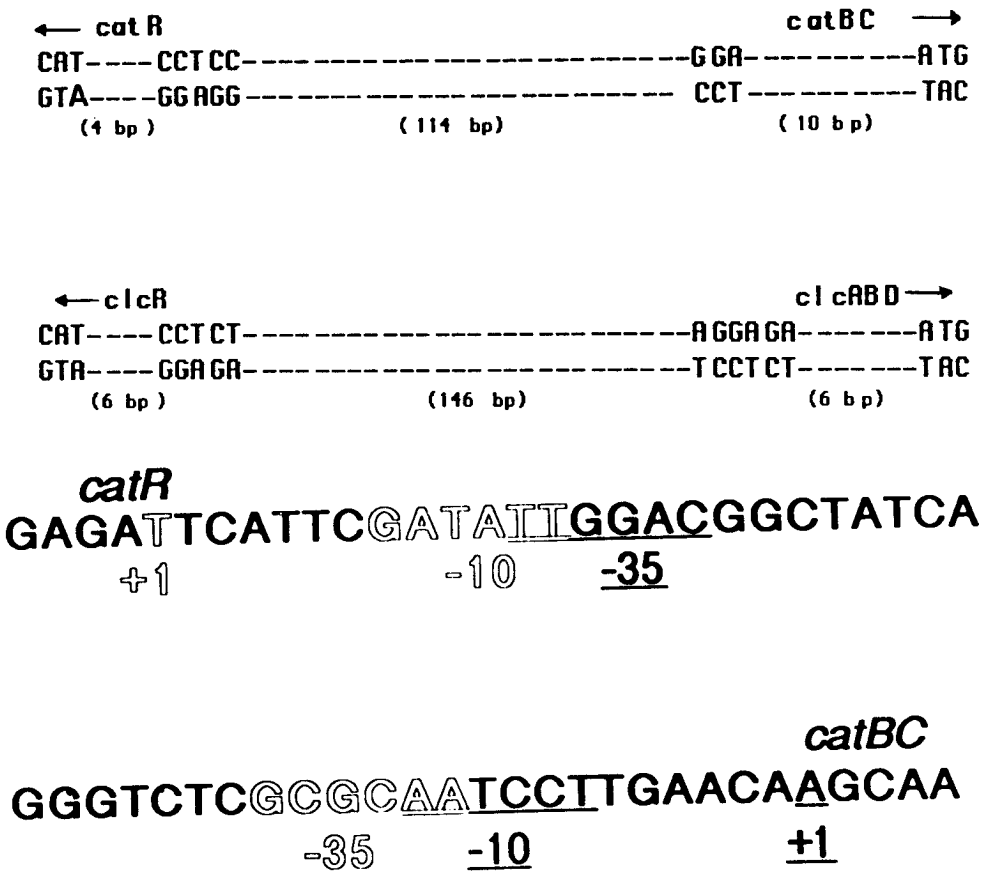


Fig. 4 **Top:** The genetic organization of the *cat*R-*cat*BC and the *clc*R-*clc*ABD operons showing the direction of transcription by arrows, the translational start sites and the Shine-Dalgarno sequences with the number of base pairs between them. **Bottom:** The DNA sequence of the *cat*R-*cat*BC promoter region showing the two transcriptional start sites and the -10 and -35 promoter regions, which overlap as shown. Highlighted sequences indicate the *cat*R promoter and underlined nucleotides the *cat*BC promoter. Site of transcription is marked as+1.

which exerts negative regulation by repressing the expression of the operon, but derepressed the operon in presence of effectors such as 2,4-D, 2,4-DCP or 4-chlorocatechol. The involvement of another regulatory gene *tfd*S has also been mentioned, even though it appears to regulate *tfd*B gene rather than the *tfd*A gene. All the 3 operons encode inducible enzymes. How do the regulatory systems evolve in relation to the structural genes to accommodate new synthetic compounds as inducers? While Fig. 2 shows the location and divergent transcription of the *cat*R and *clc*R gene with respect to the operons they control, the *tfd*R gene has been shown to map several kb away from the *tfd*CDEF operon and controls the expression of *tfd*A gene which also maps several kb away on the other side of *tfd*R gene. It is interesting to note that both CatR and ClcR belong to a large group of regulatory proteins termed the LysR family and show homology to the regulatory protein TfdS, involved in the Tfd pathway. The homology of CatR with ClcR and TfdS within the first 175 amino acids from the N-terminal end is substantial.[48] Such extensive homology among the *cat* and *clc* regulatory genes provide additional confirmation that these gene clusters have a common ancestry with regard to both structural and regulatory genes. It is interesting to also note that a similar regulatory protein termed CatM, which is also divergently transcribed and is a member of the LysR family, regulates the expression of *cat*BCEFD operon in *Acinetobacter calcoaceticus;* however, unlike CatR or ClcR, which are activators, *cat*M encodes a repressor which regulates *cat*BCEFD in a negative manner (58). CatR, TfdS and ClcR show extensive homology with CatM suggesting that regulatory genes involved in catechol and chlorocatechol metabolism in various bacteria *(Pseudomonas, Alcaligenes, Acinetobacter)* may have evolved from a common ancestor.

A question of some importance is how the regulatory systems adjust themselves to accommodate different effectors and inducers? For example, *tfd*CDEF operon containing the *clc* genes respond to 2,4-D, 2-4-DCP or 4-chlorocatechol as effectors. Assuming that the effectors bind with a regulatory protein such as TfdR to enable it to activate the *tfd*CDEF promoter to allow functional RNA polymerase binding to initiate transcription, one wonders whether the affinity for TfdR to bind any of these 3 effectors is the same, or if the binding site is the same for each of them. Ghosal *et al.*[37] pointed out that in contrast to growth with 2, 4-D, the growth rate of pJP4-containing cells on 3Cba is quite slow, and faster growing colonies that came up after incubation for a long time on 3Cba plates showed considerable amount of rearrangement in the genes of this region. The same kind of question can also be asked about the *cat* and *clc* systems. There are interesting small differences in the details of the organization of these two gene clusters (Fig. 4), presumably reflecting the fact that CatR binds to a non-chlorinated effector (such as *cis, cis*-muconate) while ClcR binds chloromuconate as an effector. Does such a binding depend upon the nature of the effector i.e., can CatR bind with chloromuconate or ClcR bind with muconate, even if such binding may or may not lead to appreciable activation of the respective promoter? In order to determine exactly where CatR binds with the upstream region of the *cat*BC operon for its activation, we have mapped the promoter region of the *cap*BC operon and the *cat*R gene, since they are divergently transcribed. There are 114 bp of DNA between the Shine-Dalgarno (SD) sequences of the *cat*BC operon and the *cat*R gene while there are 146 bp between the SD sequences of the *clc*R and *clc*ABD operon (Fig. 4). The

transcription initiation sites of both *cat*R gene and the *cat*BC operon have been determined (marked as+1, highlighted for *cat*R and underlined for *cat*BC, Fig. 4), leaving 47 bp between the two start sites. Thus, the -10 region of the *cat*R gene overlaps with the -35 region of the *cat*BC operon and the -35 region of the *cat*R gene overlaps with -10 region of the *cat*BC operon. The CatR protein has recently been partially purified by DNA-Affinity chromatography and by heparin-agarose chromatography.[59] The partially purified CatR was shown to specifically bind a 200 bp *Aval* fragment containing the *cat*R/*cat*BC overlapping promoter region. Using such partially purified CatR, recent footprinting experiments have shown three regions of protection directly upstream of the -35 site of the *cat*BC promoter and within 32 bases of the transcription initiation site of *cat*R, i.e., between + 8to + 31 of the *cat*R transcript (-54 to -78 of the *cat*BC transcription initiation site). Thus, binding of CatR in the dual control *cat*R/*cat*BC region also regulates its own transcription. A similar *nah* and *sal* promoter region protected by NahR protein has been reported by Schell and Poser.[60] Similar to the *sal*/*nah* system, the inducer *cis,cis*-muconate did not appreciably alter the extent of binding or location of binding with the upstream region of the *cat*BC operon. *Clc*R has not been purified as yet, and it is not known if the *clc*R and *clc*ABD promoter regions overlap, as in the case of *cat*R/*cat*BC. A comparison of such binding mode, as well as the affinity of CatR and ClcR to bind the non-chlorinated and chlorinated form of *cis, cis*-muconate to allow activation of the *cat*BC and *clc*ABD operons would be of significant interest to understand the mechanism of evolution of regulatory systems in the biodegradation of natural and synthetic chlorinated compounds.

2, 4, 5-T (tft) DEGRADATIVE PATHWAY GENES

The 2, 4, 5-T pathway is believed to have evolved in a strain of *P. cepacia* AC1100 during chemostatic selection in presence of 2, 4, 5-T provided as its only source of carbon.[61] Initially, it was demonstrated that the strain AC1100 can not only utilize 2, 4, 5-T as its only source of carbon and energy, but that it can remove more than 99% of 2, 4, 5-T from contaminated soil and can dechlorinate, to varying extent, a large number of chlorophenols including di, tri, tetra and pentachlorophenol.[62, 63] Karns *et al.*[64] demonstrated that 2, 4, 5-T is converted to 2, 4, 5- trichlorophenol (2, 4, 5-TCP) by constitutive enzyme (s), while further degradation of 2, 4, 5-TCP is mediated by a set of inducible enzymes (Fig. 3). Both Kilbane *et al.*[65] and Chatterjee *et al.*[66] clearly demonstrated that the Tft pathway was unstable in *P. cepacia* AC1100, which procuces spontaneously at a high frequency (10^{-2} per cell per generation) Tft^- cells during growth in a non selective medium. Whether this was due to loss of a plasmid carrying one or more essential *tft* genes or due to point mutations/deletions in the *tft* genes was, however not known at that time.

The instability of the Tft pathway posed a major problem for the cloning of the genes. Tomasek *et al.*[67] isolated a large number of *Tn5* generated Tft^- mutants, many of which accumulated pink, brown or deep red products during growth with glucose+2, 4, 5-T. However, such mutants would subsequently undergo further changes during maintenance in basal salts+glucose (BSG) medium or nutrient agar to become unable to produce such colored

intermediates during growth with glucose + 2, 4, 5-T (BSGT). Sangodkar *et al.*[68] isolated a large number of spontaneous Tft^- colonies during growth of AC1100 on BSG demonstrated that they had the same properties as the *Tn5* induced mutants, *viz.* many were colorless in BSGT media, while some produced dark brown or red colored intermediates; some could grow well with BSGT, while others grew well with BSG, but would stop growing or would show slow growth on addition of 2, 4, 5-T (because of accumulation of toxic intermediates such as 2, 4, 5-TCP, 2, 5-dichlorohydroquinone (DCHQ) or 5-chloro-2-hydroxy hydroquinone (CHQ) and their auto-oxidation product (benzoquinones). Out of such mutants, only two types of mutants could be complemented to Tft^+ by the AC1100 wild type genomic library. Large numbers of other mutants could not be complemented, presumably because of extensive deletions in the *tft* genes. It is interesting to note that such deletions occur only in the *tft* genes, but not in the essential chromosomal regions, since we have never observed auxotrophy or loss of viability in nutrient broth grown cultures, later checked for auxotrophy on BSG plates.

To understand the reasons for deletion formations and their occurrence only in the vicinity of the *tft* genes, we studied the nature of the repeated sequence RS1100 that was found to be present in the genome of AC1100 with a minimum copy number of 30 [*Eco*R1 digest of AC1100 genome and subsequent hybridization of the separated fragments by ^{32}p-labeled RS1100 element as a probe, showed 30 bands lighting up;[67]since many bands were of high molecular weight (25 kb or so) and since subsequent studies showed the presence of 3 RS1100 copies in a 25 kb cloned fragment harboring the *chg* gene,[69] it is clear that 30 is a minimum number, the actual number of copies of RS1100 is likely to be 50 to 100]. Tomasek *et al.*[67] isolated a number of *Eco*R1 fragments of the AC1100 genome that showed hybridization with ^{32}p-labeled RS1100, sequenced all of them and showed that there was a common 1477 bp sequence with 38-39 bp terminal inverted repeats immediately flanked by 8-bp direct repeats. There was a single large open reading frame (ORF) whose translated sequence showed structural homology with phage Mu transposase and other DNA binding proteins. The 8-bp direct repeats from 3 *Eco*RI fragments were all different, suggesting that the target sequences of RS1100 are not specific. The duplication of the target sequence at every site of insertion, the presence of long terminal inverted repeats and gene for a transposase suggested strongly that RS1100 might be a transposable Insertion Sequence (IS) element.

The transposability of RS1100 to a number of replicons such as pCP13 and pKT240 was demonstrted by Haugland *et al.*[70] Two very interesting observations were made by these authors. They showed that transposition of RS1100 upstream of a promoterless streptomycin resistance (*aph*C) gene in the plasmid pKT240 led to the expression of this gene, allowing selection of the cells harborning RS1100 on the pKT240 *aph*C upstream region on streptomycin plates. They also showed that occasionally, Rs1100 carried intervening DNA confined within two or more copies of itself during transposition. Thus, RS1100 has been redesignated IS931 because of its ability to transpose and is now known to carry portable promotor elements as well as to mobilize flanking DNA during transposition.[70]

What roles the transposable elements such as IS931 might have played in the recruitment or expression of *tft* genes present on the genome of AC1100? In order to see if IS931 elements

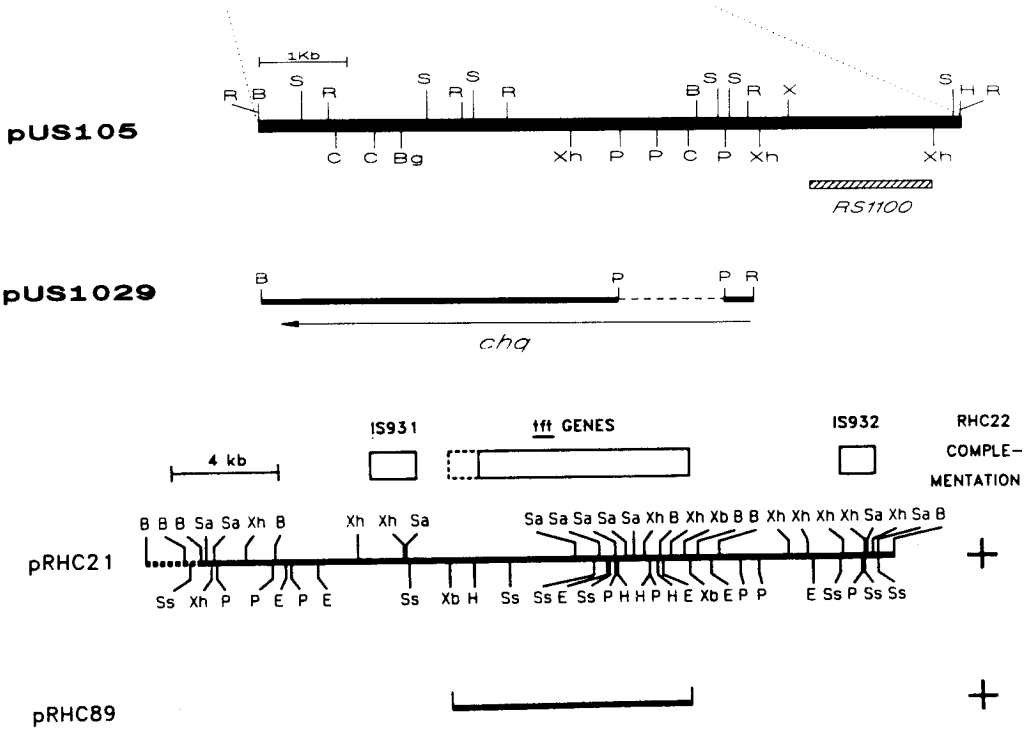


Fig 5 Restriction map of chromosomal fragments showing the localization of the *chq* genes (pUS1029) and the *tmo (tft)* genes (pRHC21). The location of the repeated sequence RS1100 upstream of the *chq* genes is shown in plasmid pUS105. The presence of the same repeated sequence (designated IS931) upstream of the *tft* genes in pRHC21 is shown. The 8.9 kb *Xbal* fragment harboring the *tft (tmo)* genes but without the upstream IS931 element is shown at the bottom (pRHC89).

occur near the *tft* genes and any possible role in their expression, we have cloned two sets of genes that complement two sets of spontaneous Tft^- mutants. For example, a mulant PT88, which accumulated a deep red pigment along with CHQ, was used as a recipient for complementation with the genomic library of the wild type AC1100 and Tft^+ colonies were selected. A 25 kb fragment from the chromosome of AC1100 was cloned (pUS1) which could complement PT88 to Tft^+ [69] Further subcloning experiments demonstrated the complementation ability to be restricted to a 4 kb *Bam*HI-*Pst*L fragment which also needed a 290 bp *Pst*I-*Eco*RI fragment located 1.3 kb upstream for its expression (pUS1029, Fig. 5). The 4 kb fragment was shown to encode CHQ oxygenase and was termed *chq* gene (s). Deletions extending to either end of the 4 kb *Bam*H1-*Pst*L fragment abolished complementation, suggesting that the entire 4 kb segment harboring the *chg* genes is necessary for complementation. The 290 bp fragment, also needed for complementation, could however be replaced by the *tac* promoter for complementation, suggesting that a promoter is present in this fragment. This fragment has been sequenced, but the sequence does not show the presence of any known *E. coli* or *Pseudomonas* type of promoter.

The presence of an oxygenase capable of further degradation of CHQ and encoded by the 4 kb *Bam*H1-*Pst*L fragment has been inferred from the ability of PT88 cells harboring pUS1 (the 25 Kb cloned insert) or pUS1029 (the 4 kb *Bam*H1-*Pst*L fragment under the 290 bp promoter, Fig. 5) to oxidize 4, 6-dichlororesorcinol to the colorless non-chlorinated hydroxybenezene derivative, while PT88 alone (without the plasmids) convert 4, 6-dichlororesorcinol to 5-chloro-1, 2,4-trihydroxy benezene (CHQ) which undergoes auto oxidation at pH 7.0 to the deep red 5-chloro-2-hydroxy benzoquinone derivative. Expression of the 4 kb *Bam*H1-*Pst*L fragment under the *tac* promoter, in presence of IPTG, allowed detection of several polypeptides in two-dimensional gels, although it is not clear if all the polypeptides are involved in the oxidative conversion of CHQ. The dechlorination reactions by *P. cepacia* AC1100, unlike those performed by anaerobes, are oxidative, *i.e.*, the chlorines from 2, 4, 5-TCP are replaced by hydroxyl groups rather than H atoms. *P. cepacia* AC1100 is unable to dechlorinate 2, 4, 5-TCP under anaerobic conditions and need molecular oxygen.

In addition to the *chq* genes, we have also cloned a 27 kb fragment (pRHC21) that complements a couple of other mutants RHC22 and RHC23. These mutants have been shown to be defective both in the uptake of 2, 4, 5-T and in the 2, 4, 5-T monooxygenase (Tmo) function; the latter catalyzes conversion of 2, 4, 5-T to 2, 4, 5-TCP. Subcloning of the 27 kb fragment and complementation of RHC22 to Tft^+ has localized the Tmo activities to an 8.9 kb fragment (pRHC89, Fig. 5). Interestingly, RHC22 cells harboring the 8.9 kb *Xba*I fragment (pRHC89, Fig. 5) lacks a copy of IS931 upstream of the *Xba*I site. This IS931 copy is, however, present in pRHC21. It is interesting to note that while both these plasmids will allow the *tmo* mutant RHC22 cells to grow with 2, 4, 5-T, when the concentration of 2, 4, 5-T is high (1.5 mM or higher), pRHC89, which lacks the upstream IS931 sequence, is unable to allow growth when the 2, 4, 5-T concentration is lower than 1.5 mM. Wild type AC1100 or RHC22 cells with pRHC21 plasmid will however show good rate of growth at 2, 4, 5-T concentrations as low as 0.6 mM.[71] It thus appears that the IS931 copy helps in the expression of the *tmo* gene cluster to allow better uptake of 2, 4, 5-T. Either the 27 kb or the 8.9 kb of fragment, when

transered to *P. aeruginosa* PAOIc cells as part of a broad host range vector will alow *P. aeruginosa* PAOIc cells to produce 2, 4, 5-TCP from 2, 4, 5-T.[71]

An important question raised in this connection is the organization of the *tmo* gene cluster relative to the *chq* genes, and the ancestry of the *tft* genes themselves, as well as the transposable element IS931. The fact that IS931 is a transposable element that carries outwardly directed promoters and can carry intervening DNA during transposition, makes it an attractive vehicle for recruitment of the *tft* genes from a common ancestor. To see if the *tft (chq, tmo)* genes and IS931 might demonstrate homology with the genomic DNA of a common ancestor, we have performed colony hybridization with several thousand isolates from soil selected on nutrient agar or PIA (*Pseudomonas* Isolation Agar, which specifically selects for pseudomonads to grow). None of these colonies hybridized with any of these probes. (*chq*, *tmo*, IS931). Since *tft* genes allow oxidative dechlorination while anaerobes allow reductive dechlorination, we thought that the *tft* genes might have been recruited from anaerobes. *P. cepacia* AC1100 can dechlorinate 2, 4, 5-TCP rapidly in presence of oxygen, but cannot perform reductive dechlorination under anaerobic conditions. DNA from anaerobic consortia capable of reductive dechlorination, as well as DNA from other anaerobic cultures, did not show hybridization with the *tft* genes or IS931. The two pentachlorophenol (PCP) degrading bacteria, *Flavobacterium* sp.[22]and *Rhodococus chlorophenolicus,*[23] which convert PCP to chlorohydroquinones before further degradation, did not show any hybridization either. Thus, we have so far been unable to detect aerobic or anaerobic bacterial cultures that show any homology to the *tft* genes and IS931. It is interesting to note that the *P. cepacia tmo* gene cluster specifies not only the production of 2, 4, 5-TCP from 2, 4, 5-T, but also 2, 4-dichlorophenol (2, 4-DCP) from 2, 4-D, and yet shows no homology with the plasmid pJP4 which harbors a 2, 4-D monooxygenase gene.

P. cepacia AC1100 is also an interesting example of how microorganisms deal with their substrates when such compounds are present in association with other compounds, particularly structurally analogous compunds. In the environment, most toxic or nontoxic chemicals are present in mixtures, particularly in waste dump sites. Different microorganisms have different capabilities with regard to utilization of the individual compounds present in the mixture. An important question in this regard is whether microbial consortia are better suited to utilize mixtures of chemicals, rather than a single culture that might be manipulated to utilize different compounds. A case in point is Agent Orange, which is a mixture of 2, 4-D and 2, 4, 5-T which was used widely by the U.S. Air Force in Vietnam. 2, 4-D biodegradation (Fig.3) is known to be mediated by plasmid pJP4, initially characterized in a strain of *Alcaligenes eutrophus* JMP134.[42] This plasmid can be transferred to a number of other organisms, including AC1100, to enable AC1100 to utilize rapidly either 2, 4-D or 2, 4, 5-T. The interesting question, therefore, is if a mixed culture of JMP134 and AC1100 will show the same efficiency of utilization of a mixture of 2, 4-D and 2, 4, 5-T than the pure culture of AC1100 harboring pJP4. Haugland *et al.*[72] demonstrated clearly that combined cell suspensions of AC1100 and JMP134 could effectively degrade either of these compounds provided as a single substrate. These combined cell susgensions, however, poorly degraded mixtures of the two componds provided at the same concentrations. Growth and viability studies revealed that such mixtures of 2, 4,-D and 2, 4,

5-T were toxic to AC1100 alone, and to combinations of AC1100 and JMP134. HPLC analyses of culture supernatants of AC1100 incubated with 2, 4-D and 2, 4, 5-T revealed the accumulation of chlorohydroquinone as an apparent dead-end catabolite of 2, 4-D and the subsequent accumulation of both 2, 4-DCP and 2, 4, 5-TCP. JMP134 cells incubated in the same medium did not catabolize 2, 4 5-T and were also inhibited in initiating 2, 4-D catabolism. On the other hand, AC1100 cells harboring pJP4 were shown to efficiently degrade mixtures of 2, 4-D and 2, 4 5-T through simultaneous metabolism of these compounds. It thus appears that genetically competent single cultures are better suited in many instances to simultaneously utilize mixtures of chemicals than mixed cultures where individual members may have the capability to utilize individual compounds but are often slow in this precess because of misrouting of some substrates into forming highly toxic intermediates that then greatly inhibit the growth of the key participating strains.

REFERENCES

1. Leisinger, T. and W. Brunner. (1986) *In* Biotechnology, vol. 8, W. Schonborn, Ed., pp. 475-513. VCH Verlagsgesellschaft, Weinheim, FRG.
2. Alexander, M. (1981). *Science* **211:** 132-138.
3. Gibson, D.T. Ed. (1981). Microbial Degradation of Organic Compounds. Marcel Dekker, New York.
4. Chapman, P.J. (1988). In Environmental Biotechnology : Reducing the Risks from Environmental Poullution through Biotechnology. G.S. Omenn, Ed. pp. 81-95. Plenum Press, New York.
5. Schneider, M.J. (1979). Persistent Poisons : Chemical Pollutants in the Environment. The New York Academy of Sciences, New York.
6. Chatterjee, D.K. and A.M. Chakrabarty. (1982). *Mol. Gen Genet.* **188:** 279-285.
7. Rothmel, R.K. and A.M. Charkrabarty. (1990). *Pure & Appl. Chem.* **62:** 769-779.
8. *Farrell, R. and A.M. Chakrabarty. (1979).* ***In*** *Plasmids of Medical, Environmental and Commercial Importance. K.N. Timmis and A. Puhler, Eds. pp. 275-285. Elsevier/North-Holland Biomedical Press, Amsterdam.*
9. *Kawasaki, H., N. Tone and K. Tonomura. (1981). Agric. Biol. Chem.* **45:** 29-34.
10. Slater, J. H., A.J. Weightman and B.G. Hall (1985). *Mol. Biol. Evol.* **2:** 557-567.
11. Nelson, M.J., S.O. Montgomery, W.R. Mahaffey and P.H. Pritchard. (1987). *Appl. Environ. Microbiol.* **53:** 949-954.
12. Wachett, L.P. and D.T. Gibson. (1988). *Appl. Environ. Microbiol.* **54:** 1703-1708.
13. Derbyshire, M.K., J.S. Karns, P.C. Kearney and J.O. Nelson. (1987). *J. Agric. Food Chem.* **35:** 871-877.
14. Tam, A.C., R.M. Behki and S.U. Khan. (1987). *Appl. Environ. Microbiol.* **53:** 1088-1093.
15. Englehardt, G., P.R. Wallnofer and R. Plapp. (1973). *Appl. Microbiol.* **26:** 709-718.
16. Cook, A.M. and R. Hutter. (1984). *J. Agric. Food Chem.* **32: 581-585.**
17. Behki, R.M. and S.U. Khan. (1986). *J. Agric. Food Chem.* **34:** 746-749.
18. Mulbry, W.M., P.C. Kearney, J.O. Nelson and J.S. Karns. (1987). *Plasmid.* **18:** 173-177.
19. Mulbry, W.W., J.S. Karns, P.C. Kearney, J.O. Nelson, C.S. McDaniel and J.R. Wild. (1986). *Appl. Environ. Microbiol.* **51:** 926-930.
20. Kishore, G.M. and G.S. Jacob. (1987). *J. Biol. Chem.* **262:** 12164-12169.
21. Balthazor, T.M. and L.E. Hallas, (1986), *Appl. Environ. Microbiol.* **51:** 432-434.
22. Steiert, J.G. and R.L. Crawford. (1986). *Biochem. Biophys. Res. Communs.* **141:** 825-830.
23. Apajalahti, J.H.A. and M.S. Salkinoja-Salonen. (1987). *J. Bacteriol.* **169:** 675-681.
24. Thiele, J.,R. Muller and F. Lingens. (1988). *Appl. Environ. Microbiol.* **54:** 1199-1202.
25. Rojo, F., D.H. Pieper, K.-H. Engesser, H.-J. Knachmuss and K.N. Timmis. (1987). *Science* **238:** 1395-1398.
26. Spain, J.C. (1990). *In Pseudomonas :* Biotransformations, Pathogenesis and Evolving Biotechnology. S. Silver, A.M. Chakrabarty, B. Iglewski and S. Kaplan, Eds. pp. 197-206. American Society for Mlicrobiology, Washington, D.C.

27. Brown, J.F., D.L. Bedard. M.J. Brennan, J.C. Carnahan, H. Feng and R.E. Wagner. (1987). *Science* **236**: 709-712.
28. Furukawa, K. (1982). *In* Biodegradation and Detoxification of Environmental Pollutants. A.M. Chakrabarty, Ed., pp. 33-57. CRC Press, Inc. Boca Raton, Florida.
29. Furukawa, K., N. Tomizuka and A. Kamibayashi. (1983). *Appl. Environ. Microbiol.* **46**: 140-145.
30. Bedard, D.L., R. Unterman, L.H. Bopp, M.J. Brennan, M.L. Heberl and C. Johnson. (1986). *Appl. Environ. Microbiol.* **51**: 761-768.
31. Bopp, L.H. (1986). *J. Ind. Microbiol.* **1**: 23-29.
32. Furukawa, K., N. Hayase and K. Taira. (1990). *In Pseudomonas* : Biotransformations, Pathogenesis and Evolving Biotechnology. S. Silver, A.M. Chakrabarty, B. Iglewski and S. Kaplan, Eds., pp. 111-120. American Society for Microbiology, Washington, D.C.
33. Ornston, L.N. and D. Parke. (1976). *Biochem. Soc. Trans.* **4**: 468-473.
34. Dorn, E., M. Hellwig, W. Reineke and H.-J. Knackmuss. (1974). *Arch. Microbiol.* **99**: 61-70.
35. Reineke, W. and H.-J. Knackmuss. (1988). *Annu. Rev. Microbiol.* **42**: 263-287.
36. Kukor, J.J., R.H. Olsen and J.-S. Siak. (1989). *J. Bacteriol.* **171**: 3385-3390.
37. Ghosal, D., I-S. You, D.K. Chatterjee and A.M. Chakrabarty. (1985). *Proc. Natl. Acad. Sci. USA.* **82**: 1638-1642.
38. Frantz, B. and A.M. Chakrabarty. (1987). *Proc. Natl. Acad. Sci. USA.* **84**: 4460-4464.
39. Aldrich, T.L., B. Frantz, J.F. Gill, J.J. Kilbane and A.M. Chakrabarty. (1987). *Gene* **52**: 185-195.
40. Aldrich, T.L. and A.M. Chakrabarty. (1988). *J. Bacteriol.* **170**: 1297-1304.
41. Aldrich, T.L., R.K. Rothmel and A.M. Chakrabarty. (1989). *Mol. Gen. Genet.* **218**: 266-271.
42. Don, R.H., A.J. Weightman, H.-J. Knackmuss and K.N. Timmis. (1985). *J. Baceriol.* **161**: 85-90.
43. Kaphammer, B., J.J. Kukor and R.H. Olsen. (1990). *J. Bacteriol.* **172**: 2280-2286.
44. Streber, W.R., K.N. Timmis and M.H. Zenk. (1987). *J. Bacteriol.* **169**: 2950-2955.
45. Henikoff, S., G.W. Haughn, J.M. Calvo and J.C. Wallace. (1988). *Proc. Natl. Acad. Sci. USA.* **85**: 6602-6606.
46. Kukor, J.J., R.H. Olsen and D.O. Ballou. (1988). *J. Bacteriol.* **170**: 4458-4465.
47. Kaphammer, B. and R.H. Olsen. (1990). *J.Bacteriol* **172**: 5856-5862.
48. Rothmel, R.K., R.A. Haugland, W.M. Coco, U.M.X. Sangodkar and A.M. Chakrabarty. (1989). In Recent Advances in Microbial Ecology. T. Hattori, Y, Ishida, Y. Maruyama, R.Y. Morita and A. Uchida, Eds., pp. 605-610, Japan Scientific Societies Press, Tokyo.
49. Schlomann, M., D.H. Pieper and H.-J. Knackmuss. (1990). *In Pseudomonas* : Biotransformation, Pathogenesis, and Evolving Biotechnology, S. Silver, A.M. Chakrabarty, B. Iglewski and S. Kaplan, Eds., pp. 185-196. Americam Society for Microbiology, Washington, D.C.
50. Ngai, K.-L. and L.N. Ornston. (1988). *J Bacteriol.* **170**: 2412-2413.
51. Chatterjee, D.K. and A.M. Chakrabarty. (1983). *J. Bacteriol.* **153**: 532-534.
52. Frantz, B., K.-L. Ngai, D.K. Chatterjee, L.N. Ornston and A.M. Chakrabarty. (1987). *J. Bacteriol.* 169: 704-709.
53. Ngai, K.-L., M. Schlomann, H.-J. Knackmuss and L.N. Ornston. (1986). *J. Bacteriol.* **169**: 699-703.
54. Perkings, E.J., M.P. Gordon, O. Caceres and P.F. Lurquin (1990). *J. Bacteriol.* **172**: 2351-2359.
55. Ghosal, D. and I.-S. You. (1988). *Mol. Gen. Genet.* **211**: 113-120.
56. Ghosal, D. and I.-S. You. (1989). *Gene* **83**: 225-232.
57. Frantz, B., T. Aldrich and A.M. Chakrabarty. (1987). *Biotech. Adv.* **5**: 85-99.
58. Niedle, E.L., C. Hartnett and L.N. Ornston, (1989). *J Bacteriol.* **171**: 5410-5421.
59. Rothmel, R.K., T.L. Aldrich, J.E. Houghton, W.M. Coco, L.N. Ornston and A.M. Chakrabarty. (1990). *J. Bacteriol.* **172**: 922-931.
60. Schell, M.A. and E.F. Poser. (1989). *J. Bacteriol.* **171**: 837-846.
61. Kellogg, S.T., D.K. Chatterjee, and A.M. Chakrabarty. (1981). *Science.* **214**: 1133-1135.
62. Kilbane, J.J., D.K. Chatterjee and A.M. Chakrabarty. (1983). *Appl. Environ. Microbiol.* **45**: 1697-1700.
63. Karns, J.S., J.J. Kilbane, D.K. Chatterjee and A.M. Chakrabarty. (1984). *In* Genetic Control of Environmental Pollutants. G.S. Omenn, and A., Hollaender, Eds., pp. 3-21. Plenum Press, N.Y.
64. Karns, J.S., S. Duttagupta and A.M. Chakrabarty. (1983). *Appl. Environ. Microbiol.* **46**: 1182-1186.

65. Kilbane, J.J., D.K. Chatterjee, J.S. Karns, S.T. Kellogg and A.M. Chakrabarty. (1982). *Appl. Environ. Microbiol.* **44:** 72-78.
66. Chatterjee, D.K., J.J. Kilbane and A.M. Chakrabarty. (1983). *Appl. Environ. Microbiol.* **44:** 514-519.
67. Tomasek, P.H., B. Frantz, U.M.X. Sangodkar, R.A. Haugland and A.M. Chakrabarty. (1989). *Gene* **76:** 227-238.
68. Sangodkar, U.M.X., T.L. Aldrich, R.A. Haugland, J., Johnson, R.K. Rothmel, P.J. Chapman and A.M. Chakrabarty. (1989). *Acta Biotechnol.* **9:** 301-316.
69. Sangodkar, U.M.X., P.J. Chapman, and A.M. Chakrabarty. (1988). *Gene* **71:** 267-277.
70. Haugland, R.A., U.M.X. Sangodkar and A.M. Chakrabarty. (1990). *Mol. Gen. Genet.* **220:** 222-228.
71. Haugland, R.A., U.M.X. Sangodkar, P.R. Sferra and A.M. Chakrabarty. (1991). Gene, in press.
72. Haugland, R.A., D.J. Schlemm, R.P. Lyons, P.R. Sferra and A.M. Chakrabarty (1990). *Appl. Environ. Microbiol.* **56**-1357-1362.