IDENTIFICATION OF THE INDUCING AGENT OF THE 2,4-DICHLOROPHENOXYACETIC ACID PATHWAY

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Bachelor of Science

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Stillwater, Oklahoma

1989

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
July, 1995

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Thesis Approved:

Thesis Adviser

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ACKNOWLEDGMENTS

I express my appreciation to Dr. Alan Harker for allowing me to begin my scientific career in his laboratory. I am grateful to my committee members—Dr. Moses Vijayakumar, Dr. Robert Miller, and Dr. George Murphy. Their suggestions and support were very helpful. I am much obliged to Dr. Jeff Hadwiger for the use of his centrifuge and computer.

I acknowledge all my lab colleagues and friends in the Department. Special recognition goes to Trish Ayoubi for her valuable insights during the last year of graduate school.

Without my parents, I. A. and Cathy, this degree would not have been possible. I can never say enough for the financial and emotional help they have provided. My sincere gratitude is extended to Garland and Kathy who have helped me to achieve my goal. And thanks to the remaining members of my family for their constant support: Brian, MeMe, Carla, P.J., Karl, Tamara, Eileen, Eddie, Tiffany, Brandon, and Logan.

I am indebted to my wife Shelly. Without her support and sacrifices I would not have succeeded. She has traveled the peaks and valleys of graduate school and has remained by my side the entire time. We made it through this experience together.

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CHAPTER I

INTRODUCTION

Production and consumption of xenobiotic organic compounds have increased in recent years (36). Xenobiotic compounds are chemicals which are foreign to the biosphere. Xenobiotics have structural groups which are not found in natural products. Over five million chemical compounds are now known and industry produces about 150 million tons of chemicals annually (54). Release into the environment has resulted from increased production and consumption of xenobiotics.

Xenobiotics become pollutants when they exert an undesirable effect. The release of large quantities of these compounds has caused major pollution problems and health hazards (24). Release directly from industry and agriculture is responsible for considerable contamination of soil and water in the United States. Contamination of soils and aquifers is a growing concern. Nearly half the United States population depends on ground water for potable water (100). These concerns have resulted in the need for quick and efficient cleanup process to be developed.

Compounds that cause contamination include herbicides, pesticides, degreasers, plastics, and solvents (25). Although many xenobiotics are recalcitrant, microbes have evolved systems to degrade a wide range of xenobiotics. The xenobiotics metabolized are aromatic and aliphatic organics, including halogenated forms. Chlorinated aromatic compounds are major environmental pollutants because they are often released in substational quantities, are toxic, are resistant to degradation, and accumulate in sediment and biota. Some xenobiotics can be mineralized by bacteria, fungi, or diverse microbial populations working as consortia (2).

Microbial degradation depends on environmental factors such as temperature, pH, oxygen, salinity, and availability of other nutrients. Important molecular properties include water-solubility, lipid water partition coefficient, volatility and also molecular size, charge, shape and presence of functional groups. All naturally occurring compounds can be utilized by a microorganism under suitable conditions (67), but many of the xenobiotic compounds that are able to be mineralized have been in the environment less than 50 years. Many of these compounds have no known natural analogs. While organisms not previously exposed to xenobiotics cannot be expected to have the ability to reduce them, relatively common bacteria have been observed to degrade xenobiotic compounds (5). Some researchers believe that given the proper conditions, microbes are capable of degrading any compound (100).

Microorganisms have shown an ability to metabolize a wide array of xenobiotic compounds once exposure has occurred. Although some xenobiotics can be metabolized, many are resistant to microbial attack (67). A problem in the degradation of some alkanes and cycloalkanes as well as other compounds is the toxicity of the substrate compound to the organism. Persistence of compounds is often linked to the presence of chemical substituents, including amines, methoxy groups, sulfonates, nitro groups, substituted benzene rings, ether linkages, and branched carbon chains. The presence of halogenated substituents in alkyl and aryl compounds, particularly the chlorinated moieties present in solvents, pesticides, plasticizers, and the trihalomethanes are important in the persistence of compounds. Metabolites such as chlorocatechols or chlorosubstituted ring fusion products may inhibit growth and may also suppress development of the marginal microbial community that utilize haloaromatics (100).

The ability of microorganisms to metabolize xenobiotic compounds, once exposure has occurred, is poorly understood at the molecular level. The TOL plasmid, which is self-transmissible and contains two or more operons, encodes enzymes required for the degradation of methylbenzenes, such as toluene, xylenes, and 1,2,4-trimethylbenzene, via methylbenzoates (4). The TOL plasmid is naturally occurring.

This suggests that the evolution of the ability to degrade xenobiotic compounds may have resulted, in part, from the interaction with molecules produced by indigenous microbes (66). It has been shown that indigenous organisms at a pristine site could readily adapt to degrade xenobiotic compounds to which they were never previously exposed (112). A wide variety of microorganisms from aquatic and soil biota have demonstrated the ability to adapt once exposed to xenobiotics, including members from the genera *Arthrobacter, Acinetobacter, Alcaligenes, Bacillus, Corynebacterium, Flavobacterium,* and *Pseudomonas.*

Microorganisms' ability to metabolize a wide variety of xenobiotics has made them valuable in biodegradation. Microbiological-based techniques for the destruction of naturally occurring organic compounds have historically been the treatment of choice, relative to physical or chemical methods (66). The ability of microorganisms to remove xenobiotics from the environment has resulted in research to elucidate the mechanisms of metabolism. Understanding the pathways may allow for the construction of novel pathways to metabolize xenobiotic compounds that are presently thought to be recalcitrant. Modifications to increase the range of capabilities of the microbes as well as the rate of degradation and survival stability of the organism under environmental stresses have been attempted. Excellent results have been obtained in laboratory situations (66). Abramawicz (1) combined genetic material from 26 different isolates to produce a single useful bacterium. The isolate demonstrated the ability to metabolize PCBs. The first organism designed for use in the field was an oil-degrading bacterium developed by Chakrabarty (66).

Molecular studies have produced information on a variety of pathways that are involved in the metabolism of xenobiotic compounds. A modified *ortho* cleavage pathway has been shown to be responsible for the biodegradation of a large array of chloroaromatic compounds. A well characterized group of modified *ortho* cleavage pathway operons that encode functions for the metabolism of catechol and chlorocatechols provides a model sytem for studying the regulation of evolutionarily related pathways. These include operons in degradative pathways for 3-chlorobenzoic

degradative pathways for 3-chlorobenzoic acid from *Pseudomonas putida*, 1,2,4-trichlorobenzene from a *Pseudomonas* sp., 2,4-dichlorophenoxyacetic acid (TFD) from *Alcaligenes eutrophus*, and benzoic acid from *P. putida* and *Acinetobacer calcoaceticus* (26).

Studies on the regulatory mechanisms of biodegradative pathways have not provided a complete understanding of regulation. In order to genetically manipulate degradative pathways the regulation of the pathways must be completely understood. An aspect of regulation not well understood is the inducer of the chloroaromatic pathways. An interaction between inducer and activator results in increased transcriptional activation of the pathway. The inducing agent and the interaction are not understood. Understanding these aspects is important in developing a full understanding of the expression characteristics of the pathway.

Purpose of the Research

The overall goal of this research is to develop a better understanding of the factors involved in the expression of genes involved in the metabolism of a chlorinated aromatic compound. This study requires identifying a method to study the expression of the structural genes in the TFD pathway of the plasmid pJP4. The plasmid pJP4 was isolated from *Alcaligenes eutrophus* JMP134. The plasmid is a broad host range, conjugative, 80 kb plasmid. The plasmid encodes for the degradation of 2,4-dichlorophenoxyacetic acid (TFD) and 3-chlorobenzoate (3CBA). Expression studies and sequence analysis will determine the inducing agent of the pathway. Understanding the regulatory mechanisms that are studied will be useful in the construction of improved bacterial strains. Such genetically engineered microorganisms could have enhanced degradative abilities and broader substrate utilization potentials. These microorganisms will be useful in bioremediation processes.

Goals and Strategies of Research

Specific goals and strategies for the accomplishment of this research are as follows:

1. Identify a system that allows the reliable production of gene fusions. Three separate transposon systems will be utilized for the production of *lacZ* gene fusions in the plasmid pJP4.

2. Use the *lacZ* gene fusions produced to estimate expression levels of the structural genes in the TFD pathway of the plasmid pJP4. The *lacZ* gene fusions that are produced by transposon mutagenesis will allow expression studies to be performed under induced and noninduced conditions.

3. Determine the inducing agent of the TFD pathway. From the expression studies that are performed and DNA sequence analysis, the inducing agent of the pathway will be able to be determined.

CHAPTER II

LITERATURE REVIEW

Halogenated Organic Compounds

Many xenobiotics that have been introduced into the environment are halogenated. This halogenation is often implicated as a possible reason for persistence (75). Chlorinated compounds have been the most extensively studied of the halogenated compounds. Chlorinated hydrocarbons that are degraded by microorganisms are grouped into three classes: (i) aliphatic, (ii) polycyclic, and (iii) aromatic (25). Chlorinated compounds serve as the basis for most of the information available on the biotransformation of xenobiotics. Most of this information has been produced from aerobic studies, since aerobic culture techniques are simple compared with anaerobic. Some chlorinated aromatics that have been demonstrated to be metabolized by soil and aquatic microorganisms are listed in Table 1.

Pathways for the Degradation of Chlorinated Aromatics

The basic strategies for the metabolism of aromatic compounds have been described. Five phases can be distinguished: (a) entry into the cell—this will usually occur by diffusion, but transport mechanisms exist for some compounds (102); (b) manipulations of the side-chains and formation of substrates for ring-cleavage; (c) ring-cleavage; (d) conversion of the products of ring-cleavage into amphibolic intermediates; and (e) utilization of the amphibolic intermediates (36).

A variety of different pathways have been described in detail for degradation of chlorinated aromatics. The crucial point in degradation is removal of halogen substituents from the organic compound. This may occur at an early stage of the degradative

TABLE 1

BIODEGRADATION OF SOME CHLORINATED AROMATIC COMPOUNDS

Compound	Microorganism	Plasmid	Reference
1,2-Dichlorobenzene	*Pseudomonas* sp.	–	(44)
1,3-Dichlorobenzene	*Alcaligenes* sp.	–	(28)
1,4-Dichlorobenzene	*Alcaligenes* sp.	–	(92)
	Pseudomonas sp.	–	(99)
	P. putida	+	(78)
2,6-Dichlorotoluene	*P. cepacia* HCV	+	(109)
2-Clorobenzoate	*P. cepacia*	–	(115)
3-Chlorobenzoate	*A. eutrophus*	+	(23)
	Pseudomonas sp. B13	+	(31)
	P. putida	+	(40)
4-chlorobenzoate	*Arthrobacter* sp.		(69)
	Pseudomonas sp. CBS3	–	(89)
	A. denitrificans NTB-1	–	(108)
4-chlorophenol	*Pseudomonas* sp.	–	(91)
	Alcaligenes sp.	+	
4-chlorophenylacetate	*Pseudomonas* sp. CBS3	–	(62)
2,4-D	*A. eutrophus*	+	(80)
	Pseudomonas sp.		(59)
	Flavobacterium sp.	+	(25)
2,4,5-T	*P. cepacia*	–	(83)

pathway with reductive, hydrolytic, or oxygenolytic elimination of the halosubstituent. Nonaromatic structures may also be generated, which spontaneously lose halide by hydrolysis of hydrogen halide by β-elimination (84).

Two well studied pathways that result in cleavage of the aromtic ring are *meta* cleavage of chloroprotocatechuate and the *ortho* cleavage of halosubstituted catechols (84), benzoates, phenols, and phenoxyacetates (12, 32, 34, 35, 61, 103). The metabolism of 2,4-dichlorophenoxyacetic acid (TFD) is performed by a modified *ortho* cleavage. The conversion is demonstrated in Figure 1.

Genetics of Chlorinated Aromatics Degradation

The genes necessary for the degradation of chlorinated aromatics may be carried on plasmids or the chromosome. Most of the genes that encode for degradation of chlorinated hydrocarbons are plasmid mediated, such as the 3-chlorobenzoate (3CBA) degrading plasmid pAC25 and the TFD degrading plasmids pJP4 and pRC10 (25). Table 2 contains the names of various plasmids that are involved in the degradation of chlorinated aromatics. The plasmid pJP4 of *Alcaligenes eutrophus* JMP134, that encodes the degradation of TFD, 3CBA, and 2-methyl-4-chlorophenoxyacetic acid (30), was used in these studies.

Expression of Chloroaromatic Degradation Pathways

Three similar *ortho* cleavage pathways have been studied in detail. The operons of the pathways include *clc*ABD, *tcb*CDEF, and *tfd*CDEF. The *clc*ABD operons converts 3-chlorocatechol to maleylacetic acid. The *tcb*CDEF encodes for the conversion of 3,4,6-trichlorocatechol to 2-chloromaleylacetic acid, and 3,5-dichlorocatechol to 2-chloro-maleylacetic acid (26). Transcription of these pathways has been shown to be controlled by regulatory proteins. The *clc*ABD operon is regulated by ClcR, TcbR regulates the *tcb*CDEF operon, and TfdR has been shown to regulate *tfd*CDEF (26, 56, 107). Each of these is a member of the LysR family of bacterial regulators. Other members of the LysR family include *cat*R, *cat*M, and *tfd*S (57, 74, 85).

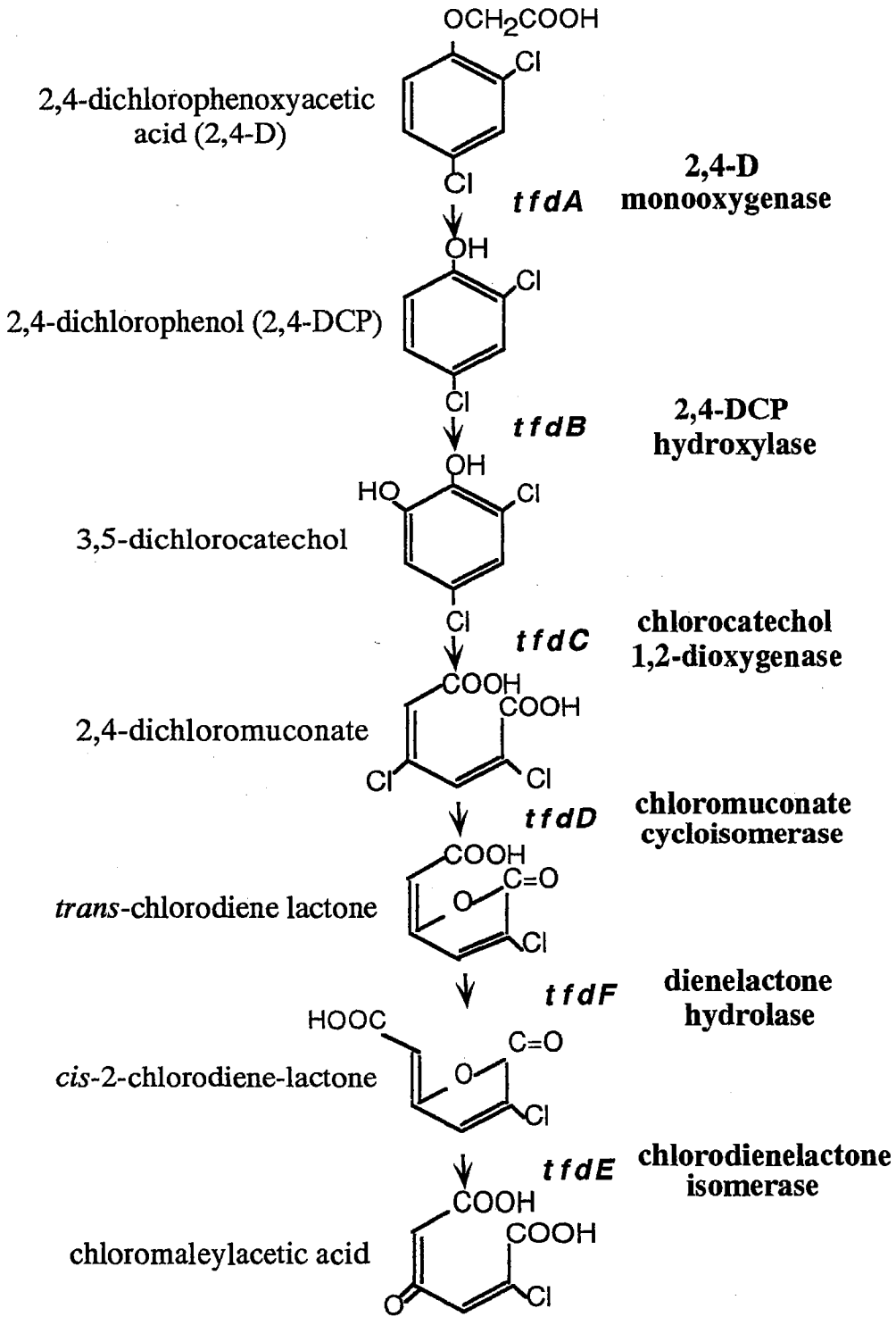


Figure 1. TFD Pathway on the Plasmid pJP4

TABLE 2

SOME DESIGNATED PLASMIDS ENCODING DEGRADATION OF CHLORINATED HYDROCARBONS

Plasmid	Compound	Molecular Size	Reference
pUU204	2-Monochloropropionate	53 kb	(45)
pKF1	4-Chlorobiphenyl	82 kb	(38)
pSS50	4-Chlorobiphenyl	53 kb	(93)
pAC21	1,4-Dichlorobiphenyl	65 MDa	(22)
pJP4	3-Chlorobenzoate 2,4-D and MCPA	80 kb	(31)
pEML159	2,4-D	‡	(3)
*	2,4-D	50-100 MDa	(82)
*	2,4,5-T	‡	(41)
pAC27	3CBA	110 kb	(23)
pAC31	3,5-Dichlorobenzoate	105 kb	(23)
pRC10	3CBA 2,4-D and MCPA	45 kb	(25)
pUO1	Flouroacetate	44 MDa	(58)
pUO11	Flouroacetate	40 MDa	

*No designation.

‡Size unknown.

The members of the LysR family typically exhibit several properties in common. These include a well-conserved amino terminus with a helix-turn-helix motif, selected conserved residues throughout the protein, and a size generally ranging from 285 to 315 residues (50). The majority of LysR family member genes are transcribed divergently from a gene or operon they control.

Gel shift analysis of CatR binding to the *cat*BC promoter region has shown that in the absence of the inducer, *cis, cis*-muconate, CatR at relatively low concentrations forms complexes with the *cat*BC promoter. When *cis, cis*-muconate is present in the binding reaction, the affinity of CatR for the promoter region is increased approximately 20-fold (79). The TrpI-*trp*BA interaction favors the formation of a second complex when incubated with the inducer indoleglycerol phosphate (20). The regulatory gene, *nah*R, controls the regulation of the napthalene and salicylate operons. Induction by *nah*R requires salicylate for activation (90).

Many of these LysR family protein members require an inducer for activation. The interaction between the regulatory protein and the inducer increases transcriptional activation of the operon which the regulator controls. The exact mechanism of the interaction and the resulting expression is not completely understood. It has been proposed that a protein-protein interaction between RNA polymerase and the transcriptional activator stabilizes RNA polymerase binding to the promoter region (39). Other suggestions are that changes in DNA topology at the –35 region brought about by the transcriptional activator-inducer complex allow proper recognition of the –35 region by RNA polymerase (111).

The mechanism of interaction between the regulator and inducer for other LysR members is not well understood either. The affinity of NahR for its respective target sites was increased only 2-fold by the inducer. This led to the proposal that the role of inducer is not to control binding of the activator to its target sites, but rather to induce a conformational change in the constitutively bound activator, which results in increased transcription from that promoter. Recent experiments using TrpI have suggested an alternative possibility. Hydroxyl radical footprinting showed that the

inducer causes: (1) extension of TrpI for protection of its target promoter from the -77 to -52 region down into the -32 region, (2) a 14-fold increase in target site affinity, and (3) appearance of an additional higher molecular weight species in gel retardation assays. This was interpreted to mean that the inducer causes binding of an additional molecule of TrpI adjacent to the one already bound at -77 to -52 resulting in transcriptional activation (53).

Some of the inducing agents of members in the LysR family have been determined. The most closely related pathway to the TFD pathway in which the inducing agent has been identified is the *cat*BC operon. The CatR protein, which regulates the *cat*BC operon, has been shown to have an increased binding affinity for the *cat*BC operon in the presence of *cis, cis*-muconate (79). In the absence of the inducer *cis, cis*-muconate, CatR at low concentrations forms two complexes with the *cat*BC promoter. When *cis, cis*-muconate is present, the affinity of CatR for the promoter region is increased approximately 20-fold (79).

The only inducing agent of any of these pathways that had been demonstrated was for the CAT pathway (79). The TFD, TCB, and CLC pathways contain chlorinated muconate compounds as intermediate products in the metabolism process. It had been speculated that the chlorinated muconate of these pathways is also the inducing agent (26, 107).

Other LysR Inducers

Other LysR family inducing agents have been identified. The LysR family member LysB is involved in the regulation of L-cystein synthesis. The inducer of the pathway is o-acetylserine. Mutants which lack the ability to make o-acetylserine can only be derepressed by the addition of o-acetylserine to the growth medium (79). NahR coordinately regulates the expression of the two naphthalene degradation operons of plasmid NAH7. NahR is induced by salicylate. OccR regulates the transcription of Ti plasmid encoded genes and is induced by octopine (79). TrpI regulates the *trp*BA genes of the tryptophan synthase pathway. Induction is provided

by indoleglycerol phosphate. All these regulatory genes are LysR family members in which the inducing agent is known (79).

Of the most closely related LysR family members to TfdR and TfdS, the CatR inducer is the only compound that has been determined. Intermediates of these pathways include chlorinated muconate compounds. These chlorinated muconates have been speculated to be the inducing agents of each pathway. The inducing agent for ClcR has been speculated to be 2-chloro-*cis,cis*-muconate (26). The inducing agent for TcbR has been suggested to be 2-chloromuconic acid (108). If these speculations are correct, then the chlorinated muconate is an important compound in the regulation of these pathways.

Degradation of TFD by the pJP4 Encoded Pathway

2,4-Dichlorophenoxyacetate

The structure of TFD is given in Figure 1. TFD is a chlorinated aromatic hydrocarbon that belongs to a group of halogenated phenoxyacetates. $C_8H_6Cl_2O_3$ is the chemical formula and the molecular weight is 221.04. TFD is used as a herbicide to control broad leafed plants. TFD acts like a plant growth hormone. The result is increased proliferation of the vascular tissues in the plant. The enlarged tissues block transport of nutrients and death of the plant occurs. Monocots and narrow leaf plants with scattered vascular tissue arrangements escape the blockage effects caused by tissue proliferation.

Degradation of TFD

TFD is rapidly degraded by soil microorganisms. It has a half life of six weeks in soil and is degraded by a modified *ortho* cleavage pathway (34, 84). TFD is converted into 2,4-dichlorophenol and then to 1,2-dichlorocatechol. The 1,2-dichloro-catechol is then converted to *cis, cis*-chloromuconate, which is converted to chloro-maleylacetate. Chloromaleylacetate is then converted to chlorosuccinate and acetyl coenzyme A via an NADH dependent reduction or to β-ketoadipate with the consumption of NADH

and liberation of chloride (33, 65). All conversions from chloromaleylacetate are encoded by the chromosome and not by pJP4 (65). Acetyl coenzyme A and succinate are formed from β-ketoadipate via the conventional β-ketoadipate pathway and enter the TCA cycle.

Plasmid pJP4

The plasmid pJP4 was isolated from *A. eutrophus* strain JMP134 (30). The plasmid pJP4 is a broad host range, conjugative, 80 kilobase plasmid and is a member of the P1 incompatability group of plasmids. The plasmid encodes resistance to mercuric chloride and phenyl mercuryl acetate. Plasmid pJP4 also encodes for the degradation of TFD and 3CB (30).

Genes of TFD Pathway

The modified *ortho* cleavage pathway genes and enzyme products of plasmid pJP4 are shown in Figure 1 (31). The restriction maps of pJP4 and the position of TFD genes are shown in Figure 2. The genes are arraged into three operons: *tfd*A, *tfd*B and *tfd*CDEF. Two regulatory genes have been identified, *tfd*R and *tfd*S (47, 56, 57). Copies of genes having a similar sequence have been located on pJP4 (40, 70, 81). These genes have been named *tfd*AII, *tfd*CI, and *tfd*DII. It has also been demonstrated that *tfd*R and *tfd*S have identical nucleotide sequences (70).

Regulation of TFD Pathway

The expression of TFD genes is controlled by regulatory elements (47, 56, 57). The nature of the expression is not well understood. Original studies proposed that TfdR regulated the expression of *tfd*A and *tfd*CDEF operons, and TfdS regulated the expression of *tfd*B operon (47, 56). This regulation would be unusual for LysR family regulatory proteins. A characteristic of LysR family member genes is transcription divergently from a gene or operon they control. If the previous regulatory pattern described is accurate, TfdR and TfdS control operons which are not divergently encoded. The sequence data that showed *tfd*R and *tfd*S are identical also suggest the

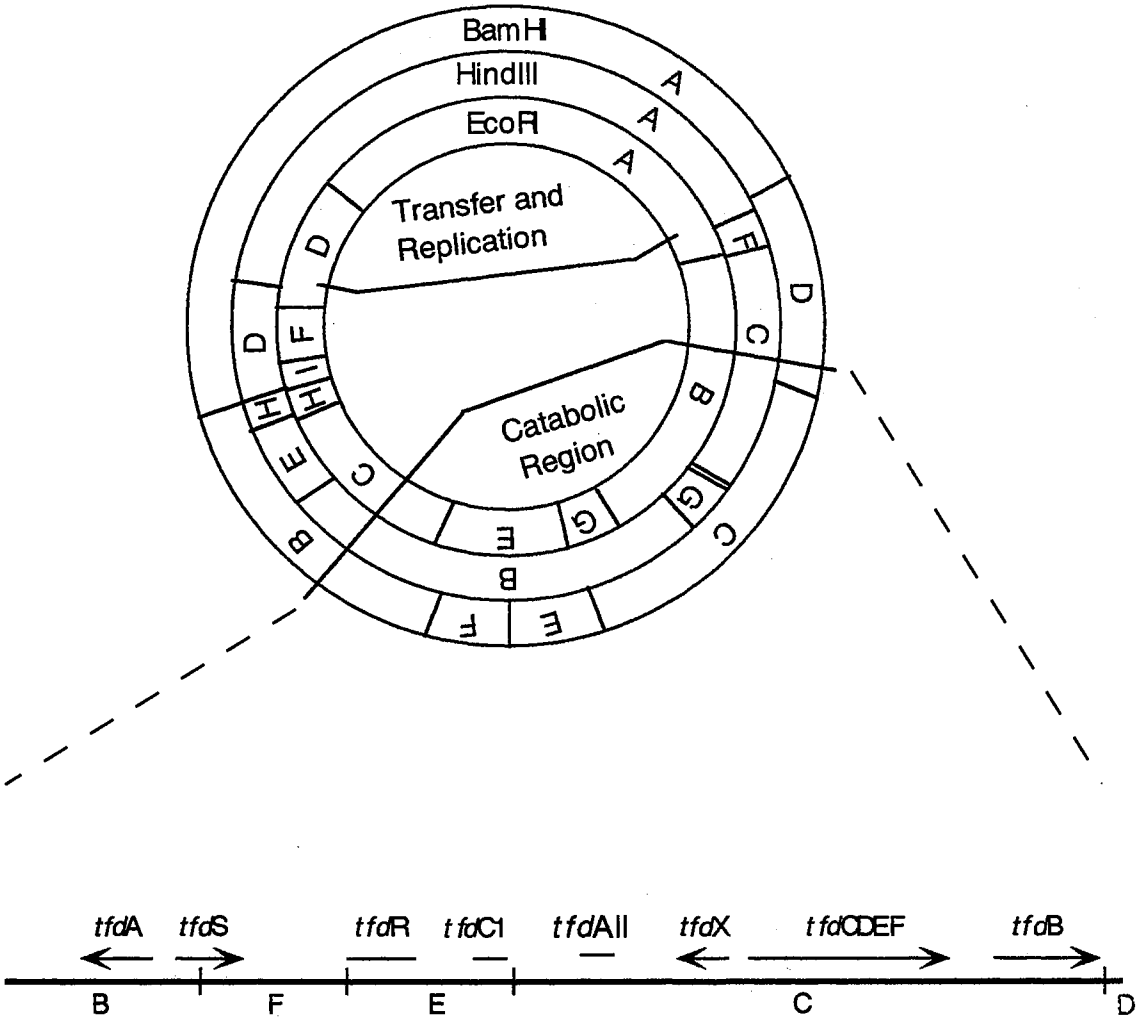


Figure 2. Physical Map of Plasmid of pJP4 and Positions of TFD Genes on *Bam*HI Fragments. Arrows indicate the direction of transcription.

previously proposed regulatory scheme is not accurate (70). Further studies need to be performed to determine the exact mechanism of control for the TFD pathway. A suggestion could be that *tfd*R, *tfd*S, and *tfd*X may regulate genes *tfd*DII, *tfd*A, and *tfd*CDEF, respectively. Gene *tfd*B may be regulated by some downstream element as proposed by others and its promoter showed no homology to other *tfd* promoters. This regulatory scheme is more consistent with other LysR family member proteins.

Gene Fusions With Transposable Elements

Genetic fusions have provided an important means of analyzing basic biological problems. Gene fusions are used for a variety of reasons. To study the regulation of a gene or operon; to detect genes which are subject to a particular regulatory signal; to study the mechanism of localization of a protein to an intracellular compartment or to the extracellular space; to detect and assay a protein which has not been identified or for which no assay exists; to detect a gene, the protein product of which is known, but for which there is no simple method for localizing or cloning the gene. Transposon mutagenesis allows the mutation, identification, and isolation of genes contained within large genetic targets, including prokaryotic (87) and eukaryotic genomes (9, 27). Gene fusions are used to allow analysis and manipulation of the expression and regulation of genes whose functions are unknown or difficult to assay (7, 37).

In gene fusions, the control sequences of a gene of interest are placed in front of the coding sequences of a reporter gene whose product can be assayed. Expression of the gene can be monitored by measuring the reporter gene activity whose product can be easily assayed. Gene fusions were used to monitor activities of the structural genes in the TFD pathway. A reporter gene that has been used under numerous conditions is the β-galactosidase *lacZ* gene of *Escherichia coli*. β-galactosidase is easily and quantitatively assayed and is active in a variety of organisms and when contained within hybrid proteins (7). Transcriptional and translation fusions can be produced. Transcriptional fusions are generated using sequences that contain the *lacZ* gene including its translational initiation signal; expression of a transcription fusion results

in the production of wild type β-galactosidase. In translational fusions, the translation initiation and the amino-terminal coding sequences of a gene are linked 5' to the *lac*Z coding sequences; expression of a translational fusion results in the production of a chimeric protein with β-galactosidase activity.

Transposable elements that contain *lac*Z coding sequences combine the techniques of transposon mutagensis and the generation of *lac* gene fusions. With transposons the random insertion of *lac*Z into a variety of DNA sequences can occur. Modified transposons containing *lac*Z have been constructed that allow the production of random gene fusions. Tn*3* (101), Tn*5* (50, 64), and Tn*10* (110) are transposons that have been manipulated to contain *lac*Z genes for use in fusions. A short list of transposable elements that are used in genetic studies are listed in Table 3. A more extensive list can be found in Berg and Howe's *Mobile DNA* (8).

TABLE 3

USEFUL TRANSPOSABLE ELEMENTS

Designation	Marker	Size (kb)	Reference
Tn*3*-Ap, Km	*amp, kan*	11.70	(42)
Tn*3*-HoHo1	*amp, 'lac*	14.25	(101)
Tn*1721*	*tet*	11.20	(106)
Tn*5*-CM	*cam*	3.90	(88)
Tn*5toxi*	*kan, tox*	10.50	(76)
Mini-Tn*5*	*kan*	5.00	(29)
Tn*7-lac*	*lac*ZYA	11.20	(6)
Mini-Tn*10*	*tet*	4.00	(110)
Mu dI1	*amp, 'lac*	37.00	(18)
Mini-Mu-*amp*	*amp*	6.00	(17)
Mu dII1734	*kan, 'lac*	9.70	(15)

CHAPTER III

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The bacterial strains and plasmids used throughout these experiments are shown in Tables 4 and 5.

Genetic Manipulations

DNA Isolation Procedures

Rapid isolation of plasmid DNA was performed by the modified Birnboim-Doly alkaline-SDS method (11). A loopful of freshly grown cells was scraped from a plate and suspended in 1 ml of phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO4, pH 7.3) and pelleted for 20 seconds in a Beckman Microcentrifuge at room temperature. The cell pellet was then suspended in 100 µl of a solution containing 2 M glucose, 0.5 M EDTA, 1 M Tris-HCl (pH 8.0), and 0.2% lysozyme. The sample was incubated for 5 minutes at room temperature. The mixture was denatured by adding 200 ml alkaline-SDS (0.2 N NaOH and 1% w/v SDS), mixed and incubated on ice for 10 minutes. To neutralize the mixture, 150 µl of 5 M potassium acetate (pH 4.8) was added and the mixture was incubated on ice for 5 minutes. The mixture was then centrifuged for 2 minutes in a Beckman Micro-centrifuge. The supernatant was transferred to a fresh microcentrifuge tube and the plasmid DNA was precipitated with 800 µl of 100% ethanol by incubating on ice for 5 minutes. The DNA was pelleted by centrifuging for 2 minutes. The pellet was washed with 200 µl of 70% ethanol and centrifuged for 1 minute. The pellet was vacuum dried and resuspended in 40 µl of TE buffer (0.05 M Tris-Cl, 10 mM EDTA, pH 7.5).

TABLE 4

BACTERIAL STRAINS

Strains	Relevant Characteristics	Reference
E. coli		
S17	thi pro hsdR recA	(96)
HB101	*rec*A, hsr, hsm, pHoHo1, pSShe	(101)
SF8000	Nal^r, *pol*A	(48)
POII1734	MC1040 with Mu dII1734 Lac	(15)
M8820	D (*pro*AB-*arg*F-*lac*IPOZYA)	(15)
JC7623	*rec*BC, *sbc*B	(55)
DPB271	l- *rec*D1903::mini-*tet*	(10)
DH5α	*rec*Al, Lac	BRL
Alcaligenes eutrophus		
JMP134	Prototroph, Tfd^+ Hg^r	(30)
AEK101	Prototroph, Tfd^-, Rif^r	(60)
Pseudomonas putida		
PPO300	Prototroph, Nal^r	ATCC 17514

TABLE 5

PLASMIDS AND PHAGE

Plasmid or Phage	Relevant Characteristics*	Reference
Plasmid		
pJP4	TFD^+, Hg^r	(30)
pUT mini-Tn*5* *lac*Z1	Ap^r Km^r; delivery plasmid for mini-Tn*5* *lac*Z1	(50)
pBS	Bluescript cloning vector, Ap^r	Stratagene Co.
pHoHo1	Tn3-*lac*Z transposon vector, Ap^r	(101)
pSShe	*tnp*A supplying vector, Cm^r	(101)
Phage		
Mu dII1734	Mu *cts*62::IS121 (Ap^r, Km^r)	(15)
	Defective *lac* transcriptional fusion	
	Phage with D (Mu A B)	

*Carbenicillin was substituted for ampicillin when *Alcaligenes* strains were involved.

Large scale plasmid isolation was by a 10-plate alkaline-SDS procedure. Each plate containing a lawn of bacteria was scraped into 5 ml of PBS. The cell mixture from 10 plates was separated into 4 Oak Ridge centrifuge tubes and centrifuged at 5,000 x g, 10 minutes, 4°C. The cell pellet was then suspended in 5 ml of a solution containing 2 M glucose, 0.5 M EDTA, 1 M Tris Cl (pH 8.0), and 0.2% lysozyme. The sample was incubated for 10 minutes at room temperature. The mixture was denatured by adding 10 ml of alkaline-SDS (0.2N NaOH and 1% w/v SDS), mixed and incubated on ice for 20 minutes. To neutralize the mixture, 7.5 ml of 5 M potassium acetate (pH 4.8) was added and the mixture was incubated on ice for 10 minutes. The mixture was centrifuged at 11,000 x g for 30 minutes at 4°C. The supernatant was transferred to a clean tube and plasmid DNA was precipitated overnight on ice by adding 0.313 volumes of 42% polyethylene glycol, 5 grams polyethylene glycol per 8 ml of water. Plasmid DNA was pelleted by centrifuging at 12,000 rpm, 10 minutes, 4°C. The DNA pellet was resuspended in TE (0.5 mM Tris·Cl pH 8.0 and 0.1 mM EDTA) and incubated on ice for 1 hour. The polyethylene glycol was removed by centrifuging at 11,000 x g, 10 minutes, 4°C. The supernatant, containing plasmid DNA, was layered onto a cesium-chloride-ethidium bromide gradient and centrifuged at 425,000 g for 4 hours at 20°C in a table-top ultracentrifuge (Optima TL, Beckman). Supercoiled plasmid DNA was extracted. The ethidium bromide was extracted with water saturated butanol. The DNA was ethanol precipitated with 100% ethanol and precipitated overnight at –20°C, and pelleted by centrifuging at 12,000 rpm, 15 minutes, 4°C. The pellet was washed with 70% ethanol, dried, and dissolved in TE buffer.

Ligations

A ligation reaction was set up in a buffer containing 30 mM Tris·HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DDT, and 1 mM ATP. In each reaction approximately 100 ng of the vector, 25 ng of insert, and 1 Weiss unit of T4 DNA ligase were used. Cohesive end ligations were performed at 15°C for overnight.

Construction of Clones

These clones were used for mini-Mu transposon mutagenesis. The *Eco*RI-B, Figure 2, fragment of pJP4 was cloned into pBluescript II KS (pBS). The pBS vector and pJP4 were digested to completion with *Eco*RI and mixed in a ligation reaction. Ligation reactions were set up as previously described. The ligation mixture was transformed into $CaCl_2$ competent DH5α *E. coli* cells. Transformation was performed by the heat shock method. The transformed cells were transferred to Luria Broth medium (LB, 10 grams Tryptone, 5 grams Yeast Extract, 10 ml NaCl, 10 grams agar for plates, pH 7.5 per 500 ml water) for 1 hour at 37°C. They were then plated on LB plates containing ampicillin (50 μg/ml), IPTG (0.5 mM), and X-Gal (40 μg/ml) to select and screen for transformants. White recombinant colonies were picked and checked for the presence of insert DNA in vector plasmid by alkaline mini-lysis method of Birnboim-Doly (68).

When the appropriate clone was obtained, a further subclone of the *Eco*RI-B fragment was created. The pBS *Eco*RI-B clone was digested completely with *Eco*RI. The digested DNA samples were electrophoresed on a 1.0% low melting agarose gel (SeaPlaque, FMC). The DNA was visualized under UV light after ethidium bromide staining. The appropriate band was cut out of the gel and the DNA electroeluted out of the agarose. The DNA was precipitated with ethanol and resuspended in TE buffer. This fragment was completely digested with *Hin*dIII and *Dra*I. The pBS vector was completely digested with *Hin*dIII and *Sma*I. A ligation reaction was incubated as described previously. The ligation mixture was transformed and clones selected as previously described.

The insertion point of the mini-Tn*5* transposon and pJP4 was cloned into the pBS plasmid. These clones were then used in DNA sequencing. The pJP4 mini-Tn*5* mutant and pBS plasmids were digested with *Bam*HI. A ligation reaction was incubated as described previously. The ligation reaction was transformed by the heat shock treatment into DH5α. The mixture was plated on LB kanamycin (50 μg/ml) and ampicillin (100 μg/ml). The plates were incubated at 37°C. Kanamycin and ampicillin

resistant clones were analyzed for insertions by alkaline mini-lysis procedures. Plasmid DNA from appropriate clones was then isolated by the 10 plate lysis procedure and purified on a CsCl gradient.

Transformation Procedures

Transformation by the calcium chloride procedure described in Maniatis (68) was utilized to transform plasmid DNA into *E. coli*. A single colony of *E. coli* was inoculated into 5 ml of LB medium, and grown at 37°C overnight with shaking. Four mls of this culture were transferred to 400 ml of fresh LB medium and grown at 37°C with shaking. When the culture reached an OD_{590} of 0.4, it was dispensed into 50 ml prechilled Oak Ridge centrifuged tubes and left on ice for 10 minutes. It was centrifuged for 5 minutes at 4000*g* at 4°C to pellet the cells. The cell pellet was suspended in half volume ice-cold, sterile $CaCl_2$ solution (50 mM $CaCl_2$ and 10 mM Tris•HCl, pH 8.0). The cell suspension was placed in an ice bath for 15 minutes. The cells were centrifuged at 4000 *g* for 5 minutes at 4°C. The pellet was resuspended in 1/15 volume $CaCl_2$ solution containing 10% glycerol. 200 µl aliquots of competent cells were dispensed into prechilled, sterile tubes and stored at –70°C until needed. When transformation was performed, the cells were thawed and placed on ice. 40 ng of DNA dissolved in 50 µl of TE was mixed with the cell suspension and incubated on ice for 30 minutes. The mixture was heat shocked in a water bath heated to 42°C, for 2 minutes. 1.0 ml of SOC (0.5% Yeast Extract, 2.0% Tryptone, 10.0 mM NaCl, 2.5 mM KCl, 10.0 mM $MgCl_2$, 10.0 mM $MgSO_4$, 20.0 mM Glucose) was added to each sample and incubated at 37°C for 1 hour. The mixture was plated on selective media and incubated overnight to allow for growth.

Cells that were used in transformation using the electroporation technique were prepared as follows. A 5 ml culture was inoculated with the appropriate cells and incubated overnight. This culture was then used to inoculate 500 ml of appropriate media. The culture was allowed to grow to an OD_{600} of 0.5 to 1.0. The cells were dispensed into prechilled centrifuge bottles and chilled on ice for 10 minutes. The cells

were centrifuged at 4000 *g* for 5 minutes at 4°C. The pellet was resuspended in equal volume of ice-cold, sterile water and centrifuged at 4000 *g* for 5 minutes at 4°C. The pellet was resuspended in half volume of ice-cold, sterile water and centrifuged at 4000 *g* for 5 minutes at 4°C. The half volume wash and centrifuge steps were repeated. The pellet was resuspended in 0.02 volumes ice-cold, sterile water and centrifuged at 4000 *g* for 5 minutes at 4°C. The final pellet was resuspended in 0.002 volumes of 10% glycerol and water. The cells were dispensed into prechilled micro-centrifuge tubes in a volume of 80 µl and stored at –70°C.

When the electroporation was perfomed all the materials were kept on ice. The cells were thawed and placed on ice. DNA was added in a volume of 4 µl. The mixture was chilled and added to an electroporation cuvette that was on ice for 5 minutes. The following conditions were used to electroporate cells in a BTX Electro Cell Manipulator 600: charging voltage 2.45 kV, resistance R5 (129 ohm), field strength 12.25 kV/cm, pulse length 5 to 6 msec. Following electroporation, 1 ml of SOC was added and the mixture incubated at 30°C for 1 hour. The cells were plated on selective media and incubated overnight.

Electroelution Techniques

Following electrophoresis on an agarose gel, the gel was visualized on a UV light source. Appropriate DNA bands were cut out of the agarose gel and placed into a dialysis membrane (Spectrapor membrane tubing, m.w. cutoff: 12,000 to 14,000). A minimal amount of TE was added to cover the gel. The membrane was placed in an IBI electrophoresis chamber. The membrane was covered with 0.5x Tris-borate electroporesis buffer (concentrated stock 5x per liter: 54 grams Tris base, 27.5 grams boric acid, 20 ml 0.5 M EDTA pH 8.0). The DNA was electrophoresed at 100 volts until the DNA had moved out of the gel. This was tested by examining the presence of DNA in the agarose gel on a UV lamp. Once the DNA had moved out of the gel, the electrodes on the electrophoresis chamber were reversed and run for 2 minutes at 100 volts. The buffer was removed from the dialysis membrane and the DNA precipitated

by adding 1/10 volume of 7.5 M ammonium acetate and 2X volume of 100% ethanol. The DNA was incubated on ice for 10 minutes. The mixture was spun in a Beckman Microcentrifuge at full speed for 5 minutes. The pellet was washed with 70% ethanol and vacuum dried for 5 minutes. The final pellet was resuspended in TE.

Transposon Mutagenesis

Several methods were chosen to produce *lac* fusions in the plasmid pJP4. In order to produce *lac* fusions within the structural genes of the TFD pathway, transposon mutagenesis using a Tn*3* transposon, developed by Stachel et al. (101), mini-Tn*5*, developed by de Lorenzo et al. (29), and a mini-Mu phage system, developed by Castilho et al. (15) were utilized. The mutagenesis was attempted in pJP4 plasmid as well as a subcloned portion of pJP4. Tn*3* and mini-Tn*5* transposons were utilized to produce gene fusions directly in pJP4, and mini-Mu was used to produce fusions in cloned segments of pJP4. Marker exchange experiments were used to replace wild type regions with the mutated mini-Mu regions.

Tn*3* Transposon Mutagenesis

Transpositions into target DNA sequences were selected by the transconjugant procedure of Heffron et al. (48). The plasmid pJP4 was conjugated into strain HB101 pHoHo1, pSSHe from AEK101 pJP4 by the method previously described (48). The pJP4 plasmid became the target sequence. HB101 pHoHo1 pSShe pJP4 isolates were selected for growth on BHI with mercury (100 μg/ml), ampicillin (100 μg/ml), and chloramphenicol (100 μg/ml). The HB101 pHoHo1 pSShe pJP4 strain was mated with SF800. HB101 pHoHo1 pSShe pJP4 was grown overnight in 5 ml BHI with mercury (100 μg/ml), ampicillin (100 μg/ml), and chloramphenicol (100 μg/ml). SF800 was grown in 5 ml of LB nalidixic acid (60 μg/ml). Both cultures were incubated shaking, overnight, at 37°C. The cultures were mixed in a 1:1 ratio and spotted on LB plates. The plates were incubated at 37°C for 6 to 8 hours. The plates were then scraped with 1 ml of PBS, the mixture pelleted, resuspended in 100 μl PBS and plated on BHI

mercury (100 μg/ml), ampicillin (100 μg/ml), and nalidixic acid (60 μg/ml). The plates were incubated overnight at 37°C.

Tn5 Transposon Mutagenesis

In a typical mating experiment, the donor strain *E. coli* S17-1 carrying the plasmid pUT mini-Tn5 *lac*Z1 was grown overnight shaking at 37°C in 5 ml of LB medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin. AEK101 containing pJP4 was grown overnight shaking at 30°C in 5 ml of TNA medium. After overnight growth the cultures were mixed and centrifuged at 1,500 g for 10 minutes in a Sorvall centrifuge. The pellet was resuspended in 0.05 ml of PBS. The suspension was dropped on a TNA plate. The plate was incubated at 30°C for 6 to 8 hours. After incubation, the cells were scraped off the plates into 1 ml of PBS. 0.1 ml of the cell resuspension was plated on BHI (18.5 grams brain heart infusion, 10 grams agar for plates per 500 ml water) containing 150 μg/ml rifampicin, 100 μg/ml mercury, and 50 μg/ml kanamycin. The plates were incubated at 30°C overnight.

Mini-Mu Transposon Mutagenesis

The pBS *tfd*DEF subclone of pJP4 was transformed into POII1734 by the heat shock method. Movement of the phage was then induced by temperature induction. A 5 ml culture was inoculted and incubated overnight at 30°C. A fresh culture was inoculated at a ratio of 50:1 and incubated for 2 hours at 30°C. The culture was then transferred to 42°C and incubated for 25 minutes. The culture was incubated at 37°C until lysis. Lysis was presumed to have occurred after 50 minutes if a clearing of the culture was not observed. Following lysis, chloroform was added to a concentration of 1% and the mixture was incubated at room temperature for 5 minutes. Cell debris and chloroform were pelleted by centrifuging at 5,000 *g* for 5 minutes, 4°C. The supernatant was removed and used immediately or stored at 4°C. After induction of the phage, infection of M8820 cells was performed. M8820 was grown to early log phase. The M8820 culture and phage lysate were mixed in a ratio of 2:1 and incubated at

22°C for 30 minutes. Following incubation, 1 ml of SOC was added and cultures incubated at 30°C for 1 hour. The cell-phage mixture was then spread onto LB plates ampicillin (100 μg/ml), kanamycin (100 μg/ml), and streptomycin (100 μg/ml). The isolated colonies were then examined for the presence of the poII1734 phage in the plasmid. This was done by isolating the plasmid using the Birnboim-Doly alkaline-SDS method (11) and restriction enzyme analysis. The restriction enzymes used were *Kpn*I and *Spe*I.

Marker Exchange Experiments

The pBS *tfd*DEF fusion plasmids were used in the marker exchange experiments. A variety of procedures were attempted to produce recombinational events. The first procedure utilized circular plasmid DNA. The pBS *tfd*DEF mini-Mu insertions were electroporated into AEK101 pJP4 as previously described. The transformants were plated on BHI mercury (100 μg/ml) plates and incubated overnight at 30°C. The lawn of bacteria that grew was scraped off these plates, serial diluted, and plated on BHI mercury (100 μg/ml) and kanamycin (50 μg/ml). Following the growth of transformants on mercury and kanamycin, isolates were picked and tested for the ability to grow on carbenicillin (50 μg/ml). The antibiotic resistance marker on pBS is ampicillin. Carbenicilin is substituted for ampicillin when *Alcaligenes* strains are used to test for growth on the antibiotic. *Alcaligenes* is naturally resistant to ampicillin. Growth studies were used to determine if a single or double crossover event occurred.

Linear transformation experiments were then attempted. The pBS *tfd*DEF fusion plasmids were digested with the restriction enzyme *Sca*I. This linear plasmid was then transformed into *E. coli.* strains JC7623 pJP4 and DPB271 pJP4 and AEK101 pJP4 by electroporation. In addition to these procedures, the fusion plasmids were digested completely with *Apa*I and *Spe*I. The restriction digests were separated on an agarose gel and the band containing the cloned portion of the pathway and mini-Mu transposon were cut out of the gel and the DNA isolated by electroelution. This linear

portion of DNA was then transformed into JC7623 pJP4 and DPB271 pJP4 by electroporation.

Following electroporation the cells were plated on BHI containing mercury and incubated overnight at 30°C. The lawn of bacteria was then scraped off with PBS and the cells plated on BHI containing mercury (100 μg/ml) and kanamycin (50 μg/ml). The isolates that were produced by transformation with linear DNA using *Sca*I were then tested for the ability to grow on carbenicillin (50 μg/ml).

Analysis of Transposon Mutants

Following the production of transposon mutants, a scheme was designed to determine if the proper transpositional event had occurred. Once it was determined that a transposition event of interest had occurred, the expression of the *lac* fusion genes under inducing and noninducing conditions was determined.

Analysis of Tn5 Transposon Mutants

Growth on TFD as a Carbon Source. Following the conjugation of *E. coli* S17-1 carrying the plasmid pUT mini-Tn5 *lac*Z1 with AEK101 pJP4 the rifampicin resistant, mercury resistant, kanamycin resistant transconjugants were tested for the ability to grow on TFD as a sole carbon source. Single colonies were picked from the BHI rifampicin (150 μg/ml), mercury (100 μg/ml), and kanamycin (50 μg/ml) plates. The colonies were replica-plated onto BHI plates containing 150 μg/ml rifampicin, 100 μg/ml mercury, and 50 μg/ml kanamycin plates and mineral medium plates (MMO; 40 ml solution A, 1 M Na_2 $HP0_4$, 1 M KH_2P0_4; 20 ml solution B, 10.0 g nitriloacetate, 14.45 g Mg•SO_4 •7H_2O, 3.34 g $CalCl_2$•2H_2O, 9.25 mg $(NH_4)_6$ MO_7O_{24}•4H_2O, 0.1 g $FeSO_4$•7H_2O, 50 ml metal "44" per 1000 ml; metal 44, 0.25 g EDTA, 0.5 g $FeSO_4$ •7H_2O, 0.154 g $MnSO_4$•H_2O, 0.039 g $CuSO_4$•SH_2O, 0.025 g CO $(NO_3)_2$•6H_2O, 0.11 Mg $2nSO_4$•7H_2O, 0.18 mg Na_2 B_4O_7•0H_2O per 100 ml; 10 ml solution C, 10% $(NH_4)_2$ SO_4) plates containing 0.3% case amino acids (CAA), 0.05% TFD. The replica pattern consisted of 128 colonies picked onto a plate. Two of the colonies were AEK101 pJP4

and two were AEK101. These were used as positive and negative controls. The plates were incubated at 30°C overnight. After growth on the MMO 0.3% CAA, 0.05% TFD plates the colonies were replica plated to MMO 0.05% TFD plates. These plates were incubated overnight at 30°C.

The colonies that were unable to grow on MMO 0.05% TFD plates were then tested for the ability to grow on TFD as a sole carbon source in liquid cultures. 5 ml of MMO 0.3% CAA, 0.05% TFD liquid cultures in 16 mm test tubes were inoculated with cultures that were unable to grow on MMO 0.05% TFD plates. The test tubes were incubated, shaking on a test tube rack at 30°C overnight. After overnight incubation the cultures were tested for the presence of TFD. One ml of the culture was analyzed using a Shimadzu UV160U UV-Visible recording spectrophotometer. A scanning photometric analysis was performed at 240 nm to 320 nm. This range of wavelengths will produce a peak that has a maximum absorbance at 277 nm when TFD is present. AEK101 and AEK101 pJP4 were grown under the same conditions and used as negative and positive controls.

Conjugation of Kanamycin Resistance. Once it was determined that mini-Tn5 transposon isolates were not able to utilize TFD as a sole carbon source, the ability to transfer kanamycin resistance through conjugation was studied. This will demonstrate the presence of mini-Tn5 on the plasmid. AEK101 pJP4 Tn5 transconjugants were grown overnight in a 16 mm test tube containing 5 ml of BHI mercury (100 μg/ml), kanamycin (50 μg/ml) broth overnight, shaking in a test tube rack at 30°C. PPO300 was grown in a 16 mm test tube containing 5 ml of TNB (2.5 grams Tryptone, 1.25 grams Yeast Extract, 0.5 grams Dextrose, 2.5 grams NaCl, 2.0 grams KNO_3 per 500 ml) containing nalidixic acid (60 mg/ml) overnight, shaking, in a test tube rack, at 30°C. After overnight growth the cultures were mixed and centrifuged in 15 ml Beckmann glass centrifuge tubes, 5,000 rpm, 5 minutes, room temperature. The pellet was resuspended in 0.05 ml of PBS and spotted onto TNA plates. The plates were incubated for 6 to 8 hours at 30°C. Following this incubation the cells were scraped off

the TNA plates using 1 ml of PBS. This mixture was plated onto BHI plates containing 60 μg/ml nalidixic acid, 100 μg/ml mercury, and 50 μg/ml kanamycin. The plates were incubated overnight at 30°C. The isolates were then replica plated to BHI 60 μg/ml nalidixic acid, 100 μg/ml mercury, and 50 μg/ml kanamycin plates. Growth on these plates revealed that kanamycin resistance was conjugated from AEK101 to PPO300.

Assay of β-Galactosidase Activity. Following the analysis by growth on TFD as a carbon source and transfer of kanamycin resistance by conjugation, expression levels of β-galactosidase were determined. Expression studies were performed under inducing and noninducing conditions using AEK101 pJP4 mini-Tn*5* isolates. Inducing conditions contained TFD in the media and noninducing conditions contained no TFD.

The assays were performed as described by Miller (72). Cultures of 5 ml MMO 0.3% CAA and 5 ml MMO 0.3% CAA, 0.05% TFD in 16 mm test tubes were inoculated and grown overnight shaking at 30°C. The overnight cultures were subcultured into fresh media of the same type. Four drops of the overnight culture were added to 5 ml of fresh media. The cultures were incubated at 30°C shaking until the cultures reached an OD_{600} of 0.28-0.70. The cultures were cooled to prevent further growth by immersing in an ice bucket containing a mixture of ice and water. After 20 minutes the cultures were assayed and the bacterial density measured.

The cell density was recorded by measuring the absorbance at 600 nm. One ml of the culture was withdrawn for the measurment. Aliquots of the cultures were added to the assay medium, Z buffer (0.06 M $Na_2HPO_4 \cdot 7H_2O$, 0.04 M $NaH_2PO_4 \cdot H_20$, 0.01 M KCl, 0.001 M $MgSO_4 \cdot 7H_20$, 0.05 M β-mercaptoethanol, pH 7.0). 0.2 ml of the culture was added to 0.8 ml of Z buffer. Two drops of chloroform and 1 drop of 0.1% SDS solution was added to each ml of assay mix. The mixtures were vortexed for 10 seconds. This process disrupts the cell membrane, allowing small molecules, such as ONPG, to diffuse into the cell. The tubes were placed in a water bath at 28°C for 5 minutes. The reaction is started by adding 0.2 ml of ONPG (4 mg/ml in Z buffer) to

each tube and shaking for a few seconds. The time of the reaction was recorded. The reaction is stopped by adding 0.5 ml of a 1 M Na_2CO_3 solution after sufficient yellow color has developed. AEK101 and AEK101 pJP4 were inoculated as negative controls.

The optical density was recorded at both 420 nm and 550 nm for each sample. The reading at 420 nm is a combination of *o*-nitrophenol and light scattering by cell debris. The reading at 550 nm represents light scattering. The following formula is then utilized:

$$\text{Units} = 1000 \text{ X } \frac{OD_{420} - 1.75 \times OD_{550}}{t \times v \times OD_{600}}$$

OD_{420} and OD_{550} are read from the reaction mixture;

OD_{600} reflects the cell density just before assay;

t = time of the reaction in minutes; and

v = volume of culture used in assay, in ml.

The units are proportional to the increase in *o*-nitrophenol per minute per bacterium.

Sequencing. Once it was established that an isolate was able to transfer kanamycin resistance and the β-galactosidase expression level was determined, the site of insertion was determined. The isolation of plasmid was performed as described by large-scale lysis in the DNA manipulation section. The insertion point of the mini-Tn5 transposon was cloned into pBS. These isolates were then sequenced.

The clones were sequenced at the Sarkeys Biochemistry Core Facility. Sequence data were produced by an Applied Biosystems automated sequencer. The sequence data were aligned using MacVector 4.1 program.

Analysis of Tn3 Transposon Mutants

SF800 pJP4::Tn3 transconjugants were mated with AEK101. SF800 pJP4 Tn3 transconjugants were grown in 5 ml of BHI mercury (100 μg/ml), ampicillin (100 μg/ml), and nalidixic acid (60 μg/ml). AEK101 was grown in 5 ml of TNA. The cultures were incubated, shaking, overnight at 37°C and 30°C, respectively. After

overnight growth, the cultures were mixed and spotted as described before. After scraping, the mixture was plated onto BHI mercury (100 µg/ml), ampicillin (100 µg/ml), and rifampicin (100 µg/ml) plates. The plates were incubated at 30°C overnight.

The AEK101 pJP4::Tn*3* transconjugants were then tested for the ability to grow on TFD as a sole carbon source. The procedure was the same as described for mini-Tn*5* insertions.

Analysis of Mini-Mu Transpositional Events

The kanamycin resistant mini-Mu transconjugants were tested for the ability to grow on TFD as a sole carbon source. This was done as previously described. Following the growth tests Southern blotting and hybridizations were performed to identify mini-Mu marker exchange events.

Southern Blotting and Hybridization

Agarose Gel Electrophoresis. Agarose gel electrophoresis was performed as described by Maniatis (68). *Bam*HI restriction digests of the plasmids were separated on a 1.0% agarose gel. The agarose gel was prepared and electrophoresed in 0.5x Tris-borate electroporesis buffer (concentrated stock 5x per liter: 54 grams Tris base, 27.5 grams boric acid, 20 ml 0.5 M EDTA pH 8.0).

Gel Pretreatment and Transfer to Nylon Membrane. Following electrophoresis, the DNA on the gel was depurinated by soaking the gel in 0.25 M HCl twice for 15 minutes each. The DNA was denatured by soaking the gel two times for 15 minutes in a solution of 0.5 M NaOH and 1.5 M NaCl. Soaking the gel twice for 15 minutes each time in 0.5 M Tris and 1.5 M NaCl (pH 7.0) resulted in neutralization. The DNA was transferred to a Nylon membrane (Zeta probe GT, BioRad) with vacuum blotter (Milliblot-V, Millipore) using 10x SSC transfer solution (1.5 M NaCl and 0.15 M sodium citrate).

Hybridization. Following transfer, the membrane was rinsed in 2x SSC and air dried. The membrane was then dried in a vacuum-oven at 80°C for 30 minutes. Prehybridization was started by sealing the membrane in a heat sealable bag containing 0.25 M Na_2HPO_4 (pH 7.2) and 7% w/v SDS hybridization solution and incubating the bag at 65°C for 2 to 4 hours. The hybridization solution was replaced with fresh solution. The probe was heat denatured by incubating in boiling water for 5 minutes. Heat denatured DNA probe was added to the solution and the bag resealed. Hybridization was conducted by incubating the bag at 65°C for 16 hours with agitation. The membrane was removed from the bag and washed twice for 30 minutes in 20 mM Na_2HPO_4 (pH 7.2) and 5% w/v SDS solution at 65°C. Two more washes for 45 minutes in 20 mM Na_2HPO_4 (pH 7.2) and 1% SDS solution at 65°C were performed next. The wet membrane was exposed to X-ray film. The film was processed according to the manufacturer's instructions.

Preparation of DNA Probe. The DNA probe used in Southern hybridization consisted of the 1.5 kb *Bgl*II-*Bam*HI fragment of the mini-Mu phage. This fragment consisted of the kanamycin resistant gene in the mini-Mu phage. The band was isolated by digesting poII1734 plasmid DNA, containing the mini-Mu phage, with *Bgl*II and *Bam*HI restriction enzymes. The DNA was separated on an agarose gel and the appropriate band was cut out of the agarose gel and isolated by electroelution. The DNA was labeled by nick translation.

Nick Translation. Nick translation was performed by a Promega nick translation kit. Reactions contained 1 µg of probe DNA, 10 µl of nucleotide mix (5 µM each of cold dCTP, dGTP, dTTP), 5 µl of nick translation buffer (50 mM Tris-HCl, pH 7.2 and 10 mM $MgSO_4$), 7 µl of [α-^{32}P] dATP (400 Ci/mM at 10 mCi/ml), 5 µl of enzyme mix (DNA polymerase I at 1 U/µl and DNase I at 0.2 ng/µl) and sterile water for a final volume of 50 µl. The reaction mixture was incubated at 15°C for 1 hour. The reaction was stopped by adding 5 µl of 0.2 M EDTA (pH 8.0). Unincorporated label was removed by selectively precipitating the DNA with ammonium acetate and

ethanol. The ethanol was pelleted after a 70% ethanol wash and dissolved in TE (10 mM Tris-HCI pH 8.0, 1 mM EDTA) buffer. The radiolabeled probe was stored at –70°C.

CHAPTER IV

RESULTS

Tn*3* Transposon Mutagenesis

Isolation and Characterization of Tn*3* Induced Mutants

A system developed by Stachel et al. (101) was used that enables the transfer of a modified Tn3 transposon. The modified Tn3 transposon contains the *lac*ZYA genes. The transposon can be used in the generation of both transcriptional and translational *lac* fusions with plasmid DNA sequences and these fusions are stable.

The target plasmid, pJP4, was transformed into the transposon donor strain, HB101 pHoHo1, pSShe. The resulting strain contained pJP4 and allows for the transposition of Tn3 out of the pHoHo1 plasmid and into pJP4. This strain was mated with SF800. Conjugation of the HB101 strain with SF800 allows the pHoHo1 and pSShe to be moved into environments in which they are unable to replicate. Replication of pHoHo1 and pSShe is dependent on DNA polymerase I, the *pol*A gene product. SF800 is a *pol*A mutant and will not support the replication of these plasmids. The Tn3 is maintained if it transposes into the pJP4 plasmid that is able to replicate in SF800. The Tn3 transposon is not able to further transpose because the transposase on pSShe is not replicated in this environment.

Following the successful conjugation of pJP4 into HB101 pHoHo1, pSShe the conjugation with SF800 was performed. The result of this conjugation was the transfer of pJP4, containing the Tn3-*lac* transposon, into SF800. The resulting isolates were mercury, ampicillin, and nalidixic acid resistant. The pJP4::Tn3 plasmids were able to be successfully conjugated to SF800. Isolates that were produced showed mercury,

ampicillin, and nalidixic acid resistance. This showed that the Tn3 transposon was able to be transposed into pJP4.

The next step was to determine if any insertions occurred in genes of the TFD pathway. *E. coli* is unable to metabolize TFD as a sole carbon source. Since growth on TFD was the easiest initial screen, these plasmids were conjugated to a strain that is able to metabolize TFD. AEK101 was used in conjugation studies to test for metabolism of TFD as a sole carbon source.

The SF800 pJP4::Tn3 isolates were conjugated to AEK101 as a mixed culture of isolates. Selection of transconjugants occurred on rifampicin (150 μg/ml), mercury (100 μg/ml), and carbenicillin (50 μg/ml). The result of this selection was transconjugants of AEK101 that contain pJP4 with the Tn3 transposon inserted. Individual isolates were then tested for the ability to grow on TFD as a sole carbon source. The replica plating technique on MMO 0.3% CAA 0.05% TFD and MMO 0.05% TFD plates was performed as described previously.

Approximately 5,000 individual isolates were picked and replica-plated. Of these isolates, none lacked the ability to grow on TFD as a sole carbon source.

Mini-Mu Phage Mutagenesis

Cloning of pJP4

The *Eco*RI-B fragment of pJP4 was succesfully cloned into pBS. The *Eco*RI-B fragment of pJP4 contains the *tfd*B and *tfd*CDEF operons. From this clone, a *Hin*dIII-*Sma*I clone was produced in pBS. This clone contains contains the 300 base pairs of the 3′ end of the *tfd*D gene sequence and the complete nucleotide sequence of the *tfd*E and *tfd*F gene sequences.

Induction of Mini-Mu

The pBS *tfd*DEF clone was transformed into POII1734 by the heat shock method. This strain was used for the production of mini-Mu *lac* fusions and contains a

temperature- sensitive transposase gene in the Mu phage present in the chromosome. The temperature-sensitive phage supplies transposition and replicative functions of Mu *in trans*. The mini-Mu prophage replicates and transposes hundreds of times in each cell and occasionally inserts into the pBS *tfd*DEF plasmid by a cointegrate transposition event (15). Mu DNA sequences are then packaged into Mu phage heads from the Mu left end to include 38 kb of DNA (14). If packaging starts from a mini-Mu sequence on the left side of a mini-Mu plasmid cointegrate stucture and if the size of the mini-Mu plus the size of pBS *tfd*DEF is less than 38 kb, then duplicated copies of mini-Mu sequences will be packaged. Multiple copies of the plasmid could also be packaged. Upon infection of a new cell with a particle containing multimeric plasmid, homologous recombination can occur between the duplicated plasmids to form a plasmid without an insertion. Insertions of mini-Mu into multimer forms of a plasmid are unstable since recombination can occur between duplicated plasmid sequences with the loss of insertion.

Analysis of Mini-Mu Insertions

Once the phage induction experiments were performed, isolates were examined for appropriate mini-Mu *lac* fusions. The cloned fragment in pBS *tfd*DEF contains an internal *Kpn*I restriction site 800 bp from the *Hin*dIII site used in cloning. Thirty base pairs from the *Hin*dII site in the multiple cloning site is another *Kpn*I site. At the other end of the clone, in the multiple cloning site of pBS, an *Spe*I restriction site is present. These two restriction sites were not found to occur elsewhere in the plasmid and were used in the analysis of clones.

Digestion of pBS *tfd*DEF with *Kpn*I and *Spe*I produced three fragments. The pBS vector produced a fragment of approximately 2.8 Kb. The insert of the clone produced two fragments. One of the fragments was approximately 1.5 kb and contains the complete *tfd*F nucleotide sequence and approximately 200 base pairs of the 3′ end of *tfd*E next to *tfd*F in the operon. The remaining fragment was approximately 800 base pairs in length. The smallest fragment contained the remaining portion of *tfd*E,

approximately 520 base pairs, and the portion of *tfd*D that was cloned. Utilizing these restriction sites, it was possible to determine if the insertion had occurred in the pBS vector or in genes of interest. In addition to determining insertions in the vector, the general location of the insert within the *tfd*DEF region was also determined.

Initial analysis of 50 mini-Mu fusion isolates showed that 49 of these possible fusion mutants did not contain an insertion. This showed that the majority of transpositional events that occurred resulted in multimeric forms of mini-Mu phage and pBS *tfd*DEF plasmids. The majority of multimers that were formed undergo a homologous recombinational event. The result was that the mini-Mu phage recombines out and the resulting plasmid was the pBS *tfd*DEF plasmid.

In order to prevent this recombinational event from being the major type of plasmids isolated, an additional plasmid isolation and transformation step was performed. Following the heat induction and transduction into M8820 a large scale plasmid prep was performed. The resulting plasmid prep contained a mixed population of pBS *tfd*DEF. Some of these contained mini-Mu phage in a variety of positions and a large portion contained no phage. The plasmids that contain no mini-Mu phage were then separated from the mixture. This was performed by transforming the plasmid mixture into M8820 by the heat schock method. By selecting for kanamycin, the plasmids that contained no mini-Mu phage were unable to grow on LB kanamycin (50 μg/ml) plates. Only those plasmids that contained a phage were able to replicate and produce a colony.

The isolates that were produced by this procedure were then analyzed. The plasmids were isolated by mini-lysis procedure and examined by restrictional enzyme analysis and agarose gel electrophoresis. Of the 100 isolates analyzed, 46 contained an insertion in the cloned region containing the *tfd*DEF genes. The remaining 54 were in the pBS plasmid. Of the 46 plasmids containing insertions in the *tfd*DEF genes, 44 of the insertions were in the 888 bp fragment. The remaining 2 isolates were in the 1.5 kb fragment. This appears to be an unusually large number of insertions in the smaller

888 bp region. Random insertion of mini-Mu should result in a larger number of insertions in the 1.5 kb region.

Homologous Recombination Experiments

Following the insertion of mini-Mu phage into the *tfd*DEF gene region in the pBS clone, the insertion mutant had to be inserted into pJP4. This was attempted by a homologous recombination event. Significant amounts of homologous DNA should exist on both sides of the mini-Mu point of insertion to allow for a recombination event. A double crossover event would result in the replacement of wild-type segments of pJP4 with the mutated fragment.

The first attempt to produce homologous recombinational events involved electroporating circular mini-Mu fusion plasmids into AEK101 pJP4. The pBS plasmid contains a ColE1 origin of replication and is unable to replicate in AEK101. A recombination event would result in incorporation of the kanamycin resistant gene of mini-Mu. A possible recombination event could then be selected for by growth on kanamycin. Growth on carbenicillin could then be analyzed to distinguish between single and double crossover events. A double–crossover event would result in incorporation of kanamycin resistance in pJP4. A single–crossover event would produce a colony that is kanamycin and carbenicillin resistant. Carbenicillin is the antibiotic resistance marker on pBS. Approximately 100 isolates were picked and found to be kanamycin and mercury resistant. The isolates were then tested for the ability to grow on carbenicillin. All of the isolates showed the ability to grow on carbenicillin. This showed that all the recombinational events were the result of single–crossover events.

Since this homologous recombination event did not produce a double–crossover event, a new approach was tried. This procedure depended on homologous recombination using linear DNA. Two *E. coli* strains have been developed for use in linear transformation: JC7623 and DPB271 (55, 113).

In addition to using these *E. coli* strains, the transformation of linear DNA was performed with AEK101. The circular plasmid DNA was linearized and electroporated into AEK101 pJP4. The mini-Mu containing plasmids were digested with *Sca*I. The pBS plasmid contains a single *Sca*I restriction site. The site is present in the ampicillin gene of pBS. The linear fragment contained the *tfd*DEF insert with a mini-Mu insertion. Depending on where the insertion occurred, homologous flanking DNA should occur on each side of the insertion. The regions are homologous to regions on pJP4. If a recombination event occurred, the mini-Mu insertion would recombine into pJP4. The wild-type segment would recombine out and the pBS DNA segments would also be lost following the recombination.

No kanamycin resistant AEK101 pJP4 transformants were produced using linear DNA. Following the growth on BHI mercury (100 μg/ml), the lawn of bacteria was plated on BHI mercury (100 μg/ml) kanamycin (50 μg/ml) plates. No isolates were produced that had the ability to grow on kanamycin. This suggested that AEK101 was unable to take up linear DNA.

Since the use of AEK101 did not result in the appropriate recombination event, the *E. coli* strains capable of taking up linear DNA were used. Two types of linear transformation experiments were performed. One involved digesting the mini-Mu fusion plasmid with *Sca*I and transforming the linearized DNA fragment. The second was used to increase the chances of producing a recombinational event, the vector DNA was separated from the cloned segment of DNA with the mini-Mu DNA fusion. The samples were digested with *Spe*I and *Apa*I. The result of this digestion was two fragments. One fragment contained the mini-Mu insertion and cloned DNA fragment; the other fragment contained the pBS vector. These isolates were separated on an agarose gel. The mini-Mu and cloned DNA fragments were isolated from the agarose gel by the electroelution process as described in Materials and Methods. This fragment was then transformed, followed by selection for a homologous recombination event. The lawn of JC7623 pJP4 and DPB271 pJP4 isolates produced after electroporation was

plated onto BHI mercury (100 μg/ml) and kanamycin (50 μg/ml) plates. Kanamycin-resistant isolates were produced on the BHI mercury kanamycin plates. The linear transformation using the single digested mini-Mu fusion plasmid with *Sca*I produced a small number of kanamycin resistant isolates. The linear transformation procedure utilizing two restriction enzymes was much more efficient. About twice as many kanamycin mercury kanamycin isolates were produced. Using the two-restriction enzyme method, 26 isolates were obtained.

The next step was to determine if the recombination event occurred in pJP4. The plasmids were isolated by 10 plate lysis. The plasmids were digested with *Bam*HI and *Hin*dIII restriction enzymes and separated on a gel. Two distinct types of plasmids were isolated. One appeared to have an identical pattern on the gel as wild-type pJP4. Southern hybridizations were then performed to determine if a recombination event could have occurred that produced a wild-type pattern of pJP4. A 1.5 kb portion of the kanamycin gene was used as the probe. The probe was isolated from the pOII1734 plasmid by using the restriction enzymes *Bam*HI and *Bgl*II. The Southern hybridization showed that no recombination event had occurred. The positive control, pOII1734 plasmid, produced a band in the audioradiogram at 9.7 kb. No other bands showed a hybridization pattern on the audioradiogram. The negative control was pJP4. These results showed that no recombination event had occurred in these plasmids.

Another type of plasmid was produced in the homologous recombination events performed. When pJP4 was digested with *Bam*HI, six bands were produced: A: 39.4 kb, B: 14.4 kb, C: 11.9 kb, D: 6.5 kb, E: 3.6kb, and F: 3.3 kb. Several homologous recombinants were produced that had an altered pattern from the wild–type pJP4. When digested with *Bam*HI, the mutants produced bands at approximately 39.4 kb, 7.0 kb, 6.0 kb, 2.0 kb, and 1.7 kb.

The restriction pattern using *Hind*III was also determined. When a restriction digest of pJP4 was electrophoresed on an agarose gel, the following bands were

produced: A: 34.7 kb, B: 22.2 kb, C: 8.0 kb, D: 6.4 kb, E: 4.1 kb, F 2.0 kb, G: 1.6 kb, and H: 1.4 kb. When the homologous recombinants were digested using *Hin*dIII and elecectrophoresed, three bands could be easily identified. The sizes were 34.7 kb, 18.0 kb and 6.4 kb. Figure 3 is an agarose gel picture of two isolates that are characteristic of the deletion observed. The molecular weight standard λ digested with *Hin*dIII is in Lane 1. Two isolates showing a deletion with the production of an 18.0 kb fragment are in Lanes 2 and 3. The plasmid pJP4 digested with *Hin*dIII is in Lane 4. The appropriate band sizes for each fragment are shown on the side of the picture.

In order to get an idea of the homologous recombination event that occurred, a Southern hybridization was performed. The samples were digested with *Hin*dIII and the Southern hybridization was performed as described in Materials and Methods. The probe that was used for these hybridization studies was an *Apa*I-*Spe*I fragment from the pBS *tfd*DEF clone. The probe contained the 3′ portion of *tfd*D and complete sequences of *tfd*E and *tfd*F. This probe was used to determine if these gene sequences were present in the mutants.

The positive control used in the Southern was pJP4. The genes of the probe were present in the 8.0 kb *Hin*dIII C fragment. This 8.0 kb fragment in the control produced a signal. The pBS plasmid was used as a negative control and produced no signal. The samples that were utilized were those mini-Mu insertions that produced the altered pattern compared to pJP4. These were the same isolates that the mini-Mu phage were inserted in the 1.5 kb *Spe*I-*Kpn*I fragment. The 18.0 kb fragment in a *Hin*dIII digest produced a signal. No other bands produced any signal on the autoradiogram. The 18.0 kb *Hin*dIII fragment that was present in these fusion mutants contained some portion of the *tfd*DEF genes in the TFD pathway. Five isolates were analyzed using the *tfd*DEF cloned segment in pBS. All showed the same pattern on the autoradiogram. Figure 4 is the audioradiograph of two of the deletion mutants. The deletion mutants were in Lanes 3 and 5. The 18.0 kb fragment produced a signal. The *Hin*dIII C fragment of pJP4 produced a signal from pJP4. A negative control, pBS, was also included.

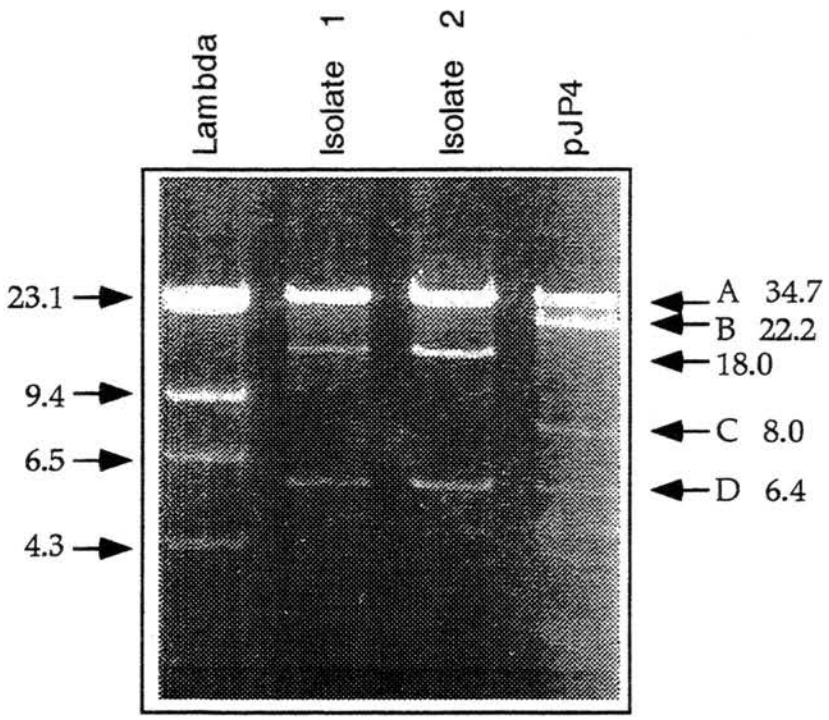


Figure 3. Agarose Gel of Deletion Recombinants That Were Produced During Homologous Recombination Studies. The samples were digested with the restriction enzyme *Hin*dIII. Numbers represent fragment sizes in kilobases; letters represent fragment designations of a pJP4 *Hin*dIII digest. The 18.0 kb band is the band that was created during the recombinational event.

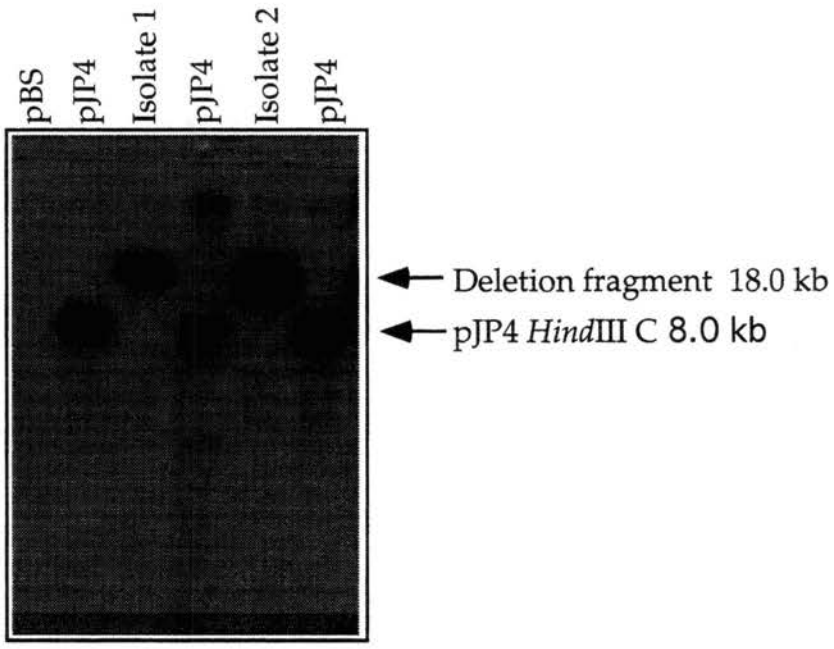


Figure 4. Audioradiograph of Deletion Mutants. The probe contains *tfd*DEF genes of pJP4 labeled with α-^{32}P-ATP; positive controls are pJP4. A negative control pBS is in the first lane.

Tn5 Transposon Mutagenesis

Isolation and Characterization of Mini-Tn5 Transposons

The mini-Tn5 system utilized in these experiments was developed by de Lorenzo et al. (29). The system used contains kanamycin resistance and was used to produce transcriptional fusions.

AEK101 and *E. coli* S17-1 containing the mini-Tn5 were conjugated. Approximately 5,000 isolates that were kanamycin, rifampicin, and mercury resistant were isolated. These isolates were tested for the ability to metabolize TFD as a sole carbon source. Of the 5,000 isolates, 30 were unable to metabolize TFD as a sole carbon source.

The ability of kanamycin resistance to be conjugated to PPO300 was then studied. Conjugation demonstrated the transposon had inserted into the plasmid. Of the 30 isolates unable to metabolize TFD as a sole carbon source, 27 were able to be conjugated to PPO300.

The lack of metabolism of TFD and conjugation studies demonstrated these isolates had an insertion in pJP4. Since the isolates were unable to metabolize TFD, the insertions occurred in a region that was critical for growth on TFD. Induction studies were then performed on 15 of the isolates that were able to be conjugated. The results of the induction studies are shown in Table 6. The β-galactosidase activities are characteristic of three independent expression studies.

The isolates were grouped into four categories following the β-galactosidase assays. Group 1 demonstrated activities of approximately 2,000 units and an induction ratio of 1.00. Group 2 had induction ratios of 2.00 to 2.78 and uninduced units of about 2,000 units. Group 3 had induced and uninduced β-galactosidase levels of about 50 units. The induction ratio was approximately 1.00. Group 4 had induction ratios of approximately 0.50.

Five isolates were in Group 1. The β-galactosidase activity of isolate number 1 under noninducing conditions was 2,271 units and 2,289 units under inducing condi-

TABLE 6

β-GALACTOSIDASE INDUCTION STUDIES

Isolate	β-Galactosidase Units		Induction Ratio
	Noninduced	Induced	
1	2,271	2,289	1.01
2	1,954	2,023	1.04
3	1,856	5,151	2.78
4	59	61	1.03
5	2,107	4,586	2.18
6	1,539	1,576	1.02
7	46	43	0.93
8	2,297	4, 569	1.99
9	2,458	5,646	2.30
10	1,294	1,317	1.02
11	2,087	1,080	0.52
12	2,019	4,707	2.33
13	2,241	2,248	1.00
14	2,567	5,319	2.07
15	1,350	550	0.41

tions. Isolate number 2 produced 1,954 units of β-galactosidase under noninducing conditions and 2,023 units under inducing conditions. Isolate number 6 had β-galactosidase activity that was 1,539 units under noninducing conditions and 1,576 under inducing conditions. The β-galactosidase activity of isolate number 10 was 1,294 units under noninducing conditions and 1,317 units under inducing conditions. Isolate number 13 showed β-galactosidase activity under noninducing conditions of 2,241 and when grown in inducing conditions 2,248 units. These β-galactosidase activities are very similar to the isolates that were inducible.

Six isolates were in Group 2. Isolate number 3 showed almost a 3-fold induction rate at 2.7. This isolate showed the highest amount of induction, although the amount of induction was similar to the other inducible isolates. Isolates 9 and 12 showed a slightly lower amount of induction at 2.3-fold. Isolate number 5 had a very similar amount of induction at 2.2-fold. The remaining isolates, numbered 8 and 14, showed a 2-fold rate of induction. The induction levels of all these isolates were in the same range. Each isolate showed noninduced β-galactosidase levels of approximately 2,200 units. The induced level of β-galactosidase activity for each of these isolates was approximately 5,000 units. The average amount of induction was approximately 2.3-fold.

Two isolates were in Group 3. The β-galactosidase activity was 2,087 units under noninducing conditions and 1,080 units under inducing conditions for isolate number 11. Isolate number 15 produced β-galactosidase activity that was similar to number 11. When isolate number 15 was grown in noninducing conditions, 1,350 units were produced and 550 units under inducing conditions.

Two isolates were in Group 4.. Isolate number 4 had β-galactosidase activity of only 59 units under noninducing conditions and 61 under inducing conditons. Isolate number 7 had similar β-galactosidase activities as number 4. Under noninducing conditions, 46 units of β-galactosidase activity were produced and 43 units under inducing conditions.

Isolates in Groups 1 and 2 had activities that were explained by the hypothesis. Three of the isolates were randomly selected from Grup 1 and two from Group 2; these were studied in greater detail.

Detailed studies involved determining the point of insertion of the Tn5 transposon. This was done by DNA sequence analysis. The plasmid DNA of isolates number 1, 2, 3, 5, and 6 were initially isolated. The isolates were then cloned into pBS. Each isolate was digested with *Bam*HI and a ligation reaction set-up as described in Materials and Methods. The ligation mixture was transformed into DH5α . The transformants were selected on LB kanamycin (50 μg/ml) ampicillin (100 μg/ml). This would insure growth of clones that contained the mini-Tn5 transposon and the point of insertion. These plasmids could then be used in sequence analysis.

A 17 base pair primer was used in sequence analysis. The sequence of the primer was taken from the 3' end of the kanamycin gene in the mini-Tn5 transposon. Sequence data of the clones are shown in Figure 5. Additional sequence data of each clone is not shown in this figure. Just the insertion point for mini-Tn5 is shown. The point of insertion for the mini-Tn5 fusions that were determined is shown. The sequence data showed where the insertion occurred. The sequence data obtained were aligned with sequence data for the *tfd* genes by the computer program MacVector 4.1. These alignment data were utilized to determine the position of the insertions. Figure 6 shows alignment of the insertion point with wild type pJP4 sequences.

Isolate number 1 had an insertion in the *tfd*C gene. The insertion occurred 585 base pairs from the 5' end of the gene. Isolate number 2 had an insertion in the *tfd*A gene. The point of insertion in this gene was 250 base pairs from the 5' end of the gene. Isolate number 3 had an insertion in the *tfd*D gene. This insertion was 70 base pairs from the 5' end of the gene. The insertion in isolate number 5 was in the *tfd*E gene. This insertion was 65 base pairs from the 5' end of the gene. The insertion in isolate number 6 was in the *tfd*B gene. This insertion was 160 base pairs from the 5' end of the gene. The relative positions of the insertions are shown in Figure 7.

ISOLATE 1

GCCCCCGC**ACTTGTATAAGAGTCAG** TCTGG TCGATGACCGTCTCAAGACTT

ISOLATE 2

GCCCCCGC**ACTTGTATAAGAGTCAG** CGATATCGAGTTTGCCGATCTCGCCCC

ISOLATE 3

GCCCCCGC**ACTTGTATAAGAGTCAG** TCACTACCGTGCACCAGCAGAGCTACG

ISOLATE 5

GCCCCCGC**ACTTGTATAAGAGTCAG** TCGCTTTGATGCCTAACTCGGATAGCC

ISOLATE 6

GCCCCCGC**ACTTGTATAAGAGTCAG** CAACGCACATTGGACAT CCTGCGTGA

Figure 5. Nucleotide Sequence Data of the Clones. The underlined sequence data is the *Not*I site present in the mini-Tn*5* transposon. The sequence data in bold is the O end of the mini-Tn*5* transposon. The remaining sequence data are the gene in which the insertion has occurred. Isolate number 1 shows an insertion in the *tfd*C gene. Insertion number 2 is an insertion in the *tfd*A gene. Insertion 3 is in the *tfd*D gene. Insertion number 5 is in the *tfd*E gene and insertion 6 in the *tfd*B gene.

Isolate No. 1

No. 1. GCCCCCGCACTTGTATAAGAGTCAG TCTGG TCGATGACCGTCTCAAGACTTACGA

*tfd*C GACCCTACT ATCGCGACGACGCCCC TCTGGTCGATGACCGGCTCAAGACTTACGA

Isolate No. 2

No. 2. GCC CCCGCACTTG TATAAGAGTCAG CGATATCGAGTT TG CCGATCTCGCCCCGGG

*tfd*A AGAGACAGCGCCGTGCCGAGGTAG C CGATATCGAGTTGG CCGATCTCGCCCCCGG

Isolate No. 3

No. 3. GCCCCCGCACTTGTATAAGAGTCAG TCACTACCGTGCACCAGCAGAGCTACGTTA

*tfd*D ACCAAGCGGCCGATCCAGATGTCGA TCACTACCGTGCACCAGCAGAGCTACGTTA

Isolate No. 4

No. 4. GCCCCCGCACTTGTATAAGAGTCAG TCGCTTTGATGCCTAACTCGGATAGCCGACG

*tfd*E TTGAGATCACGTCGCGCTCGGGTGG TCGCTTTGGTGCCTACCTCGGAAAGCCGACG

Isolate No. 5

No. 5. GCCCCCGCACTTGTATAAGAGTCAG CAACGCACATTGGACAT CCTGCGTGATCGC

*tfd*B CGACGCCGCGTGCCACATCACCAAC CAACGCACAATGGAGATCCTGCGTGATCGC

Figure 6. Sequence Alignment Data. Top sequence represents data from each of the clones; bottom segment represents data from wild type plasmid; underlined sequence data represent mini-Tn5 sequences. (Symbols: ^ identical sequences, V non-identical sequences.)

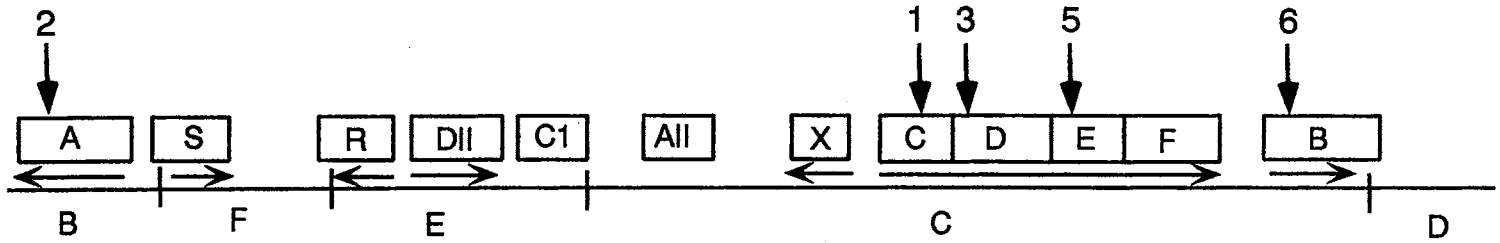
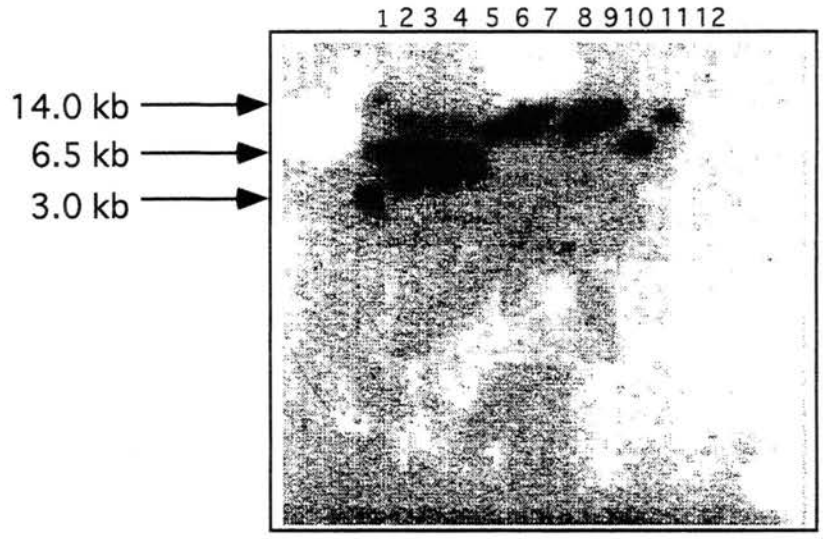


Figure 7. Illustration of Mini-Tn5 Transposon Insertion Sites. The *Bam*HI-B, F, E, C, and D fragments of pJP4 and locations of known tfd genes are shown. The open boxes represent position of genes; the genes are identified by the names inside the open boxes; the arrows represent positions of mini-Tn*5* transposon insertions that were identified by cloning and sequencing.

A second insertion of Tn5 could prevent production of the inducing agent. This could alter the interpretation of the β-galactosidase assays. To ensure that two insertions had not occurred, hybridization studies were performed. An internal *Hin*dIII fragment of Tn5 was used as a probe. Figure 8 represents the audioradiograph from the hybridization. Each pJP4 mutant was digested with *Bam*HI and *Eco*RI. The samples are in Lanes 2 through 11. The *Bam*HI and *Eco*RI digests are run next to each other. A single band from each isolate demonstrated that a single insertion was present. If a second insertion had occurred, two bands would be present in the audioradiograph. Positive control is present in Lane 1 and pJP4 is present in Lane 12 as a negative control. The single band in each lane represents a single insertion of Tn5.

Utilizing the sequence data and β-galactosidase induction assay numbers, it was determined that the most likely inducing agent for the TFD pathway is 2,4-dichloro-muconate.



Lane 1: Positive Control
Lane 2: Mutant 2, BamHI Digest
Lane 3: Mutant 2, EcoRI Digest
Lane 4: Mutant 6, BamHI Digest
Lane 5: Mutant 6, EcoRI Digest
Lane 6: Mutant 1, BamHI Digest
Lane 7: Mutant 1, EcoRI Digest
Lane 8: Mutant 3, BamHI Digest
Lane 9: Mutant 3, EcoRI Digest
Lane 10: Mutant 5, BamHI Digest
Lane 11: Mutant 5, EcoRI Digest
Lane 12: pJP4

Figure 8. Audioradiograph of Tn5 Mutants

CHAPTER V

DISCUSSION

Gene Fusions and Transposon Mutagenesis

Molecular genetic techniques are utilized in this study to determine the inducing agent of the TFD pathway. Other inducing agents for members of the LysR family of proteins have been discovered. The inducing agent for the benzoate pathway was discovered by gel shift analysis of the regulatory gene in the presence of the inducer. When the intermediate of the pathway such as muconate, 3-chlorocatechol, and 2-chloromuconic can be purchased, gel shift analysis can be utilized in determining the inducing agent of the pathway. The inducing agent for the TFD pathway is more difficult to determine. The intermediates in the pathway have short half-lives and cannot be purchased commercially. As a result of this, a different technique had to be employed to determine the inducing agent.

A system that would enable the genes in the pathway to be assayed under inducing and noninducing conditions needed to be utilized. Inducing conditions were the presence of TFD and noninducing conditions did not contain TFD. TFD is not the substrate that interacts with the regulatory gene to enhance transcriptional activation. Each gene product has some level of constitutive expression which ensures the production of all metabolites when TFD is present. This allows one of the down-stream metabolites, the inducing agent, to interact with the regulatory genes. This interaction results in increased transcriptional activation of the genes that the regulator controls. Since the intermediates cannot be purchased or easily synthesized, being able to assay the expression of these gene products is important for determining the inducing agent. A simple assay does not exist for each of the genes in the pathway.

Assaying for each gene product under physiological conditions is not a feasible option either.

Not being able to purchase the intermediates of the pathway and not having a simple assay were obstacles that had to be overcome. A solution to these problems was producing gene fusions with a reporter gene that can be easily assayed. A reporter gene that is commonly used in the construction of gene fusions is the *E. coli* β-galactosidase structural gene, *lac*Z. The β-galactosidase structural gene was chosen for gene fusions because of the wide variety of delivery mechanisms available and the ease of assaying for activity.

A delivery system had to be utilized that would allow for production of gene fusions. Transposons have been used in numerous studies to produce gene fusions and study gene organization and expression in a variety of organisms (15, 29, 101). A modified transposon system was the most convenient way to produce *lac* fusions.

The production of *lac* fusions allowed the inducing agent of the TFD pathway to be determined. At least two regulatory genes have been identified for the TFD pathway (47, 56, 57). The regulatory genes are thought to have inducing activity as well as regulatory activity. The two proteins are members of the LysR family of activator proteins. Other members in this family have been shown to require effector molecules for activity.

Finding the inducing agent that interacts with the regulatory genes of the TFD pathway is the purpose of this research. The inducing agent may interact with the regulator in the same way as other LysR family members. Transposon mutagenesis was utilized because the intermediates in the pathway cannot be purchased. The intermediate compounds are not synthesized commercially.

The *lac*Z gene fusions were assayed under inducing and noninducing conditions. Under inducing conditions the expression of gene fusions occurred at two distinct levels depending on where the insertion occurred. Once the inducing agent of the pathway is produced, the genes in the pathway transcribed prior to the inducing agent are transcribed at an increased level. If the *lac*Z gene fusion inserted in a gene that is

transcribed before the inducing agent, the inducing agent is not produced. If the inducing agent is not produced, the pathway is not induced. A lack of induction was demonstrated by the inability of β-galactosidase activity to be increased under inducing conditions. Any gene fusion not induced when grown in the presence of TFD occurred before the production of the inducing agent. If an increase in β-galactosidase activity results when the insertion mutant is grown in the presence of TFD, the inducing agent was produced. The inducing agent was able to interact with the regulator gene and induce the pathway. Increased expression of the pathway is a result of induction. By utilizing a combination of noninducible and inducible transposon mutants, the inducing agent was determined.

Tn*3* Transposon Mutagenesis

The Tn*3* *lac*Z transposon was developed to produce random insertions of *lac* fusions useful in the study of gene organization and expression. Tn*3* is able to transpose at a high frequency into plasmid DNA versus chromosomal DNA, generates both transcriptional and translational hybrid *lac*Z gene fusions and can be stable after transposition by supplying transposase activity *in trans*.

The *lac*Z coding sequences within Tn3-HoHo1 are open and in-frame to the end of the Tn*3* left terminal repeat. When Tn3-HoHo1 inserts into a gene so that the coding sequences of the gene are in-frame to the *lac*Z coding sequences, expression of the gene will result in the production of a hybrid β-galactosidase protein. If the coding sequences are not in-frame following an insertion, a nonhybrid β-galactosidase protein will be the result of gene expression. Translation will be initiated within the transposon *lac*Z sequences. Six methionine codons occur in frame 5' of the *lac*Z structural gene. Translation can be initiated at one or more of these codons if transcription occurs across this region (101). Transcriptional control sequences do not precede the *lac* sequences within Tn*3*. The production of β-galactosidase is dependent on expression that initiates outside of and reads into the element.

Tn3-HoHo1 is defective for transposase activity. To allow Tn3-HoHo1 transposition, pSShe was constructed to supply *tnp*A activity *in trans*. The pSShe plasmid encodes transposase activity but cannot be transposed.

These properties of the Tn3-HoHo1 system made it appear suitable for the production of *lac* fusions into the plasmid pJP4. The plasmid pJP4 was able to be maintained in the same environment as the pHoHo1 and pSShe plasmids. Since pJP4 was able to be maintained in the same bacteria as the pHoHo1 and pSShe plasmids, it would seem that transposition to pJP4 would occur at a rate that could be detected.

The antibiotic resistance marker ampicillin was able to be conjugated from the HB101 strain to the SF800 strain. This shows that the Tn3 transposon could be transposed into the pJP4 plasmid. When the plasmid pJP4 was conjugated into *A. eutrophus*, the ability to grow on TFD as a sole carbon source was detected. Using TFD as selection it would be easy to determine if an insertion in the pathway had occurred. If an isolate was unable to grow, then the pathway must have been interrupted by a transposon insertion. Subsequent analysis would then be used to determine where the insertion had occurred.

None of the isolates that were picked were unable to metabolize TFD as a sole source of carbon. Using the modified Tn3 transposon, no isolates were able to be identified that were blocked in the TFD pathway. A large number of isolates were tested, so this should not have been a factor.

Some aspect of the plasmid pJP4 could be preventing the appropriate insertions of the Tn3-pHoHo1 plasmid. Studies have been produced that show clustering of Tn3 at certain DNA locations is determined by the nucleotide sequence of the recipient genome (17). Analysis of insertions showed the presence of several distinct hotspots. Determinination of the DNA sequence in the vicinty of the hotspot region for Tn3 insertion indicated that a major homology with a sequence within Tn3 existed. A regional specificity of Tn3 insertion with a strong preference for AT-rich segments has been proposed (63). The site of insertion of Tn3 is partially determined by the primary nucleotide sequence of the recipient genome.

These transpositional facts about Tn3 suggest a possible reason why no insertions were isolated in the TFD pathway. It may be possible that hotspots exist in the pJP4 plasmid. The hotspots could be the point where insertions have occurred. These hotspots would not be in any genes of the TFD pathway since growth was not interrupted. The entire sequence of pJP4 is not known. This does not allow any possible sequences to be identified from the sequence data. The insertion sequence was not determined for any of the transposon mutants. It was not determined if any hotspots are present in pJP4.

From these experiments it does appear that Tn3 is not a suitable transposon for the production of the desirable mutants. The transposon was not able to insert into any regions of interest in pJP4. As a result of these two observations, another sytem was used to produce gene fusions.

Bacteriophage Mu Transposition

Although Mu is actually a bacteriophage, movement of the phage is similar to a transpositional event by a transposon. Several properties of bacteriophage Mu have made it useful for genetic manipulations (13, 52, 105). Mu is a temperate phage which replicates by a process that involves DNA transposition. Transposition to new sites can be induced at a high rate by lytic growth, or it can be repressed in the lysogenic state. Mu has a headful mechanism for packaging its DNA. A total of approximately 38 kb including the Mu genome can be taken up into a viral particle and carried to a new cell upon infection (14). New DNA sequences can be incorporated between the ends of the Mu genome and carried as part of the phage genome. Segments of the Mu genome can be removed without affecting phage growth or intracellular transposition. These functions can be complemented by a nondefective helper Mu phage as long as the DNA sequences at each Mu end are maintained (104).

Modified Mu phages have been constructed that allow for the production of *lac* fusions. The properties of bacteriophage Mu allowed the construction of the mini-Mu

phage used in these studies. The mini-Mu system used in these studies was developed by Castilho et al. (15) and contains a selectable drug resistance gene, kanamycin. The mini-Mu phage is also missing the transposition genes necessary for movement and the packaging genes used in the life cycle. These functions are supplied *in trans* by insertion of a temperature-sensitive Mu phage in the chromosome. Insertions following the initial transposition are genetically stable and incapable of subsequent transposition. These elements permit the use of the Mu transduction process to select Mu insertions, including insertions in high copy-number plasmids.

Using mini-Mu phages for the production of *lac* fusions requires a different scheme than using a transposon such as Tn*3* or Tn*5*. The mini-Mu system is unable to package the entire pJP4 plasmid. The transposition of mini-Mu cannot be used with pJP4. To overcome this situation portions of pJP4 were subcloned. The fusions were produced in a region of pJP4 that was thought to contain the gene that coded for the enzymes responsible for the production of the inducing agent. This region was small enough, 2.5 kb, so that the insertion could be easily mapped.

Introduction into pJP4 was attempted by a homologous recombination event. This event allowed the introduction of the fusion gene into pJP4. Several different techniques were employed to induce the recombinational event involving *A. eutrophus* and *E. coli* strains. In contrast to some other bacteria, *E. coli* is not able to be transformed by linear pieces of DNA (98). No references were found about possible linear transformation of *A. eutrophus*.

The inability to take up linear DNA by *E. coli* is due in part to degradation of the incoming DNA by intracellular exonucleases (51). *E. coli* strains lacking exonuclease V by virtue of *rec*B or *rec*C mutations can be transformed by linear DNA if they carry the *sbc*B and *sbc*C mutations that as well restore recombination proficiency to the *rec*B *rec*C mutant. A *rec*D mutant also lacking exonuclease activity can be transformed with linear DNA. Since these stains have been reported to be useful for linear transformation, they were utilized in these studies.

The initial plasmids that were produced contained the same pattern on an agarose gel as wild-type pJP4 and showed no hybridization with any portion of the mini-Mu phage. These were produced by linearizing the fragment with a single restriction enzyme. The remaining pBS *tfd*DEF mini-Mu fusion mutants did produce a recombinational event. These linear fragments were produced by digesting with two restriction enzymes and separating the vector DNA from the mini-Mu and cloned DNA. The recombination event did not replace the desired fragment in pJP4 but appeared to produce a deletion of pJP4. The *tfd*DEF genes that are present in the clone are contained in the *Hin*dIII-C fragment of pJP4. The recombination event that occurred resulted in a large deletion of pJP4. A *Hin*dIII digest of these fusion mutants results in a band at 18 kb on an agarose gel. The size of this band is between *Hin*dIII fragments B and C of pJP4. The pJP4 *Hin*dIII-B and C fragments are not present in these mini-Mu fusion mutants. The mini-Mu fusion plasmids have lost approximately 15 kb of DNA.

A second copy of the *tfd*D gene is present in the *Hin*dIII-B fragment (70). The distance between the second copy of *tfd*D and the *tfd*D gene in the *tfd*CDEF operon is approximately 15 kb. The removal of this fragment results in the loss of three *Hin*dIII restriction sites. If these sites were lost, the *Hin*dIII-B and C fragments would become one fragment. The restriction sites that are lost produce the B and C fragments from a *Hin*dIII restriction digest. The loss of these sites would produce a fragment that is approximately 18 kb. From the Southern hybridizations it was shown that the 18 kb fragment did hybridize to the *tfd*DEF fragment in a probe. The possible position of the mini-Mu phage in the deleted plasmids was not determined.

It appeared from the restriction digest and Southern hybridizations that a large deletion has occurred. If the deletion occurred between the two *tfd*D genes, it may be possible that a recombinational event occurred as a result of possible homology between the two genes. A looping out of the DNA segment between the two genes may have occurred. The Southern hybridization does not prove that the deletion occurred

as described, but it does offer a possible suggestion of the event that did occur. Further hybridization studies using the mini-Mu phage and possible missing sequences as a probe could give more evidence to support the possible recombination event.

When fusions of pBS *tfd*DEF were analyzed, a pattern of insertions developed. These isolates had an unusually high insertion rate in an 888 bp region of the pBS *tfd*DEF. Ideally for the most efficient homologous recombination to occur, at least 200 bp of homologous DNA should exist on each side of the fusion. Further analysis of these isolates in the 888 bp region showed that the insertion was within 250 bp of the fragment end. It was possible that a hotspot for mini-Mu phage existed in this region. Some insertions have been shown to occur in the same location by recombination tests (15). Experiments have shown that a shortened version of the pBR322 plasmid had hotspots for insertion (15). The insertions in the pBS *tfd*DEF clone could be very close to the end of the fragment. This could be preventing a homologous recombination event from occurring.

From these studies it appeared that a mini-Mu phage system was not adequate for producing *lac* fusions in areas of interest. The mini-Mu phage was able to transpose into the *tfd*DEF region that had been cloned. From a rough screening of the isolates it appeared that a possible hot spot for insertion was present in this small region. This region must have been close to the end of the *tfd*DEF sequence that was cloned. A recombination event was not able to be produced. When a recombination event did occur, the result appeared to be a large deletion.

The mini-Mu phage was useful in the production of insertions. Limitations seem to have occurred in the homologous recombination event. The production of a homologous rcombination event was difficult to produce in this system. As a result of this, the mini-Mu phage could not be used for successful production of *lac* fusions in pJP4.

Tn*5* Transposition

The Tn*5* transposon has proven to be of great utility for the insertion mutagenesis of a variety of gram-negative bacteria (8, 60, 71, 96, 97). A variety of mini-Tn*5* derivatives have been constructed for generating gene and operon fusions (29). The mini-Tn*5* utilized in these experiments has a promotorless *trp*'-'*lac*Z fusion with its Shine-Dalgarno sequence downstream of the Tn*5* I end of the transposon to generate operon fusions. The mini-Tn*5* utilized in these experiments has a promotor-less *trp*'-'*lac*Z fusion with its Shine-Dalgarno sequence downstream of the Tn*5* I end of the transposon to generate operon fusions. The fusions begin with a '*lac*Z moeity at codon 9, which is separated from the interrupted gene by a 49-base-pair linker sequence composed of the 19-base-pair I end of Tn*5* and an additional 30 base pairs resulting from different steps in the construction (29). The creators of this system suggest that it is much simpler to use for the creation of transcriptional fusions than Tn*3*, other Tn*5* systems, and Mu phage constructs (29).

Using the mini-Tn*5* system a large number of insertions were produced. These were screened and a small portion of these were found to be in pJP4. The simplest way to determine if the TFD pathway has been interrupted is to test for growth on TFD as a sole carbon source. If the isolate is unable to grow on TFD, the pathway must have been interrupted. The isolates that were unable to grow on TFD were analyzed for the ability to conjugate the kanamycin resistance of mini-Tn*5*. If the marker can be conjugated, the insertion must have occurred on pJP4. It would be possible to produce insertions in the chromosome that would inhibit growth on TFD. Isolates that were unable to grow on TFD and kanamycin resistance could be conjugated were analyzed for β-galactosidase activity.

The β-galactosidase activity of a number of these was determined. Several different types of induction patterns resulted when expression assays were performed. It was expected that a certain constitutive level of expression would occur for each of the fusions. An uninduced level of protein product is present even when no TFD is

present. When TFD was present, it was expected that a number of the isolates would show some level of increase in β-galactosidase activity. This would mean that the inducing agent had been produced and increased activation of the pathway had occurred. Eleven isolates showed β-galactosidase activity that fit the hypothesis. The levels under noninducing conditions were similar and six of these showed increased levels of induction in the presence of TFD.

Detailed studies of Groups 1 and 2 involved determining where the point of insertion of the trans-poson had occurred. This was done by sequence analysis. In order to sequence these constructs a primer had to be developed that would allow the end of the transposon into the insertion point to be sequenced. The sequence of the gene that is responsible for resistance to kanamycin has been determined (77). A primer for sequencing was able to be created from the sequence data. The primer hybridized to the 3' end of the gene. This allowed sequencing through the end of the kanamycin gene, to the end of the transposon, through the point of insertion, and into the gene where insertion had occurred. Identifying the point of insertion was the data needed from these sequences. These sequence data were then aligned with sequence data that had been produced for the TFD pathway. The three operons of the pathway have been completely sequenced.

In order to get sequence data of the insertions it was necessary to isolate the plasmid DNA. The plasmid pJP4 is a very large plasmid and difficult to isolate. It was very difficult to isolate a significant quantity of highly purified and concentrated DNA for sequencing. As a result of this the point of insertion needed to be cloned into a high copy number vector. This allowed for a high concentration of highly purified DNA to be isolated and sequenced. By selecting for kanamycin resistance it was easy to directly select for clones that contained the mini-Tn*5* transposon and the point of insertion. A single *Bam*HI site was present at the 5' end of the transposon. This allowed virtually the entire mini-Tn*5* transposon to be cloned. The restriction pattern of pJP4 when digested with *Bam*HI was also known. From these sites, it could be

determined that the largest fragment that would be produced, if the mini-Tn5 had inserted into one of the genes of the pathway, was approximately 5 kb. The mini-Tn5 was approximately 5 kb. The largest fragment that would be cloned would be approximately 10 kb. This size fragment could be cloned into a high copy number vector such as pBS.

The plasmids of five of the isolates from Groups 1 and 2 were isolated and attempts were made to clone the desired region. Clones from these five isolates were produced and sequenced. The sequence data were analyzed and the point of insertion determined. From these data the inducing agent of the pathway was determined. Of the isolates that were sequenced, three were noninducible and two were inducible. The isolates that were inducible must have inserted into genes that are transcribed after the inducing agent is produced. The noninducible isolates must have insertions in genes that are transcribed before the production of the inducer.

If a second copy of the transposon did insert in pJP4, the analysis of the β-galactosidase assays would be different. If two insertions occurred in isolate 1, and the second insertion was in fdc, removal of the insertion could allow production of the inducing agent. The second insertion would have resulted in an inappropriate identification of the inducing agent. A Southern analysis was performed to determine how many copies of Tn5 were present in pJP4. Figure 8 shows that only one copy of Tn5 is present in pJP4. If a second copy was present, two bands would have been produced on the audioradiograph. The second insertion would cause a change in the restriction pattern, producing two bands. The single band demonstrates that only one insertion of Tn5 has occurred.

The noninducing isolates were in the *tfd*A, *tfd*B, and *tfd*C genes. The inducing agent of the pathway must be a product that is produced after the *tfd*C gene is transcribed. A lack of induction showed that the inducing agent had not been produced. The products after the *tfd*C gene in the pathway are 2,4-dichloromuconate, 2-chlorodiene lactone, and chloromaleylacetic acid. One of these products must be the

inducing agent of the pathway. The isolates that showed induction in the presence of TFD were found to be inserted in the *tfd*D and *tfd*E genes. The inducing agent was produced and the result was an increased β-galactosidase activity.

The gene product of *tfd*C produces 2,4-dichloromuconate from 3,5-dichlorocatechol. When the dichloromuconate is not produced, the pathway is not induced. This is shown by the *tfd*C insertion mutant. The pathway has been blocked before the inducing agent is produced. β-Galactosidase activity is not increased because the inducing agent was not produced. The two insertions that have occurred in the *tfd*A and *tfd*B genes have also blocked the pathway before the inducing is produced.

When an insertion has occurred in the *tfd*D gene, a new pattern of β-galactosidase activity is produced. When this mutant is grown under inducing conditions, the amount of β-galactosidase activity that is produced is more than double the β-galactosidase activity that is produced when the mutant is grown under noninducing conditions. The pathway in this mutant must be blocked after the inducing agent is produced. This would explain the increased production of β-galactosidase activity under inducing conditions. The gene product of *tfd*D converts dichloromuconate to chlorodiene lactone. The inducing agent was produced in this mutant. The last product that is produced by this mutant is chlorodiene lactone. The inducing agent must be a product in the pathway that precedes chlorodiene lactone.

From the *tfd*C mutant it was shown that the inducing agent must be a product that is produced after the dichlorocatechol. With the information from the *tfd*D mutant, that the inducing agent must be produced before the chlorodiene lactone, the inducing agent of the pathway can be determined. The product that is produced immediately after the dichlorocatechol and before the chlorodiene lactone is dichloromuconate. The dichloromuconate is not produced by the *tfd*C mutant, but is produced by the *tfd*D mutant. The dichloromuconate is the product that is produced by the *tfd*D mutant that allows induction of the pathway. The lack of production of this product in the

*tfd*C mutant prevents the induction of the pathway. The chloromuconate is most likely the inducing agent of the pathway.

Isolates in Groups 3 and 4 had β-galactosidase activities that were different than Groups 1 and 2. A possible reason could be that an insertion in a regulatory gene could have occurred. The expression levels of the regulatory genes may be different than the structural genes. This could explain the expression levels characteristic of Group 3. An insertion in the carboxy-terminal region of a regulatory gene could explain the expression patterns in Group 4. An insertion could have occurred that allowed binding of the regulator, such as TfdR, to the promoter region, but would not allow transcriptional activation of the operon. This would result in less expression when TFD is present. In the presence of TFD down regulation of the regulator would occur. Group 4 shows less expression in the presence of TFD.

The fact that the chloromuconate is the inducing agent of the pathway is not a complete surprise. The inducing agent for the degradation of benzoate was found to be muconate. Benzoate is converted to β-ketoadipate with catechol being an intermediate. Catechol is converted to muconate and then to muconolactone. The conversion occurs by the *cat*B and *cat*C gene products. The protein CatR is an activator of the catechol degradation genes *cat*BC. This pathway is similar to the TFD. The products of pathway are identical except for the fact that the products are chlorinated in the TFD pathway. The regulatory proteins CatR and TfdR are very similar (70). These are similar pathways and the inducing agent of the *cat*BC operon has been shown to be muconate.

The fact that a muconate derivative is the inducing agent for the TFD and CAT pathways suggests that the TFD pathway may have evolved from the CAT pathway. The TFD pathway may have evolved from the catechol pathway after the chlorinated compound entered the environment. When TFD was introduced into the environment, random mutation of genes would result in genes that would encode for the metabolism of a chlorinated product. If muconate had evolved as a regulatory mechanism, it

would be likely that it would continue to be utilized in the same manner. A new inducing agent would not be selected, but the mutation of an inducing agent that existed. This would result in similar regulatory mechanisms. Benzenoid residues such as those found in lignin are among the most common units encountered in nature (36). The conversion of benzoate to β-ketocadipate is widely distributed and may have served as a starting point for the evolution of chlorinated aromatic pathways.

The chloromuconate of other pathways similar to the TFD pathway has also been suggested to be the inducing agent. In these pathways the inducing agent has been speculated to be a chloromuconate intermediate in the pathway. With all these pathways being very similar, the inducing agent in each of these pathways may also be very similar. The work produced in these studies does suggest that the inducing agent of each pathway may be the chloromuconate intermediate of the pathway.

The nature of the specific activation mechanism is not understood. Understanding the nature of inducer-activator interactions, and the role they play in transcriptional activation, will help in the engineering of biodegradative pathways for compounds that currently cannot be degraded by bacteria.

The mini-Tn*5* transposon was a very useful tool for genetic studies using *A. eutrophus*. With this system *lac*Z gene fusions were produced in genes of the TFD pathway. With the other systems used in these studies no usable gene fusions were obtained. The mini-Tn*5* transposon system was the only system that was able to be used successfully. With this system the inducing agent of the pathway was determined.

Sequence analysis has shown that several different pathways involved in the metabolism of chlorinated aromatics are very similar. The structural and regulatory genes have shown sequence similarity and the regulatory patterns of the pathways appear to be similar (26, 70, 107). These pathways include TFD, CAT, TCB, and CLC.

The data presented here have shown that the most likely inducing agent of the TFD pathway is the chlorinated muconate intermediate of the pathway. Since this

pathway is similar to other chlorinated aromatic degrading pathways discussed above, it is also possible that the chlorinated muconate intermediate found in these pathways are the inducing agents. If the inducing agents for all of these pathways are found to be chlorinated muconates, the importance of these compounds in the regulatory scheme of these pathways will be shown.

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