SITE DIRECTED MUTAGENESIS OF DIENELACTONE HYDROLASE

THESIS

Presented to the Graduate Council of the University of North Texas in Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Haifa Yousef Al-Khatib, B.Sc., M.Sc.

Denton, Texas

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The *clcD* gene encoding dienelactone hydrolase (DLH) is part of the *clc* gene cluster for the utilization of the ß-ketoadipate pathway intermediate chlorocatechol. The roles that individual amino acids residues play in catalysis and binding of the enzyme were investigated. Using PCR a 1.9 kbp *clcD* fragment was amplified and subcloned yielding a 821 bp *Bam*HI to *Eco*RI subclone in the plasmid pUC19. Specifically altered mutants of DLH (Arg81 to Alanine; pUNTR81A, and Glu36 to Glutamine; pUNTE36Q), were created using primer extension from modified mutagenic oligonucleotides. Enzyme assays of the modified proteins showed that activity of the DLHE36Q was totally abolished; while the k_M value of the mutant protein expressed from pUNTR81A was two fold higher (40 µmol) compared to native DLH (22.22 µmol).

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INTRODUCTION

Man-made chemicals used as refrigerants, fire retardants, paints, solvents, herbicides, and pesticides cause considerable environmental pollution and human health problems as a result of their persistence, toxicity, and transformation into hazardous metabolites. Many environmentally important xenobiotics introduced for industrial use are halogenated, and halogenation is often implicated as a reason for persistence. Many of these man made compounds are carcinogenic, teratogenic or mutagenic. Halogenated organics find use in modern society as herbicides, plastics, solvents, and degreasers. Many biogenic compounds are naturally occurring and have existed for millions of years, which has allowed microorganisms capable of their degradation and assimilation to evolve and adapt to the use of these compounds as growth substrates in their environment. However, even though many of the synthetic man-made halogenated aromatic compounds have only been around for a few decades, microbes capable of their degradation have already been identified, and one can isolate pure cultures capable of utilizing these compounds as sole sources of carbon and energy. Evidently the compounds have natural counterparts with analogous structures so that the bacteria can metabolize them. It is also possible that the bacterial enzyme systems have sufficiently broad specificity of binding and catalysis

so as to handle a wide range of compounds. In addition to degradation, several biotransformation reactions, such as methylation and polymerization, may take place and produce more toxic or recalcitrant metabolites.

The degree of degradation varies for compound to compound. Some are apparently resistant to microbial attack, while others may be partially broken down to non-degradable intermediates, or even transformed to possibly more toxic products. Several excellent reviews have been published regarding this topic (Chakrabarty, 1982; Reineke and Knackmuss, 1988; Schlömann *et al.*, 1990a; Chaudhry and Chapalmadugu, 1991; Engesser and Fischer, 1991; Häggblom, 1990 and 1992; Golovela *et al.*, 1992). Complete biodegradation ultimately results in the mineralization of the compound to carbon dioxide or methane, and in the case of haloaromatics, with release of the halogen atom(s). Several halogenated aromatic pesticides and related compounds have been shown to be biodegradable, under either aerobic or anaerobic conditions. In some cases the compounds are also naturally labile so that persistence in the environment is not a problem given appropriate conditions of moisture, temperature and sunlight to speed their spontaneous breakdown. Degradation of toxic xenobiotic compounds from a practical standpoint by bioremediation may ultimately involve both physical processes and microbial degradation.

The ß-ketoadipate pathway is a metabolic pathway found in bacteria and fungi (Ornston and Yeh, 1982). This pathway comprises enzymes responsible for catalyzing the degradation of toxic aromatic compounds found in the environment, both as natural byproducts and as industrial effluent, into the innocuous citric acid cycle intermediates, acetyl CoA and succinyl CoA. Different branches of the ß-ketoadipate pathway contain related enzymes that process structurally similar substrates.

The degradative pathways of aromatics and haloaromatics converge at a few common intermediates, such as catechol or derivatives of catechol, which are the substrates for the cleavage of the aromatic ring. They are further catabolized through the ß-ketoadipate pathway to yield the tricarboxylic acid cycle metabolites acetyl CoA and succinyl CoA (Ornston, 1966). There are three predominant branches of the ß-ketoadipate pathway; the catechol branch, the protocatechuate branch and the chlorocatechol branch. The catechol branch is used by the bacteria for the degradation of catechol and compounds giving rise to catechol, such as benzoate. The protocatechuate branch is used for the catabolism of protocatechuate and compounds giving rise to protocatechuate such as *para*-hydroxybenzoate. The chlorocatechol branch is used for the assimilation and degradation of chlorinated aromatics, such as the breakdown products of the herbicide 2,4 dichlorophenoxyacetic acid. In addition, aromatic compounds are degraded in bacteria through one of two different general schemes. All of the branches of the ß-

ketoadipate pathway catalyze the *ortho* cleavage of the aromatic ring. That is to say, the ring cleavage is performed by oxygenases between adjacent diols in the basic catechol ring nucleus. A distinct pathway has evolved to degrade catechol *via* a *meta* cleavage where the ring scission occurs adjacent to the ring diols (hence the *meta* designation). In the catechol branch and the analogous protocatechuate branch catechol and protocatechuate, derived from aromatic acids such as benzoate and *para*-hydroxybenzoate respectively, are degraded via *ortho* cleavages. Methyl catechols derived from xylenes and toluene are typically degraded *via* the *meta* pathway. When aromatic compounds are degraded through the *ortho* pathway they are converted to the common intermediate ß-ketoadipate enol lactone. This compound is hydrolyzed by the enzyme enol lactone hydrolase to ß-ketoadipate, whence the pathway is named. On the other hand chlorocatechols are usually degraded by a modified *ortho* pathway. Degradation of chlorocatechols by this pathway results in the formation of the dienelactone which is catabolized to maleylacetate by dienelactone hydrolase (DLH). Maleylacetate is further converted to ß-ketoadipate by a reductase. The ß-ketoadipate from both branches is raised to the level CoA by a CoA-transferase using succinyl-CoA as the CoA donor, and the ß-ketoadipyl-CoA is subsequently cleaved thiolytically to acetyl CoA and succinyl CoA. These compounds then enter intermediary metabolism to provide carbon and energy to the cell. Ring cleavage by the *ortho* pathway was found to be a critical

step in the metabolism of halogenated catechols. In general, *meta*-cleavage of halocatechols by 2,3-dioxygenases produced dead end or suicide metabolites (Bartels *et al.*, 1984). However, an *Achromobacter* sp. was shown to cleave 4-chlorocatechol and 3,5-dichlorocatechol by *meta*-cleavage enzymes (Hovarth, 1970).

Two main schemes can be differentiated in the cleavage of the halogen carbon bond of halogenated aromatic compounds: (i) the halogen substituent is removed as an initial step in degradation, *via* reductive, hydrolytic or oxygenolytic mechanisms, or (ii) chlorocatechols are formed by the action of a dioxygenase with dehalogenation occurring after ring cleavage.

A search of the current literature on degradation of xenobiotics indicates that the major degraders of persistent pollutants are bacteria. Among bacteria, those most frequently mentioned as effective degraders are members of the genus *Pseudomonas*. The great potential of the pseudomonads to degrade xenobiotic compounds lies in their vast range of catabolic pathways and of their adaptive changes, which is promoted by their capacity of regulation of these pathways (Houghton and Shanley, 1994). The ß-ketoadipate pathway is a metabolic mechanism for assimilation of aromatic and hydroaromatic growth substrates that is universally shared by fluorescent *Pseudomonas* species (Stanier *et al.*, 1966).

One of the most metabolically versatile strains with good ability to degrade halogenated aromatics is the bacterium *Pseudomonas* sp. B13 and its derivatives that has been studied by

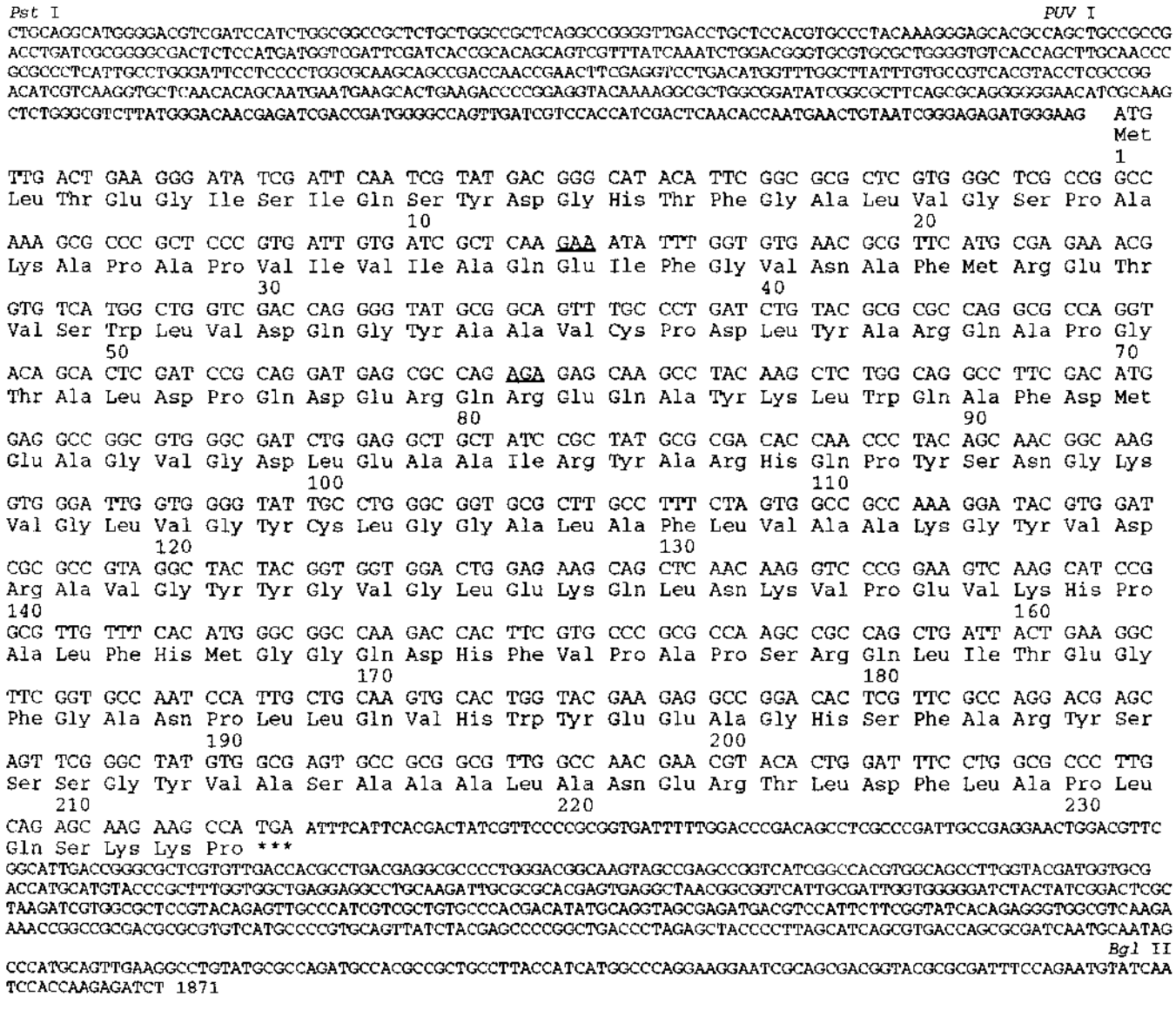
Knackmuss and co-workers. This strain was initially isolated for its ability to degrade 3-chlorobenzoate (Dorn *et al.*, 1974), but was later shown to be able to utilize 4-chlorophenol as a sole carbon and energy source (Knackmuss and Hellwig, 1978). The strain also cooxidized 2- and 3-chlorophenol and also some dichlorophenol. The modified *ortho*-cleavage pathway of chlorinated catechols first described for *Pseudomonas* sp. strain B13 (Dorn and Knackmuss, 1978a; Weisshaar *et al.*, 1987), has also been found in the bacteria *P. putida* carrying the plasmid pAC27 (Chatterjee *et al.*, 1981), and *Alcaligenes eutrophus* JMP134 that carries the plasmid pJP4 (Don *et al.*, 1985).

Complete degradation of the compound 3-chlorobenzoate by the soil bacteria *P. putida* and *Pseudomonas* sp. B13 is specified by enzymes encoded by genes on the plasmids pAC27 (a deletion derivative of pAC25) (Chatterjee *et al.*, 1981; Chatterjee and Chakrabarty, 1982 and 1984) and pWR1 (Weisshaar *et al.*, 1987) respectively. Plasmids pAC27 and pWR1 have extensive homology (Chatterjee and Chakrabarty, 1983). The plasmid pAC25 has been characterized (Chatterjee *et al.*, 1981) and a circular map has been established (Chatterjee and Chakrabarty, 1984). Genes for chlorocatechol metabolism present on plasmid pJP4 in *Alcaligenes eutrophus* show sequence homology with a 4.2 kbp region of pAC27 (Ghosal *et al.*, 1985). While the plasmid pJP4 encodes the complete 2,4-dichlorophenoxyacetate degradative pathway, the plasmid pAC27 encodes only a chlorocatechol degradative pathway. The

organization and complete nucleotide sequence of the three genes involved in the degradation of chlorocatechols namely catechol oxygenase II (chlorocatechol dioxygenase), muconate cycloisomerase II and dienelactone hydrolase, are found in a 4.2 kbp *Bgl*II fragment cloned from the plasmid pAC27 (Frantz and Chakrabarty, 1987; Frantz *et al.*, 1987). The 4.2 kbp fragment contains the three genes and ribosome binding sites for those genes but no transcriptional promoter for expression of the genes. However, the genes on the 4.2 kbp fragment are organized into and operon and believed to be expressed coordinately under the control of a single promoter. A 1.9 kbp *Pst*I-*Eco*RI segment subcloned from the *Bgl*II fragment was shown to carry the *clcD* gene which encodes a dienelactone hydrolase that resembles the dienelactone hydrolase from *Pseudomonas* sp. B13 (Frantz *et al.*, 1987). The complete nucleotide sequence of the 1.9 kbp *Pst*I-*Eco*RI fragment is shown in Figure 1. The *clcR* regulatory gene, which activates the *clcABD* operon, was cloned recently from the region immediately upstream of the operon. The nucleotide sequence analysis predicted a polypeptide of 32.5 kDa. ClcR protein activated the *clcA* promoter, which controls the expression of the *clcABD* operon, and it shares a high sequence identity with the LysR family of bacterial regulatory proteins (Coco *et al.*,1993).

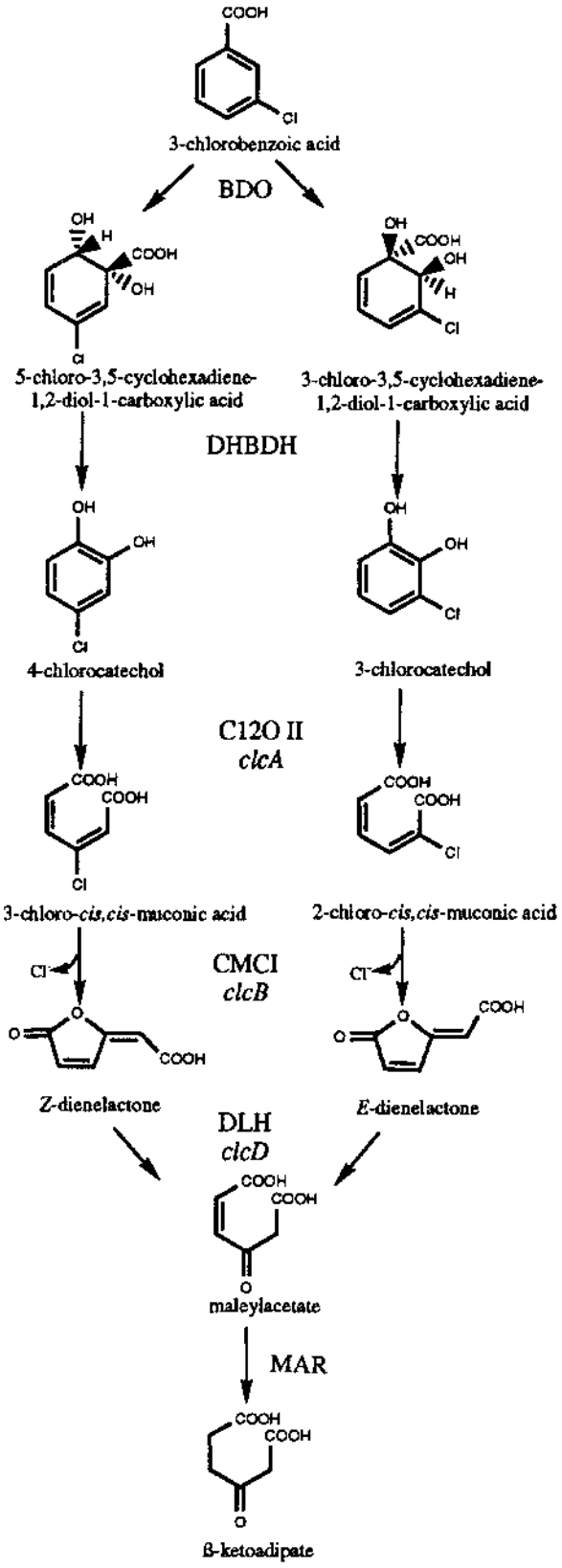
Intense investigations of the biochemistry of 3- and 4-chlorobenzoate degradation by the 3-chlorobenzoate-degrading organism *Pseudomonas* sp. strain B13 (Dorn and Knackmuss, 1978a,

Figure 1. Nucleotide sequence of the *clcD* gene. The *clcD* gene encodes the enzyme dienelactone hydrolase. The adjacent 5' and 3' regions are shown. The first amino acid residue is designated number 1.


Pst I
PUV I
ATG
Met
1
Bgl II

1978b; Schmidt and Knackmuss, 1980; Schmidt *et al.*, 1980; Reineke, 1984) have indicated that the catabolic sequence is as depicted in Figure 2. *Pseudomonas* sp. B13 converted 3-chlorobenzoate by a nonselective dioxygenation to a mixture of 3- and 5-chloro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acids, which were dehydrogenated to 3- and 4-chlorocatechol, respectively (Reineke and Knackmuss,1978a and b). Catechol 1,2,-dioxygenase type II (EC 1.13.11.1) and chloromuconate cycloisomerase (EC 5.5.1.7) catalyze reactions analogous to those of the ß-ketoadipate pathway. Chloro-substituted muconolactones are formed after cycloisomerization. Dehalogenation is postulated to be a spontaneous secondary reaction yielding *E*-dienelactone (from 2-chloro-*cis, cis*-muconate) or *Z*-dienelactone (from 3-chloro-*cis, cis*-muconate) by an a,ß elimination of HCl (Schmidt and Knackmuss, 1980). These dienelactones are directly hydrolyzed by dienelactone hydrolase (DLH, EC 3.1.1.45), whereas the more stable muconolactone in the 3-oxoadipate pathway first must be isomerized to 3-oxoadipate by muconolactone isomerase (MI, EC 5.3.3.4) before it is subject to hydrolysis by enol-lactone hydrolase (ELH, EC 3.1.1.24) (Ornston and Stanier, 1966). However because of predominance of 1,2-dioxygenation over 1,6-dioxygenation of 3-chlorobenzoate (Reineke and Knackmuss, 1978a), *Pseudomonas* sp. strain B13 degrades most of its growth substrate *via* 3-chlorocatechol, 2-chloro-*cis, cis*-muconate, and *E*-4-carboxymethylenebut-2-en-4-olide. Only minor amounts of 3-chlorobenzoate are catabolized through the parallel route. This

Figure 2. Pathway for the microbial degradation of 3-chlorobenzoate. Specific designations for the *clc* genes and the enzymes they encode are shown with the corresponding metabolic steps. The *clcA* gene codes for catechol 1,2-dioxygenase type II (EC 1. 13. 11. 1); the *clcB* gene encodes muconate cycloisomerase II (EC 5. 5. 1. 7); the *clcD* gene encodes dienelactone hydrolase (EC 3. 1. 1. 45). Abbreviations not given in the text are: BDO, benzoate 1,2-dioxygenase; DHBDH, 3,5-cyclohexadiene-1,2-diol-1-carboxylate dehydrogenase; C12O II, catechol 1,2-dioxygenase type II; CMCI, chloromuconate cycloisomerse; DLH, dienelactone hydrolase; MAR, maleylacetate reductase.


3-chlorobenzoic acid
BDO
5-chloro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid
3-chloro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid
DHBDH
4-chlorocatechol
3-chlorocatechol
C12O II
clcA
3-chloro-cis,cis-muconic acid
2-chloro-cis,cis-muconic acid
CMCI
clcB
Z-dienelactone
E-dienelactone
DLH
clcD
maleylacetate
MAR
ß-ketoadipate

corresponds to the substrate specificity of the DLH from strain B13, which has considerably higher affinity for the *E* dienelactone (k_M =15 µM) than for the *Z* isomer (k_M = 400 µM). The 4-carboxymethylenebut-2-en-4-olides are converted to maleylacetate and degraded further *via* ß-ketoadipate and thence to acetyl-CoA and succinyl-CoA (Schmidt and Knackmuss, 1980).

Pseudomonas sp. B13 failed to utilize 4-chlorobenzoate or 3,5-dichlorobenzoate because of the narrow substrate specificity of the initial enzyme, benzoate 1,2-dioxygenase (Reineke and Knackmuss, 1978a). Conjugants obtained by recruitment of a non-specific dioxygenase from *P. putida* mt-2 degraded 4-chlorobenzoate and 3,5-dichlorobenzoate (Reineke and Knackmuss, 1979). However *Pseudomonas* sp. B13 had the pathway for the degradation of 4-chlorocatechol and 3,5-dichlorocatechol, the expected intermediates of 4-chlorobenzoate and 3,5-dichlorobenzoate respectively. The compound 3,5-dichlorocatechol was first cleaved by a type II catechol 1,2-dioxygenase. The tentative ring cleavage product 2,4-dichloro-*cis, cis*-muconate was degraded by lactonization and concomitant dechlorination to 2-chloro-4-carboxymethylenebut-2-en-4-olide which was decarboxylated to 2-chloromaleylacetate and further converted to maleylacetate (Schwien *et al.*, 1988). Kaschabek and Reineke (1992) purified maleylacetate reductase from 3-chlorobenzoate grown *Pseudomonas* sp. strain B13 that converted 2-chloromaleylacetate, the metabolite from the degradation of 3,5- and

3,6-dichlorocatechol, into 3-oxoadipate with maleylacetate as an intermediate after chloride elimination.

Degradation of chlorobenzoates with dechlorination occurring as the initial step by hydroxylation was reported by Johnston *et al.* (1972). A *Pseudomonas* sp. degraded 3-chlorobenzoate by dehalogenation and hydroxylation to 3-hydroxybenzoate, followed by hydroxylation *para* to the newly introduced hydroxyl group, producing 2,5-dihydroxybenzoate.

In some bacteria the pathway of chlorobenzoate degradation is similar to that of chlorophenol degradation, proceeding through chlorocatechols, and some strains are able to degrade both compounds. Dehalogenation of chlorocatechols takes place only after ring cleavage. The mechanisms whereby chlorocatechols are formed are, however, different. A dioxygenase system converts chlorobenzoates to *cis*-1,2-dihydrodiols which are subsequently dehydrogenated, whereas a hydroxylase system generally converts chlorophenols to chlorocatechols. However this is not exclusive, since degradation of chlorophenols via chlorocatechols through the action of a dioxygenase has also been reported (Spain and Gibson, 1988). *Alcaligenes eutrophus* JMP134 which harbors plasmid pJP4 has three different phenol hydroxylases that differ in their substrate specificity (Pieper *et al.*, 1988). Chlorophenols are metabolized by *Pseudomonas* sp. B13 to 3- and 4-chlorocatechols, which are cleaved at the *ortho* position to form chloromuconic acid. The chlorine substituent is removed spontaneously during cycloisomerization into

4-carboxymethylenebut-2-en-4-olide (Dorn *et al.*, 1974; Schmidt and Knackmuss, 1980). The *A. eutrophus* strain JMP134, resembles *Pseudomonas* sp. strain B13 in synthesizing two separate sets of enzymes for the degradation of catechol and halocatechols via *ortho* cleavage.

The herbicide 2,4-dichlorophenoxy acetic acid (2,4-D) is readily degraded in soil. Evidence of the degradation pathway of 2,4-D was obtained with an *Arthrobacter* sp. isolated from soil, which utilized 2,4-D as a growth substrate (Loos, *et al.*, 1967a). Degradation of 2,4-D was demonstrated to be initiated by cleavage of the ether linkage to produce 2,4-dichlorophenol (Loos *et al.*, 1967b; Tiedje and Alexander, 1969) which was then hydroxylated to 3,5-dichlorocatechol by the *Arthrobacter* sp. (Bollag *et al.*, 1968). 2,4-dichlorophenol was also converted to 4-chlorocatechol. The compound 3,5-dichlorocatechol was proposed to be degraded by *ortho* ring-cleavage to yield *cis, cis*-2,4-dichloromuconic acid, which then underwent lactonization to 2-chloro-4-carboxymethylenebut-2-en-4-olide with release of chloride, and further conversion to chloromaleylacetate (Bollag, *et al.*, 1968; Tiedje *et al.*, 1969). Similarly, 4-chlorocatechol was degraded through *cis,cis*-3-chloromuconic acid, 4-carboxymethylene-but-2-en-4-olide and maleylacetate (Tiedje *et al.*, 1969).

The degradation pathway of 2,4-D described above, by initial oxidative cleavage of the ether bond to produce the corresponding phenol and subsequent hydroxylation to a catechol followed by *ortho*

ring-cleavage, has been demonstrated or proposed for isolates belonging to such genera as *Alcaligenes*, *Azotobacter*, *Pseudomonas*, *Acinetobacter*, *Xanthobacter*, and *Flavobacterium* (Pemberton and Fisher, 1977; Beadle and Smith, 1982; Greer *et al.*, 1990).

Catechol is degraded in many bacteria along the well established *ortho* or 3-oxoadipate (ß-ketoadipate) pathway (Ornston and Stanier 1966; Ornston 1966). Amongst all halocatechols this mechanism was found to be effective only in the catabolism of fluorocatechol (Clarke *et al.*, 1975; Engesser *et al.*, 1980; Engesser *et al.*, 1990; Schreiber *et al.*, 1980) which is cleaved, in contrast to other halocatechols, by regular catechol 1,2-dioxygenases at sufficiently high rates to allow growth on 4-fluorobenzoate (Dorn and Knackmuss, 1978a and b; Schreiber *et al.*, 1980).

Ortho cleavage of 4-fluorocatechol yields 3-fluoromuconate which can be cycloisomerized 4-fluoromuconolactone (Harper and Blakley, 1971). This lactone is metabolized to maleylacetate by dienelactone hydrolases and/or 3-oxoadipate (ß-ketoadipate) enol lactone with concomitant formation of fluoride (Schlömann *et al.*, 1990b and c).

Dienelactone hydrolase from the chlorobenzoate arm of the ß-ketoadipate pathway has been isolated in purified form from the strain *Pseudomonas* sp. B13 that degrades 3-chlorobenzoate (Dorn *et al.*, 1974; Dorn and Knackmuss, 1978; Schmidt *et al.*, 1980; Schmidt and Knackmuss, 1980). Purified protein was crystallized in a form suitable for high resolution X-ray diffraction study (Ollis and Ngai,

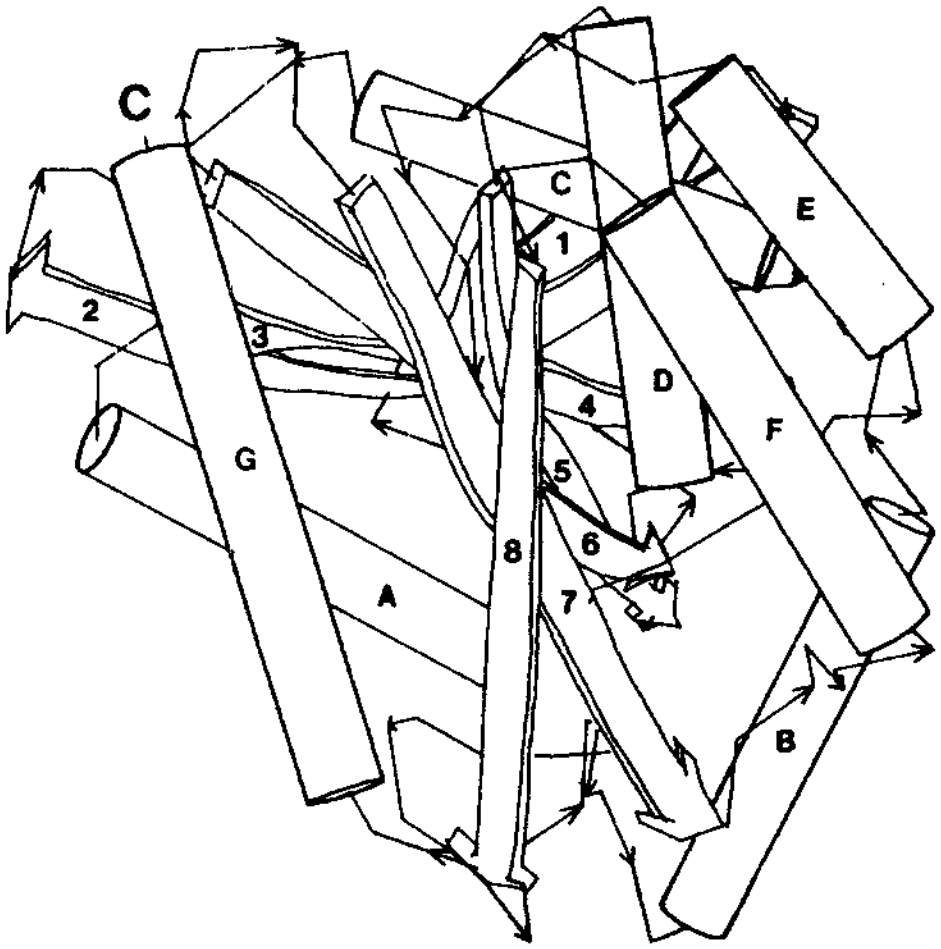
1985). The crystals are orthorhombic, the space grouping being $P2_12_12_1$, with unit cell dimensions a=48.9 Å, b=71.2 Å, and c=77.5 Å.

DLH from *Pseudomonas* sp. B13 is a monomeric protein containing 236 amino acid residues and having a molecular weight 25,500 kDa. as determined from the predicted amino acid sequence (Pathak *et al.*, 1988). An X-ray crystallographic study of DLH has recently resulted in the determination of the three dimensional structure at a nominal resolution of 2.8 Å (Pathak *et al.*, 1988). DLH is an α/ß protein composed of eight strands of ß-pleated sheet and seven helical segments (Fig. 3). Strand 2 of ß-pleated sheet occupies the central core of the enzyme which is surrounded by seven alpha helices. Approximately one third of DLH is made up of ß-sheet while another third is helical (Pathak *et al.*, 1988).

Prior to its structure determination, little was known about the catalytic mechanism of DLH. Titration with *para*-chlorobenzoate had implicated one of the two cysteine residues (Cys60 and Cys123) in catalysis and it was concluded from limited sequence similarity with enol lactone hydrolase that Cys60 was the essential cysteine (Ngai *et al.*, 1987).

In a more recent study a stereochemically restrained least refinement at 1.8 Å resolution, is described. An examination of the structure of DLH showed that Cys123 resides in a cleft in the molecule, nestled against the N-terminal end of an α-helix that is peculiar in its consisting entirely of hydrophobic residues (except for a C-terminal lysine). The ß-sheet is composed of parallel strands

Figure 3. The structure of dienelactone hydrolase. This schematic view of DLH shows the splayed strands of ß-sheet occupying the center of protein. Strand 2 is the single anti-parallel strand.


C
C
1
2
3
4
5
6
7
8
A
B
D
E
F
G

except for strand 2, which gives rise to a short antiparallel region at the N-terminal end of the central ß-sheet. In close proximity to this cysteine residue were found His202 and Asp171, in an arrangement reminiscent of the catalytic triad of the serine and cysteine proteases The positions of 279 water molecules are included in the final model (Pathak and Ollis, 1990). It should be pointed out that the catalytic triad seen in the viral cysteine proteases is identical in its composition to the active site triad of DLH (Bazan and Fletterick, 1988). The catalytic triad present in DLH and the viral proteases is a hybrid of the triads seen in the papain class of thiol proteases and the trypsin and subtilisin classes of serine proteases. Papain contains cysteine, histidine, and asparagine residues, while both of serine protease families contain serine, histidine, and aspartic acid at their active sites.

In a recent study a model is proposed to explain why this triad is active in DLH but not in the proteases (Cheah *et al.*, 1993a). The mechanism of binding the substrate and hydrolysis of DLH was investigated using a mutant protein DLHC123S and dienelactam which was found to be a competitive inhibitor (Cheah *et al.*, 1993b).. In the mutant protein DLHC123S the catalytic cysteine was replaced by serine as described by Pathak *et al.*, (1991) to give an enzyme that has only 10-15% of the wild type activity.

The α/ß hydrolase fold was found to be common to several hydrolytic enzymes of widely differing phylogenetic origin and catalytic function. The core of each of these enzymes is similar and it

consists of α/ß sheet,(and not a barrel) of eight ß sheets connected by α-helices. These enzymes have diverged from a common ancestor so as to preserve the catalytic residues, not the binding sites. The α/ß hydrolases, the cysteine proteases, subtilisins and serine proteases contain catalytic triads and are related by convergent evolution (Ollis *et al.*, 1992). The dienelactone hydrolase acts on both *E* and *Z* isomers of dienelactone but exhibits no activity when incubated with the substrate analogs muconolactone or ß-ketoadipate enol lactone (Ngai et al., 1987). The dienelactone hydrolases and the 3-oxoadipate enol lactone hydrolases (ß-ketoadipate enol lactone hydrolases, ELH) seem to share an α/ß hydrolase fold, but the sequences comprising the fold are quite dissimilar. In a recent review the evolutionary relationship between 3-oxoadipate enol lactone and dienelactone hydrolases was addressed (Schlömann, 1994, personal communication and in press).

Dienelactone hydrolases were originally differentiated based on their substrate specificities (Schlömann *et al.*, 1990b). Those detected, for example, in *A. eutrophus* 335, *A. eutrophus* H16, and *A eutrophus* JMP222 (a cured derivative of JMP134) convert *E*- but not *Z*- dienelactone and were designated as type I The dienelactone hydrolase of *P. cepacia* has the opposite preference, hydrolyzing only the *Z*-isomer, and was termed type II. The plasmid-encoded (pAC27 and pJP4) dienelactone hydrolases for chlorocatechol catabolism showed turnover of both isomers, although with some variation in the kinetic parameters (see above), these were designated type III.

Type II dienelactone from *P. cepacia* was purified to apparent homogeneity and was found to differ from dienelactone hydrolase (type III) in certain properties, such as pH optimum of activity, inhibition by *p*-chloromercuribenzoate, and amino acid composition (Schlömann *et al.*, 1993). A recently described dienelactone hydrolase of chlorophenol degrading *Rhodococcus erythropolis* 1CP resembled the *P. cepacia* hydrolase with respect to substrate specificity (Maltseva *et al.*, personal communication). Other properties, however, suggested that it might be more closely related to the plasmid encoded type III dienelactone hydrolases, implying that substrate specificity is not necessarily a valid property for differentiation of the dienelactone hydrolases.

The alteration of genes and the proteins they encode through the substitution of specific nucleotide within a gene sequence by the technique of site-directed mutagenesis represents a fundamental tool of modern recombinant DNA technology (Rossi and Zoller, 1987). It not only allows for the analysis of the structural basis of gene and protein function, but also facilitates the generation of novel gene products. Mechanistic studies of enzyme function are now frequently supplemented with structural evidence from X-ray diffraction studies so that specific hypotheses about the contribution of individual amino acid side chains to substrate binding or catalysis can be made. In some cases, such as for the lactone hydrolases, these studies have been strengthened by cooperative studies pointing out residues that have been conserved as protein structures evolved

(Ornston and Yeh, 1982; Yeh *et al.*, 1978; Yeh and Ornston, 1980). These hypotheses can be tested by site directed mutagenesis. Substitution of an amino acid residue in a protein can contribute to an understanding of the relationship between protein structure and enzyme function.

The objective of this work is to determine the functions of amino acid sequences in the lactone hydrolases of the ß-ketoadipate pathway. Dienelactone hydrolase from *Pseudomonas* sp B13 is a particularly appropriate candidate for this type of study. The enzyme has been extensively studied and characterized. Dienelactone hydrolase has been purified and its physical properties reported (Ngai *et al.*, 1987). The X-ray crystallographic structure has been reported. The enzyme has been shown to have a reactive cysteine at the active site. The gene for the enzyme has been cloned and expressed in *E. coli*. The protein has been purified from recombinant *E. coli* strains. Comparative studies are possible since other dienelactone hydrolases from other species, and the related enol lactone hydrolases have been characterized (purified, cloned, sequenced, etc.). The DNA sequence of *clcD* encoding dienelactone is known, also the crystal structure has been refined to the limits of the technique. The physical and chemical properties of the protein did not appear to be altered when the gene was expressed in *E .coli* . Because the protein can be readily purified from *E .coli* it is a promising subject for analysis of the structural basis of its substrate specificity by site-directed mutagenesis (Frantz *et al.*, 1987).

As detailed in the Methods and in the Results, I sought to specifically alter selected amino acid residues in the enzyme by site-directed mutagenesis in order to identify those residues involved in binding and catalysis (Bostein and Shortle, 1985). Based on suggestions from the crystal structure two amino acid residues were chosen for modification (Caruthers, 1987). The resulting amino acid substitutions that were made in the course of this investigation and the hypothesized effects are as follows:

Conversion of the residue Arg81 to Alanine should reduce the affinity of the enzyme for the substrate.

Changing the residue Glut36 to Glutamine could prove interesting since glutamate 36 is involved in activating the nucleophile.

METHODS

Media and chemicals. Antibiotics (ampicillin, tetracycline, and kanamycin), ethidium bromide, Tris base, urea, isopropylthio-ß-D-galactopyranoside (IPTG) were all purchased from Sigma Chemical Company. Agarose was purchased from GIBCO BRL. Phenol, chloroform, and inorganic salts were purchased from Fisher Scientific. Restriction enzymes, and sequencing kits were purchased from United States Biochemical. Polyacrylamide, *bis*-acrylamide, and X-ray film were obtained from Kodak. All solutions, buffers, and media were formulated with double distilled deionized water of the highest purity available.

The proprietary Altered Sites *in vitro* Mutagenesis System was purchased from Promega. The system included the plasmid pAlter-1 DNA, pAlter-control DNA, ampicillin repair oligonucleotide, control *lacZ* repair oligonucleotide, annealing buffer, synthesis buffer, T4 DNA polymerase, T4 DNA ligase, bacterial strains BMH 71-18 *mutS*, and JM109, and helper phage R408 and M13KO7.

The substrate for the enzyme reactions *E*-4-carboxymethylenebut-2-en-4-olide (dienelactone) was synthesized enzymatically (Reineke and Knackmuss, 1984), and was a gift from David Ollis (Australian National University).

Plasmids. As mentioned in the Introduction, a 1.9 kbp *Pst*I-*Eco*R I fragment containing the *clcD* gene was subcloned in pKK223-3 (Frantz *et al.*,1987) The 1.9 kbp *Pst*I-*Eco*RI fragment containing the *clcD* gene was carried on this plasmid which was obtained from David Ollis. The plasmid was digested and the fragment was purified and subcloned into pUC19 generating the plasmid pDO. Using PCR new restriction sites (*Bam*HI-*Eco*RI) were introduced into the 1.9 kbp resulting in a sized down version of the fragment, carrying the *clcD* gene. The construction of the plasmid is shown in Figure 4. The sized-down *Bam*HI-*Eco*RI fragment carrying the cloned *clcD* gene encoding DLH, was 821 bp. This new plasmid was termed pNDO. The entire DNA sequence of the subcloned fragment was determined to ensure the identity of the fragment, as well as to confirm its orientation. The single clone selected for all subsequent experiments was confirmed by DNA sequence analysis to have no changes through the length of the DNA sequence of the *clcD* gene

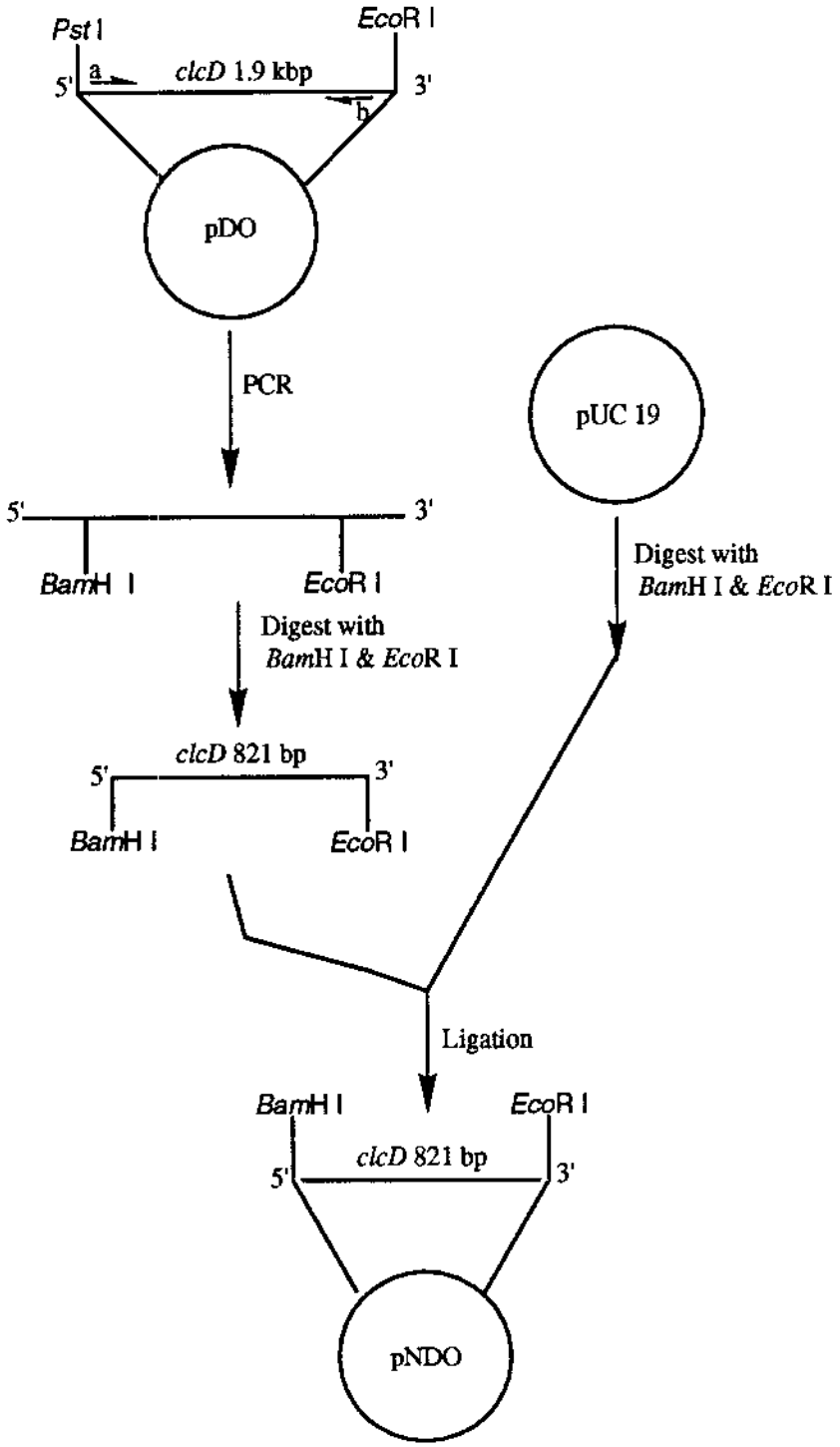
Though PCR was used to size down the fragment of DNA carrying the *clcD* gene, the fidelity of the thermostable polymerases used for PCR amplification of DNA was deemed insufficient to effectively allow for PCR techniques to be used to introduce of specific mutations into the *clcD* gene sequence. The plasmid pAlter was used for mutagenesis reactions. This plasmid is 5.68 kbp in length and contains the origin of a single stranded DNA

Figure 4. Schematic diagram of the construction of the recombinant plasmid pNDO. The oligonucleotides used as primers for the PCR reactions are shown as 'a' and 'b' in the figure. The sequences of these oligos are as follows:

'a', 5' AAAAAAAGGATCCTCCACCATCGACTCAAC 3';

'b', 5' AAAAAAGAATTCGGGCGAGGCTGTCGGGTCC 3'.

The primer 'a' anneals to residues 476-497 in the sequence of the 1.9 kbp *Pst*I-*Eco*RI fragment. Primer 'b' anneals to residues 1277-1298 of the sequence of the 1.9 kbp *Pst*I-*Eco*RI fragment. The PCR product is a *Bam*HI-*Eco*RI fragment with a length of 821 bp. This fragment was digested with *Bam*HI and *Eco*RI and ligated into plasmid pUC19.


Pst I
EcoR I
a
clcD 1.9 kbp
5'
3'
b
pDO
PCR
pUC 19
5'
3'
BamH I
EcoR I
Digest with
BamH I & EcoR I
Digest with
BamH I & EcoR I
clcD 821 bp
5'
3'
BamH I
EcoR I
Ligation
BamH I
EcoR I
clcD 821 bp
5'
3'
pNDO

bacteriophage. It produces ssDNA upon infection of the cells with the helper phage R408, or M13KO7. The vector contained a multiple cloning site flanked by the SP6 and T7 RNA polymerase promoters and inserted into the coding sequence for the *lacZ* α-peptide. The 821 bp *Bam*HI-*Eco*RI fragment carrying *clcD* was subcloned into p-Alter-1. The resulting plasmid that was generated plasmid was termed pUNT. The p-Alter-Control plasmid provided white/blue positive control for the mutagenesis reactions.

Bacterial strains. Three bacterial strains of *Escherichia coli* were used in this study. DH5α from Gibco-BRL laboratories was used for carrying and maintaining the plasmids pDO and pNDO. DH5α has the genotype F^-, λ^-, *recA1*, Δ(*lacZYA-argF*) U169, *hsdR17*, *thi-1*, *gyrA96*, *supE44*, *endA1*, *relA1*, ϕ 80d*lacZ* ΔM15.

BMH 71-18 *mutS* was used to transform the heteroduplex DNA after the mutagenesis procedure. Transformation into this strain prevented repair of the newly synthesized unmethylated strand, leading to higher mutation efficiencies. The genotype of BMH 71-18 *mutS* is *thi*, *sup E*, *D*(*lac-pro* AB), [*mut*S::Tn10] [F', $proA^+B^+$, $lacI^q$ *ZΔM15*].

E. coli JM109 was used to clone pUNT and for the production of SS-DNA. Undesirable restriction of cloned DNA and recombination with host chromosomal DNA are also prevented by using this strain. The genotype of JM109 is *endA1*, *recA1*, *gyrA96*, *thi*, *hsdR17*(r_k^-,

m_K^+), *relA1*, *sup*E44, λ^-, Δ(*lac-proAB*), [F', *traD36*, *proA*$^+$*B*$^+$, *lac*I^qZΔM15].

Media, growth and cultivation. JM109 and BMH 71-18 *mutS* were always maintained on M9 minimal plates supplemented with 1mM thiamine-HCl. This medium maintains selection for the presence of the F' which carries a nutritional requirement for growth (proline biosynthesis). M9 plates were prepared with the following ingredients per liter: 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl. These salts were dissolved in 500 ml of deionized distilled water. In a separate flask 15 g of agar was dissolved in 500 ml of deionized, distilled water and autoclaved separately. When salts cooled down the following was added: 2 ml of 1M $MgSO_4$, 0.1 ml of 1M $CaCl_2$, 10 ml of 20% glucose (w/v), 1 ml of 1 M thiamine-HCl. The agar and salts were mixed and the plates were poured.

Lurea Bertani rich medium was used in this study to grow transformants after mutagenesis reactions, and to grow cells for determination of enzyme activity. LB medium contained per liter of distilled deionized water: 10 g Bacto yeast extract, 5 g Bacto tryptone, and 10 g NaCl. To prepare solid medium, 1.5 percent agar (w/v) was added. Whenever needed for selection the antibiotics ampicillin and/or tetracycline were added to a final concentration of 125 mg per ml and 15 mg per ml respectively. All cultures were incubated at 37°C, and liquid cultures were provided with shaking at 200 rpm.

TYP medium was used to grow and titer the helper phage M13KO7. TYP medium contains per liter of distilled deionized water: 16 g Bacto-tryptone, 16 g Bacto-yeast extract, 5 g NaCl, and 2.5 g K_2HPO_4. To prepare solid medium, 1.5 per cent agar (w/v) was added. Kanamycin was added to a final concentration of 70 μg per ml.

Oligonucleotide design and purification. Oligonucleotides of 21 to 30 nucleotides chain length were synthesized on a Milligen DNA synthesizer or a Pharmacia System 1 Gene Assembler DNA synthesizer using ß-cyanoethyl phosphoramidite chemistry. The sequence of the mutagenic oligonucleotides is shown in Figure 4. Addition, detritylation, oxidation, and activation reactions were performed according to the instrument's recommended run parameters. Oligonucleotides were synthesized on 0.2 μmol columns. All oligonucleotides sequences were designed and checked against the *clcD* gene sequence to determine that the level of homology was sufficiently stringent to prevent non-specific priming during DNA sequencing or PCR amplification reactions.

Oligonucleotides were stored attached to the column, desiccated at -20°C until used. Oligonucleotides were removed from the column by treatment with fresh, concentrated, reagent grade ammonium hydroxide. Then the blocking groups on the amino groups of the bases were removed by incubation at 55°C for 12 to 18 hours in 1-2 ml concentrated ammonium hydroxide. Ammonium hydroxide was

removed by evaporation and the oligonucleotide was evaporated to dryness and resuspended in 1 ml of sterile distilled water. This oligonucleotide solution was applied to a Sephadex G25 column (0.8 cm X 5 cm) and diluted with sterile distilled water. The void volume of the column was discarded and the oligonucleotide collected in approximately 2 ml. This removed the freed benzoyl and isobutyrl blocking groups as well as short failure sequences present in the preparation. A 1:50 dilution in water of the oligonucleotide solution was measured for UV absorbance at 260 nm in order to determine the concentration of the oligonucleotide solution. The concentration was determined using the following formula:

$$\text{number of nmoles} = \frac{A_{260} \times 10^2}{\text{length of oligonucleotide}}$$

The purity of each oligonucleotide was checked by loading 30 µl on a 20 per cent (w/v) polyacrylamide. The oligonucleotide was dried down by centrifugal evaporation and resuspended in water to give a concentration of 20 pmol per ml. This preparation was stored at -80°C and was used to make a working solution of 1 pmol per ml.

Oligonucleotides used for mutagenesis were phosphorylated prior to annealing to the SS-DNA template obtained from pUNT. Phosphorylation was achieved by using 10 units of T_4 polynucleotide kinase, 5 ml of 10X kinase buffer, 300 pmol of oligonucleotide, 5 ml

of 5 mM spermidine, 8 ml of 100 mM ATP, and sterile deionized distilled water to a final volume of 50 ml. The reaction mixture was incubated at 37°C for 15 minutes. The reaction was terminated by heating at 65°C for 5 minutes. The phosphorylated oligonucleotide was stored at -20°C for future use.

Generation of PCR products. The polymerase chain reaction (PCR) is a method by which a specific DNA sequence can be amplified *in vitro*. The primers anneal at either end of the targeted nucleotide sequence and are oriented in opposite directions. Exponential amplification of the target sequence occurs over the course of multiple rounds of denaturation, annealing, and 5' to 3' extension by a heat stable DNA polymerase (*Taq* polymerase).

PCR was used to introduce *Bam*H1-*Eco*RI cutting sites. As shown in Figure 4, primer "a" and primer "b" will hybridize with complementary regions lying outside of the *clcD* gene sequence. Amplification of DNA fragments from the plasmid pDO template (1.9 kbp *Pst*I-*Eco*RI *clcD* fragment in pUC19) was achieved by adding 10 ng of template DNA, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 0.1 per cent (w/v) gelatin, 20 pmol of each dNTP, 20 pmol of each primer (one primer that carried the 5' *Bam*H1 cutting site and the other primer that carried the *Eco*RI cutting site) and 2.5 units of *Taq* DNA polymerase in a final volume of 100 µl. This mixture was overlaid with 100 µl of light mineral oil (Sigma) and subjected to 28 cycles of denaturation (1 minute at 94°C), annealing (2 minutes at

58°C), and extension (3 minutes at 72°C) using a Techne thermocycler machine to perform the PCR amplification. The products of the reaction were analyzed on an 1.5 per cent (w/v) agarose gel (Bethesda Research Laboratories). A 1.9 *clcD* fragment was amplified and cut with *Bam*HI and *Eco*RI, yielding the 821 bp *Bam*HI to *Eco*RI *clcD* fragment. Later these fragments were ligated into pUC19 cut with *Bam*HI and *Eco*RI to yield the recombinant plasmid pNDO (refer to Figure 4). The recombinant plasmid was transformed into *E. coli* strain DH5α. The sequence of the sized down fragment was confirmed by single stranded sequencing after subcloning into M13mp19.

Plasmid purification. Plasmid preparation was one of two types. For rapid plasmid analysis of transformants, a rapid plasmid preparation was performed as follows: cells were grown in 10 ml LB plus 0.4 per cent (w/v) glucose, and selective drug at 37°C incubator on a New Brunswick TB1 tube roller at 50 rpm. Cells were resuspended in 0.4 ml of 15 per cent sucrose, 50 mM Tris-HCl, and 50 mM Na_2EDTA (pH 8.5). Lysis of cells was performed in a freshly prepared lysozyme solution 5 mg per ml in the above buffer at 0°C for 10 minutes. Cell lysis was completed by adding 0.3 ml of 0.1 per cent Triton X-100, 50 mM Tris-HCl, and 50 mM Na_2EDTA (pH 8.5) at 0°C for 10 minutes. Lysed cells were centrifuged for 2 minutes at 10,000 rpm in a microfuge centrifuge in a cold room. Supernatant was transferred to a new centrifuge tube and 2 µl of

diethyloxydiformate was added. The microfuge tubes were heated to 70°C for 15 minutes, cooled on ice for 15 minutes and centrifuged at 10,000 rpm in an Eppendorf Centrifuge 5415 at room temperature. The supernatant was transferred to a new microfuge centrifuge and DNA was precipitated with absolute ethanol and centrifuged in a microfuge centrifuge at room temperature. Samples were dried in a speed vacuum, and precipitated DNA was dissolved in 50 µl of 10 mM Tris-HCl (pH 7.5), 1 mM Na_2EDTA, and 10 mg per ml of RNase A (Davis *et al.*, 1980). Plasmid DNA samples were checked by running 5 ml on 0.7 per cent (w/v) agarose in TBE buffer.

For preparation of a large amount of plasmid DNA, a bulk plasmid preparation from amplified samples was performed. For bulk plasmid preparation one liter of L-broth with the appropriate antibiotic was inoculated with 10 ml of overnight culture, grown to an A_{600} of 0.4, and 0.2 g of chloramphenicol was added. The culture was allowed to incubate on a rotary shaker for 12-14 hours. Cells were pelleted and resuspended in 12.5 ml of 25 per cent (w/v) sucrose in 0.05 M Tris-HCl (pH 8.0). Lysis of cells was accomplished initially by adding 5 ml of a mixture of 10 mg per ml lysozyme in 0.05 M Tris-HCl (pH 8.0). In the second step 20 ml of Triton mix was added drop by drop with continuous stirring to achieve uniform lysis. The triton mix is composed of 0.5 ml Triton X-100, 5 ml of 1 M Tris-HCl (pH 8.0), 25 ml of 0.25 M EDTA (pH 8.0), and 69.5 ml of sterile deionized distilled water to get a total volume of 100 ml. Lysis mixture was centrifuged at 19,000 xg for 30 minutes. Next, 40

ml of lysate was poured into 50 ml graduated cylinder containing 37.5 g CsCl and dissolved gently at room temperature. Next 2.5 ml of ethidium bromide (10 mg/ml in deionized distilled water) was added. The mixture was loaded into Ti1270 ultracentrifuge tubes. The tube was sealed and spun at 40,000 xg for 40 hours to obtain a stable gradient. The lower band of DNA was pulled with 18-gauge hypodermic needle. The top of the tube was vented by using 21-gauge needle. Plasmid DNA was extracted with 1-butanol saturated with water. Extraction was repeated until all pink color from the aqueous phase and the organic phase had disappeared. Cesium chloride was removed by dialysis for 24-48 hours against several changes of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The first change was done within the first hour.

Transformation. The plasmids pDO, and pNDO were transformed into *E coli* DH5α. The plasmids pUNT, pUNTR81A, and pUNTE36Q were transformed into *E. coli* JM109 and BMH 71-18 *mutS*. Two different methods were used to prepare competent cells. The first method involved inoculation of 5 ml of LB with a single colony and incubate at 37°C overnight. An aliquot of the overnight culture was diluted one to 100 in LB, and grown to an absorbance of 0.45-0.55 at 600 nm. The culture tube was placed on ice for 20 minutes, and centrifuged at 2500 xg for 15 minutes at 4°C. The pelleted cells were suspended in half of the original volume of sterile ice-cold 100 mM $CaCl_2$, and incubated on ice for 30 minutes. Cells were pelleted again

at 2500 xg for 15 minutes at 4°C, and resuspended in one tenth of the original volume of ice-cold 100 mM $CaCl_2$. Cells were kept on ice, and were used the same day (BRL M13 Cloning Dideoxy/Sequencing Manual, 1986).

The other method of preparing competent cells, allowed for storage of competent cells at -80°C for up to 3 months. The competent cells were prepared as follows: 0.5 ml of an overnight culture was inoculated into 20 ml of LB, and grown at 37°C to an absorbance of 0.13-0.15 at 600 nm. Cells were pelleted at 5000 xg at 4°C. The pelleted cells were resuspended in 1 ml of solution A (10 mM MOPS, pH 7.0 and 10 mM rubidium chloride), and the volume was brought up to 10 ml. Cells were pelleted at 5000 xg, and resuspended in 1 ml of solution B (100 mM MOPS, pH 6.5; 50 mM $CaCl_2$; and 10 mM rubidium chloride), and volume was brought up to.10 ml. Cells were incubated on ice for 30 minutes, and pelleted at 5000 xg. Supernatant was decanted, and the pellet was resuspended in 2 ml of solution B. For long term storage of cells 10 per cent final concentration of glycerol was added. Cells were frozen at -80°C in 200 µl aliquots (Promega Altered Sites *in vitro* Mutagenesis System Technical Manual, 1992).

For transformation of the plasmids pDO, and pNDO into competent *E. coli* DH5α cells, 100 ng of plasmid DNA in a volume of 5-10 ml was used to transform 200 µl of competent cells. The mixture was incubated on ice for 15 minutes, heat-shocked at 42°C for 90 seconds, 2 ml of LB was added, and incubated at 37°C on a

New Brunswick TB1 tube roller at 50 rpm for one hour. Portions of 10 µl and 100 µl were plated on LB plates containing 100 µg per ml ampicillin per ml.

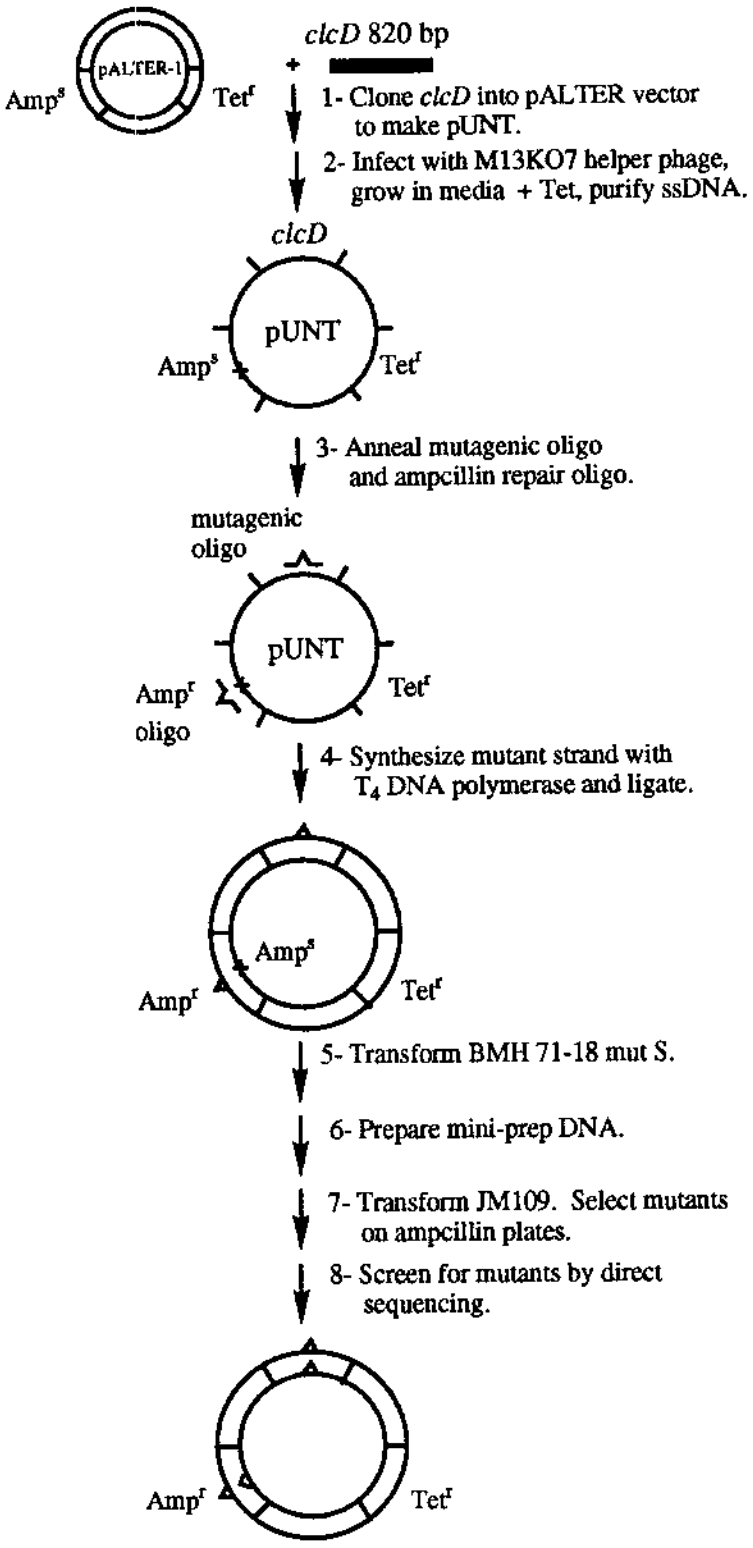
After performing the mutagenesis reactions on pUNT single stranded template, the entire synthesis reaction was added to 200 µl of competent BMH 71-18 *mutS* to which 3 ml of DMSO was added The DMSO was stored as frozen aliquots and not reused once thawed. The competent BMH 71-18 *mutS* cells and synthesis reaction mixture were incubated on ice for 30 minutes. A heat-shock was performed at 42°C for one minute. A volume of 4 ml of LB was added and the transformed cells were incubated in a tube roller for one hour. Ampicillin was added to a final concentration of 125 µg per ml, and cells were allowed to grow for 12-14 hours at 37°C. A rapid plasmid preparation was made as described previously from the 4 ml culture of the BMH 71-18 *mutS*. Approximately 50-100 ng of DNA was used to transform 200 µl of JM109 competent cells as described above for transformation of BMH71-18 *mutS*. After the heat-shock 2 ml of LB medium were added and cells were incubated at 37°C for one hour to allow cells to recover. The culture was divided into two microfuge centrifuge tubes and spun down for one minute. The cells from each tube were resuspended in 50 µl of LB. Cells from each tube were plated on LB plates containing 125 µg ampicillin per ml and incubated at 37°C for 12-14 hours. Transformant colonies were screened by direct double stranded sequencing as will be described below.

Plasmid construction. The amplified PCR product of *Bam*HI-*Eco*RI *clcD* fragment was electroeluted, cut with *Bam*HI and *Eco*RI restriction enzymes, and ligated into pUC19 as described in Sambrook *et al*, 1989. The newly constructed plasmid was named pNDO (Figure 4). To perform the mutagenesis reactions the *Bam*HI-*Eco*RI fragment was cut, electroeluted, and ligated into pAlter-1 (5.681 kbp). The newly constructed plasmid was given the name pUNT.

Mutagenesis reactions. Mutagenesis reactions involved annealing the mutagenic primer to a single stranded template of pUNT (Figure 5). The single stranded template was prepared as follows. An overnight culture of JM109 cells containing pUNT was prepared by inoculating 2 ml of TYP broth containing 15 mg per ml tetracycline, and incubating in a tube roller at 37°C. The next day 5 ml of TYP broth in 50 ml flask containing 15 µg per ml tetracycline was inoculated with 100 µl of the overnight culture, and shaken vigorously at 37°C for 30 minutes. The culture was infected with helper phage M13KO7 at a multiplicity of infection (m.o.i.) of 10, K_2HPO_4 was added to a final concentration of 20 mM, and vigorous agitation and good aeration was continued overnight. The helper phage M13KO7 stock was prepared and titrated as described in the Promega Protocols and Application Guide. Cells were harvested at

12,000 xg for 15 minutes, the supernatant was transferred to a fresh tube, and spun again for 15 minutes. The phage was precipitated by

Figure 5. Schematic diagram of the mutagenesis procedure. The system employs a phagemid, the pAlter-1 vector, which contains two genes for antibiotic resistance. One of these genes, for tetracycline resistance, is always functional. The other, for ampicillin resistance, has been inactivated. An oligonucleotide is provided which restores ampicillin resistance to the mutant strand during the mutagenesis reaction. This oligonucleotide is annealed to the single-stranded DNA (SS-DNA) template at the same time as the mutagenic oligonucleotide, and subsequent synthesis and ligation of the mutant strand links the two. The DNA is transformed into a repair minus strain of *E coli* (BMH 71-18 *mutS*) and the cells are grown in the presence of ampicillin, yielding large numbers of colonies. A second round of transformation in JM109 or a similar host ensures proper segregation of mutant and wild type plasmids and results in a high proportion of mutants.



pUNTR81A or pUNTE36Q

adding 0.25 volume of phage precipitation solution which consisted of 3.75 M ammonium acetate (pH 7.5), and 20 per cent polyethylene glycol (MW 8,000). The resulting preparation incubated on ice for 30 minutes, then centrifuged at 12,000 xg for 15 minutes. The pelleted phage was resuspended in TE buffer (10 mM Tris-HCl, pH 8, and 1 mM EDTA). The phage preparation was lysed by adding 0.4 ml of chloroform: isoamyl alcohol (24:1), mixed by vortex mixer for 1 minute, and centrifuged at 12,000 xg for 5 minutes. The upper aqueous phase was extracted with equal volume of TE-saturated phenol repeatedly until there was no visible material at the interface. Excess phenol was removed by extracting with an equal volume of chloroform: isoamyl alcohol (24:1). The upper aqueous phase containing phagemid and pUNT ssDNA was transferred to a fresh tube. The ss-DNA template was precipitated by adding 0.5 volume of 7.5 M ammonium acetate plus 2 volumes of ice-cold absolute ethanol, mixed and left at -80°C for 30 minutes. The mixture was centrifuged at 12,000 xg for 15 minutes, the supernatant was removed, and the pellet was dried under vacuum. The pellet was resuspended in 20 ml of sterile deionized distilled water. Next the SS-DNA was checked by electrophoresis through a 1 per cent agarose gel. For further confirmation, SS-DNA dideoxy sequencing was performed as will be described later. The same procedure was followed to prepare the pAlter- Control ssDNA to be used as positive control for the mutagenesis reactions.

Two mutagenic primers were used to create two mutant enzymes. The first mutation was to change Arg81 to Alanine, this involved changing the codon TCT to TGC on the first mutagenic primer. The second mutation was to change Glu 36 to Glutamine, this involved changing a TTC to TTG on the second mutagenic primer. The sequence of the two mutagenic primers is shown in Figure 6. Mutagenesis annealing reaction was performed by mixing the following ingredients: pUNT ss-DNA (100 ng); 1 µl (0.25 pmol) of ampicillin repair oligonucleotide; phosphorylated mutagenic oligonucleotide (1.25 pmol); 2 µl of 10X annealing buffer (200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, and 500 mM NaCl). The control annealing reaction contained 100 ng of pAlter-Control, 0.25 pmol of phosphorylated ampicillin repair oligonucleotide, 1.25 pmol of phosphorylated *lacZ* control oligonucleotide, and 2 µl of 10X annealing buffer. Both reaction mixtures were heated to 90°C for 3 minutes. The annealing mixture was placed on ice and the following was added: 3 µl of 10X synthesis buffer (100 mM Tris-HCl pH 7.5, 5 mM dNTPs, 10 mM ATP, 20 mM DTT), 10 units of T4 DNA polymerase 10 units of T4 DNA ligase, and sterile deionized distilled water to a final volume of 30 µl. The reaction mixture was incubated at 37°C for 90 minutes to perform mutant strand synthesis and ligation. The entire synthesis reaction was used to transform competent *E. coli* BMH 71-18 *mutS*.

Figure 6. Sequence of Arg 81 to Alanine, and Glu 36 to Glutamine mutagenic oligonucleotides.

R81A: 5' GAGGCGCAGGCAGAGCAAGCC 3'

E36Q: 5' ATCGCTCAACAAATATTTGGT 3'

DNA sequencing. DNA sequencing was performed using either a single or double stranded DNA to generate the template for the sequencing reactions. The reactions were performed according to established procedures for DNA sequencing by Sanger's dideoxynucleotide method (1977) using ^{35}S-labeled dATP (Biggin *et al.*, 1983) and Sequenase brand of DNA polymerase from USB Corporation. Both standard 8 per cent and buffer gradient 6 per cent polyacrylamide gels (Tris-borate-EDTA buffer or TBE concentration from 2.5X to 0.5X) were employed to run the sequencing products. For double stranded sequencing of plasmid DNA templates, the plasmid was denatured by treatment with base and annealed to sequencing primers. Chain extension was performed according to the instructions included in the Sequenase version 2.0 kit. As the limit of resolution for chain extension of each primer was reached, specific oligonucleotide primers were synthesized and used to walk through the sequence. Typically, dGTP termination and labeling mixtures were used. To resolve compressions dITP termination and labeling mixtures were added and run with the dGTP mixtures. After the DNA sequencing gels were completely dry, the gels were exposed to a sheet of Kodak XAR5 x-ray film. After 1 to 5 days exposure, the films were developed. Since ^{35}S will not penetrate the film support to expose the emulsion on the distal side of the film, this emulsion was removed by stripping with a 1 to 10 dilution of commercial bleach. Care was taken not to wet the exposed side of the film with the bleach. The film was rinsed, dried and illuminated. The

sequence was read to confirm that there were no substitutions after the PCR amplification, and no other mutations were introduced other than the desired ones when the mutagenesis reactions were done.

Enzyme assays. Bacterial cultures (*E. coli* JM109 carrying wild type control and mutant pUNT plasmids) were grown at 37°C in L-broth containing 15 μg of tetracycline per ml (growth medium for mutant pUNT also contained 125 μg of ampicillin per ml) and 0.5 mM final concentration of the inducer IPTG. Cells were grown to an A_{600} between 0.8-1.0, harvested by centrifugation and stored at -20°C. The pellets were resuspended with 5 ml of enzyme extraction buffer and sonicated with six 10 seconds bursts in Braun Model 2000 ultrasonic disrupter at a power setting of 4 using a standard probe tip. The enzyme extraction buffer consists of 50 mM ethylene diamine, 1 mM $MnCl_2$, 1 mM 2-mercaptoethanol (pH 7.3). Extracts were kept on ice at 4°C and assayed the same day.

Activity of wild type DLH and mutant DLH was measured spectrophotometrically at 280 nm in a 1 ml cuvette containing 0.1 M phosphate buffer (pH 6.5) and 100 mM (*E*) dienelactone. Enzyme activities was determined using a Beckman Model 25 recording/scanning UV-visible spectrophotometer. The absorbance decreases were plotted directly on an Epson printer and calculated manually. A molar absorption coefficient of 15,625 liter $M^{-1}cm^{-1}$ was used. One enzyme unit is defined as the amount of dienelactone hydrolase that catalyzes the formation of 1 μmol of product or the

disappearance of 1 μmol of substrate per minute under the assay conditions described (Schmidt *et al.*, 1980).

RESULTS and DISCUSSION

Sizing down of the *clcD* gene fragment

Much of the work described in this study involved the construction of appropriate recombinant strains, the amplification and subcloning of mutant DNA sequences, and consequently the introduction of specific alterations into the primary sequence of the enzyme dienelactone hydrolase. The amplification of specific DNA sequences using the polymerase chain reaction was performed to first obtain subclones of a specific sequence containing the *clcD* gene from the plasmid pDO. Small DNA fragments are easier to manipulate and involve less expense to sequence. In addition, it was hoped that the smaller DNA would have other advantages such as increased level of expression as discussed in the following section. Oligonucleotide primers were used that introduced a 5' *Bam*HI linker and a 3' *Eco*RI linker into sequences that framed the DNA encoding *clcD*. At the same time, the original *Pst*I-*Eco*RI 1.9 kbp fragment containing the *clcD* was sized down to 821 bp. The amplified fragment carried only 54 bp (including the *Bam*HI site) on the 5' end of the coding sequence, and 56 bp (including the *Eco*RI site) from the 3' end of the open reading frame. The *clcD* open reading frame became repositioned in this construct from residue 55 to residue 765, encoding 236 amino acids (including a UGA stop codon) using the new sequence's position for the numbering system (Figure 7).

Figure 7. Nucleotide sequence of the *Bam*HI to *Eco*RI fragment which includes *clcD* open reading frame and the flanking regions. The entire 821 bp fragment is shown. A 5' *Bam*HI linker and a 3' *Eco*RI linker were used to clone out by PCR a sized down version of the *clcD* gene with approximately 50 bases on either end of the coding sequence. As indicated in the figure the *clcD* open reading frame (ORF) is from position 55 to 765 (encoding 236 amino acids and UGA stop). The alterations in the DNA sequence (and hence the amino acid sequence) made in this study are underlined.

```
                    GAT CCT CCA CCA TCG ACT CAA CAC CAA TGA ACT GTA ATC

GGG AGA GAT GGG AAG ATG TTG ACT GAA GGG ATA TCG ATT CAA TCG TAT GAC GGG  39
                    Met Leu Thr Glu Gly Ile Ser Ile Gln Ser Tyr Asp Gly  13
                     1                                   10

CAT ACA TTC GGC GCG CTC GTG GGC TCG CCG GCC AAA GCG CCC GCT CCC GTG ATT  93
His Thr Phe Gly Ala Leu Val Gly Ser Pro Ala Lys Ala Pro Ala Pro Val Ile  31
                         20                                  30

                   CAA
GTG ATC GCT CAA GAA ATA TTT GGT GTG AAC GCG TTC ATG CGA GAA ACG GTG TCA 147
Val Ile Ala Gln Glu Ile Phe Gly Val Asn Ala Phe Met Arg Glu Thr Val Ser  49
                   Gln             40

TGG CTG GTC GAC CAG GGG TAT GCG GCA GTT TGC CCT GAT CTG TAC GCG CGC CAG 201
Trp Leu Val Asp Gln Gly Tyr Ala Ala Val Cys Pro Asp Leu Tyr Ala Arg Gln  67
50                                       60

                                                  GCA
GCG CCA GGT ACA GCA CTC GAT CCG CAG GAT GAG GCG CAG AGA GAG CAA GCC TAC 255
Ala Pro Gly Thr Ala Leu Asp Pro Gln Asp Glu Ala Gln Arg Glu Gln Ala Tyr  85
        70                                       80  Ala

AAG CTC TGG CAG GCC TTC GAC ATG GAG GCC GGC GTG GGC GAT CTG GAG GCT GCT 309
Lys Leu Trp Gln Ala Phe Asp Met Glu Ala Gly Val Gly Asp Leu Glu Ala Ala 103
                   90                                  100

ATC CGC TAT GCG CGA CAC CAA CCC TAC AGC AAC GGC AAG GTG GGA TTG GTG GGG 363
Ile Arg Tyr Ala Arg His Gln Pro Tyr Ser Asn Gly Lys Val Gly Leu Val Gly 121
                         110                                 120

TAT TGC CTG GGC GGT GCG CTT GCC TTT CTA GTG GCC GCC AAA GGA TAC GTG GAT 417
Tyr Cys Leu Gly Gly Ala Leu Ala Phe Leu Val Ala Ala Lys Gly Tyr Val Asp 139
                              130

CGC GCC GTA GGC TAC TAC GGT GGT GGA CTG GAG AAG CAG CTC AAC AAG GTC CCG 471
Arg Ala Val Gly Tyr Tyr Gly Val Gly Leu Glu Lys Gln Leu Asn Lys Val Pro 157
140                                      150

GAA GTC AAG CAT CCG GCG TTG TTT CAC ATG GGC GGC CAA GAC CAC TTC GTG CCC 525
Glu Val Lys His Pro Ala Leu Phe His Met Gly Gly Gln Asp His Phe Val Pro 175
        160                                      170

GCG CCA AGC CGC CAG CTG ATT ACT GAA GGC TTC GGT GCC AAT CCA TTG CTG CAA 579
Ala Pro Ser Arg Gln Leu Ile Thr Glu Gly Phe Gly Ala Asn Pro Leu Leu Gln 193
                   180                                 190

GTG CAC TGG TAC GAA GAG GCC GGA CAC TCG TTC GCC AGG ACG AGC AGT TCG GGC 633
Val His Trp Tyr Glu Glu Ala Gly His Ser Phe Ala Arg Tyr Ser Ser Ser Gly 211
                         200                                 210

TAT GTG GCG AGT GCC GCG GCG TTG GCC AAC GAA CGT ACA CTG GAT TTC CTG GCG 687
Tyr Val Ala Ser Ala Ala Ala Leu Ala Asn Glu Arg Thr Leu Asp Phe Leu Ala 229
                              220

CCC TTG CAG AGC AAG AAG CCA TGA ATT TCA TTC ACG ACT ATC GTT CCC CGC GGT 741
Pro Leu Gln Ser Lys Lys Pro ***

GAT TTT TGG ACC CGA CAG CCT CGC CG
```

This sized-down *Bam*HI-*Eco*RI fragment carrying *clcD* and little else, was ligated into correspondingly cut plasmid pUC19 and the resulting plasmid was named pNDO. Next, SS-DNA sequencing was performed on the whole *Bam*HI-*Eco*RI PCR fragment in order to confirm that the sequence had not been changed throughout the experimental manipulations in the construction of pNDO. A number of clones were sequenced and one with the known, correct and proven DNA sequence was chosen for all further work,. Other subclones from this procedure were discarded and a single pUNT from unique origin was chosen for all subsequent genetic manipulations. In the meantime, the same region in the parent plasmid pDO was sequenced as a control.

Sequence analysis of the sized-down *clcD* clone contained sequence discrepancies compared to the published DNA sequence of *clcD*. Results from the DNA sequence analysis of the clone showed that there were two nucleotide bases different from the sequence of the open reading frame compared to the published sequence. The two differences are: one "G" nucleotide is added between "G288" and "C289" relative to the original *clcD*5' strand, and there is no "C" at position 292 in the original *clcD*5' strand (numbering on the plus strand). The net result of these two nucleotide base changes (one an insertion, and one a deletion was a single change in the amino acid sequence from arginine to alanine at position 79 in the protein's primary amino acid sequence (Figure 7). Thus, if the amino acid sequence for the enzyme of the the pNDO (821 bp) is compared with

the published sequence of the 1.9 kbp fragment, the sequence at this region changes from Glu-Arg-Gln-Arg- to Glu-Ala-Gln-Arg (compare the sequence about residue 79 in Figure 1 and Figure 7).

Thus the sequence of the sized-down clone in pNDO differed from the published sequence. But the DNA sequence of *Bam*HI-*Eco*RI PCR fragment was identical to the sequence of the parent plasmid over the whole length of the gene and its flanking regions. The DNA sequence of *clcD* region in the sized-down fragment of pNDO was identical to the corresponding sequence of pDO when sequenced in this study. Each strand of both template DNAs was sequenced to confirm the identity and resolve any ambiguities. This indicated that there was not a single base pair substitution introduced by the PCR reaction. Differences seen were errors in the published DNA sequence or the result of changes in the gene that may have occurred in the strain since publication of the sequence.

In addition, more errors in the published DNA sequence were discovered during the course of our work. In our amplified, clone (pNDO) as well as in the parent (pDO) there were differences in the nucleotide sequence relative to the published sequence. However, these differences still gave the reported amino acid sequence for the protein. Indeed, if the reported nucleotide sequences were translated as written, they would not yield the amino acids reported. For example Asn 154 is encoded by the sequence AAC when sequenced in this study. But the original nucleotide sequence is reported as an AAG. This would give a Lys at 154, but an Asn was

still recorded for the amino acid in the primary sequence of the protein. Secondly, amino acid residue 224 is a Thr residue in our sequence, and in the published protein sequence. But we discovered that the nucleotide sequence at this position is ACA, not the reported AGA (which would actually code for Arg).

Introduction of mutations into the *clcD* gene encoding dienelactone hydrolase.

To perform the mutagenesis reactions the sized down *Bam*HI-*Eco*RI fragment was cloned into pAlter, and the recombinant plasmid was given the name pUNT. This represents another advantage of the sized-down fragment. The new restriction sites allow the fragment to be easily cut out and ligated into different vectors. The fact that the two ends are asymmetric allows the orientation of the clone to be "forced" relative to the promoter and thus ensure proper orientation for efficient transcription. Two mutants were created by introducing mismatches into the oligonucleotides used to prime the SS-DNA produced from the pUNT phagemid. Two mutations (Arg 81 to alanine and Glu 36 to glutamine) were introduced separately into the *clcD* sequence (Fig. 8 & 9). The new plasmids were named pUNTR81A and pUNTE36Q. The DS-DNA dideoxy sequence showed

Figure 8. Sequence analysis of pUNTR81A generated by Altered site *in vitro* mutagenesis system compared to the sequence of pUNT. The mutated position in the nucleotide sequence is denoted by a ldouble arrow.

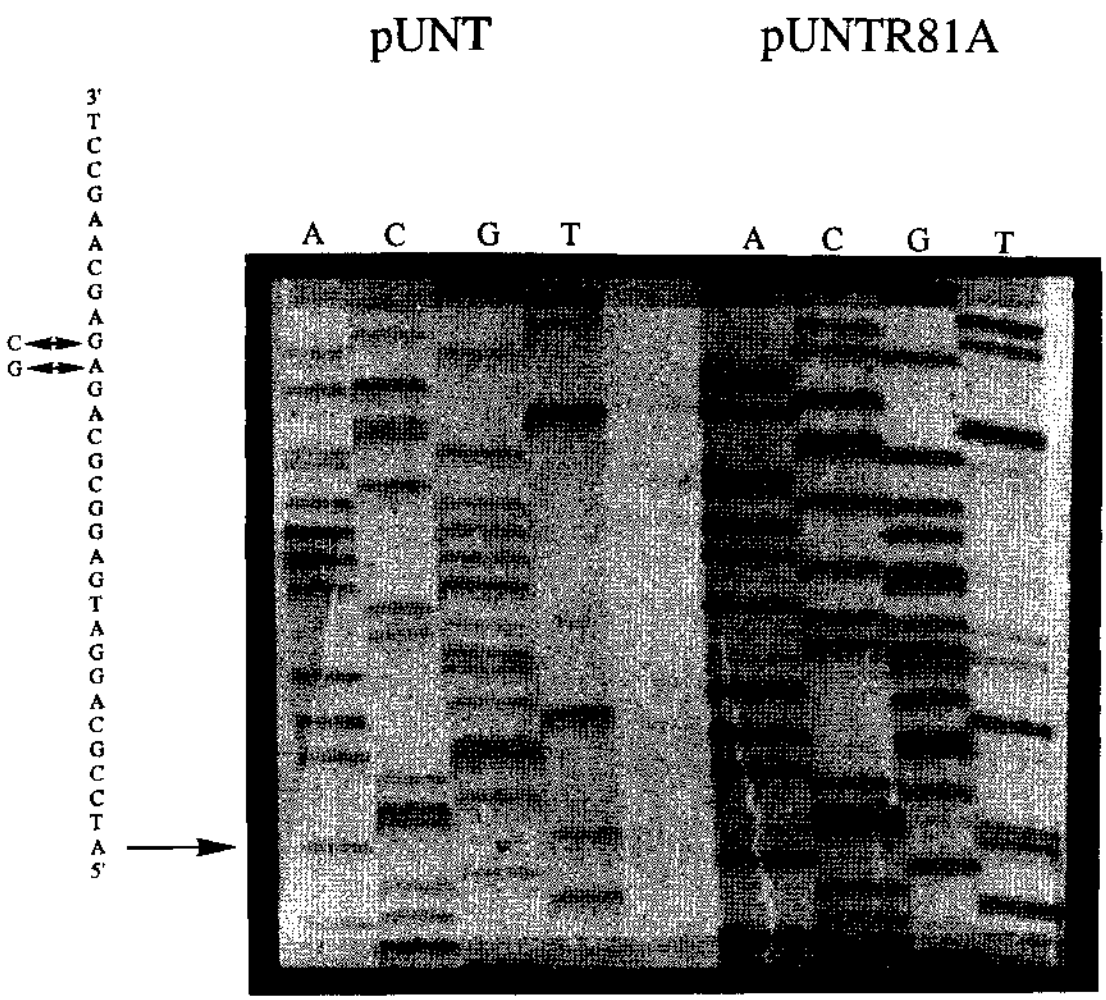
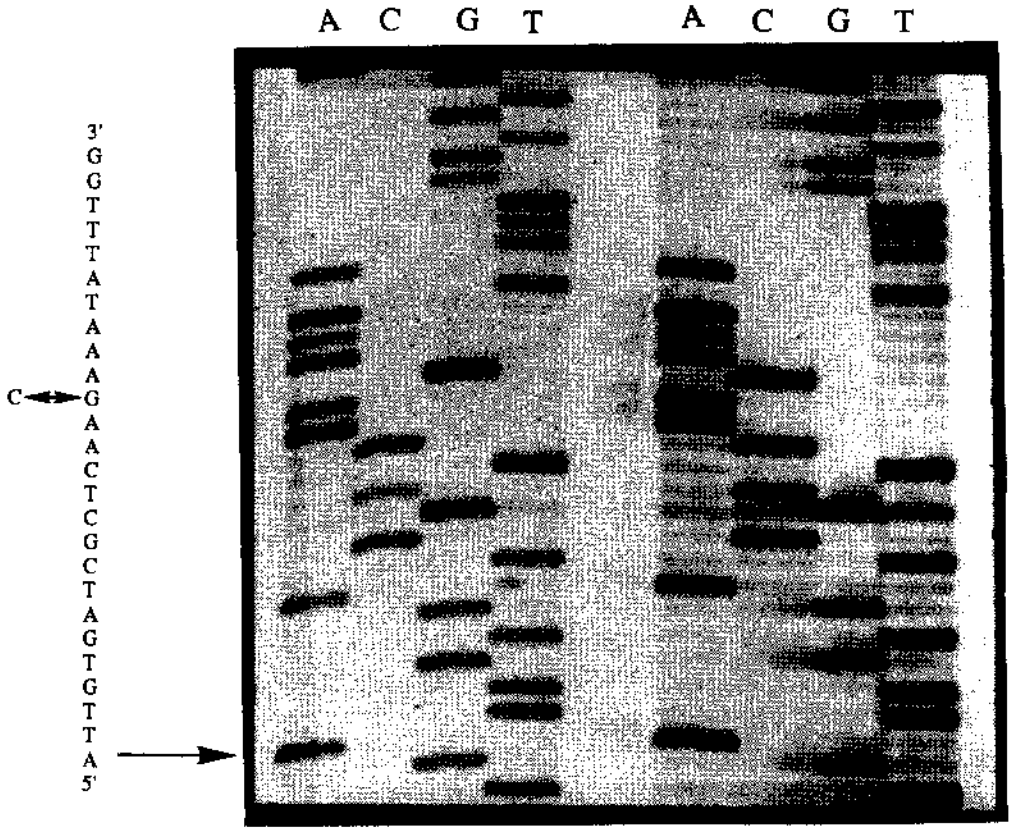

pUNT
pUNTR81A
A C G T
A C G T
3' T C C G A A C G A G A G A C G C G G A G T A G G A C G C C T A 5'
C
G

Figure 9. Sequence analysis of pUNTE36Q generated by Altered site *in vitro* mutagenesis system compared to the sequence of pUNT. The mutated position in the nucleotide sequence is denoted by a double arrow.

pUNT pUNTE36Q



that only the desired nucleotide substitutions to change these specific residues were introduced and that no other changes have been made.

Enzyme kinetics of specifically altered dienelactone hydrolases

Enzyme assays were performed on cell extracts prepared from *E. coli* JM109 carrying the plasmids pUNT, pUNTR81A, and pUNTE36Q as described in the Methods. The enzyme specific activity of wild type dienelactone hydrolase, and the mutant dienelactone hydrolases DLHR81A and DLHE36Q was measured and is listed in Table I.

Table I. Specific Activities of dienelactone hydrolase encoded by different *clcD* fragments. The specific activity for each enzyme is given in terms of nmol min^{-1} (mg protein)$^{-1}$.

DLH encoded by different *clcD* fragments	Specific Activity
pUNT	48.96
pUNTR81A	123.73
pUNTE36Q	0

From the data presented in Table I, *E. coli* JM109 carrying the plasmid pUNTE36Q lacked any dienelactone hydrolase activity. Changing Glu 36 to glutamine abolished the dienelactone activity completely. *E. coli* JM109 was shown to contain the plasmid by rapid

plasmid screens, and selection for the plasmid was maintained during growth by addition of ampicillin and tetracycline to all culture media. In addition, IPTG was added to induce the enzyme which was transcribed from the endogenous *lac* promoter operator of the parent plasmid pAlter.

The DLH enzyme activity of the mutant pUNTR81A is higher than the enzyme from the parent pUNT when grown under identical conditions. However, the k_M value for DLHR81A (40 μM, Fig. 10) is two fold higher than that of DLH (22.2 μM, Fig. 11). The higher specific activity of the mutant can not be ascribed to increased efficiency of expression since the kinetic properties of the enzyme are altered in the mutant DLHR81A, but it does seem to have decreased affinity for substrate and yet higher overall levels of enzyme activity in the cell. An increase in the specific activity alone is inconclusive proof of the level of gene expression and purified enzyme was not used for the determination of the kinetic parameters of the wild type and mutant enzymes. However, conditions of growth and the actual assay between the two samples, mutant and parent strains were kept constant. The mutant enzyme DLHR81A had a lower affinity for its substrate, the increase in specific activity is probably due to an increase in the level of the enzyme's concentration in the cells. Little variation was observed within samples from trial to trial, only between different samples of the two strains.

Figure 10. The Lineweaver-Burk plot of DLHR81A. The k_M value is 40 μM.

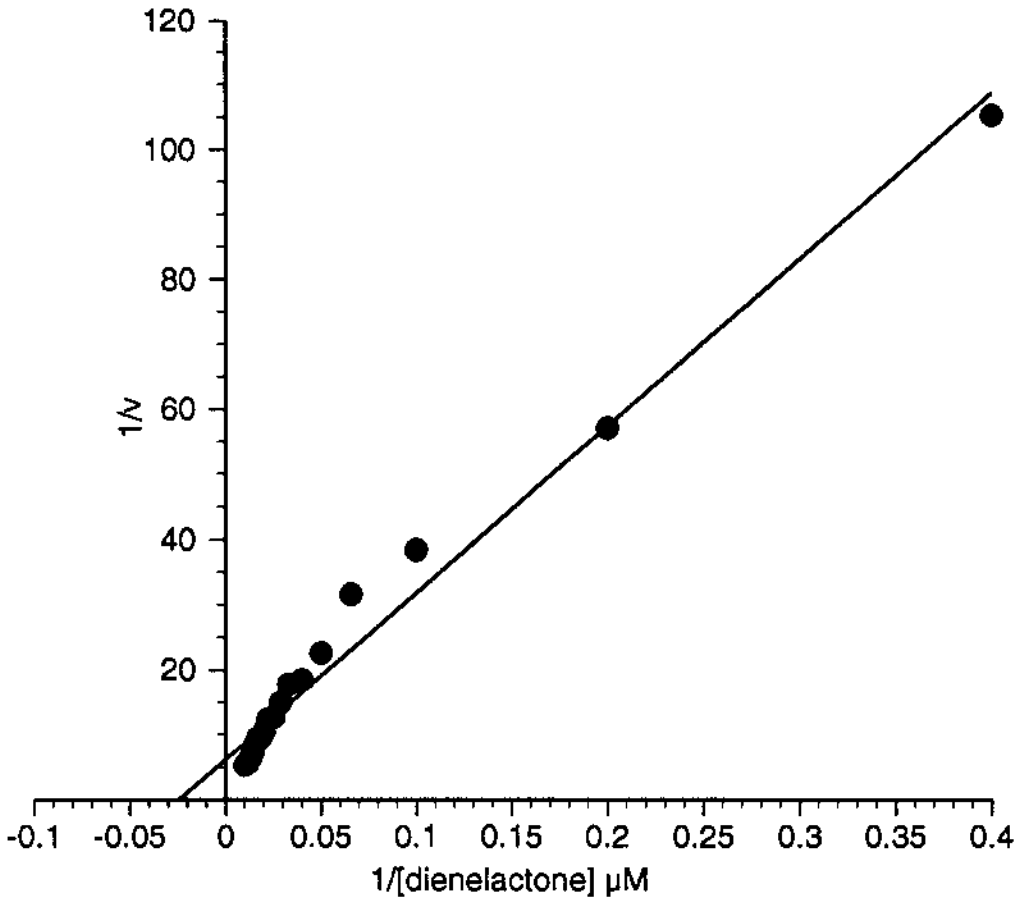
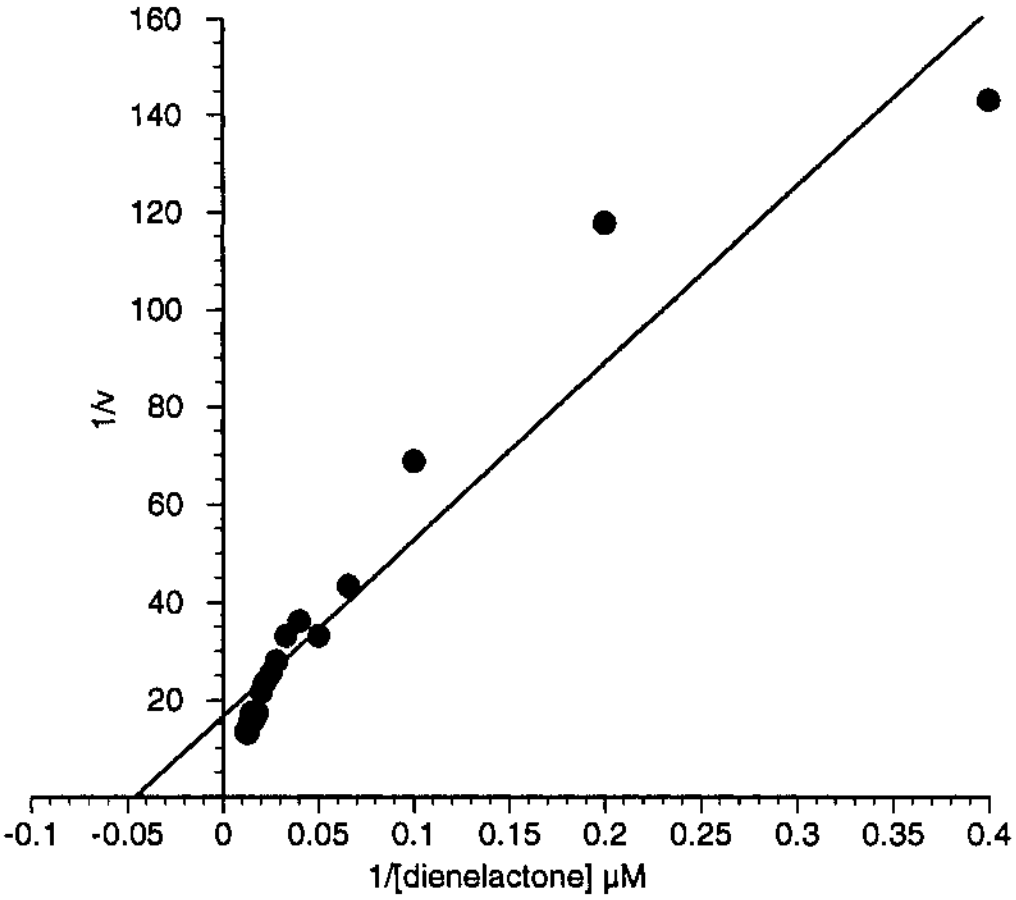

120
100
80
60
40
20
1/v
-0.1
-0.05
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
1/[dienelactone] µM

Figure 11. The Lineweaver-Burk plot of DLH. The k_M value is 22.2 μM.


160
140
120
100
80
60
40
20
1/v
-0.1
-0.05
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
1/[dienelactone] µM

CONCLUDING COMMENTS

This research was performed to investigate the role that individual amino acids in the active site of dienelactone hydrlase play in the catalytic mechanism of the enzyme. The structure of dienelactone hydrolase is well characterized with published reports of Ollis and his collaborators at a resolution of 2.8 Å (Pathak *et al.*, 1988), and at a resolution of 1.8 Å (Pathak and Ollis, 1990). The X-ray crystallographic structure reveals the enzyme to be an α/ß protein consisting of eight parallel strands of ß pleated sheets, except for strand 2 which runs antiparallel. The ß-sheets are themselves surrounded by seven α-helices (Figure 3). Studies of the structure of DLH showed that Cys 123 reside lies in a cleft in the globular structure of the enzyme nestled against the N-terminal end of an α-helix consisting entirely of hydrophobic residues. The Cys 123 is favorably aligned in close proximity to His 202 and Asp 171, in an arrangement reminiscent of the catalytic triad of the serine and cysteine proteases.

One problem associated with the determination of the crystal structure is the ability to obtain sufficient material for crystallization and further study. The enzyme is poorly expressed in *E. coli* hence the level of enzyme activity is less than the activity obtained from fully induced cultures of *Pseudomonas* B13. Low activity is observed even though the enzyme is expressed from multicopy number plasmids. In the original subclone pDO, the enzyme is

expressed from the strong *E. coli lac* promoter. In this clone however, the gene must be transcribed approximately 900 bp before the *clcD* open reading frame is reached. This section of DNA is very GC rich (60 per cent GC) relative to the host and this high GC content may result in frequent stalling by RNA polymerase during transcription. Slowing of RNA polymerase might then result in premature termination. It was assumed that the removal of much of the flanking DNA would remove any sequences contributing to the premature termination of the messenger RNA in *E. coli*. Thus' besides being easy to work with and manipulate, the sized-down *clcD* gene fragment should yield increased level of expression. This is so because many polar effects eliminated by the removal of upstream sequences that might interfere with the expression of the *Pseudomonas* gene in the heterospecific host. Other host vector systems were not considered since vectors for this type of experimentation are not available as broad host range plasmids for use in *Pseudomonas* strains. The purification of DLH from *E. coli* also eliminates the possibility of contaminating activities from other forms of the enzyme present in the original host strain, even at the expense of decreased levels of expression in the heterospecific host.

The *clcD* was sized down also because higher expression of the gene was sought. If the long GC rich leader sequence was causing premature termination, then elimination of this sequence might result in increased expression of the enzyme (Caruthers, 1987). An *Eco*RI site was introduced 50 bp downstream of the *clcD* open

reading frame to further size down the clone and provide for the ability to do directional cloning.

Although an unusually low G+C content (46.9 per cent) with a unique codon usage preference of the ML2 plasmid encoded benzene dioxygenase has been reported recently (Tan *et al.*, 1993) the codon composition of other *Pseudomonas* genes show preferential usage of G and C at the wobble base position (West and Igleweski, 1988). Therefore, the dienelactone hydrolase gene expression may result from a preferential use of codons in the *clcD* open reading frame that are handled by minor species of tRNAs in *E. coli*. The GC content of *E. coli* is 50 per cent. But the codon usage is not random, even when there is no bias in base composition. In *E coli*, many codons for amino acids where degeneracy is observed show marked differences in their usage from one another. Some codons for a specific amino acid are used at a much higher frequency than others, while some codons for the same amino acid may be used infrequently. There is a strong correlation between the frequency of use for a codon and the level of its corresponding tRNA. Thus, proteins which may be expressed at low levels may experience translational control by limiting amounts of tRNA required for translation. A study of the effect of codon usage on translation rate in *E. coli* showed that there is a difference of about 6-fold in translation rate between infrequent codons and common codons (Sørensen *et al.*, 1989). The dienelactone hydrolase gene has a large number of codons that are typically classed as low-use-codons in *E coli*. For example, there are four

codons for the amino acid proline. In the codon usage table obtained from the GenBank genetic sequence data (1992) *E. coli* uses CCG at a frequency of 23.8 (values are given in the use of the codon per 100 residues) and with a combined frequency of19.3 by the other three codons (CCU, CCC, CCA). But in *clcD*, only 4 out of 14 proline residues are coded by the preferred CCG. The largest number of codons for proline in the *clcD* gene open reading frame were CCC (5 out of 14). In *E. coli* this codon had the lowest frequency usage (4.3) amongst all codons for proline. Dienelactone hydrolase uses the minor CCC most frequently, though this codon may be recognized by a tRNA present only at low levels in *E. coli* cells. If sufficient levels of the tRNA for proline that recognizes CCC were not available, expression of dienelactone hydrolase would be limited by the availability of the charged corresponding tRNA. Other codons for minor tRNA species (in *E. coli*) used significantly by the *clcD* gene are ACA and ACG for threonine, GUC and GUG for valine, and UCA and UCG for serine. In addition to using codons for minor tRNAs frequently, little use may be made by the *clcD* gene of favored codons, corresponding to tRNAs present in high levels in *E. coli*. For example, the codons UCU and UCC are most frequently used in *E. coli* but the dienelactone hydrolase gene does not contain a single case of these two common, high usage codons.

The plasmid pAC27 encoding the 3-chlorobenzoate degradative enzymes on *P. putida* AC866 has been thoroughly investigated (Chatterjee and Chakrabarty, 1984). From *Pseudomonas* sp. B13, the

genes for chlorocatechol metabolism have been shown to transmissible to various recipients (Reineke *et al.*, 1982). However, the plasmid, named pB13 or pWR1, was difficult to isolate and gave low yields (Chatterjee and Chakrabarty, 1983; Weisshaar *et al.*, 1987). It was found to be very similar to pAC25, the precursor of pAC27 (Chatterjee and Chakrabarty, 1982, 1983). Furthermore, the dienelactone hydrolases purified from *Pseudomonas* sp. B13 and from a clone pAC27 genes were found to be identical in every discernible respect (Frantz *et al.*, 1987; Ngai *et al.*, 1987). The dienelactone hydrolases of *Pseudomonas* sp. B13 and pAC27 are therefore equivalent in this study.

The *clcD* gene used in these studies was generated by PCR amplification. Synthesis of the DNA occurs by primer extension of specifically designed oligonucleotides. Since the clone was ultimately to be used for the purification of the enzyme for protein crystallization and X-ray analysis, the resulting clone (pNDO) had to be shown to contain no sequence changes from the known, characterized sequence. DNA sequence analysis of the sized-down clone in pNDO showed sequence disparity at four different positions. Two nucleotide errors were discovered that resulted in a single codon change and therefore a single amino acid change in the translated protein sequence. The sequence beginning at residue 286 (residue 761, using the numbering system of the published *clcD* gene sequence by Frantz *et al. in* 1987) reads GAG **GCG** CAG. The parent plasmid (pDO) gave the identical sequence about this region, but the

published sequence reads GAG **CGC** CAG. This can best be viewed as actually a two base change, not the immediately apparent three base difference between the two sequences. The experimental determination is a G insertion and a C deletion. This region of the *clcD* gene is extremely GC rich and thus difficult to sequence. This was even more so at the time when the original sequences were determined because since techniques to avoid GC compression were not yet so well developed. DNA that is GC rich shows the phenomenon of GC compression when sequencing procedures are performed. The sequences of the clones reported in this work require verification because the genes are not native genes but artificially produced by *in vitro* DNA synthesis using PCR. In the synthesis of DNA during PCR cycling, a thermostable polymerase is used. The *Taq* polymerase possesses the ability to survive multiple rounds of temperature cycling and efficiently replicates DNA. But the *Taq* polymerase does not possess a 3' to 5' proofreading activity typical of other DNA polymerases. Use of *Taq* polymerase requires caution when the accuracy of the DNA produced is required. In order to confirm the sequence of the sized-down *clcD* clone in pNDO, both strands were sequenced, and the parent was sequenced concomitantly. The sequence about the region discussed above yields an amino acid sequence of Ala-Gln-Arg. The corrected nucleotide sequence has been independently confirmed in the parent plasmid (pDO) by Cheah *et al.* (1993). Alanine at position 79 fits the

electron density pattern about this region of the crystal structure perfectly (Cheah *et al.*, 1993).

In addition there were two other nucleotide changes in the *clcD* open reading frame from plasmid pNDO relative to the published sequence (see Results). Both of these changes produced no change in the amino acid sequence, and require no reexamination of the electron density map for correctness. Indeed, the sequences are probably in error in the published account due to typographical errors, since the published nucleotide sequence does not yield the published amino acid sequence when translated.

In this study residues were modified in order to characterize the active site with greater precision than is presently possible. All of the chosen residues are located at or near the active site. The active site is found at the N-terminal end of helix D which is composed entirely of non-polar residues, except for Lys 135. These residues are believed to be involved in catalysis, binding of the anionic substrate, or stability of transition state.

The goal of modifying a protein's amino acid sequence at will is becoming a reality. When amino acid substitutions are introduced into the primary sequence of a protein, the assumption is made that nothing radical has been changed in the enzyme. That is to say, the protein has experienced no drastic changes in structure and the catalytic importance of individual residues may be probed. For residues that obviously participate in catalysis, substitution will result in complete loss of activity. Changes in amino acid residues

that influence binding may alter only the enzyme's kinetics. Other properties that might be affected besides the kinetic properties k_{cat} and k_M include specificity, pH optimum, temperature stability and the isoelectric point.

Modification of Glu36 to Gln destroyed all enzyme activity. This observation substantiates the model proposed by Cheah *et al.* (1993) to explain the triad activity in DLH. The resting form of DLH has Cys 123 in an inactive conformation where the protonated thiol is rotated away from the histidinium cation and is hydrogen bonded to the carboxylate of Glu 36. The Glu 36 is also ion paired with the guanidinium group of Arg 206. In this model, Glu 36 is the critical residue which couples the binding of the substrate with conversion from the inactive to the active conformation. Binding of the anionic substrate weakens the ion pairing between Arg 206 and Glu 36, resulting in proton transfer from Cys123 to Glu36. As the thiolate anion is not energetically favored in the hydrophobic interior of the enzyme, it swings into the active site where it can be stabilized by His 202 imidazolium and the dipole of helix C. The Cys 123 thiolate which now lies adjacent to the acyl carbon of the substrate, is thus generated only in the presence of substrate.

Modification of Arg 81 to alanine resulted in an enzyme with a k_M value that is two-fold higher than that of the parent enzyme. This indicates that Arg 81 is involved in binding the substrate. In a recent study involving an amide analog (dienelactam) of the ester substrate bound to the modified enzyme DLHC123S it was shown

that dienelactone is held in the active site by hydrophobic interactions around the lactone ring and by the ion pairs between its carboxylate and Arg 81 and Arg 206. Therefore, the active site mirrors the amphipathic nature of the substrate in that it is divided into a hydrophobic and a hydrophilic region. The nucleophile lies at the heart of the hydrophobic region. The polar region of the active site is distal to the nucleophile and it includes the side chains of Arg 81, Arg 206, and Ser 203.

The specific activity of DLHR81A is around 2.5 fold higher than that of DLH. This however, may be attributed to codon usage as discussed earlier. The codon AGA has the second lowest frequency usage (2.1) amongst all 6 codons that code for arginine whereas GCA has the third highest frequency usage (20.8) of all 4 codons for alanine. Hence, this higher specific activity of DLHR81A could be due to having more enzyme being expressed in this mutant because the wild type rare codon for arginine was limiting expression and the change to the common alanine codon relieved this translational restraint.

In summary, the role of individual amino acid residues of the enzyme dienelactone hydrolase was investigated. Using the polymerase chain reaction (PCR) to amplify mutagenic DNA primers, a 1.9 kbp *clcD* fragment was amplified and subcloned yielding a 821 bp *Bam*HI to *Eco*RI *clcD* subclone in the plasmid pUC19. This plasmid was termed pNDO. Site-specific mutants of dienelactone hydrolase were then created by using mismatched oligonucleotides to prime

DNA synthesis in the plasmid subclone pUNT. Specifically modified protein mutants of dienelactone hydrolase from mutated *clcD* genes (Arg 81 to alanine, Glu 36 to Gln) were constructed and cloned. Enzyme assays showed that dienelactone hydrolase activity of the mutant Glu 36 to Gln was totally abolished. The DLHase mutant of Arg 81 to alanine resulted in a two fold increase in the k_M value. These mutant proteins will be useful for future studies of the enzyme. When the mutations are characterized in purified preparations of each modified enzyme, the changes in catalytic and physical properties such as stability, pH dependence, substrate specificity, substrate binding, catalytic efficiency, and physical structure can be investigated. The mutant proteins provide material for comparative studies on the structural analysis of dienelactone hydrolase by X-ray crystallography. This assortment of mutant proteins will be valuable to define and further refine our understanding of the mechanism of the enzyme and the relationship between structure and function within the enzyme dienelactone hydrolase.

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