Mandelate Dehydrogenases From *Rhodotorula graminis*

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DEDICATION

To my parents, my wife Faezah and my family.

DECLARATION

I hereby declare that this thesis was composed by myself and that I carried out the work presented here, except indicated in the text.

Rosli Md Illias

May 1997

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ABSTRACT

Rhodotorula graminis is a yeast with the ability to utilise mandelate as a source of carbon and energy. Mandelate exists in two enantiomeric forms; D-mandelate and L-mandelate. *R. graminis* possesses both D(-)-mandelate dehydrogenase and L(+)-mandelate dehydrogenases which enable this organism to use both forms of mandelate. The L(+)-mandelate dehydrogenase (LMDH) is a soluble component of the mitochondrial intermembrane space and is similar to L(+)-lactate dehydrogenase (LLDH) from *S. cerevisiae* and *H. anomala* which is a flavocytochrome b_2. The D(-)-mandelate dehydrogenase is an unrelated enzyme which is a soluble NAD-dependent enzyme.

In order to study these two proteins the genes encoding LMDH and DMDH were isolated, sequenced and expressed. An 81 bp fragment was amplified by RT-PCR and used to probe the gene encoding the LMDH from *R. graminis*.The genomic DNA was isolated from a genomic library and then the cDNA was amplified by RT-PCR. Comparison of the genomic DNA and the cDNA sequences reveals the presence of eleven introns in the genomic DNA encoding LMDH. The conserved intron sequences of LMDH in *R. graminis* are similar to the intron sequences from the phenylalanine-ammonia lyase (PAL) gene *in Rhodosporidium toruloides* and *Rhodotorula rubra*. The LMDH from *R. graminis* is predicted to contain a 74 amino acid extension at the N-terminus. This presequence is highly basic and contains a long stretch of non-polar amino acids, typical of a subclass of mitochondrial targeting sequences. The 1479 bp cDNA specifies a mature LMDH of 492 amino acids including the incorporated N-terminal methionine. The calculated Mr is 54,604. Computer search with other proteins in the database shows similarity with other FMN-dependent 2-hydroxyacid oxidising enzymes. LMDH is very closely related to flavocytochrome b_2 from *S. cerevisiae* and *H. anomala*. Based on the crystal structure and the amino acid sequence comparison with L(+)-lactate dehydrogenase from *S. cerevisiae*, LMDH from *R. graminis* could be divided into two domains, the haem binding domain and the flavin binding domain. The amino acid sequence of the predicted cytochrome domain shows high similarity with microsomal b_5.

Comparison of the amino acid sequence of LMDH and LLDH from *S. cerevisiae* shows that all the residues important in catalysis and substrate binding of the enzyme are highly conserved. Amino acid sequence comparison also reveals that Leu230 in LLDH from *S. cerevisiae*, which determins the substrate specificities of the enzyme, is replaced by a smaller glycine in LMDH from *R. graminis.*

The genomic DNA and the cDNA of D(-)-mandelate dehydrogenase have been isolated and amplified respectively. Comparison of the genomic DNA and the cDNA revealed the presence of three introns. All the introns start with GT, have the sequence of CAG at the 3' end and have an internal consensus sequence of CTGAC. The entire 1053 bp sequence of the amplified cDNA specifies a protein (DMDH) of 351 amino acids with the calculated Mr of 38,591. A computer search with other proteins in the database shows that DMDH from *R. graminis* belongs to the D-isomer-2 hydroxyacid dehydrogenase family. Based on the crystal structure and amino acid sequence from D-glycerate dehydrogenase from *Hyphomicrobium methylovorum* it is predicted that the polypeptide chain of DMDH is divided into two domains, the catalytic domain which is formed by approximately residues 1 to111 and 308 to 351 and the second coenzyme-binding domain which is located in the middle of the polypeptide chain approximately between residue 111 to 307. Arg259 in DMDH is believed to be important in substrate binding as in the other D-isomer specific dehydrogenases. His304 acts as an acid/base catalyst. The highly conserved sequence G-X-G-X-X-G-17X-D which is common to the NAD-binding domain is conserved in DMDH except that the third glycine is replaced by glutamine. The function of glutamine here is not clear and can only be determined when the DMDH crystal structure becomes available.

Both LMDH and DMDH were succesfully expressed by using the expression vector pRC23 which is thermoinducible. The calculated k_{cat} and Km of the partially purified recombinant LMDH is 350 s^{-1} and 0.35 mM respectively. The specific activity for DMDH is 0.0528 units/mg.

ABBREVIATIONS

ADP	adenosine diphosphate
APS	ammonium persulphate
ATCC	American Typestrain Culture Collection
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CoA	coenzyme A
dNTP	deoxynucleotide triphosphate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
dITP	deoxyinosine triphosphate
ddATP	dideoxyadenine triphosphate
ddCTP	dideoxycytosine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddTTP	dideoxythymidine triphospphate
dH_2O	distilled water
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetate
EMBL	European Molecular Biology Laboratory
FMN	flavin mononucleotide
IPTG	isopropyl thiogalactoside
kb	kilo base
kDa	kilo dalton

LB	Luria broth
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
RNA	ribonucleic acid
Rnase	ribonuclease
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

For nucleic acid sequences:

A	adenine
C	cytosine
G	guanosine
T	thymidine
Y	C or T
R	A or G
N	T or C or G or A

Abbreviations for amino acids

Amino acid	Three letter abbreviation	One letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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Introduction

1.1 Mandelic acid.

Mandelic acid (2-hydroxy-2-phenylacetic acid) is an aromatic α-hydroxy acid which occurs naturally in plants for example in grape (Cologrande, 1959). There are two enantiomeric forms of mandelate: D-mandelate and L-mandelate. Much of the mandelate found in nature is product by degradation of other compounds or as an excretory product in animal urine (Luthe *et al.*, 1983). Mandelic acid is also found in the body after industrial or domestic exposure to styrene (Chakrabarti, 1979) or from using various pharmaceutical products eg 3,3,5-trimethylcyclohexanyl mandelate, a vasoactive drug (Middleton *et al.*, 1983).

Mandelate does not seem to be a particularly common compound in most natural environments, but several microorganisms have been isolated that are able to metabolise mandelate (Fewson, 1988). This apparent paradox could be because many of the mandelate enzymes tolerate ring-substituted mandelates which could be the principle substrates for degradation. D(-)-4-hydroxy-3-methoxy mandelate, 3,4-dihydroxymandelate, 4-hydroxy-3-methoxy phenylglycol and 3,4-dihydroxymandelate are metabolites of adrenaline and noradrenaline (Goodhal and Alton, 1969). These compounds are continually being excreted into the environment in the urine and faeces of normal animals (Lun *et al.*, 1976) and greater amounts are excreted in certain pathological conditions (Tuchman *et al.*, 1985). Octopamine, an invertebrate neurotransmitter also found in mammalian brain, is metabolised to 4-hydroxymandelate which is excreted (Axelrod and Saavedra, 1977). Finally, the fungi *Polyporus hispidus* (Perrin and Towers, 1973), *Penicillium chrysogenum* (Hockenhall *et al.*, 1952) and *Aspergillus niger* (Kishore *et al.*, 1974) can convert L-phenylalanine, phenylacetate and L-tyrosine into mandelate and 4-hydroxymandelate. All these contribute to the presence of mandelate and substituted mandelate in the natural environment.

1.2 Microorganisms that can utilise mandelate.

Mandelate can be utilised by a limited range of bacteria and fungi, including some yeasts. In prokaryotes, studies on the ability of *Pseudomonas aeruginosa* to utilise mandelate showed that all strains of this organism which have been tested can grow on L(+)-mandelate but not on D(-)-mandelate (Stanier *et al.*, 1966). However, in *Pseudomonas putida*, from 41 strains tested 3 can grow on both enantiomers and 5 can only grow on the D form of mandelate (Stanier *et al.*, 1966). Mandelate utilising strains of various species eg *Arthrobacter*, *Azotobacter*, *Bacillus*, *Nocardia*, *Rhizobium* and *Rhodopseudomonas spp* have been reported (Fewson, 1988; Chen *et al.*, 1989). There are also some organisms that cannot utilise either enantiomer of mandelate, but can grow on phenylglyoxylate (the initial product of mandelate oxidation). These include a number of strains of *Acinetobacter calcoaceticus*, *Arthrobacter spp*, *Pseudomonas fluorescens*, *P. multivorans* and *P. putida* (Fewson, 1988). In eukaryotes, *Aspergillus flavus*, *Byssochlamys fulva* and the yeast *Rhodotorula graminis* can grow well on both enantiomers of mandelate (Iyayi and Dart, 1980; Dart and Iyayi, 1981; Durham *et al.*, 1984). In addition strains of *Aspergillus niger* and *Neurospora crassa* can grow on mandelate with the presence of other carbon sources (Jamaluddin *et al.*, 1970; Ramakrishna and Vaidyanathan, 1977).

1.3 *Rhodotorula graminis*

The yeast *Rhodotorula graminis* was first isolated by Di Menna (1958) from the leaves of pasture grasses in New Zealand. Durham *et al.* (1984) have isolated a strain of *R. graminis* from soil which can use mandelate as a source of carbon and energy. The strain of *R. graminis* which was isolated does not form true mycelia or ballistospores and can utilise nitrate or nitrite as a source of nitrogen. Durham *et al.* (1984) showed that *R. graminis* differs from other yeasts such as *Trichosporon cutaneum* and *Rhodotorula mucilaginosa* in that it contains both branches of the β-

ketoadipate pathway. The ability to utilise mandelate is not a universal characteristic within the genus *Rhodotorula* (Fewson *et al.*, 1993).

1.4 Pathways for the metabolism of mandelate.

Mandelate is initially attacked through stereospecific dehydrogenation to phenylglyoxylate. Mandelate utilisation can also be initiated by racemisation or ring hydroxylation (Fig. 1.1; Fewson, 1988, 1992).

In the bacterium *Pseudomonas putida,* D-mandelate is converted into L-mandelate by mandelate racemase. The L-mandelate then is oxidised by L(+)-mandelate dehydrogenase to form phenylglyoxylate. Phenylglyoxylate will then be converted to benzaldehyde by phenylglyoxylate decarboxylase. Some strains of *Pseudomonas putida* (Stanier *et al.*, 1966) possess only D-mandelate dehydrogenase but lack mandelate racemase (Kenyon and Hageman, 1979) and L(+)-mandelate dehydrogenase, so can only metabolise D-mandelate. There are also strains of *Pseudomonas putida* which have mandelate racemase and only D(-)-mandelate dehydrogenase so can grow on both enantiomers of mandelate. Benzoate enters the β-ketoadipate pathway through catechol and is further metabolised to form succinate and acetyl CoA (Stanier and Ornston, 1973). *Pseudomonas aeruginosa* (Rosenberg, 1971) metabolises mandelate through the same pathway as in *Pseudomonas putida* except that it can only metabolise L-mandelate. *Acinetobacter calcoaceticus* metabolises mandelate just like the other two bacteria mentioned above. Not all strains of *Acinetobacter calcoaceticus* can metabolise both forms of mandelate. Some strains have only L(+)-mandelate dehydrogenase so can only metabolise L-mandelate and some have only D(-)-mandelate dehydrogenase and can only grow on D-mandelate. There are also strains which can use both D- and L-mandelate (Baumann *et al.*, 1968; Hill and Fewson, 1983). In *Pseudomonas convexa,* mandelate undergoes ring hydroxylation rather than dehydrogenation to form 4-hydroxymandelate which is then converted directly to 4-hydroxybenzaldehyde. 4-hydroxyphenylglyoxylate does not act as intermediate. In *Pseudomonas convexa* it

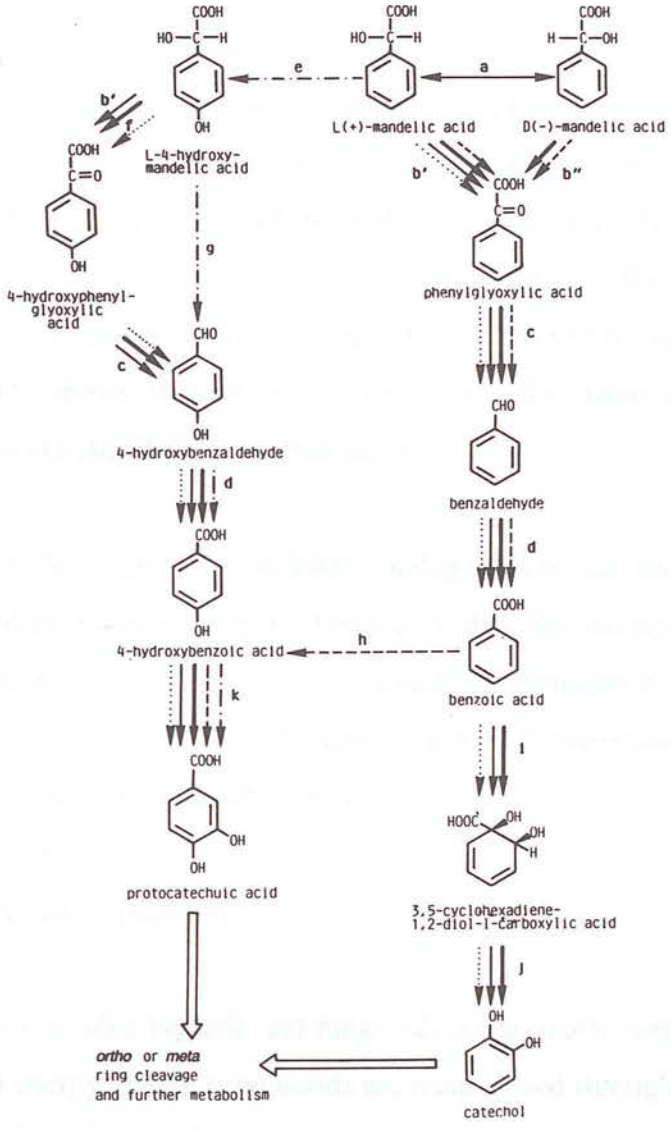

Figure 1.1. Pathway for metabolism of mandelate in: *Acinetobacter calcoaceticus* (⟶); *Pseudomonas putida* (⟶); *P. convexa* (-·-·⟶); *Rhizobium leguminosarum* (·····⟶); *Aspergillus niger*, *Neurospora crassa* and *Rhodotorula graminis* (----⟶) enzyme: a, mandelate racemase; b', L-mandelate dehydrogenase; b'',D-mandelate dehydrogenase; c, phenylglyoxylate decarboxylase; d, benzaldehyde dehydrogenase; e, L-mandelate 4-hydroxylase; f, L-4-hydroxymandelate dehydrogenase; g, L-4-hydroxymandelate oxidase; h, benzoate 4-hydroxylase; i, benzoate 1,2-oxygenase; j, 3,5-cyclohexadiene-1,2-1-carboxylate dehydrogenase; i+j = 'benzoate oxidase'; k, 4-hydroxybenzoate 3-hydrolase. (Fewson, 1988)

follows a slightly different pathway from other bacteria. The substrate for ring cleavage is protocatechuate rather than catechol (Bhat *et al.*, 1973; Bhat and Vaidyanathan, 1976).

Filamentous fungi such as *Aspergillus niger* (Jamaluddin *et al.*, 1970) and *Neurospora crassa* (Ramakrishna and Vaidyanathan, 1977) and the yeast *Rhodotorula graminis* (Durham *et al.*, 1984) contain two stereospecific mandelate dehydrogenases to enable them to grow on both enantiomers of mandelate. In these organisms D,L-mandelate is metabolised to benzoate through the same pathway as in bacteria but then it enters the protocatechuate branch for further ring cleavage. Studies on *Aspergillus flavus* (Iyayi and Dart, 1980) and *Byssochlamyfulva* (Dart and Iyayi, 1981) shows that these two organisms also have a similar pathway for metabolism of mandelate as in other fungi.

A wide range of mandelate analogues can be metabolised or partially metabolised by some organisms (Fewson, 1988). For example, as shown in figure 1.1, 4-hydroxymandelate can be degraded to protocatechuate by *Acinetobacter calcoaceticus* (Kennedy and Fewson, 1968), *Pseudomonas putida*, *Rhizobium leguminosarum*, and *Aspergillus niger*.

1.5 β-ketoadipate pathway

Many aerobic bacteria and fungi can use aromatic compounds as a source of carbon and energy. These compounds are metabolised through specialised metabolic pathways where these substrates are converted to aliphatic compounds. One of the major microbial pathways for the dissimilation of aromatic compounds is the β-ketoadipate pathway, which provides a mechanism for the utilisation of many different primary substrates in bacteria and fungi (Ornston and Stanier, 1964; Stanier and Ornston, 1973; Harwood and Parales, 1996). The β-ketoadipate pathway has been shown by biochemical study to be used by most yeast and fungi (Cain *et al.*,

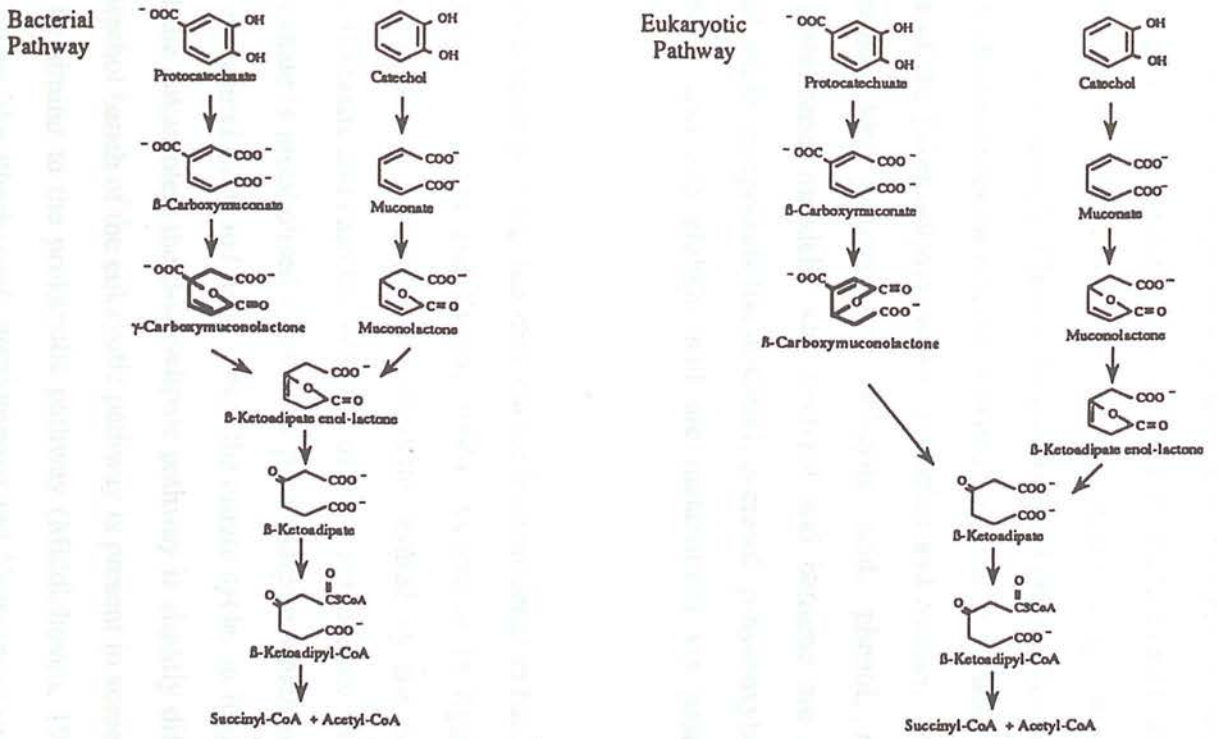

Figure 1.2. The β-ketoadipate pathway in bacteria and eukaryote (Harwood and Parales, 1996).

1968; Cook *et al.*, 1974) including *R. raminis* (Duham *et al.*, 1984) for aromatic substrate metabolism.

Various primary substrates dissimilated through this pathway are initially converted either to protocatechuate or catechol. These two compounds are the entry sites of the two parallel and convergent branches of the β-ketoadipate pathway (Fig. 1.2). Catechol and protocatechuate undergo ring fission either by meta- or ortho-cleavage. The ring fission is catalysed by dioxygenases and is termed ortho-cleavage when it occurs between the hydroxyl groups and meta-cleavage when it occurs adjacent to one of the hydroxyls. Cleavage of catechol and protocatechuate by ortho-cleavage leads to the formation of muconic acid and channels its metabolism into one of the branches of the β-ketoadipate pathway (Ornston and Stanier, 1964; Gibson, 1968). Compounds like benzoic acid, salicylic acid, phenol, naphthalene, phenanthrene, anthracene, mandelic acid, o-cresol and benzene are metabolised through catechol while compounds like m-cresol, ρ-cresol, ρ-hydroxybenzoic acid, ρ-hydroxymandelic acid and phthalic acid are metabolised via protocatechuate (Gibson, 1968).

The β-ketoadipate pathway has been studied in most detail in bacteria such as *Pseudomonas putida* (Ornston and Stanier, 1966). As shown in figure 1.2, β-ketoadipate enol-lactone is a common intermediate formed by the two separate branches (protocatechuate and catechol pathway) of the β-ketoadipate pathway. This common intermediate is metabolised to form the β-ketoadipate which is cleaved to yield succinate and acetyl CoA and hence enters the citrate cycle. In fungi and yeast which represent the eukaryotes, the β-ketoadipate pathway is slightly different from bacteria. The catechol banch of the eukaryotic pathway is present in some eukaryotes and appears to be similar to the prokaryotic pathway (Middlehoven, 1993; Cain *et al.*, 1968). Organisms like *Rhodotorula mucilaginosa* and *Neurospora crassa* appear to have only the protocatechuate branch of the β-ketoadipate pathway (Cook and Cain, 1974; Cain, 1969). The protocatechuate branch in eukaryotes differs from the pathway in prokaryotes in that 3-carboxymuconate is converted to β-

carboxymuconolactone but in prokaryotes the product is γ-carboxymuconolactone (Harwood and Parales, 1996). Another difference is that the two branches of the fungal β-ketoadipate pathway converge at β-ketoadipate rather than at β-ketoadipate enol lactone, as in the bacterial pathway (Fig. 1.2).

1.6 Mandelate dehydrogenases

1.6.1 Introduction

There are several different types of mandelate dehydrogenases, depending on the enantiomer that is oxidized and the nature of the organism (Fewson, 1992). Mandelate dehydrogenases can be divided into two major groups: the NAD-independent enzymes and the NAD-dependent enzymes (Fig.1.3). Some bacterial and yeast mandelate dehydrogenases and lactate dehydrogenases are clearly related (Fewson,1992; Fewson *et al.*, 1993). Both are 2-hydroxy acid dehydrogenases and catalyse the same chemical reaction, but their substrates have different side groups; a methyl group in the case of lactate whereas mandelate has a phenyl ring.

1.6.2 Bacterial mandelate and lactate dehydrogenases

D- and L-mandelate dehydrogenases and D- and L-lactate dehydrogenases from *Acinetobacter calcoaceticus* have been purified and characterised. All four enzymes are NAD(P)-independent and are integral components of the cytoplasmic membrane (Allison *et al.*, 1985). D-mandelate dehydrogenase and D-lactate dehydrogenase are similar to each other. Both enzymes are monomeric proteins of similar Mr (60,000), containing non-covalently bound FAD as prosthetic group (Allison *et al.*, 1985b) and in this respect they resemble the membrane-bound D-lactate dehydrogenase from *E. coli* (Futai, 1973) which also contains FAD and has an Mr of 65,000. The N-terminal sequences of the three enzymes also show striking similarities (Fewson *et al.*, 1993). In contrast, purification of L-mandelate dehydrogenase (Hoey *et al.*, 1987) and L-lactate dehydrogenase (Allison and

Fewson, 1980) from *A. calcoaceticus* shows that both enzymes contain non-covalently bound FMN as cofactor and are very similar to each other and to the L(+)-lactate dehydrogenase from *E. coli* (Hoey *et al.*, 1987; Futai and Kinura, 1977). All these three L-isomer dehydrogenases are very similar in their Mr which is about 40,000 (Fewson *et al.*, 1993). The D-lactate dehydrogenases from *A. calcaoceticus* and *E. coli* exist as oligomers but the L-mandelate dehydrogenase from *A. calcaoceticus* is a monomer.

Purification of the L-mandelate dehydrogenase from *P. putida* shows that the Mr value is 49,000 which is very similar to the L(+)-mandelate dehydrogenase from *A. calcaoceticus.* The former contains FMN bound non-covalently as a cofactor. Tsou *et al.* (1990) have isolated the gene encoding L-mandelate dehydrogenase from *P. putida* and amino acid sequence comparison shows 62% identity between the 50 N-terminal amino acids of the L-mandelate dehydrogenases from *A. calcaoceticus* and *P. putida* (Fewson *et al.*, 1993). Tsou *et al.* (1990) also discovered that L(+)-mandelate dehydrogenase from *P. putida* has extensive sequence identity with the FMN-containing enzyme glycolate oxidase and the FMN domain of L(+)-lactate dehydrogenase from *S. cerevisiae* and *H. anomala.* All of the bacterial mandelate and lactate dehydrogenases which have been discussed above are membrane bound.

1.6.3 Mandelate dehydrogenases from *R. graminis*

The situation in *R. graminis* is rather different from that in bacteria. The D-mandelate dehydrogenase is a soluble NAD-dependent enzyme (Baker and Fewson, 1989) which has superficial resemblance to the NAD-dependent D(-)-mandelate dehydrogenase from *Lactobacillus curvatus* (Hummel *et al.*, 1988) and D(-)-2-hydroxyisocaproate dehydrogenase from *Streptococcus faecalis* (Yamazaki and Maeda, 1986). All three enzymes exists as dimers with identical subunits.

The L(+)-mandelate dehydrogenase from *R. graminis* is a totally different enzyme from the D(-)-mandelate dehydrogenase. LMDH is a flavocytochrome b_2

similar to L(+)-lactate dehydrogenase from *Saccharomyces cerevisiae* and *Hansenula anomala* (Yasin and Fewson, 1993). The similarities between mandelate dehydrogenase and lactate dehydrogenase have led to the suggestion that mandelate dehydrogenase probably arose by the duplication and mutation of gene for another 2-hydroxyacid dehydrogenase (Tsou et *al.*, 1990).

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1. NAD-independent

(a) FMN-dependent

(i)FMN, non-haem, Mr= approx. 44000

L(+)-mandelate dehydrogenase of *Acinetobacter calcoaceticus*
L(+)-mandelate dehydrogenase of *Pseudomonas putida*
L(+)-lactate dehydrogenase of *Acinetobacter calcoaceticus*
L(+)-lactate dehydrogenasse of *Escherichia coli*

(ii)FMN, haem, Mr = approx. 59000

L(+)-mandelate dehydrogenase of *Rhodotorula graminis*
L(+)-lactate dehydrogenase of *Saccharomyces cerevisiae*
L(+)-lactate dehydrogenase of *Hansenula anomala*

(b) FAD-dependent, Mr = approx. 60000

D(-)-mandelate dehydrogenase of *Acinetobacter calcoaceticus*
D(-)-lactate dehydrogenase of *Acinetobacter calcoaceticus*
D(-)-lactate dehydrogenase of *Escherichia coli*

2. NAD-dependent

D(-)-mandelate dehydrogenase of *Rhodotorula graminis*
D(-)-mandelate dehydrogenase of *Lactobacillus curvatus*
D(-)-hydroxyisocaproate (mandelate) dehydrogenase of *Streptococcus faecalis*
D(-)-hydroxyisocaproate dehydrogenase of *Lactobacillus casei*

Table 1.1. Relationship among some mandelate and lactate dehydrogenases (Fewson *et al.*, 1993)

1.7 Flavocytochrome b_2

1.7.1 Introduction

Flavocytochrome b_2 was first purified by crystallization from *Saccharomyces cerevisiae* by Appleby and Morton (1954), but the protein was found to be unstable. Jacq and Lederer (1972) isolated the intact form of enzyme in the presence of

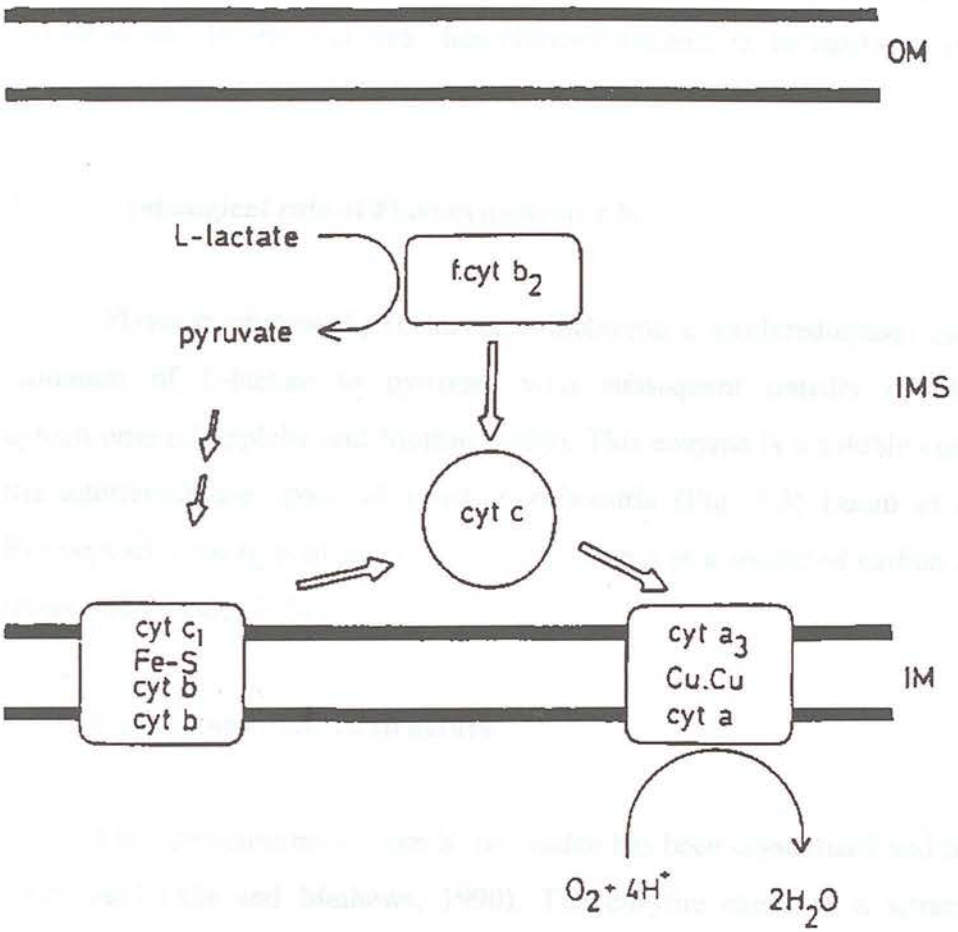


Figure 1.3. Flavocytochrome b_2 at the intermembrane space of yeast mitochondria. OM, outer membrane; IMS, inter membrane space; IM, inner membrane; cyt, cytochrome; f.cyt b_2, flavocytochrome b_2.

phenylmethylsufonyl fluoride, a serine protease inhibitor because when originally isolated the flavocytochrome b_2 had undergone proteolysis by endogenous yeast proteases. The intact polypeptide has a Mr of about 57,500. Flavocytochrome b_2 was also isolated from *Hansenula anomala* (Labeyrie and Baudras, 1972). The genes encoding flavocytochrome b_2 from *S. cerevisiae* and *H. anomala* have both been isolated and the sequences determined (Guiard *et al.*, 1985; Black *et al.*, 1989a). Flavocytochrome b_2 from *S. cerevisiae* has been succesfully expressed in *E. coli* (Black *et al.*, 1989b) and this has allowed mutants to be made by site directed mutagenesis (Reid *et al.*, 1988).

1.7.2 Physiological role of Flavocytochrome b_2

Flavocytochrome b_2 (L-lactate:cytochrome c oxidoreductase) catalyses the oxidation of L-lactate to pyruvate with subsequent transfer of electrons to cytochrome c (Appleby and Morton, 1954). This enzyme is a soluble component of the intermembrane space of yeast mitochondria (Fig. 1.3; Daum *et al.*, 1982). Flavocytochrome b_2 enables yeast to use L-lactate as a source of carbon and energy (Pajot and Claisse, 1974).

1.7.3. Flavocytochrome b_2 structure

Flavocytochrome b_2 from S. *cerevisiae* has been crystallised and its structure determined (Xia and Mathews, 1990). The enzyme exists as a tetramer of Mr 230,000 (Labeyrie and Baudras, 1972) which consists of four identical subunits arranged about a molecular four fold axis (Fig. 1.4). Each subunit (Fig. 1.5) is divided into two structural domains, a smaller haem binding domain (cytochrome domain) which comprises residues 1 to 99 and a larger flavin binding domain (residues 100 to 486).

The cytochrome domain of flavocytochrome b_2 is folded in a similar fashion to the homologous soluble fragment of cytochrome b_5 (Xia and Mathews, 1990).

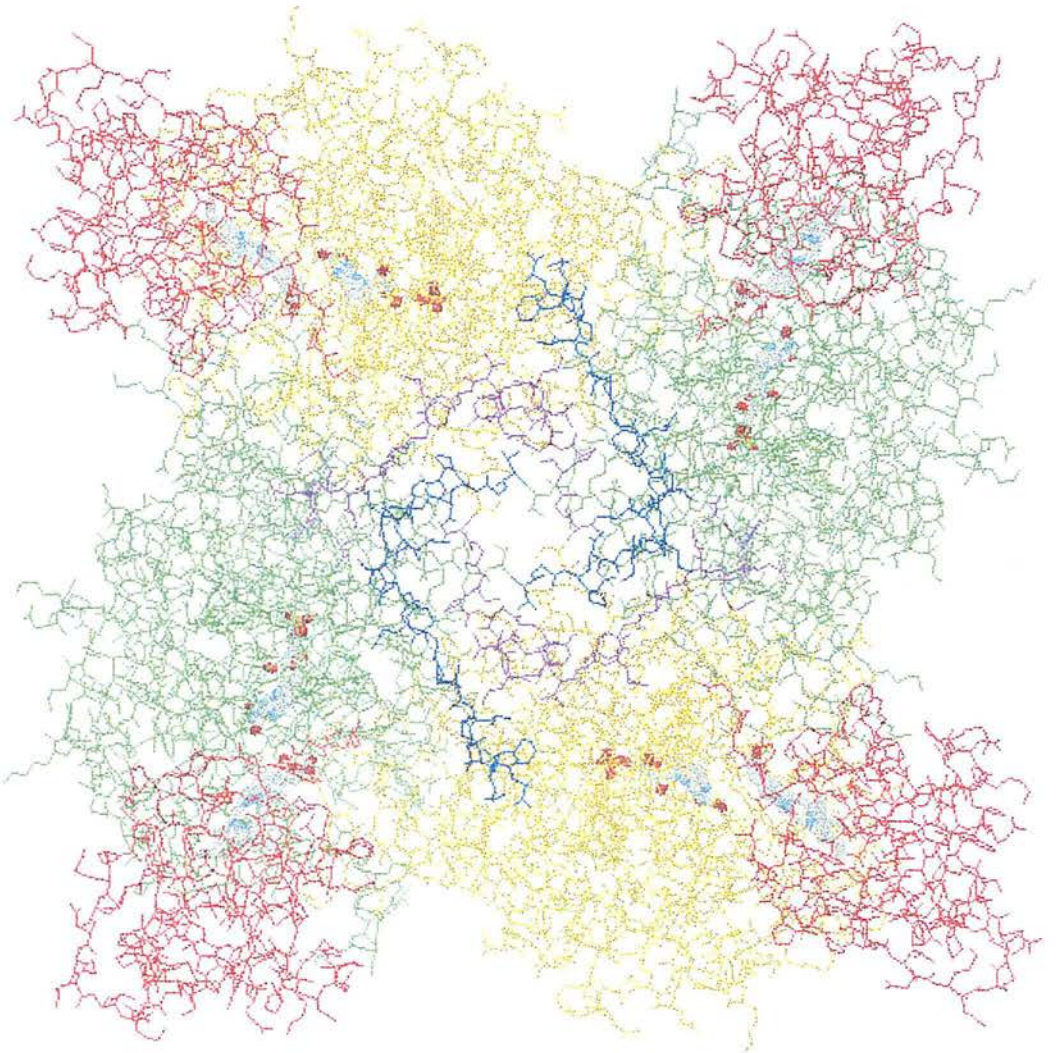


Figure 1.4. Flavocytochrome b_2 in tetrameric form. Yellow and green represent the FMN-binding domain while the cytochrome domain is in red for each subunit. The FMN and haem are in grey. Blue and purple represent the C-terminal tail. (Xia and Mathews, 1990)

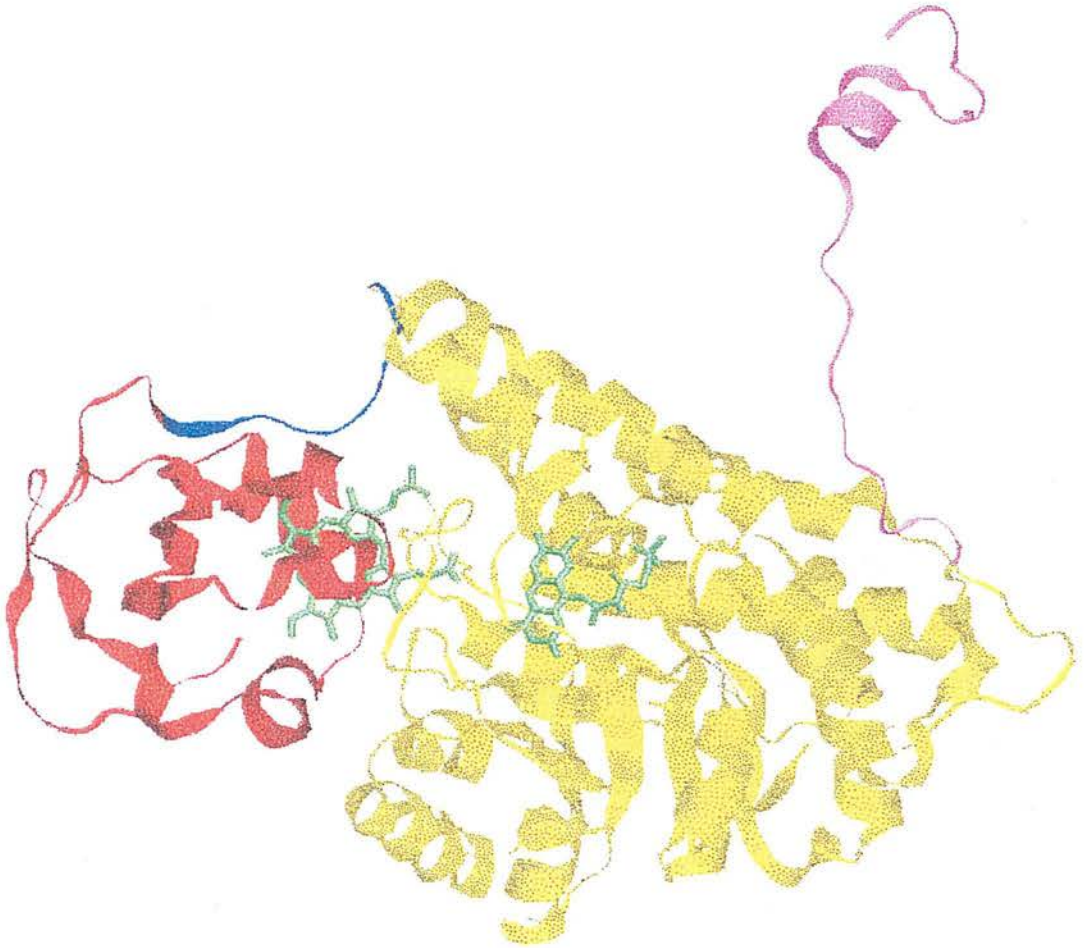


Figure. 1.5. Subunit of flavocytochrome b_2. The cytochrome domain is in red and the FMN-binding domain is in yellow. Blue, hinge region; purple, C-terminal tail. FMN and haem are shown in green.

The cytochrome domain is located on the outer edge of the tetramer protruding away from the four fold axis. The flavin binding domain is connected with the cytochrome domain through a single polypeptide (hinge region).The flavin binding domain contains a parallel $\alpha_8\beta_8$ structure (Banner *et al.*, 1975) similar to glycolate oxidase from spinach (Lindqvist and Branden, 1985) and trimethylamine dehydrogenase from the methylotrophic bacterium W_3A_1 (Lim *et al.*, 1986). Extended from the flavin binding domain is the C-terminal tail which wraps around the four fold axis and makes contact with each of the other three subunits.

There are two crystallographically different subunits observed in the flavocytochrome b_2 structure. In subunit one both the cytochrome and flavin binding domain are visible in the electron density map, but in the second subunit the cytochrome domains are disordered. NMR spectra (Labeyrie *et al.*, 1988) indicate that the cytochrome domain is relatively mobile. The subunit interface between the flavodehydrogenase and cytochrome domain is dominated by nonpolar contacts. Ten residues of the flavodehydrogenase domain are involved in nonpolar van der Waals interaction with nine amino acid side chains and the haem group. Lys296 has been shown to form an interdomain salt bridge with one of the haem propionate groups in subunit 1, whereas Tyr143 is hydrogen bonded to an oxygen of the other haem propionate group. In subunit 2, where the cytochrome domain is disordered, Tyr143 is now hydrogen bonded to the carboxylate group of pyruvate (Xia and Mathews, 1990).

1.7.4 Active Site

Pyruvate (the product of lactate dehydrogenation) is present in subunit 2 of the flavocytochrome b_2 crystal where the cytochrome domain is absent. Pyruvate is oriented so that the two carbonyl oxygens can form a hydrogen bond with the hydroxyl group of Tyr143 and to the Nε of Arg376 respectively. Tyr254 OH and His373 Nε are in contact with the keto oxygen of pyruvate. His373 also interacts with the carboxylate of Asp282 through the Nδ of the former. Figure 1.6 shows the

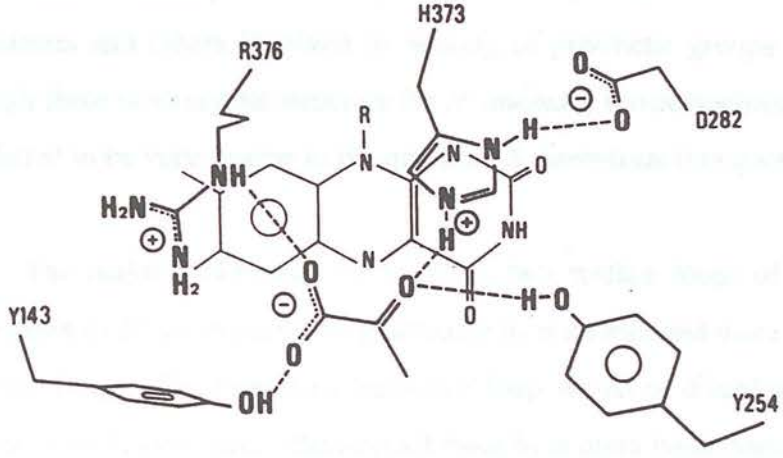


Figure 1.6. Pyruvate at the active site of flavocytochrome b_2 (Xia and Mathews, 1990)

structure at the active site. The functions of the amino acid side-chains at the active site are discussed in chapter 3.

1.7.5 Comparison of *S. cerevisiae* and *H. anomala* flavocytochrome b_2.

The gene encoding flavoctochrome b_2 from *Hansenula anomala* has been isolated and sequenced (Black *et al.*, 1989a). Activity comparison of flavocytochrome b_2 from *H. anomala* and *S. cerevisiae* shows that the former is several fold faster than the latter (Tegoni *et al.*, 1984; Capeillère-Blandin *et al.*, 1986). Comparisons of the amino acid sequence of flavocytochrome b_2 from both organisms have been made to identify the basis for this difference. There is overall 60 % sequence identity between these two flavocytochromes b_2 with all the active site residues and others involved in binding of prosthetic groups being conserved. Although there is no crystal structure for *H. anomala* flavocytochrome b_2, this protein is predicted to be very similar to the one from *S. cerevisiae* (Haumont *et al.*, 1987).

The major differences are found in two surface loops of the protein. The hinge region of *H. anomala* flavocytochrome b_2 is shorter and more acidic than the *S. cerevisiae* hinge. The proteinase sensitive loop which is disordered in the crystal structure from *S. cerevisiae* flavocytochrome b_2 is quite basic compared with the *H. anomala* protein which is acidic. These structural differences are likely to have a significant effect on the catalytic differences between the two enzymes(Black *et al.*, 1989a).

1.7.6 Catalytic cycle of flavocytochrome b_2

The catalytic cycle of flavocytochrome b_2 is shown in figure 1.7. The first step is oxidation of L-lactate to pyruvate with subsequent transfer of two electrons to FMN. Then in step 2, one electron is transferred to haem from fully reduced FMN which results in the reduction of haem and flavin semiquinone. In step 3 oxidised cytochrome *c* receives one electron from the reduced haem and this reaction

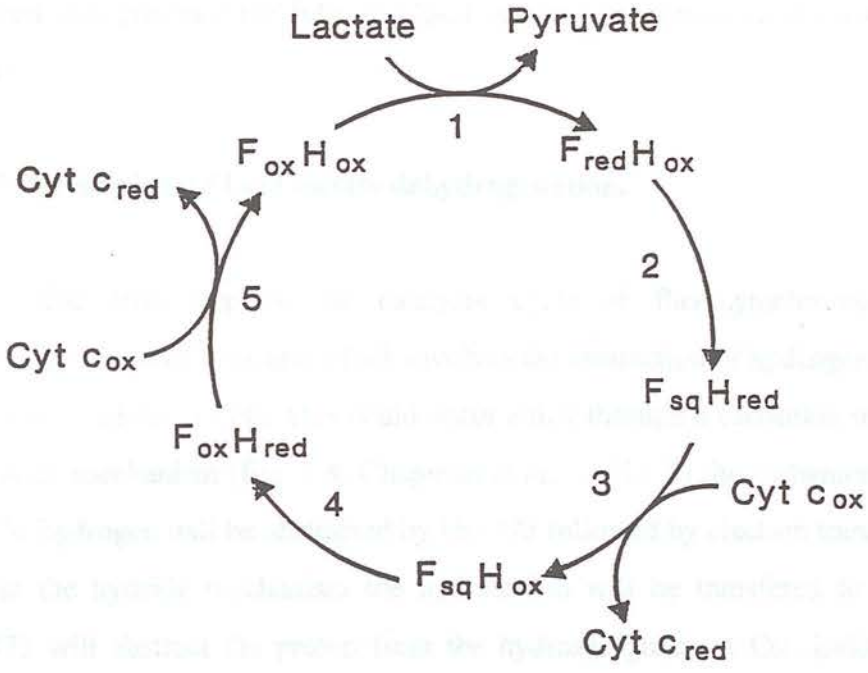


Figure 1.7. Catalytic cycle of flavocytochrome b_2

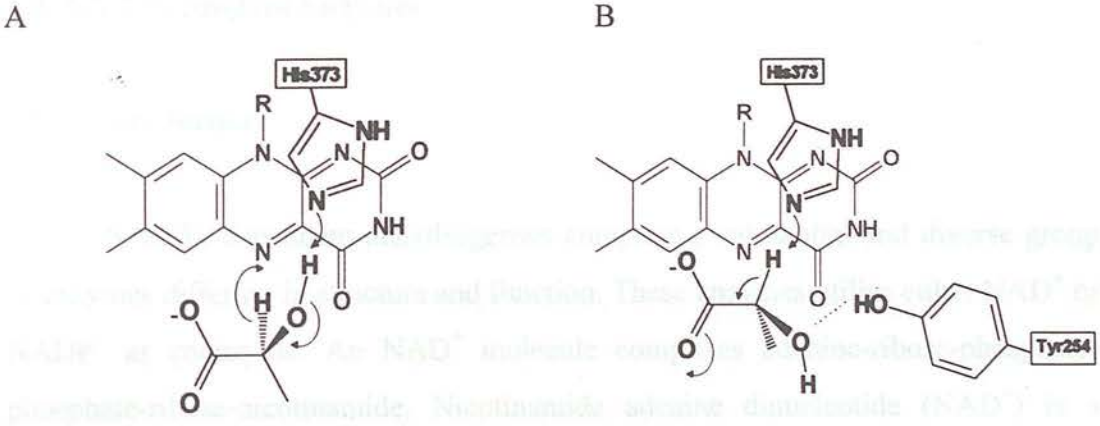


Figure 1.8. Mechanism of L-lactate dehydrogenation. Two possible mechanism for abstraction of αH at C2 of the substrate. A: Hydride transfer, B: Carbanion formation.

produces reduced cytochrome *c* and oxidised b_2 haem. Step 4 involves transfer of one electron from flavin semiquinone to oxidise haem. Both steps 2 and 4 involve interdomain electron transfer. Finally in step 5 a second cytochrome *c* will be reduced and generate the fully oxidised enzyme (Chapman *et al.*, 1991; Lederer, 1991).

1.7.7 Mechanism of L(+)-lactate dehydrogenation.

The first step in the catalysis cycle of flavocytochrome b_2 is the dehydrogenation of L-lactate which involves the abstraction of hydrogen at Cα of the substrate (Lederer, 1974). This could occur either through a carbanion mechanism or a hydride mechanism (Fig. 1.8; Chapman *et al.*, 1991). In the carbanion mechanism the Cα hydrogen will be abstracted by His 373 followed by electron transfer to FMN, but in the hydride mechanism the hydride ion will be transfered to FMN while His373 will abstract the proton from the hydroxyl group at Cα. Evidence for the carbanion mechanism have been proposed for flavocytochrome b_2 (Lederer, 1991; Lederer and Mathews, 1987), but there is currently controversy surrounding the interpretation of much of the experimental evidence.

1.8 NAD-Dependent Enzymes

1.8.1 Introduction

NAD(P)-dependent dehydrogenses comprise a substantial and diverse group of enzymes differing in structure and function. These enzymes utilise either NAD^+ or $NADP^+$ as coenzyme. An NAD^+ molecule comprises adenine-ribose-phosphate-phosphate-ribose-nicotinamide. Nicotinamide adenine dinucleotide (NAD^+) is a major electron acceptor in the biological oxidation of molecules. The reactive part of NAD^+ is its nicotinamide ring.

1.8.2 Structure of NAD-dependent dehydrogenases

Rossmann *et al.* (1975) made a comparison of the three dimensional structure of four NAD-dependent dehydrogenases; liver alcohol dehydrogenase (LADH) from horse (Eklund *et al.*, 1976), lactate dehydrogenase (LDH) from dogfish (Adams *et al.*, 1970), malate dehydrogenase (MDH) from pig heart (Hill *et al.*, 1972) and glyceraldehyde-3-dehydrogenase (GAPDH) from lobster (Buehner *et al.*, 1973) to see the structural and evolutionary relationships among the NAD-dependent dehydrogenase enzymes. Sequence comparison of the amino acid sequences from these enzyme show no significant sequence similarity but comparison of the three dimensional structures shows a striking structure function relationship.

LDH and GAPDH exist as tetramers of identical subunits, whereas MDH and LADH are dimers. The lengths of their polypeptide chains vary slightly, but they are all around 350 residues. These long polypeptides fold into two clearly separated domains, each associated with a particular function. One of the domains is a coenzyme-binding domain and the second one is the catalytic domain. The nucleotide binding domains, which have the common function of binding NAD, exhibit fundamental similarities in their structure and the way the coenzyme binds (Ohlsson *et al.*, 1974) whereas the substrate binding or catalytic domain have a very different structure in each of the different enzymes. The active site of these enzymes is in a cleft between the two domains (Fig. 1.9).

The coenzyme-binding domain in LDH, GAPDH and LADH polypeptide is not located at the same position in the primary structure. In LADH and GAPDH the NAD-binding domains are formed from the N-terminal portion of the polypeptide chain (residue 1 to 147 in GAPDH and residue 22 to 164 in LDH) whereas this domain is formed by the C-terminal region in LADH (residues 193 to 318). This shows that the functionally similar NAD-binding domain can occur in different regions of the polypeptide chain in these dehydrogenases (Rossmann *et al.*, 1975). Comparison of the structure from LDH, GAPDH and LADH leads to the suggestion

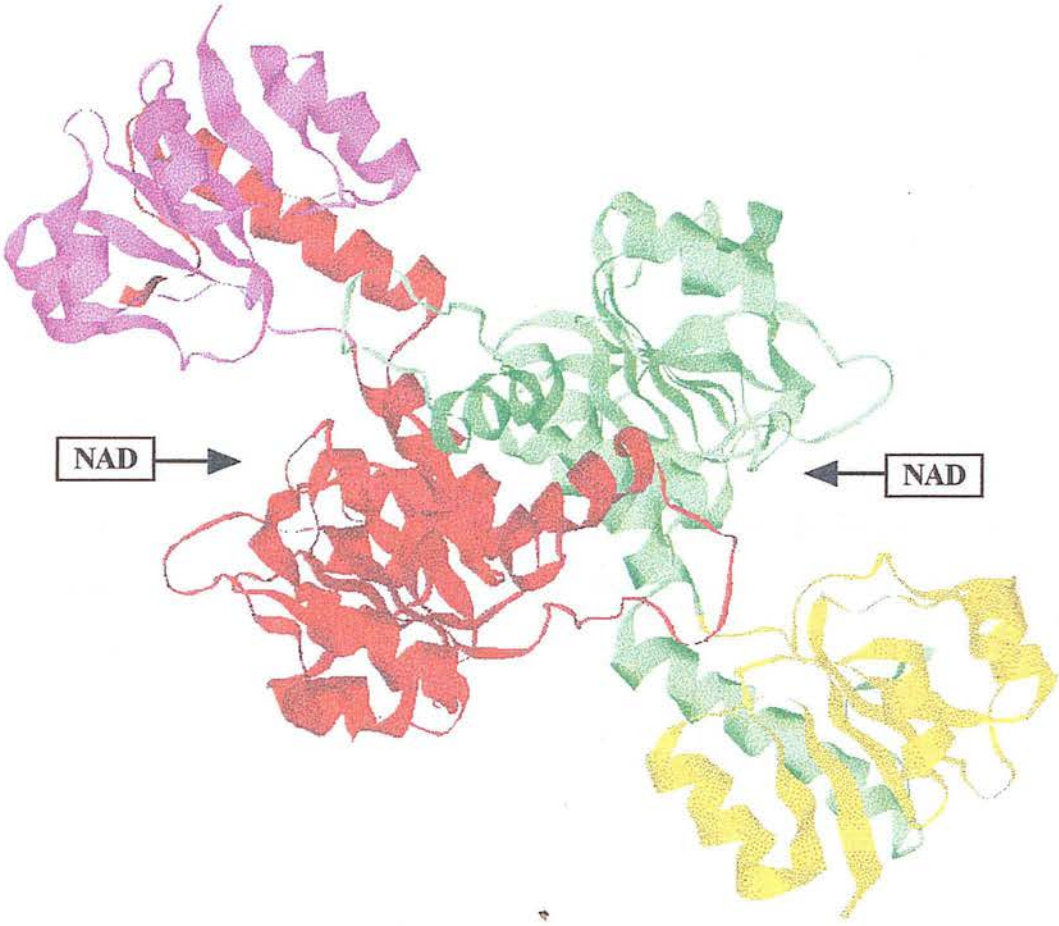


Figure 1.9. Structure of D-glycerate dehydrogenase from *Hyphomicrobium methylovorum* shows the common structure of NAD-dependent enzyme. Each subunit contains two domains, the coenzyme binding domain is in green and the catalytic domain is in yellow for one of the subunit. The N-terminal of the polypeptide forms part of the catalytic domain. (Goldberg *et al.*, 1994).

that the ancestors of each of the dehydrogenases evolved by gene fusion, one gene for the nucleotide binding protein common to all dehydrogenases fusing with one gene for a substrate binding protein different for all the dehydrogenases (Ohlsson *et al.*, 1974)

1.8.3 The NAD-binding domain

The NAD-binding domain is an open, parallel six-stranded β sheet with helices on both sides of the sheet (Fig. 1.10). Rao and Rossmann (1973) showed that the coenzyme binding domain consists of two roughly identical units, with an approximate 2 fold axis running parallel to the strands between βA and βD. Each half of this symmetrical domain is a mononucleotide binding domain, binding one of the two nucleotides in the dinucleotide NAD. The first half forms an adenine nucleotide binding fragment and the second forms a nicotinamide nucleotide binding fragment (Rossmann *et al.*, 1974).

There are certain conserved amino acid sequence motifs for the prediction of the regions of the polypeptide domain that are involved in NAD binding in proteins of unknown three dimensional structure. There are three conserved glycine residues with the sequence of G-X-G-X-X-G-X17-D, where X is any residue and there are six conserved hydrophobic residues. Finally there is one conserved aspartate at the carboxy end of β strand (Fig 1.11). This glycine rich region plays a crucial role in positioning the central part of NAD in its correct conformation close to the protein framework and the aspartate makes a hydrogen bond to the 2'-OH of the adenine ribose of the NAD (Wierenga *et al.*, 1985; Wierenga *et al.*, 1986; Rossmann *et al.*, 1971).

1.8.4 The Hydride Transfer.

The chemical reaction catalysed by the NAD-dependent enzyme involves hydride transfer. In this reaction the alcohol group of the substrate is oxidised by

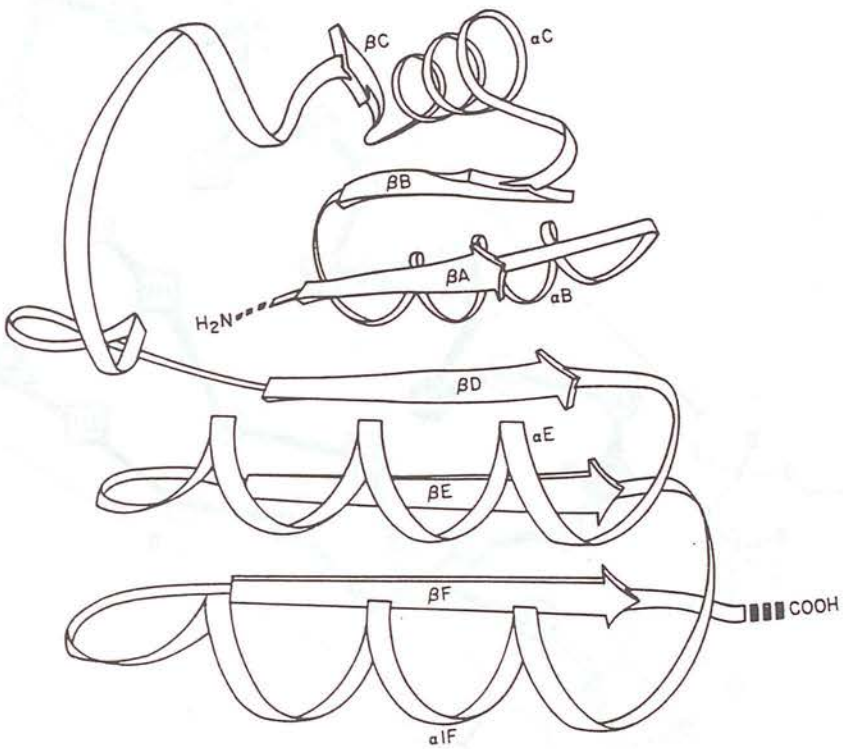


Figure 1.10. Schematic of the structure for the coenzyme binding domain in dehydrogenases.

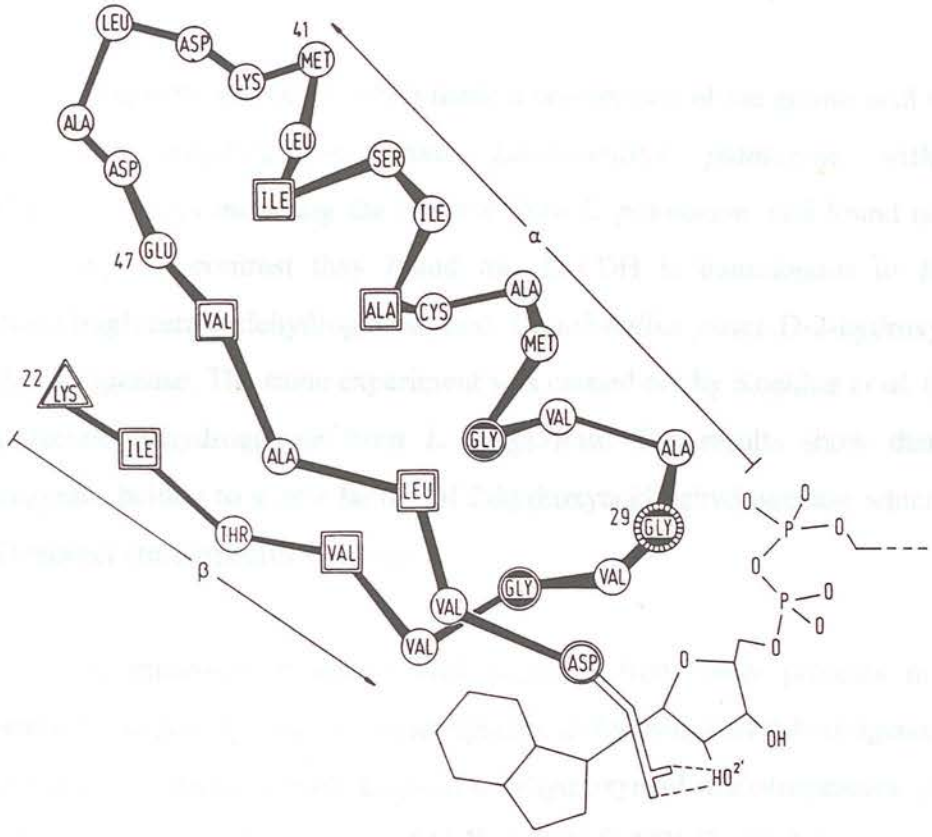


Figure 1.11. Schematic of the ADP-binding βαβ fold of L-lactate dehydrogenase from dogfish (Wierenga *et al.*, 1986). Small and hydrophobic residues are indicated in the box. The three glycines are in round frame.

transfer of a hydride ion to the oxidised form of the coenzyme, NAD^+. A proton is also removed from the alcohol hydroxyl group. In LDH for example His at the active site will receive the proton released from the hydroxyl group at Cα of the substrate and the hydride ion will transfer to the C4 of the nicotinamide ring (Clarke *et al.*, 1989).

1.9 D-isomer-specific 2-hydroxy acid dehydrogenase enzyme

Taguchi and Ohta (1991) made a comparison of the amino acid sequence for D-lactate dehydrogenase from *Lactabacillus plantarum* with L-lactate dehydrogenases including the enzyme from *L. plantarum*, and found no significant similarity. In contrast they found that D-LDH is homologous to *E. coli* D-3-phosphoglycerate dehydrogenase and *Lactobacillus casei* D-2-hydroxyisocaproate dehydrogenase. The same experiment was carried out by Kochhar *et al.* (1992b) with D-lactate dehydrogenase from *L. bulgaricus*. The results show that the above enzymes belong to a new family of 2-hydroxyacid dehydrogenase which consists of D-isomer stereospecific enzymes.

Comparison of amino acid sequence from other proteins in this group strongly suggest that the D-isomer-specific 2- hydroxyacid dehydrogenase family do not share homology with L-specific 2-hydroxyacid dehydrogenases (Vinal *et al.*, 1993). A conserved sequence of G-X-G-X-X-G-17X-D which is common in NAD-dependent dehydrogenases is found at the same position of all members of the D-isomer-specific dehydrogenase family, indicating that these families of enzyme have similar NAD-binding domain structure (Taguchi and Ohta, 1991), but they have divergent structures of the catalytic domain due to different substrate specificities of each protein (Kochhar *et al.*,1992b). On the other hand the position of the conserved sequence for NAD-binding differs between D- and L-isomer-specific 2-hydroxyacid dehydrogenase family, showing a structural difference between them. Kochhar *et al.* (1992b) has also suggested that L- and D-specific 2-hydroxyacid dehydrogenase genes evolved from two different ancestors and thus represent two different families.

Relatively little work has been done on D-isomer 2-hydroxyacid dehydrogenase enzymes compared to the L-isomer family. However the crystal structure of formate dehydrogenase (FDH) from *Pseudomonas sp.101* (Lamzin *et al.*, 1992) and D-glycerate dehydrogenase (GDH) from *Hyphomicrobium methylovorum* (Fig. 1.9; Goldberg *et al.*, 1994) gives some idea of progress with the D-isomer dehydrogenase family. FDH does not utilise a D-isomer as substrate because there is no chiral centre in formate, but sequence comparison shows homology with other D-isomer-specific 2-hydroxyacid dehydrogenases.

1.10 Biology of *Rhodotorula*.

Rhodotorula is a non-fermentative imperfect yeast which belongs to the basidiomycetes. The cells are sphaeroidal, ovoidal or elongate with a capsule. This yeast reproduces vegetatively by budding. *Rhodotorula* can produce a true mycelium and the cells are red in colour due to the presence of carotenoid (Phaff and Ahearn, 1971)

The sexual stage in the genus *Rhodotorula* was first reported by Banno (1963). He later described a new genus as *Rhodosporidium* as a sexual stage of *Rhodotorula*. Fell *et al.* (1973) found that *Rhodosporidium dacryoidum* appears to be closely related to members of the genus *Rhodotorula*. The close relationship between *Rhodotorula* and *Rhodosporidium* has been strengthened byYamazaki and Komagata (1981), who made a comparison of seven enzymes in 108 strains of *Rhodotorula* and *Rhodosporidium* by an electrophoretic technique. Nucleotide composition of DNA in fungi has been used for classification (Storch, 1966). The G+C content of basidiomycetous yeasts is approximately 50-70% (Kurtzman and Phaff, 1987). Storch *et al.* (1969) showed that species of *Rhodotorula* contain a very high GC content which ranging from 52 to 70%. Genomic DNA encoding the phenylalanine ammonia-lyase (PAL) from *Rhodotorula rubra* (Filpula *et al.*, 1988) and the closely related *Rhodosporidium toruloides* (Anson *et al.*, 1987) has been isolated and shown

to contain high GC and several introns. Comparison of introns in the PAL gene from these two organisms revealed conserved intron sequences in both organisms (Vaslet *et al.*, 1988). Another genus of yeast which show similarities to *Rhodotorula* is the genus *Cryptococcus*. The difference between them is that *Rhodotorula* is unable to assimilate inositol and also unable to form starch-like compounds (Kregen-van Rij, 1987).

Rhodotorula has been isolated largely in the aquatic environment especially in fresh water. This genus is also found in other habitats associated with plants, animals and soil. These yeasts utilise a wide range of compounds as carbon sources (Hagler and Ahearn, 1987).

1.11 Aims of the project

(1) To isolate and sequence the complete coding sequences of L(+), and D(-)-mandelate dehydrogenases from *Rhodotorula graminis*.
(2) To determine the amino acid sequence of the enzymes to enable the study of the structural basis of enzymes' substrate specificity.
(3) To design and construct expression systems for both genes to produce large amounts of the enzymes for further biochemical and structural analysis.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2

Materials and Methods

2.1 GROWTH AND MAINTENANCE OF STRAINS

2.1.1 Bacterial and Yeast Stocks

Name	Genotype	Reference
E. coli TG1	*supE*, *hsd*Δ5, *thi*, Δ(*lac-proAB*), F′[*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]	Gibson, 1984
E. coli JM105	*thi*, *rpsL*, *endA*, *sbcB*15, *hsdR*4, *supE*, Δ(*lac-proAB*), F′[*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]	Yanisch-Perron, 1985
E. coli NF1	*K*12Δ*H*1, Δ*trp*, *lacZ* λ*Nam*7, *Nam*53, *c*I857, Δ*H*1	Stanley and Luzio, 1984
R. graminis (ATCC20804)		Durham, 1984

2.1.2 Growth of bacteria and yeast cultures

Bacteria and yeast were grown by inoculating a single colony into appropriate broth in a given volume of liquid cultures. *R. graminis* culture was grown at 30°C and *E. coli* were grown at 30°C to 37°C depending on strains and application.

2.1.3 Maintenance and storage of cultures

Bacterial and yeast colonies were maintained for 2 to 4 weeks on agar plates and stored at 4°C for short term storage. For long term storage, 1 ml of fresh bacteria culture was transfered into 1.5 ml microcentrifuge tube and to this sterile glycerol was added to 15 % (v/v) final concentration. These cultures were stored at -80°C. To subculture, the frozen culture was thawed and streaked out on plate containing appropriate agar medium or transferred to liquid medium.

2.1.4 Growth media for *Rhodotorula graminis*

Complex medium

	Per litre
Nutrient Broth	26 g
D,L-mandelic acid	1.5 g
L-glutamic acid	0.9 g
KH_2PO_4	2.0 g
$(NH_4)_2SO_4$	1.0 g
$MgSO_4.7H_2O$	0.4 g

Adjust to pH 7 with 10N NaOH

This medium was used to induce *R. graminis* cells for the production of mandelate dehydrogenases mRNA for RNA extraction.

YPD medium

	Per litre
Yeast extract	10 g
Peptone	20 g
Glucose	20 g

This medium was used to grow *R. graminis* for genomic DNA extraction.

Sabouraud-Dextrose agar (Oxoid)

	Per litre
Mycological peptone	10g
Glucose	40g
Agar	15g

To prepare the plates, 65 g of the above was mixed with 1litre of dH_2O, dissolved then autoclaved for 15 minutes. Sabouraud-Dextrose agar was used to store *R. graminis* culture.

2.1.5 Media for *Escherichia coli*

Luria Broth

	Per litre
Difco Bacto tryptone	10 g
Difco Bacto yeast extract	5 g
NaCl	5 g
$MgSO_4$	6 g

Plates of the above medium were made by adding 2% agar prior to autoclaving

Minimal medium

	Per litre
20 % glucose solution	10 ml
K_2HPO_4	12 g
KH_2PO_4	3 g
NH_4Cl	1 g
NaCl	0.5 g
dH_2O	800 ml

The above was autoclaved and cooled to 4°C, then the following were added:

1 M $CaCl_2$	100 µl
1M $MgSO_4$	2 ml
2 mg/ml vitamin B_1(thiamine.HCl)	200 µl

2.1.6 Antibiotics

	Stock Solution	Working Concentration
Ampicillin	100 mg/ml	100 µg/ml
Kanamycin	70 mg/ml	70 µg/ml

Stock solutions were sterilized by filtration through a 0.22 µm filter and stored at minus 20°C. Antibiotics were added after the media had been autoclaved and cooled.

2.2 SOLUTIONS

2.2.1 TNE buffer

Tris. HCl pH 7.5	50 mM
NaCl	100 mM
EDTA	5 mM

2.2.2 10 x Formaldehyde gel running buffer

MOPS	0.2 M
Na acetate pH 7.0	0.05 M
EDTA	0.01 M

2.2 M formaldehyde was added in agarose gel

2.2.3 Formaldehyde gel loading buffer

Glycerol	50 %
EDTA	1 mM
Bromophenol blue	0.25 %
Xylene cyanol FF	0.25 %

2.2.4 10 x TBE

	Per litre
Tris base	108 g
Boric acid	55 g
0.5 M EDTA pH 8.0	40 ml

2.2.5 6 x loading buffer type II

Bromophenol blue	0.25 %
Xylene cyanol FF	0.25 %
Ficoll (Type 400; Pharmacia) in water	15 %

2.2.6 Denaturing solution

	Per litre
NaCl	87.66 g
NaOH	20 g

2.2.7 Neutralizing solution

	Per litre
NaCl	1.5 mM
Tris.HCl pH 7.5	0.5 M
EDTA	0.001 M

2.2.8 20 x SSC

	Per litre
NaCl	3 M
Na_3citrate	0.3 M

2.2.9 100 x Denhardt's solution

Bovine serum albumin	2 %
Ficoll	2 %
Polyvinylpyrrolidone	2 %

2.2.10 Pre-hybridisation solution

20 x SSC	7.5 ml
100 x Denhart's solution	1.25 ml
10 % SDS	1.25 ml

2.2.11 DNA Sequencing Gel (6 % acrylamide)

	Per 60 ml
Urea	25.2 g
3 % acrylamide; 0.8 % bis-acrylamide	12ml
10 x TBE	6 ml
dH_20	to 60 ml
10 % APS	140 ul
TEMED	140 ul

APS and TEMED were added immediately before pouring the gel

2.2.12 4 x Resolving buffer for SDS-PAGE

	Per litre
Tris.base	181.6 g
SDS	4.0 g
dH_20	600 ml
conc.HCl	10 ml
Adjust pH to 8.8	

2.2.13 4 x Stacking buffer for SDS-PAGE

	Per 500 ml
Tris.base	30.28 g
SDS	2 g
dH_2O	450 ml

conc.HCl	15 ml
Adjust pH to 6.8	

2.2.14 2 x SDS-PAGE loading buffer

	Per 100 ml
1 M Tris.HCl pH 6.8	3.31 ml
SDS	2 g
Glycerol	9 ml
2-mercaptoethanol	5 ml
1 % bromophenol blue	1 ml
dH_20	80 ml

2.2.15 10 % Resolving Gel for SDS-PAGE

	Per 30 ml
Protogel(30 % acrylamide; 0.8% N',N' methylene bis-acrylamide)	10 ml
4 x Resolving buffer	7.5 ml
dH_20	12.3 ml
10 % APS	190 µl
TEMED	50 µl

2.2.16 5 % Stacking gel for SDS-PAGE

	Per 10 ml
30 % acrylamide; 0.8% bis-acrylamide	1.6 ml
4 x Stacking Buffer	2.5 ml
dH20	5.86 ml
10 % APS	30 µl
TEMED	10 µl

2.2.17 5 x Tris-glycine electrophoresis buffer

	Per litre
Tris.base	15.1 g
Glycine	94 g
10 % SDS	50 ml

2.2.18 10 x Transfer buffer

	Per litre
1 M Tris.HCl pH 8.3	250 ml
Glycine	112.6 g

2.2.19 Tris-buffered saline (TBS)

	Per litre
1 M Tris.HCl pH 7.5	10 ml
4 M NaCl	37.5 ml

2.2.20 0.1 M Phosphate buffer pH 7

	Per litre
NaH_2PO_4 (0.2 M)	195 ml
$NaHPO_4$ (0.2 M)	305 ml
dH_20	500 ml

2.2.21 0.1 M Phosphate buffer pH 5.85

	Per litre
NaH_2PO_4 (0.2 M)	460 ml
Na_2HPO_4 (0.2 M)	40 ml
dH_2O	500 ml

2.2.22 10 x NTB Buffer

Tris.HCl pH 7.2	500 mM
$MgSO_4$	100 mM
DTT	1 mM

2.2.23 TE buffer

Tris.HCl	10 mM
EDTA	1 mM

2.3 SUPPLIERS

2.3.1 Enzymes

T_4 DNA ligase, DNase I, restriction endonucleases, reverse transcriptase and Klenow fragment of DNA polymerase were obtained from Gibco-BRL, Paisley, UK. High Fidelity Taq Polymerase for PCR was obtained from Boehringer Mannheim. Pancreatic ribonuclease A was obtained from Sigma. Sequenase was obtained from United State Biochemical Corporation, Cleveland, Ohio.

2.3.2 Antisera

HRP-conjugated goat-anti rabbit IgG was obtained from Bio Rad.

2.3.3 Radiolabelled Nucleotides

All were obtained from Amersham International

2.3.4 General laboratory chemicals

These were obtained from either Sigma chemical company, Poole, Dorset or, BDH, Poole, Dorset.

2.4 PHAGE

Name	Description	Reference
MI3KO7	Helper phage	Vieira and Messing, 1987

2.5 PLASMIDS

Name	Description	Reference
pTZ18R and pTZ19R	phagemid cloning vectors	Rokeach *et al.*, 1988
pKK223-3	expression vector	Brosius and Holy, 1984
pRC23	expression vector	Crowl *et al.*, 1985

2.6 OLIGONUCLEOTIDE PRIMERS

(I) L-mandelate dehydrogenase:

Sequence	Name	Function
GGAATTCGAYGCNCARCTNCCNGTNAARCA	H1549	PCR
TCGAAGCTTRTGYTTNGCNACYTTCNGC	N7501	PCR
CAACCCGGGATGGATGCTCAGCTGCCGGT	RI1	PCR
CGAAGCTTCTACTCGGGCACCCACCG	RI2	PCR
Sequence	**Name**	**Complementary to**
CAGGAAACAGCTATGAC	Reverse Primer	*see below
AAGCAGCACCGCACGACGCC	LMDH1	216 to 232
GAGCTGAGTAGAAGCAG	LMDH2	2392 to377
CGAGGTGACCAAGATTG	LMDH3	827 to 843
GACACGACTTGCAAGAT	LMDH4	1479 to 1463
ATCTTGCAAGTCGTGTC	LMDH5	1463 to1479
TGCCGACCTTGCGAAG	LMDH6	474 to 489
GTACCTATGGTCATCAAG	GR1	2044 to 2061
AATTGCTGAGGTTGCT	GR2	1135 to 1150
CGATGTGTCGCATCTT	GR3	1258 to 1443
CTACGTTGACGGACA	MC1	2351 to 1365

(II) D-mandelate dehydrogenase:

Sequence	Name	Function
CCACTGCAGTCAGTAGGCGCGAAAAGC	RI3	PCR
CAAGGAATTCATGCCTCGCCCTCGCG	N1179	PCR
Sequence	**Name**	**Complementary to**
TCTCGACCAGTTCC	M8160	1 to 14
TCTCGCACCTCCCTTC	M5122	445 to 460
GATCCAGAAGGAGAT	M7347	743 to 757
GACTGTGTCAGCGTGTC	M7741	888 to 904
AGCACGTCACGCTCAC	M8006	1256 to 1271
CTGAGTTTCATTGCGAC	M8155	1595 to 1579
GGCTTGGCCTTGCAGGA	M8156	1364 to 1349
GCAGTATTGACAATGC	M8157	1057 to 1042
CAGGCGCGACGTCGT	M8158	816 to 802
AGACGGACAGGATGAG	M8159	609 to 594
AGGGCCTGTTTAAACC	M5123	269 to 254

*Reverse primer binds 34 nucleotides before the multiple cloning site on pTZ19/18R

2.7 TRANSFORMATION AND SELECTION PROCEDURES

2.7.1 Preparation of competent *E.coli* cells

10 ml of LB broth was inoculated with a single colony and incubated overnight with shaking at 37°C. 0.4 ml of the overnight culture was then inoculated into 40 ml (1:100 dilution) LB broth and incubated at 37°C with vigorous shaking until OD_{600} reached 0.3-0.5 (about 2-2.5 hours). Then the cells were centrifuged at 6,000 rpm (Sorvall GSA rotor) for 5 minutes at 4°C.The supernatant was removed and 20 ml (0.5 of original volume) of 100 mM cold $CaCl_2$ was added and the pellet was resuspended gently. This was then allowed to incubate on ice for 20 minutes or longer, followed by centrifugation for 5 minutes at 6,000 rpm at 4°C. The supernatant was decanted and the pellet resuspended in 4 ml (0.1 of original volume) of 100 mM $CaCl_2$. Glycerol was added to final concentration of 15 %(v/v). The competent cells were then aliquoted into 200 µl volume in sterile tubes, snap frozen in liquid nitrogen and stored at -80°C.

2.7.2 Transformation of *E. coli*

The frozen competent cells were thawed at room temperature and immediately put on ice. To the 200 µl of competent cells, all or a fraction of the ligation reaction mixture was added and incubated on ice for 15-20 minutes. The cells were then heat-shocked at 42°C for 90 seconds and returned to ice for another five minutes. To this was added 800 µl of LB broth and the tubes were then incubated at 37°C for one hour. The cells were then spun down and the supernatant decanted. The pelleted cells were then resuspended in 50 µl of LB broth and then spread onto a Luria agar petri dish containing 100 µg/ ml Ampicillin. The plates were incubated overnight at the appropriate temperature.

2.8 ISOLATION OF RNA

2.8.1 Isolation of RNA from *R. graminis*

A 100 ml culture of *R. graminis* was incubated overnight at 30°C in the complex medium containing D,L -mandelate until the OD_{600} reached about 0.6 (mid log phase). The cells were pelleted at 6,000 rpm(Sorvall-GSA rotor) for 15 minutes. The supernatant was removed and the cells were resuspended in 1 ml TNE solution. The suspension was then removed into a clean sterile 30 ml corex tube which has been treated with DEPC to removed any contamination with RNase. Then about 30 mg acid-washed glass beads were added to the cells suspension in TNE and vortexed vigorously for 2 minutes to disrupt the cells. 4 ml of TNE, 0.2 ml of 20 % SDS and 4 ml phenol were rapidly added to and vortexed for another 2 minutes. The mixture was then spun at 8,500 rpm (Sorvall-SS34 rotor) for 15 minutes. The aqueous phase containing RNA was then removed and extracted with the same volume of phenol by centrifugation at 8,500 rpm for 15 minutes. The upper aqueous phase was removed and reextracted with phenol/chloroform until a clear interface was achieved. The clear, upper aqueous phase containing the RNA was removed. 2 volumes of 100 % ethanol and 0.1 volume of 3M Na acetate were added to the aqueous containing the RNA and incubated at -20°C for 30 minutes to one hour to precipitate the RNA. The sample was spun at 8,500 rpm for 15 minutes and the pellet was washed carefully in 70 % ethanol. The pelleted RNA was resuspended in 100 µl of DEPC-treated dH_2O and stored at -80°C. All the centrifugation procedures were carried out at 4°C.

2.8.2 Agarose Gel Electrophoresis for RNA.

RNA was separated in 1.2 %(w/v) agarose (BRL electrophoresis grade) with 0.5 µg/ml ethidium bromide in 1 x formaldehyde buffer. About 12 µl (5-10 µg) RNA was mixed with 25 µl formamide, 5 µl 10 x MOPS and 8 µl formaldehyde and then incubated at 65°C for 5 minutes. The sample was then immediately chilled on ice. Prior to loading the RNA mixture was mixed with 0.1 x volume of loading buffer [50 % (v/v) glycerol, 1mM EDTA, 0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene

cyanol FF]. Electrophoresis was carried out using a horizontal gel tank at 40-70 volts for 1-1.5 hours. RNA was visualised by UV illumination and photographed.

2.9 ISOLATION OF DNA

2.9.1 Isolation of plasmid DNA from *E. coli*

The alkaline lysis method of Birnboim and Doly (1979) was used for the isolation of plasmid DNA from *E. coli*.

2.9.1.1 Small scale isolation

A single colony was inoculated into a 5 ml LB broth containing 100 μg/ml ampicillin and incubated at 37°C overnight with constant shaking. The culture was pelleted by centrifugation and the supernatant removed. The pelleted cells were resuspended in 0.1 ml TEG solution (25 mM Tris.HCl pH 8.0, 10 mM EDTA and 50 mM glucose) and transferred to a 1.5 ml eppendorf tube. 0.2 ml of lysis buffer (0.2 M NaOH, 1%SDS) was added to the pellet and gently mixed and then left on ice for 5 minutes. 0.15 ml of 3M Na acetate pH 5.0 was then added to the lysed cells, mixed by inversion and left for another 5 minutes on ice to precipitate the chromosomal DNA, SDS and protein. The precipitate was then removed by centrifugation in a microfuge for 10 minutes at 4°C. To the supernatant 1 ml of absolute ethanol was added, mixed and left 5 minutes at -20°C to precipitate the plasmid DNA. The plasmid DNA was pelleted by centrifugation for 10 minutes at 4°C. The pelleted plasmid DNA was washed with 70% ethanol, dried under vacuum, and resuspended in 50 μl of TE buffer or in dH_2O. The plasmid DNA was stored at -20°C.

2.9.1.2 Large scale isolation

A single colony was inoculated into 5 ml LB broth with 100 μg/ ml ampicillin and grown overnight at 37°C. 2 ml was inoculated from the 5 ml overnight culture into 250 ml LB broth with 100 μg/ml ampicilin and incubated overnight at 37°C with shaking. Cells were pelleted by centrifugation at 6,000 rpm (Sorvall GSA rotor) for 10 minutes at 4°C. The pelleted cells were resuspended in 4 ml TEG

(25mM Tris.HCl pH 8.0, 10 mM EDTA and 50 mM glucose). Then 8 ml of 0.2 M NaOH, 1 % SDS was added and the tubes left on ice for 20 minutes to lyse the cells. To this was added 6 ml of 3 M sodium acetate pH 5.0 to precipitate the chromosomal DNA, SDS and protein which were then spun down at 15,000 rpm (Sorvall SS-34 rotor) for 15 minutes at 4°C. The supernatant was then transferred to a sterile SS-34 tube, carefully avoiding carrying over material from pellet. 11 ml of isopropanol was added to the supernatant and incubated at room temperature for 30 minutes to precipitate the plasmid DNA. The plasmid DNA was pelleted by centrifugation at 18,000 rpm (Sosvall SS-34 rotor)for 15 minutes at 4°C. Supernatant was poured off and the pellet was washed with 70 % of ethanol. The pellet was dissolved in 3 ml of 1 x TE and spun again to removed any undissolved material. 2.8 ml of plasmid DNA was transferred to a fresh tube to which 400 µl TE and 3.8 g of CsCl was added and dissolved. Then 150 µl of 10 mg/ml of ethidium bromide was added and this solution was loaded into 2 ml heat seal tubes. The DNA was banded by centrifugation at 80,000 rpm for more than 8 hours at 18°C in the Beckman TL-100 ultracentrifuge. DNA was visualized by side illumination with UV light. The lower band containing supercoiled plasmid DNA was removed by puncturing the tube with a 19 gauge needle and syringe. Another 19 gauge needle was previously inserted at the top of the tube to allow pressure release. Extraction with equal volumes of butanol was carried out several times to remove the ethidium bromide. The CsCl was removed by dialysis against 2 litres of TE buffer for 8 to 15 hours at room temperature. The TE buffer was changed 3 to 4 times during dialysis. The DNA was stored at 4°C for up to12 months or at -80°C for longer storage.

2.9.2 Isolation of single stranded DNA from *E. coli*

M13KO7 helper phage was used to prepare single stranded DNA from plasmids with the F1 origin of replication (phagemids) from *E. coli*. This method yielded about 1 µg single stranded DNA for sequencing.

A single colony of *E. coli* host containing the phagemid was grown in 2 ml of LB broth containing 100 µg/ml ampicillin to mid log phase (OD_{600} of 0.5-0.8). Then 1 µl of M13KO7 helper phage was added to the 2 ml culture and shaken vigorously

at 37°C for 1 hour. After 1 hour 400 µl of infected cells were mixed with 10 ml of LB broth with 100 µg/ml ampicillin and 70 µg/ml of kanamycin. The culture was grown overnight at 37°C with vigorous shaking to give good aeration.

1.5 ml of the overnight culture was spun down at 8,000 rpm for 5 minutes to remove the cells. The supernatant containing the phage was removed into a 1.5 ml tube and to this 0.3 ml of NaCl/PEG solution (2.5 M NaCl, 20 % polyethylene glycol 600) was added, shaken and then left 15 minutes at room temperature. The phage was pelleted by centrifugation at 8,000 rpm for 5 minutes. The supernatant was discarded and the tubes then spun again briefly, and any remaining supernatant removed. The pelleted phage was resuspended in 100 µl of TE and 50 µl phenol was then added vortexed for 10 seconds, allowed to stand for 1 minute then vortexed again and finally was spun again for 1 minute. The upper aqueous phase was removed into another tube and 0.5 ml of chloroform was added to this. This was vortexed, then spun for 1 minute and the upper aqueous phase was removed to a fresh tube. To this upper phase 10 µl of 3M Na acetate pH 5.5 and 250 µl ethanol were added and left for 1 hour at -20°C, then spun for 5 minutes at 4°C, the supernatant was removed and the pelleted single stranded DNA was dried under vacuum. The single stranded DNA was then dissolved in 50 µl TE.

2.9.3 Preparation of *R. graminis* chromosomal DNA

R. graminis was grown in 10 ml YPD medium overnight to stationary phase. The cells were pelleted by centrifugation at 6,000 rpm for 10 minutes.The pelleted cells were washed with sterile dH_20 and spun down again. Then the cells were resuspended in 0.5 ml breakage buffer (0.9M sorbitol, 0.05 M sodium phosphate buffer pH 7.5 and 14 mM 2-mercaptoethanol) and then transferred to an eppendorf tube. A small amount of acid washed glass beads was added to the mixture and vortexed for 1 minute. To this 50 µl 0.5 M EDTA pH 8.0 was added, vortexed briefly and then 50 µl of 10 % SDS and 100 µl proteinase K solution (5 mg/ml) was added. The mixture was mixed well and incubated at 65°C for 15 to 30 minutes. The sample was allowed to cool and 500 µl phenol/chloroform was added and the tube spun for 5 minutes to removed the cell debris. The upper aqueous phase was transferred to a

new sterile 1.5 ml tube. 500 µl of ethanol was added and mixed by inversion left 5 minutes at room temperature and then spun down the precipitate DNA. The ethanol was removed and the pelleted DNA was resuspended in 500 µl TE and to this 5 µl RNase (2 mg/ml) was added and incubated at 65°C for 15 to 30 minutes. RNase was removed by extraction with an equal volume of phenol/chloroform and the aqueous phase was then ethanol precipitated. The precipitate was spooled out with a sterile pasteur pipette into TE buffer, allow to dissolve and then stored at 4°C.

2.9.4 Extraction of protein from DNA with phenol and chloroform

Distilled phenol was equilibrated with buffer at pH 8.0 for extraction of protein from DNA, by mixing with an equal volume of 1 M Tris.HCl (pH 8). The phases were allowed to separate and the upper aqueous phase was discarded. This process was repeated until the pH of the phenol reached 8. TE buffer was added to replace the Tris.HCl layer. 1 x volume of phenol was mixed with the DNA to be extracted and centrifuged at 5,000 rpm(Sorvall SS-34 rotor) for 5 minutes at 4°C. The upper aqueous phase was removed into a fresh tube and re-extracted with phenol if necessary. Then the DNA was extracted with phenol-chloroform. An equal volume of chloroform was then used to extract the aqueous phase to remove any remaining phenol. Then the mixture was centrifuged at 5,000 rpm and the aqueous phase was removed to a fresh tube.

2.9.5 Precipitation of DNA with ethanol

0.1 volume of 3M sodium acetate (pH 5) and 3 volumes of absolute ethanol were added to the nucleic acid-containing solution to precipitate the nucleic acid. The solution was mixed thoroughly and left at room temperature for 10 minutes, -20°C for 20 minutes, or -70°C for 15 minutes depending on DNA concentration. The DNA was pelleted by centrifugation at 18,000 (Sorvall SS-34 rotor) for 15 to 30 minutes. The supernatant was removed and the pelleted DNA was washed with 70 % ethanol and centrifuged as above for 2 minutes. Then the supernatant was discarded and the pellet dried under vacuum until no visible traces of ethanol remained. The dried pelleted DNA was dissolved in sterile distilled water or TE buffer.

2.10 GEL ELECTROPHORESIS OF DNA

2.10.1 Agarose gel electrophoresis of DNA

0.8-2 % (w/v) of BRL electrophoresis grade agarose containing 0.5 μg/ml ethidium bromide in 1 x TBE was used to separate DNA. DNA samples were mixed with 0.1 x volume of DNA loading buffer prior to loading into the wells. The gel was run in a horizontal-bed gel apparatus at 40 to 70 volts depending on the size of the gel. Bacteriophage lambda CI*857* DNA cut with *Hin*dIII or the 1 kb DNA ladder marker were used as size markers. The DNA was visualised directly on a UV transilluminator and photographed.

2.10.2 Isolation of DNA from agarose gel

DNA was electrophoresed through 0.8-1 % BRL electrophoresis grade agarose containing 0.5 μg/ml ethidium bromide. The gel was viewed on a UV transilluminator and the desired DNA band was sliced out of the gel using a sterile surgical blade and placed in eppendorf tube and then extracted from the agarose using Geneclean™. The sliced agarose containing the DNA was weighed and to this 0.5 volume of TBE modifier and 4.5 volume of 6 M NaI solution were added. Then the mixture was heated at 55°C for 5 minutes to dissolve the agarose, and then cooled on ice for 5 minutes. To this 5 μl of 'glass milk' (a silica matrix suspended in water) was added, and left for 5 minutes on ice with occasional mixing to allow the DNA to bind to the silica matrix. The mixture was centrifuged in a microcentrifuge for 30 seconds to pellet the glass milk bound with DNA. The supernatant was discarded and the pellet was washed three times with 500 μl of 'New Wash'(NaCl/ethanol/water mix). After a final spin all the New Wash was discarded and the pelleted 'glass milk ' was resuspended in 10 -20 ml of TE buffer or sterile dH_2O and incubated at 55°C for 2 to 3 minutes to elute the DNA. The mixture was then spun in a microfuge, and the supernatant containing the DNA was removed into a fresh eppendorf tube and stored at -20°C.

2.11 DNA MANIPULATION TECHNIQUES

2.11.1 Restriction digest of DNA

BRL restriction enzymes and buffers were used to digest DNA. 0.1 to 10 μg of DNA was cut in 10 to 50 μl of 1 x appropriate 'React' buffer 2 to 3 hours or overnight at the appropriate temperature. Double digestion involving enzymes with different buffers was done using the buffer which gives the most efficient digestion.

2.11.2 DNA 5' end filling

1 μg DNA in 25 μl dH_20 containing 2.5 μl NTB buffer (0.5 M Tris.HCl pH 7.5, 0.1 M $MgSO_4$, 10 mM DTT, 500 μg/ml BSA), 1 μl 2 mM dNTPs and 2 units of Klenow fragment was incubated at 25°C for 30 minutes. Then the mixture was incubated at 70°C for 5 minutes to inactivate the Klenow fragment.

2.11.3 Dephosphorylation of DNA

To prevent self ligation of single cut vector DNA, calf intestinal phosphatase (CIP) was used. Ligation of vector DNA can only occur when a DNA fragment with an intact 5' phosphate group was inserted. 0.01 unit of CIP obtained from Promega was used to removed the 5' phosphate group of 1 pmol of linearised vector DNA. After digestion of DNA with the appropriate restriction enzyme, CIP was added directly to the digested mixture and incubated at 37°C for 30 minutes. CIP was inactivated by heating the mixture at 65°C. Phenol/chloroform extraction was carried out to remove protein material and after centrifugation in the microfuge the aqueous phase was removed to a new eppendorf tube. The DNA was precipitated by adding absolute ethanol and then spun down. The pelleted DNA was finally resuspended in TE or sterile dH_2O before ligation.

2.11.4 Ligation of DNA

About 0.01 to 0.1 μg of vector cut with appropriate restriction enzymes was incubated with a four-fold molar excess of cut fragment in 1 x digestion buffer (10 mM Tris.HCl pH 7.2, 1 mM EDTA, 10 mM $MgCl_2$, 10 mM DTT and 1 mM ATP)

with 10 units of T_4 DNA ligase. Sterile dH_2O was added up to 10 µl final volume and the mixture was incubated overnight at 16°C.

2.12 POLYMERASE CHAIN REACTION

2.12.1 Treatment of RNA with DNase I

100 µg of total RNA was incubated for 1 hour in 100 µl reaction mixtures containing 10 µl of 10 x DNase I reaction buffer (200mM Tris-HCL pH8.4, 120 mM $MgCl_2$, 500 mM KCl), 10 µl of Dnase I (1U/µl) and sterile H_2O. The reaction was stopped by inactivation of Dnase I, by heating at 65°C for 10 minutes. Then phenol/chloroform extraction was carried out to purify the RNA and finally precipitated by ethanol precipitation. The RNA was spun down, dried under vacuum and resuspended in 50 µl of sterile H_2O and stored at -80°C.

2.12.2 First strand cDNA synthesis

Superscript™ RNase H⁻ Reverse Transcriptase and 5 x First Strand Buffer(Gibco BRL) were used to synthesise the first strand cDNA.

1 µl of oligo$(dt)_{12-28}$(500 µg/ml) was added to 15 to 20 µg of total RNA from *R.graminis* in 10 µl of sterile dH_2O. The mixture was heated to 70°C for 10 minutes and then quickly chilled on ice. The contents of the tube were collected by brief centrifugation and then mixed with 4 µl 5 x first strand buffer, 2 µl of 0.1 M DTT, 2 µl of 5mM dNTPs (prepared by mixing equal volumes of 20 mM stocks of dATP,dTTP, dGTP, dCTP) and 1 µl (200 units) Superscript™ II Rnase H⁻ Reverse Transcriptase. The mixture was mixed gently and incubated at 37°C for 1 hour. The products were used immediately for PCR or stored at -20°C.

2.12.3 Reverse Transcription-Polymerase Chain Reaction(RT-PCR)

RT-PCR was carried out in 0.5 ml eppendorf tubes in a 50 µl reaction mixture containing: 5 µl 10 x PCR buffer with 15mM $MgCl_2$ (Boehringer Mannheim); 2.6 units of Expand™ High Fidelity Polymerase (Boehringer Mannheim); 5 pmol each

of forward and reverse primers; 5 % (2.5 µl) DMSO; 2 ul of 5 mM dNTPs (mix of 20 mM each dTTP, dCTP, dGTP, dATP), 1 µl of reverse transcription reaction (as 2.11.1). 30 µl of mineral oil was layed on top of the 50 µl reaction mixture. Then the tube was placed in the Techne PHC-2 and the reaction was carried out for 35-40 cycles under appropriate denaturing, annealing and extension temperature with appropriate length of time for each steps.

2.13 SEQUENCING OF SINGLE STRANDED DNA

Sequenase™ Version 2.0 kit (United State Biochemicals) which uses the Sanger dideoxy chain termination method was used to sequence DNA on both strands.

The appropriate sequencing primer (1 µl of 3 ng/µl) was annealed to 7 µl of template DNA (2.5-5 µg/ml) in 2 µl of 5 x reaction buffer (200 mM Tris.HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl) by heating at 65°C for 2 minutes, and then allowing to cool to 35°C over 30 minutes. When the mixture had cooled to 35°C the extension reaction was carried out by adding 1 µl 0.1 M DTT, 2 µl dITP label mix (a 1in 4 dilution of 7.5 µM dCTP, dTTP and 15 uM dITP), 0.5 µl of α-[^{35}S]-dCTP (400 Ci/mole) and 2 µl of diluted sequenase™ (a 1 in 8 dilution of sequenase at 130 U/µl in 10 mM Tris.HCl pH 7.5, 5mM DTT and 0.5 mg/ml BSA). The extension reaction was left at room temperature for 2-5 minutes. Then 3.5 µl of extension mix was dispensed into each of four tubes preheated to 37°C containing 2.5 µl of one of the four (GATC) termination mixes for further extension and termination. The termination mixes are:

ddGTP mix - 80 µM dNTPs; 160 µM dITP; 1.6 µM ddGTP; 50mM NaCl
ddATP mix - 80 µM dNTPs; 80 µM dITP; 8 µM ddATP; 50 mM NaCl
ddCTP mix - 80 µM dNTPs; 80 µM dITP; 8 µM ddCTP; 50 mM NaCl
ddTTP mix - 80 µM dNTPs; 80 µM dITP; 8 µM ddTTP; 50 mM NaCl

The mixture was incubated at 37°C for 5 minutes for the termination reaction. The reaction was stopped by the addition of 4 µl of stop solution (95 % formamide,

20 mM EDTA and 0.05 % bromophenol blue). Aliquots of the final sequencing reaction mix were then separated by electrophoresis through a 6 % denaturing polyacrylamide gel made up by mixing 25.2 g of urea, 6 ml 10 x TBE, 12.5 ml Protogel (30 % acrylamide, 0.8 % bis-acrylamide) made up to a final volume of 60 ml with water. 140 µl of 10 % ammonium persulphate and 140 µl of TEMED were added immediately prior to pouring the gel to polymerise the acrylamide. The sequencing reaction mixtures were heated at 75°C for 2 minutes and then loaded onto the gel and electrophoresed in 1 x TBE buffer at 65 W for 1 hour and 40 minutes to 5 hours. Then the gel was fixed in solution containing 5 % acetic acid (v/v) and 15 % methanol (v/v) and then dried under vacuum at 80°C for 1 hour. The gel was then autoradiographed at room temperature.

2.14 DETECTION OF COMPLEMENTARY DNA

2.14.1 Southern transfer

The capillary blotting method used for transfer was essentially that described by Southern (1975).

R. graminis chromosomal DNA was digested with restriction endonucleases and then electrophoresed on a 0.8 % agarose gel in 1 x TBE buffer. After electrophoresis was completed and the DNA was viewed on the UV transilluminator, the gel was soaked in denaturing solution (0.5 M NaOH and 1.5 M NaOH)for 30 minutes with constant shaking to denature the DNA. The gel was neutralised by soaking in the neutralising solution (1.5 M NaCl, 0.5 M Tris.HCl pH 7.2 and 1mM Na_2EDTA) for another 30 minutes with constant shaking. Then the gel was placed upside down on a piece of 3MM filter paper, the ends of which were resting in a reservoir of 20 x SSC. A sheet of nylon membrane (Hybond-N from Amersham International) was cut to the size of the gel and placed on top of the gel. Any air bubbles between the gel and the membrane were removed as the bubbles can prevent migration of the DNA. 3 pieces of presoaked 3 MM Whatman filter paper were then placed on top of the nylon membrane followed by a stack of absorbent paper towels (5-10 cm high). A glass plate was then put on top of the stack of papers towel and

weighted down with a 500 gram weight to ensure even transfer. Transfer was allowed to proceed for 12-24 hours. To prevent short circuiting of liquid between papers towel and the 3 MM Whatman, the gel was surrounded with a water-tight border of cling wrap. Then the 3 MM filter paper and paper towel were removed and the filter was washed in 2 x SSC and then air dried. UV crosslinking was carried out by exposing the nylon membrane with the bound DNA on a UV transilluminator for 3 to 5 minutes. The filter was wrapped in saran wrap and stored at 4°C prior to hybridization.

2.14.2 Random-primed labelling of DNA

(Feinberg and Vogelstein, 1983)

About 25 ng of linear DNA dissolved in 5-20 µl of water was heat denatured by boiling for 5 minutes then cooled on ice immediately. To this 2 µl each of 0.5 mM dATP, dGTP, dTTP;15 µl random primer buffer mixture [0.67 M HEPES, 0.17 M Tris.HCl, 17 mM $MgCl_2$, 33 mM 2-mercaptoethanol, 1.33 mg/ml BSA, 18 OD_{260} units/ml oligodeoxyribonucleotide primers (hexamer fraction), pH 6.8]; 5 µl α-^{32}P-dCTP (3000 Ci/mmol, 10 µCi/µl) was added. Then sterile distilled water was added to give a total volume of 49 µl. The mixture was mixed briefly. 1 µl Klenow fragment was added and mixed gently. The mixture was incubated at 25°C for 1 to 3 hours. Finally 5 µl of stop buffer (0.2 M Na_2EDTA, pH7.5) was added to stop the reaction. The probe was denatured immediately prior to use by heating to 95°C for 5 minutes.

2.14.3 Hybridization

25 ml of pre-hybridization solution was added to the hybridization tube containing the nylon membrane to which the DNA was bound. Then 0.5 ml of 1 mg/ml solution of sonicated salmon sperm DNA was heated in a boiling water bath for 5 minutes and chilled on ice.The salmon sperm DNA was then added to the tube above and incubated for at least 1 hour at 65°C with constant rotation. Then the pre-hybridization solution was removed and an identical fresh one was added. Hybridization was then carried out by adding the denatured labelled probe and the

fresh denatured salmon sperm to the tube and incubated for at least 12 hours at 65°C with constant rotation. After hybridization the membrane was washed with increasing stringency (lower and lower salt concentration) until all the background radiation had been washed off. The membrane was then allowed to dry, wrapped in saran wrap and autoradiographed at -70°C.

2.15 CONSTRUCTION AND SCREENING OF *R. graminis* DNA LIBRARY

2.15.1 Library construction

Plasmid pTZ19R was digested to completion with either *Hin*dIII or *Sac*I and then ligated with chromosomal DNA from *R. graminis* digested with *Hin*dIII and *Sac*I respectively. Aliquots of the ligation mix were transformed into *E. coli* TG1.

2.15.2 Selection of clones containing inserted DNA.

Transformants were screened for the presence of inserts by plating out onto LB agar plates, containing 100 µg/ml ampicillin and which had already been spread with 40 µl of X-gal (20 mg/ml in dimethylformamide) and 4 µl of isopropylthio-β-D-galactoside (IPTG)(200 ng/ml) solution. The plates were incubated at 37°C overnight and recombinants were identified by the lack of blue colour in the colonies.

2.15.3 Colony blotting

A nylon membrane (Hybond-N) cut to the size of the plate was placed carefully on to the agar surface with colonies to be screened. The membrane and agar were marked by piercing both using the sterile needle. The membrane was removed after 1 minute and placed colony side up for 7 minutes on a pad of absorbent filter paper soaked in denaturing solution. The membrane was then placed colony side up for 3 minutes on a pad of absorbent filter paper soaked in neutralizing solution. This step was repeated with a fresh pad soaked in the same solution. Finally the membrane was washed in 2 x SSC and air dried, colony side up, on dry filter paper and then UV fixed as for Southern blottting.

2.15.4 Colony hybridization

The colony blots were screened using the same radioactive probes (L-MDH or D-MDH) and hybridization conditions as the Southern blottting and the positive colonies were identified by autoradiography. The corresponding colony could be identified on the agar plate using the orientation marks.

2.16 ONE DIMENSIONAL SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEINS

The method of Laemmli (1970) was used to separate proteins according to their molecular weight. Two phases of polyacrylamide gel were prepared, a resolving gel for the separation of the protein samples and a stacking gel for the concentration of the protein samples before separation. The resolving gel was mixed well, poured between two glass plates and overlaid with water and left to polymerise for 30 minutes to 1 hour. After the gel had polymerised the water was rinsed off with fresh distilled water and the stacking gel was prepared. The stacking gel was poured on top of the resolving gel. A comb was then inserted into the top of the stacking gel and the gel was allowed to polymerise for 30 minutes to 1 hour. Once polymerised the gel was clamped into a vertical electrophoresis tank filled with 1 x Tris-glycine electrophoresis buffer. Then the comb was removed and the protein samples were loaded into the wells. The electrophoresis was carried at 150 volts for 3 hours. Finally the gel was stained with 1 % PAGE Blue Electran in 20% (v/v) methanol, 5 % (v/v) acetic acid to visualise the separated proteins.

2.16.1 Western transfer

An SDS-PAGE gel was run and then soaked in 1 x transfer buffer for two minutes. It was then assembled into a 'sandwich' with the gel adjacent to a piece of nitrocellulose membrane (Hybond-C) and this placed between two double layers of 3 MM filter paper and foam sponge. All these materials were presoaked in 1 x transfer buffer. The 'sandwich' was then placed in a transfer tank containing 1 x transfer

buffer and the lid fitted making sure that the gel was nearest to the -ve terminal and that all the wires are covered. The proteins were then transferred for 1 to 2 hours at 0.6 to 2 amps. The 'sandwich' was then removed, opened carefully and the membrane was then air dried or used immediately for immunodetection.

2.16.2 Filters blocked with skimmed milk proteins

The filters were incubated overnight in 100 ml of 20 % milk powder made up in TBS buffer (10 mM Tris.HCl pH 7.5, 150 mM NaCl) to block the unbound sites on the membrane. The solution was then replaced with 20-40 ml of 5 % (w/v) milk solution in TBS. The primary antibody, anti L-mandelate dehydrogenase (or anti D-mandelate dehydrogenase) polyclonal serum from rabbit (30 µl) (the antibody was supplied by Charles Fewson, University of Glasgow) was added and incubated with shaking at room temperature for 3 hours or more. After incubation with antibody the membrane was washed in 100 ml of TBS, four times for 5 minutes. The membrane was then placed in fresh 20-40 ml 5 % milk in TBS and the second antibody was added (see 2.16.3).

2.16.3 Primary antibody detection with HRP-conjugated secondary antibody

Horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio Rad) was used as secondary antibody. 10 µl of antibody was added to a small volume of the blocking agent and incubated with the washed membranes at room temperature for 2 hours or more. The solution containing the HRP-conjugated anti-rabbit antibody was removed and the membrane was then washed thoroughly. The membrane was developed by shaking in 10 ml of developing solution (0.5 ml of 5 mg/ml dianisidine, 1ml of 0.1 M imidazole (pH 7.4), 0.1 ml of 30 % H_2O_2 and 8.4 ml water) until an orange band appeared. The reaction was stopped by rinsing the membrane in distilled water and then left to dry. Finally the developed membrane was photographed.

2.17 PURIFICATION OF L-MANDELATE DEHYDROGENASE

2.17.1 Cell lysis

Each batch of frozen cells (recombinant *E. coli* expressing LMDH were grown in LB media) was defrosted and suspended in approximately 100 ml of 0.1 M phosphate buffer pH 7.0 containing 10 mM L-mandelic acid and 1mM EDTA and the cells then lysed by sonication. The presence of L-mandelate ensures that the enzyme is maintained in its more stable reduced form on release from the cells and EDTA aids lysis by chelating Ca^{2+} ions as they are released from the cell membrane. After lysis the cell debris was pelleted by centrifugation at 18,000 rpm for 10 minutes (Sorvall SS-32 rotor). The supernatant was retained.

2.17.2 Ammonium sulphate fractionation

Fractionation of the supernatant was done by addition of ammonium sulphate $(NH_4)_2SO_4$, which was added slowly to 30 % saturation and left for an appropriate time with stirring. The solution was then centrifuged at 18,000 rpm for 10 minutes at 4°C. Further addition of ammonium sulphate to the supernatant to 50 % saturation, caused precipitation of L-MDH. This was collected by centrifugation as described above. The supernatant was decanted and the pelleted fraction collected was dissolved in a minimal volume of 10 mM phosphate buffer pH 7. This was dialysed overnight at 4°C against several changes of 10 mM phosphate buffer to remove the ammonium sulphate.

2.17.3 DE-52 ion exchange column

Whatman DE-52 ion exchange resin was equilibrated at pH 7.0 by adjusting the pH of the suspension of the column material in phosphate buffer by addition of 1 M HCl. The column (20 x 2.5 cm) was then washed by elution with two column volume of buffer to remove any unbound material. The protein solution was loaded onto the column and elution with an increasing linear gradient of phosphate buffer pH 7 (10 mM to 100 mM concentration). Fractions with L-MDH activity were

pooled and dialysed against several changes of 10 mM phosphate buffer pH 7 overnight.

2.17.4 Hydroxyapatite column

The hydroxyapatite was equilibrated in 10 mM phosphate buffer pH 7 and poured to form a column 10 x 2.5 cm. The L-MDH fractions were loaded onto the column and then washed with an increasing linear gradient of phosphate buffer (10 mM to 100 mM concentration). Fractions with L-MDH activity were pooled and the enzyme was precipitated by the addition of ammonium sulphate up to 70 % saturation. The precipitate was collected and dissolved in a minimum volume of Tris buffer pH8.0.

2.17.5 Sephadex G-25

The L-MDH solution was passed down a Sephadex G-25 column (15 x1.5 cm) equilibrated in Tris buffer pH 8.0. The L-mandelate dehydrogenase fractions were pooled and drops were immediately snap frozen in liquid nitrogen and stored at -194°C.

2.18 ENZYME CONCENTRATION

LMDH concentration was calculated from the reduced haem peak at 423 nm with ε=183,000 M^{-1} cm^{-1}(Chapman *et al.*, 1991). The DMDH was estimated by using a 1 cm path-length cell, measuring the absorbance of an appropriately diluted protein solution at 260 nm and 280 nm, [Protein concentration (mg/ml) = $1.55A_{280}$-$0.76A_{260}$].

2.19 ENZYME ASSAY

2.19.1 L-mandelate dehydrogenase assay

L-mandelate dehydrogenase activity was measured in 3 ml assay mixtures containing 1 mM ferricyanide as electron acceptor, 2 ml of 10 mM mandelate in Tris buffer pH 8.0 and the enzyme. Enzyme activity was calculated with an extinction coefficient for ferricyanide of 1010 M^{-1} cm^{-1}.

2.19.2 D-mandelate dehydrogenase assay

D-mandelate dehydrogenase activity (reversed reaction) was carried out in 1.4 ml assay mixtures containing 200 mM phosphate buffer (pH 5.85), 200 μM- NADH, 1 mM phenylglyoxylate and enzyme. The progress of the assay was monitored at 340 nm, 27°C by monitoring the decrease in absorbance. Enzyme activity was calculated with an extinction coefficient for NADH of 6200 M^{-1} cm^{-1}.

2.20 Electronic absorption spectra of recombinant L(+)-mandelate dehydrogenase.

A 50 μl aliquot of stock enzyme was made up to 1 ml with 10 mM Tris buffer pH 8.0 in a cuvette with 1 cm pathlength. The visible spectrum was recorded from 300 to 600 nm (using Shimadzu UV-2101PC). The reduced peak of the enzyme was observed in the presence of L-mandelate (substrate) and the oxidised peak was observed in the absence of L-mandelate. The baseline for the reactions was determined using the 10 mM Tris-buffer pH 8.0 alone.

CHAPTER 3

L(+)-MANDELATE DEHYDROGENASE CLONING, SEQUENCING AND EXPRESSION

CHAPTER 3

Cloning, sequencing and expression

3.1 Introduction

L(+)-mandelate dehydrogenase from *Rhodotorula graminis* has been purified and characterised previously by Yasin and Fewson, (1993). The sequence of 32 amino acid residues from the N-terminus of L(+)-mandelate dehydrogenase has been determined and exhibits 50 % identity with the N-terminus of flavocytochrome b_2 from *S. cerevisiae* (Fig. 3.1). Attempts to isolate the gene encoding L(+)-mandelate dehydrogenase were initially performed by probing a Southern blot of genomic DNA from *R. graminis* with *S. cerevisiae* and *H. anomala* genes (heterologous probes), but no bands were detected (Miles *et al.*, unpublished)

```
1.          E P K L D M N K Q K I S P A E V A K H N K P D D C W V V
2. D A Q L P V K Q R G R A R S I S A A E V A K H N S R D X M W V V
3.          D V - P H W K D I E L T P E I V S Q H N K K D D L W V V
4.                S K A V K Y Y T L E Q I E K H N N S K S T W L I
```

Figure 3.1 Comparison of the N-terminal amino acid sequences of L(+)-lactate dehydrogenase from *S. cerevisiae* (1), L(+)-mandelate dehydrogenase from *R. graminis* (2), L(+)-lactate dehydrogenase from *H. anomala* (3) and microsomal cytochrome b_5 of beef (4).

In order to carry out further biochemical and structural characterisation it is necessary to isolate the gene encoding L(+)-mandelate dehydrogenase to determine the complete amino acid sequence and also to construct an expression system to obtain large amounts of the enzyme. In this chapter the isolation, sequencing and expression of the L(+)-mandelate dehydrogenase gene are described. The amino acid sequence derived from the cDNA is compared with sequences of other proteins in the database. Preliminary purification and characterisation of the recombinant enzyme are also described.

3.2 Results and Discussion

3.2.1 Synthesis of the L(+)-mandelate dehydrogenase probe

Based on the N-terminal sequence of the *R. graminis* L(+)-mandelate dehydrogenase, two suitable degenerate primers for PCR have been designed. An *Eco*RI restriction site was incorporated for the forward primer and a *Hin*dIII restriction site for the reverse primer to facilitate cloning of the product. The forward primer (H1549) corresponds to the amino acids DAQLPVKQ and the reverse primer (N7501) corresponds to the amino acids AEVAKHN of the 32 amino acids available, respectively (Fig. 3.1).

Single stranded cDNA made by reverse transcription of total RNA from *R. graminis* was used as a template in the PCR. The PCR was carried out at 95°C for 5 minutes initial denaturing then 40 cycles of: 94°C for 1 minutes denaturing, 52°C annealing for 1 minute and 72°C extension for 2 minutes. Finally another 5 minutes extension at 72°C was carried out to complete the reaction. A fragment of 81 bp was amplified. The fragment was cut with *Eco*RI and *Hind*III and then cloned into pTZ19R cut with *Eco*RI and *Hind*III to generate the recombinant plasmid pLM1. The 81 bp fragment in pLM1 was recloned into pTZ18R to obtain the alternative orientation (pLM2) for sequencing the second strand. Reverse primer was used to sequence (on both strands) the above fragment. The amino acid sequence deduced from the nucleotide sequence of the amplified 81 bp fragment (Fig. 3.2) matched exactly the amino acid sequence from the N-terminal of LMDH.

3.2.2 Isolation of the L(+)-Mandelate Dehydrogenase Gene

Chromosomal DNA from *Rhodotorula graminis* was isolated (see Materials and Methods) and digested with seven different restriction enzymes. Southern blot analysis was carried out on the digested chromosomal DNA. This was then probed with ^{32}P-labeled 81 bp fragment, labelled by random priming (see Materials and

*Eco*RI

GAATTCGCTGCTCAACTGCCCGTGAAACAACGCGGGCGAGCCCGCAGTATCTCGGCTGCCGAAGTGGCAAAACACAAGCTT

CTTAAGCGACGAGTTGACGGGCACTTTGTTGCGCCCGCTCGGGCGTCATAGAGCCGACGGCTTCACCGTTTTGTGTTCGAA

AspAlaGlnLeuProValLysGlnArgGlyArgAlaArgSerIleSerAlaAlaGluValAlaLysHis

*Hind*III

Figure 3.2. The sequence of a cloned 81 base pair PCR fragment amplified from single stranded cDNA from *R. graminis*.The PCR primer sequences are underlined.

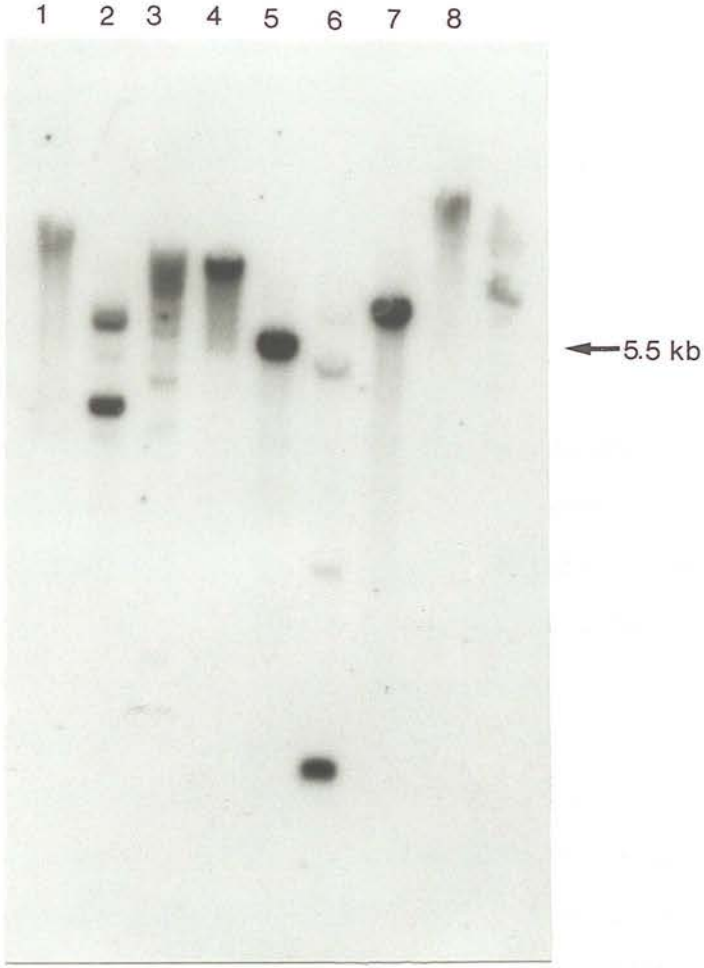


Figure 3.3. Autoradiograph of the Southern Blot of total genomic DNA probed with radiolabelled oligonucleotide. Lane 1: cut with *Xba*I, Lane 2: cut with *Sph*I, Lane 3: cut with *Sac*I, Lane 4: cut with *Bam*HI, Lane 5: cut with *Hind*III, Lane 6: cut with *Pst*I, Lane 7: cut with *Eco*RI, Lane 8: uncut genomic DNA.

Methods). The autoradiograph of the Southern blot showed a hybridising band in each lane. For the purpose of cloning it is desirable to select a fragment of 2 to 6 kb. Apparently the L(+)-mandelate dehydrogenase gene is, at least partly, contained within a 5.5 kb *Hin*dIII fragment (Fig. 3.3). An *R. graminis* genomic library was therefore constructed from chromosomal DNA digested with *Hin*dIII. The digested DNA was ligated to *Hin*dIII-cut pTZ19R and then transformed into *E. coli* TG1. Transformants containing plasmid with insert were identified as white colonies on X-gal/IPTG plates. Approximately 6,000 recombinants were screened by colony blotting using the same probe as for the Southern blot. A single positive clone was identified. Plasmid (pLM3) from this positive clone was purified, cut with *Hin*dIII and shown to contain an insert of the expected size (Fig. 3.4).

3.2.3 Sequencing of the cloned fragment

Plasmid pLM3 containing the 5.5 kb fragment was isolated and cut with several different restriction enzymes. Southern blot analysis probed as in 3.2.2 was carried out on the digested fragment to roughly locate the gene encoding LMDH within the 5.5 kb fragment and also to identify restriction sites within the gene. The reverse primer was first used to sequence the 5.5 kb insert as far as possible. Based on the available sequence, new primers were designed to sequence the whole LMDH gene. The 5.5 kb insert from plasmid pLM3 was also recloned to obtain a recombinant with the opposite insert orientation (called pLM4) for sequencing the second strand. The inserts in pLM3 and pLM4 were also cut with *Eco*RI, *Sac*I or *Pst*I to remove part of the 5.5 kb fragment and then religate again in the same vector, so that reverse primer could be used to sequence parts of the gene which were not covered by the other primers. About 2788 bp have been sequenced (on both strands) from the 5.5 kb insert. The LMDH coding sequence ends at position 2603.

L(+)-lactate dehydrogenase from *Saccharomyces cerevisiae* is synthesised as a precursor form with an 80 residue N-terminal presequence (Guiard, 1985). This N-terminal extension directs the enzyme into the mitochondrion (Gasser *et al.*, 1982;

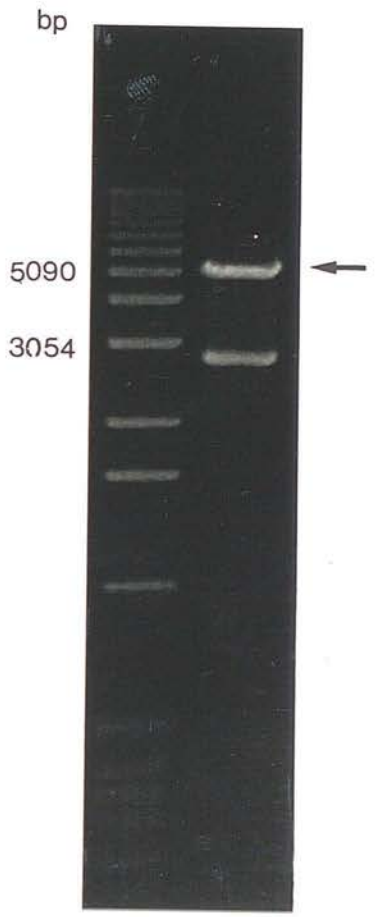
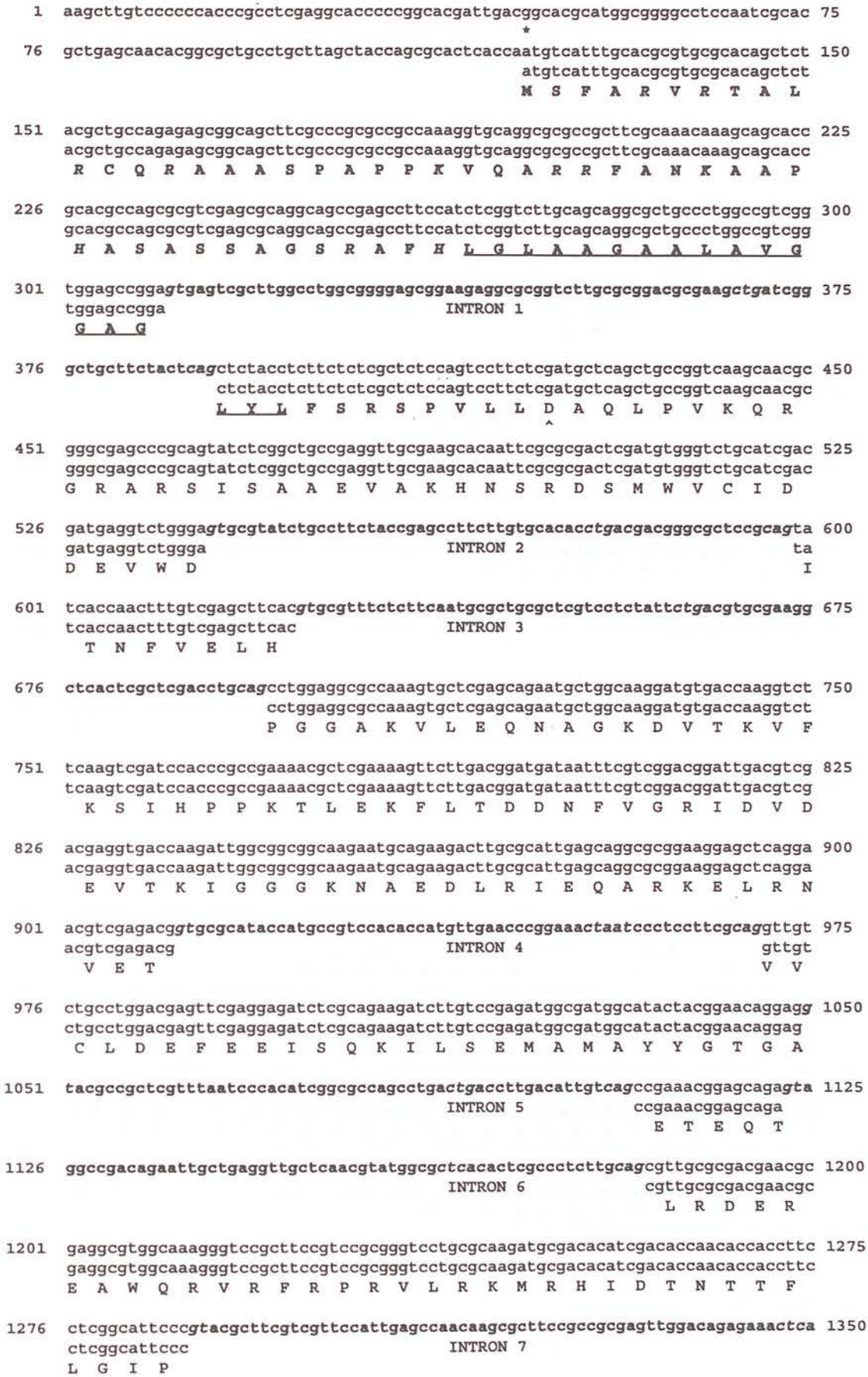


Figure 3.4. Restriction digest of the pLM3 with *Hin*dIII showing the 2.9 kb plasmid (pTZ19R) and the insert of 5.5 kb which contains the gene for L(+)-mandelate dehydrogenase.

Figure 3.5. The genomic sequence of the cloned L(+)-mandelate dehydrogenase and the protein translation. The start of LMDH gene encoding the mature protein and the stop codon (TAG) are marked with a caret (^). The predicted start codon for the gene encoding the LMDH(including the presequence) is marked with an asterisk (*). The predicted presequence is in bold with the basic amino acids in italic and the stretch of non-polar amino acids underlined. The predicted intron and the ten introns are written in single line bold with the conserved 5', 3' and branchpoint sequences in italic. The sequence was determined on both strands.



```
1351  tgttgctgccgtgcagactcccctccccatctttgttgctcccgccggcctcgctcgtctcggccaccccgacgg 1425
                    actcccctccccatctttgttgctcccgccggcctcgctcgtctcggccaccccgacgg
                    T  P  L  P  I  F  V  A  P  A  G  L  A  R  L  G  H  P  D  G

1426  cgagcaaaatatcgttcgcggcgtcgccaagcacgacatcttgcaagtcgtgtcatcgggcgcaagttgctcgat 1500
      cgagcaaaatatcgttcgcggcgtcgccaagcacgacatcttgcaagtcgtgtcatcgggcgcaagttgctcgat
       E  Q  N  I  V  R  G  V  A  K  H  D  I  L  Q  V  V  S  S  G  A  S  C  S  I

1501  cgacgagatcttcgaggtgaaggagccagaccagaacctggcgtggcagttctacgtccattcggacaagaagat 1575
      cgacgagatcttcgaggtgaaggagccagaccagaacctggcgtggcagttctacgtccattcggacaagaagat
       D  E  I  F  E  V  K  E  P  D  Q  N  L  A  W  Q  F  Y  V  H  S  D  K  K  I

1576  cgcggaggagaagctgaagagggctctcgcgctgggcgccaaggccatcttcgtcacggtggacgtgcccgtcct 1650
      cgcggaggagaagctgaagagggctctcgcgctgggcgccaaggccatcttcgtcacggtggacgtgcccgtcct
       A  E  E  K  L  K  R  A  L  A  L  G  A  K  A  I  F  V  T  V  D  V  P  V  L

1651  gggcaagcgcgagcgcgatctcaagctcaaggcgcgcagccagaactacgagcatccgatcgcagcggtacgtct 1725
      gggcaagcgcgagcgcgatctcaagctcaaggcgcgcagccagaactacgagcatccgatcgcagcg
       G  K  R  E  R  D  L  K  L  K  A  R  S  Q  N  Y  E  H  P  I  A  A

1726  tgcgaattccggttcttttcgcggctgcgactgattcaagtgtgtggcttgcagcaatggaaagcggcaggaagc 1800
                                        INTRON 8        caatggaaagcggcaggaagc
                                                         Q  W  K  A  A  G  S

1801  aaggtagaggagaccattgccaaacgtggagtgtccgacattcccgacagtgcgtcctttccatgcctctcaggt 1875
      aaggtagaggagaccattgccaaacgtggagtgtccgacattcccgaca
      K  V  E  E  T  I  A  K  R  G  V  S  D  I  P  D  T

1876  cgagccgtcgttgactgctgctgatggatgattttaccggcagctgctcacatagacgccaacttgaactgggat 1950
                INTRON 9                        ctgctcacatagacgccaacttgaactgggat
                                                 A  H  I  D  A  N  L  N  W  D

1951  gttcgcttgctgtcactttgctctcgccctccgcgtagctgacaccaaaaactcacctaggacatcgcttggatc 2025
                                        INTRON 10               gacatcgcttggatc
                                                                D  I  A  W  I

2026  aaggagcgcgctccgggcgtacctatcgtcatcaagggtgtcggatgtgttgaggacgtcgaactggcgaagcaa 2100
      aaggagcgcgctccgggcgtacctatcgtcatcaagggtgtcggatgtgttgaggacgtcgaactggcgaagcaa
      K  E  R  A  P  G  V  P  I  V  I  K  G  V  G  C  V  E  D  V  E  L  A  K  Q

2101  tatggggcggacggcgttgtcttggtgcgtctcatgtctccttcgctgcgctgtctagctcgccagtacatccgg 2175
      tatggggcggacggcgttgtcttg              INTRON 11
      Y  G  A  D  G  V  V  L

2176  acgccgtatggagaatcgcgttagatcccgagactgcatgacattgtacgcttacgccagacctgctctcgcagt 2250
                                                                                t
                                                                                S

2251  ccacgcacggcgcacggcagcttgatggcgctcgtgcacctctcgatgtgctcattgaggtccggcgcaagaacc 2325
      ccacgcacggcgcacggcagcttgatggcgctcgtgcacctctcgatgtgctcattgaggtccggcgcaagaacc
        T  H  G  A  R  Q  L  D  G  A  R  A  P  L  D  V  L  I  E  V  R  R  K  N  P

2326  cggctcttctcaaggaaatagaagtctacgttgacggacaagcccgccgtggaaccgacgtgctcaaggcactgt 2400
      cggctcttctcaaggaaatagaagtctacgttgacggacaagcccgccgtggaaccgacgtgctcaaggcactgt
        A  L  L  K  E  I  E  V  Y  V  D  G  Q  A  R  R  G  T  D  V  L  K  A  L  C

2401  gcctcggcgcccgcggcgtcggcttcggccgaggattcctctacgcccagtcggcgtacggagcagatggcgttg 2475
      gcctcggcgcccgcggcgtcggcttcggccgaggattcctctacgcccagtcggcgtacggagcagatggcgttg
        L  G  A  R  G  V  G  F  G  R  G  F  L  Y  A  Q  S  A  Y  G  A  D  G  V  D

2476  acaaggcgatccgcatccttgagaacgagattcagaacgcaatgcgcctcctcggcgccaacacgttggcagatc 2550
      acaaggcgatccgcatccttgagaacgagattcagaacgcaatgcgcctcctcggcgccaacacgttggcagatc
        K  A  I  R  I  L  E  N  E  I  Q  N  A  M  R  L  L  G  A  N  T  L  A  D  L

2551  ttaagccggaaatggtcgagtgcagcttcccggagcggtgggtgcccgagtagcagttccgttcgtgtggaaccg 2625
      ttaagccggaaatggtcgagtgcagcttcccggagcggtgggtgcccgag  ^
        K  P  E  M  V  E  C  S  F  P  E  R  W  V  P  E

2626  gagttcggtcacgaccattgcaatacacgagcaccgagactgcgattcttgggactttgttgaggctaagcggca 2700
2701  gactgatgtgaccttcgcaacttgcattccgccgtctgccggaaattcgtacaacggttgcatcccgaatcaagt 2775
2776  ctgagatgaaaaa 2788
```

Figure 3.6. Comparison of presequence of amino acid sequences

Polypeptide/Location	N-Terminal amino acid sequence of precursor	Reference
Cytochrome c peroxidase (intermembrane space)	+ + +++ MTTAVRLLPSLGRTAHKRSLYLFSAAAAAAAAAATFAY +++ + SQSHKRSSSSPGGGSNHGWNNWGKAAALASTTPLV...	Kaput *et al.* (1982)
Cytochrome c_1 (intermembrane space)	++ + + + + + MFSNLSKRWAQRTLSKSFYSTATGAASKSGKLTQKLVT - - AGVAAAGITASTLLYADSLTAEA	Sadler *et al.* (1984)
Citrate synthase (matrix)	+ + + + ++ MSAILSTTSKSFLSRGSTRQCQNMQKALFALLNARHYSS..	Suissa *et al.* (1984)
LLDH (intermembrane space)	+ + + + - + + + + + + - - MLKYKPLLKISKNCEAAILRASKTRLNTIRAYGSTVPKSKSFE - +++ + + - - QDSRKRQSWTALRVGAILAATSSVAYLNWHNGQIDN..	Guiard, (1985)
LLDH(*H. anomala*) (intermembrane space)	+ + + + + + + MFKSQLRTATARSSFRSLASKLNPQRFNSSKTPLLNA + + + + - + TRGSNRSKNLIALAISLSAVSSSYYLYQKDKFISA...	Black *et al.* (1989a)
LMDH(*R. graminis)* (intermembrane space)	+ + + + + ++ + + MSFARVRTALRCQRAAASPAPPKVQARRFANKAAPHASA + + + SSAGSRAFHLGLAAGAALAVGGAGLYLFSRSPVLL	

The basic amino acid are marked +; the acidic are marked -. All the sequence are from *S. cerevisiae* except LLDH from *H. anomala* and LMDH from *R. graminis.*

Lill *et al.*, 1996). The position of the start codon and the presequence for L(+)-mandelate dehydrogenase cannot be confirmed because of the presence of inframe stop codons in the genomic DNA, which is due to the presence of introns. We have not amplified cDNA corresponding to the presequence so the location of an intron in this region cannot be directly confirmed. However, there are a number of relevant features found in the presequences of mitochondrial proteins e.g flavocytochrome b_2 from *S. cerevisiae* and *H. anomala* (Black *et al.*, 1989a; Guiard, 1985) cytochrome *c* peroxidase (Kaput *et al.*, 1982) and cytochrome c_1 from yeast (van Loon *et al.*, 1986). These presequences are strongly basic and contain a long stretch of non-polar residues (Reid, 1985; van Loon *et al.*, 1986) (Fig. 3.6). It is also found that the first amino acid of the mature sequence is conserved as an acidic amino acid (Black *et al.*, 1989a). Based on these features it is predicted that the start codon of LMDH is at position 122 and the presequence consist of 74 amino acids as shown in figure 3.5 and 3.6. Based on the conserved intron sequences in the LMDH gene from *Rhodotorula graminis* (see below and fig 3.7) there could be an intron at position 311 to 390 bp. Based on the N-terminal amino acid sequences available (Fig. 3.1) the sequence encoding the mature form of L(+)-mandelate dehydrogenase starts at position 424 (Fig. 3.5).

The *Rhodosporidium toruloides* and *Rhodotorula rubra* genes encoding the phenylalanine ammonia lyase (PAL) have been isolated and sequenced (Anson *et al.*, 1987; Filpula *et al.*, 1988). These organisms are closely related to *R. graminis*. Comparison of cDNA and genomic sequences of the PAL genes from both organisms revealed the presence of six and five introns in *R. toruloides* and *R. rubra* respectively (Vaslet *et al.*, 1988). All the introns in both PAL genes contained the nucleotides GT at their 5' end and CAG at the 3' ends. These introns have the same internal consensus sequence of CTGAC. The presence of inframe stop codons in the *R. graminis* genomic DNA indicates the presence of several introns in the L(+)-mandelate dehydrogenase coding sequence. The positions of the introns in L(+)-mandelate dehydrogenase were confirmed only after the isolation of the cDNA (as described below) and comparison between the sequence of the genomic DNA and the

(A). Introns in LMDH gene from *R. graminis*

Intron	Position	5'	branchpoint	3'
1	311-391	GT.....	.CTGAT.....	.CAG
2	540-598	GT.....	.CTGAC.....	.CAG
3	624-695	GT.....	.CTGAC.....	.CAG
4	912-970	GT.....	.CTAAT.....	.CAG
5	1050-1107	GT.....	.CTGAC.....	.CAG
6	1123-1183	GT.....	.CTCAC.....	.CAG
7	1288-1366	GT.....	.CTCAT.....	.CAG
8	1717-1779	GT.....	.CTGAT.....	.CAG
9	1850-1918	GT.....	.CTGAT.....	.CAG
10	1952-2011	GT.....	.CTGAC.....	.TAG
11	2125-2249	GT.....	.CTTAC.....	.CAG

(B). Introns in PAL genes from *R. toruloides* and *R. rubra* (Vaslet *et al.*, 1988)

GT.....CTGAC.....CAG

(C). Introns in *S. cerevisiae* (Orbach *et al.*, 1986)

GT.....TACTAAC.....CAG

Figure 3.7. Conservation of intron sequences in *Rhodotorula graminis*

cDNA. There are 11 introns present in the L(+)-mandelate dehydrogenase genomic sequence (Fig. 3.7). Most of these are relatively small with sizes ranging from 59 to 79 bp, which is in common with a number of introns from yeast and filamentous fungi (Anson *et al.*, 1987). The last intron (position 2125 to 2249) is 125 bp long. All of the introns` 5' ends have the invariant sequence of GT. All of the introns also contain the nucleotides CAG at their 3' ends except intron 10 at positions 1952 to 2011 which has TAG at the 3' splice site. Introns 2, 3, 5 and 10 contain the internal consensus sequence of CTGAC as the phenylalanine ammonia-lyase gene intron. For introns 1, 4, 6, 7, 8, 9 and 11, the closest match to the internal consensus sequence is CTGAT, CTAAT, CTCAC, CTCAT, CTGAT, CTGAT and CTTAC respectively. The conserved intron sequences of LMDH gene in *R. graminis* are similar to the intron sequences from the PAL genes from *R. toruloides* and *R. rubra*. Internal sequences of LMDH gene in *R. graminis* presumably have the same function as the internal consensus sequence TACTAAC in *S. cerevisiae* which forms a branch point during splicing (Langford and Gallwitz, 1983).

3.2.4 Isolation of the L(+)-mandelate dehydrogenase cDNA

Isolation of cDNA was undertaken to confirm the intron boundaries and thus to determine the whole sequence of L(+)-mandelate dehydrogenase which could then be used to direct expression of recombinant L(+)-mandelate dehydrogenase. Two specific primers were designed based on the known N-terminal sequence of the protein and the C-terminal sequence predicted from the genomic DNA. An *Xma*I restriction site and a start codon (ATG) were incorporated in the forward primer (RI1) and a *Hin*dIII restriction site for the reverse primer (RI2) to facilitate cloning of the product and to allow later expression of the cDNA. Single-stranded cDNA, reverse transcribed from total RNA of *R. graminis*, was used as a template in the polymerase chain reaction.

PCR was carried out at 95°C for 5 minutes initial denaturing then 3 cycles of: 95°C for 40 seconds denaturing, 50°C annealing for 30 seconds, 72°C extension for 2

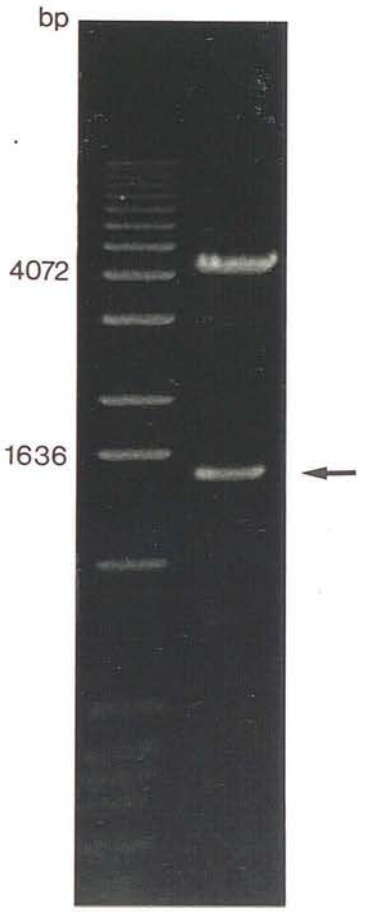
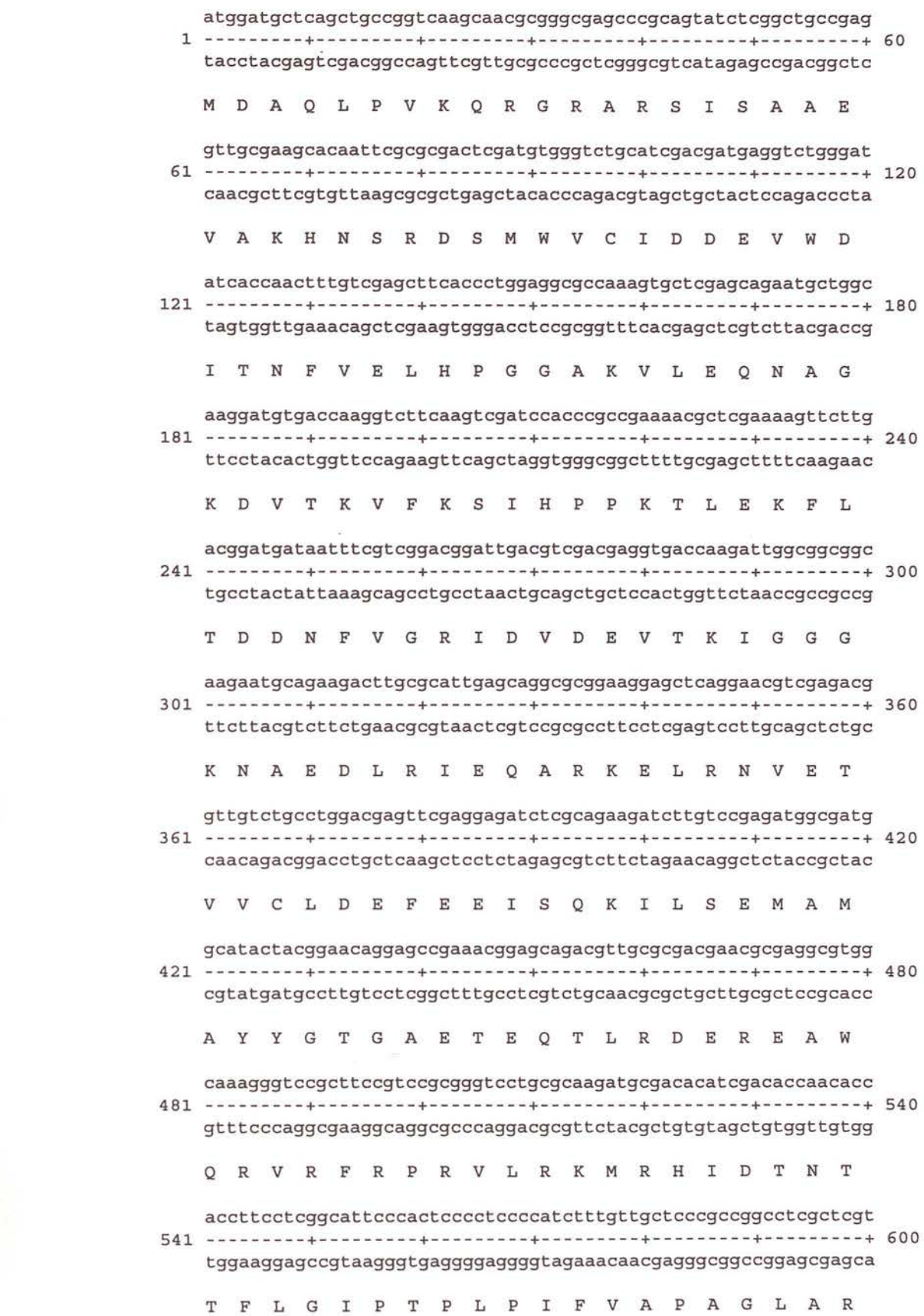


Figure 3.8. The above figure shows the fragment of LMDH cDNA (1479bp/marked by arrow) which was amplified by PCR. The cDNA fragment was subcloned into the expression vector pRC23 (4.6kb) to produce recombinant plasmid pLM7 (see appendix).

Figure 3.9. The cDNA sequence of L(+)-mandelate dehydrogenase and the deduced protein translation. The ATG start codon was incorporated in the cDNA using a primer (RI1) during amplification by PCR. The aspartate residue (D2) after the first methionine in this sequence is D75 of the primary gene product (see figure 3.5). The sequence was determined on both strands.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tacctacgagtcgacggccagttcgttgcgcccgctcgggcgtcatagagccgacggctc
M D A Q L P V K Q R G R A R S I S A A E
gttgcgaagcacaattcgcgcgactcgatgtgggtctgcatcgacgatgaggtctgggat
caacgcttcgtgttaagcgcgctgagctacacccagacgtagctgctactccagacccta
V A K H N S R D S M W V C I D D E V W D
atcaccaactttgtcgagcttcaccctggaggcgccaaagtgctcgagcagaatgctggc
tagtggttgaaacagctcgaagtgggacctccgcggtttcacgagctcgtcttacgaccg
I T N F V E L H P G G A K V L E Q N A G
aaggatgtgaccaaggtcttcaagtcgatccacccgccgaaaacgctcgaaaagttcttg
ttcctacactggttccagaagttcagctaggtgggcggcttttgcgagcttttcaagaac
K D V T K V F K S I H P P K T L E K F L
acggatgataatttcgtcggacggattgacgtcgacgaggtgaccaagattggcggcggc
tgcctactattaaagcagcctgcctaactgcagctgctccactggttctaaccgccgccg
T D D N F V G R I D V D E V T K I G G G
aagaatgcagaagacttgcgcattgagcaggcgcggaaggagctcaggaacgtcgagacg
ttcttacgtcttctgaacgcgtaactcgtccgcgccttcctcgagtccttgcagctctgc
K N A E D L R I E Q A R K E L R N V E T
gttgtctgcctggacgagttcgaggagatctcgcagaagatcttgtccgagatggcgatg
caacagacggacctgctcaagctcctctagagcgtcttctagaacaggctctaccgctac
V V C L D E F E E I S Q K I L S E M A M
gcatactacggaacaggagccgaaacggagcagacgttgcgcgacgaacgcgaggcgtgg
cgtatgatgccttgtcctcggctttgcctcgtctgcaacgcgctgcttgcgctccgcacc
A Y Y G T G A E T E Q T L R D E R E A W
caaagggtccgcttccgtccgcgggtcctgcgcaagatgcgacacatcgacaccaacacc
gtttcccaggcgaaggcaggcgcccaggacgcgttctacgctgtgtagctgtggttgtgg
Q R V R F R P R V L R K M R H I D T N T
accttcctcggcattcccactcccctccccatctttgttgctcccgccggcctcgctcgt
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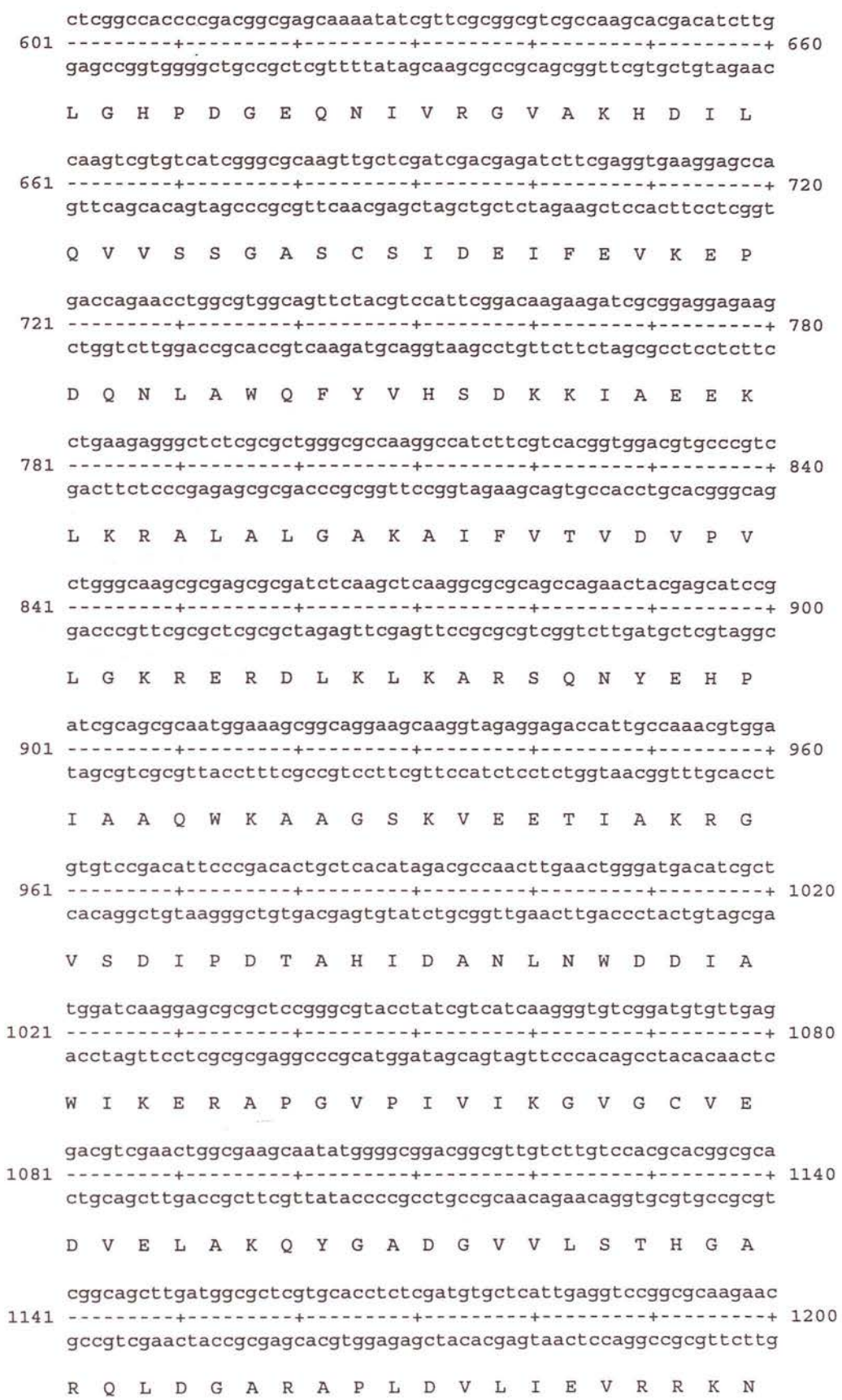
T F L G I P T P L P I F V A P A G L A R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L G H P D G E Q N I V R G V A K H D I L
caagtcgtgtcatcgggcgcaagttgctcgatcgacgagatcttcgaggtgaaggagcca
661 720
gttcagcacagtagcccgcgttcaacgagctagctgctctagaagctccacttcctcggt
Q V V S S G A S C S I D E I F E V K E P
gaccagaacctggcgtggcagttctacgtccattcggacaagaagatcgcggaggagaag
721 780
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D Q N L A W Q F Y V H S D K K I A E E K
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781 840
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L K R A L A L G A K A I F V T V D V P V
ctgggcaagcgcgagcgcgatctcaagctcaaggcgcgcagccagaactacgagcatccg
841 900
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L G K R E R D L K L K A R S Q N Y E H P
atcgcagcgcaatggaaagcggcaggaagcaaggtagaggagaccattgccaaacgtgga
901 960
tagcgtcgcgttacctttcgccgtccttcgttccatctcctctggtaacggtttgcacct
I A A Q W K A A G S K V E E T I A K R G
gtgtccgacattcccgacactgctcacatagacgccaacttgaactgggatgacatcgct
961 1020
cacaggctgtaagggctgtgacgagtgtatctgcggttgaacttgaccctactgtagcga
V S D I P D T A H I D A N L N W D D I A
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1021 1080
acctagttcctcgcgcgaggcccgcatggatagcagtagttcccacagcctacacaactc
W I K E R A P G V P I V I K G V G C V E
gacgtcgaactggcgaagcaatatggggcggacggcgttgtcttgtccacgcacggcgca
1081 1140
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D V E L A K Q Y G A D G V V L S T H G A
cggcagcttgatggcgctcgtgcacctctcgatgtgctcattgaggtccggcgcaagaac
1141 1200
gccgtcgaactaccgcgagcacgtggagagctacacgagtaactccaggccgcgttcttg
R Q L D G A R A P L D V L I E V R R K N

```
     ccggctcttctcaaggaaatagaagtctacgttgacggacaagcccgccgtggaaccgac
1201 ---------+---------+---------+---------+---------+---------+ 1260
     ggccgagaagagttcctttatcttcagatgcaactgcctgttcgggcggcaccttggctg

     P  A  L  L  K  E  I  E  V  Y  V  D  G  Q  A  R  R  G  T  D

     gtgctcaaggcactgtgcctcggcgcccgcggcgtcggcttcggccgaggattcctctac
1261 ---------+---------+---------+---------+---------+---------+ 1320
     cacgagttccgtgacacggagccgcgggcgccgcagccgaagccggctcctaaggagatg

     V  L  K  A  L  C  L  G  A  R  G  V  G  F  G  R  G  F  L  Y


     gcccagtcggcgtacggagcagatggcgttgacaaggcgatccgcatccttgagaacgag
1321 ---------+---------+---------+---------+---------+---------+ 1380
     cgggtcagccgcatgcctcgtctaccgcaactgttccgctaggcgtaggaactcttgctc

     A  Q  S  A  Y  G  A  D  G  V  D  K  A  I  R  I  L  E  N  E

     attcagaacgcaatgcgcctcctcggcgccaacacgttggcagatcttaagccggaaatg
1381 ---------+---------+---------+---------+---------+---------+ 1440
     taagtcttgcgttacgcggaggagccgcggttgtgcaaccgtctagaattcggcctttac

     I  Q  N  A  M  R  L  L  G  A  N  T  L  A  D  L  K  P  E  M

     gtcgagtgcagcttcccggagcggtgggtgcccgagtag
1441 ---------+---------+---------+--------- 1479
     cagctcacgtcgaagggcctcgccacccacgggctcatc

     V  E  C  S  F  P  E  R  W  V  P  E  *
```

minutes. Then continued with another 35 cycles of: 95°C for 40 seconds denaturing, 56°C annealing for 30 seconds and 72°C extension for 2 minutes. Finally another 7 minutes extension at 72°C was carried out to complete the reaction. A fragment of about 1.5 kb was amplified (Fig. 3.8). The fragment was then cut with *Xma*I and *Hin*dIII and ligated to pTZ19R cut with *Xma*I and *Hin*dIII to generate the recombinant plasmid pLM5. The cDNA in pLM5 was recloned into pTZ18R (called pLM6) to obtain the alternative orientation for sequencing the second strand. Primers that were used to sequence the genomic DNA encoding L(+)-mandelate dehydrogenase were used again to sequence (on both strands) the cDNA (Fig. 3.9), with single stranded pLM5 and pLM6 templates as appropriate.

3.2.5 Amino acid sequence comparisons

Figure 3.9 shows the entire 1479 bp sequence of the amplified L(+)-mandelate dehydrogenase cDNA. This open reading frame specifies a mature protein (LMDH) of 492 amino acids including the incorporated N-terminal methionine. The calculated Mr is 54,604 compared with Mr of 59,100 estimated from SDS-PAGE (Yasin and Fewson, 1993).

A computer search of the Swissprot protein sequence data bank with the programe FASTA, using the L(+)-mandelate dehydrogenase as the query sequence indicated amino acid sequence similarity with other L-2-hydroxy acid dehydrogenases. Aligment with other protein sequences in the database using the PILEUP programme (Fig. 3.11) demonstrated that *Rhodotorula graminis* L(+)-mandelate dehydrogenase exhibits 26-42 % identity to each of: L(+)-lactate dehydrogenase from *Saccharomyces cerevisiae*, L(+)-lactate dehydrogenase from *Hansenula anomala*, glycolate oxidase from spinach, L-lactate dehydrogenase from *E. coli*, L(+)-mandelate dehydrogenase from *Pseudomonas putida* and lactate-2-monooxygenase from *Mycobacterium smegmatis*. All these enzymes are members of the family of FMN-dependent 2-hydroxyacid-oxidising enzymes (Lê and Lederer, 1991).

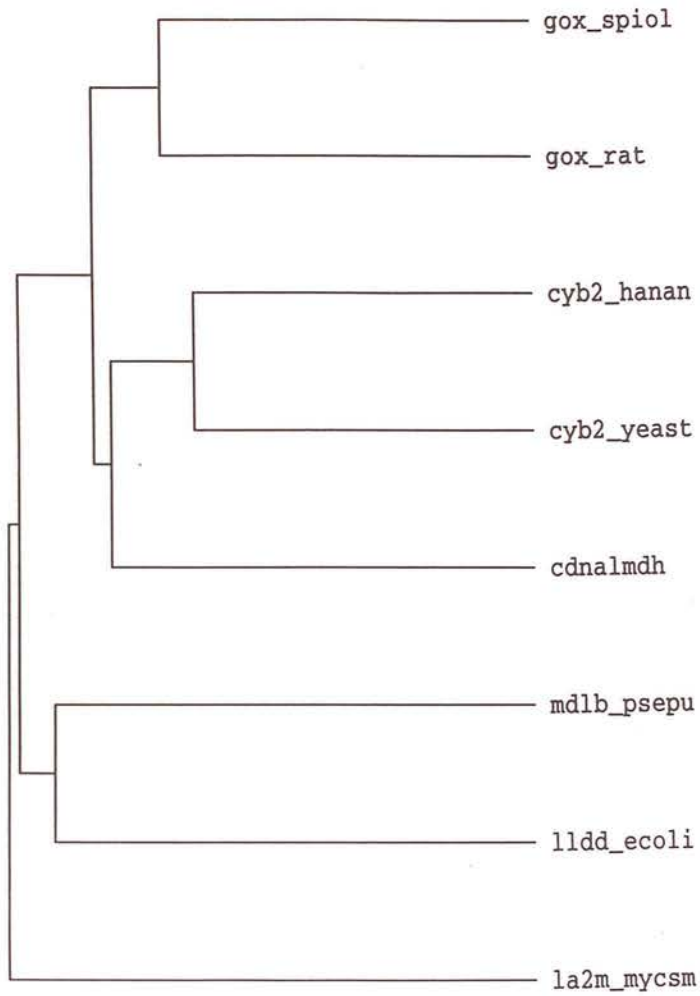

Figure 3.10. Family tree of the FMN-dependent α-hydroxy acid-oxidizing enzymes. The dendrogram shows the output of the UWGCG programme PILEUP.The dendrogram indicates a clustering order from a cluster of sequences based on the similarity. gox_spiol: glycolate oxidase from spinach, gox_rat: rat kidney hydroxy-acid oxidase, cyb2_hanan: L(+)-lactate dehydrogenase from *Hansenula anomala*, cyb2_yeast: L(+)-lactate dehdrogenase from *Saccharomyces cerevisiae*, cdnalmdh: L(+)-mandelate dehydrogenase from *Rhodotorula graminis*, mdlb_psepu: mandelate dehydrogenase from *Pseudomonas putida*, lldd_ecoli: lactate dehydrogenase from *E. coli*, la2m_mycsm: lactate mono-oxygenase from *Mycobacterium smegmatis*. The diagram shows that L(+)-mandelate dehydrogenase is more closely related to L(+)-lactate dehydrogenase from *S. cerevisiae* and *H. anomala* which is a flavocytochrome b_2

Figure 3.11: Sequence alignment of the L-2-hydroxyacid dehydrogenase family. The sequences were aligned using the PILEUP programme in the University of Wisconsin Genetics Computer Group (UWGCG) package. Conserved residues (identical in all sequences) are marked with an asterisk (*) and the semi-invariant residues (allowing two mismatches) are marked with (+) below the alignment. Flavocytochrome b_2 from *S. cerevisiae* hinge region and proteinase sensitive loop are in bold. Amino acids which are known to be functionally important are marked with `f` on top. The sequences are:

SEQUENCE NAME	ENZYME / ORGANISMS	REFERENCES	SWISSPROT ACCESSION NUMBER
gox_spiol	glycolate oxidase/ spinach	Volokita & Somerville, (1987)	P05414
gox_rat	hydroxy-acid oxidase/ rat	Le & Lederer, (1991)	Q07523
cyb2_hanan	L(+)-lactate dehydrogenase/ *H. anomala*	Black *et al.*, (1989a)	P09437
cyb2_yeast	L(+)-lactate dehydrogenase/*S. cerevisiae*	Guiard, (1985)	P00175
cdnalmdh	L-mandelate dehydrogenase/*R. graminis*		
mdlb_psepu	mandelate dehydrogenase/ *P. putida*	Tsou *et al.*, (1990)	P20932
lldd_ecoli	lactate dehydrogenase/ *E. coli*	Dong *et al.*, (1993)	P33232
la2m_mycsm	lactate mono-oxygenase/ *M. smegmatis*	Giegel *et al.*, (1990)	P21795

```
 gox_spiol  ..........  ..........  ..........  ..........  ..........
   gox_rat  ..........  ..........  ..........  ..........  ..........
cyb2_hanan  ..........  ..........  ..........  .DVPHWKDIE  LTPEIVSQHN 19
cyb2_yeast  ..........  ..........  ..........  EPKLDMNKQK  ISPAEVAKHN 20
  cdnalmdh  ..........  ..........  ......DAQL  PVKQRGRARS  ISAAEVAKHN 24
mdlb_psepu  ..........  ..........  ..........  ..........  ..........
lldd_ecoli  ..........  ..........  ..........  ..........  ..........
la2m_mycsm  ..........  ..........  ..........  ..........  ..........
                                                          *  **
                                        f                 f
 gox_spiol  ..........  ..........  ..........  ..........  ..........
   gox_rat  ..........  ..........  ..........  ..........  ..........
cyb2_hanan  KKDDLWVVLN  GQVYDLTDFL  PNHPGGQKII  IRYAGKDATK  IFVPIHPPDT 69
cyb2_yeast  KPDDCWVVIN  GYVYDLTRFL  PNHPGGQDVI  KFNAGKDVTA  IFEPLHAPNV 70
  cdnalmdh  SRDSMWVCID  DEVWDITNFV  ELHPGGAKVL  EQNAGKDVTK  VFKSIHPPKT 74
mdlb_psepu  ..........  ..........  ..........  ..........  ..........
lldd_ecoli  ..........  ..........  ..........  ..........  ..........
la2m_mycsm  ..........  ..........  ..........  ..........  ..........
              *  **      * * * *     ****        **** *       * *

 gox_spiol  ..........  ..........  ..........  ..........  .........M 1
   gox_rat  ..........  ..........  ..........  ..........  ..........
cyb2_hanan  IEKFIPPEKH  LGPLVGEFEQ  E......EEE  LSDEEIDRLE  RIER.KPPLS 112
cyb2_yeast  IDKYIAPEKK  LGPLQGSMPP  ELVCPPYAPG  ETKEDIARKE  QLKSLLPPLD 120
  cdnalmdh  LEKFLTDDNF  VGRIDVDEVT  KIGGGKNAED  L......RIE  QARKELRNVE 118
mdlb_psepu  ..........  ..........  ..........  ..........  .......MSQ 3
lldd_ecoli  ..........  ..........  ..........  ..........  .........M 1
la2m_mycsm  ..........  ..........  .........S  NWGDYENEIY  GQGLVGVAPT 21

                                      f
 gox_spiol  EITNVNEYEA  IAKQKLPKMV  YDYYASGAED  QWTLAENRNA  FSRILFRPRI 51
   gox_rat  PLVCLADFKA  HAQKQLSKTS  WDFIEGEADD  GITYSENIAA  FKRIRLRPRY 50
cyb2_hanan  QMINLHDFET  IARQILPPPA  LAYYCSAADD  EVTLRENHNA  YHRIFFNPKI 162
cyb2_yeast  NIINLYDFEY  LASQTLTKQA  WAYYSSGAND  EVTHRENHNA  YHRIFFKPKI 170
  cdnalmdh  TVVCLDEFEE  ISQKILSEMA  MAYYGTGAET  EQTLRDEREA  WQRVRFRPRV 168
mdlb_psepu  NLFNVEDYRK  LRQKRLPKMV  YDYLEGGAED  EYGVKHNRDV  FQQWRFKPKR 53
lldd_ecoli  IISAASDYRA  AAQRILPPFL  FHYMDGGAYS  EYTLRRNVED  LSEVALRQRI 51
la2m_mycsm  LPMSYADWEA  HAQQALPPGV  LSYVAGGSGD  EHTQRANVEA  FKHWGLMPRM 71
                        *           +    ++ + +      +            +
                                              f
 gox_spiol  LIDVTNIDMT  TTILGFKISM  PIMIAPTAMQ  KMAHP.EGEY  ATARAASAA. 99
   gox_rat  LRDMSKVDTR  TTIQGQEISA  PICISPTAFH  SIAWP.DGEK  STARAAQEA. 98
cyb2_hanan  LIDVKDVDIS  TEFFGEKTSA  PFYISATALA  KLGHP.EGEV  AIAKGAGRE. 210
cyb2_yeast  LVDVRKVDIS  TDMLGSHVDV  PFYVSATALC  KLGNPLEGEK  DVARGCGQGV 220
  cdnalmdh  LRKMRHIDTN  TTFLGIPTPL  PIFVAPAGLA  RLGHP.DGEQ  NIVRGVAKH. 216
mdlb_psepu  LVDVSRRSLQ  AEVLGKRQSM  PLLIGPTGLN  GALWP.KGDL  ALARAATKA. 101
lldd_ecoli  LKNMSDLSLE  TTLFNEKLSM  PVALAPVGLC  GMYAR.RGEV  QAAKAADAH. 99
la2m_mycsm  LMAATERDLS  VELWGKTWAA  PMFFAPIGVI  ALC.AQDGHG  DAASAQASAR 120
            *      +       +       *                +  *+
                     f                                f
 gox_spiol  .GTIMTLSSW  ATSSVEEVAS  TGP.G..IRF  FQLYVYKDRN  VVAQLVRRAE 145
   gox_rat  .NICYVISSY  ASYSLEDIVA  AAPEG..FRW  FQLYMKSDWD  FNKQMVQRAE 145
cyb2_hanan  .DVVQMISTL  ASCSFDEIAD  ARIPGQQ.QW  YQLYVNADRS  ITEKAVRHAE 258
cyb2_yeast  TKVPQMISTL  ASCSPEEIIE  AAPSDKQIQW  YQLYVNSDRK  ITDDLVKNVE 270
  cdnalmdh  .DILQVVSSG  ASCSIDEIFE  VKEPDQNLAW  .QFYVHSDKK  IAEEKLKRAL 264
mdlb_psepu  .GIPFVLSTA  SNMSIEDLAR  QCDGDL...W  FQLYVIH.RE  IAQGMVLKAL 146
lldd_ecoli  .GIPFTLSTV  SVCPIEEVAP  AIKRPM...W  FQLYVLRDRG  FMRNALERAK 145
la2m_mycsm  TGVPYITSTL  AVSSLEDI..  RKHAGDTPAY  FQLYYPEDRD  LAESFIRRAE 168
                  *   +  + +                +   *+*+   *+          +
```

```
                    f
 gox_spiol  RAGFKAIALT VDTPRLGRRE ADIKNRFVL. ...PPFLTLK N..... 182
   gox_rat  ALGFKALVIT IDTPVLGNRR RDKRNQLNL. ...EANILLK D..... 182
cyb2_hanan  ERGMKGLFIT VDAPSLGRRE KDMKMK.... .FEADSDVQG ..... 293
cyb2_yeast  KLGVKALFVT VDAPSLGQRE KDMKLK.... .FSNTKAGPK A..... 306
  cdnalmdh  ALGAKAIFVT VDVPVLGKRE RDLKLKARSQ NYEHPIAAQW K..... 305
mdlb_psepu  HTGYTTLVLT TDVAVNGYRE RDLHNRFKIP MSYSAKVVLD GCLHPRWSLD 196
lldd_ecoli  AAGCSTLVFT VDMPTPGARY RDAHSGMSGP NA.AMRRYLQ AVTHPQWAWD 194
la2m_mycsm  EAGYDGLVIT LDTWIFGWRP RDLTI..... ...SNFPFLR GLCLTNYVTD 210
              *   +  *  * +  * *   *

 gox_spiol  .........F EGIDLGKMDK ANDSGLSSYV AGQIDRSLSW KDVAWLQTIT 223
   gox_rat  .........L RAL...KEEK PTQSVPVSFP KA....SFCW NDLSLLQSIT 216
cyb2_hanan  .......... DDEDIDRSQG ASRALSSF.. ...IDPSLSW KDIAFIKSIT 398
cyb2_yeast  .........M KKTNVEESQG ASRALSKF.. ...IDPSLTW KDIEELKKKT 342
  cdnalmdh  .........A AGSKVEET.I AKRGVSDIPD TAHIDANLNW DDIAWIKERA 345
mdlb_psepu  FVRHGMPQLA NFVS...SQT SSLEMQAALM SRQMDASFNW EALRWLRDL. 242
lldd_ecoli  VGLNGRPHDL GNISAYLGKP TGLEDYIGWL GNNFDPSISW KDLEWIRDF. 243
la2m_mycsm  PVFQKKFKAH SGVEAEGLRD NPR.LAADFW HGLFGHSVTW EDIDWVRSIT 259
                                              + +  *  +
                                             f  f
 gox_spiol  S.LPILVKGV ITAEDARLAV QHGAAGIIVS NHGARQLDYV PATIMALEEV 272
   gox_rat  R.LPIILKGI LTKEDAELAM KHNVQGIVVS NHGGRQLDEV SASIDALREV 265
cyb2_hanan  K.MPIVIKGV QRKEDVLLAA EHGLQGVVLS NHGGRQLDYT RAPVEVLAEV 377
cyb2_yeast  K.LPIVIKGV QRTEDVIKAA EIGVSGVVLS NHGGRQLDFS RAPIEVLAET 391
  cdnalmdh  PGVPIVIKGV GCVEDVELAK QYGADGVVLS THGARQLDGA RAPLDVLIEV 395
mdlb_psepu  WPHKLLVKGL LSAEDADRCI AEGADGVILS NHGGRQLDCA IS...PM... 286
lldd_ecoli  WDGPMVIKGI LDPEDARDAV RFGADGIVVS NHGGRQLDGV LSSARAL... 290
la2m_mycsm  K.MPVILKGI QHPDDARRAV DSGVDGIYCS NHGGRQANGG LPALDCLPEV 308
               +   **     +*        +  *   * +**+**++

 gox_spiol  VK.....AAQ GRIPVFLDGG VRRGTDVFKA LALGAAGVFI GRPVVFSLAA 317
   gox_rat  VA.....AVK GKIEVYMDGG VRTGTDVLKA LALGARCIFL GRPILWGLAC 310
cyb2_hanan  MPILKERGLD QKIDIFVDGG VRRGTDVLKA LCLGAKGVGL GRPFLYAMSS 427
cyb2_yeast  MPILEQRNLK DKLEVFVDGG VRRGTDVLKA LCLGAKGVGL GRPFLYANSC 441
  cdnalmdh  RR..KNPALL KEIEVYVDGQ ARRGTDVLKA LCLGARGVGF GRGFLYAQSA 443
mdlb_psepu  .EVLAQSVAK TGKPVLIDSG FRRGSDIVKA LALGAEAVLL GRATLYGLAA 335
lldd_ecoli  .PAIADAV.K GDIAILADSG IRNGLDVVRM IALGADTVLL GRAFLYALAT 338
la2m_mycsm  VK......AS GDTPVLFDSG IRTGADVVKA LAMGASAVGI GRPYAWGAAL 352
                          *++  * * *+ ++ + +**  +   **  +

 gox_spiol  EGEAGVKKVL QMMRDEFELT MALSGCRSLK EISRSHIAAD WDGPSSRAVA 367
   gox_rat  KGEDGVKEVL DILTAELHRC MTLSGCQSVA EISPDLIQFS RL..... 352
cyb2_hanan  YGDKGVTKAI QLLKDEIEMN MRLLGVNKIE ELTPELLDTR SIHNRAVPVA 477
cyb2_yeast  YGRNGVEKAI EILRDEIEMS MRLLGVTSIA ELKPDLLDLS TLKARTVGVP 491
  cdnalmdh  YGADGVDKAI RILENEIQNA MRLLGANTLA DLKPEMVE.C SFPERWVPE. 491
mdlb_psepu  RGETGVDEVL TLLKADIDRT LAQIGCPDIT SLSPDYLQNE GVTNTAPVDH 385
lldd_ecoli  AGQAGVANLL NLIEKEMKVA MTLTGAKSIS EITQDSLVQG LGKELPAALA 388
la2m_mycsm  GGSKGIEHVA RSLLAEADLI MAVDGYRNLK ELTIDALRPT R..... 393
              *  *+          +     +   *       +

 gox_spiol  RL..... ..... ... 369
   gox_rat  ..... ..... ...
cyb2_hanan  KDYLYEQNYQ RMSGAEFRPG IED 500
cyb2_yeast  NDVLYNEVYE GPTLTEFEDA ... 511
  cdnalmdh  ..... ..... ...
mdlb_psepu  LIGKGTHA.. ..... ... 393
lldd_ecoli  PMAKGNAA.. ..... ... 396
la2m_mycsm  ..... ..... ...
```

In the dendrogram (Fig. 3.10) it can be seen that L(+)-mandelate dehydrogenase is very closely related to L(+)-lactate dehydrogenases from *Saccharomyces cerevisiae* and *Hansenula anomala*, which are flavocytochromes b_2. L(+)-mandelate dehydrogenase from *R. graminis* represents a new type of microbial mandelate dehydrogenase which is a flavocytochrome b_2. Since LMDH belongs to the family of flavocytochromes b_2, it is predicted that it will have a similar structure to flavocytochrome b_2 from *S. cerevisiae* and *H. anomala*. Comparison of amino acid sequence of LMDH from *R. graminis* with those of the mature LLDH from *S. cerevisiae* and *H. anomala* shows that there are two regions in which the sequence differs greatly: residues 92 to 103 and 298 to 314 (numbering as in LLDH in *S. cerevisiae*). These regions are the 'hinge region' which joins the cytochrome and flavodehydrogenase domain of the enzyme together (Chapman *et al.*, 1991) and the second corresponds to a protease-sensitive loop in *S. cerevisiae* flavocytochrome b_2 (Ghrir and Lederer, 1981).

3.3 The L(+)-mandelate dehydrogenase Protein

3.3.1 Cytochrome Domain

Saccharomyces cerevisiae flavocytochrome b_2 L(+)-lactate dehydrogenase has been crystallised and its structure determined (Xia and Mathews, 1990). The flavocytochrome b_2 polypeptide consists of two different regions which form the haem binding domain (cytochrome domain) and the flavin binding domain (flavodehydrogenase domain). The cytochrome domain is located at the N-terminus of the flavocytochrome b_2 polypeptide chain from residue 1 to 99 (Xia and Mathews, 1990). Based on a comparison with the sequence of flavocytochrome b_2 from *S. cerevisiae*, the cytochrome domain of L(+)-mandelate dehydrogenase from *R. graminis* consists approximately of residues 1 to 103. There are 21 invariant residues conserved in this region (Fig. 3.11). The amino acid sequence of the cytochrome domain from *S. cerevisiae* shows extensive similarity with the sequence of bovine

```
                                                          *
cdnalm DAQLPVKQRGRARSISAAEVAKHNSRDSMWVCIDDEVWDITNFVELHPGGAKVLEQNAGK 60
                        |::|||:::| |: :: :|:|:|:|:| ||||::|| ::||
cyb5_b     AEESSKAVKYYTLEEIQKHNNSKSTWLILHYKVYDLTKFLEEHPGGEEVLREQAGG 50


                 *
cdnalm DVTKVFKSI.HPPKTLEKFLTDDNFVGRIDVDEVTKIGGGKNAEDLRIEQARKELRNVETV 120
       |:|: |::: |:     :: |::: ::| :: |: :||:
cyb5_b DATENFEDVGHSTD..ARELSKTFIIGELHPDDRSKITKPSESIITTIDSNPSWWTNWLIP 110


cdnalm VCLDEFEEISQKILSEMAMAYYGTGAETEQTLRDEREAWQRVRFRPRVLRKMRHIDTNTT 180

cyb5_b AISALFVALIYHLYTSEN 130
```

Figure 3.12. Sequence alignment of the first 120 residues of L(+)-mandelate dehydrogenase from *R. graminis* (cdnalm) with amino acid sequence from bovine microsomal cytochrome b_5 (cyb5_b) (Cristiano and Steggles, 1989). Asterisks mark the two histidine which may be the ligands to the haem iron.

microsomal cytochrome b_5 (Guiard *et al.*, 1974; Mathews and Xia, 1987) and the three dimensional structures are also well conserved (Xia and Mathews, 1990).

The haem group of flavocytochrome b_2 is located close to the interface between the cytochrome domain and the flavin binding domain. It is tucked into a hydrophobic crevice in the cytochrome domain formed by two pairs of antiparallel helices and a six stranded β-sheet (Xia and Mathews, 1990). Two histidine side chains ligate the haem iron via their Nε atoms. The residues are His43 and His66 which are also conserved in LMDH from *R. graminis* (His47 and His70). Tyr143 and Lys296 in LLDH from *S. cerevisiae*, which make hydrogen bonds to a haem propionate group are also conserved in LMDH from *R. graminis* (Tyr141 and Lys290). Tyr97 (in flavocytochrome b_2) which is hydrogen bonded to the other haem propionate group is replaced by asparagine in LMDH from *R. graminis*.

The cytochrome domain is connected to the flavodehydrogenase domain through a hinge region from residue 92 to 103 (numbering as in flavocytochrome b_2 from *S. cerevisiae*). In LMDH this region could be from residues 96 to 105 predicted from amino acid sequence comparison with flavocytochrome b_2 from *S. cerevisiae*. Mutant *S. cerevisiae* enzymes have been made where the hinge has been truncated, extended (Sharp *et al.*, 1994,1996) and replaced by the hinge region of *Hansenula anomala* flavocytochrome b_2 (White *et al.*, 1993). In all mutants inter-domain electron transfer has been disrupted indicating the importance of this region in facilitating intramolecular electron transfer.

3.3.2 The Flavodehydrogenase Domain

The flavodehydrogenase domain of L(+)-lactate dehydrogenase from *S. cerevisiae* consists of residues 100 to 486 (Xia and Mathews, 1990). Alignment of the L(+)-mandelate dehydrogenase from *R. graminis* with flavocytochrome b_2 from *S. cerevisiae* indicated that the flavin binding domain of LMDH consists of residues 104 to 487. The flavodehydrogenase domain of flavocytochrome b_2 has been shown

to be structurally related to other FMN-containing enzymes as described above (Fig. 3.11).

About 35 residues are conserved throughout all of the aligned sequences at this region of the polypeptide (Fig.3.11). Almost all of the residues identified as functionally important by Lederer and Mathews (1987) are identical except for Ala196 and Ala198 in LLDH from *S. cerevisiae* which are in contact with the FMN are replaced by Pro194 and Gly196 in LMDH from *R. graminis.*

3.3.2.1 Active Site Residues

In the crystal structure of flavocytochrome b_2 from *S. cerevisiae* (Xia and Mathews, 1990) Arg376 is well positioned to interact with the substrate carboxylate both electrostatically and by forming a hydrogen bond between Nε of Arg376 and one of the carboxylate oxygen atoms. This residue is conserved in LMDH from *R. graminis* (Arg380) and throughout the aligned sequences (Fig.3.11) and apparently plays an important role to bind and orient the substrate along with Tyr143 (Reid *et al.*, 1988).

Mutation of Arg376 in *S. cerevisiae* to lysine resulted in total loss of enzymic activity (Reid *et al.*, 1988), whereas replacement of Arg171 by lysine in a nicotinamide-linked lactate dehydrogenase did not result in inactive enzyme, though k_{cat} was lower by about 4×10^4 fold (Hart *et al.*, 1987). In the latter enzyme the interaction of Arg171 and the substrate carboxylate involves the two terminal nitrogens of the guanidinium group and is symmetrical (Grau *et al.*, 1981) and this is contrast with flavocytochrome b_2 in which the interaction involves the Nε and one of the terminal nitrogens of Arg376. A possible explanation for the total loss of activity may be that the Arg to Lys substitution removes the interaction between the guanidinium group and neighbouring atoms of the protein which would be essential for the integrity of the active site (Reid *et al.*, 1988). Mutation of Arg293 to lysine in

lactate monooxygenase from *M. smegmatis* decreased the k_{cat} for L-lactate but the binding affinity only shows a small change (Müh *et al*, 1994a).

Tyr143 (Tyr141 in LMDH from *R. graminis*) is also making a hydrogen bond to the oxygen at the carboxylate end of the substrate and plays an important role in stabilising the Michaelis complex (Rouvière-Fourmy *et al.*, 1994). The three dimensional structure also reveals that Tyr143 is hydrogen bonded to a haem propionate (Xia and Mathews, 1990). Mutation of Tyr143 to phenylalanine resulted in a larger K_m value than the wild type, indicating a decrease in substrate binding affinity and it also disrupted electron transfer between FMN and haem (Miles *et al.*, 1992).

Tyr254 in flavocytochrome b_2 from *S. cerevisiae* which is also conserved throughout the aligned sequences, was predicted to act by making a hydrogen bond to the substrate OH at all stages of the reaction and facilitate electron departure to the flavin by deprotonating the substrate hydroxyl (Reid *et al.*, 1988). Mutation of this residue in flavocytochrome b_2 from *S. cerevisiae* to phenylalanine shows that Tyr254 takes part in transition state stabilisation but it is not essential for electron transfer (Dubois *et al.*, 1990)

His373 is important in catalysis by acting as a general base. Mutation of His 373 to glutamine reduced the catalytic activity by a factor of at least 5×10^5 compared to the wild type (Gaume *et al.*, 1995). Mutation of His290 in lactate monooxygenase from *Mycobacterium smegmatis* which is equivalent to His373 in LLDH from *S. cerevisiae* has also been made. The mutant enzyme shows 10^7-10^8 fold less activity then the wild type enzyme (Müh *et al.*, 1994b). It appeared that replacement of His290 by glutamine has not resulted in a conformational disruption since substrate and inhibitors bind to the mutant enzyme in a similar fashion to their binding to wild type enzyme (Müh *et al.*, 1994b). L(+)-mandelate dehydrogenase has the identical histidine residue at position 377 which could have the same function as His373 in LLDH from *S. cerevisiae*.

Asp282 has been shown in the crystal structure of flavocytochrome b_2 (Xia and Mathews, 1990) to make a hydrogen bond to of His373 through one of the carboxylate oxygens and it plays an important role in stabilizing the imidazolium ion of His373 (Lederer and Mathews, 1987). Identical interactions are also formed by the active-site aspartate(D157) in glycolate oxidase (Lindqvist and Brändèn, 1989; Lindqvist *et al.*, 1991). Mutation of Asp282 to asparagine has been shown to cause a decrease in the activity of L(+)-lactate dehydrogenase from *S. cerevisiae* (Gondry and Lederer, 1996). Asp282 is also conserved in LMDH from *R. graminis*.

Finally Lys349 in flavocytochrome b_2 from *S. cerevisiae* is believed to facilitate electron transfer by stabilising the N1 anion of the reduced flavin. Mutation of this residue to arginine caused a complete loss of activity in lactate dehydrogenase. L(+)-mandelate dehydrogenase from *R. graminis* contains an equivalent lysine at position 353.

3.3.2.2 FMN Interaction

The flavin binding domain of flavocytochrome b_2 is composed of a parallel $\beta_8\alpha_8$ barrel motif (Xia and Mathews, 1990) similar to that first observed in triose phosphate isomerase (Phillips *et al.*, 1978). This barrel structure is contained within the segment of residue 191 to 465 (numbering as in flavocytochrome b_2 from *S. cerevisiae*). LMDH from *R. graminis* possesses this segment of residues at positions 189 to 467. The FMN is located at the C-terminal end of the central β-barrel and makes contact with main and side chain atoms from residues located on six of the eight β-strands (Mathews and Xia, 1987). The flavodehydrogenase domain of flavocytochrome b_2 has been shown to be structurally related to other FMN-containing enzymes (Fig. 3.11; Xia and Mathews, 1990). Comparison of the three dimensional structure from flavocytochrome b_2 and glycolate oxidase in spinach shows that FMN is bound in a similar fashion in these two enzymes (Lindqvist *et al.*, 1991; Lederer *et al.*, 1991). Since L(+)-mandelate dehydrogenase from *R. graminis* is very closely related to flavocytochrome b_2 from *S. cerevisiae*, and its predicted

flavodehydrogenase domain has a high similarity to glycolate oxidase it is predicted that LMDH from *R. graminis* will form similar protein:FMN interactions.

A comparison of the amino acids which make contact with FMN in L(+)-lactate dehydrogenase from *S. cerevisiae* (Lederer and Mathews, 1987) to the corresponding amino acids in L(+)-mandelate dehydrogenase from *R. graminis* shows that all the important residues are also conserved. In particular, Lys349 which is important in the catalytic mechanism of LLDH from *S. cerevisiae* and makes contact with the isoalloxazine ring and ribose moiety of FMN, is conserved in LMDH (Lys353) as well as throughout the family of FMN-dependent 2-hydroxyacid dehydrogenases.

3.3.3. C-terminal tail

The C-terminal tail of flavocytochrome b_2 from *S. cerevisiae* consists of residues 487 to 511 (Xia and Mathews, 1990). Based on the above, the C-terminal tail for L(+)-mandelate dehydrogenase from *R. graminis* probably comprises residue 488 to 491. This indicates that the C-terminal tail of LMDH is much shorter than that from LLDH of *S. cerevisiae*. However the exact length of the C-terminal tail and the whole structure of LMDH can only be confirmed after determination of the crystal structure of the enzyme.

3.3.4. Substrate Specificity

There are many similarities between *R. graminis* L(+)-mandelate dehydrogenase and the flavocytochrome b_2 from *S. cerevisiae*. Both enzymes are tetramers of identical subunits, they have similar Mr values and contain flavin mononucleotide and protohaem IX prosthetic groups and have identical electronic absorption spectra (Smekal *et al.*, 1993). However the most striking difference between them is that they have different substrate specificity although both are 2-hydroxyacid dehydrogenases (Fig 3.13). *R. graminis* L(+)-mandelate dehydrogenase

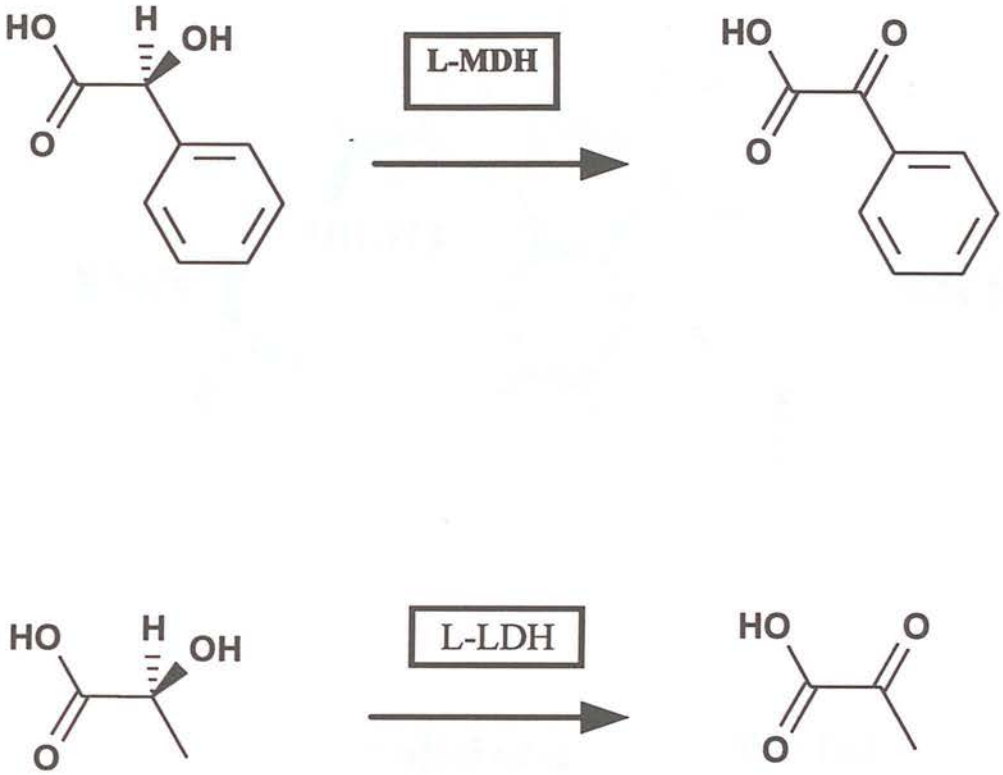

Figure 3.13. The reaction catalyse by L(+)-mandelate dehydrogenase and L(+)-lactate dehydrogenase.

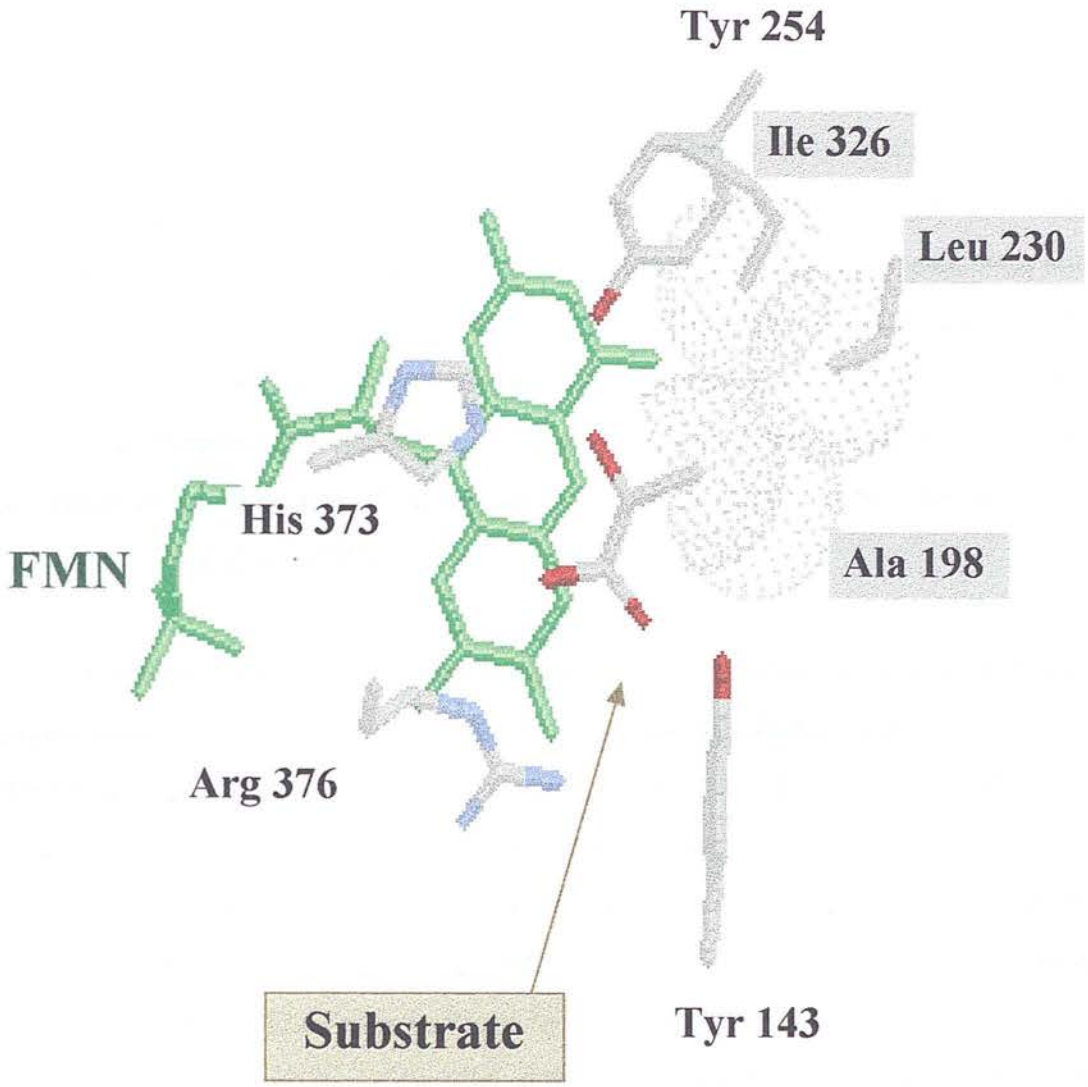

Figure 3.14. The three dimensional crystal structure at the active site of flavocytochrome b_2 from *S. cerevisiae*. The grey spheres represent Van der Waal's contact ranges for the side-chains of Ala198, Leu230 and Ile326 with substrate methyl group (Xia and Mathews, 1990; Daff, 1996).

is unable to oxidise lactate whereas L(+)-lactate dehydrogenase from *S. cerevisiae* is unable to oxidise mandelate. Moreover lactate is a competitive inhibitor for L(+)-mandelate dehydrogenase and L-mandelate is a competitive inhibitor for L(+)-lactate dehydrogenase.

Smekal *et al.*, (1993) carried out a molecular modelling study on the known three-dimensional sructure of L(+)-lactate dehydrogenase from *S.cerevisiae*. The authors suggest that productive binding of L-mandelate at the active site of LLDH might be impeded by steric interaction between the phenyl ring of L-mandelate and the side chains of Leu230 and Ala198 (Fig. 3.14). They also suggest that the side chains of Phe325 and Leu286 might be involved. Because steric interaction does not prevent the binding of L-mandelate (competitive inhibition occured) it is possible that steric interactions force L-mandelate to adopt an unfavourable orientation for catalysis (Smekal *et al.*, 1993).

Comparison of the amino acid sequence of L(+)-lactate dehydrogenase with several related flavoenzymes with different substrate specificities haas been made to identify the key residues required for substrate recognition (Fig. 3.15) by Daff *et al.* (1994). The comparison shows that residues Ala198 and Leu230, found in the crystal structure to have contact with the methyl group of pyruvate (the product of lactate oxidation) are not well conserved. Ala198 is replaced by glycine in some members of the family, whereas Leu230 is replaced by the larger tryptophan side side chain in glycolate oxidase and by a smaller alanine in mandelate dehydrogenase from *Pseudomonas putida*. This indicates that when the substrate is small the side chain of the residue at the position equivalent to Leu230 is larger and vice versa. To determine the importance of these residues Daff *et al.* (1994) constructed three mutants of flavocytochrome b_2: Ala198 to glycine, Leu230 to Ala and the double mutants of Ala198 to Gly/Leu230 to Ala. Results from mutation of Leu230 to Ala shows that selectivity of LLDH from *S. cerevisiae* increased in favour of long chain 2-hydroxyacids over lactate. A similar study by Wilks *et al.* (1990) involved the mutation of NAD^+-dependent L-lactate dehydrogenase from *Bacillus*

```
          198                                   230
           ↓                                     ↓
Scb2 PFYVSATALC KLGNPLEGEK DVARGCGQGV TKVPQMISTL ASCSPEEIIE
Hab2 PFYISATALA KLGHP.EGEV AIAKGAGRE. .DVVQMISTL ASCSFDEIAD
 Gox PIMIAPTAMQ KMAHP.EGEY ATARAASAA. .GTIMTLSSW ATSSVEEVAS
 Hao PICISPTAFH SIAWP.DGEK STARAAQEA. .NICYVISSY ASYSLEDIVA
 Mdh PLLIGPTGLN GALWP.KGDL ALARAATKA. .GIPFVLSTA SNMSIEDLAR
 Lox PMFFAPIGVI ALC.AQDGHG DAASAQASAR TGVPYITSTL AVSSLEDIRK
LctD PVALAPVGLC GMYAR.RGEV QAAKAADAH. .GIPFTLSTV SVCPIEEVA.
LMDH PIFVAPAGLA RLGHP.DGEQ NIVRGVAKH. .DILQVVSSG ASCSIDEIFE
```

Figure 3.15. Sequence comparison of L(+)-mandelate dehydrogenase from *R. graminis* (LMDH) and other 2-hydroxy acid dehydrogenase. The numbering as in flavocytochrome b_2 from *S. cerevisiae* (Scb2). The other sequences are: *Hansenula anomala* flavocytochrome b_2 (Hab2), spinach glycollate oxidase (Gox), rat hydroxy acid oxidase (Hao), *Pseudomonas putida* mandelate dehydrogenase (Mdh), *Mycobacterium smegmatis* lactate oxidase (Lox) and L-lactate dehydrogenase from *E. coli* (LctD). The position of Ala198 and Leu230 are indicated.

stearothermophilus to remove a large amount of steric bulk from the active site to convert the enzyme into a broad-specificity 2-hydroxyacid dehydrogenase.

When the amino acid sequence from L(+)-mandelate dehydrogenase from *R. graminis* became available, comparison with other flavodehydrogenase enzymes (Fig.3.15) showed that the positions of Ala198 and Leu230 in LLDH from *S. cerevisiae* are occupied by glycine at both positions in LMDH from *R. graminis*. This could explain the importance of the amino acid at position 230, especially in determining the substrate specificity of the enzyme and also why LLDH from *S. cerevisiae* is unable to utilise L-mandelate. Another explanation that can be given as to why L(+)-mandelate dehydrogenase from *R. graminis* is unable to use L-lactate is because this substrate is too small to occupy the pocket and interact with Gly225 (in LMDH of *R. graminis*) via its methyl group, this might cause the substrate to be improperly oriented for catalysis. Based on the amino acid sequence of LMDH from *R. graminis*, a mutation has been made which replaced Leu230 with glycine in LLDH from *S. cerevisiae*. The effects of the mutation include a 40-fold increase in kcat for mandelate compared to the wild type enzyme (R. Sinclair *et al*, unpublished results). This result is consistent with the importance of Leu230 (in LLDH from *S.cerevisiae*) and presumably Gly225 (in LMDH from *R. graminis*) in determining the substrate specificity of both enzymes.

3.4. Expression of L(+)-mandelate dehydrogenase

Two expression vectors were used in attempts to express L(+)-mandelate dehydrogenase. Firstly the cDNA insert in pLM5 was cut with *Xma*I and *Hind*III then cloned into the expression vector pKK223-3 cut with the same restriction enzymes to generate the recombinant plasmid pLM7, which was then transformed into *E. coli* JM105. The pKK223-3 expression vector contains a strong tac promoter (Amann *et al.*, 1983) which is regulated by the lac repressor supplied by the host JM105 (Yanisch-Perron, 1985). Transformants harbouring pLM7 were grown in LB medium containing 100 μg/ml ampicillin to OD_{600} about 0.6 then induced with IPTG

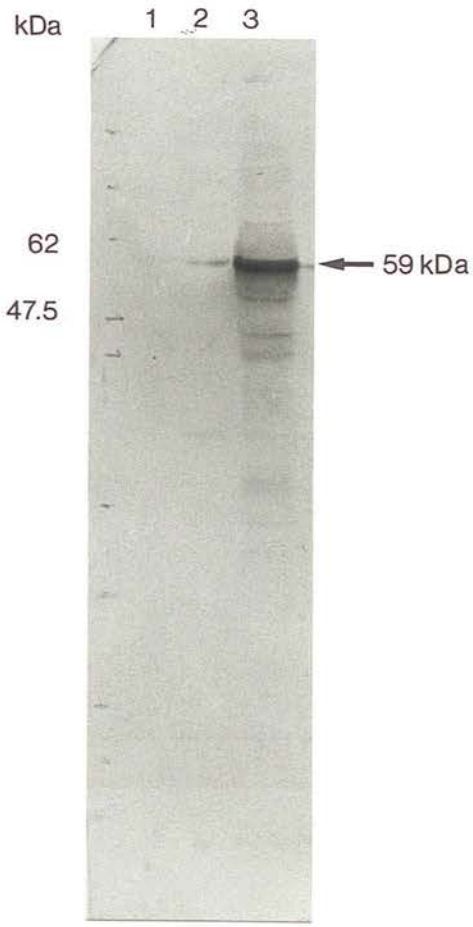

Figure 3.16. Immunological detection of L(+)-mandelate dehydrogenase after electrophoresis. L(+)-mandelate dehydrogenase was detected by probing with anti-LMDH followed by HRP-conjugated 2nd antibody as described in Materials and Methods. Lane 1: pre stain molecular marker, Lane 2 NF1 cell extract without any plasmid, Lane 3 extract from NF1 cell with plasmid pRC23 containing the cDNA of L(+)-mandelate dehydrogenase.

with concentration ranging from 0.5 to 2.0 mM. Western blotting of the cell extracts with anti-LMDH antibody followed by HRP-conjugated 2nd antibody failed to detect L(+)-mandelate dehydrogenase expression.

The cDNA insert from pLM7 was removed by cutting with *Eco*RI and *Hind*III, isolated and cloned into pRC23 cut with *Eco*RI and *Hind*III as a second vector to generate the recombinant plasmid pLM8. This plasmid was then transformed into *E. coli* NF1. Because pRC23 contains the thermoinducible lamda P_L promoter (Crowl *et al.*, 1985) which is repressed by the lamda *c*I857 at 30°C, the NF1 cells culture containing plasmid pLM8 were grown in LB medium at 30°C then shifted to 42°C to express the L(+)-mandelate dehydrogenase when the OD_{600} reached 0.6. A single strong band with Mr about 59,000 was detected on a Western blot probed with anti-LMDH antibody (Fig.3.16).

A batch of 5 litres of *E. coli* NF1 cells expressing the recombinant LMDH were grown overnight in LB medium. The recombinant LMDH from the grown 5 litre culture was purified as in Materials and Methods. Samples were taken at each step of the purification and checked for enzyme concentration (haem absorbance at 423 nm) and the enzyme activity. At the end of the purification samples from each steps were run on SDS-PAGE for enzyme purity estimation. A recombinant LMDH of about 30 % purity was obtained. Concentration for the impure recombinant LMDH is 3.4×10^{-5} M and from this a 50 µl aliquot was used to obtain the absorption spectrum.

The absorption spectrum of the recombinant LMDH shown in figure 3.17 is indistinguishable from that of LMDH isolated from *R. graminis* (Yasin and Fewson, 1993). Preliminary steady state kinetic analysis was performed on the partially purified recombinant L(+)-mandelate dehydrogenase. The data (Fig. 3.18) indicate a calculated k_{cat} of about 350 s^{-1} and K_m of 0.35 mM for L-mandelate. In LMDH from *R. graminis* the calculated k_{cat} and K_m was 50.8 s^{-1} and 0.27 mM respectively. The amount of LMDH produced in *R. graminis* is small and the purification of this

enzyme involves a long process which finally yield small amount of purified LMDH (Yasin and Fewson, 1993). During purification the loss of flavin could have caused the k_{cat} of LMDH from *R. graminis* to be lower than the recombinant LMDH. The concentration of enzyme stock was estimated from the haem absorbance at 423 nm where ε=183000 $M^{-1}cm^{-1}$.

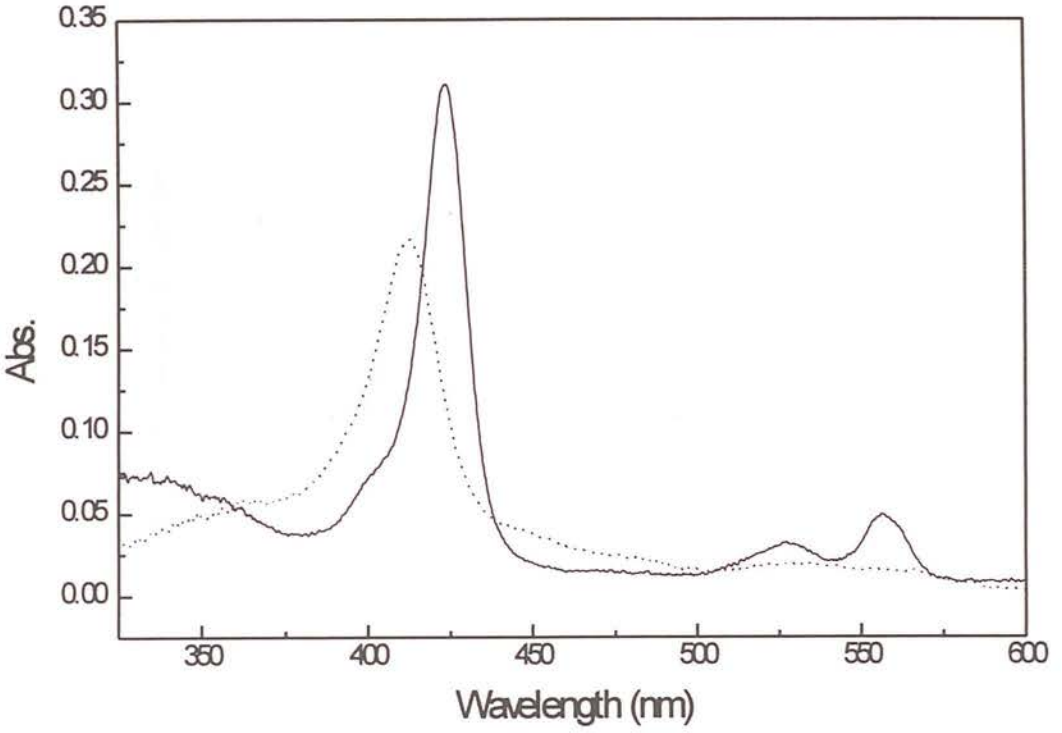

Figure 3.17. The spectra of oxidised (.....) and reduced (—) recombinant L(+)-mandelate dehydrogenase expressed in *E. coli*. The absorption spectra were obtain using partially purified recombinant LMDH with a concentration of 1.7 X 10^{-6} M in 1 ml solution.

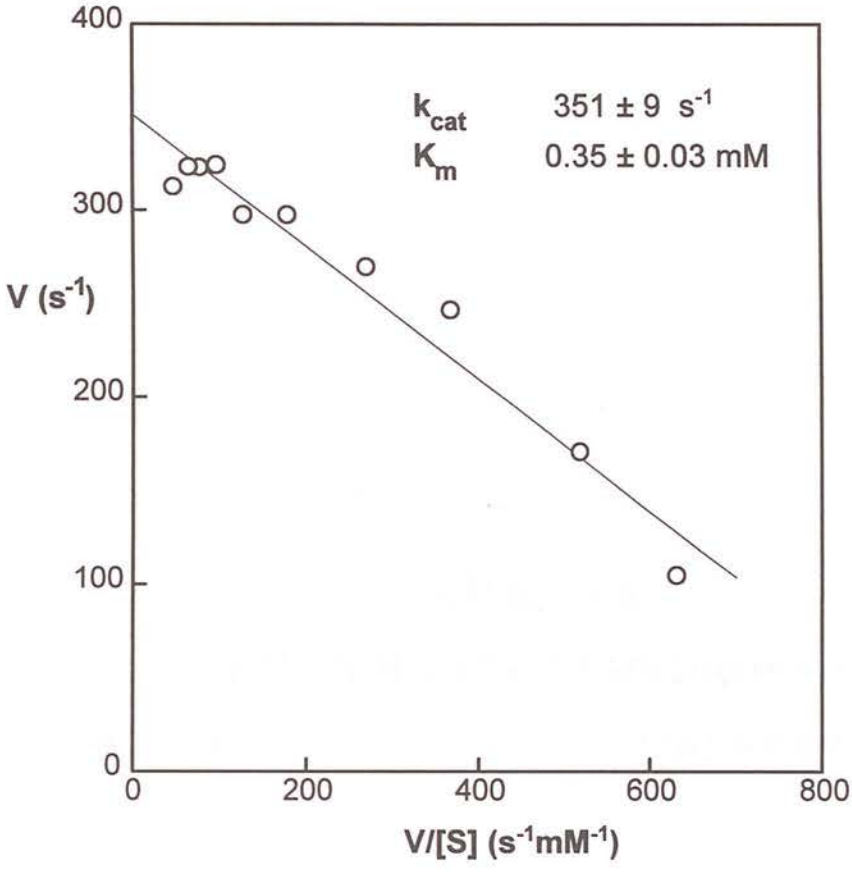

Figure. 3.18. An Eadie-Hofstee plot of recombinant L(+)-mandelate dehydrogenase expressed in *E. coli.* The result was obtain from partially purified enzyme.

CHAPTER 4

D(-)-MANDELATE DEHYDROGENASE CLONING, SEQUENCING AND EXPRESSION

CHAPTER 4

Cloning, sequencing and expression

4.1 Introduction

D-Mandelate dehydrogenase from *Rhodotorula graminis* has been purified and characterised previously by Baker and Fewson, (1989). Approximately 30 % of the amino acid sequence from this enzyme has been determined from the native protein and from three tryptic peptides (Miles, J.S. unpublished result). No obvious similarities were observed with the sequences of other soluble D-isomer specific 2-hydroxyacid dehydrogenases (Fewson *et al.*, 1993). Based on the amino acid sequences available, two fully degenerate oligonucleotides were made for PCR to synthesise a probe for D-mandelate dehydrogenase. A Polymerase Chain Reaction on genomic DNA of *R. graminis* amplified a 320bp DNA fragment which was then cloned into M13mp19. The sequence of this fragment was shown to encode the N-terminal region of D-mandelate dehydrogenase. A small intron was also detected within the sequence by comparing the DNA sequence with the amino acid sequence (Fig. 4.1).

Not much is known about the D-mandelate dehydrogenase from *Rhodotorula graminis*. Characterization of this enzyme showed that it is a NAD-dependent enzyme and does not contain bound flavin or cytochrome as cofactor (Baker and Fewson, 1989). In order to carry out further biochemical characterization it is important to isolate the gene encoding D-mandelate dehydrogenase to determine the complete amino acid sequence and also to construct an expression system to obtain large amounts of the enzyme. This will enable the study of the structural basis of the enzymes' substrate specificity.

In this chapter the isolation and sequencing of the D-mandelate dehydrogenase gene are described. The amino acid sequence derived from the cDNA

P33
*Eco*RI
GAATTCGACTTTCAGCAGAAATTTGAAGTCATCCCTGCCAACCTGACCACGCACGACGGG
AspPheGlnGlnLysPheGluValIleProAlaAsnLeuThrThrHisAspGly

TTTAAACAGGCCCTGCGCGAGAAGCGGTGCGTTCTGTTCCTGGCCCTCACGCGGTTCTTC
PheLysGlnAlaLeuArgGluLysAr

CGCTGACCGCATTCTCCGCCGCATCACTTGTGCTTCTCCCCGTCGTACGCAGCTATGGCG
gTyrGlyA

ACTTCGAAGCCATCATCAAGCTTGCCGTCGAGAACGGCACCGAGAGCTATCCCTGGAAGC
spPheGluAlaIleIleLysLeuAlaValGluAsnGlyThrGluSerTyrProTrpAsnA

CCGACCTCATCTCGCACCTCCCTTCGTCCCTCAAAGTCTTTGCCGCCGCCGGCGCAGGTT
laAspLeuIleSerHisLeuProSerSerLeuLysValPheAlaAlaAlaGlyAlaGlyP

TTGATTGGCCGGATCC
heAspTrp *BamHI*
P34

Figure 4.1 The sequence of 320 basepair fragment of D-mandelate dehydrogenase gene from *Rhodotorula graminis*. The PCR primer (P33 and P34) sequences are underlined. For P34 this is the complementary sequence. This work was done by Miles, J.S.

is compared with sequences of other proteins in the database. The cDNA was cloned into an expression vector for production of D-mandelate dehydrogenase in *E. coli.*

4.2 Result and Discsusion

4.2.1 Isolation of the D(-)-Mandelate Dehydrogenase Gene

Rhodotorula graminis chromosomal DNA was isolated (see Materials and Methods) and digested with seven different restriction enzymes. None of the enzymes used to digest the genomic DNA cut within the 320 bp fragment. Southern Blot analysis was carried out on the digested chromosomal DNA. This was then probed with the ^{32}P-labelled 320 bp fragment (Fig. 4.1) labelled by random priming. The autoradiograph of the Southern blot showed that the D-mandelate dehydrogenase gene was contained within a 4.4 kb *Sac*I fragment (Fig. 4.2).

A genomic library was constructed from *R. graminis* DNA digested with *Sac*I. The digested DNA was ligated to *Sac*I-cut pTZ19R. Transformants containing plasmids with inserts were identified as white colonies on X-gal/IPTG plates. Approximately 10,000 recombinants were screened by colony blotting using the same probe as for the Southern blot. A single positive clone (pRI1) was identified. Plasmid from this positive clone was purified, cut with *Sac*I and shown to contain an insert of the expected size (Fig. 4.3).

4.2.2 Sequencing of the Cloned Fragment

Two primers were designed based on the known sequence from the 320 bp fragment to start sequencing the D-mandelate dehydrogenase gene. The 4.4 kb insert was recloned to obtain a recombinant with the opposite insert orientation (pRI2). The DNA was sequenced from these primers as far as possible. Then new primers were designed based on the available sequence till all the expected D-mandelate dehydrogenase gene had been sequenced. The DMDH sequences were determined on

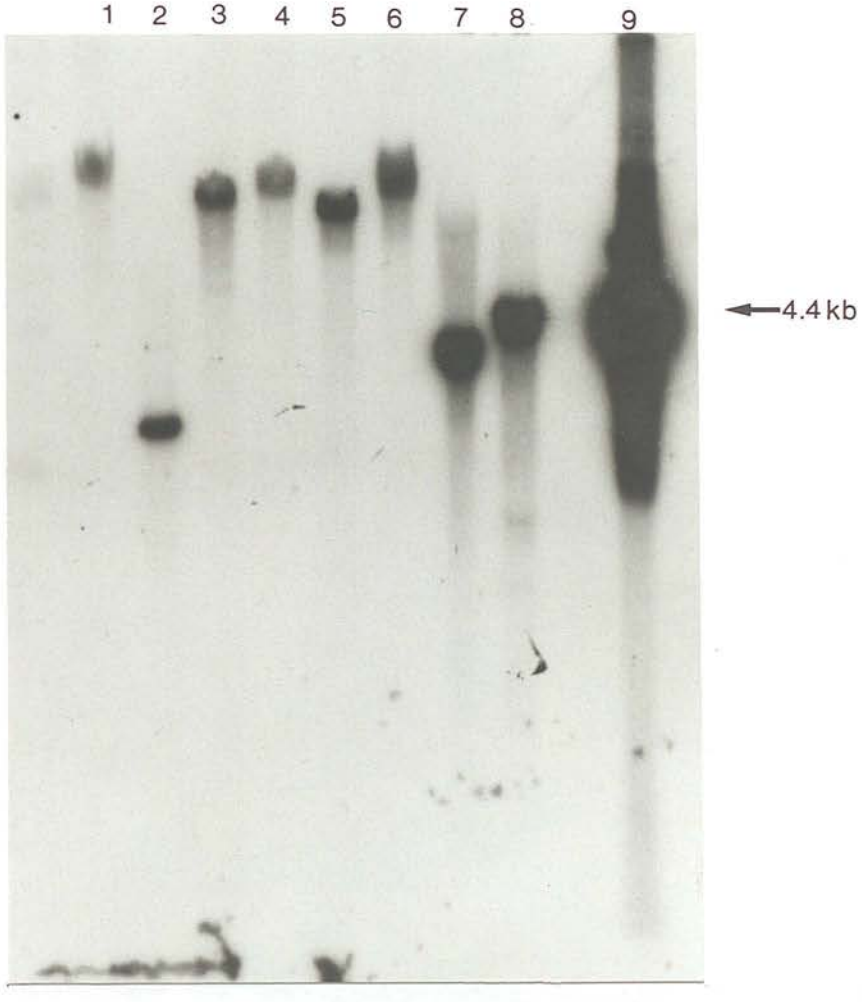

Figure 4.2. Autoradiograph of the Southern Blot of total genomic DNA and the positive colony probed with radiolabelled oligonucleotide. Lane 1; undigested genomic DNA, Lane 2; cut with *Pst*I, Lane 3: cut with *Bam*HI, Lane 4: cut with *Eco*RI, Lane 5: cut with *Sma*I, Lane 6: cut with *Xba*I, Lane 7, cut with *Sph*I, Lane 8 cut with *Sac*I, Lane 9; the positive colony.

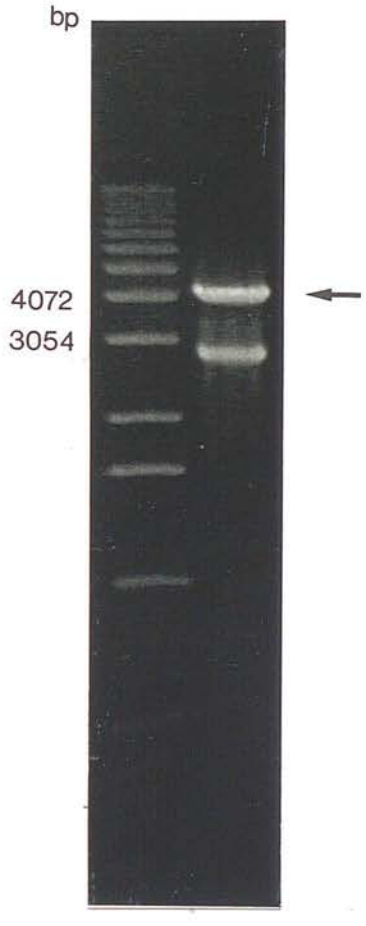

Figure 4.3. Restriction digest of pRI1 with *Sac*I showing the 2.9 kb plasmid (pTZ19R) and the insert of 4.4 kb which contains the gene for D-mandelate dehydrogenase.

Figure 4.4. The sequence of the cloned D-mandelate dehydrogenase gene. The DMDH gene starts at ATG and ends with TGA, which are marked with a caret (^). The three introns are underlined with the internal sequence consensus marked with an asterisk (*). Each intron starts with GT and ends with CAG. The sequence was determined on both strands.

```
   1  tctcgaccag ttcccgcaca aagtgggggc gatttgtgct ggtggggcgc
  51  ccggagctgg cccctctgct tcttacagtc cacttgcgct acgctgcttc
 101  ttgtcgtgcc cctttcttgc tgctgtccga cttccgccat gcctcgccct
 151  cgcgtccttc tcctcggcga ccccgctcgg cacctcgacg acctctggag
 201  cgacttccag caaaagttcg aagtcatccc tgccaacctg accacgcacg
 251  acgggtttaa acaggccctg cgcgagaagc ggtgcgttct gttcctggcc
 301  ctcacgcggt tcttccgctg accgcattct ccgccgcatc acttgtgctt
                          *** **
 351  ctccccgtcg tacgcagcta tggcgacttc gaagccatca tcaagcttgc
 401  cgtcgagaac ggcaccgaga gctatccctg gaacgccgac ctcatctcgc
 451  acctcccttc gtccctcaaa gtctttgccg ccgccggcgc aggcttcgac
 501  tggctcgacc tcgacgcact caacgagcgc ggagtcgcgt ttgccaactc
 551  gcgcggcgca ggcgacacgg cgacatccga tctcgcgctg tacctcatcc
 601  tgtccgtctt ccgcctcgcg agctactcgg agcgcgccgc gcgcacgggc
 651  gaccccgaga cgttcaaccg cgtgcacctc gagattggca agtcggcgca
 701  caacccgcgc gggcacgtcc tcggcgcggt cgggctcggc gcgatccaga
 751  aggagatcgc gaggaaggcg gtgcatggcc ttgggatgaa gctcgtgtac
 801  tacgacgtcg cgcctgccga cgcggagacg gagaaggcgc tcggtgctga
 851  gcgcgtcgac tcgctcgaag agctggcgag gaggagcgac tgtgtcagcg
 901  tgtcgggtga gtctttccac ttcgtcatcg gggaacccgt cctcttggca
 951  ctcaccagta tgcaccacac agtgccgtat atgaagttga cgcaccatct
      *****
1001  cattgacgaa gccttcttcg ccgcgatgaa gcccggctcg cgcattgtca
1051  atactgcgcg tggccccgtc atctcgcagg acgcacttat cgccgcgctc
1101  aagtcgggca agctgctcag tgcaggcctc gacgtgcacg agttcgagcc
1151  acaggtgtcc aaggaactca tcgagatggt gcgttcgagc cctgccactg
1201  ctctgcccgc cttccatact ttcagatcgt gctgacctat ccatgtgtct
                                          *****
1251  gcagaagcac gtcacgctca cgacgcacat cggcggcgtg gcgatcgaga
1301  ccttccacga gttcgagcgg ctcaccatga ccaacatcga ccgcttcctc
1351  ctgcaaggca agcccttgct gaccgtgcgt ctttgcccga actctctgcg
1401  ccccgctttt cgcgcctact gacgctctgg cgctgctgca gcctgcgggc
1451  aaggtgtttg cgccgtccag cgctgcatga cctgctcgga cagattcaga
1501  tcgatactgc aggaaggagt gcacgaggag ggcagcaccc tgacaagagc
1551  gctccgtctg caatgaacct tacaggatgt cgcaatgaaa ctcagggaga
1601  cagcggaaca gcggaagttg ctgctgaaat
```

both strand. About 1630 bp have been sequenced from the 4.4 kb insert. The D-mandelate dehydrogenase coding sequence starts at position 139 and ends at position 1417 (Fig. 4.4). The initiation codon of the D-mandelate dehydrogenase is ATG and TGA is used as a stop codon. The D-mandelate dehydrogenase gene contains a high GC content, about 63 %.

There are several introns present in phenylalanine-ammonia lyase gene from *Rhodosporidium toruloides* and *Rhodotorula rubra* as explained in chapter three. These organisms are closely related to *Rhodotorula graminis*. The presence of introns in the coding sequence of the D-mandelate dehydrogenase gene from *Rhodotorula graminis* was obvious from the presence of in-frame stop codons in the genomic DNA. The first intron in the gene sequence is located at position 281 to 367 and had already been detected from the 320 bp fragment (Fig. 4.1). There are two other introns predicted to be present in the genomic sequence based on the presence of in-frame stop codons and the presence of conserved intron sequence as above. The positions of these two introns were confirmed after the isolation of the cDNA. These are located at position 907 to 972 and 1180 to 1255 respectively (Fig. 4.4). In common with a number of genes from yeast and filamentous fungi (Anson *et al.*, 1987), the introns are relatively small with sizes ranging from 66 to 86 bp. In all cases the 5'ends of the introns have the invariant sequence of GT. All the introns also contain the nucleotides CAG at their 3' end, indicating perfect agreement to the consensus intron acceptor sequence generally observed in eukaryote genes (Mount, 1982). *Rhodotorula graminis* introns also contain the internal consensus sequence of CTGAC like the phenylalanine ammonia-lyase gene intron, except that in the second intron in *Rhodotorula graminis* the closest match to the internal consensus sequence is CTCAC. The 3' splice site CAG of *Rhodotorula graminis* is located between 13 and 42 nucleotides downstream from the internal consensus sequence. The conserved

(A). Introns in some organisms

(a). *Neurospora crassa* intron consensus (Orbach *et al.*, 1986)

```
GTAAGT.......ACTAACA......7-18......CAG
   C           G  G  T
```

(b). *Schizosaccharomyces pombe* intron consensus (Hindley and Phear, 1984)

```
GTANGT.......TNCTAACCNT.....9-12.....AAG
```

(c). Higher eukaryote intron consensus (Orbach *et al.*, 1986)

```
GTATGT..................CAG
  G
```

(B). Comparison of introns in *R. graminis* and *S. cerevisiae*(Orbach *et al.*, 1986):

```
                                      C
R. graminis :  .......GT........CTGAC........CAG.......
S. cerevisiae: .......GT......TACTAAC........CAG.......
               exon      5'        branch point        3' exon
```

Figure 4.5. A comparison of *R. graminis* introns with other introns.

intron sequences of DMDH gene in *R. graminis* are very similar to the intron sequence from the phenylalanine ammonia-lyase genes from *Rhodosporidium toruloides* and *Rhodotorula rubra*. In *Saccharomyces cerevisiae* introns, internal sequences conforming to the consensus TACTAAC, which form a branch point during splicing, are always present (Langford and Gallwitz, 1983). This sequence has been shown by mutagenesis to be an essential element of the yeast intron splicing mechanism. The CTGAC/CTCAC internal sequences in *R. graminis* probably have the same function as the branch point sequence in *S. cerevisiae* (Fig. 4.5).

4.2.3 Isolation of the D-Mandelate Dehydrogenase cDNA

Isolation of cDNA was undertaken to confirm the intron boundaries and thus to determine the whole sequence of D-mandelate dehydrogenase. The cDNA could then be used to direct expression of recombinant D-mandelate dehydrogenase. Total RNA from *Rhodotorula graminis* was used as a template to make a single stranded cDNA by reverse transcription (see Materials and Methods) which was then used in the PCR. Two primers (RI3 and N1179) have been made based on the known N-terminal sequence of the protein and the C-terminal sequence predicted from the genomic DNA. An *Eco*RI restriction site was incorporated for the forward primer and *Pst*I restriction site for the reverse primer to facilitate cloning of the product.

The polymerase chain reaction was carried out at 95°C for 5 min initial denaturing then 3 cycles of: 95°C for 40 sec denaturing, 50°C annealing for 30 sec, 72°C extension for 1.5 min. Then proceed with another 40 cycles of: 95°C for 40 seconds denaturing, 64°C annealing for 30 seconds and 72°C extension for 1.5 minutes. Finally another 5 min extension at 72°C was carried out to complete the reaction. A fragment of about 1053 bp was amplified (Fig. 4.6). The fragment was then treated with Klenow fragment to create a blunt 3'end and cut with *Eco*RI at the 5' site. This was done to provide the restriction site close to the *Sma*I site in the pTZ19R to be used in later cloning of the cDNA into the expression vector. The treated fragment was then cloned into pTZ19R cut with *Eco*RI and *Sma*I to generate

Figure 4.6. The above figure shows the fragment of DMDH cDNA (1053 bp/marked by arrow) which was amplified by PCR. The cDNA fragment was subcloned into the expression vector pRC23 (4.6kb) to produce recombinant plasmid pRI6 (see appendix).

Figure 4.7. The cDNA sequence of D-mandelate dehydrogenase and the deduced protein translation. Sequence was determined on both strands.

```
   atgcctcgccctcgcgtccttctcctcggcgaccccgctcggcacctcgacgacctctgg
 1 ---------+---------+---------+---------+---------+---------+ 60
   tacggagcgggagcgcaggaagaggagccgctggggcgagccgtggagctgctggagacc

   M  P  R  P  R  V  L  L  L  G  D  P  A  R  H  L  D  D  L  W

   agcgacttccagcaaaagttcgaagtcatccctgccaacctgaccacgcacgacgggttt
61 ---------+---------+---------+---------+---------+---------+ 120
   tcgctgaaggtcgttttcaagcttcagtagggacggttggactggtgcgtgctgcccaaa

   S  D  F  Q  Q  K  F  E  V  I  P  A  N  L  T  T  H  D  G  F

   aaacaggccctgcgcgagaagcgctatggcgacttcgaagccatcatcaagcttgccgtc
121 ---------+---------+---------+---------+---------+---------+ 180
   tttgtccgggacgcgctcttcgcgataccgctgaagcttcggtagtagttcgaacggcag

   K  Q  A  L  R  E  K  R  Y  G  D  F  E  A  I  I  K  L  A  V

   gagaacggcaccgagagctatccctggaacgccgacctcatctcgcacctcccttcgtcc
181 ---------+---------+---------+---------+---------+---------+ 240
   ctcttgccgtggctctcgatagggaccttgcggctggagtagagcgtggagggaagcagg

   E  N  G  T  E  S  Y  P  W  N  A  D  L  I  S  H  L  P  S  S

   ctcaaagtctttgccgccgccggcgcaggcttcgactggctcgacctcgacgcactcaac
241 ---------+---------+---------+---------+---------+---------+ 300
   gagtttcagaaacggcggcggccgcgtccgaagctgaccgagctggagctgcgtgagttg

   L  K  V  F  A  A  A  G  A  G  F  D  W  L  D  L  D  A  L  N

   gagcgcggagtcgcgtttgccaactcgcgcggcgcaggcgacacggcgacatccgatctc
301 ---------+---------+---------+---------+---------+---------+ 360
   ctcgcgcctcagcgcaaacggttgagcgcgccgcgtccgctgtgccgctgtaggctagag

   E  R  G  V  A  F  A  N  S  R  G  A  G  D  T  A  T  S  D  L

   gcgctgtacctcatcctgtccgtcttccgcctcgcgagctactcggagcgcgccgcgcgc
361 ---------+---------+---------+---------+---------+---------+ 420
   cgcgacatggagtaggacaggcagaaggcggagcgctcgatgagcctcgcgcggcgcgcg

   A  L  Y  L  I  L  S  V  F  R  L  A  S  Y  S  E  R  A  A  R

   acgggcgaccccgagacgttcaaccgcgtgcacctcgagattggcaagtcggcgcacaac
421 ---------+---------+---------+---------+---------+---------+ 480
   tgcccgctggggctctgcaagttggcgcacgtggagctctaaccgttcagccgcgtgttg

   T  G  D  P  E  T  F  N  R  V  H  L  E  I  G  K  S  A  H  N

   ccgcgcgggcacgtcctcggcgcggtcgggctcggcgcgatccagaaggagatcgcgagg
481 ---------+---------+---------+---------+---------+---------+ 540
   ggcgcgcccgtgcaggagccgcgccagcccgagccgcgctaggtcttcctctagcgctcc

   P  R  G  H  V  L  G  A  V  G  L  G  A  I  Q  K  E  I  A  R
```


aaggcggtgcatggccttgggatgaagctcgtctactacgacgtcgcgcctgccgacgcg
541 --------+--------+--------+--------+--------+--------+ 600
ttccgccacgtaccggaaccctacttcgagcagatgatgctgcagcgcggacggctgcgc
K A V H G L G M K L V Y Y D V A P A D A
gagacggagaaggcgctcggtgctgagcgcgtcgactcgctcgaagagctggcgaggagg
601 --------+--------+--------+--------+--------+--------+ 660
ctctgcctcttccgcgagccacgactcgcgcagctgagcgagcttctcgaccgctcctcc
E T E K A L G A E R V D S L E E L A R R
agcgactgtgtcagcgtgtcggtgccgtatatgaagttgacgcaccatctcattgacgaa
661 --------+--------+--------+--------+--------+--------+ 720
tcgctgacacagtcgcacagccacggcatatacttcaactgcgtggtagagtaactgctt
S D C V S V S V P Y M K L T H H L I D E
gccttcttcgccgcgatgaagcccggctcgcgcattgtcaatactgcgcgtggccccgtc
721 --------+--------+--------+--------+--------+--------+ 780
cggaagaagcggcgctacttcgggccgagcgcgtaacagttatgacgcgcaccggggcag
A F F A A M K P G S R I V N T A R G P V
atctcgcaggacgcacttatcgccgcgctcaagtcgggcaagctgctcagtgcaggcctc
781 --------+--------+--------+--------+--------+--------+ 840
tagagcgtcctgcgtgaatagcggcgcgagttcagcccgttcgacgagtcacgtccggag
I S Q D A L I A A L K S G K L L S A G L
gacgtgcacgagttcgagccacaggtgtccaaggaactcatcgagatgaagcacgtcacg
841 --------+--------+--------+--------+--------+--------+ 900
ctgcacgtgctcaagctcggtgtccacaggttccttgagtagctctacttcgtgcagtgc
D V H E F E P Q V S K E L I E M K H V T
ctcacgacgcacatcggcggcgtggcgatcgagaccttccacgagttcgagcggctcacc
901 --------+--------+--------+--------+--------+--------+ 960
gagtgctgcgtgtagccgccgcaccgctagctctggaaggtgctcaagctcgccgagtgg
L T T H I G G V A I E T F H E F E R L T
atgaccaacatcgaccgcttcctcctgcaaggcaagcccttgctgaccgtgcgtctttgc
961 --------+--------+--------+--------+--------+--------+ 1020
tactggttgtagctggcgaaggaggacgttccgttcgggaacgactggcacgcagaaacg
M T N I D R F L L Q G K P L L T V R L C
ccgaactctctgcgccccgcttttcgcgcctactga
1021 --------+--------+--------+------ 1056
ggcttgagagacgcggggcgaaaagcgcggatgact
P N S L R P A F R A Y *

the recombinant plasmid pRI3. The cDNA in pRI3 was recloned into pTZ18R (then called pRI4) to obtain the alternative orientation for sequencing the second strand. The same primers that were used to sequence the genomic DNA encoding D-mandelate dehydrogenase were also used to sequence the cDNA (Fig. 4.7), with single-stranded pRI3 and pRI4 templates as appropriate. The sequences were determined on both strands.

4.2.4 Amino acid Sequence Comparison

The entire 1053 bp sequence of the amplified D-mandelate dehydrogenase cDNA is shown in figure 4.7. This open reading frame specifies a protein (DMDH) of 351 amino acids with a calculated molecular weight of 38,591 Daltons. This agreed with the Mr of 38,000 which was estimated from the SDS-PAGE (Baker and Fewson, 1989).

A computer search of the EMBL and Swissprot protein sequence data banks with the program FASTA, using the D-mandelate dehydrogenase as the query sequence indicated amino acid sequence similarity with D-2-hydroxyacid dehydrogenases. Alignment with other proteins in the data base using the PILEUP programme (Fig. 4.9.) demonstrated that *Rhodotorula graminis* D-mandelate dehydrogenase exhibits 27-33% identity to each of: the D-3-phosphoglycerate dehydrogenase from *Haemophilus influenzae*, D-glycerate dehydrogenase from *Hyphomicrobium methylovorum*, D-lactate dehydrogenase from *Lactobacillus delbrueckii*, formate dehydrogenase from *Hansenula polymorpha,* D-3-glycerate dehydrogenase from *E. coli*, formate dehydrogenase from *Emericella nidulans*, formate dehydrogenase from *Neurospora crassa,* D-lactate dehydrogenase from *Lactobacillus casei*, D-3-phosphoglycerate dehydrogenase from yeast *S. cerevisiae* (serx-yeast) and D-3-phosphoglycerate dehydrogenase from *S. cerevisiae* (sery-yeast). All these enzymes utilise D-2-hydroxyacids as substrates except for formate which have no chiral centre. D-mandelate dehydrogenase from *Rhodotorula graminis* clearly belongs to the D-isomer specific 2-hydroxyacid dehydrogenase family.

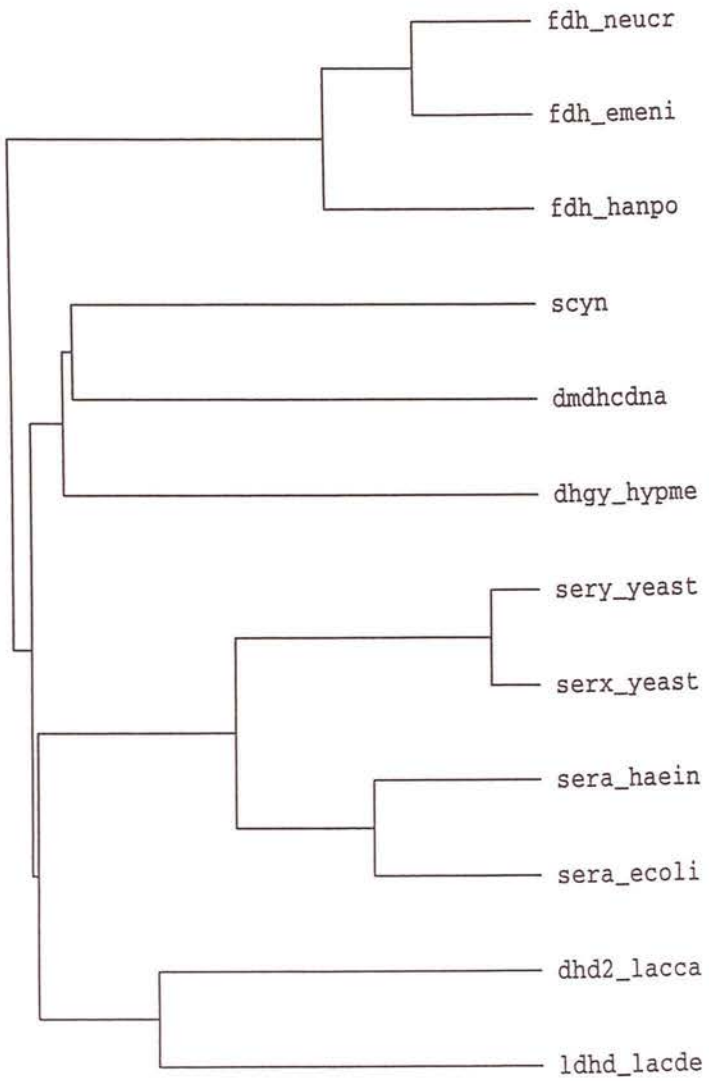

Figure 4.8. Family tree of the D-isomer specific dehydrogenase enzymes. The dendogram shows the output of the UWGCG programme PILEUP. dmdhcdna and scyn represent the deduced amino acid sequence of DMDH of *R. graminis* and the product of *S. cerevisiae* chromosome XIV respectively. fdh means formate dehydrogenase. fdh_neucr from *Neurospora crassa*, fdh_emeni from *Emericella nidulans*. fdh_hanpo from *Hansenula polymorpha*. dhd2_lacca; D-2-hydroxyisocaproate dehydrogenase of *Lactobacillus casei*, ldhd_lacde; D-lactate dehydrogenase of *Lactobacillus delbureckii*. sery_yeast, serx_yeast, sera_haein, sera_ecoli represent D-3-phosphoglycerate dehydrogenase from *S. cerevisiae*, *Haemophilus influenza* and *E. coli*. dhgy_hypme; D-glycerate dehydrogenase of *Hyphomicrobium methylovorum*.

Figure 4.9. The following are the sequences alignment of the D-isomer specific 2 - hydroxyacid dehydrogenase family. The sequence were aligned using the PILEUP programme in the University of Wisconsin Genetics Computer Group (UWGCG) package. The conserved residues (identical in all sequences) are marked with an asterisk (*) and semi-invariant residues (allowing two mismatches) are marked with (+) below the alignment. The functionally important residues are marked with f on top. The sequences are:

SEQUENCE NAME	ENZYME / ORGANISMS	REFERENCES	SWISSPROT ACCESSION NUMBER
fdh_neucr	formate dehydrogenase/*N. crassa*	Chow and BajBhandry,(1993)	Q07103
fdh_emeni	formate dehydrogenase/*E. nidulans*	Saleeba *et al.*, (1992)	Q03134
fdh_hanpo	formate dehydrogenase/*H. polymorpha*	Trishkov *et al.*, (1993)	P33677
scyn	chromosome XIV product/*S. cerevisiae*		Z71550(EMBL)
	(ORFYNL274c)		
dmdhcdna	D-mandelate dehydrogenase/*R. graminis*		
dhgy_hypme	D-glycerate dehydrogenase/*H. methylovorum*	Yoshida *et al.*, (1994)	P36234
sery_yeast	D-3-phosphoglycerate dehydrogenase/*S. cerevisiae*		P40510
serx_yeast	D-3-phosphoglycerate dehydrogenase/*S. cerevisiae*		P40054
sera_haein	D-3-phosphoglycerate dehydrogenase/*H.influenzae*	Fleischmann *et al.*, (1995)	P43885
sera_ecoli	D-3-phosphoglycerate dehydrogenase/*E. coli*	Tobey and Grant, (1986)	P08328
dhd2_lacca	D-2-hydroxyisocaproate dehydrogenase/*L. casei*	Lerch *et al.*, (1989)	P17584
ldhd_lacde	D-lactate dehydrogenase/*L. delbrueckii*	Bernard *et al.*, (1991)	P26297

```
 fdh_neucr  ..........  ..........  ..........  ..........  ..........
 fdh_emeni  ..........  ..........  ..........  ..........  ..........
 fdh_hanpo  ..........  ..........  ..........  ..........  ..........
      scyn  ..........  ..........  ..........  ..........  .......MSK 3
   dmdhcdna  ..........  ..........  ..........  ..........  .......MPR 3
dhgy_hypme  ..........  ..........  ..........  ..........  ........SK 2
sery_yeast  MSYSAADNLQ  DSFQRAMNFS  GSPGAVSTSP  TQSFMNTLPR  RVSITKQPKA 50
serx_yeast  MTSIDINNLQ  NTFQQAMNMS  GSPGAVCTSP  TQSFMNTVPQ  RLNAVKHPKI 50
sera_haein  ..........  ..........  ..........  ..........  .......MTN 3
sera_ecoli  ..........  ..........  ..........  ..........  .........A 1
dhd2_lacca  ..........  ..........  ..........  ..........  ..........
ldhd_lacde  ..........  ..........  ..........  ..........  ..........

 fdh_neucr  .....MVKVL  AVLYDGGKHG  EEVPELLGTI  QNELGLRKWL  EDQGHTLVTT 45
 fdh_emeni  ..........  MVLYDGGSHA  KDQPGLLGTT  ENELGIRKWI  EEQGHTLVTT 40
 fdh_hanpo  .......KVV  LVLYDAGKHA  QDEERLYGCT  ENALGIRDWL  EKQGHDVVVT 43
      scyn  KPIVLKLGKD  AFGDQAWGEL  EKIADVITIP  EST......T  REQFLREVKD 47
   dmdhcdna  PRVLLLGDPA  RHLDDLWSDF  QQKFEVIP.A  NLT......T  HDGFKQALRE 46
dhgy_hypme  KKILITW...  PLPEAAMARA  RESYDVIAHG  DDP......K  IT..IDEMIE 41
sery_yeast  LKPFSTGDMN  ILLLENVNAT  A..IKIFKDQ  GY.......Q  VEFHKSSLPE 91
serx_yeast  LKPFSTGDMK  ILLLENVNQT  A..ITIFEEQ  GY.......Q  VEFYKSSLPE 91
sera_haein  KVSLDKSKIK  FVLFEGVHQS  A..LDTLHAA  GYT......N  IDYYKKALDG 45
sera_ecoli  KVSLEKDKIK  FLLVEGVHQK  A..LESLRAA  GYT......N  IEFHKGALDD 43
dhd2_lacca  ........MK  IIAYGARVDE  IQYFKQWAK.  DTG......N  TLEYHTEFLD 35
ldhd_lacde  ........TK  IFAYAIREDE  KPFLKEWEDA  HKD......V  EVEYTDKLLT 36

 fdh_neucr  CDKDGENSTF  DKELEDAEII  ITTPFHPGYL  TAERLARAKK  LKLAVTAGIG 95
 fdh_emeni  SDKDGENSTF  DKELVDAEVI  ITTPFHPGYL  TAERLAKAKN  LKLAVTAGIG 90
 fdh_hanpo  SDKEGQNSVL  EKNISDADVI  ISTPFHPAYI  TKERIDKAKK  LKLLVVAGVG 93
      scyn  PQNKLSQVQV  ITRTA.RSVK  NTGRFDEELA  LALPSS....  VVAVCHTGAG 92
   dmdhcdna  ..KRYGDFEA  IIKLAVENGT  ESYPWNADLI  SHLPSS....  LKVFAAAGAG 90
dhgy_hypme  TAKSVDALLI  TLNEKCRK..  ........EVI  DRIPEN....  IKCISTYSIG 78
sery_yeast  D....ELIEK  IKDVHAIGIR  SKTRLTEKIL  QHARN.....  LVCIGCFCIG 132
serx_yeast  E....ELIEK  IKDVHAIGIR  SKTRLTSNVL  QHAKN.....  LVCIGCFCIG 132
sera_haein  D....ELKEA  IKDVHFIGLR  SRTHLTAEMI  EAAPK.....  LIAVGCFCIG 86
sera_ecoli  E....QLKES  IRDAHFIGLR  SRTHLTEDVI  NAAEK.....  LVAIGCFCIG 84
dhd2_lacca  E....NTVEW  AKGFDGINSL  QTTPYAAGVF  EKMHAYG...  IKFLTIRNVG 78
ldhd_lacde  P....ETAAL  AKGADGVVVY  QQLDYTAETL  QALADNG...  ITKMSLRNVG 79
                                                            *

 fdh_neucr  SDHVDLNAAN  KTNGGITVAE  VTGSNVVSVA  EHVLMTILVL  VRNFVPAHEQ 145
 fdh_emeni  SDHVDLDAAN  KTNGGITVAE  VTGSNVVSVA  EHVVMTILLL  VRNFVPAHDQ 140
 fdh_hanpo  SDHIDLDYIN  QSGRDISVLE  VTGSNVVSVA  EHVVMTMLVL  VRNFVPAHEQ 143
      scyn  YDQIDVEPFK  K..RHIQVAN  VPDLVSNATA  DTHVFLLLGA  LRNFGIGNRR 140
   dmdhcdna  FDWLDLDALN  E..RGVAFAN  SRGAGDTATS  DLALYLILSV  FRLASYSERA 138
dhgy_hypme  FDHIDLDACK  A..RGIKVGN  APHGVTVATA  EIAMLLLLGS  ARRAGEGEKM 126
sery_yeast  TNQVDLKYAA  S..KGIAVFN  SPFSNSRSVA  ELVIGEIISL  ARQLGDRSIE 180
serx_yeast  TNQVDLDYAT  S..RGIAVFN  SPFSNSRSVA  ELVIAEIISL  ARQLGDRSIE 180
sera_haein  TNQVDLNAAK  A..RGIPVFN  APFSNTRSVA  ELVLGEILLL  MRNVPQANAE 134
sera_ecoli  TNQVDLDAAA  K..RGIPVFN  APFSNTRSVA  ELVIGELLLL  LRGVPEANAK 132
dhd2_lacca  TDNIDMTAMK  Q..YGIRLSN  VPAYSPAAIA  EFALTDTLYL  LRNMGKVQAQ 126
ldhd_lacde  VDNIDMAKAK  E..LGFQITN  VPVYSPNAIA  EHAAIQAARI  LRQAKAMDEK 127
                *          +              + +          *
```

```
                                                  f f  f
 fdh_neucr  IQEGRWDVAE ......AAKN EFDLEGKVVG TVGVGRIGER VLRR.LKPFD 188
 fdh_emeni  IRNGDWNVAA ......VAKN EFDLENKVVG TVGVGRIGER VLRR.LKPFD 183
 fdh_hanpo  IISGGWNVAE ......IAKD SFDIEGKVIA TIGAGRIGYR VLER.LVAFN 186
      scyn  LIEGNWPEAG PACGSPFG.. .YDPEGKTVG ILGLGRIGRC ILER.LKPFG 186
  dmdhcdna  ARTGD.PETF NRVHLEIGKS AHNPRGHVLG AVGLGAIQKE IARKAVHGLG 187
dhgy_hypme  IRTRSWPG.. ...WEPLELV GEKLDNKTLG IYGFGSIGQA LAKRA.QGFD 170
sery_yeast  LHTGTWNKVA ........AR CWEVRGKTLG IIGYGHIGSQ LSVLA.EAMG 221
serx_yeast  LHTGTWNKVA ........AR CWEVRGKTLG IIGYGHIGSQ LSVLA.EAMG 221
sera_haein  VHRGVWNKSA ........TG SHEVRGKKLG IIGYGHIGSQ LSIIA.ESLG 175
sera_ecoli  AHRGVWNKLA ........AG SFEARGKKLG IIGYGHIGTQ LGILA.ESLG 173
dhd2_lacca  LQAGDYEKAG .......TFI GKELGQQTVG VMGTGHIGQV AIKLF.KGFG 168
ldhd_lacde  VARHDLRWAP ........TI GREVRDQVVG VVGTGHIGQV FMQIM.EGFG 168
               +                          +   * * *+
                  f
 fdh_neucr  CKELLYYDYQ PLSAEKEAEI GCRRVADLEE MLAQCDVVTI NCPLHEKTQG 238
 fdh_emeni  CKELLYYDYQ PLRPEVEKEI GARRVDSLEE MVSQCDVVTI NCPLHEKTRG 233
 fdh_hanpo  PKELLYYDYQ SLSKEAEEKV GARRVHDIKE LVAQADIVTI NCPLHAGSKG 236
      scyn  FENFIYHNRH QLPSEEEH.. GCEYVG.FEE FLKRSDIVSV NVPLNHNTHH 233
  dmdhcdna  M.KLVYYDVA PADAETEKAL GAERVDSLEE LARRSDCVSV SVPYMKLTHH 236
dhgy_hypme  M.DIDYFDTH RASSSDEASY QATFHDSLDS LLSVSQFFSL NAPSTPETRY 219
sery_yeast  L.HVLYYDIV TIMALGTARQ ....VSTLDE LLNKSDFVTL HVPATPETEK 266
serx_yeast  L.HVLYYDIV TIMALGTARQ ....VSTLDE LLNKSDFVTL HVPATPETEK 266
sera_haein  M.DVYFYDIE NKLPLGNAKQ ....VRSLEE LLSSCDVVSL HVPELPSTKN 220
sera_ecoli  M.YVYFYDIE NKLPLGNATQ ....VQHLSD LLNMSDVVSL HVPENPSTKN 218
dhd2_lacca  A.KVIAYDPY PMKG.DHPDF D...YVSLED LFKQSDVIDL HVPGIEQNTH 213
ldhd_lacde  A.KVIAYDIF RNPELEKKGY Y...VDSLDD LYKQADVISL HVPDVPANVH 213
               ++                    +        +       *
                                                  f
 fdh_neucr  LFNKELISKM K.........  ........KG SWLVNTARGA IVVKEDVAEA 271
 fdh_emeni  LFNKELISKM KPGKSALLYL IIPMLMYHKG SWLVNTARGA IVVKEDVAEA 283
 fdh_hanpo  LVNAELLKHF K.........  ........KG AWLVNTARGA ICVAEDVAAA 269
      scyn  LINAETIEKM K.........  ........DG VVIVNTARGA VIDEQAMTDA 266
  dmdhcdna  LIDEAFFAAM K.........  ........PG SRIVNTARGP VISQDALIAA 269
dhgy_hypme  FFNKATIKSL P.........  ........QG AIVVNTARGD LVDNELVVAA 252
sery_yeast  MLSAPQFAAM K.........  ........DG AYVINASRGT VVDIPSLIQA 299
serx_yeast  MLSAPQFAAM K.........  ........DG AYVINASRGT VVDIPSLIQA 299
sera_haein  LMNVARIAQL K.........  ........QG AILINAARGT VVDIDALAQA 253
sera_ecoli  MMGAKEISLM K.........  ........PG SLLINASRGT VVDIPALCDA 251
dhd2_lacca  IINEAAFNLM K.........  ........PG AIVINTARPN LIDTQAMLSN 247
ldhd_lacde  MINDKSIAKM K.........  ........QD VVIVNVSRGP LVDTDAVIRG 247
                       +                    +     *  *+          +
                             f                                    f
 fdh_neucr  LKSGHLRGYG GDVWFPQPAP QDHPLRYAKN PFG.......  .GGNAMVPHM 313
 fdh_emeni  LKSGHLRGYG GDVWFPQPAP KEHPLRYAEH PWG.......  .GGNATVPHM 325
 fdh_hanpo  VKSGQLRGYG GDVWFPQPAP KDHPWRSMAN KYG.......  .AGNAMTPHY 311
      scyn  LRSGKIRSAG LDVFEYEPKI SKE.......  ........LLS MSQVLGLPHM 302
  dmdhcdna  LKSGKLLSAG LDVHEFEPQV SKE.......  ........LIE MKHVTLTTHI 305
dhgy_hypme  LEAGRLAYAG FDVFAGEPNI NEG.......  ........YYD LPNTFLFPHI 288
sery_yeast  VKANKIAGAA LDVYPHEPAK NGEGSFNDEL .NSWTSELVS LPNIILTPHI 348
serx_yeast  VKANKIAGAA LDVYPHEPAK NGEGSFNDEL .NSWTSELVS LPNIILTPHI 348
sera_haein  LKDGKLQGAA IDVFPVEPAS INEE......  ...FISPLRE FDNVILTPHI 294
sera_ecoli  LASKHLAGAA IDVFPTEPAT NSDP......  ...FTSPLCE FDNVLLTPHI 292
dhd2_lacca  LKSGKLAGVG IDTYEYETED LLNLAKHGSF KDPLWDELLG MPNVVLSPHI 297
ldhd_lacde  LDSGKVFGYA MDVYEGEVGV FNEDREGKEF PDARLADLIA RPNVLVTPHT 297
                        *+    +                                 +*
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 fdh_neucr  SGTSLDAQKR YAAGTKAIIE SYLSGKHDYR PEDLIVYGGD YATKSYGERE 363
 fdh_emeni  SGTSLAAQIR YANGTKAILD SYFSGRFDYQ PQDLIVHGGD YATKAYGQRE 375
 fdh_hanpo  SGSVIDAQVR YAQGTKNILE SFFTQKFDYR PQDIILLNGK YKTKSYGADK 361
      scyn  GTHSVETRKK MEELVVENAK NVILTGKVLT IVPELQNEDW PNESKPLV*. 350
  dmdhcdna  GGVAIETFHE FERLTMTNID RFLLQGKPLL TVRLCPNSLR PAFRAY.... 351
dhgy_hypme  GSAATQARED MAHQANDLID ALFGGADMSY ALA..... ..........  321
sery_yeast  GGSTEEAQSS IGIEVATALS KYINEGNSVG SVNFPEVSLK SLDYDQENTV 398
serx_yeast  GGSTEEAQSS IGIEVATALS KYINEGNSVG SVNFPEVALK SLSYDQENTV 398
sera_haein  GGSTAEAQEN IGFEVAGKFV KYSDNGSTLS SVNFPEVSLP ....EHEGTK 340
sera_ecoli  GGSTQEAQEN IGLEVAGKLI KYSDNGSTLS AVNFPEVSLP ....LHGG.R 337
dhd2_lacca  AYYTETAVHN MVYFSLQHLV DFLTKFKPAR KLLVQQVVN. ..........  336
ldhd_lacde  AFYTTHAVRN MVVKAFDNNL ELVEGKEAET PVKVG..... ..........  332
                  +

 fdh_neucr  RAKAAAAAAK SA..... ..........  .......... .......... 375
 fdh_emeni  KK..... ..........  .......... .......... .......... 377
 fdh_hanpo  ..........  .......... .......... .......... ..........
      scyn  ..........  .......... .......... .......... ..........
  dmdhcdna  ..........  .......... .......... .......... ..........
dhgy_hypme  ..........  .......... .......... .......... ..........
sery_yeast  RVLYIHRNVP GVLKTVNDIL SDH..NIEKQ FSDSHGEIAY LMADISSVNQ 446
serx_yeast  RVLYIHQNVP GVLKTVNDIL SNH..NIEKQ FSDSNGEIAY LMADISSVDQ 446
sera_haein  RLLHIHENRP GILNKLNQIF VEANLNIAAQ YLQTDPKIGY VVVDVET.ND 389
sera_ecoli  RLMHIHENRP GVLTALNKIF AEQGVNIAAQ YLQTSAQMGY VVIDIEA.DE 386
dhd2_lacca  ..........  .......... .......... .......... ..........
ldhd_lacde  ..........  .......... .......... .......... ..........

 fdh_neucr  ..........  .......... ...
 fdh_emeni  ..........  .......... ...
 fdh_hanpo  ..........  .......... ...
      scyn  ..........  .......... ...
  dmdhcdna  ..........  .......... ...
dhgy_hypme  ..........  .......... ...
sery_yeast  SEIKDIYEKL NQTSAKVSIR LLY 479
serx_yeast  SDIKDIYEQL NQTSAKISIR LLY 479
sera_haein  ..ASPLLTKL KEIDGTIRAR VLY 410
sera_ecoli  DVAEKALQAM KAIPGTIRAR LLY 409
dhd2_lacca  ..........  .......... ...
ldhd_lacde  ..........  .......... ...
```

Apart from known D-2-hydroxyacid dehydrogenases, another protein sequence was found in the data base which had extensive sequence identity with D-mandelate dehydrogenase from *Rhodotorula graminis*. This is the putative product of a gene (ORFYNL274c) on the *Saccharomyces cerevisiae* chromosome XIV (EMBL accession number: Z71550). This sequence shows 33% identity and 339 amino acids overlapping with D-mandelate dehydrogenase. In the dendrogram it can be seen that DMDH is most closely related to the product of the Z71550 reading frame from *Saccharomyces cerevisiae* (Fig. 4.8). This reading frame was identified by genome sequencing, no information on its function is currently available.

Since the D-mandelate dehydrogenase belongs to the D-isomer specific 2-hydroxyacid dehydrogenase family, it is predicted that these enzymes will have a similar structure. Most of the invariant and semivariant residues which are conserved in the alignment are clustered in the middle of the sequence between positions 112 to 307 which is believed to be the coenzyme-binding domain except for Gly90, Gly103, Lys96, Asp95 at the N-terminus and Ala312 (in GDH) at the C-terminus, which is predicted to form the catalytic domain (Fig. 4.10).

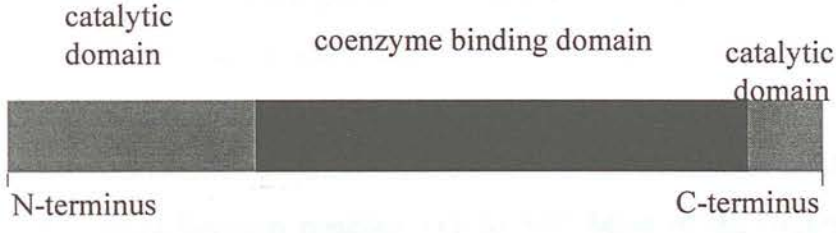

Figure 4.10: The domain structure of the D-MDH predicted from the sequence alignment with D-glycerate dehydrogenase of *H. methylovorum*

4.3 The D-Mandelate Dehydrogenase Protein

4.3.1 The Catalytic Domain

D-glycerate dehydrogenase from *Hyphomicrobium methylovorum* has been crystallized and its structure determined (Goldberg *et al.*, 1994). The structure showed a striking similarity to formate dehydrogenase from *Pseudomonas sp101* which has also been structurally characterised (Lamzin *et al.*, 1992). These two enzymes have a domain structure that is typical for the NAD-dependent dehydrogenases. Each subunit of the dimeric D-glycerate dehydrogenase molecule is divided into two domains separated by a deep cleft. One of these is the catalytic domain, the other is a typical NAD-binding domain. Comparison of amino acid sequences suggested that DMDH has a similar structure. Based on the D-glycerate dehydrogenase sequence, the DMDH catalytic domain comprises approximately residues 1 to 111 and 308 to 351 . Sequence in these two regions seems to be much more diverse within the family compared with the NAD-binding domain. Both termini of DMDH are predicted to be located in the catalytic domain.

4.3.2 The Coenzyme-Binding Domain

In D-glycerate dehydrogenase of *Hyphomicrobium methylovorum* the coenzyme-binding domain is constructed from a single, central portion of the polypeptide chain, comprising residues 100 to 290. Alignment of the D-glycerate dehydrogenase and DMDH sequences indicated that the coenzyme-binding domain of DMDH is located between residues 111 to 307. Most of the highly conserved residues in DMDH are located in this region of the polypeptide.

Nine invariant residues are conserved throughout all the aligned sequences in this region of the polypeptide. These residues are marked with asterisks (*) in the sequence alignment (Fig. 4.9). The crystal structure of D-glycerate dehydrogenase shows that this enzyme has the same basic polypeptide fold as L-lactate

dehydrogenase for the coenzyme-binding domain of these NAD-dependent enzymes (Goldberg *et al.*, 1994). The essential character of the fold is the formation of a cleft that specifically binds a pyridine nucleotide molecule. Several conserved residues around this cleft are involved in NAD binding.

4.3.2.1 Conserved Residues

In the aligned sequences of other D-2-hydroxyacid dehydrogenases (Fig 4.9), one aspartate residue (Asp281) is conserved. Two invariant arginine residues (Arg130 and Arg257) are conserved and one is believed to be involved in substrate binding (Goldberg *et al.*, 1994). His304 is conserved throughout the sequences and is believed to have a catalytic function and in making a His-Asp pair (Birktoft and Banaszak, 1983) with one of the conserved aspartic residues. There are two glycine residues which are conserved. In DMDH at position 175 the glycine is replace by glutamine and at position 277 is replaced by serine. The glycines at positions 154, 156 and 159 in D-glycerate dehydrogenase are believed to be involved in the formation of the NAD-binding motif (Goldberg *et al* , 1994).

Other residues which are conserved in DMDH as well as in other D-isomer-specific 2 hydroxyacid dehydrogenase are Ile174, Pro229 and Asn254. Two alanine residues (Ala106 and Ala295 numbering as in *H. methylovorum* D-glycerate dehydrogenase) are identical in the other sequences but are replaced by serine and threonine respectively in DMDH. Glutamate107 and proline286 (numbering as in *H. methylovorum* D-glycerate dehydrogenase) are identical in the aligned sequences except that in DMDH they are replaced by aspartate and threonine respectively.

4.3.2.2 Active Site Residues

L-2-hydroxyacid dehydrogenases utilise an arginine and an aspartic acid /histidine pair in substrate binding and catalysis (Holbrook *et al.*, 1975). Investigation using computer analysis (Kochhar *et al.*, 1992b), chemical modification (Kochhar *et al.*, 1992c) and site-directed mutagenesis (Kochhar *et al.*, 1992a) indicate that a similar triad exists in the D-isomer specific enzymes. The 2-hydroxyacid dehydrogenase activity of lactate dehydrogenase and malate dehydrogenase is shared by D-glycerate dehydrogenase and other D-isomer specific dehydrogenases and it is reasonable to suggest that these enzymes also employ an active site histidine/ carboxylate and a substrate orienting arginine residue (Goldberg *et al.*, 1994).

An active-site role for the conserved Arg240 in D-glycerate dehydrogenase has been suggested by the work of Kochhar *et al.* (1992c) on D-lactate dehydrogenase from *Lactobacillus bulgaricus*. Chemical modification of this enzyme with the arginine-specific reagent 2,3-butanedione caused almost complete inactivation (98%). This residue is conserved throughout the family of D-2-hydroxyacid dehydrogenase and is important for substrate binding, orientation and recognition in the substrate-binding site (Taguchi and Ohta, 1991). The role of Arg235 (Arg259 in DMDH) has also been examined using site-directed mutagenesis (Taguchi and Ohta, 1994) with D-lactate dehydrogenase from *Lactobacillus plantarum*. Substitutions of the conserved arginine with Lys and Gln drastically decreased the catalytic efficiency of the *Lactobacillus plantarum* D-lactate dehydrogenase. The authors suggested that Arg235 (Arg259 in DMDH) is essential for tight and correct substrate binding to the D-lactate dehydrogenase. The guanidinium group of Arg235 in D-lactate dehydrogenase is suggested to interact with the carboxyl group of the substrate. Sequence alignment indicates that Arg259 in DMDH could have the the same function as in the other D-isomer specific dehydrogenases in the activity of the enzyme.

His195 in L-lactate dehydrogenase is essential for its catalysis and acts as an acid /base catalyst. In lactate oxidation, His195 accepts a proton from the substrate hydroxyl group, facilitating hydride transfer to NAD and converting the hydroxyacid to a keto acid (Clarke *et al.*, 1989a,b). The sequence alignment of D-lactate dehydrogenase from *Lactobacillus plantarum* with other D-isomer specific dehydrogenase showed that His296 (His304 in DMDH) is conserved and thus provides a target for site-directed mutagenesis (Taguchi and Ohta, 1991,1993). Substitution of this residue by Tyr induced a drastic decrease in the catalytic activity of the enzyme. Their results suggest that His296 is essential and acts as an acid/base catalyst in D-lactate dehydrogenase like His195 in L-lactate dehydrogenase and suggests that the L- and D-lactate dehydrogenase have a similar catalytic mechanism despite the evolutionary isolation of these two enzymes. Alignment of the sequences of DMDH with other D-isomer specific dehydrogenases indicated that His304 in DMDH might play a similar important role in the catalytic activity of the enzyme.

The conserved aspartate residues in the aligned sequences could be candidates for an Asp-His pairs. These oriented His-Asp pairs linked by a hydrogen bond may function as a proton relay system during catalysis (Birktoft and Banaszak, 1983). This catalytic arrangement has been found not only in 2-hydroxyacid dehydrogenases but also in serine proteases, thermolysin and in phospholipase (Kraut, 1977; Weaver *et al.*, 1977; Dijkstra *et al.*, 1981). There is evidence that an active-site histidine residue in D-glycerate dehydrogenase is coupled not with an aspartate but with a glutamate residue. At the active-site of the D-glycerate dehydrogenase enzyme, His287 forms a hydrogen bond with the carboxylate side-chain of the conserved Glu269. This glutamate is also conserved in DMDH. Mutation of Glu264 (Glu269 in D-glycerate dehydrogenase) in D-lactate dehydrogenase of *Lactobacillus bulgaricus* to Gly suggest that the conserved Glu, although not critical for enzyme catalysis, can influence the function of an acid/base group at the active site of the enzyme (Kochhar *et al.*, 1992a). They also suggest that Glu264 (Glu286 in DMDH) is situated very close to the essential amino acid residues at the active site.

4.3.2.3 Interaction With NAD

A βαβ-fold which is involved in the binding of the ADP-moiety of the dinucleotide is common to the NAD-binding domain in many NAD-dependent dehydrogenase and is centred around a highly conserved sequence, G-X-G-X-X-G-I7X-D, where X can be any amino acid (Wierenga *et al.*, 1985). D-mandelate dehydrogenase from *Rhodotorula graminis* possesses this sequence at position 170 to 194. In all members of the D-isomer specific dehydrogenase family reported, the conserved sequence exists at the equivalent position (Fig. 4.9), indicating that these D-isomer specific enzymes have a similar NAD-binding domain structure. Lamzin *et al.* (1992) have made a sequence alignment of alcohol dehydrogenase from horse liver, formate dehydrogenase from *Pseudomonas sp101*, glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus*, lactate dehydrogenase from lobster muscle and cytoplasmic malate dehydrogenase from porcine heart at the equivalent places in the coenzyme binding domain. They found that the first glycine (Gly170 in DMDH) is replaced by alanine in formate dehydrogenase in *Pseudomonas sp101* and alanine at the third glycine position (Gln175 in DMDH) in malate dehydrogenase. Scrutton *et al.*, (1990) extended the work of Wierenga *et al.* (1986) by including several other proteins, showing for example the lack of conservation of the third glycine in Wierenga's fingerprint in the NAD(P)H-dependent dehydrogenases. This could explain why at position 175 in the DMDH sequence the amino acid is not glycine but glutamine. This also could indicate that DMDH has a different pattern of interaction with NAD. This can only be explained after the crystal structure of DMDH has been obtained. Asp194 in DMDH is conserved in all of the proteins aligned (Fig. 4.9). This residue forms a hydrogen bond to the 2-hydroxyl of the adenine ribose moiety, it provides a mechanism for discrimination between NAD and NADPH (Wierenga *et al.*, 1986). In D-glycerate dehydrogenase from *Hyphomicrobium methylovorum*, Asp177 occupies this position and the enzyme is only active towards NAD (Izumi *et al.*, 1990).

4.4 Expression of the D-mandelate dehydrogenase

To express the D-mandelate dehydrogenase, two expression vectors were used. Firstly the cDNA insert in pRI3 was cut with *Eco*RI and *Pst*I then cloned into the expression vector pKK223-3 cut with the same restriction enzymes to generate the recombinant plasmid pRI5, which was then transformed into *E. coli* JM105 as a host. The pKK223-3 expression vector contains a strong tac promoter (Brosius and Holy, 1984) which is regulated by the lac repressor supplied by the host JM105 (Yanisch-Perron *et al.*, 1985). The expression is induced by the addition of IPTG when added to the medium. In this experiment the recombinant cells were grown in LB medium containing 100 μg/ml ampicillin to OD_{600} about 0.6 then induced with IPTG ranging from 0.5 to 2.0 mM. Immunological detection of the cell extract with anti-DMDH antibody followed by HRP-conjugated 2nd antibody failed to detect D-mandelate dehydrogenase expression.

A second vector was used to express DMDH. The cDNA insert from pRI3 cut with *Eco*RI and *Bam*HI was isolated and cloned into pRC23 cut with *Eco*RI and *Bam*HI to generate the recombinant plasmid pRI6. This plasmid was then transformed into *E. coli* NF1. pRC23 contains the thermoinducible lambda P_L promoter (Crowl *et al.*, 1985) which is repressed by the lambda *c*I857 protein supplied by the host NF1 system (Stanley and Luzio, 1983). The *c*I857 repressed the P_L promoter at the temperature 30°C and lower but when the temperature is shifted to 42°C the repressor is inactivated and the P_L promoter is induced. To express DMDH, the NF1 cells containing pRI6 plasmid were grown at 30°C in LB medium containing 100 μg/ml ampicillin until the OD_{600} reached about 0.6 then the temperature was shifted to 42°C and left overnight. A single strong band with size about 38,000 Daltons was detected on a Western blot probed with anti-DMDH antibody (Fig. 4.11). D-mandelate dehydrogenase activity was readily detected by measuring the decrease in absorbance at 340 nm (oxidation of NADH) in a reaction mixture containing 200uM-NADH, 1mM-phenylglyoxylate, crude cell extract and 200mM-

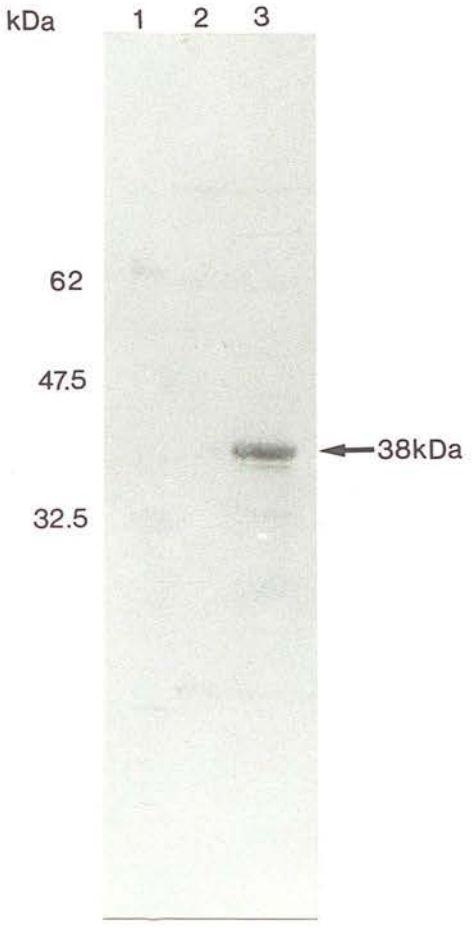

Figure 4.11. Immunological detection of the D-mandelate dehydrogenase after electrophoresis. D-mandelate dehydrogenase was detected by probing with anti-DMDH followed by HRP-conjugated 2nd antibody as described in Material and Methods. Lane 1; pre stain molecular weight marker, Lane 2; NF1 cell without any plasmid, Lane 3; extract from NF1 cell with plasmid pRC23 containing the cDNA of D-mandelate dehydrogenase.

potassium phosphate buffer (pH 5.85). Specific activity of recombinant D-mandelate dehydrogenase in crude cells extract is 0.0528 units per mg of total protein present. This activity was produced from a yield of 1370 mg total protein obtained from 17 gram wet cells.

CHAPTER 5

CONCLUSION

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Conclusion

5.1 Genes encoding mandelate dehydrogenases in *R. graminis*

Comparison of the isolated genomic and cDNA sequences from the L(+)-mandelate dehydrogenase and D(-)-mandelate dehydrogenase reveals the presence of eleven and three introns respectively. The presence of these introns in *R. graminis* seems to be common due to the presence of several introns also in the phenylalanine ammonia-lyase (PAL) genes from *Rhodotorula rubra* and *Rhodosporidium toruloides* which are closely related to *R. graminis*. All the introns in the DMDH gene have a similar 5' end of GT and 3' end of CAG as in the PAL gene of *R. rubra* and *R. toruloides*. Introns in LMDH gene also contain a 5' end of GT but one of the eleven introns contains the nucleotides of TAG at the 3' end instead of CAG as in the others. In the PAL genes from *R. rubra* and *R. toruloides* all the introns have an internal consensus sequence of CTGAC. However this does not occur in all introns from mandelate dehydrogenase genes in *R. graminis*. Takahashi *et al.* (1996) found that introns in the small nuclear RNA (snRNA) genes in *Rhodotorula hasegawae* have a consensus of CTrAC, where r is purine (A or G), with a 5' end of GT and 3' end of CAG (one of the introns has TAG). The sequences of the introns in the mandelate dehydrogenase genes suggest even greater flexibility in the internal (branch-point) sequence, with all introns containing the sequence of CTnAy.

5.2 Mandelate dehydrogenases from *R. graminis*.

Purification and preliminary characterization of L(+)-mandelate dehydrogenase (Yasin and Fewson, 1993) and D(-)-mandelate dehydrogenase (Baker and Fewson, 1989) showed that both enzymes belong to different families and are quite distinct from each other. Amino acid sequences deduced from the isolated genes for both mandelate dehydrogenase in this work confirmed this. Comparison of LMDH with other sequences (Fig. 3.11) clearly showed that the polypeptide is

composed of two domains as in other flavocytochromes b_2: a cytochrome domain and a flavin-binding domain. DMDH amino acid sequence shows that its belongs to the D-isomer specific 2-hydroxyacid dehydrogenase family. Although LMDH and DMDH are distinct, they catalyse the same reaction where the substrate αH at C2 is abstracted to form phenylglyoxylate.

5.3 Further Work

Now that recombinant L(+)-mandelate dehydrogenase and D(-)-mandelate dehydrogenase can both be expressed in *E. coli*, the step towards further characterization of these enzymes is to purify both enzymes. Once the purified enzymes are obtained, kinetic analysis experiments will be essential to study the mechanism of each enzyme. It is very helpful in the purification process if the expression of the enzyme could be improved. A suitable and efficient expression would be an advantage in order to obtain sufficient enzyme for crystallization and also to be used for construction of mutant enzymes. Site directed mutagenesis can be carried out to produce mutants of L(+)-mandelate dehydrogenase and D(-)-mandelate dehydrogenase to pinpoint important residues at the active site and involvement of certain residues for electron transfer.

In flavocytochrome b_2 the physiological pathway of the electron transfer is from lactate to FMN, then from FMN to haem and finally to cytochrome *c*. Construction and expression of independent domains of LMDH would be helpful to facilitate the investigation of each domain's biochemical properties. Rhona Sinclair has expressed the recombinant flavin domain from LMDH without the cytochrome domain and this will enable the study of redox changes at the flavin to be monitored spectrophotometrically without interference from the haem.

The substrate specificity and involvement of amino acids at the active site which are involved in the substrate specificity in LMDH and DMDH have yet to be determined. Molecular modelling of the active site of L(+)-lactate dehydrogenase

from *Saccharomyces cerevisiae* (Smekal *et al.*, 1993) and mutation of Leu230 (Daff *et al.*, 1994) shows the involvement of Leu230 in substrate specificity of LLDH and why this enzyme is unable to use L-mandelate. Comparison of amino acid sequences from *R. graminis* LMDH with other proteins (Fig. 3.15) indicate the possible involvement of glycine 255 in substrate specificity of LMDH. However mutation of this residue to other amino acids will help to confirm its role and explain why LMDH is unable to utilise L-lactate. Work on D(-)-mandelate dehydrogenase substrate shows that the substrates contain some form of aromatic ring structure, but substrate analogues with hydrophobic side chains inhibit activity (Baker and Fewson, 1989). The authors also suggested that the hydrophobic ring is important for the correct orientation of the substrate at the active site and for 2-hydroxy and 2-oxocarboxylic acids with hydrophobic side chains, although they can bind at the active site, they cannot undergo catalysis because they lack the ring structure. Vinal *et al.* (1995) made a model of three dimensional structure of D-lactate dehydrogenase from *Lactobacillus bulgaricus* and determined amino acid involved in stabilising the methyl group of the substrate. The same method will also help to determine amino acids involved in substrate specificity of DMDH.

Finally, a crystal structure of mandelate dehydrogenases from *R. graminis* is important in oder to get a clear understanding about the exact structure of both enzymes. The crystal structure will give information about amino acid residues involved in binding of substrate, cofactor or coenzyme, catalysis and electron transfer. The structure at the active site would help in determining how these two enzymes bind the same substrate (mandelate) but as different enantiomers. Preliminary crystallization of D(-)-mandelate dehydrogenase from *R. graminis* has been done by Basak *et al.*(1993), and with the expression of the recombinant DMDH it would be an advantageous for the crystallization to proceed faster.

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APPENDIX

List of Plasmids Constructed

A. L(+)-mandelate dehydrogenase.

Names	Description
pLM1	81 bp PCR fragment cloned in pTZ19R
pLM2	81 bp PCR fragment cloned into pTZ18R
pLM3	5.5 kb fragment of *R. graminis* genomic DNA cloned into pTZ19R
pLM4	5.5 kb fragment of *R. graminis* genomic DNA cloned into pTZ19R (opposite orientation from pLM3)
pLM5	1.5 kb of PCR fragment (LMDH cDNA) cloned into pTZ19R
pLM6	1.5 kb of PCR fragment (LMDH cDNA) cloned into pTZ18R
pLM7	1.5 kb of LMDH cDNA fragment from pLM5 subcloned into expression vector pKK223-3
pLM8	1.5 kb LMDH cDNA fragment from pLM7 subcloned into expression vector pRC23

B. D(-)-mandelate dehydrogenase.

Names	Description
pRI1	4.4 kb fragment of *R. graminis* genomic DNA cloned into pTZ19R
pRI2	4.4 kb fragment of *R. graminis* genomic DNA cloned into pTZ19R (opposite orientation from pLM1)
pRI3	DMDH cNA fragment amplified by PCR cloned into pTZ19R
pRI4	DMDH cDNA fragment amplified by PCR subcloned into pTZI8R

pRI5	DMDH cDNA fragment from pRI3 subcloned into expression vector pKK223-3
pRI6	DMDH cDNA fragment from pRI3 subcloned into expression vector pRC23