Cloning and expression of the *Propionibacterium shermanii* methylmalonyl-CoA epimerase gene in *Escherichia coli*

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'Etuate Lui Saafi

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Dedication

This thesis is dedicated to my dear parents
Sione Kiteau and Meliame Saafi

Thank you very much for all your
love and sacrifices for me.

ABSTRACT

Genomic DNA was isolated from *Propionibacterium shermanii* (52W). A 454 bp DNA fragment coding for the methylmalonyl-CoA epimerase (EC 5.1.99.1, subsequently referred to as epimerase) was amplified from genomic DNA by the polymerase chain reaction using primers designed from the known DNA sequence of the gene.

The *P. shermanii* epimerase gene was ligated into the 2.47 kbp expression vector pT7-7. The ligation reaction mixture was transformed into electroporation competent *E. coli* XL1-Blue cells. Plasmid DNA prepared from several transformants was analysed, by agarose gel electrophoresis of restriction enzyme digestions, and transformed into *E. coli* SRP84/pGP1-2 cells to identify potential epimerase expression constructs (pTEEX) by heat shock induction. The insert DNA of one of the putative pTEEX epimerase constructs was fully sequenced and shown to be identical to the known DNA sequence of the epimerase gene described by Davis (1987).

Using the sequenced expression construct pTEEX, recombinant epimerase was expressed to 20-35% of the total cell protein in the protease deficient *E. coli* strain SRP84 using the dual plasmid expression system of Tabor and Richardson (1985). The recombinant epimerase was ~95-100% soluble in *E. coli*.

The recombinant epimerase and the 'wild-type' epimerase produced by *P. shermanii* were purified using the procedures developed for the 'wild-type' epimerase. The addition of a heat-treatment step (70°C for 15 min) early in the purification of the recombinant enzyme successfully exploited the unusually high thermostability of the epimerase protein.

The epimerase protein was found to have an anomalously low electrophoretic mobility in a modified Laemmli discontinuous Tris-glycine alkaline buffer system for SDS-PAGE gels compared to the Weber and Osborn continuous phosphate buffer system. Using the latter system, a subunit molecular weight of 16.6 kDa was obtained. This is consistent with the molecular weight of 16.72 kDa (methionine on) calculated from the inferred amino acid sequence.

The *N*-terminal sequence of the purified 'wild-type' and recombinant epimerases were identical although only half of *N*-terminal methionine residues were removed from the recombinant protein. The subunit molecular weight, specific activity, activation by divalent metal ions and behaviour in crystallization trials of the 'wild-type' and recombinant epimerases were very similar. Recombinant epimerase crystals were grown in a buffer containing 0.2 M ammonium acetate and 0.1 M citrate, pH 5.6, containing 30% PEG 4000 as precipitant. These crystals were relatively poorly ordered and diffracted to only 4.5 Å resolution, but crystals of the recombinant epimerase that diffract to 2.6Å can be grown under appropriate conditions.

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A_x	absorbance, e.g. A_{280}
Amp	ampicillin
bovine CA	bovine Carbonic Anhydrase
BSA	bovine serum albumin
DNA	deoxyribonucleic acid
DTT	dithiothreitol
cDNA	complementary DNA
CoA	coenzyme A
EDTA	ethylenediaminetetraacetic acid
g	gravitational field, unit of
G-3-P DeH	Glyceraldehyde-3-Phosphate Dehydrogenase
HEPES	*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid
kan	kanamycin
kb(p)	kilobase (pairs)
KP	potassium phosphate
MOPS	3-(*N*-morpholino)propanesulphonic acid
MPD	2-methyl-2,4-pentanediol
M_r	relative molecular mass
mRNA	messenger ribonucleic acid
NADH	nicotinamide-adenine dinucleotide, reduced
N.C.I.B	National Collection of Industrial Bacteria
NaP	sodium phosphate
N-terminal	amino terminal
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	poly(ethylene) glycol
PEI	polyethylenimine
PMSF	phenylmethylsulphonyl fluoride
ppt	precipitate
r_{av}	average centrifugal radius
RNAase	ribonuclease
SDS	sodium dodecyl sulphate
soyabean TI	soyabean Trypsin Inhibitor
TCA cycle	tricarboxylic acid cycle
TE (*x*:*y*)	*x* mM Tris/HCl pH 8.0, *y* mM EDTA
TEMED	*N*, *N*, *N'*, *N'*-tetramethylethylenediamine
Tris	tris-(hydroxymethyl)-aminomethane
UV	ultra violet

AMINO ACID	SINGLE LETTER ABBREVIATION
Alanine	A
Arginine	R
Asparagine	N
Aspartic acid	D
Cysteine	C
Glutamic acid	E
Glutamine	Q
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

CHAPTER 1: INTRODUCTION

1.1 GENERAL INTRODUCTION

Mammals and some bacteria, notably the propionibacteria (propionate-producing bacteria) and the rumen bacterium *Selenomonas ruminantium*, possess a metabolic pathway that interconverts succinyl-CoA (carboxypropionyl-CoA) and propionyl-CoA. The direction in which this pathway operates depends on the role of the pathway and differs in mammals and bacteria. In fermentations by propionibacteria, succinyl-CoA is converted to propionyl-CoA as part of a complex catalytic cycle in which pyruvate (produced by anaerobic glycolysis) is reduced to propionate and NADH oxidised to NAD^+ to regenerate the electron acceptor required for continuous glycolysis (Rétey, 1982). In contrast, mammals utilise the same pathway to convert propionyl-CoA into succinyl-CoA which is then able to enter the TCA (tricarboxylic acid) cycle where it may be oxidized to produce ATP. For good ruminant nutrition the pathway must operate in both directions; from succinyl-CoA to propionyl-CoA in the principle propionate-producing rumen bacterium, *Selenomonas ruminantium*, and from propionyl-CoA to succinyl-CoA in ruminant liver to utilise the propionate generated in the rumen. In all mammals the pathway is also required for the complete degradation of odd-chain fatty acids, the amino acids isoleucine, valine, methionine and threonine, and some products of cholesterol metabolism (Kamoun, 1992).

Figure 1.1 shows how the interconversion of succinyl-CoA and propionyl-CoA is catalysed by the three enzymes methylmalonyl-CoA mutase (EC 5.4.99.2), methylmalonyl-CoA epimerase (EC 5.1.99.1) and a carboxylase enzyme (oxaloacetate transcarboxylase (methylmalonyl-CoA carboxyltransferase, EC 2.1.3.1) in propionibacteria, and propionyl-CoA carboxylase (EC 6.4.1.3) in mammals). The terms epimerase, mutase and transcarboxylase will often be used in this work when referring to methylmalonyl-CoA epimerase, methylmalonyl-CoA mutase and oxaloacetate transcarboxylase respectively.

Mutase isomerises succinyl-CoA and the (*R*) stereoisomeric form of methylmalonyl-CoA. This reaction is dependent upon adenosylcobalamin (coenzyme B_{12}) (Kellermeyer *et al.*, 1964). Epimerase plays the pivotal role of racemising the (*R*) and the (*S*) stereoisomeric forms of the metabolite methylmalonyl-CoA (Allen *et al.*, 1962; Mazumder *et al.*, 1962). In propionibacteria, transcarboxylase converts (*S*)-methylmalonyl-CoA into propionyl-CoA. Coupled to this reaction is the utilization of pyruvate to produce oxaloacetate (Allen *et al.*, 1962). In contrast, mammalian tissues use propionyl-CoA carboxylase to convert propionyl-CoA to (*S*)-methylmalonyl-CoA (Mazumder *et al.*,

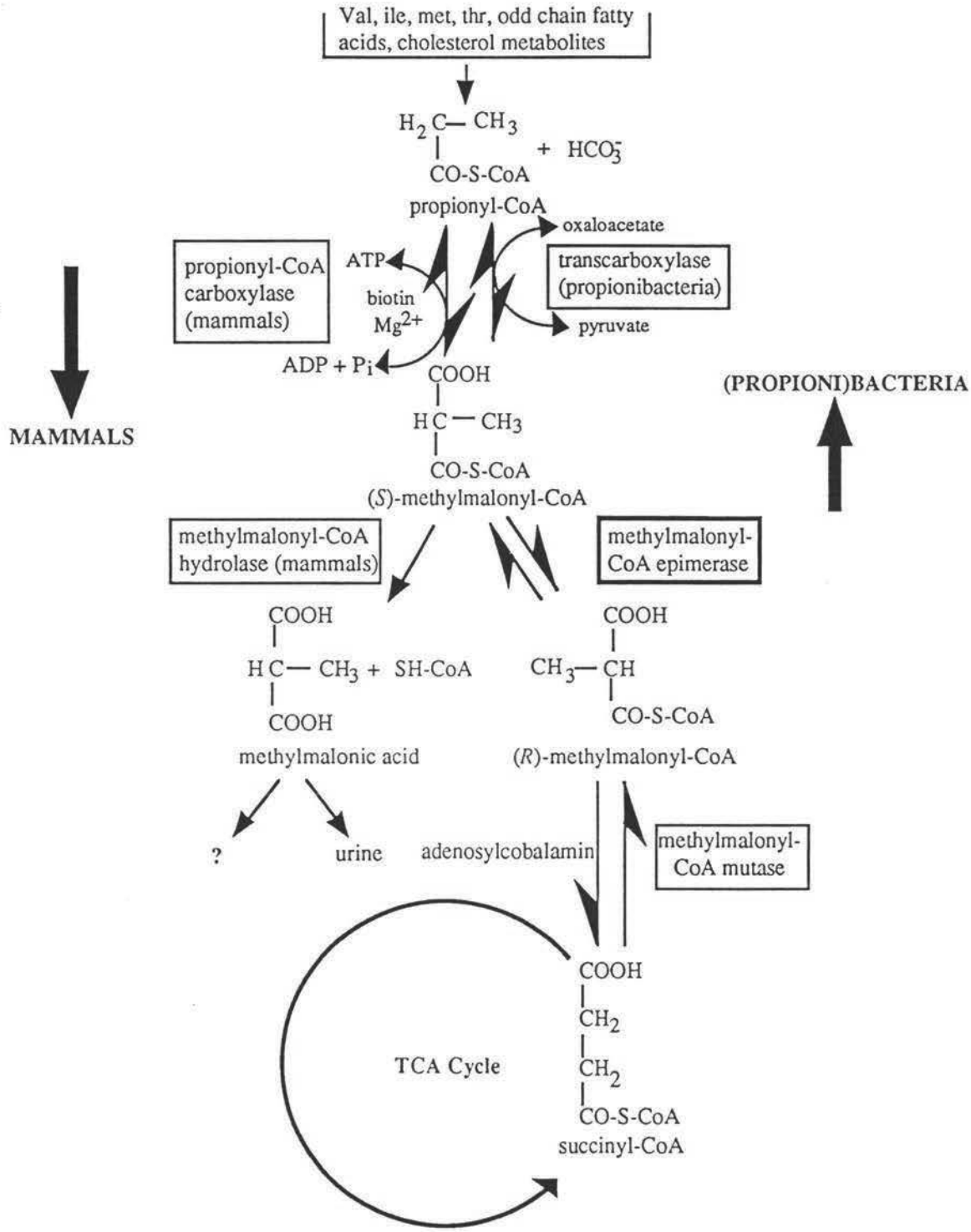

Figure 1.1: The reaction pathways involved in the interconversion of succinyl-CoA and propionyl-CoA. In propionibacteria the pathway functions from bottom to top. (i.e. succinyl-CoA is converted to propionyl-CoA, and subsequently propionate). In mammalian tissues the pathway works from the top to the bottom and the product of succinyl-CoA enters the TCA cycle (the cycle for the oxidation of fuel molecules in cellular mitochondria). Alternatively, (*S*)-methylmalonyl-CoA may be hydrolysed by methylmalonyl-CoA hydrolase (Kovachy *et al.*, 1988) to form methylmalonic acid which is excreted in the urine. The directions the pathway operates in mammalian and (propioni)bacteria are indicated by the bold arrows on either side of the diagram.

1962). Transcarboxylase and propionyl-CoA carboxylase are biotinyl-proteins, and transcarboxylase also contains cobalt and zinc.

1.2 METHYLMALONYL-CoA EPIMERASE FROM MAMMALS

The epimerase enzyme has been purified and characterised from sheep liver and kidney (Mazumder *et al.*, 1962), and also rat liver (Stabler *et al.*, 1985), but no mammalian epimerase has been cloned. The rat liver and the rat and human white blood cell epimerase are suggested to be immunologically related (Stabler *et al.*, 1985). These animal epimerase enzymes have a M_r of approximately 32,000. Studies on the rat liver epimerase by Stabler *et al.* (1985) indicated the presence of two subunits, with a M_r of 16000, that are not connected by disulfide bonds. The optimum pH for rat liver epimerase activity was 7.0, and 50% of maximal activity was observed at pH 5.0 and 9.0. The epimerase activity was shown to be completely inactivated by the presence of EDTA and later reactivated with the addition of Co^{2+}. Other divalent metal ions such as Zn^{2+}, Cu^{2+}, Cu^{+}, and Cd^{2+} completely inhibited epimerase activity, while Mn^{2+}, Co^{3+} and Fe^{2+} were mild activators. The purified epimerase from rat liver binds 1 mole of Co per subunit. However, the specific metal that binds the epimerase *in vivo* is yet to be established. The fact that Co^{2+} provides the greatest degree of activation for both mammalian and *P. shermanii* epimerase (Leadlay, 1981) is interesting since cobalt has been considered to be an essential trace element only as a component of the cyanocobalamin (vitamin B_{12}) molecule, which is only synthesized by certain microorganisms, including some rumen bacteria. The K_m of rat liver epimerase for methylmalonyl-CoA is 0.1 mM and the k_{cat} was found to be 250,000 molecules of substrate per minute (Stabler *et al.*, 1985). The mammalian enzyme in located in the mitochondrial matrix, together with the other two enzymes of this pathway converting propionyl-CoA to succinyl-CoA. Because the methylmalonyl-CoA hydrolase (Figure 1.1) acts only on the (*S*) isomer of methylmalonyl-CoA, whereas mutase acts only on the (*R*) isomer, the epimerase plays an important role in determining the fate of (*S*)-methylmalonyl-CoA after it is formed from propionyl-CoA.

Three alternative mechanisms have been suggested for the epimerase catalysed-epimerization of the methylmalonyl-CoA: (i) the intermolecular transfer of the coenzyme A moiety to methylmalonic acid, (ii) the intramolecular CoA transfer from one carboxyl group to another, or (iii) a shift of the α-hydrogen atom (Mazumder *et al.*, 1962). Experiments by Mazumder *et al.* (1962) have ruled out possibilities (i) and (ii) and have suggested that the epimerization occurs via mechanism (iii) where the epimerase enzyme relocates the carboxyl group on the C-2 position of (*S*)-methylmalonyl-CoA to form the (*R*)-methylmalonyl-CoA stereoisomer by the exchange of the methylmalonyl-CoA C-2

hydrogen atoms with protons in the medium.

1.3 METHYLMALONYL-CoA EPIMERASE FROM *P. SHERMANII*

Propionibacterium shermanii (52W) is a propionate-producing species of the propionibacterium genus from the family propionibacteriaceae. Propionibacteria are gram positive, chemo-organotrophic, non-spore-forming, non-motile, usually rod-shaped bacteria that anaerobically metabolize carbohydrates (e.g. glucose), polyols (e.g. glycerol) and organic acids such as lactate. Propionibacteria are generally anaerobic to aerotolerant anaerobes (Moore and Holdman, 1975).

Propionibacteria fermentation products include a combination of propionic and acetic acids and frequently lesser amounts of mono- and dicarboxylic acids, such as isovaleric, formic, succinic or lactic acids, and CO_2. All species of propionibacteria produce acids when grown on glucose. Although most strains in this genus grow most rapidly under strictly anaerobic conditions, many strains grow well in a peptone/yeast-extract/glucose broth exposed to air when a large inoculum is used. Bacterial growth is most rapid between 30-37°C at a pH near 7.0 (Moore and Holdman, 1975). Propionibacteria produce pigments and colonies may be white, gray, pink, red, yellow or orange. Propionibacteria are isolated from and used in the manufacture of dairy products such as cheese. The CO_2 produced by *P. shermanii* strains during cheese maturation is responsible for the holes in some types of Swiss cheese. Species have also been isolated from the skin of animals. Some propionibacteria species may be pathogenic, and have been implicated in the development of facial acne. Until recently propionibacteria were cultivated as commercial sources of vitamin B_{12} and derivatives. The GC content of the DNA of most propionibacteria species ranges between 59% and 66% (Moore and Holdman, 1975).

Methylmalonyl-CoA epimerase has been purified to homogeneity from *P. shermanii* strain 52W (Allen *et al*., 1963; Leadlay, 1981). The purified epimerase protein was indefinitely stable indefinitely when stored at -20°C and pH 7.0.

Studies on the subunit structure of the epimerase have shown no evidence for the formation of any aggregates larger than dimers. Histidine was the free *N*-terminal residue identified when *S*-carboxymethylated epimerase was subjected to the dansyl procedure. This is consistent with the two subunits being identical. These results were further supported by the peptide-mapping experiments of Leadlay (1981), although given the *N*-terminal sequences obtained in the current study (see Figure 3.20), an *N*-terminal histidine residue seems unlikely. It was also suggested that there may be at least two

active sites per epimerase dimer. Each of the two identical dimer subunits has an approximate M_r of 16,500 and a total M_r of 33,000 (Leadlay, 1981).

The activity of the epimerase enzyme is increased by preincubation with certain divalent metal ions, especially Co^{2+}, and to a lesser extent by Ni^{2+}, Zn^{2+} and Mn^{2+} (Roeder and Kohlaw, 1980; Leadlay, 1981). Several metal ion chelating reagents, such as EDTA, have been found to inactivate epimerase activity although this inactivation was reversible in most instances (Leadlay, 1981). This suggests the presence of tightly bound metal ions, and a role for metal ions in epimerase catalysis or thermostability, although this is yet to be proven.

Methylmalonyl-CoA epimerase is unusually thermostable. Studies have indicated that 50% inactivation of epimerase required approximately 5 min in a boiling water bath and 10 to 20 min at 100°C to achieve complete inactivation (Allen *et al.*, 1963). Sedimentation velocity studies found that the epimerase sediments as a single, apparently symmetrical, boundary (Leadlay, 1981). This study was in good agreement with that done earlier by Allen *et al.* (1963). A discrepancy exists, however, with regard to the stability of epimerase activity in acid solutions. While Allen *et al.* (1963) stated that the *P. shermanii* epimerase was resistant to 1 M perchloric for up to 30 min at 0°C, Leadlay (1981) found that the enzyme activity was not unusually acid stable.

In *P. shermanii* the epimerase enzyme catalyses the epimerization of (*R*)- and (*S*)-methylmalonyl-CoA within a larger metabolic cycle that reduces pyruvate to propionate with the oxidation of NADH to NAD^+ (Figure 1.2). This production of propionate is important for the propionibacteria in that it ensures the continuity of its nutrition by allowing it to utilise the high amount of glucose present in its environment as food. Without propionate production the regeneration of NAD^+, necessary for continuous glycolysis, could not occur.

The sequence of events in the larger metabolic cycle begins with the carboxylation of pyruvate to oxaloacetate by the biotin-containing transcarboxylase using (*S*)-methylmalonyl-CoA as carboxyl donor. Oxaloacetate is then reduced to malate, which in turn is dehydrated to fumarate by the action of the enzyme couple malate dehydrogenase and fumarase. The second reduction is performed by a flavin-containing fumarate reductase, reaction leading to succinate, which is then activated to succinyl-CoA by a coenzyme A transferase making use of propionyl-CoA as CoA donor. The other product of this transesterification is propionate that will be released into the medium as waste. The role of mutase becomes apparent in the conversion of succinyl-CoA to (*R*)-methylmalonyl-CoA. Since transcarboxylase, the closing member of the cycle, is specific for (*S*)-methylmalonyl-CoA, an epimerization of the (*R*)-stereoisomer is required, and is

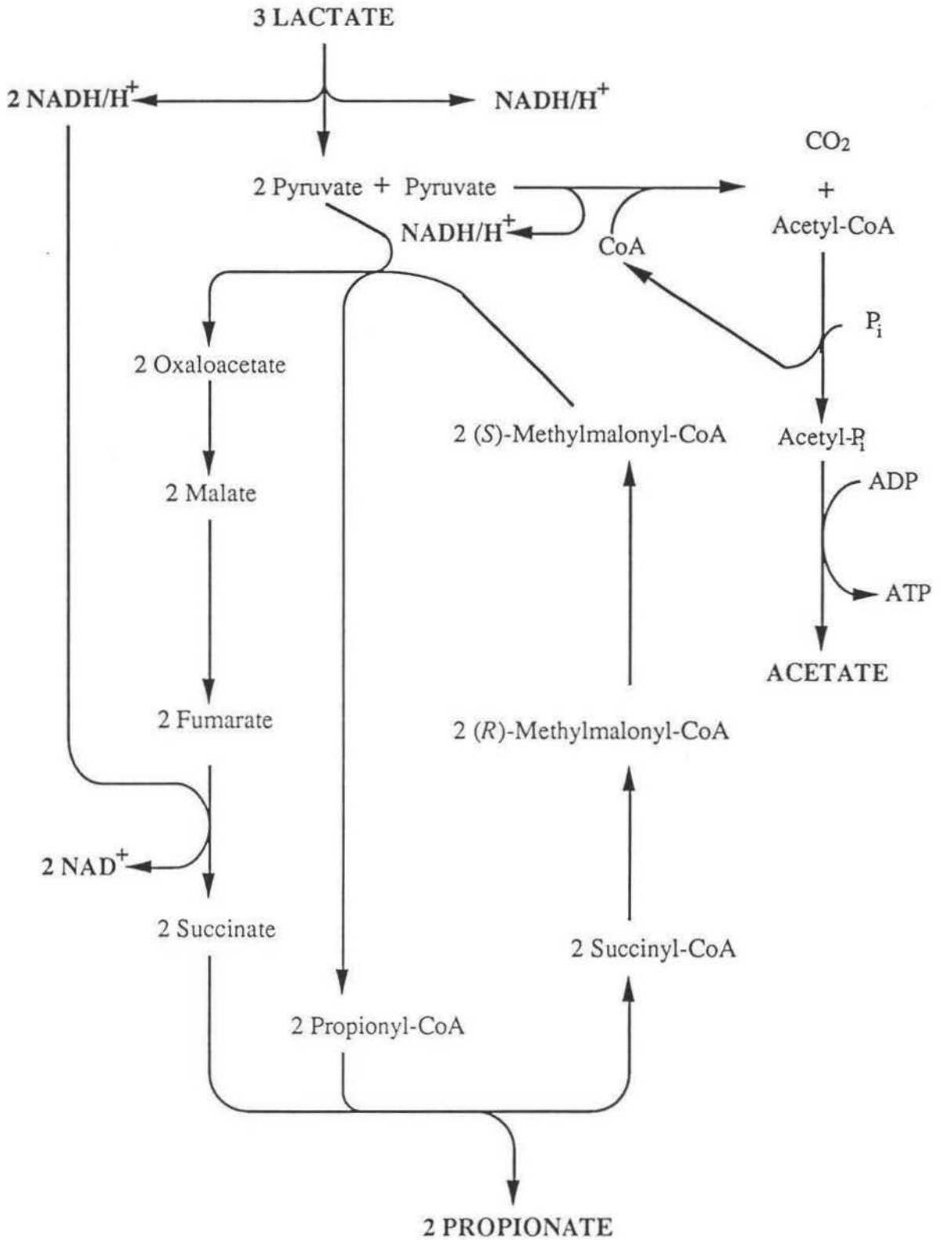

Figure 1.2: The metabolism of lactate in propionibacteria (after Asmundson (1982)).

catalysed by epimerase (Rétey, 1981).

From an evolutionary point of view, it is interesting to question why three different enzymes are required to interconvert succinyl-CoA and propionyl-CoA in both mammalian tissue and *P. shermanii*. It would have been more efficient if evolution had evolved mutase and transcarboxylase enzymes with a common reactant, either the (*R*) or (*S*) stereoisomer of methylmalonyl-CoA, thus eliminating the need for an epimerase enzyme. Instead, nature has opted for an unusual three enzyme pathway in which initially CO_2 has to be attached to the C-2 position of propionyl-CoA, to give the (*S*)-stereoisomer of methylmalonyl-CoA, by propionyl-CoA carboxylase. Methylmalonyl-CoA epimerase then has to invert the configuration at C-2 of the methylmalonyl portion of the CoA thioester. The bulky -CO-S-CoA group then has to be moved from the C-2 to the C-3 position by the mutase enzyme by exchanging it with the C-3 hydrogen atom to form succinyl-CoA.

1.4 CLONING AND EXPRESSION OF THE *P. SHERMANII* EPIMERASE GENE

The genes for the *P. shermanii* epimerase (Davis, 1987), mutase (Marsh *et al.*, 1989), and transcarboxylase (Samols *et al.*, 1988) enzymes have all been cloned and sequenced. The genes for the mutase (McKie *et al.*, 1990) and the transcarboxylase (Samols *et al.*, 1988) enzymes have also been expressed in *E. coli*.

1.4.1 METHYLMALONYL-CoA EPIMERASE GENE EXPRESSION IN *STREPTOMYCES LIVIDANS*

Davis (1987) cloned and sequenced the epimerase gene from *P. shermanii*. The approach taken involved the purification of the epimerase from *P. shermanii* (52W), followed by proteolysis using Arg-C and Lys-C proteases (Boehringer) to generate peptides for *N*-terminal sequencing. Selected RP-HPLC-purified peptides and the intact enzyme were subjected to *N*-terminal sequencing and the amino acid sequence information was used to design redundant oligonucleotides probes for the epimerase gene.

A *P. shermanii* mini-library was prepared by digestion of *P. shermanii* genomic DNA with the restriction enzyme *Kpn*I and the ligation of DNA fragments larger than 1 kbp into pUC18 (digested with *Kpn*I and treated with CIAP). The ligation mix was transformed into *E. coli* to produce the *P. shermanii* mini-library. A redundant 18-mer oligonucleotide probe designed to hybridize to an internal portion of the epimerase gene was used to

probe the library by colony hybridization. Plasmid DNA isolated from hybridizing colonies was sequenced using primers designed from the protein sequence and an open reading frame coding for a protein with the epimerase *N*-terminal protein sequence and subunit M_r was identified. At the time this work was done, there was some evidence to suggest that *P. shermanii* promoters did not function well, if at all, in gram-negative bacteria such as *E. coli* (Murtif *et al.*, 1985). The gram positive *Streptomyces lividans* was therefore chosen as the host for the heterologous expression of *P. shermanii* epimerase from its own promoter. The epimerase gene was subcloned into a high copy number *S. lividans* vector, that conferred resistance to the antibiotic thiostrepton, to create the epimerase expression plasmid pND2. *S. lividans* protoplasts transformed with pND2 expressed moderate levels of *P. shermanii* epimerase when grown in liquid medium containing thiostrepton.

Although the *S. lividans* heterologous expression system for epimerase was functional, there were several disadvantages compared with a possible heterologous expression system in *E. coli*. Firstly, the level of expression obtained in *S. lividans* was considerably lower than could normally be expected for a foreign bacterial gene expressed in *E. coli* under the control of a strong promoter. In addition, the pND2 epimerase expression plasmid was not stable in *S. lividans*; it would often be lost or modified so that *P. shermanii* epimerase was not expressed. This plasmid instability may have been due in part to the fact that *S. lividans* has its own epimerase gene. The presence of low levels of a host epimerase also complicated the purification of recombinant epimerase. The purification of recombinant epimerase from *S. lividans* was further complicated by difficulties in lysing *S. lividans*, which is quite resistant to conventional lysis techniques, and by the presence of aggressive proteolytic activities in the cell extract. The latter caused some degradation of the recombinant epimerase before and during purification. Heterologous expression in *E. coli* offered several advantages: (i) no host epimerase gene; (ii) faster growth of the host organism (12 h versus 2-3 days for *S. lividans*); (iii) easier plasmid transformation procedures; (iv) cheaper antibiotic costs (thiostrepton is 6x more expensive than kanamycin and 25x more expensive than ampicillin); (v) easier cell lysis; (vi) the possibility of taking advantage of the intrinsic thermostability of the epimerase by adding a heat-treatment step to the epimerase purification scheme developed by Leadlay (1981).

1.4.2 EPIMERASE GENE EXPRESSION IN *E. COLI*

The instability of a functional *P. shermanii* epimerase expression plasmid in *S. lividans*, and difficulties with epimerase purification, led to the idea of expressing the epimerase gene in *E. coli*. The availability of protease-deficient *E. coli* strains is an attractive feature

of choosing *E. coli* for heterologous expression of proteins. The T7-based expression system of Tabor and Richardson (1985) was to be used. This system places the gene to be expressed under the control of the ϕ10 T7 promoter, a strong promoter for T7 RNA polymerase. Induction is either by heat shock or isopropyl β-D-thiogalactopyranoside (IPTG). In addition, the pT7-7 expression vector ensures that the mRNA contains a consensus *E. coli* ribosome binding site at an optimal spacing from the ATG start codon of the gene to be expressed (see Figure 2.1). This system had worked well for the expression of *P. shermanii* methylmalonyl-CoA mutase in *E. coli* (McKie *et al.*, 1990).

1.5 EXPERIMENTAL STRATEGIES AND OBJECTIVES

The aim of this study was to express the *P. shermanii* methylmalonyl-CoA epimerase gene in *E. coli* and to conduct a preliminary characterization of the purified recombinant epimerase protein obtained, comparing its properties to the 'wild-type' epimerase purified from *P. shermanii*.

This required the growing of *P. shermanii* cultures and isolation of genomic DNA, the amplification of the *P. shermanii* epimerase gene by PCR, the ligation of the epimerase gene PCR product into the *E. coli* expression vector pT7-7, and the identification of the desired expression construct (called pTEEX). Because the epimerase gene inserted into the pT7-7 expression vector had been produced by PCR, it was necessary to check the sequence of the epimerase gene in pTEEX to guard against the possibility of errors having been introduced by the infidelity of *Taq* polymerase in the PCR amplification process. The epimerase gene was expressed by heat shock induction of *E. coli* SRP84/pGP1-2/pTEEX. Purification of the expressed recombinant and the 'wild-type' epimerase, together with the purification of the coupling enzymes, mutase and transcarboxylase, needed to assay epimerase, allowed a comparison of the specific activity of the epimerases. It was vital to obtain the *N*-terminal sequence of both the 'wild-type' and recombinant epimerases, their specific activities and subunit M_r, and to perform crystallisation trials in order to assess how similar the 'wild-type' and recombinant epimerase proteins were.

The immediate goal of this study was to provide a convenient and abundant recombinant source of methylmalonyl-CoA epimerase, free of contaminating methylmalonyl-CoA mutase and oxaloacetate transcarboxylase activities, for use in enzyme coupled assays of the mutase and transcarboxylase enzymes. It was also hoped that it would be possible to crystallize both the 'wild-type' and the recombinant epimerase, and that they would behave similarly in crystallization trials. As a longer term goal, it was hoped that X-ray crystallographic studies of the enzyme might provide a structural foundation for further

investigation of possible metal ion-mediated catalysis and stability in the epimerase enzyme.

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 BACTERIAL GROWTH

Propionibacterium shermanii (strain 52W) was obtained from the National Collection of Industrial Bacteria (U.K.) N.C.I.B. 9885. The medium in which it was grown (Brown *et al.*, 1973) consisted of yeast extract (Gibco BRL), D-glucose, L-methionine and vitamins from Sigma Chemical Co., $CoSO_4$ (BDH), and potassium/sodium phosphate salts from Ajax Chemicals.

The bacteriological agar used in the growth of *E. coli* was supplied by Difco Laboratories. The tryptone and casamino acids were supplied by Difco Laboratories. Ampicillin (Sigma) and kanamycin (Gibco BRL) were prepared as 100 mg/ml stock solutions in Milli-Q water and filter-sterilized using Millex®-GS 0.22 μm filters (Millipore).

2.1.2 DNA PREPARATION AND ANALYSIS

Agarose (type I-A:Low EEO, Sigma) was used for horizontal agarose gel electrophoresis. The 1 kb DNA ladder (Gibco BRL) was used as a marker of molecular sizes in agarose gels. Restriction enzymes were from Gibco BRL except for *Nde*I (New England Biolabs). T4 DNA polymerase, Klenow and T4 DNA ligase were from Gibco BRL. Lysozyme (L 6876) and RNAase A (R 5503) were from Sigma. Proteinase K was from Boehringer *Taq* polymerase and the accompanying reaction buffers were from Promega. The *f-mol* DNA sequencing system was from Promega and the X-OMAT AR (35 x 43 cm) type Kodak film was from Radiographic Supplies. PCR primers were synthesized by the Separation Science Unit in the Department of Chemistry and Biochemistry at Massey University. The vectors used for expression of methylmalonyl-CoA epimerase in *Escherichia coli*, pT7-7 and pGP1-2, were kindly provided by S. Tabor (Tabor and Richardson, 1985). *E. coli* SRP84 was used for expression of recombinant *P. shermanii* methylmalonyl-CoA mutase and epimerase. Standard reagents used in the manipulation of DNA, such as neutral phenol, were prepared as described by Sambrook *et al.* (1989). Lyophilisation of DNA and protein was done in a Speed Vac (model SC100, Savant). Submerged horizontal agarose gels were cast and electrophoresed in 1x TAE (Sambrook *et al.*, 1989) as a running buffer at 40-60 V using a Bio-Rad "sub mini" electrophoresis unit. Gels were photographed on a transilluminator (model TM 20, UVP).

2.1.3 PROTEIN PURIFICATION

The SDS-PAGE low-M_r markers (Dalton VII-L, Sigma cat. no. SDS-7), bicinchoninic acid protein determination system, streptomycin sulphate and bovine serum albumin (BSA, A7906) were from Sigma. Acrylamide, bis-acrylamide and SDS were from Serva. Stock acrylamide/bis-acrylamide solutions were stored at 4°C in the dark over a few grams of mixed ion-exchange resin (AG® 501-X8(D), 20-50 mesh, Bio-Rad). Ammonium sulphate (AR) was from Riedel-de Haën, and poly(ethylene)glycol (PEG) 6000 (biochemical grade) from BDH. Cellulose-phosphate (P11, Whatman), Sephadex G-75 (40-120 μm, Pharmacia), Q-sepharose fast-flow (Pharmacia), hydroxyapatite (Bio-Rad), and Bio-gel A-1.5m (100-200 mesh, Bio-Rad) chromatographic matrices were used in the purification of methylmalonyl-CoA epimerase or coupling enzymes. MOPS was supplied by United States Biochemical Corporation. The Centriprep units for the concentration of proteins by ultrafiltration were from Amicon. The silver staining system (Bio-Rad) used was kindly supplied by Dr K. Stowell. The Econo-system and columns used were from Bio-Rad. A MSE Soniprep 150 ultrasonic disintegrator was used to lyse cells.

2.1.4 MISCELLANEOUS MATERIALS

Methanol, acetic acid, TEMED and NADH were from BDH. CsCl, isopropanol, ethanol, sodium acetate and ammonium acetate were from Ajax Chemicals. Magnesium chloride was from Riedel-de Haën, Tris (basic form) and dithiothreitol (DTT) from Serva, boric acid from Prolabo Supplies, and ammonium persulphate from Gibco BRL. Coenzyme A, lithium salt (C3019), was from Sigma. All other compounds and chemical reagents used were of the highest grade available. Unless stated otherwise, the water used in all solutions was supplied from a NANOpure II water purification unit (Barnstead). This water is referred to as Milli-Q water in this work, and is reagent grade deionized water with a resistivity greater than 16 MΩ·cm. Dialysis tubing (12000 M_r-cutoff, Union Carbide) was prepared by autoclaving for 10 min in 15 mM sodium bicarbonate, 10 mM EDTA. The tubing was stored at 4°C in TE (10:0.1) buffer. Before use, the tubing was washed both inside and outside with Milli-Q water. The tubing was only handled when wearing gloves.

2.2 METHODS

2.2.1 DNA MANIPULATION

Genomic DNA was isolated from *P. shermanii* (strain 52W) and was used as a template in the amplification of the methylmalonyl-CoA epimerase gene by the polymerase chain reaction (PCR). The PCR product was then ligated into the expression vector pT7-7 to give the expression construct pTEEX. The pTEEX transformants were identified by agarose gel electrophoresis of uncut 'mini-prep' plasmid DNA and restriction endonuclease digestion of 'mini-prep' DNA, and also by cycle sequencing of plasmid DNA and SDS-PAGE analysis of the whole cell protein from induced cultures.

2.2.1.1 THE ISOLATION OF *P. SHERMANII* GENOMIC DNA

P. shermanii was grown in a sterile glucose/salts/yeast-extract medium, subsequently referred to as Brown medium (Brown *et al.*, 1973) (described in section 2.2.2.1), at 30°C with occasional gentle shaking or stirring to prevent the cells from settling out. A 100 µl sample from a 20% (w/v) glycerol deep of *P. shermanii* was used to inoculate 10 ml of sterile Brown medium in a sealed 20 ml Universal bottle. After 120 h of incubation, *P. shermanii* growth was indicated by the increased turbidity of the medium and the presence of a tangy acid scent in the culture headspace. This culture was used to inoculate 200 ml of sterile Brown medium in a sealed 250 ml conical flask which was grown under the same conditions. The pH of the culture was occasionally checked and adjusted back to pH 7.0 using ammonia whenever it dropped below pH 6. After a period of 60 h, the *P. shermanii* cells were harvested by centrifugation at 8000 rpm in a GSA rotor for 15 min at 10°C. The yield (wet weight) was approximately 4 g of cell paste from the 200 ml culture.

2 g of *P. shermanii* cell paste was suspended in 5 ml 25% sucrose, 50 mM Tris/HCl and 0.25 M EDTA (pH 8.0). Lysozyme was added to 0.25 mg/ml. After incubation at 37°C for 10 min, 90 ml of pretreated (37°C for 20 min) proteinase K solution (10 mM Tris/HCl, 10 mM EDTA (pH 8.0), 10 mM NaCl, 0.5% SDS, 50 µg/ml proteinase K] was added. The mixture was incubated for 16 h at 37°C, then extracted twice with an equal volume of neutral phenol, and once with an equal volume of 24:1 chloroform:isoamyl alcohol. The aqueous solution was dialysed with stirring against several changes (20 L total) of TE (10:0.1) buffer containing 10 mM NaCl for 2 days. The dialysate was treated with 25 µg/ml RNAase A (DNAase free) for 4 h at 37°C and extracted with neutral phenol and chloroform:isoamyl alcohol. The DNA was precipitated from solution (80 ml) by adding sodium acetate to 0.3 M final concentration, and layering 160 ml of 95 % ethanol over the aqueous solution. Threads of DNA that precipitated at the

interface were spooled onto a glass rod, rinsed in 70% ethanol and 95% ethanol, air dried and rehydrated in 0.5 ml TE (10:1) overnight. The genomic DNA was aliquoted and stored at -70°C. The presence of high-M_r DNA in samples before and after the dialysis step was confirmed by agarose gel electrophoresis of the genomic DNA.

2.2.1.2 THE AMPLIFICATION OF THE EPIMERASE GENE BY PCR

The optimum set of conditions for the amplification of the gene coding for the 148 amino acid methylmalonyl-CoA epimerase enzyme (methionine on) was obtained by setting up trial PCR experiments in which several parameters were varied including concentrations of template DNA and Mg^{2+}, annealing temperature, and the number of PCR cycles. The effect of pre-heating the PCR machine reaction block before loading the samples was also tested.

A typical PCR, with a total reaction volume of 20 µl in a 0.5 ml Eppendorf tube, was set up by adding 1.6 µl of $MgCl_2$ (25 mM) to give a final concentration of 2 mM, 2 µl of 10x *Taq* polymerase buffer, 1.6 µl of dNTP's (2.5 mM in each dNTP), 2.5 µl of 5-fold diluted genomic DNA template (80 ng/µl), 0.85 µl of forward primer EPCRF (45 ng/µl), 2.5 µl of the reverse primer EPCRB (20 ng/µl), 0.2 µl of *Taq* polymerase (5 U/µl) and made up to 20 µl total volume with 8.8 µl of sterile Milli-Q water. The PCR reaction tube was kept on ice while these additions were made, and the reaction mix was then overlaid with sterile paraffin oil before placing the tube in the reaction block of the PCR machine (DNA thermocycler, Perkin-Elmer Cetus). PCR products were analysed by loading and running 5-10 µl samples of a reaction mix on 1% or 1.2% agarose gels alongside the 1 kb DNA ladder of molecular size markers. A 1.2% agarose gel was prepared by making up 80 ml of a 1.2% agarose mix (0.96 g agarose, 80 ml 1x TAE buffer, 5 ml water). The agarose slurry was melted in a microwave, cooled to 45°C, and 2 µl of a 10 mg/ml ethidium bromide stock solution was added immediately before pouring the gel. After agarose gels had set, they were transferred to an appropriate electrophoresis tank, submerged in 1x TAE buffer and loaded with samples (made dense by adding one fifth volume of gel loading buffer type III (Sambrook *et al.*, 1989)) using an autopipette.

The oligonucleotide PCR primers EPCRF [5' **TGA** GTA ATG AGG ATC TTT TCA TCT G 3' ➡] and EPCRB(Eco) [5' G<u>GAATTC</u> **GTC** AGT TCT TCG GGT ACT GGG TGA GC 3' ➡] were designed from the known sequence of the *P. shermanii* methylmalonyl-CoA epimerase gene (Davis, 1987). The second and third bases of the start codon in the EPCRF forward primer, and the stop codon in the EPCRB(Eco) reverse primer are highlighted in **bold** letters. The arrows indicate the direction of DNA sequence from 5' to 3'. The primers were purified by urea-PAGE and used in PCR to

amplify the DNA sequence coding for the epimerase enzyme using *Taq* polymerase. EPCRF was supplied detritylated whereas the EPCRB(Eco) was supplied with the trityl group on.

2.2.1.3 PURIFICATION OF THE PCR PRIMERS

Detritylation of the EPCRB oligonucleotide was carried out by adapting, with slight modification, the method of Walker and Gaastra (1983). The lyophilised EPCRB pellet was dissolved in 150 µl 80% acetic acid and incubated at 25°C for 20 min. 75 µl of water was added and the solution was extracted three times with 600 µl water saturated diethyl ether. The aqueous phase was retained and then centrifuged at 5000 rpm in a microcentrifuge for 5 min. The supernatant was transferred to a clean 1.5 ml Eppendorf tube and the detritylated oligonucleotide was lyophilised.

The EPCRF and EPCRB oligonucleotides were purified on a 15% urea-PAGE gel. The gel mix (15% acrylamide, 0.68% bis-acrylamide, 7 M urea, 50 mM Tris-borate buffer, pH 8.3, 1mM EDTA) was polymerized with 1.5 µl of 20% (w/v) ammonium persulphate and 1.5 µl of TEMED per ml of gel mix. The gel was allowed to set for 3 h at room temperature and then pre-electrophoresed for 1 h at 33 mA. The running buffer used was 50 mM Tris-borate containing 2.5 mM EDTA, pH 8.3.

100 µg amounts of the lyophilised oligonucleotide primers were dissolved in 50 µl of sample buffer (1 ml deionized formamide, 22 µl of 1.2% bromophenol blue in ethanol, 22 µl of xylene cyanol in water, 22 µl of 1 M Tris-borate buffer, pH 8.3, containing 50 mM EDTA) and heated for 2 min at 100°C prior to electrophoresis. The urea in the wells was flushed out and samples loaded on to the gel (20 cm wide, 18 cm high, 0.15 cm thick). The gel was run until the lower bromophenol blue dye front had just run off the gel.

The oligonucleotides were observed by UV shadowing at 254 nm (Ogden and Adams, 1987). The single predominant band in each lane was sliced out using a sterile scalpel blade and each gel slice was soaked in 1 ml of TE (10:0.2) buffer at 37°C overnight. The liquid was filtered through sterile glass wool packed into a syringe. Each oligonucleotide was precipitated from the filtered solution by adding one tenth volume of 3 M sodium acetate, pH 6.5, and 3 volumes of 95% ethanol and incubating at -20°C overnight. The solution was centrifuged at 12000 rpm for 15 min at 4°C in a microcentrifuge. The pellet was washed with 200 µl of 85% ethanol and then centrifuged as above. The ethanol was poured off and the pellet was lyophilised. The dried pellet was dissolved in 200 µl of TE (10:0.1) and the concentration of the oligonucleotide determined spectrophotometrically at 260 nm.

2.2.1.4 LIGATION OF THE PCR PRODUCT INTO THE *E. COLI* EXPRESSION VECTOR pT7-7

A simple map of the pT7-7 vector is shown in Figure 2.1, and Figure 2.2 summarises the strategy used in constructing the epimerase expression plasmid pTEEX (Figure 2.3). For expression in *E. coli*, cDNA sequences are often ligated into pT7-7 by introducing an *Nde*I recognition site (CATATG) at the start of the gene so that it overlaps the ATG start codon. The gene is then ligated into pT7-7 that has been digested with *Nde*I and another enzyme that recognises an appropriate polylinker site. The presence of an internal *Nde*I site in the epimerase gene meant that this method could not be used. Instead, the ATG start codon in the pTEEX plasmid was reconstructed by the blunt-ended ligation of the digested and end-filled *Nde*I site in the pT7-7 polylinker (supplying the A of the start codon) to the PCR product consisting of the rest of the epimerase gene (supplying the TG of the start codon).

(A) PREPARATION OF THE PCR PRODUCT FOR LIGATION INTO pT7-7

The 454 bp PCR product was treated with T4 DNA polymerase to ensure that the blunt end (at the start of the gene) required for the ligation was present. This treatment was needed because of the tendency of *Taq* polymerase to add a single non-template-directed nucleotide to blunt-ended duplex DNA (Clark, 1988). The PCR product was then digested with the restriction endonuclease *Eco*RI to create a staggered end at the 5' end of the gene. This ensured that the prepared insert could only ligate to the pT7-7 vector in one orientation.

The aqueous PCR products from 200 µl of reaction mixes were combined and extracted with 100 µl of 24:1 chloroform:isoamyl alcohol. This was followed by extracting twice with 100 µl of neutral phenol-chloroform, then repeating the chloroform:isoamyl alcohol extraction before precipitating with 1/10 volume sterile 3 M sodium acetate (pH 5.8), and 2.5 volumes of ethanol at -70°C for 1 h. The mix was centrifuged at 12000 rpm in a microcentrifuge at 4°C for 15 min, before carefully aspirating all the supernatant with a drawn out Pasteur pipette. The large gelatinous pellet was allowed to air-dry at room temperature for 1 h. The dried pellet was dissolved in 20 µl of sterile 10 mM Tris-acetate buffer containing 0.1 mM EDTA (pH 8.0), and drop dialysed (Wallace, 1987) on a 0.025 µm VS filter (Millipore) against 25 ml sterile T4 DNA polymerase buffer (33 mM Tris-acetate pH 8.0, 66 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT) with stirring for 2 h at room temperature. The dialysed drop was recovered and dNTP's were added to a final concentration of 0.125 mM for each dNTP. T4 DNA polymerase (1 U) was added and the reaction mix was incubated at 12°C for 30 min. The reaction tube was then transferred to ice, and 120 µl of ice-cold TE (10:0.1) was added. The reaction mix

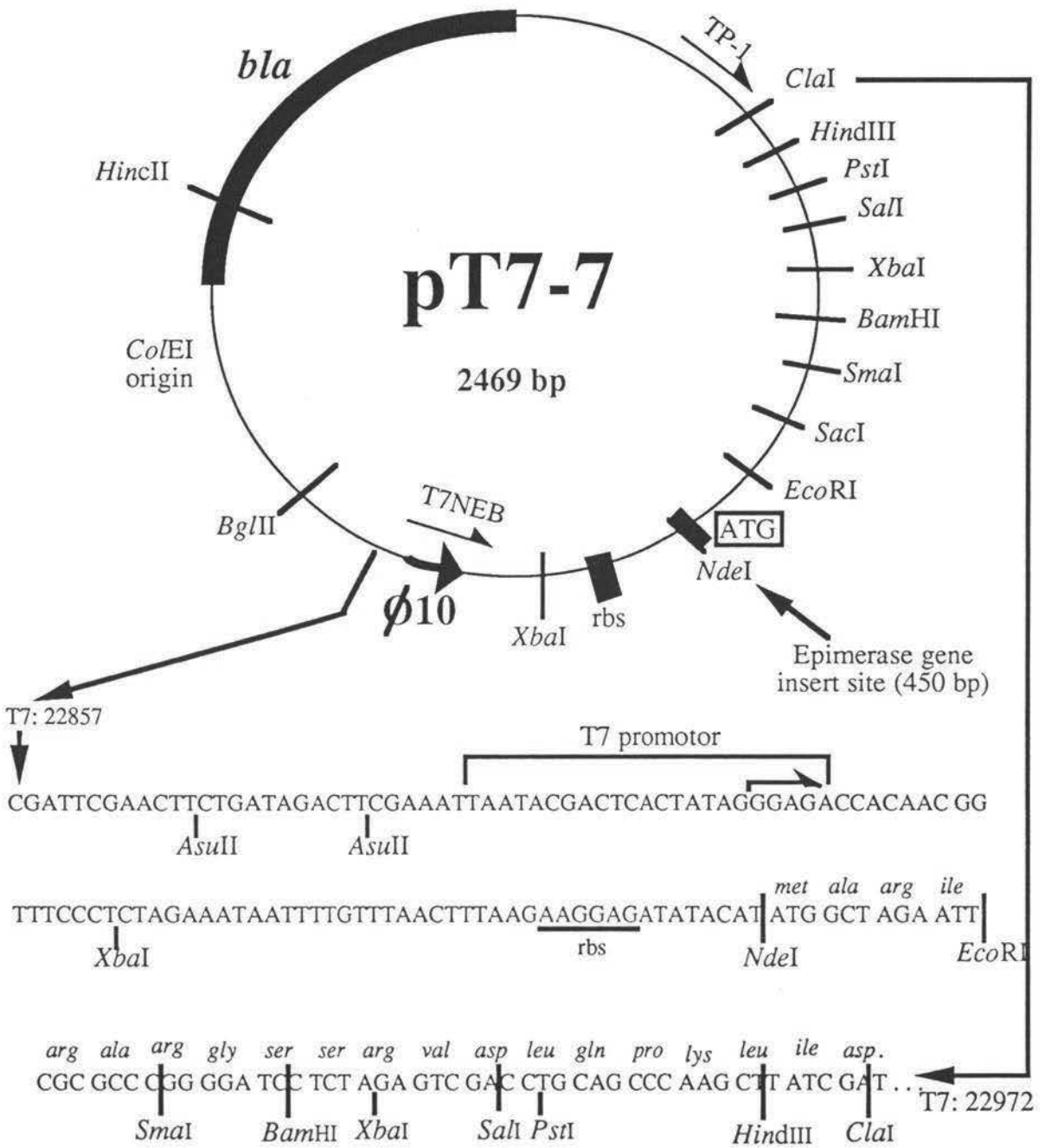

Figure 2.1: The pT7-7 expression vector into which the epimerase gene was cloned. The pT7-7 vector was kindly supplied by Stan Tabor of the Department of Biological Chemistry, Harvard Medical School.

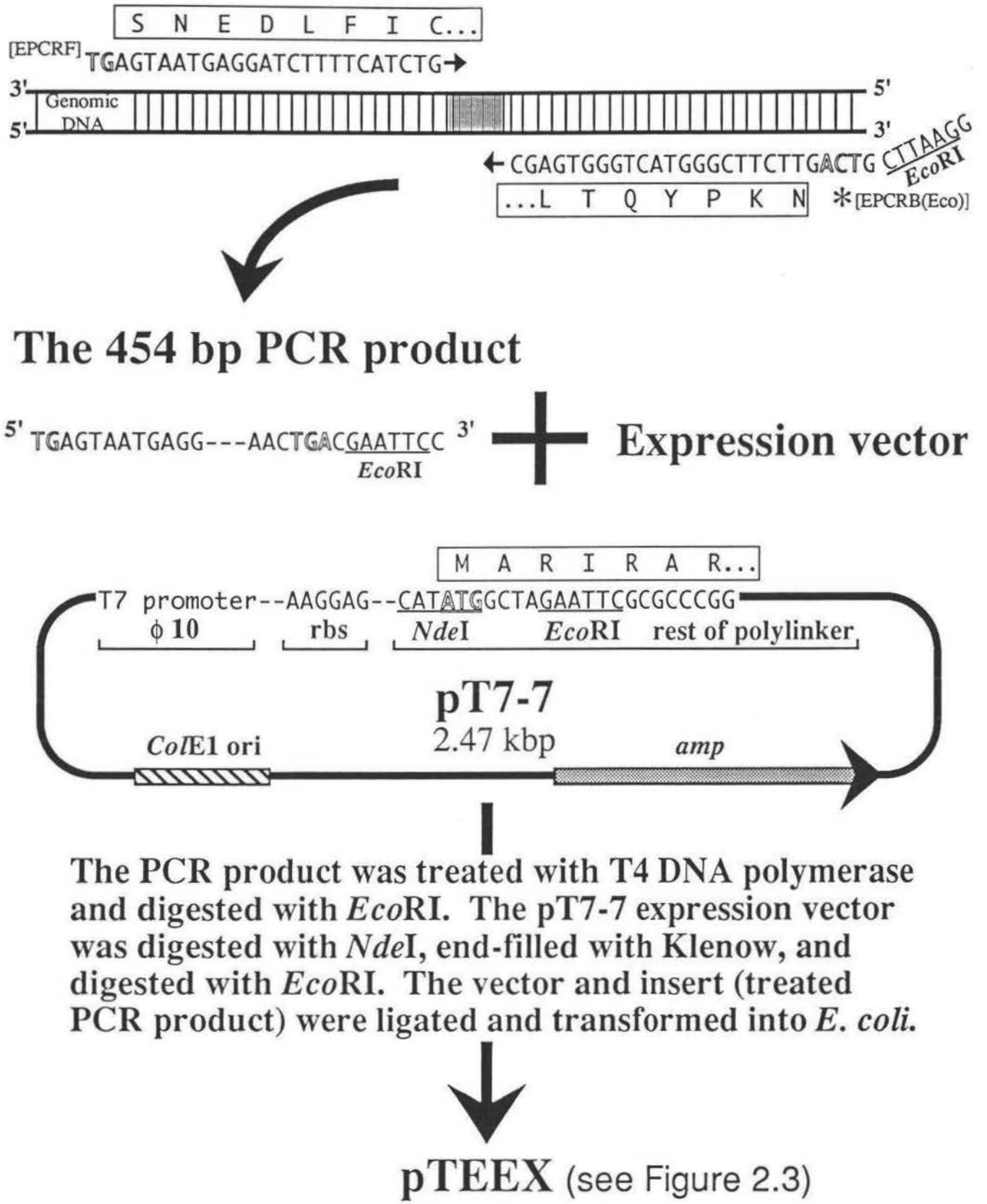

Figure 2.2: A summary of the strategy for obtaining the epimerase expression construct pTEEX for the expression of *P. shermanii* methylmalonyl-CoA epimerase in *E. coli*. The PCR primers EPCRF and EPCRB(Eco) are shown, and the nucleotide bases of the start and stop codons are shown in outline. The known *N*-terminal and predicted *C*-terminal amino acid sequences of the methylmalonyl-CoA epimerase are boxed.

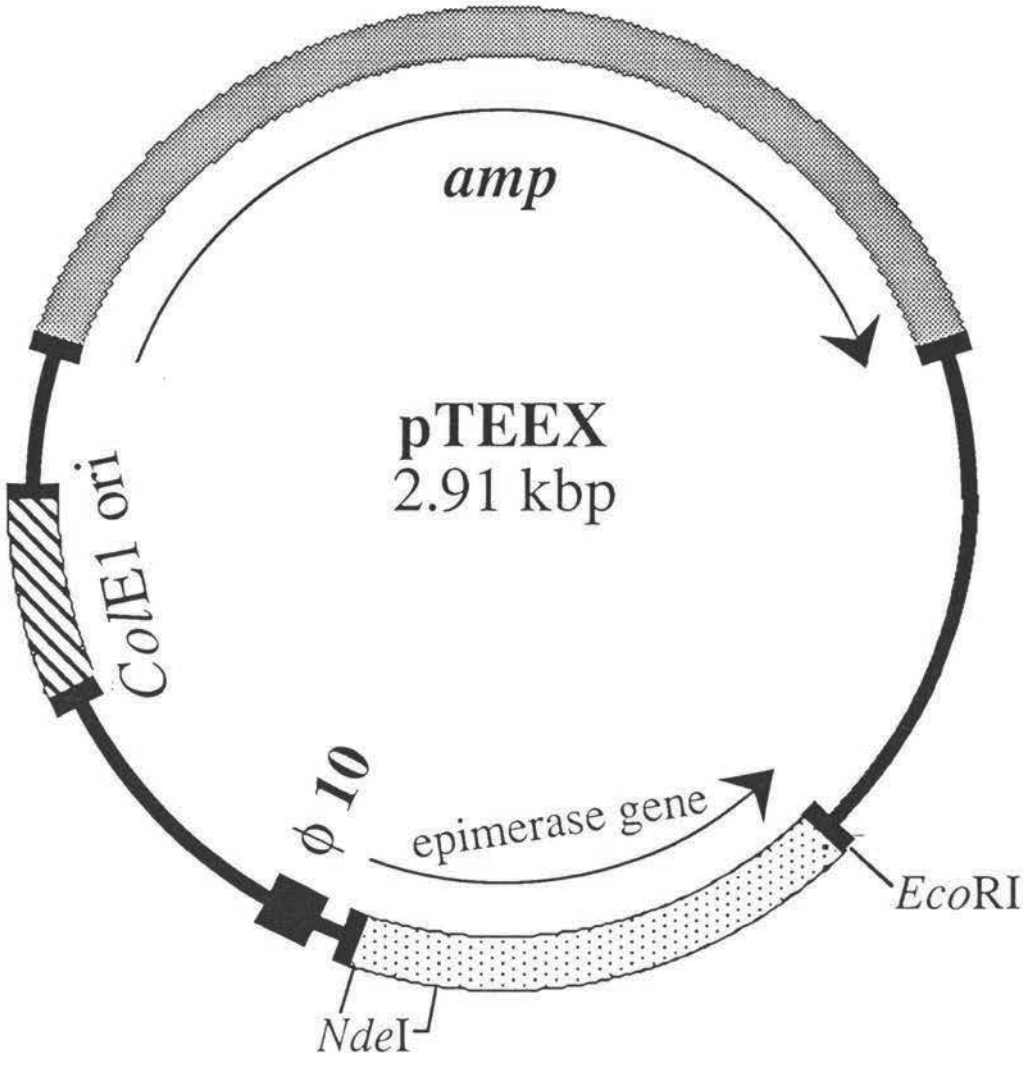

Figure 2.3: A simple plasmid map of the *E. coli* expression construct for *P. shermanii* methylmalonyl-CoA epimerase, pTEEX, showing the *Nde*I site internal to the gene.

was immediately extracted twice with 100 µl of neutral phenol-chloroform and once with 100 µl chloroform-isoamyl alcohol. The aqueous layer was transferred to a sterile 0.5 ml Eppendorf tube and the DNA precipitated by adding 1/10 volume of 3 M sodium acetate (pH 6.0) and 2.5 volumes of ethanol and incubating at -70°C for 1 h. The mixture was centrifuged at 12000 rpm in a microcentrifuge at 4°C for 15 min, before carefully removing all the supernatant by aspiration with a drawn out Pasteur pipette. The almost invisible pellet was air-dried at room temperature for 1 h.

The pellet was dissolved in 10 µl of TE (10:0.1) and drop dialysed against 40 ml of TE (10:0.1) with stirring for 2 h at room temperature. The dialysed PCR product was recovered and 10x *Eco*RI buffer added to give a 1x concentration. The DNA was digested with 5 U of *Eco*RI for 70 min at 37°C, then incubated for 15 min at 65°C. EDTA was added to a final concentration of 15 mM and the incubation was continued for another 6 min at 65°C. The digest (approximately 7 µl at this stage) was cooled to room temperature and drop dialysed against 40 ml of TE (10:0.1) for 2.5 h with stirring at room temperature. 10.5 µl of the resulting 12 µl was stored at -70°C. 1.5 µl was electrophoresed on a 1% agarose gel and this indicated a DNA concentration of approximately 5 ng/µl.

(B) PREPARATION OF *Nde*I-KLENOW-*Eco*RI TREATED pT7-7 FOR LIGATION

The 2.47 kbp pT7-7 expression vector was prepared for ligation by digestion with *Nde*I, end-filling with Klenow to create a blunt end that corresponded to the PCR product blunt end, and then digestion with *Eco*RI to create a staggered end that corresponded to the staggered end of the PCR product.

3 µg of CsCl-purified pT7-7 vector DNA was digested to completion with *Nde*I (20 U) in a total reaction volume of 20 µl at 30°C for 1 h. The digest was placed on ice and 1 µl of *Nde*I digested sample was analysed by agarose gel electrophoresis to confirm that the digest was complete. The digest was heated at 65°C for 15 min, then 1.5 µl 0.25 M EDTA, pH 8, was added and the 65°C incubation continued for 5 min. The heat-inactivated reaction mix was drop dialysed against 40 ml sterile Klenow buffer (22 mM Tris/HCl pH 7.6, 6 mM $MgCl_2$, 5 mM DTT) for 2.5 h at 25°C with stirring. The resulting DNA solution (18 µl) was recovered and dATP and dTTP were each added to a final concentration of 0.1 mM. The solution was made up to 20 µl with 1x Klenow buffer, and 3 U Klenow (0.3 µl) was added. After a 15 min incubation at 25°C, 1.3 µl of 0.25 M EDTA, pH 8, was added and the tube incubated at 75°C for 11 min. The reaction mix was cooled to room temperature and drop dialysed against 40 ml TE (10:0.1) for 2 h at room temperature with stirring. The DNA solution (15 µl) was transferred to a 0.5 ml sterile Eppendorf tube and 4 µl of 5x *Eco*RI reaction buffer was added, followed by 1 µl

*Eco*RI (10 U). The reaction was incubated at 37°C for 2.5 h, then at 65°C for 20 min. 1.5 µl 0.25 M EDTA, pH 8, was added and the incubation continued for 5 min. The reaction mix was drop dialysed against 40 ml TE (10:0.1) and the resulting 35 µl had a DNA concentration of ~ 50 ng/µl. This treated pT7-7 preparation was used in the ligation reaction described below.

(C) LIGATION OF THE TREATED PCR PRODUCT (A) AND pT7-7 VECTOR (B)

The treated PCR product was concentrated to ~ 8 ng/µl in a Speed Vac. 4 µl of this PCR product and 1 µl of ~ 50 ng/µl treated pT7-7 were mixed in a sterile 0.5 ml Eppendorf tube, heated at 45°C for 5 min then placed on ice. Added 1.5 µl 5x BRL T4 DNA ligase ligation buffer, mixed, then added 1 µl T4 DNA ligase (1 U/µl), mixed by pipetting up and down and incubated at 16°C for 4 h. The ligation mixtures were drop dialysed against 10% (v/v) glycerol for 30 min at room temperature with stirring (volume increased slightly from 7.5 µl to ~9 µl). 2 µl of the ligation mix was electroporated into a 40 µl suspension of electroporation-competent XL1-blue *E. coli* cells. Electroporation cuvettes (*E. coli* pulser cuvettes, 0.2 cm electrode gap, Bio-Rad) and a Bio-Rad Gene Pulser apparatus set at 800 Ω resistance, 25 µFD capacitance, and 2.5 V, were used for electroporation. The time constants seen in these electroporation transformations were typically 17-18 ms. Electroporation-competent *E. coli* cells were prepared by the method of Dower *et al.* (1988). The electroporated cells were transferred to a 1.5 ml Eppendorf tube, 0.5 ml 2TY (2xYT, Sambrook *et al.*, 1989) was added, and the culture was incubated at 37°C with shaking for 30 min. 20 µl and 200 µl volumes of culture were plated on to 2TY/Amp plates (100 µg/ml ampicillin). The plates were allowed to stand right side up for 10 min, allowing the mixture to soak into the agar, before being incubated upside-down at 37°C overnight. At least 200 colonies per plate were seen when 20 µl of the culture was plated, and individual colonies were picked and cultured in 2.5 ml 2TY/Amp (100 µg/ml) at 37°C overnight for the preparation of 'mini-prep' plasmid DNA.

2.2.1.5 PREPARATION AND ANALYSIS OF PLASMID DNA PREPARED FROM TRANSFORMANTS

'Mini-prep' plasmid DNA was prepared using the rapid boil method for the isolation of plasmid DNA (Holmes and Quigley, 1981). A 1.5 ml sample of each 2.5 ml culture was centrifuged in a 1.5 ml Eppendorf tube at 13000 rpm for 1 min at room temperature in a microcentrifuge. The supernatant was poured off and the pellet was drained well. Each cell pellet was completely resuspended by vortexing in 350 µl of sterile STET buffer (8% sucrose, 5% Triton X-100, 50 mM Na_2EDTA pH 8.0, 50 mM Tris/HCl pH 8.0). 25 µl of

a fresh 10 mg/ml solution of lysozyme in 10 mM Tris/HCl, pH 8.0, was mixed with the resuspended pellet. Each tube was heated in a boiling water bath for 40 seconds and then immediately centrifuged for 10 min. The gelatinous pellet was removed with a sterile toothpick. An equal volume of isopropanol (≤ 300 µl) was added to the remaining solution. After mixing, the solution was incubated at -20°C for 20 min. The DNA was pelleted by centrifugation at 13000 rpm for 5 min at 4°C. The supernatant was drained off and each pellet was washed with 350 µl of 80% ethanol. The DNA was again centrifuged at 13000 rpm for 5 min at 4°C. Each DNA pellet was drained and dried under vacuum. Each lyophilised DNA pellet was dissolved in 50 µl of TE (10:0.1) by vortexing. 0.4 µl of 10 mg/ml RNAase was added to each tube. The DNA was stored at -20°C.

The 'mini-prep' plasmid DNA samples were analysed for the presence of a PCR insert in pT7-7 by electrophoresing undigested plasmid DNA samples alongside the 1 kb DNA ladder on a 1% agarose gel. The results showed that about one out of every 15 plasmids appeared to be slightly larger that pT7-7. To confirm this result, 4 of these slightly larger plasmids were digested with restriction endonucleases that should have linearised the plasmids. These 4 larger plasmids were also used to transform the *E. coli* expression strain SRP84/pGP1-2 by electroporation, and cultures of transformant colonies were induced and the whole cell protein was analysed by SDS-PAGE. All 4 plasmids were shown to make recombinant protein, and so large-scale plasmid preparations of the 4 plasmids were done. These 4 plasmids were putative methylmalonyl-CoA epimerase expression plasmids, i.e. putative pTEEX plasmids.

Large-scale isolation of the plasmid DNA using a CsCl-density gradient was carried out according to the method described by Maniatis *et al.*(1982). The only major difference was that after the removal of ethidium bromide the plasmid DNA was ethanol precipitated, washed with 5 ml of ice-cold 80% ethanol and air dried. The plasmid DNA was resuspended in 400 µl of TE (10:0.5). The $A_{260/280}$ ratio was determined and a small sample of the plasmid DNA was sized by digestion with *Eco*RI to linearise the plasmid. The plasmid DNA was then aliquoted and stored at -70°C. Some of these large-scale preparations of putative pTEEX constructs were subsequently used in DNA sequencing.

2.2.1.6 DNA SEQUENCING OF THE PUTATIVE EXPRESSION PLASMID pTEEX

The insert of one of the putative pTEEX plasmids (#4) was fully sequenced using the dideoxy-chain-termination method of Sanger *et al* (1977). The oligonucleotide T7NEB (the T7 primer produced by New England Biolabs) was the forward primer. The oligonucleotide TP-1, designed from the known pT7-7 sequence was used as the reverse

primer (see Figure 2.4). The primer oligonucleotide sequences were checked for potential stem-loop structures and possible mis-priming before used.

(A) THE *F-MOL* DNA SEQUENCING SYSTEM

The protocol for DNA sequencing using direct incorporation of ^{35}S-αATP, described in the *f-mol* DNA sequencing system technical manual, 5th edition (product Q4100, Promega) was followed. CsCl gradient-purified DNA templates were used. The thermo-cycling conditions given in profile 1 of the manual were followed (i.e. 95°C for 2 min to denature the template, then 50 cycles of 95°C for 30 s (denaturation), 42°C for 30 s (annealing), 70°C for 1 min (extension)). The total number of thermo-cycles (50) represents an increase on the recommended 25 cycles. After 50 cycles the reaction mixes were held at 4°C until the tubes were removed from the PCR machine for processing.

The samples for loading were heated at 90°C for 2 min immediately before loading 2.5-3 µl onto the DNA sequencing gel. The gels were prepared, run and processed as described in the DNA sequencing gel electrophoresis section 2.2.1.7.

Forward sequencing primer T7NEB: $^{5'}$.TAATACGACTCACTATAGGGAGA.$^{3'}$, 23-mer

Reverse sequencing primer TP-1: $^{5'}$.CTTTAGATTGATTTAATTC.$^{3'}$, 19-mer

Figure 2.4: The DNA sequences of the forward and the reverse sequencing primers designed and used in the DNA sequencing of the putative pTEEX plasmids. The forward primer T7NEB anneals to the T7 promoter region of the pT7-7 vector. The reverse primer TP-1 is located 20 bp downstream of the *Cla*I site of the pT7-7 polylinker.

2.2.1.7 DNA SEQUENCING GEL ELECTROPHORESIS

Preparation of DNA sequencing gels, and gel electrophoresis, was carried out using the standard method described by Maniatis *et al.* (1982). A Gibco BRL Model SA 60 sequencing apparatus was used to electrophorese samples in the DNA sequencing gels.

(A) STOCK REAGENTS

The stock reagents of 20% acrylamide, urea mix solution and 5x TBE buffer contained the following ingredients:

(i) 20% acrylamide solution:	Acrylamide	96.5 g
	Bis-acrylamide	3.35 g
	Urea	233.5 g
	5x TBE	100 ml
	H_2O	to 500 ml
(ii) Urea mix solution:	Urea	233.5 g
	5x TBE	100 ml
	H_2O	to 500 ml
(iii) 5x TBE solution:	Tris base	54 g
	Boric acid	27.5 g
	0.5 M Na_2EDTA (pH 8.8)	20 ml
	H_2O	to 1 L

The stock reagents were stored in air-tight bottles. The 20% acrylamide stock was stored at 4°C, while the other reagents were stored at room temperature.

A typical 8% DNA sequencing gel was prepared by adding 20 ml of 20% acrylamide to 30 ml of urea, mixing, and then degassing the solution for 10 min. 50 µl of TEMED and 0.4 ml of fresh 10% ammonium persulphate were added to polymerize the gel. The gel mix was mixed by rapid swirling before being poured into a sequencing gel mould preferably in one continuous flow whilst making sure that air bubbles were avoided. Any air bubbles trapped within the gel mould were gently but firmly persuaded to the gel surface by calmly tapping the glass plates with a solid object. The gel was allowed to set at room temperature for at least 60 min.

(B) PREPARATION AND ELECTROPHORESIS OF SAMPLES

Samples were prepared for loading by denaturation. Samples were heated to 90°C for 2 min then immediately placed on ice to avoid reannealing of single stranded DNA until the samples were loaded. The urea in each well of the gel (which was pre-run for 30 min at 2600 V (75 W, 55 mA) using 1x TBE as the running buffer) was carefully flushed out using a Pasteur pipette prior to loading 2.5 µl samples into each well. Typically gels were electrophoresed for about 3 h at 2600 V, and the gel was allowed to run an extra 15 min after the bromophenol blue dye front had run off the bottom edge of the gel before the gel was stopped. For 'long runs' the electrophoresis time was doubled or in a few cases trebled to 9 h. For the extended electrophoresis times the upper tank TBE buffer was replaced once every 3 h.

(C) FIXING, DRYING AND EXPOSING THE GEL

The gel, still in the glass mould, was removed from the DNA sequencing apparatus. One glass plate was carefully lifted off and the other plate still with the intact gel was carefully lowered into the fixative (10% acetic acid/10% methanol mixture). The gel was allowed to soak in the fixative for 15 minutes. The gel was then carefully lifted out of the fixative on the glass plate, and the excess fixative was drained off. A sheet of Whatman 3-MM filter paper cut to be slightly larger than the gel was lowered onto the gel. With the gel sticking onto the paper, the gel was carefully lifted off the glass plate. The gel was covered with glad-wrap plastic film and dried under vacuum using a Bio-Rad model 583 gel drier for 40 min at 80°C. A portable Geiger counter was passed over the dry gel to determine the approximate amount of radioactive label incorporated into the DNA products of the sequencing reactions. In the dark room, the gels were exposed to X-OMAT AR (35 x 43 cm) type Kodak film in an autoradiography cassette, usually for between 2-5 days, before the film was developed manually and any DNA sequence read from the gel.

2.2.2 ENZYME PURIFICATION

The aim of this section of work was to produce, extract and purify the 'wild-type' and the recombinant *P. shermanii* methylmalonyl-CoA epimerases. In order to assay the epimerase enzymes, it was also necessary to purify the coupling enzymes methylmalonyl-CoA mutase (recombinant source, *P. shermanii* enzyme), and oxaloacetate transcarboxylase (purified from *P. shermanii*).

The column chromatography purification steps for epimerase, and later on in section 2.2.3 for the mutase and transcarboxylase purifications, were carried out using a Bio-Rad Econo-system, with a 280 nm filter in the detector, set-up at in a 4°C cold room.

2.2.2.1 PURIFICATION OF 'WILD-TYPE' *P. SHERMANII* EPIMERASE

(A) GROWTH OF 'WILD-TYPE' *PROPIONIBACTERIUM SHERMANII*

P. shermanii was grown in Brown medium, prepared from the following stock reagents:

(i) 50x KP/NaP stock (100 ml):	K_2HPO_4	4.35 g
	NaH_2PO_4	3 g
	H_2O	to 100 ml

(ii) 1000x Cobalt stock (10 ml):	$CoSO_4.7H_2O$	100 mg
	H_2O	to 10 ml

(iii) 100x Vitamin stock (100 ml):	Dimethylbenzimidazole	0.25 g
	Riboflavin	10 mg
	Ca-pantothenate	10 mg
	Biotin	10 mg
	Nicotinic acid	10 mg
	Thiamine	10 mg
	L-methionine	0.5 g
	H_2O	to 100 ml

(iv) 23% (w/v) glucose stock (1 L):	Glucose	230 g
	H_2O	to 1 L

The vitamin stock solution was filter sterilized through a Millex®-GS 0.22 μm filter. The rest of the stock reagents were sterilized by autoclaving.

5 L of Brown medium was prepared by autoclaving 100 g of yeast extract dissolved in 3.85 L of water, adjusted to pH 7 with NaOH, in a 5 L conical flask with a magnetic stir-bar in the flask. While the stir-bar was stirring, 1 L of 23% (w/v) sterile glucose, 100 ml of sterile 50x KP/NaP stock, 5 ml of sterile 1000x cobalt stock and 50 ml of sterile 100x vitamin stock were slowly added and stirred well. The following amounts of Brown medium were then aliquoted out into sterile containers: 5 ml into a bijou bottle; two lots of 20 ml each into two Universal bottles; 400 ml into a 500 ml flask with a bung inserted in the flask mouth and the rest of the 4.565 L of Brown medium left in the 5 L flask with a bung inserted in the flask mouth. All the bungs (non-absorbent cotton-wool wrapped with cheese-cloth) had been sterilized by autoclaving.

5 ml of Brown media in a bijou bottle was inoculated with 100 μl of a 20% (w/v) glycerol-deep of *P. shermanii*, capped tightly and grown in a 30°C incubator with occasional shaking until the culture was cloudy and a notable drop in pH to between 5.0-5.5 was measured using pH paper. Initial signs of growth may take between 1-5 days depending on how fast *P. shermanii* adjusts to the conditions of the medium.

The 5 ml culture was used to inoculate the two 20 ml volumes of Brown medium by transferring a 2.5 ml aliquot into each using aseptic technique. The bottles were capped tightly and again grown at 30°C with occasional shaking until the bacteria started to grow. The pH was again measured using pH indicator paper as an indicator of growth.

The 20 ml cultures were used as an inoculum for the 400 ml volume of Brown medium which was grown at 30°C with occasional gentle swirling. Once growing well, the 400 ml culture was used to inoculate the rest of the Brown medium in the 5 L flask and this culture was grown at 30°C with gentle stirring from the magnetic stir-bar until the A_{650} was greater than 10. The pH was maintained at pH 7.0, the optimum pH for *P. shermanii* growth, by careful addition of concentrated ammonia (specific gravity 0.880 g/ml).

The cells were harvested by centrifugation at 10000 rpm for 5 min at 4°C in a Sorvall GSA rotor. The total yield (wet weight) of cells was determined (114 g) and the cells were stored at -70°C.

(B) PURIFICATION OF 'WILD-TYPE' *P. SHERMANII* EPIMERASE

The purification of 'wild-type' *P. shermanii* was carried out by adapting the method described by Leadlay (1981) to a smaller wet weight of cells. Samples were removed at different stages of the purification for later analysis by SDS-PAGE.

Step 1: Initial Extraction of Cells

40 g of bacterial cell paste was suspended in 57 ml of lysis buffer (0.2 M KP (pH 7.2), 2 mM DTT, 1 mM EDTA). The cells were ruptured by sonication at an amplitude of 20 microns using a 1.5 cm diameter probe for a period of 10 min (ten 1 min sonication bursts). The lysate was cooled in liquid N_2 down to about 5°C following each 1 min sonication burst. The temperature of the lysate was monitored and did not exceed 15°C. During sonication PMSF was added to a final concentration of 0.2 mM from a 0.1 M PMSF stock solution in acetone. Estimation of adequate cell lysis was made by observing the cells under a light microscope and also by holding the beaker with the sonicated cells up to a source of bright light. The sufficiently ruptured cell solution appeared red-grey in colour and semi-transparent. The cell lysate was placed on ice.

The ruptured cell mixture was centrifuged at 16000 rpm in a Sorvall SS-34 rotor at 4°C for 30 min. The supernatant was decanted and stored on ice. The cell pellet was resuspended in 25 ml of lysis buffer and the sonication process was repeated for another 10 min with 45 s sonication bursts. The second extract was centrifuged as before and the supernatant was decanted and combined with supernatant from the first centrifugation. The combined supernatant (100 ml) was centrifuged in three Sorvall AH-627 rotor tubes at 20000 rpm for 30 min at 4°C in an OTD-combi Sorvall ultracentrifuge. The supernatant was decanted and stored on ice.

Step 2: $(NH_4)_2SO_4$ salt fractionation

100 µl of a 0.1 M PMSF solution in acetone was added with mixing to the supernatant from step 1. The $(NH_4)_2SO_4$ salt fractionation guide described in Scopes (1987) was followed. 20.84 g of solid $(NH_4)_2SO_4$ was slowly added to the 100 ml of supernatant on ice over a period of 10 min with slow stirring to give 35% saturation. The solution was stirred slowly on ice for 20 min. The solution was centrifuged at 16000 rpm for 30 min at 4°C. The supernatant (94 ml) was decanted and solid $(NH_4)_2SO_4$ (15.28 g) was slowly added to give 60% saturation while being gently stirred on ice. The pellet from this centrifugation (the 35-60% saturation $(NH_4)_2SO_4$ cut) was stored at -70°C for later purification of transcarboxylase. The supernatant (98 ml) was decanted and solid $(NH_4)_2SO_4$ (21.46 g) was added slowly while being stirred on ice to give 90% saturation. The precipitate was removed by centrifugation as above.

The 60-90% $(NH_4)_2SO_4$ saturated pellet was dissolved in 15 ml of ice-cold 50 mM Tris/HCl, pH 7.5 (containing 0.1 mM PMSF and 0.1 mM EDTA). The protein solution was dialysed with stirring against three 1 L changes of the same 50 mM Tris/HCl buffer described above (without PMSF) at 4°C, with buffer changes every 2 h. After dialysis the volume of the protein solution had increased to 20.8 ml. The dialysed solution was stored on ice.

Step 3: Streptomycin Sulphate treatment

At 4°C, the dialysed protein solution (20.8 ml) was treated by adding 0.2 volumes (4.16 ml) of 10% (w/v) streptomycin sulphate in 50 mM Tris/HCl, pH 7.4, with gentle stirring. An extra 15 min of stirring was allowed and the precipitate was removed by centrifugation at 16000 rpm in an SS-34 rotor for 15 min at 4°C. The supernatant was decanted and dialysed against two 1 L changes of 50 mM Tris/HCl, pH 7.5 (containing 0.1 mM PMSF and 0.1 mM EDTA) over a period of 6 h. The final volume (26 ml) of dialysed solution was stored on ice.

Step 4: Poly(ethylene glycol) (PEG) fractionation

The dialysed protein solution (26 ml) from step 3 was stirred gently at 4°C while successive 0.03 volumes of 50% (w/v) PEG 6000 were added. The soluble epimerase activity was monitored after each addition of 50% PEG by assaying the epimerase activity in the supernatant fraction of a small sample. Once the epimerase activity started to decline by 5-10% (i.e. with more than 90% of the activity still remaining in the supernatant), the entire solution was centrifuged at 16000 rpm in an SS-34 rotor for 15 min at 4°C. At this stage, 3.25 ml of 50% PEG 6000 had been added to produce 5.6% PEG concentration.

The pellet was discarded and the supernatant (29 ml) was retained. More 50% PEG 6000 (7.5 ml) was slowly added to the supernatant to increase the final PEG concentration to 15% (w/v). The material was centrifuged as before and the pellet was stored on ice.

Step 5: Hydroxyapatite chromatography

The pellet from step 4 was resuspended in 40 ml of buffer A (10 mM NaP, pH 6.5) and then centrifuged at 16000 rpm for 15 min at 4°C. The supernatant (42 ml) was decanted and loaded onto a hydroxyapatite column (11 cm x 2.5 cm) equilibrated in buffer A. The sample was loaded at 1 ml/min and the column washed through with buffer A for 1 h at 1 ml/min. A 300 ml gradient of 0-100% buffer B (0.2 M NaP buffer, pH 6.5) was run at a flow rate of 1 ml/min. 7.0 ml fractions were collected and assayed for epimerase activity at 25°C. The method used for assaying of epimerase activity is described in section 2.2.5.1. Samples from the fractions were also analysed by electrophoresis on a 20% SDS-PAGE gel. Fractions containing a high level of epimerase activity were pooled together (140 ml) and concentrated down to 3.7 ml using two Amicon Centriprep 10 units. The concentrated protein was stored on ice.

Step 6: Gel Filtration Chromatography (Sephadex G-75)

The concentrated pool of active fractions (3.7 ml) from step 5 was applied to a Sephadex G-75 gel filtration column (90 cm x 2.5 cm) equilibrated with 50 mM Tris/HCl buffer, pH 7.5, containing 0.1 mM EDTA and 0.1 mM PMSF, at 0.8 ml/min. The column was prepared by swelling 38 g of Sephadex G-75 (40-120 microns) in 700 ml of room temperature 50 mM Tris/HCl, pH 7.5, containing 0.1 mM EDTA. The 3.7 ml of concentrated protein was loaded at 0.67 ml/min and eluted at 0.5 ml/min. 7.0 ml fractions were collected. The fractions containing highly purified epimerase protein were identified by running a 20% SDS-PAGE gel of samples from active fractions. Highly purified epimerase fractions were pooled together and then concentrated, using an Amicon Centriprep 10 ultrafiltration unit, down to 1 ml. The protein concentration of the concentrated solution was about 12 mg/ml. This concentrated solution was aliquoted into five 0.2 ml amounts, snap frozen in liquid nitrogen and stored at -70°C.

2.2.2.2 THE PURIFICATION OF RECOMBINANT *P. SHERMANII* METHYLMALONYL-CoA EPIMERASE

The scheme for the purification of the recombinant epimerase from *E. coli*, while differing in minor details, was essentially the same as that for the purification of the 'wild-type' epimerase from *P. shermanii*, with one major exception. This was the

addition of a heat-treatment step in the purification of the recombinant epimerase.

(A) GROWTH AND EXPRESSION OF RECOMBINANT EPIMERASE

E. coli SRP84/pGP1-2/pTEEX producing recombinant epimerase was cultured in M1 medium (McKie *et al.*, 1990) containing 15 g of casamino acids, 5 g of yeast extract, 10 ml of 0.4 M sodium phosphate buffer (pH 7.2), 10 ml of solution A (1 M NH_4Cl, 50 mM K_2SO_4, 50 mM $MgSO_4$ and 2 mM $CaCl_2$) and 1 ml of solution B (0.1 M HCl, 10 mM $FeSO_4$, 2 mM $MnCl_2$, 2 mM $ZnSO_4$, 0.2 mM $CoSO_4$, 0.1 mM $CuSO_4$ and 0.1 mM $NiCl_2$) per litre. The pH was adjusted to 7.2 with NaOH before autoclaving the M1 medium.

The recombinant methylmalonyl-CoA epimerase enzyme was expressed in the protease deficient *E. coli* strain SRP84 which was transformed with both the epimerase expression plasmid pTEEX and pGP1-2, a plasmid that contains the T7 RNA polymerase gene under the control of the λ P_L promoter and the gene for the heat-sensitive λ repressor *cI857* (Tabor and Richardson, 1985). Once the culture A_{650} values were above 2.0, the epimerase gene was induced by heat-shock treatment and the cells grown and harvested as described in section 2.2.3.1 for the recombinant methylmalonyl-CoA mutase enzyme.

(B) PURIFICATION OF RECOMBINANT *P. SHERMANII* EPIMERASE

Samples were removed at different stages of the purification for analysis by SDS-PAGE.

Step 1: Preparation of cell extract

10 g of induced *E. coli* SRP84/pGP1-2/pTEEX cell paste was suspended in 45 ml of lysis buffer (0.1 M MOPS/NaOH pH 7.5, 2 mM EDTA, 0.1 mM PMSF (added during sonication from a 0.2 M stock solution in acetone)). The cell suspension was sonicated using a 1.5 cm diameter probe at an amplitude of 18 microns for a total of 10 min (10x 1 min bursts with intermittent cooling in liquid N_2 down to about 5°C). The maximum temperature during each sonication burst was measured and did not exceed 18°C. The cells were judged to be sufficiently disrupted when the suspension looked semi-transparent when held up to a bright light. Cell disruption was confirmed by examination of the cell lysate under a light microscope. The lysate was centrifuged in a Sorvall AH-627 rotor at 26000 rpm for 1 h at 5°C in a Beckman L2-65B ultracentrifuge. The cell-free lysate supernatant (46.85 ml) was stored on ice.

Step 2: Heat-treatment

The supernatant from step 1 was transferred to a 50 ml beaker and was incubated in a 70°C shaking waterbath (140 cycles per min) for 15 min. To bring the temperature of the protein solution up to 70°C as rapidly as possible, the solution was initially heated in a 90°C waterbath with constant stirring for 2 min. Upon reaching a solution temperature of 70°C, the solution was immediately transferred to a 70°C waterbath for 15 min, then cooled by stirring on ice for 2 min after which time the temperature had dropped below 10°C. The denaturation of *E. coli* proteins turned the solution cloudy when heated. The heat-treated solution was centrifuged using an SS-34 rotor at 16000 rpm for 30 min at 4°C. The supernatant (37 ml) was decanted and stored on ice. The supernatant pH was 7.6.

Step 3: $(NH_4)_2SO_4$ salt fractionation

The $(NH_4)_2SO_4$ salt fractionation guide described by Scopes (1987) was followed. The first step was to bring the protein solution to 55% saturation. The supernatant (37 ml) was gently stirred on ice using a magnetic stirrer while 12.99 g of solid $(NH_4)_2SO_4$ was gradually added over a period of 10 min. The solution was further stirred for an extra 20 min on ice, then centrifuged to remove the precipitate using an SS-34 rotor at 16000 rpm for 20 min at 4°C. The supernatant (35.5 ml) was stored on ice.

The second step was to bring the 55% saturated supernatant up to 90% saturation. The 55% saturated supernatant (35.5 ml) was transferred into a 50 ml beaker and gently stirred on ice, while 9.15 g of solid $(NH_4)_2SO_4$ was gradually added. It was quite difficult to achieve a 90% saturation on ice. The precipitate was pelleted by centrifugation as above and the pellet was stored at -20°C. The 90% pellet was dissolved in 15 ml of ice-cold 50 mM Tris/HCl buffer, pH 7.5, containing 0.05 mM PMSF and 0.1 mM EDTA. This protein solution was dialysed with stirring for 6 h against 3x 1 L changes of 50 mM Tris/HCl buffer, pH 7.5 (without PMSF but with EDTA), at 4°C. The dialysed protein solution (22 ml) was stored on ice.

Step 4: Streptomycin sulphate precipitation

The 22 ml of dialysed protein solution from step 3 was gently stirred and 0.2 volumes of 10% (w/v) streptomycin sulphate in 50 mM Tris/HCl, pH 7.5, was added. The protein solution was stirred for a further 30 min on ice before centrifugation at 16000 rpm in an SS-34 rotor for 25 min at 4°C. The supernatant (26.5 ml) was dialysed with stirring against 2x 1 L changes of 50 mM Tris/HCl buffer containing 0.1 mM EDTA, pH 7.5, over a period of 2 h. The dialysed solution (30.5 ml) was stored on ice.

Step 5: PEG precipitation

0.11 volumes of 50% PEG 6000 was slowly added to the dialysed protein solution from step 4 (30.5 ml), and the solution was stirred gently on ice for 20 min. The solution was centrifuged at 16000 rpm for 20 min at 5°C in an SS-34 rotor. The supernatant was decanted and a further 9.34 ml of 50 % PEG 6000 was slowly added and stirred into the supernatant for 20 min. The preparation was centrifuged as before. The supernatant was discarded and the pellet was dissolved in 20 ml of 10 mM NaP buffer, pH 6.5, and centrifuged at 12000 rpm for 15 min at 5°C in an SS-34 rotor. The supernatant (20 ml) was carefully removed and stored on ice.

Step 6: Hydroxyapatite Chromatography

The protein solution (20 ml) from step 5 was diluted with two volumes of filtered 12.5 mM NaP buffer pH 7.2. The final pH of the diluted protein solution was about 7.2. The diluted protein solution was loaded at a rate of 0.5 ml/min onto a 12.5 x 2.5 cm hydroxyapatite column (Bio-Rad). The column had earlier been equilibrated for 30 min with 10 mM NaP buffer (pH 6.5) at a rate of 2.5 ml/min. The protein was eluted by running an 800 ml linear gradient from 10 mM to 300 mM NaP buffer, pH 6.5, at a flow-rate of 1 ml/min. 8 ml fractions were collected from the column. The most active epimerase fractions were located by assaying. A 20% SDS-PAGE gel of these fractions was run to determine the degree of purity of the epimerase in the most active fractions. Fractions were selected, pooled together (216 ml) and concentrated to 15 ml with an Amicon 400 ml ultrafiltration pressure cell fitted with a YM10 membrane and operating at 25 psi and 4°C. The protein solution was further concentrated to 5 ml using an Amicon Centriprep 10 unit at 4°C. Epimerase assays of the filtrate showed that the loss of epimerase during ultrafiltration had been negligible.

Step 7: Gel filtration chromatography on Sephadex G-75

The 5 ml of concentrated protein from step 6 was loaded onto a Sephadex G-75 (Pharmacia) gel filtration column (93 x 2.5 cm, Bio-Rad) equilibrated with 50 mM Tris/HCl buffer, pH 7.5 (containing 0.1 mM EDTA and 0.1 mM PMSF). 7.5 ml fractions were collected and analysed for epimerase by running fraction samples on a 20% SDS-PAGE gel. The least contaminated epimerase fractions were pooled together and concentrated to 3.3 ml using a Centriprep 10 unit by centrifugation at a speed of 3000 rpm at 4°C in an SS-34 rotor. No loss of epimerase activity through the membrane during ultrafiltration could be detected. The final concentrate of 3.3 ml purified recombinant epimerase protein was aliquoted into 0.4 ml amounts in screw-capped 1.5 ml Eppendorf

tubes, then snap-frozen in liquid N_2 before storing at -70°C.

2.2.2.3 DETERMINATION OF PURIFIED *P. SHERMANII* 'WILD-TYPE' AND RECOMBINANT METHYLMALONYL-CoA EPIMERASE PROTEIN CONCENTRATIONS

Three different assay methods were used to determine the purified *P. shermanii* 'wild-type' and recombinant epimerase protein concentrations. These were the Biuret, bicinchoninic acid, and UV methods.

(A) THE BIURET METHOD

A standard curve was prepared by adding 0.75 ml Biuret reagent (Scopes, 1987) to 0.15 ml of 0, 2, 4, 6 and 8 mg/ml BSA in 1.5 ml Eppendorf tubes. The same amount of Biuret reagent was also added to a 3x diluted 'wild-type' epimerase preparation (50 µl epimerase + 100 µl H_2O) and the 3.75x diluted recombinant epimerase preparation (40 µl epimerase + 110 µl H_2O).

The Eppendorf tubes were allowed to stand at room temperature for 30 min before the absorbance at 540 nm was measured using a "CARY 1" UV-visible spectrophotometer (Varian). Concentrations of the 'wild-type' and recombinant epimerases were determined from the standard curve.

(B) BICINCHONINIC ACID PROTEIN ASSAY METHOD

The suppliers instructions for the use of the bicinchoninic acid protein assay kit (Sigma kit No. BCA-1, cat. no. B9643) were followed exactly. A standard curve was prepared. 50 µl samples containing 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml BSA were pipetted into 1.5 ml Eppendorf tubes. To each 50 µl sample 1 ml of bicinchoninic acid reagent was added, mixed by vortexing and incubated at 37°C for 30 min. 1 ml of bicinchoninic acid reagent was also added to 50 µl samples of 20-fold diluted preparations of the 'wild-type' and the recombinant epimerase protein. The epimerase samples were also incubated at 37°C for 30 min. The absorbance due to each 50 µl sample at 562 nm was measured using a "CARY 1" UV-visible spectrophotometer. A standard curve was plotted and used to calculate epimerase protein concentrations.

(C) UV $A_{205/280}$ METHOD (Scopes, 1987)

Each measurement was carried out by adding 0.79 ml of dilution buffer (5 mM sodium

phosphate buffer, pH 7.0, containing 50 mM sodium sulphate) to a 1 ml quartz cuvette, zeroing the "CARY 1" UV-visible spectrophotometer and then adding 10 µl of appropriately diluted epimerase to the cuvette. The contents of the cuvette were mixed by inversion. The absorbance at 205 nm and 280 nm were recorded for appropriately diluted samples, and the absorbance of appropriate blank solutions were also determined. The A_{205} and the A_{280} absorbance values for each epimerase were used to calculate the protein concentration of each epimerase.

2.2.3 PURIFICATION OF COUPLING ENZYMES FOR THE METHYLMALONYL-CoA EPIMERASE ASSAY

The goal of this section of work was to provide the partially purified methylmalonyl-CoA mutase and oxaloacetate transcarboxylase enzymes required for: (i) generating the epimerase substrate, (*R*)-methylmalonyl-CoA, from succinyl-CoA (mutase), and (ii) coupling the epimerase reaction to the NADH-dependent malate dehydrogenase (oxaloacetate transcarboxylase).

The *E. coli* expression strain SRP84/pGP1-2/pMEX2 produces recombinant *P. shermanii* mutase (McKie *et al.*, 1990). This strain was cultivated and mutase expression induced by heat-shock. The mutase was purified by treatment with PEI followed by anion exchange column chromatography. The purification of the transcarboxylase enzyme was carried out using the method of Wood *et al.* (1969, 1977).

All the buffer stocks used in the column chromatography were filtered and made up in Milli-Q water that was also filtered (and partially degassed) through Millipore HA 0.45 µm filters. Buffers were stored in amber glass 2.5 L Winchester bottles at 4°C.

2.2.3.1 RECOMBINANT METHYLMALONYL-CoA MUTASE

(A) GROWTH OF THE MUTASE EXPRESSION STRAIN SRP84/pGP1-2/pMEX2

The *E. coli* strain SRP84/pGP1-2/pMEX2 capable of expressing *P. shermanii* mutase was streaked out onto 2TY plates containing ampicillin (100 µg/ml) and kanamycin (70 µg/ml) from a 20% glycerol deep stored at -70°C. The plates were grown overnight by incubation at 30°C. Single colonies were picked from the plate and cultured separately in 2 ml M1 medium in culture tubes containing the antibiotics ampicillin (100 µg/ml) and kanamycin (70 µg/ml), and also glucose (10 mM), overnight at 30°C.

A 2 ml culture was used to inoculate 50 ml (in a 250 ml flask) of M1 medium with the same ampicillin, kanamycin and glucose concentrations. The culture was grown at 28-30°C while shaking at 300 rpm until the culture A_{650} value reached 2.0. The 50 ml culture was used to inoculate five 2 L flasks each containing 600 ml M1 medium with the same concentrations of ampicillin, kanamycin and glucose. The cultures were grown at 28-30°C while shaking at 300 rpm until the A_{650} value reached 2.0.

(B) THE INDUCTION OF RECOMBINANT METHYLMALONYL-CoA MUTASE EXPRESSION IN *E. COLI* SRP84/pGP1-2/pMEX2

Once the A_{650} values of the five 600 ml cultures had each reached 2.0, expression of the mutase protein from the pMEX2 plasmid was induced by heat-shock treatment. 400 ml of M1 media (without antibiotics but with glucose), pre-heated to 65°C, was added to each 2 L flask containing 600 ml of culture, and the contents of the flasks immediately swirled around vigorously for 1 min. The increased temperature of the culture was monitored with a thermometer in the flask, and was usually between 42 and 43°C. The flask was then swirled vigorously in iced water for 1 min to decrease the temperature to about 30°C. The culture was allowed to grow for an additional 3-4 h at 28-30°C while shaking at 300 rpm. At harvesting the A_{650} value was approximately 3.8, and the pH of the medium was 6.4. The cells were harvested by centrifugation in a GSA rotor at 10000 rpm for 5 min at 4°C. The cell pellets were frozen at -70°C. The successful expression of the recombinant methylmalonyl-CoA mutase was confirmed by running whole cell protein samples on a 15% SDS-PAGE gel.

(C) THE PARTIAL PURIFICATION OF METHYLMALONYL-CoA MUTASE

The purification of the recombinant mutase involved making an extract from the cells, polyethylenimine treatment and then Q-sepharose fast-flow column chromatography.

Step 1: Initial extraction of cells

Bacterial cell paste (25 g) was suspended in 140 ml of lysis buffer (0.1 M Tris/HCl, pH 7.5, 2 mM EDTA, 4 mM DTT). PMSF was added to a final concentration of 0.2 mM during the sonication process from a 0.1 M PMSF solution in acetone. The cells were ruptured by sonication at an amplitude of 22 microns for 10 min (i.e. 5 x 2 min bursts) using a 1.5 cm diameter probe with rapid cooling of the cells to 5°C between sonication bursts using liquid N_2. The temperature during sonication did not exceed 18°C. Sufficient cell lysis was judged to have occurred once the solution became grey in colour and semi-transparent when held up to bright light, and also when very few intact cells were visible under a light microscope. The cell lysate was divided into five 31.5 ml portions and

centrifuged at 26000 rpm (at an acceleration rate of 4) for 1 h 50 min at 4°C using a Sorvall AH-627 rotor in an OTD-combi Sorvall ultracentrifuge. The supernatant (primary lysate) was carefully decanted.

Step 2: Polyethylenimine (PEI) treatment

The primary lysate (~142 ml) from step 1 was gently stirred on ice at 4°C using a magnetic stirrer. From a 10% PEI stock (neutralized to pH 7.5 with HCl), 1.42 ml of 10% PEI was slowly added drop-wise and the lysate gently stirred on ice for 20 min. Precipitation of nucleic acid was obvious during this process. The PEI treated solution was centrifuged as in step 1 for 35 min, and the supernatant was decanted.

Step 3: Q-Sepharose Fast-Flow Chromatography

The supernatant (140 ml) from step 2 was diluted with two volumes of 10 mM Tris/HCl, pH 7.5, containing 1 mM DTT. The pH of the sample before loading was 7.7. The column had previously been equilibrated with 2 L of buffer A (50 mM Tris/HCl, pH 7.5, 1 mM DTT, 0.1 mM EDTA) and the column eluent was monitored at 280 nm. The diluted protein solution was loaded onto a 7.8 cm x 5 cm Q-sepharose fast-flow column (Pharmacia) at a rate of 4 ml/min. A linear gradient programme was run from 0-100% buffer B (which contained 1 M NaCl dissolved in Buffer A) and 7.5 ml fractions were collected. The most active fractions, determined by assaying each fraction (McKie *et al.*, 1990), were eluted in approximately 0.3 M NaCl and were analysed for mutase protein abundance and purity on a 15% SDS-PAGE gel. Fractions containing mutase of sufficient purity for use in enzyme-coupled assays of epimerase were pooled (82.5 ml) and concentrated down to 3 ml using an Amicon Centriprep 30 unit. The final 3 ml of concentrated mutase protein was made 50% (w/v) in glycerol, and this solution was aliquoted out into 0.5 ml amounts in 1.5 ml Eppendorf tubes and stored at -70°C.

2.2.3.2 PURIFICATION OF TRANSCARBOXYLASE FROM *P. SHERMANII*

The strategy for the purification of the transcarboxylase enzyme involved the dialysis of the 35-60% $(NH_4)_2SO_4$ pellet from step 2 of the 'wild-type' methylmalonyl-CoA epimerase purification scheme (section 2.2.2.1 (B)), followed by cellulose phosphate and gel filtration chromatography with a final $(NH_4)_2SO_4$ precipitation.

Step 1: Dialysis of the 35-60 % $(NH_4)_2SO_4$ pellet

The 35-60% saturated $(NH_4)_2SO_4$ pellet (14 g) was dissolved in 25 ml of a 0.2 M KP buffer, pH 6.8, containing 0.1 mM EDTA. This protein solution was dialysed with stirring against 3x 1 L changes of the same 0.2 M KP buffer, pH 6.8, containing 0.1 mM EDTA over a period of 6 h at 4°C. The dialysate was centrifuged in an SS-34 rotor at 16000 rpm for 20 min at 4°C and the supernatant (46 ml) stored on ice.

Step 2: Cellulose Phosphate Chromatography

A 9.6 cm x 2.5 cm phospho-cellulose column was prepared by hydrating 80 g of dry Whatman P11 phospho-cellulose in water for 3 h at room temperature and allowing it to settle and drain to a moist cake in a sintered glass funnel. The phospho-cellulose was then washed on the funnel with 1 L of 0.1 M NaOH and suspended in 300 ml of 0.1 M NaOH for 30 min. It was then washed with copious amounts of water until the pH of the washings was less than pH 10. The phospho-cellulose was then washed on the funnel with 1 L of 0.1 M HCl and suspended in 300 ml 0.1 M HCl for 30 min at room temperature, before washing with water until the pH of the washings was >5.0. Finally the phospho-cellulose was washed with buffer A (50 mM KP buffer, pH 6.8, containing 0.1 mM EDTA). The column was packed by draining sufficient slurry under gravity into the column at 4°C for a 10 cm high bed, then starting the flow of buffer A at 2 ml/min to equilibrate the column, eventually giving a final column bed height of 9.6 cm.

The supernatant from the dialysed $(NH_4)_2SO_4$ pellet (46 ml) from step 1 was divided into two equal volumes. The first 23 ml was diluted to 92 ml with ice-cold water and was loaded onto the column at 1.8 ml/min. The second 23 ml of the dialysate was then diluted and loaded onto the column in the same way. The transcarboxylase was washed through with 1.5 L of buffer A. The column was eluted with a step-wise fashion with a 0.15 M KP buffer initially, and then a 0.3 M KP buffer, both pH 6.8 containing 0.1 mM EDTA. 7.5 ml fractions were collected and analysed by assaying for transcarboxylase activity (as described in section 2.2.5.2) in each fraction. The active fractions were pooled together and concentrated down to 3.5 ml using an Amicon Centriprep 30 unit.

Step 3: Gel Filtration Chromatography on Bio-gel A-1.5m

A 90 cm x 2.5 cm gel filtration column (Bio-gel A-1.5m; 100-200 mesh, Bio-Rad) was prepared by mixing 460 ml of the pre-swollen gel material with 200 ml of filtered 0.5 M KP buffer, pH 6.8, to give a gel slurry for packing the column. The gel slurry was degassed at 4°C. The slurry was poured into the column and was allowed to pack with 0.15 M KP running buffer containing 0.1 mM EDTA, pH 6.8 (buffer A), at 1.2 ml/min.

The column was equilibrated with 1.5 L of buffer A for 17 h at a flow rate of 1.5 ml/min. The concentrated transcarboxylase solution from step 2 was applied to the gel filtration column at 0.72 ml/min. The transcarboxylase was eluted with buffer A. 7.5 ml fractions were collected, assayed for the transcarboxylase activity and the active fractions pooled (37.5 ml).

The transcarboxylase was precipitated by adding $(NH_4)_2SO_4$ to 65% saturation by slow gradual addition of 16.2 g of $(NH_4)_2SO_4$ while gently stirring on ice. The transcarboxylase solution was stirred for another 15 min and the precipitate was recovered by centrifugation at 16000 rpm for 20 min at 4°C in an SS-34 rotor. The pellet was dissolved in 0.5 ml of 0.5 M KP buffer, pH 6.8, (containing 0.1 mM EDTA and 0.1 mM PMSF) giving approximately 0.24 mg/ml of protein. A final (low) yield of about 1.2 mg of total purified transcarboxylase protein was obtained. The purified transcarboxylase was made 50% (w/v) in glycerol, and then aliquoted out and stored at -70°C.

2.2.4 *N*-TERMINAL SEQUENCING OF THE *P. SHERMANII* 'WILD-TYPE' AND RECOMBINANT EPIMERASES

(A) SAMPLE PREPARATION

Buffer salts can interfere with *N*-terminal sequencing of proteins. Purified epimerase samples for *N*-terminal sequencing were desalted by drop-dialysing each sample against 1 L of Milli-Q H_2O with stirring at room temperature for 3 h. After drop dialyses, the samples were lyophilised. Samples for *N*-terminal sequencing were reconstituted by dissolving each dried down sample in sterile Milli-Q H_2O to give the required concentration of 8 nmoles of epimerase subunit per µl. 13.3 µl of the 8 nmoles/µl sample was prepared.

(B) *N*-TERMINAL SEQUENCING

Automated *N*-terminal sequencing of the purified 'wild-type' and recombinant epimerase by the Edman degradation method (Hewick *et al.*, 1981), followed by separation of the phenylthiohydantoin derivatives of the amino acids, was carried out using an Applied Bio-systems model 470A protein sequencer. The first 14 amino acids of each protein were determined.

2.2.5 SPECIFIC ACTIVITY DETERMINATION

The aim of this section of work was to determine the specific activity of the purified 'wild-type' and recombinant epimerases.

2.2.5.1 ASSAY PROCEDURES FOR METHYLMALONYL-CoA EPIMERASE

Methylmalonyl-CoA epimerase activity was assayed at 340 nm using a modification (Leadlay, 1981) of the method of Allen *et al.* (1963). The (*R*)-methylmalonyl-CoA substrate for methylmalonyl-CoA epimerase was formed in a 1 cm-path-length 1 ml quartz cuvette by the action of mutase on ~0.1 mM succinyl-CoA. The epimerase activity was monitored continuously at 340 nm for 2 min by coupling the formation of (*S*)-methylmalonyl-CoA to NADH oxidation using oxaloacetate transcarboxylase and malate dehydrogenase enzymes.

The assay mix was made fresh and stored on ice until immediately before the assay. Each epimerase sample to be assayed was diluted in 0.3 M KP buffer, pH 6.8, to give a ΔA_{340}/min not greater than 0.05.

To prepare 10 ml of the assay mix:

0.5 M KP (pH 6.8)	6 ml
40x NADH/Na-pyruvate stock (4 mM NADH/0.4 M Na-pyruvate)	250 µl
Methylmalonyl-CoA mutase in 50% (w/v) glycerol (0.03 U/µl)	70 µl
Malate dehydrogenase in 50% (w/v) glycerol (6 U/µl)	5 µl
Transcarboxylase in 50% (w/v) glycerol (1 U/µl)	1.5 µl
H_2O	to 10 ml

Because CoA (coenzyme A) is expensive, the succinyl-CoA substrate was prepared in 1 ml batches. The method was as follows: weigh 10 mg coenzyme A (lithium salt), 9 mg $NaHCO_3$, and 8 mg succinic anhydride into a 1.5 ml screw-cap Eppendorf tube. Add 0.99 ml of the ice-cold water and vortex immediately for 10 s then return to ice. Vortex for 1 second at 20 s intervals, to keep the succinic anhydride in suspension, for 30 minutes; return the tube to ice when not vortexing. Keeping the solution cold measure the pH (should be 6-6.2) then transfer the supernatant to another Eppendorf tube and add 60 µl 2 M HCl. This lowers the pH to about 3. Aliquot and store at -70°C. The concentration of succinyl-CoA in the preparation can be determined by an enzyme-based method (section 2.2.5.3), and is typically between 9 and 10 mM.

To perform each assay, 0.7 ml of the fresh assay mix, 7 µl of ~10 mM succinyl-CoA and

1 µl of 1 mM adenosylcobalamin was added to the cuvette and mixed well by covering the cuvette opening with parafilm and inverting several times. The cuvette was allowed to equilibrate in the "CARY 1" UV-visible spectrophotometer cuvette holder to the set temperature (25°C). A blank rate was measured and was always less than a ΔA_{340} of -0.004/min. Epimerase samples (0.5-3.5 µl) were added and the cuvette was mixed by inversion several times. The rate of decrease of A_{340} was recorded over a period of 2 min.

2.2.5.2 ASSAY PROCEDURES FOR TRANSCARBOXYLASE

The assay solution for oxaloacetate transcarboxylase consisted of 0.3 M potassium phosphate buffer, pH 6.8, 10 mM Na-pyruvate, 0.1 mM NADH and 2 U/ml malate dehydrogenase. The assay solution was prepared in 5-10 ml volumes immediately before it was needed and stored on ice. Immediately before the assay, 1.05 ml of the assay solution was equilibrated at room temperature. 1 ml of the equilibrated assay mix was then transferred to a 1 ml quartz cuvette. The spectrophotometer was zeroed with a water blank at 340 nm and the absorbance of the assay solution at the start of the assay was typically between 0.65 and 0.70. The enzyme sample was added (maximum volume of 20 µl; a volume or dilution that gave a ΔA_{340} of between -0.025 and -0.05/min was best), and the contents of the cuvette were mixed well by inversion (the cuvette was sealed with parafilm). 30 s after mixing, A_{340} was recorded for 2 min. This established the background or control rate for the transcarboxylase assay. 15 µl of a methylmalonyl-CoA preparation (containing 2 mM (*R*)/(*S*)-methylmalonyl-CoA in 10% tetrahydrofuran, prepared according to Flavin (1963)) was added and mixed well. 30 s later A_{340} was recorded for another 2 min.

Sample calculation of transcarboxylase activity:
In the purification of transcarboxylase from *P. shermanii*, the activity of the unbound transcarboxylase in the wash-through during loading of the phospho-cellulose column was determined. 1 µl of the wash-through was added to 1 ml of assay solution. The background rate was -0.0015 A/min. 15 µl of methylmalonyl-CoA was added, and the new rate was -0.036 A/min over the first 90 s. Correcting for the background rate, the rate due to transcarboxylase activity is -0.0345 A/min. So, [0.0345/6.22] x 1.016 ml = 0.0056 µmoles NADH oxidised/min with 1 µl of wash-through, therefore 5.6 µmoles NADH oxidised/min/ml, or 5.6 U/ml. One unit (1 U) is the amount of enzyme that will oxidise 1 µmole NADH per minute under the stated assay conditions. 6.22 is the millimolar absorption coefficient for NADH used in all activity calculations.

2.2.5.3 DETERMINATION OF SUCCINYL-CoA CONCENTRATION

0.7 ml of assay mix (as described for the epimerase assay in section 2.2.5.1) was added to a 1 ml quartz cuvette and the A_{340} measured. 1 µl of a 1 mM solution of

adenosylcobalamin and 0.5 µl of purified 'wild-type' epimerase (10.55 mg/ml) was added and mixed well by inverting the cuvette several times with the open end sealed with parafilm. The A_{340} was recorded for 1.5 minutes to obtain a control rate for NADH oxidation. 3 µl of the succinyl-CoA preparation was added to the cuvette, mixed well, and the final absorbance was recorded when the rate of NADH oxidation had returned to the control rate, i.e. when all the succinyl-CoA substrate was used up. To calculate the concentration of succinyl-CoA in the 3 µl added, the ΔA_{340} was calculated by subtracting the final from the initial absorbance value and the answer was divided by $\varepsilon = 6200$ (molar absorption coefficient) to give the concentration in moles/L, multiplied by 0.0007 L (0.7 ml volume of assay mix) to give the moles of succinyl-CoA used, and divided by $3x10^{-6}$ L (3 µl volume of succinyl-CoA added in this assay) to give the molar concentration of the succinyl-CoA per litre of substrate stock. This stock concentration was then corrected by multiplying by 1000 to give the millimolar amount of succinyl-CoA per litre of stock. In most 1 ml preparations the succinyl-CoA concentration was 10 mM, making the final concentration in epimerase and mutase assays approximately 0.1 mM.

2.2.5.4 THE EFFECT OF METAL IONS ON EPIMERASE SPECIFIC ACTIVITY

To determine the effect of metal ions on the activity of the two epimerases, samples of the 'wild-type' and the recombinant epimerase were incubated with various metal ions and then assayed to see how the activity of each enzyme was affected.

Epimerase samples were prepared for incubation by diluting the purified 10.55 mg/ml 'wild-type' epimerase (2000x) and the purified 20.15 mg/ml recombinant epimerase (4000x) into 50 mM MOPS/NaOH, 20 mM aspartic acid (pH 7.0) containing 1 mM concentrations of various divalent metal ions. These buffers were prepared by adding each metal ion to the pH-adjusted MOPS/NaOH/aspartic acid buffer, pH 7, from a 1 M solution of each metal ion.

'Wild-type' epimerase samples were incubated with 1 mM Co^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+} or Cd^{2+} at 60°C for 5 h. An extra Co^{2+} incubation was carried out for 10 h at 60°C. A sample with no metal ion added and a sample with 1 mM EDTA added were also incubated at 60°C for 5 h, and a control of diluted enzyme with no metal ion was left on ice during the incubations.

Samples of the recombinant epimerase were incubated with 1 mM Ni^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+}, VO^{2+}, Mn^{2+}, Cd^{2+}, Mg^{2+}, Fe^{2+} or Cu^{2+} at 60°C for 5 h. An extra Co^{2+} incubation

was carried out for 10 h at 60°C. A sample with no metal ion added and a sample with 1 mM EDTA added were also incubated at 60°C for 5 h, and a control of diluted enzyme with no metal ion was left on ice during the incubations. At the completion of the 60°C incubations, all samples were placed on ice and assayed for epimerase activity as described in section 2.2.5.1.

2.2.6 SUBUNIT MOLECULAR WEIGHT DETERMINATION BY THE LAEMMLI AND THE WEBER AND OSBORN SDS-PAGE BUFFER SYSTEMS

As both the 'wild-type' and the recombinant methylmalonyl-CoA epimerase exhibited lower than expected electrophoretic mobilities for a protein of their predicted size in SDS-PAGE gels using a modified Laemmli (1970) buffer system, it was decided to use an alternative SDS-PAGE buffer system to determine the molecular weight of the epimerase subunits. Firstly, samples were run alongside the appropriate protein standards using the modified Laemmli (1970) discontinuous Tris-glycine buffer system. Secondly, epimerase samples were run alongside the appropriate protein standards using the Weber and Osborn continuous phosphate buffer system (Weber and Osborn, 1969). Unless stated otherwise, all the SDS-PAGE gels run in this thesis were based on the modified Laemmli Tris-glycine buffer system detailed in section 2.2.6.3.

2.2.6.1 PREPARATION OF THE PROTEIN MOLECULAR WEIGHT MARKERS

A 9 protein SDS-PAGE molecular weight marker standard was prepared by adding lysozyme and β-lactoglobulin to the seven proteins included in the Dalton VII-L standard supplied by Sigma (cat. no. SDS-7). The 9 protein standards span the molecular weight range between 14.2 and 66.3 kDa (Table 2.1).

The molecular weight marker proteins for the Laemmli and the Weber and Osborn gel systems were prepared by dissolving each lyophilised marker protein or mixture of marker proteins in water as shown in Table 2.2.

THE 9 MOLECULAR WEIGHT MARKER PROTEINS	PROTEIN M_r (kDa)
BSA (bovine serum albumin)	66.3
Ovalbumin (chicken egg albumin)	44
Glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle)	35.7
Carbonic anhydrase (bovine erythrocytes)	28.8
Trypsinogen (PMSF treated, bovine pancreas)	23.6
Trypsin inhibitor (soyabean)	20.1
β-Lactoglobulin (bovine milk)	18.4
Lysozyme (chicken egg white)	14.3
α-Lactalbumin (bovine milk)	14.2

Table 2.1: Molecular weights of the nine marker proteins used in the Laemmli and the Weber and Osborn SDS-PAGE molecular weight determination of the 'wild-type' and recombinant methylmalonyl-CoA epimerases.

LYOPHILISED PROTEIN	AMOUNT OF PROTEIN (mg)	H_2O DISSOLVED IN (ml)
Dalton VII-L mix	3.5	0.75
β-lactoglobulin	25.0	1.5
lysozyme	8.2	0.5

Table 2.2: The molecular weight marker protein standards (Sigma) used for SDS-PAGE experiments with both the Laemmli and the Weber and Osborn buffer systems and the amount of water each protein was dissolved in.

Each of the protein standards dissolved in water (Table 2.2) was divided into two equal parts. The first part was diluted with an equal volume of Laemmli 2x SDS sample buffer (section 2.2.6.3). The second part was diluted with an equal volume of Weber and Osborn 2x SDS sample buffer (section 2.2.6.2). The samples for the Laemmli gels were incubated at 100°C for 3 min before they were ready for use. The samples for the Weber and Osborn gels were incubated at 37°C for 2 h before they were ready for use. Both the Laemmli and the Weber and Osborn samples were stored at -70°C. The 9 protein molecular weight marker was prepared by combining 25 µl of the ready-to-load Dalton VII-L mix with 10 µl of mixture containing 0.55 µg/µl lysozyme and 0.61 µg/µl β-lactoglobulin in 1x sample buffer.

2.2.6.2 RUNNING AND PROCESSING WEBER AND OSBORN CONTINUOUS PHOSPHATE SDS-PAGE GELS

The "Instruction manual for mighty small II slab gel electrophoresis unit SE 250" (Hoeffer) was followed to prepare and run all SDS-PAGE mini-gels. The 10% Weber and Osborn gel acrylamide mix was prepared by dissolving and diluting 22.2 g of acrylamide and 0.6 g of bis-acrylamide to 100 ml with H_2O. 0.25 g of analytical grade ion-exchange resin (AG 501-X8(D), 20-50 mesh, Bio-Rad) was added to the gel mix and stirred gently for 20 min. The beads were allowed to settle. The gel mix was filtered and stored in an amber-glass bottle at 4°C.

The Weber and Osborn 2x SDS sample buffer was prepared by dissolving the following ingredients in, and making up to 10 ml with, H_2O:

$NaH_2PO_4 \cdot H_2O$	88.4 mg
$Na_2HPO_4 \cdot 2H_2O$	255.7 mg
SDS	200 mg
DTT	220 mg
Bromophenol blue	3 mg
Urea	5.4 g

The 2x SDS sample buffer was stored at -70°C. With 5.4 g of urea in 10 ml, the Weber and Osborn buffer solution was only 1.5x with respect to urea, but nevertheless this buffer was effective when used as a 2x SDS sample buffer. All other solutions and protocols pertaining to the Weber and Osborn gels are described in Sigma technical bulletin MWS-877.

A small Weber and Osborn gel (8.2 cm wide, 6.5 cm high and 0.075 cm thick) was run to determine the optimum protein loadings for a larger gel run to determine the molecular weight of the epimerase subunit.. All the protein samples except for the molecular weight

marker proteins (already prepared in section 2.2.6.1) were incubated at 37°C for 2 h in 1x sample buffer before electrophoresis. 2.5 µl of Dalton VII-L marker, 1 µl of purified 'wild-type' epimerase (0.5 µg), 2.5 µl of a mixture containing 0.55 µg/µl lysozyme and 0.61 µg/µl β-lactoglobulin and 1 µl of recombinant epimerase (0.5 µg) were loaded onto the gel. The gel was run at a current of 18 mA (32 V, with the voltage dropping during the run) for about 5 h. The 2x gel running buffer (pH 7 at 25°C) shown below was used in the preparation of the gel mix, and also served as the electrode reservoir buffer when diluted with an equal volume of water:

NaH_2PO_4	6.8 g
Na_2HPO_4	20.45 g
SDS	2 g
H_2O	to 1 L

The buffer was stored in an amber-glass Winchester bottle at 4°C.

A 16 cm wide x 20 cm high x 0.15 cm thick 10% Weber and Osborn continuous gel was poured in a PROTEAN II xi (Bio-Rad) slab gel mould, according to the manufacturers instructions. The samples were incubated the same way as for the smaller gel. The 15 samples were loaded in the following order: 7 µl of Dalton VII-L, 2.5 µl of 'wild-type' epimerase (1.25 µg), 7 µl of a 0.55 µg/µl lysozyme and 0.61 µg/µl β-lactoglobulin mixture, 2.5 µl of recombinant epimerase (1.25 µg), 7 µl of Dalton VII-L, 5 µl of 'wild-type' epimerase (2.5 µg), 5 µl of recombinant epimerase (2.5 µg), 5 µl of 1x Weber and Osborn sample buffer, 4.5 µl of Dalton VII-L, 1.7 µl 'wild-type' epimerase (0.85 µg), 4.5 µl of a 0.55 µg/µl lysozyme and 0.61 µg/µl β-lactoglobulin mixture, 1.7 µl recombinant epimerase (0.85 µg), 4.5 µl of Dalton VII-L, 5 µl of 'wild-type' epimerase (2.5 µg) and 5 µl of recombinant epimerase (2.5 µg). The gel was run at 10 mA (16 V) for the first 3 h before slowly increasing the current to 30 mA (44 V) and eventually increasing it further to 60 mA (78 V). The current tended to drop over time. The gel was run for about a total of 5 h. When the gel was stopped the position of the dye front was permanently marked by stabbing the dye front with a glass capillary tube to remove a small disc of gel.

The gels were fixed and stained in a methanol:acetic acid:water mix, in the volume ratio of 5:1:5, containing 2.27 g/L Coomassie Brilliant Blue R-250 dye, for 1 h while gently shaking at 30 rpm. The gels were destained with shaking in 2-3 changes of the methanol:acetic acid:water 5:1:5 mix for 1-3 h, and were then transferred to Welcome destain (110 ml methanol, 130 ml acetic acid made up to 2.5 L with water) for final destaining and storage.

2.2.6.3 CONSTITUTING, RUNNING AND PROCESSING THE MODIFIED LAEMMLI DISCONTINUOUS SDS-PAGE GELS

The modified Laemmli gels were constituted as follows:

Solutions: Resolving gel acrylamide solution: 32 g acrylamide
0.2 g bis-acrylamide
to 100 ml with Milli-Q water.

Stacking gel acrylamide solution: 4 g acrylamide
0.105 g bis-acrylamide
to 50 ml with Milli-Q water.

Add 0.25% (w/v) of analytical grade mixed ion-exchange resin (e.g. AG 501-X8(D), 20-50 mesh, Bio-Rad) and store solutions in brown bottles at 4°C. This resin will settle to the bottom of the bottle, so take liquid from the top of the solution.

Resolving gel buffer solution (4x): 1.5 M Tris/HCl pH 8.7 (20°C), 0.4% SDS

Stacking gel buffer solution (8x): 1 M Tris/HCl pH 6.7 (20°C), 0.8% SDS

Electrode tank/reservoir buffer: 72 g glycine, 15 g Tris, 2.5 g SDS, to 2.5 L

For example, to prepare 10 ml of a 20% resolving gel mix, mix (20% / 32%) × 10 ml = 6.25 ml of resolving gel acrylamide solution and 2.5 ml of the resolving gel buffer solution and make up to 10 ml with Milli-Q water. To prepare 10 ml of stacking gel, use 5 ml of stacking gel acrylamide solution, 1.25 ml of the stacking gel buffer solution and make up to 10 ml with Milli-Q water.

Allow the complete gel mix to come to room temperature before polymerizing. To polymerize the resolving gel mix use 7 µl TEMED and 40 µl 10% (w/w) ammonium persulphate solution per 10 ml of gel mix. Pour the resolving gel mix, overlay with water-saturated butanol, and allow polymerization to continue for 60 min. To polymerize the stacking gel mix use 12 µl TEMED and 60 µl 10% (w/w) ammonium persulphate solution per 10 ml of gel mix.

2x SDS sample buffer: 15% (v/v) glycerol, 2% (w/v) DTT, 0.005% bromophenol blue, 6% (w/v) SDS, 0.125 M Tris/HCl pH 6.7. Stored in aliquots at -20°C.

The 'wild-type' epimerase sample for loading was prepared by combining 33 µl of the 2x Laemmli SDS sample buffer, 30 µl of H_2O and 3 µl of 'wild-type' epimerase (10.55 mg/ml). The recombinant epimerase sample for loading was prepared by combining 40 µl of the 2x Laemmli SDS sample buffer, 38 µl of H_2O and 2 µl of recombinant epimerase (20.15 mg/ml). The samples were incubated at 100°C for 3 min and cooled. They were then ready to load.

Samples were loaded onto a preliminary 20% Laemmli discontinuous gel (8.2 cm wide, 6.5 cm long and 0.075 cm thick) and run at 8 mA. The result was used to determine appropriate amounts to load on a larger gel run using the PROTEAN II xi gel apparatus.

A large 20% Laemmli SDS-PAGE discontinuous 16 x 20 x 0.15 cm gel was loaded with samples of the marker proteins and also the 'wild-type' and recombinant epimerase proteins from left to right in the order shown in Table 2.3

WELL NUMBER	SAMPLE LOADED	AMOUNT LOADED
1	9 band molecular weight marker	9 µl
2	'Wild-type' epimerase (1 µg)	2 µl
3	Recombinant epimerase (1 µg)	2 µl
4	9 band molecular weight marker	9 µl
5	'Wild-type' epimerase (6 µg)	12 µl
6	Recombinant epimerase (6 µg)	12 µl
7	9 band molecular weight marker	15 µl
8	'Wild-type' epimerase (1.75 µg)	3.5 µl
9	Recombinant epimerase (1.75 µg)	3.5 µl
10	9 band molecular weight marker	15 µl
11	2x sample buffer	10 µl
12	'Wild-type' epimerase (6 µg)	12 µl
13	Recombinant epimerase (6 µg)	12 µl
14	9 band molecular weight standard	1.5 µl
15	2x sample buffer	5 µl

Table 2.3: Samples loaded on to the large discontinuous modified Laemmli SDS-PAGE gel.

Each sample was loaded more than once to give duplicates. The gel was run at 20 mA (78 volts) for about 4 h. Once the gel was stopped, the gel dye front was marked and the gel was removed and cut into 2 halves between lane 9 and 10 using a razor blade. One half of the gel (lanes 1 to 9) was stained with Coomassie Brilliant Blue R-250, destained in 5:1:5 destain followed by Welcome destain. During the staining and destaining process the gel was shaken at 30 rpm. The other half (lanes 10-15) was silver-stained using a Bio-Rad Silver stain Plus kit (cat. no. 161-0449). The instruction manual protocol was followed.

2.2.7 RECOMBINANT EPIMERASE SOLUBILITY

The solubility of the recombinant epimerase protein expressed in *E. coli* was investigated. 0.5 g of induced SRP84/pGP1-2/pTEEX cell paste was resuspended in 5 ml of lysis buffer (0.1 M KP buffer, pH 6.8, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF). The cells were lysed by ultrasonication applied in twenty 30 s bursts at an amplitude of 20 microns using a 0.5 cm diameter probe with cooling on dry ice between each sonication burst thereby cooling the lysate to about 5°C as soon as possible after sonication bursts. The cell lysate was centrifuged at 35,000 rpm (100000 x *g* at r_{av}) and 4°C for 1 h in a SW-39L rotor in a Beckman L2-65B ultracentrifuge. Samples from the cell lysate (before centrifugation), the supernatant and washed resuspended pellet were analysed by 20% SDS-PAGE to test the solubility of the epimerase protein. The pellet was washed with 1 ml of ice-cold Milli-Q water and resuspended in 5 ml of 0.1 M KP buffer, pH 6.8.

2.2.8 CRYSTALLIZATION OF METHYLMALONYL-CoA EPIMERASE

The aim of this section of work was to screen for the optimum conditions for growing crystals of the purified 'wild-type' and recombinant epimerase proteins. The conditions that gave the most promising crystals were further studied by varying the components in the reservoir buffer in an attempt to improve the crystals. The best crystals were placed in an X-ray beam in an attempt to obtain some preliminary diffraction data.

2.2.8.1 SCREENING FOR EPIMERASE PROTEIN CRYSTALS

(i) SCREEN 1:

The first screening attempt was done by using the conditions from the fast-screening method for protein crystallization developed by Jancarik and Kim (1991). These conditions are shown in Table 2.4. Only 'wild-type' epimerase was used in this screen.

All the precipitant solutions were filtered using Millipore 0.45 μm filters. The hanging-drop technique (Wlodawer and Hodgson, 1975) for crystallization by vapour diffusion was selected and 24 welled Linbro tissue culture trays were used to set-up the crystallization trials. A 0.6 ml reservoir solution of buffered precipitant was transferred into each well. 1 μl of 10.55 mg/ml 'wild-type' epimerase and 1 μl of reservoir buffer solution were combined on each cover-slip and gently mixed by pipetting up and down to constitute the hanging drop. The cover-slips were placed drop-side down over each well.

No.	SALT	BUFFER	PRECIPITANT
1	0.2 M Calcium chloride	0.1 M Acetate	30% MPD
2	----	----	0.4 M Na/K Tartrate
3	----	----	0.4 M Ammonium phosphate
4	----	0.1 M Tris	2.0 M Ammonium sulfate
5	0.2 M Sodium citrate	0.1 M Hepes	40% MPD
6	0.2 M Magnesium chloride	0.1 M Tris	30% PEG 4000
7	----	0.1 M Cacodylate	1.4 M Sodium acetate
8	0.2 M Sodium citrate	0.1 M Cacodylate	30% Isopropanol
9	0.2 M Ammonium acetate	0.1 M Citrate (pH 5.6)	30% PEG 4000
10	0.2 M Ammonium acetate	0.1 M Acetate	30% PEG 4000
11	----	0.1 M Citrate	1.0 M Ammonium phosphate
12	0.2 M Lithium sulfate	0.1 M Hepes	30% Isopropanol
13	0.2 M Sodium citrate	0.1 M Tris	30% PEG 400
14	0.2 M Calcium chloride	0.1 M Hepes	28% PEG 400
15	0.2 M Ammonium sulfate	0.1 M Cacodylate	30% PEG 8000
16	----	0.1 M Hepes	1.5 M Lithium sulfate
17	0.2 M Lithium sulfate	0.1 M Tris	30% PEG 4000
18	0.2 M Magnesium acetate	0.1 M Cacodylate	20% PEG 8000
19	0.2 M Ammonium acetate	0.1 M Tris	30% Isopropanol
20	0.2 M Ammonium sulfate	0.1 M Acetate (pH 4.6)	25% PEG 4000
21	0.2 M Magnesium acetate	0.1 M Cacodylate	30% MPD
22	0.2 M Sodium acetate	0.1 M Tris	30% PEG 4000
23	0.2 M Mg chloride	0.1 M Hepes	30% PEG 400
24	0.2 M Calcium chloride	0.1 M Acetate	20% Isopropanol
25	----	0.1 M Sodium imidazole	1.0 M Sodium acetate
26	0.2 M Ammonium acetate	0.1 M Citrate	30% MPD
27	0.2 M Sodium citrate	0.1 M Hepes	20% Isopropanol
28	0.2 M Sodium acetate	0.1 M Cacodylate	30% PEG 8000
29	----	0.1 M Hepes	0.8 M Na/K tartrate
30	0.2 M Ammonium sulfate	----	30% PEG 8000
31	0.2 M Ammonium sulfate	----	30% PEG 4000
32	----	----	2.0 M Ammonium sulfate
33	----	----	4.0 M Sodium formate
34	----	0.1 M Acetate	2.0 M Sodium formate
35	----	0.1 M Hepes	2.0 M Na/K Tartrate
36	----	0.1 M Tris	8% PEG 8000
37	----	0.1 M Acetate	8% PEG 4000
38	----	0.1 M Hepes	1.4 M Sodium citrate
39	----	0.1 M Hepes	2% PEG 400, 2.0 M $(NH_4)_2SO_4$
40	----	0.1 M Citrate	20% Isopropanol, 20 % PEG 4000
41	----	0.1 M Hepes	10% Isopropanol, 20 % PEG 4000
42	0.05 M K phosphate	----	20% PEG 8000
43	----	----	30% PEG 1500
44	----	----	0.2 M Magnesium formate
45	0.2 M Zinc acetate	0.1 M Cacodylate	18% PEG 8000
46	0.2 M Calcium acetate	0.1 M Cacodylate	18% PEG 8000

Table 2.4: The fast screening method for protein crystallization developed by Jancarik and Kim (1991). The above buffer conditions were used for the first screen.

Vaseline petroleum jelly was used to seal each well. The trays were stored at 4°C undisturbed for 1 week. After 1 week, daily monitoring of crystal growth was started. Hanging drops were examined at 4°C under a light microscope.

(ii) SCREEN 2:

A second crystallization screen was carried out using conditions that varied slightly around the condition (number 9 in Table 2.4) that gave the best looking crystals in screen 1. These conditions are given in Table 2.5. Both 'wild-type' and recombinant epimerase were tested in this screen.

NUMBER	[ammonium acetate] (moles/L)	[citrate] (moles/L)	PEG 4000 (%)	pH
1	0.2	0.1	30	5.0
2	0.3	0.1	30	5.5
3	0.2	0.1	30	6.0
4	0.1	0.1	30	5.5
5	0.2	0.1	20	5.5
6	0.2	0.1	25	5.5
7	0.1	0.1	20	5.0
8	0.3	0.1	20	5.0
9	0.3	0.1	20	6.0
10	0.1	0.1	20	6.0
11	0.1	0.1	25	6.0
12	0.3	0.1	25	6.0
13	0.3	0.1	25	5.0
14	0.1	0.1	25	5.0

Table 2.5: The buffer conditions used in the second methylmalonyl-CoA epimerase crystallization screen.

(iii) SCREEN 3:

A third screen was done using conditions in which the types and percentages of PEG precipitant were varied. Previously successful conditions were used as a guide in choosing conditions for the third screen shown in Table 2.6. Both the 'wild-type' and recombinant epimerases were used in this screen.

Number	Precipitant type	Concentration (w/v)	pH	Buffer type
1	PEG 6000	7	5.5	0.2 M Succinic acid/KOH
2	PEG 6000	14	5.5	0.2 M Malic acid/KOH
3	PEG 6000	21	5.5	0.2 M Succinic acid/KOH
4	PEG 6000	28	5.5	0.2 M Malic acid/KOH
5	Methoxy-PEG 5000	7	5.5	0.2 M Malic acid/KOH
6	Methoxy-PEG 5000	14	5.5	0.2 M Succinic acid/KOH
7	Methoxy-PEG 5000	21	5.5	0.2 M Malic acid/KOH
8	Methoxy-PEG 5000	28	5.5	0.2 M Succinic acid/KOH

Table 2.6: The conditions of the third methylmalonyl-CoA epimerase crystallization screen.

A variation of screen 1 conditions in which the size of the hanging drop (using condition number 9 from the first screen, Table 2.4) was increased to 2 µl buffer and 2 µl recombinant epimerase (20.15 mg/ml) was attempted.

X-ray diffraction data from the best crystals was collected at room temperature on a Rigaku R-Axis II C image plate detector using a Rigaku RU-200B rotating anode source.

CHAPTER 3: RESULTS

3.1 RESULTS

3.1.1 THE GROWTH AND ISOLATION OF 'WILD-TYPE' *PROPIONIBACTERIUM SHERMANII* TOTAL GENOMIC DNA

The 200 ml of *P. shermanii* (Strain 52W) culture grown in Brown medium was harvested by centrifugation and 4 g of cells (wet weight) was obtained. The genomic DNA which contained the epimerase gene was isolated from 2 g of cells in a low yield of ~200 µg of purified genomic DNA. The template DNA $A_{260/280}$ ratio was 1.9 and the concentration was ~0.4 µg/µl. The purified DNA was stored at -20°C prior to its use as a template in PCR experiments.

3.1.2 AMPLIFICATION OF THE EPIMERASE GENE BY PCR

The aim of this experiment was to amplify the epimerase gene within the *Propionibacterium shermanii* genomic DNA and to ligate this amplified gene product into the *E. coli* expression vector pT7-7.

The forward primer oligonucleotide EPCRF and reverse primer EPCRB (see section 2.2.1.2) had been gel purified (section 2.2.1.3) to give 45 ng/µl and 20 ng/µl stocks respectively. The genomic DNA solution was diluted 5-fold in water to 80 ng/µl and used in PCR experiments.

The optimum conditions for the amplification of the epimerase gene were obtained through trial and error PCR experiments in which the effect of variables such as annealing temperature, $[Mg^{2+}]$, primer and template concentrations, and number of cycles on PCR product yield were investigated. The optimum PCR conditions for the amplification of the epimerase gene were: final concentrations of 4 mM $MgCl_2$, 10 ng/µl of template DNA, the use of SDS-PAGE purified oligonucleotide primers (1.9 ng/µl EPCRF, 2.5 ng/µl EPCRB), 0.2 mM of each dNTP, 1x Promega *Taq* polymerase buffer, 0.05 U/µl *Taq* polymerase, and pre-heating of the PCR machine heating block to 94°C before loading the sample tubes. The thermo-cycling programme that gave best results was 3 minutes at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 52°C and 1 min at 72°C. After one more cycle, in which the extension time at 72°C was increased to 2 min, the block was cooled to 4°C.

Pre-heating of the PCR machine block to 94°C before loading the reaction tubes was found to be a very important step in order to obtain high yields of a single specific PCR product. This may be because if the reaction tubes were heated up too slowly then the primers that have annealed to the template non-specifically at low temperatures will be extended as the block heats up relatively slowly. This could produce lengths of perfectly matched DNA that might act as primers in subsequent rounds of the PCR. Despite the high concentration of template present in the PCR mix, products were usually visible on agarose gels only after at least 25 cycles.

Figure 3.1 shows the PCR product obtained using the optimum conditions described above. The PCR mix was analysed on a 1.2% agarose gel. Lane 1 shows the PCR product running at about 0.45 kbp, slightly below the 506/517 bp molecular size marker in the 1 kb ladder (Gibco BRL) run in lane 2. Figure 3.2 shows the DNA sequence of the *P. shermanii* epimerase gene that was amplified in the PCR experiments.

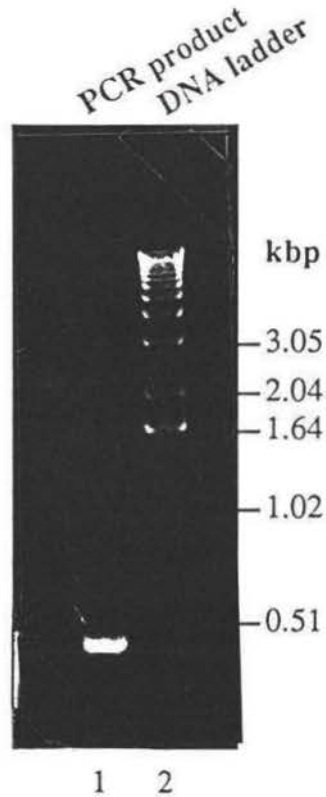

Figure 3.1: Photograph of an ethidium bromide-stained 1.2% agarose gel showing the *P. shermanii* epimerase gene amplified by PCR. Lane 1: PCR product (8 µl) running at the predicted size of 0.45 kbp; lane 2: 1 kb ladder molecular size markers. The marker DNA size corresponding to each band is indicated to the right of the gel.

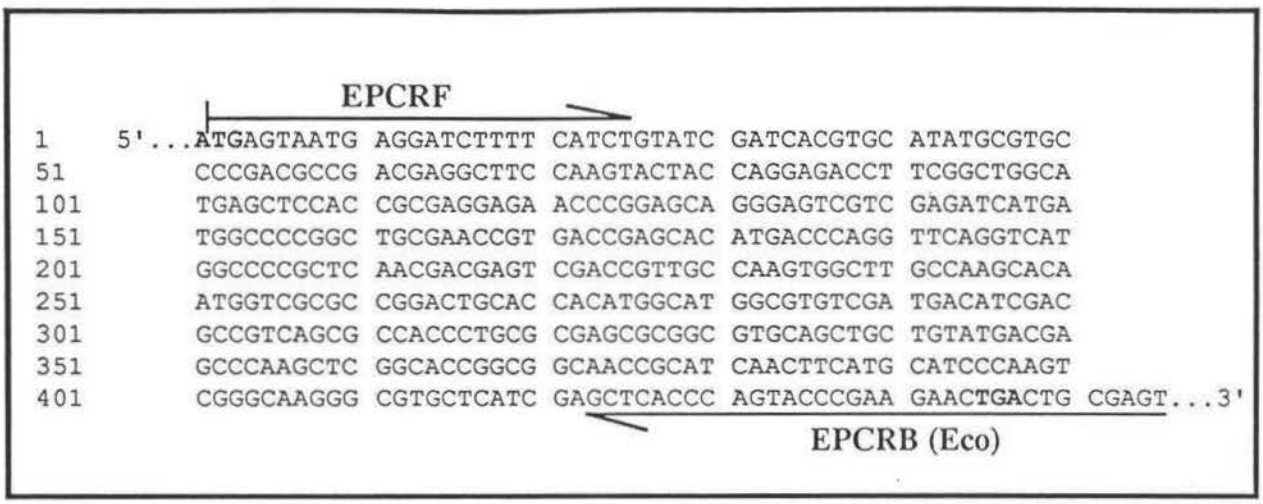

Figure 3.2: The DNA sequence of the *P. shermanii* epimerase gene. The ATG start codon and the TGA stop codon are highlighted in bold letters. The native sequence of 8 nucleotides 3' of the TGA stop codon is also included. The 24-mer EPCRF primer is identical to the sequence shown, and the 33-mer EPCRB(Eco) primer is complementary to the sequence shown with the exception for the 7 nucleotides at the 3' end of the sequence. In this region the EPCRB(Eco) primer is not complementary to the DNA sequence shown because it was used to introduce an *Eco*RI site into the PCR product.

3.1.3 LIGATING THE PCR PRODUCT INTO pT7-7 AND CHARACTERIZATION OF THE RESULTING PLASMIDS

The PCR amplified 454 bp DNA fragment containing the epimerase gene (minus the A nucleotide of the methionine start codon) was ligated into the *E. coli* expression vector pT7-7. The PCR product was prepared for ligation by incubation with T4 DNA polymerase to give a blunt-ended product, followed by digestion with *Eco*RI. The resulting insert was blunt at the 5' end of the gene, and 'sticky' at the 3' end due to the *Eco*RI digestion.

The pT7-7 vector was prepared for ligation by digestion with *Nde*I and end-filling with Klenow to produce a blunt end. The vector DNA was then digested with *Eco*RI to generate a vector with one blunt end and one 'sticky' *Eco*RI-compatible end.

The insert DNA, prepared from the PCR product, was ligated into the prepared pT7-7 vector. The product of the ligation was drop-dialysed against 10% glycerol to remove any low molecular weight compounds that might decrease the efficiency of transformation by electroporation. Dialysed ligation mixes were electroporated into *E. coli* strain XL1-Blue.

Electroporation produced an abundance of ampicillin resistant transformant colonies. Several attempts were made at ligating the 0.45 kbp insert into pT7-7. Eventually the ligation worked, albeit at low efficiency, and about 100 plasmid DNA 'mini-preps' were prepared from ampicillin-resistant transformants and analysed by agarose gel electrophoresis to check for the presence of uncut plasmids that looked larger than uncut pT7-7. About 1 in 15 plasmids appeared larger. Figure 3.3 shows an agarose gel of 40 electrophoresed uncut 'mini-preps'. Plasmids #23 and #30 are clearly larger than the other plasmids, and #4 is a mixed plasmid preparation (two transformant colonies were tooth-picked to the same culture tube by mistake) containing both a larger and a 'normal' sized plasmid. 'Mini-prep' plasmids that appeared larger were digested with restriction endonucleases that should linearise the plasmids to give a 2.91 kbp product. This analysis is shown for the mixed 'mini-prep' plasmid #4 in Figure 3.4. The two uncut plasmids in this mixed 'mini-prep' can be seen in the lane immediately to the left of the 1 kb ladder lane. In the lane immediately to the right of the 1 kb ladder lane, both plasmids are linearised with *Hin*dIII to give 2.5 kbp and 2.95 kbp products, sizes that are consistent with a failed ligation (ligation without insert) and a successful ligation, respectively. Digestions with *Eco*RI and *Pvu*II linearised only the larger plasmid. Clearly the ligation reaction has not regenerated an *Eco*RI site in smaller product plasmid (this is likely to be a vector-only ligation product). The *Pvu*II digest could only linearise a product plasmid which contained an insert, as the unique *Pvu*II site in the DNA in the ligation reaction is in the insert DNA (pT7-7 lacks a *Pvu*II site). The sizes of the products of these digestions are therefore consistent with the smaller plasmid in the #4 mixed plasmid 'mini-prep' being a ligation product of the treated pT7-7 vector only, while the larger plasmid contains a single 0.45 kbp insert. The larger plasmid in 'mini-prep' #4 was re-isolated as a pure plasmid and digested with *Hin*dIII (Figure 3.5). Although the gel is overloaded, the digested plasmid is completely linearised and has a molecular size of ~2.9 kbp.

Any larger plasmids were putative epimerase expression plasmids, i.e. putative pTEEX plasmids. Four of these putative pTEEX plasmids, re-isolated plasmid #4 and plasmids #23, #30 and #45, were transformed into *E. coli* SRP84/pGP1-2. Transformant colonies were cultured and induced by heat shock. The whole cell protein of these induced cells was analysed by SDS-PAGE. Figure 3.6 shows that the four selected plasmids were all able to produce recombinant protein, as evidenced by a single strong band in each of the four lanes that was not present in the control lane of the whole cell protein from induced SRP84/pGP1-2/pT7-7. On induction, SRP84/pGP1-2 transformed with plasmids #4 and #45 produced a recombinant protein with an apparent M_r of 21000, while #23 and #30 transformants produced a recombinant protein with an apparent M_r of 22000. At this stage it was of some concern that all the recombinant proteins had apparent M_r's that were greater than the 16700 M_r predicted for the subunit of *P. shermanii* epimerase. To address

this concern large scale plasmid preparations of all four expressing constructs were done with a view to sequencing the insert DNA in these constructs. Initial problems with DNA sequencing led to focusing on the putative pTEEX construct #4. The insert of this plasmid was fully sequenced and shown to code for the *P. shermanii* epimerase. No errors had been introduced by the PCR amplification of the gene; the DNA sequence was identical to that obtained by Davis (1987) (see Figure 3.2).

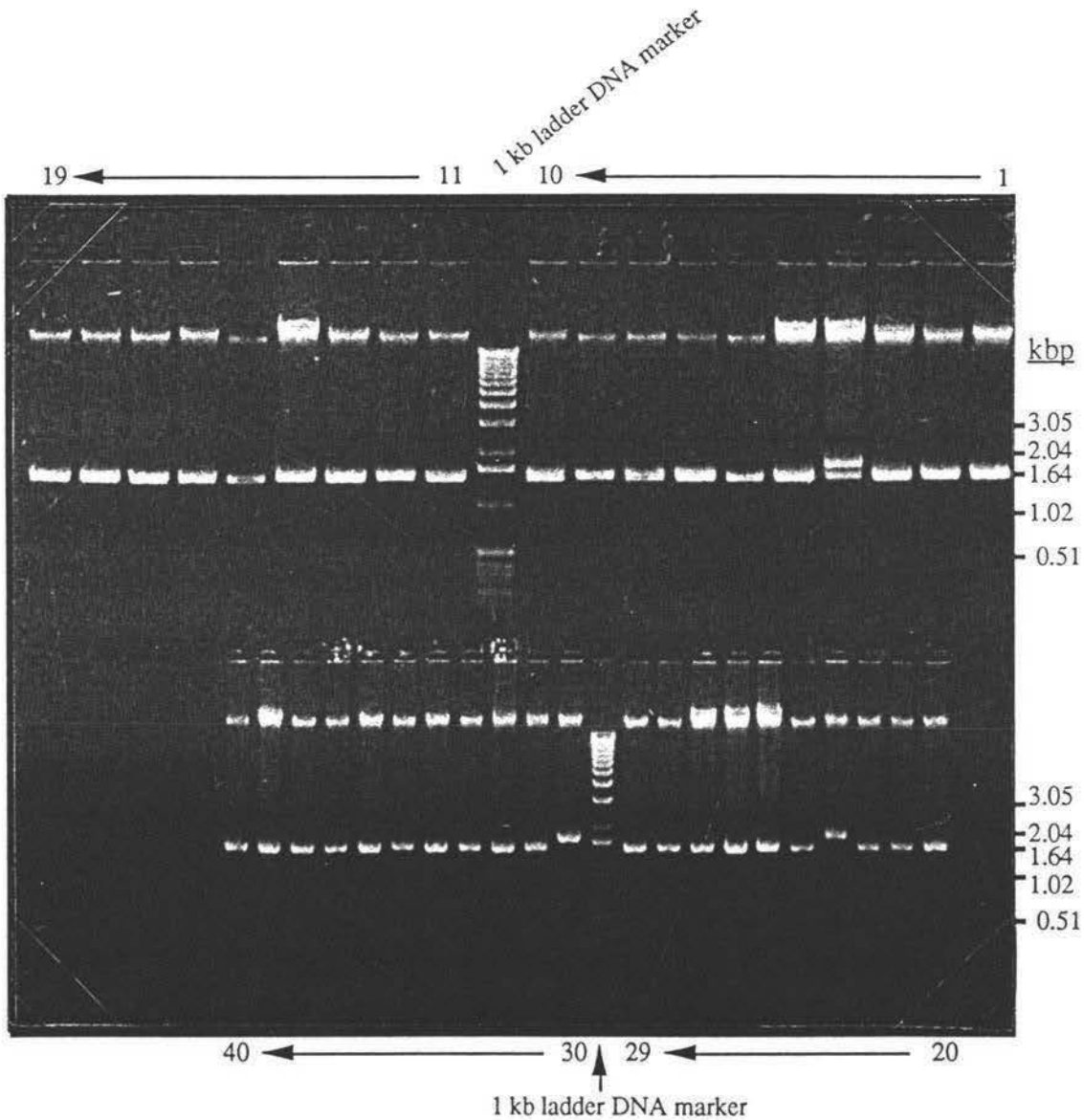

Figure 3.3: Photograph of an ethidium bromide-stained 1% agarose gel showing the screening of uncut plasmid 'mini-preps' (STET-preps) of some transformants (#1-40) from the successful PCR product insert-pT7-7 ligation. The gel shows that 3 of the 40 uncut 'mini-preps' (#4, #23 and #30) contain plasmids that are slightly larger than the others, indicating that they may contain the PCR-generated insert.

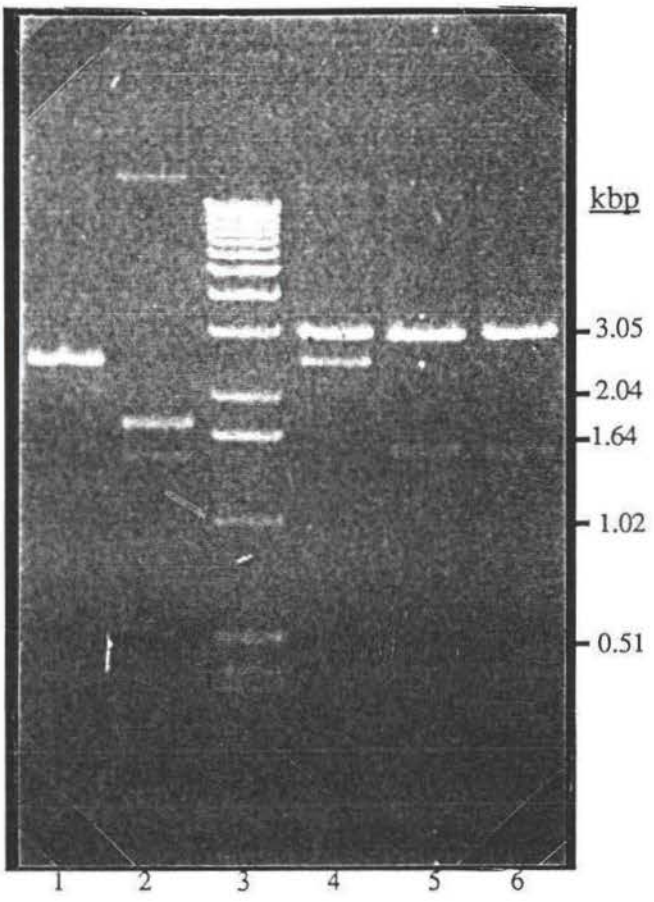

Figure 3.4: Photograph of an ethidium bromide-stained 1% agarose gel showing one of the plasmid 'mini-preps' that contained a larger plasmid, 'mini-prep' #4, digested with various restriction enzymes to test the size of the insert DNA. The pT7-7 vector is 2.46 kbp and the PCR product insert is 0.45 kbp. Lane 1: pT7-7 digested with *Hin*dIII; lane 2: undigested 'mini-prep' #4; lane 3: 1 kb ladder molecular size markers; lane 4: 'mini-prep' #4 digested with *Hin*dIII; lane 5: mini-prep' #4 digested with *Eco*RI; lane 6: 'mini-prep' #4 digested with *Pvu*II. 'Mini-prep' #4 is a mixed plasmid, i.e. it contains two plasmids. See text for an interpretation of the results of this analysis.

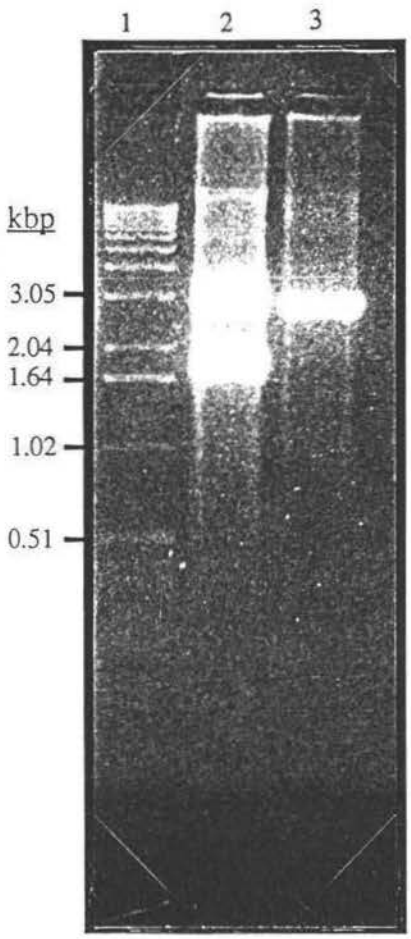

Figure 3.5: Photograph of an ethidium bromide-stained 1% agarose gel showing the re-isolated large plasmid from the mixed plasmid 'mini-prep #4. Lane 1: 1 kb molecular size ladder (Gibco BRL); lane 2: undigested 'mini-prep' #4; lane 3: 'mini-prep' #4 digested with *Hin*dIII.

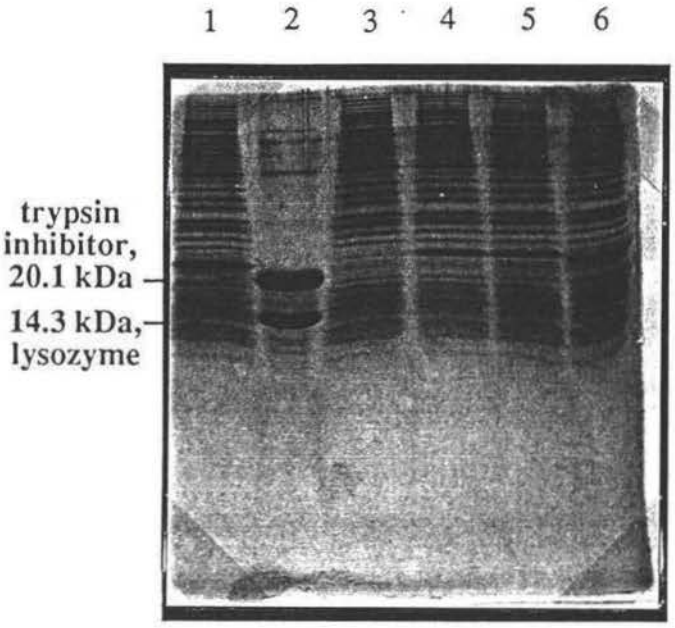

Figure 3.6: Photograph of a Coomassie Brilliant Blue R-250-stained 20% SDS-PAGE gel of whole cell proteins from induced SRP84/pGP1-2 cultures transformed with the four putative pTEEX plasmids, or pT7-7 (control). Lane 1: SRP84/pGP1-2/#4; lane 2: soyabean trypsin inhibitor and chicken lysozyme molecular weight markers; lane 3: SRP84/pGP1-2/pT7-7 (control); lane 4: SRP84/pGP1-2/#23; lane 5: SRP84/pGP1-2/#30; lane 6: SRP84/pGP1-2/#45.

This gel shows that four of the putative epimerase expression constructs (pTEEX), identified as shown in Figures 3.3 and 3.4, support expression of a recombinant protein when they are transformed into SRP84/pGP1-2 and induced by heat-shock. Plasmids #4 and #45 express proteins with an apparent M_r of 21000, while #23 and #30 produce a recombinant protein with an apparent M_r of 22000. These apparent M_r's are larger than the 16.7 kDa predicted for the epimerase subunit.

3.1.4 SEQUENCING OF THE DNA INSERT IN pTEEX

The epimerase PCR product insert, ligated into pT7-7 and transformed into the *E. coli* XL1-Blue, produced six putative epimerase expression constructs (pTEEX). Four of these (#4, #23, #30 and #45) supported the expression of recombinant protein when transformed into the expression strain SRP84/pGP1-2. Large-scale plasmid preparations of these plasmids were done with a view to sequencing the insert of at least one putative pTEEX plasmid.

The insert of the putative expression plasmid pTEEX (#4) was fully sequenced to ensure that no PCR errors had been introduced by PCR amplification of the gene from genomic DNA. It was also important to determine that the correct number of base pairs existed at the start codon ligation site, because SDS-PAGE analysis of whole cell protein from induced SRP84/pGP1-2 cultures bearing the 4 putative pTEEX plasmids (Figure 3.6) showed that the apparent molecular weight of the recombinant proteins was significantly greater than the expected 16.7 kDa. The DNA sequence of the epimerase gene in pTEEX (#4) was shown to be identical to the known sequence of the epimerase gene described by Davis (1987) (Figure 3.2).

An initial attempt at sequencing the pTEEX plasmid inserts was carried out using the "Sequenase version 2.0" kit (United States Biochemical Company). The protocol in the Sequenase Version 2.0 instruction manual (5th Edition) for plasmid templates was essentially followed. However, technical difficulties led to the use of the *f-mol* DNA sequencing system (Promega). The switch to the *f-mol* DNA sequencing system was mainly brought about by the lack of results produced while using the Sequenase Version 2.0 system. The autoradiograph X-ray films failed to show any bands at all at the end of each sequencing experiment. The *f-mol* DNA sequencing system has the advantage of requiring only 1 µg of template DNA compared to 3-5 µg per sequencing experiment required by the Sequenase Version 2.0 system.

However, it was later found that contamination of the TP-1 primer tube was the cause of the problem and probably not the Sequenase Version 2.0 DNA sequencing system, as early experiments with the *f-mol* DNA sequencing kit also failed to produce results immediately until a new TP-1 primer stock was used.

3.1.5 INDUCTION AND SOLUBILITY OF THE RECOMBINANT EPIMERASE INDUCED IN SRP84/pGP1-2/pTEEX

The treated PCR product ligated into the expression vector pT7-7 gave the desired expression plasmid construct. This construct, named pTEEX, was transformed into the *E. coli* expression strain SRP84/pGP1-2 in which the protein product of the epimerase gene was produced by heat-shock induction (Tabor and Richardson, 1985). The induction of *E. coli* SRP84/pGP1-2/pTEEX resulted in the expression of the *P. shermanii* epimerase at levels ranging from 20-35% of the total cell protein as assessed by analysis on a Laemmli 20% SDS-PAGE gel (Figure 3.7). As expected, the control strain bearing pT7-7, instead of the expression plasmid pTEEX, showed no expression of the epimerase.

The solubility of the recombinant epimerase cloned into *E. coli* was tested by centrifuging a cell lysate of induced SRP84/pGP1-2/pTEEX at 150000 x *g* for 1 h at 4°C. Samples of the supernatant fractions and the resuspended pellet were then analysed by SDS-PAGE (Figure 3.7). Judging from the distribution of the epimerase enzyme in the supernatant and the pellet, the recombinant epimerase was 95-100% soluble in *E. coli*.

3.1.6 PURIFICATION OF 'WILD-TYPE' EPIMERASE FROM *P. SHERMANII*

'Wild-type' epimerase was purified from 40 g of *P. shermanii* cells (wet weight) as described in the Materials and Methods chapter section 2.2.2.1. Samples taken at various stages of the purification were analysed on a 20% SDS-PAGE gel (Figure 3.8). The major stained protein band of the purified 'wild-type' epimerase preparation had a lower electrophoretic mobility than the 20.1 kDa soyabean trypsin inhibitor standard marker protein. This contradicts the predicted size of the epimerase gene product which according to the DNA sequence should be 16.72 kDa (*N*-terminal methionine on). The anomalous migration is similar to that seen for the recombinant epimerase, suggesting that the 'wild-type' and recombinant enzymes have similar molecular weights (see section 3.1.9.2 for further discussion). The elution profile of the final 'wild-type' epimerase purification step, gel filtration on Sephadex G-75, is shown in Figure 3.9. The data for the purification of 'wild-type' epimerase is given in Table 3.1. After the final Sephadex G-75 gel filtration step, 11.8 mg of purified epimerase was recovered with an overall 215-fold purification which yielded a 63% recovery of epimerase activity. The purified 'wild-type' epimerase had a specific activity of 86 U/mg.

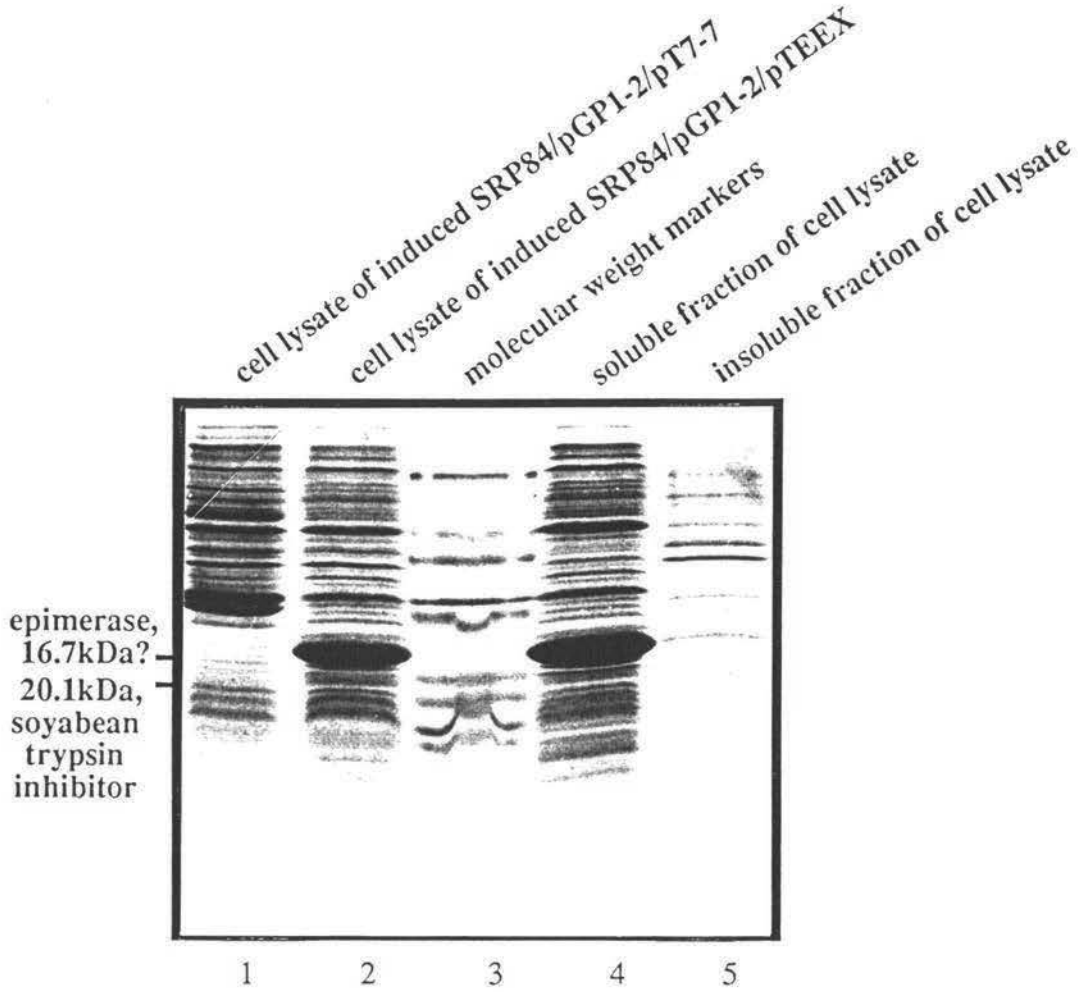

Figure 3.7: Photograph of a Coomassie Brilliant Blue R-250-stained 20% SDS-PAGE gel showing an analysis of the size and solubility of the recombinant epimerase produced in *E. coli* SRP84/pGP1-2/pTEEX(#4), after confirming that the DNA sequence of the epimerase gene insert in pTEEX(#4) was identical to the epimerase gene sequence determined by Davis (1987). Lane 1: cell lysate of induced SRP84/pGP1-2/pT7-7; lane 2: cell lysate of induced SRP84/pGP1-2/pTEEX; lane 3: 9 protein molecular weight marker standard (see Table 2.1); lane 4: soluble fraction of cell lysate; lane 5: insoluble fraction of the cell lysate. See section 2.2.7 for experimental details.

The predicted size of the translated product of the epimerase gene is 16.7 kDa, so this gel suggests that either the epimerase has an anomalously low electrophoretic mobility in the modified Laemmli Tris-glycine buffer system used for this gel, or that there is an error in the expression construct . The results also show that the recombinant epimerase is 95-100% soluble when produced in *E. coli*.

Purification of 'Wild-Type' Epimerase from *P. shermanii*

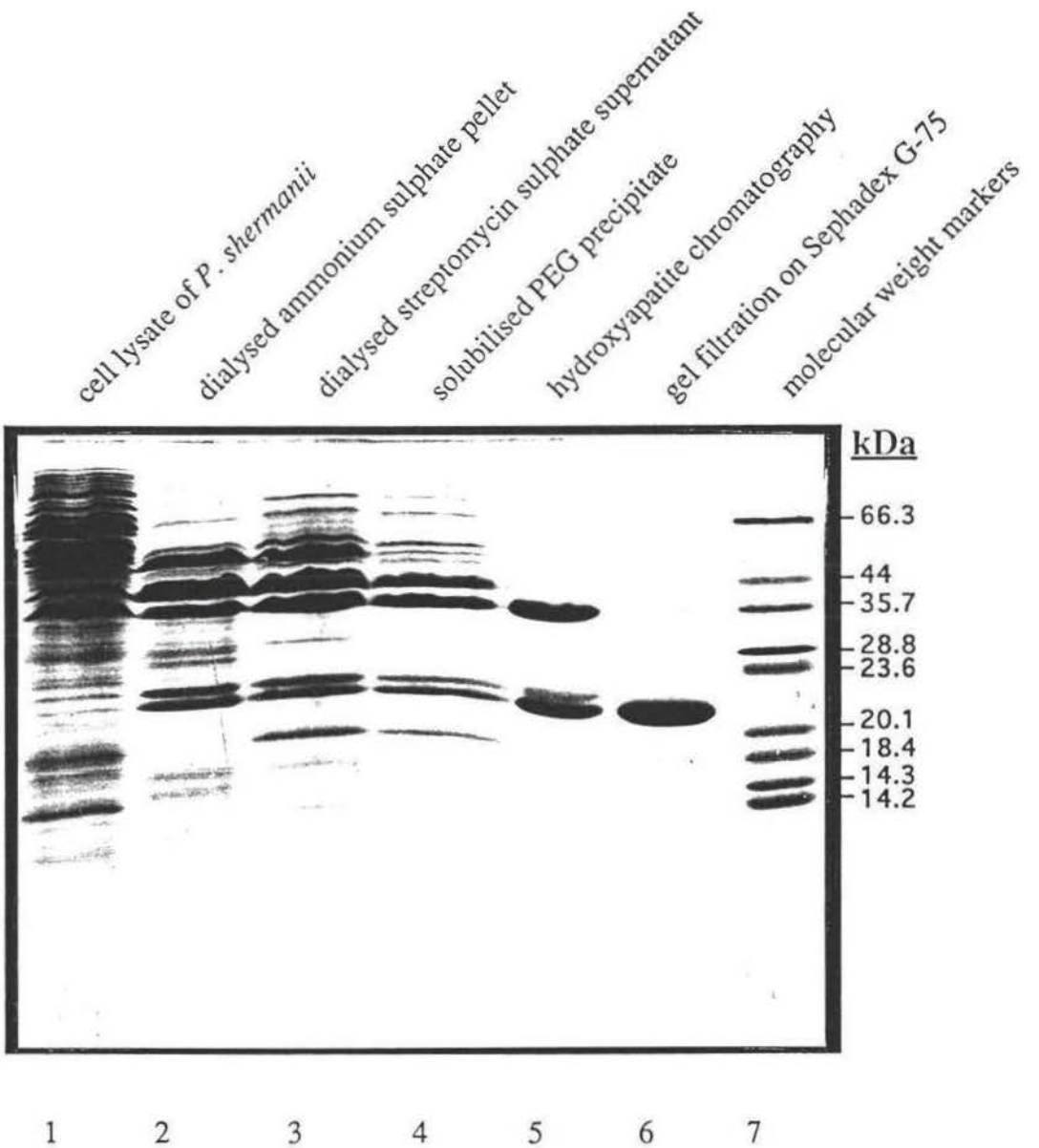

Figure 3.8: Photograph of Coomassie Brilliant Blue R-250-stained 20% SDS-PAGE gel showing the purification of the 'wild-type' epimerase from *P. shermanii*. Each lane corresponds to a sample taken after each purification step. Lane 1: cell lysate from *P. shermanii*; lane 2: dialysed ammonium sulphate pellet; lane 3: dialysed streptomycin sulphate supernatant; lane 4: re-dissolved PEG precipitate; lane 5: pooled fractions from two major protein peaks after hydroxyapatite chromatography; lane 6: pooled fractions from gel filtration chromatography; lane 7: molecular weight markers. The molecular weights of the marker proteins, in kDa, are shown to the right of the gel.

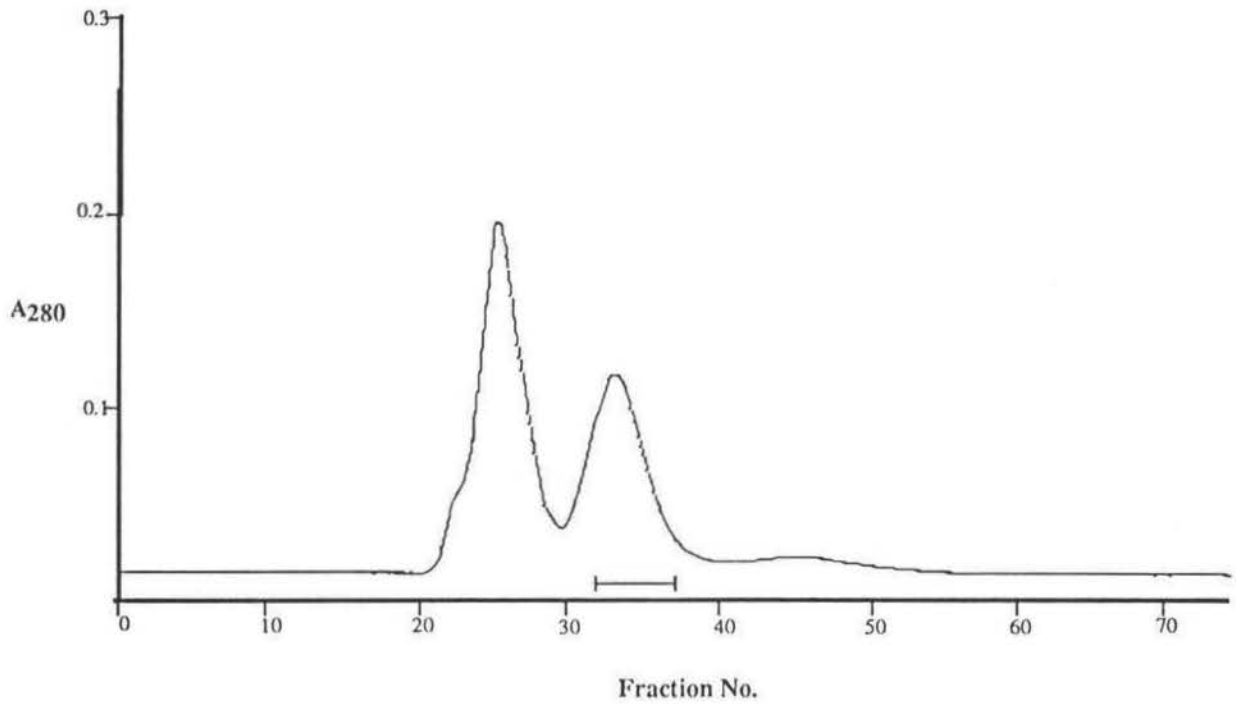

Figure 3.9: 'Wild-type' epimerase purification. Elution profile from the Sephadex G-75 chromatography step. Fractions 22-38 were analysed by SDS-PAGE and fractions 32-37 were pooled (⊢——⊣). The larger peak contained only a small proportion of the total epimerase protein and this was contaminated with other proteins.

PURIFICATION STEP	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (U/mg)	-FOLD PURIFICATION	% RECOVERY
1. Cell free lysate	4000	0.4	1.0	100
2. Ammonium sulphate ppt	220	5.8	15	80
3. Streptomycin sulphate ppt	210	5.4	14	71
4. PEG 6000 ppt	105	11	28	72
5. Hydroxyapatite column	30	35	88	66
6. Sephadex G-75 column	11.8	86	215	63

Table 3.1: A summary of the purification of the 'wild-type' epimerase from *P. shermanii*. The protein concentration was determined by the Biuret method. The abbreviation "ppt" stands for precipitate.

3.1.7 PURIFICATION OF RECOMBINANT EPIMERASE PRODUCED BY HEAT-SHOCK INDUCTION OF SRP84/pGP1-2/pTEEX

Samples taken after each step in the purification of recombinant epimerase from 10 g of induced *E. coli* SRP84/pGP1-2/pTEEX cells were analysed by SDS-PAGE (Figure 3.10). An extra heat-treatment step was employed early in the purification scheme, just after the initial cell lysis. Apart from this heat-treatment step, all the other purification steps were the same as those employed for the *P. shermanii* 'wild-type' epimerase purification. The heat-treatment step, which involved the heating of the cell lysate at 70°C for 15 min, was found to be very effective in denaturing and precipitating the majority of contaminating *E. coli* proteins present (Figure 3.10). The supernatant from the heat-treatment is free of NADH oxidase activity. This activity increases the blank rate in the enzyme-coupled assay of methylmalonyl-CoA mutase, epimerase and transcarboxylase, in which enzyme activity is coupled to oxidation of NADH. The heat-treated supernatant is therefore an excellent source of epimerase coupling enzyme for the assay of mutase and transcarboxylase.

As in the earlier recombinant epimerase expression experiments, the recombinant epimerase band was found to migrate more slowly than the 20.1 kDa standard marker protein on a Laemmli 20% SDS-PAGE gel. As for the 'wild-type' epimerase, this result is again inconsistent with the epimerase molecular weight of 16.72 kDa predicted from the inferred amino acid sequence. The elution profiles for the hydroxyapatite (Figure 3.11) and gel filtration (Figure 3.12) purification steps are shown. The hydroxyapatite elution profile is surprisingly complex given the near-homogeneous appearance of the epimerase protein on an SDS-PAGE gel at this stage of the purification. Most of the major peaks in this elution profile were due to epimerase protein (confirmed by SDS-PAGE analysis of individual fractions), suggesting that this step may be capable of separating different forms of the epimerase and that the protein may be microheterogeneous in nature. Some additional peaks may have been due to non-protein contaminants in the epimerase preparation, e.g. PEG, carbohydrate, lipid or DNA. The two major epimerase protein peaks seen in the hydroxyapatite elution profile were combined and concentrated for the final gel filtration step. The purification of recombinant epimerase is summarised in Table 3.2. 66 mg of pure recombinant epimerase protein was obtained with a specific activity of 84 U/mg. The overall purification factor was 5.3, with a 29% recovery of epimerase activity.

It is puzzling that the percentage recovery of the recombinant protein was less than half that for the 'wild-type' epimerase. This may be related to the extra step, a heat-treatment, in the recombinant epimerase purification. Although recovery for this step was greater than 100%, indicating that some activation of the enzyme had taken place, the recovery in

the subsequent ammonium sulphate precipitation step was only 40% compared to 80% for the 'wild-type' epimerase purification. The heat-treatment may have caused this next step to be less effective. Despite the greater overall losses in the recombinant epimerase purification (29% recovery versus 63% for the 'wild-type' epimerase), the final yield of 66 mg pure recombinant epimerase from 10 g *E. coli* cells (versus 11.8 mg from 40 g *P. shermanii* cells), represents a 22-fold improvement in yield of recombinant epimerase compared to the 'wild-type' source.

The appearance of the two purified epimerases on Coomassie Brilliant Blue R-250- and silver-stained gels (Figure 3.14) is slightly different. The 'wild-type' epimerase has a one discrete minor protein contaminant, and the epimerase band is not blurred. In contrast, the recombinant enzyme has no distinct protein contaminants, but the band is slightly blurred, especially above the most intense portion of the band. This 'halo' above the band seems to develop during the purification, becoming increasingly evident after the streptomycin sulphate and PEG 6000 purification steps. This blurring of the epimerase protein band could be an indication of heterogeneity in the purified protein. This may be due to the heat-treatment early in the purification. Although this step is useful, it may not be necessary given the abundance of epimerase in the starting material. It would be interesting to try to improve the purification of the recombinant epimerase to avoid the band blurring phenomenon, and to see if this affected other properties of the enzyme.

The first attempt at purifying the recombinant epimerase involved a PEI treatment to precipitate out nucleic acids present in the preparation. This step had been used successfully in the purification of the recombinant methylmalonyl-CoA mutase (section 2.2.3.1). Unfortunately the PEI precipitated out both the nucleic acids and the recombinant epimerase. In subsequent purifications of recombinant epimerase PEI treatments were omitted.

3.1.8 DETERMINATION OF THE PURIFIED 'WILD-TYPE' AND RECOMBINANT EPIMERASE PROTEIN CONCENTRATIONS

The protein concentrations of the purified concentrated 'wild-type' and recombinant epimerase solutions were determined by the Biuret, bicinchoninic acid and UV $A_{205/280}$ methods (Table 3.3).

The average of the measurements obtained from the Biuret and UV $A_{205/280}$ methods were taken as the final concentrations of the purified epimerases (Table 3.4). The Biuret method shows little protein-to-protein variation, so an accurate determination of protein concentration should be possible using BSA for the protein standard curve. Similarly, the

Purification of Recombinant Epimerase from *E. coli*

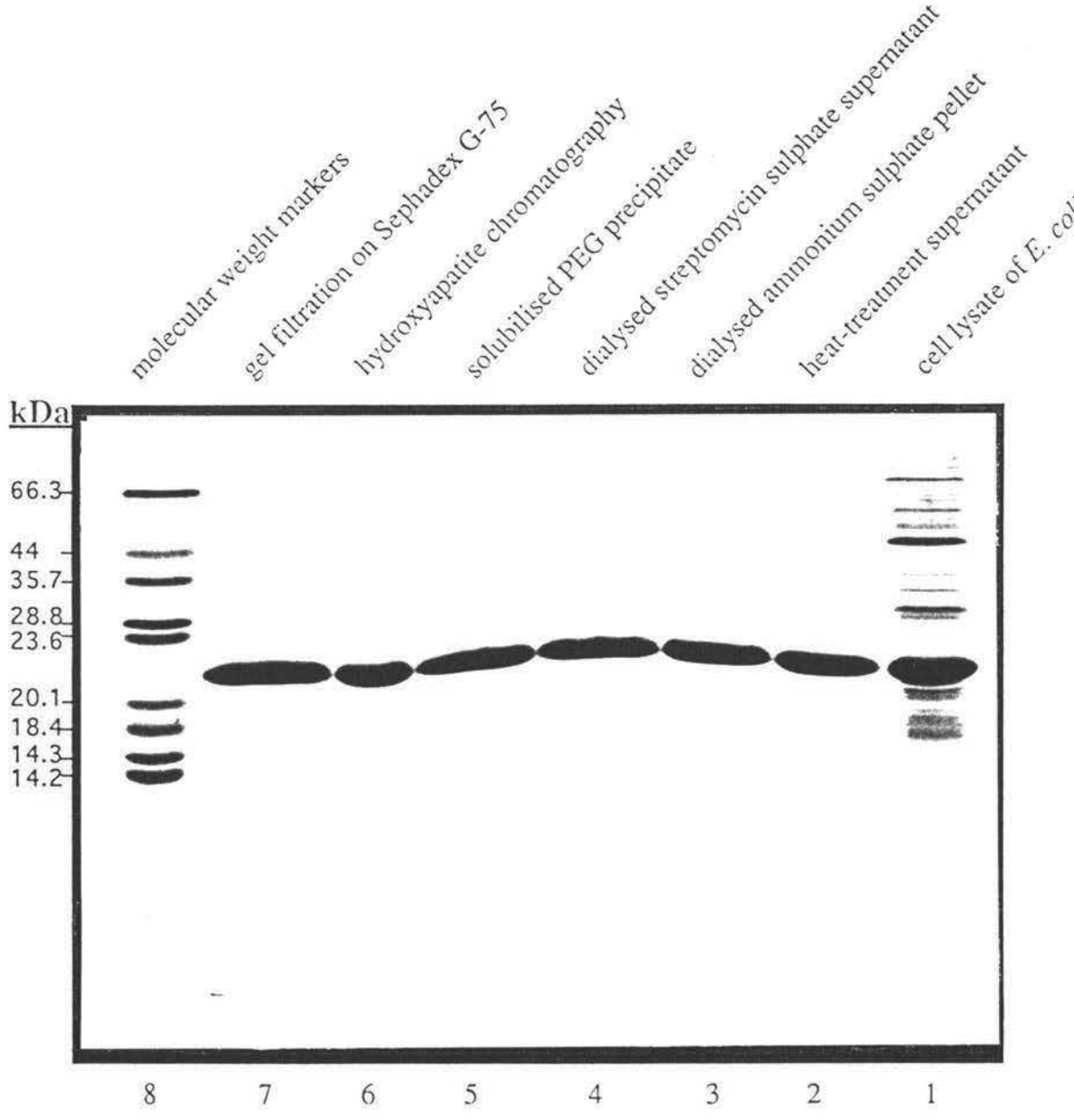

Figure 3.10: Photograph of a Coomassie Brilliant Blue R-250-stained 20% SDS-PAGE gel showing the purification of the recombinant epimerase produced in *E. coli* SRP84/pGP1-2/pTEEX. Lane 1: cell lysate; lane 2: heat-treatment supernatant; lane 3: dialysed ammonium sulphate pellet; lane 4: dialysed streptomycin sulphate supernatant; lane 5: re-dissolved PEG precipitate; lane 6: pooled fractions from hydroxyapatite chromatography; lane 7: pooled fractions from gel filtration chromatography on Sephadex G-75; lane 8: molecular weight markers. The molecular weights, in kDa, of the proteins used as molecular weight markers are shown to the left of the photograph.

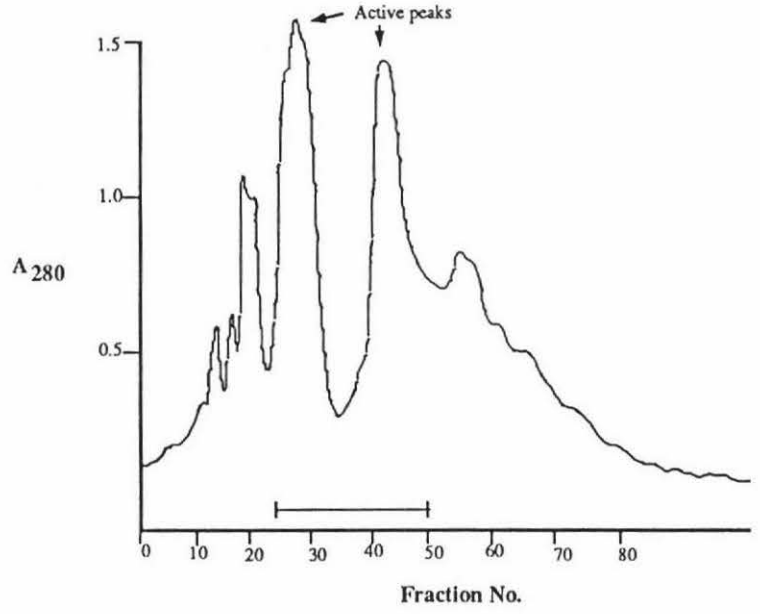

Figure 3.11: Recombinant epimerase purification. Elution profile from the hydroxyapatite chromatography step. Fractions 24-50 were pooled as indicated by (⊢——⊣).

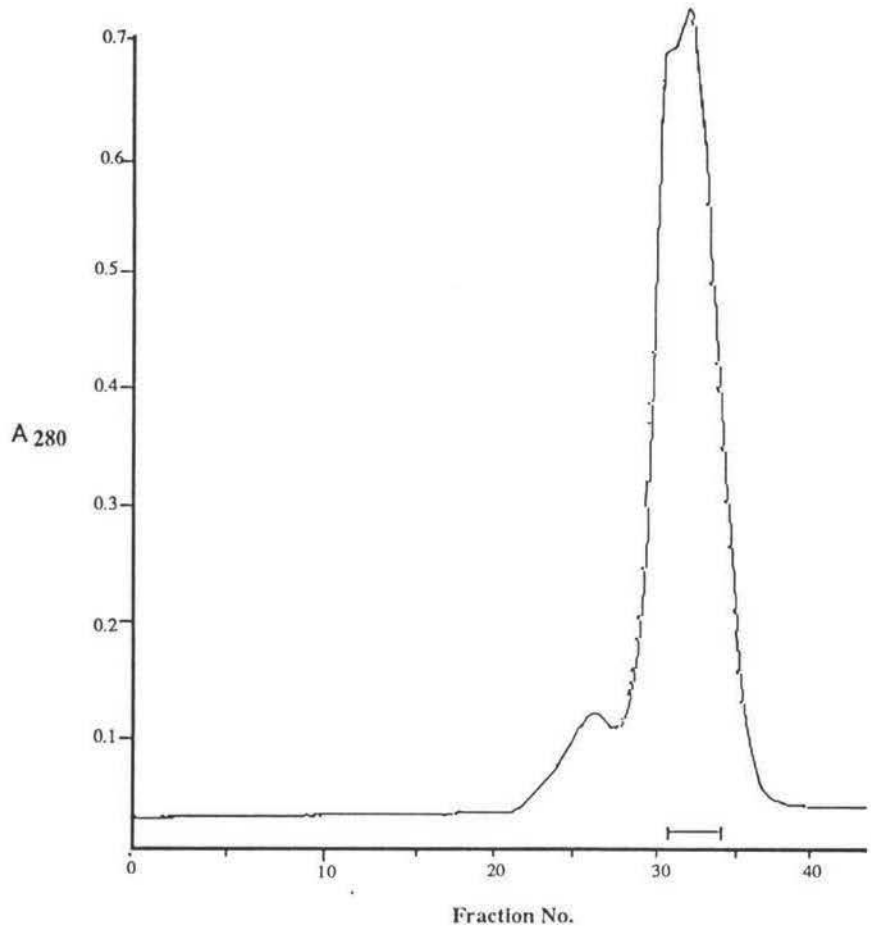

Figure 3.12: Recombinant epimerase purification. Elution profile from the Sephadex G-75 gel filtration chromatography step. Fractions 31-34 (⊢——⊣) were pooled.

PURIFICATION STEP	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (U/mg)	-FOLD PURIFICATION	% RECOVERY
1. Cell free lysate	1175	16	1.0	100
2. Heat-treatment	260	78	4.9	108
3. Ammonium sulfate ppt	94	86	5.4	43
4.Streptomycin sulfate ppt	95	80	5.0	40
5. PEG 6000 ppt	90	84	5.3	40
6. Hydroxyapatite column	70	86	5.4	32
7. Sephadex G-75 column	66	84	5.3	29

Table 3.2: A summary of the purification of the recombinant epimerase expressed in SRP84/pGP1-2/pTEEX from the plasmid pTEEX. Note that apart from the heat-treatment step all the steps are exactly the same as those employed for the 'wild-type' epimerase purification. The abbreviation "ppt" stands for precipitate.

PROTEIN METHOD OF DETERMINATION	'WILD-TYPE' EPIMERASE CONCENTRATION (mg/ml)	RECOMBINANT EPIMERASE CONCENTRATION (mg/ml)
(1) Biuret Method	11.8	20.8
(2) Bicinchoninic acid	7.7	13.5
(3) UV $A_{205/280}$	9.3	19.5

Table 3.3: The final protein concentrations of the purified 'wild-type' and recombinant epimerases were measured using the Biuret method, bicinchoninic acid method and the UV $A_{205/280}$ method. Each value is the average of three determinations.

PURIFIED PROTEIN	PURIFIED EPIMERASE CONCENTRATION (mg/ml)
'wild-type' epimerase	10.55 (± 10%)
recombinant epimerase	20.15(± 3%)

Table 3.4: The concentrations of the purified 'wild-type' epimerase and the purified recombinant epimerase. The values shown are the average of the measurements taken using the Biuret method and the UV $A_{205/280}$ method.

UV method show little variation between proteins and does not require a protein standard. In contrast, the bicinchoninic acid method, although very sensitive, does show protein-to-protein variability. Smith *et al.* (1985) have speculated that this is due to the colour development in this assay being partly due to the presence of readily oxidizable protein components such as cysteine, tyrosine and tryptophan. The mole percent of these three residues in the BSA standard protein is 9.777, whereas the epimerase protein, with it's lower cysteine content, is only 6.756. Thus, based on cysteine, tyrosine and tryptophan content alone, the epimerase protein might be expected to have a colour yield of about $(\frac{6.756}{9.777} \times 100 =)$ 69% that seen for BSA. Although speculative, this is the percentage seen when the bicinchoninic acid determination is compared to the average of the other to methods. In any case, the variation in colour yield between proteins means that the bicinchoninic acid method is only appropriate for determining relative protein concentrations. Figure 3.13 shows the inferred amino acid sequence of the epimerase protein (methionine on). The cysteine, tyrosine and tryptophan residues are underlined.

```
1     MSNEDLFICI DHVAYACPDA DEASKYYQET FGWHELHREE NPEQGVVEIM
51    MAPAAKLTEH MTQVQVMAPL NOESTVAKWL AKHNGRAGLH HMAWRVDDID
101   AVSATLRERG VOLLYDEPKL GTGGNRINFM HPKSGKGVLI ELTQYPKN
```

Figure 3.13: The amino acid sequence of the epimerase enzyme. The five tyrosine (Y), three tryptophan (W) and 2 cysteine (C) amino acid residues are underlined.

It is difficult to reconcile the different values obtained with the different methods, and the different discrepancies between methods, for the recombinant and 'wild-type' epimerase concentrations. If the proteins are identical, the difference between the Biuret and UV $A_{205/280}$ methods should be the same for both epimerases, yet the difference is significantly greater for the 'wild-type' protein.

For protein purification, the protein concentration was measured by the Biuret method. However, when calculating the specific activities of the purified epimerases, the average values in Table 3.4 were used.

3.1.9 COMPARISON OF 'WILD-TYPE' AND RECOMBINANT EPIMERASE SPECIFIC ACTIVITY, ELECTROPHORETIC MOBILITY, METAL ION ACTIVATION AND *N*-TERMINAL SEQUENCE

The 'wild-type' and recombinant epimerases were characterized with respect to specific activity, electrophoretic mobility in SDS-PAGE gels, activation by metal ions, and *N*-terminal amino acid sequence. It was hoped that the recombinant epimerase would behave similarly to the 'wild-type' enzyme, thus justifying the use of the more abundant recombinant epimerase in crystallization experiments.

3.1.9.1 ACTIVITY MEASUREMENTS AND SPECIFIC ACTIVITY DETERMINATIONS

The specific activity of the final purified concentrated epimerase was determined for both the 'wild-type' and recombinant enzyme (see section 2.2.5.1 for assay method). The results were of 86 U/mg and 84 U/mg respectively. Considering that the experimental error in these values is at least ±10%, these values agree within the limits of experimental error.

3.1.9.2 THE ELECTROPHORETIC MOBILITY OF EPIMERASE IN SDS-PAGE GELS RUNNING A MODIFIED DISCONTINUOUS TRIS-GLYCINE LAEMMLI BUFFER OR A CONTINUOUS PHOSPHATE WEBER AND OSBORN BUFFER SYSTEM

The aim of this section was to determine and compare the electrophoretic mobilities of the purified 'wild-type' and recombinant epimerase enzymes in two different SDS-PAGE buffer systems, a modified discontinuous Tris-glycine buffer system of Laemmli (1970) and the continuous phosphate buffer system of Weber and Osborn (1969). This comparison was made in an attempt to attribute the anomalously low electrophoretic mobility exhibited by both 'wild-type' and recombinant epimerase in modified Laemmli buffers to the buffer system. Other proteins have been shown to migrate anomalously in Laemmli SDS-PAGE gels (e.g. Patchett *et al.*, 1991).

(A) MODIFIED LAEMMLI DISCONTINUOUS TRIS-GLYCINE BUFFER SYSTEM

The discontinuous 20% SDS-PAGE gels in Figure 3.14 using a modified Laemmli Tris-glycine buffer system (section 2.2.6.3) show the 'wild-type' and recombinant epimerase

electrophoresed alongside molecular weight standard marker proteins. Gel (A) was stained with Coomassie Brilliant Blue R-250 dye, and gel (B) was silver stained.

The 'wild-type' and recombinant epimerase bands were found to have the same electrophoretic mobility, migrating between the 20.1 kDa soyabean trypsin inhibitor marker protein and the 23.6 kDa trypsinogen marker protein. That the 'wild-type' and recombinant epimerases had the same electrophoretic mobility, even though this is lower than the electrophoretic mobility expected for a 16.72 kDa protein, was a great relief, because it confirmed that the migration of the recombinant epimerase in SDS-PAGE gels running the modified Laemmli Tris-glycine buffer system is truly anomalous. That is, the low electrophoretic mobility is a property of the epimerase (both 'wild-type' and recombinant) in the Tris-glycine buffer system, rather than being due to any error in the pTEEX expression construct that might have given rise to a recombinant epimerase that was larger than the 'wild-type' epimerase. Figure 3.15 shows a plot of $\log(M_r/1000)$ versus R_f for the Coomassie Brilliant Blue R-250-stained modified Laemmli gel in Figure 3.14. This plot gives an apparent epimerase M_r of 21600.

(B) WEBER AND OSBORN CONTINUOUS PHOSPHATE BUFFER SYSTEM

The 10% continuous gel in Figure 3.16 was run using the Weber and Osborn phosphate buffer system. As in the modified Laemmli system, the 'wild-type' and the recombinant epimerases co-migrate, but in this buffer system both epimerases migrate between the 14.3 kDa lysozyme marker protein and the 18.4 kDa β-lactoglobulin marker protein. This behaviour is consistent with the expected epimerase M_r of 16720 (methionine on) predicted from the deduced amino acid sequence.

The plot of $\log(M_r/1000)$ versus R_f shown in Figure 3.17 gave an apparent epimerase M_r of 16600. This agrees very well with the predicted epimerase size, and suggests that the Weber and Osborn continuous phosphate buffer system would be the system of choice when determining the M_r of an unknown protein.

3.1.9.3 THE EFFECT OF INCUBATION WITH METAL IONS ON EPIMERASE ACTIVITY

Previous investigations of the effect of metal ions on epimerase activity found that high concentrations of several metal ion chelating reagents progressively inactivated both *P. shermanii* (Leadlay, 1981) and rat liver (Stabler and Allen, 1988) epimerases.

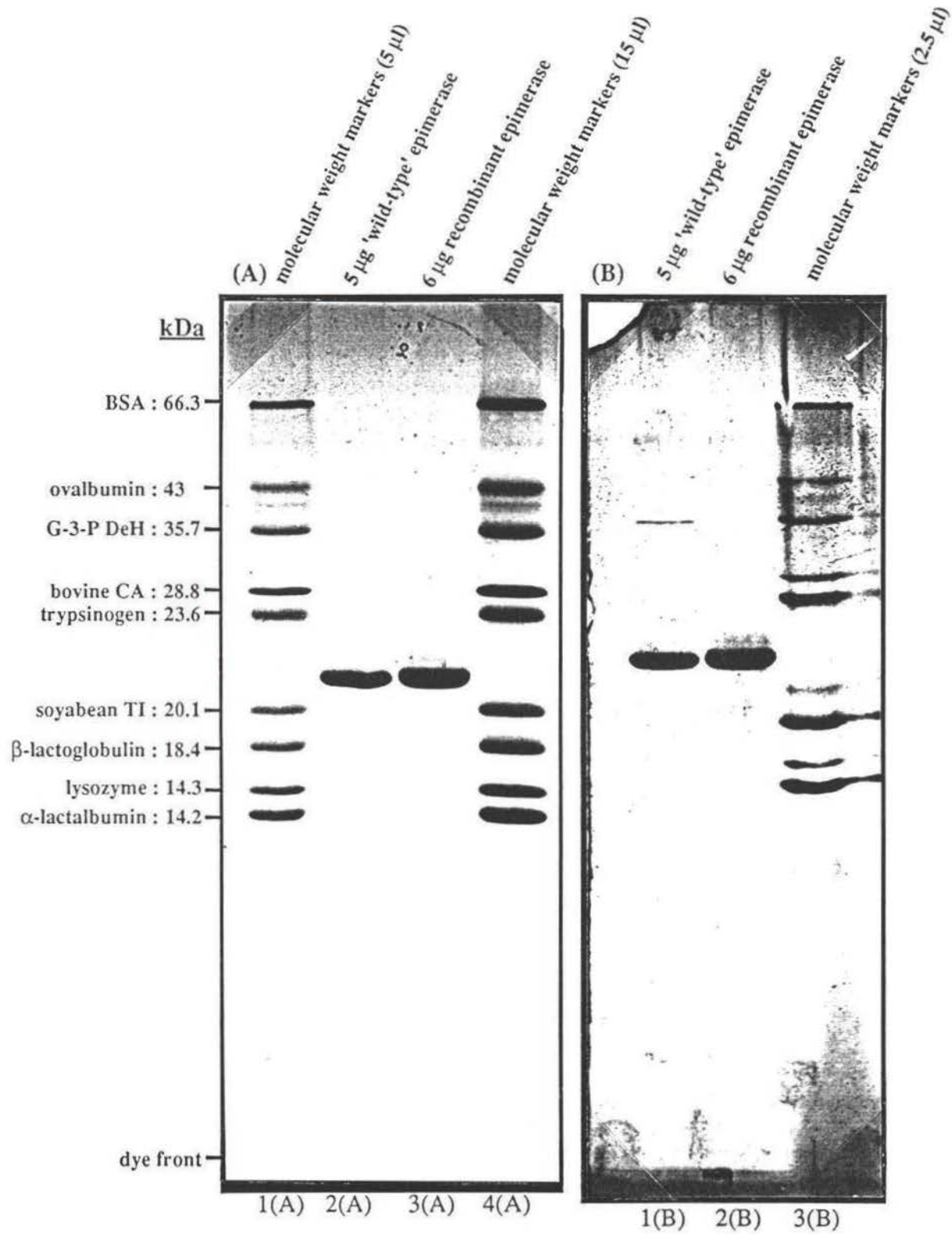

Figure 3.14: Photographs of modified Laemmli Tris-glycine 20% SDS-PAGE gels. Gel (A) was stained with Coomassie Brilliant Blue R-250. Gel (B) was silver stained. Lane 1(A): 9 protein molecular weight marker; lane 2(A): 'wild-type' epimerase; lane 3(A): recombinant epimerase; 4(a): as for lane 1(A); lane 1(B): 'wild-type' epimerase; lane 2(B): recombinant epimerase; lane 3(B): as for lane 1(A). Molecular weights of the marker proteins and the position of the dye front are shown to the left of gel (A).

PLOT OF LOG (M_r/1000) vs R_f
FOR THE LAEMMLI BUFFER SYSTEM

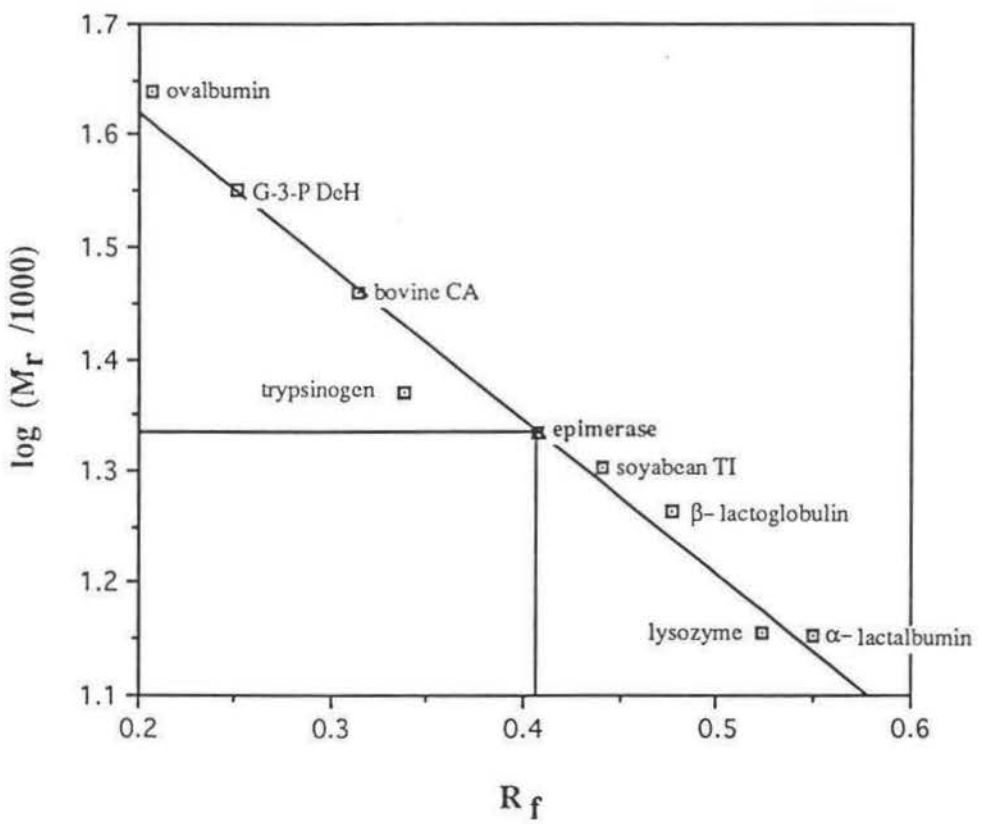

Figure 3.15: Plot of log (M_r/1000) versus R_f for 8 of the protein molecular weight markers used in the modified Laemmli Tris-glycine buffer system. The BSA data point (not shown) was not included in the data points used for the line of best fit as it deviated too far from the line of best fit for the other data points.

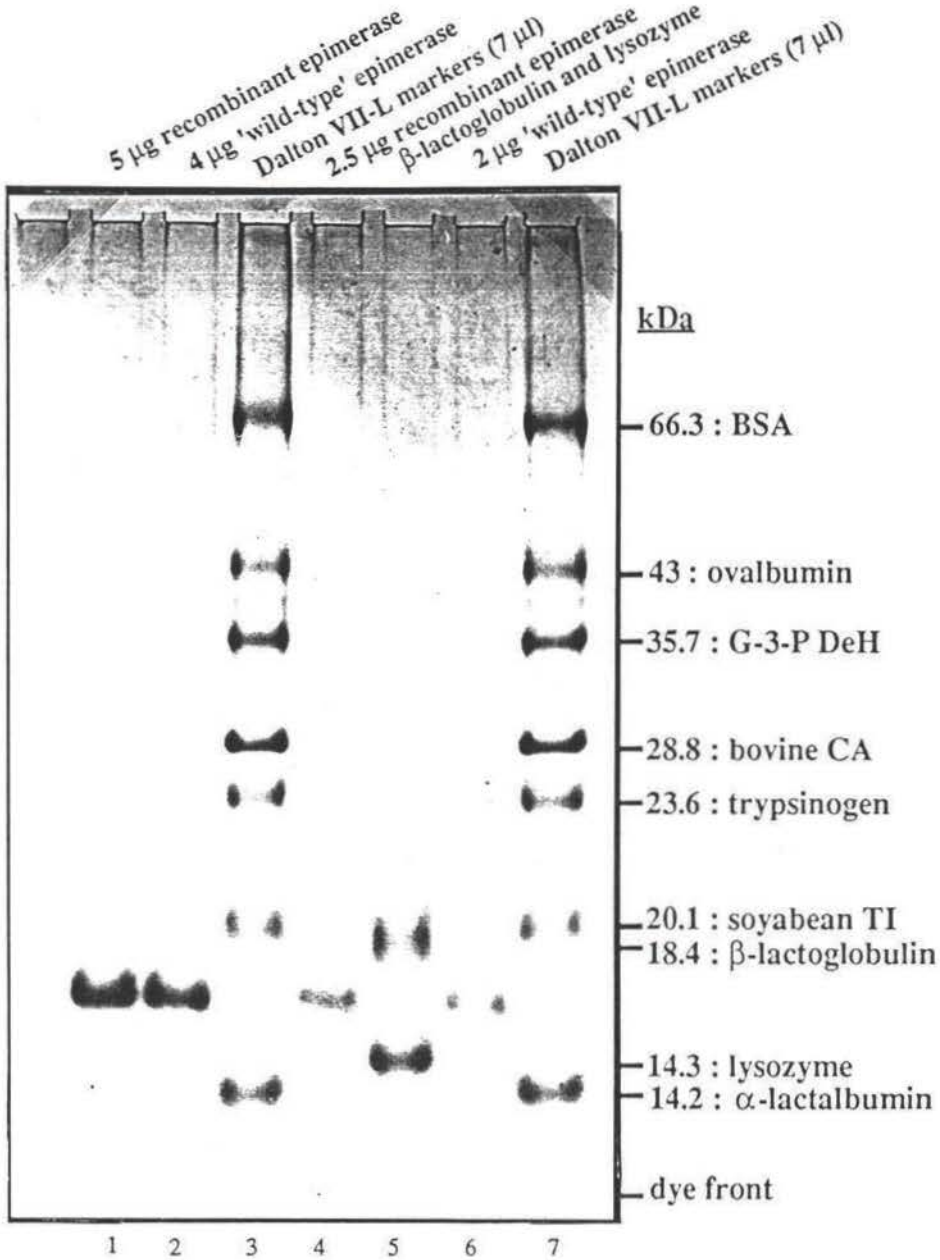

Figure 3.16: Photograph of a Coomassie Brilliant Blue R-250-stained Weber and Osborn continuous phosphate-buffer system 10% SDS-PAGE gel. Lane 1: recombinant epimerase; lane 2: 'wild-type' epimerase; lane 3: Dalton VII-L molecular weight markers; lane 4: recombinant epimerase; lane 5: β-lactoglobulin and lysozyme molecular weight markers; lane 6: 'wild-type' epimerase; lane 7: Dalton VII-L markers.

PLOT OF LOG (M_r/1000) vs R_f
FOR THE WEBER AND OSBORN BUFFER SYSTEM

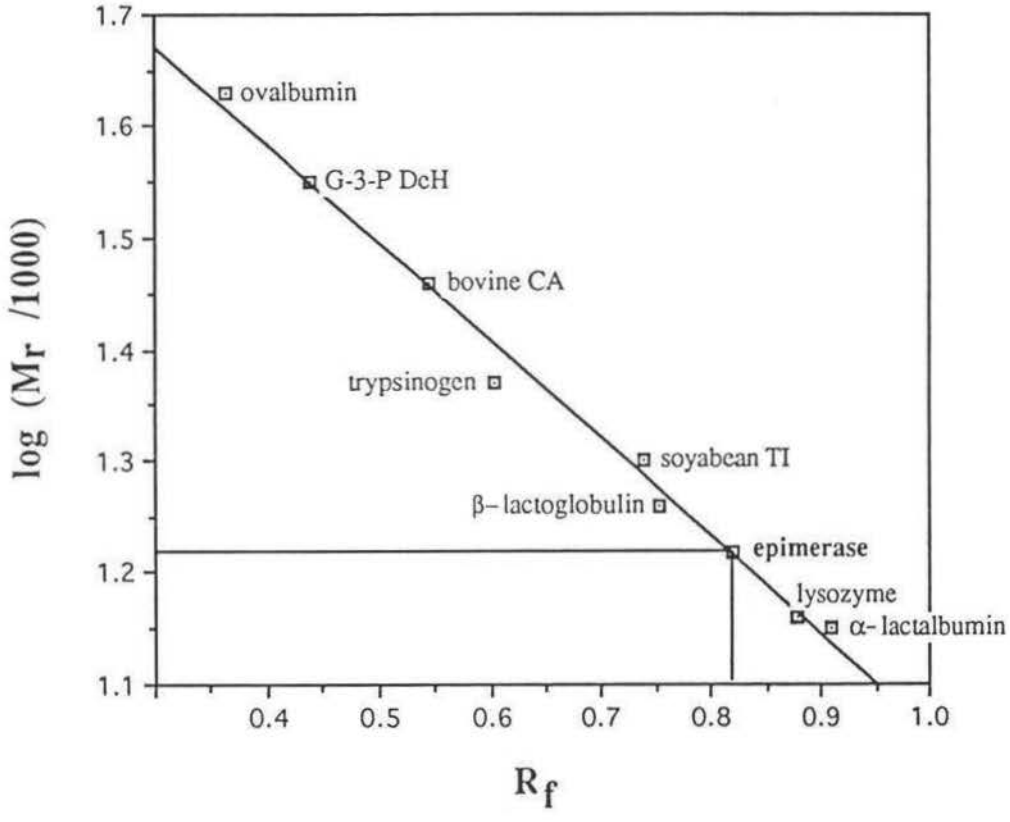

Figure 3.17: Plot of log (M_r/1000) versus R_f for 8 of the protein standard molecular weight markers used in the Weber and Osborn buffer system. The BSA data point (not shown) was not included in the data points used for the line of best fit as it deviated too far from the range of lines of best fit generated for the other data points.

The effects of pre-incubation with divalent metal ions on the activity of the *P. shermanii* 'wild-type' and recombinant epimerase was investigated. The study was done to compare the activation of the 'wild-type' and the recombinant epimerases by specific divalent metal ions. The results of the various treatments are shown in Figures 3.18 and 3.19 for 'wild-type' and recombinant epimerase respectively.

These epimerase metal ion activation results are quite intriguing. They show both similarities and differences between the two epimerase enzymes. Both the 'wild-type' and the recombinant epimerases had their activity increased approximately 3-fold by incubation with Co^{2+} and to a lesser extent by incubation with Mn^{2+}, Zn^{2+} and Cd^{2+}. The activation by the latter three ions was more pronounced for the 'wild-type' epimerase. The activation by Co^{2+} was substantially complete after 5 hours for the 'wild-type' enzyme, but may be slightly slower for the recombinant enzyme. Ni^{2+} was also effective in increasing the activity of 'wild-type' epimerase, but unfortunately an Ni^{2+} incubation was not performed with the recombinant enzyme. This pattern on metal ion activation is similar to that seen for many arginase enzymes (Patchett *et al.*, 1991).

Both enzymes were inactivated by incubation with EDTA, although the 'wild-type' enzyme was more resistant to this treatment, retaining 13% activity. This may reflect a lower thermostability for the recombinant enzyme, as incubation without any metal ions added decreased the activity of the recombinant epimerase but caused activation of the 'wild-type' enzyme. This may be a reflection of a different metal ion status for the two enzymes, with the 'wild-type' enzyme being more saturated with a stabilising metal ion. This could arise if there was a difference in available metal ion concentrations between the two bacteria in which the epimerase enzymes are produced. However, more work clearly needs to be done to characterize the effects of metal ions on activity and thermostability, and to establish any *in vivo* requirement for a metal ion cofactor. This work does suggest that the inclusion of Co^{2+} ions in the heat-treatment step of the epimerase purification could have been beneficial for the maintenance of epimerase activity and stability during this step. Finally, it is interesting to note that all three enzymes of the pathway that interconverts succinyl-CoA and propionyl-CoA may be cobalt dependent. Epimerase is activated by, and may have a cofactor requirement for, Co^{2+}, transcarboxylase binds Co^{2+}, and the mutase coenzyme, adenosylcobalamin, contains a cobalt atom.

Other metal ions had a relatively small or insignificant effects on epimerase activity. Fe^{2+} was an exception, but this ion is not stable in neutral solutions and tended to precipitate during the incubation. This precipitation could have inactivated the enzyme, or the epimerase may have been adsorbed to the surface of the precipitate.

THE EFFECT OF METAL IONS ON THE SPECIFIC ACTIVITY OF 'WILD-TYPE' EPIMERASE

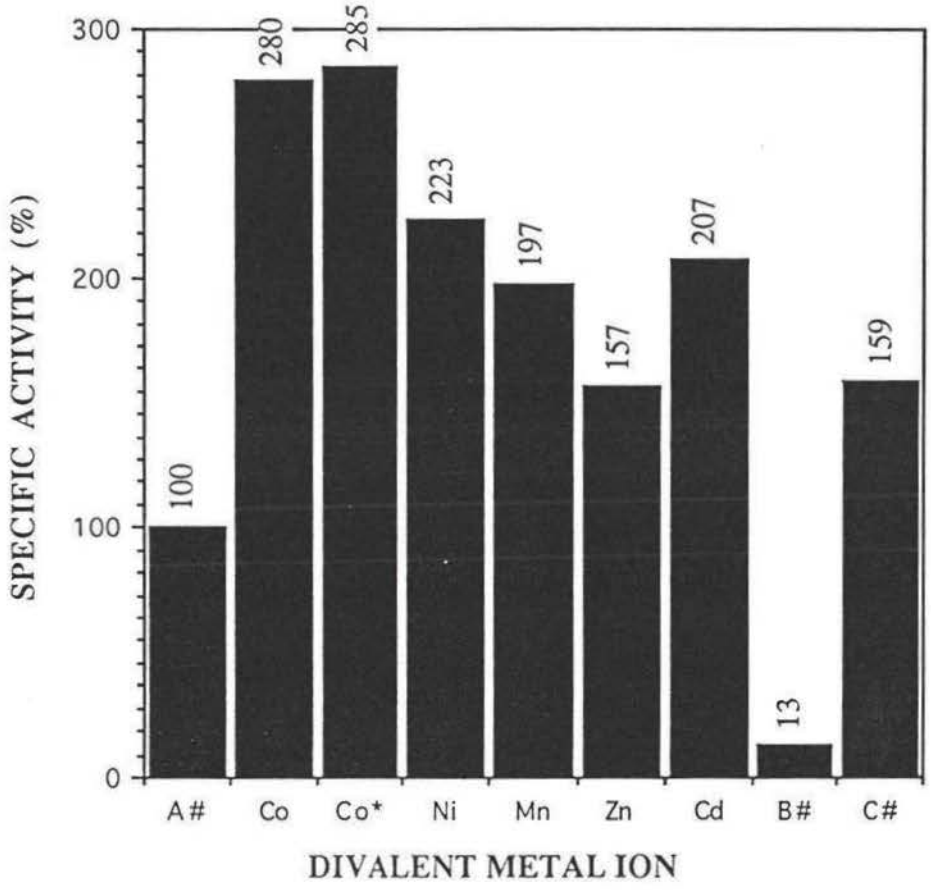

Figure 3.18: Bar graph showing the effect of pre-incubating the 'wild-type' epimerase with selected divalent metal ions on the activity of epimerase. The epimerase was incubated with 1 mM Co^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, EDTA ($B^{\#}$) and with no metal ion added ($C^{\#}$). A control sample ($A^{\#}$) with no metal ion added was stored on ice, while the other samples were incubated at 60°C for 5 h. The Co^{2+} treatment represented by Co* was incubated at 60°C for 10 h. At the completion of the incubations the samples were placed on ice and assayed for epimerase activity. The percentage activity of each sample relative to the control ($A^{\#}$) is shown above each bar.

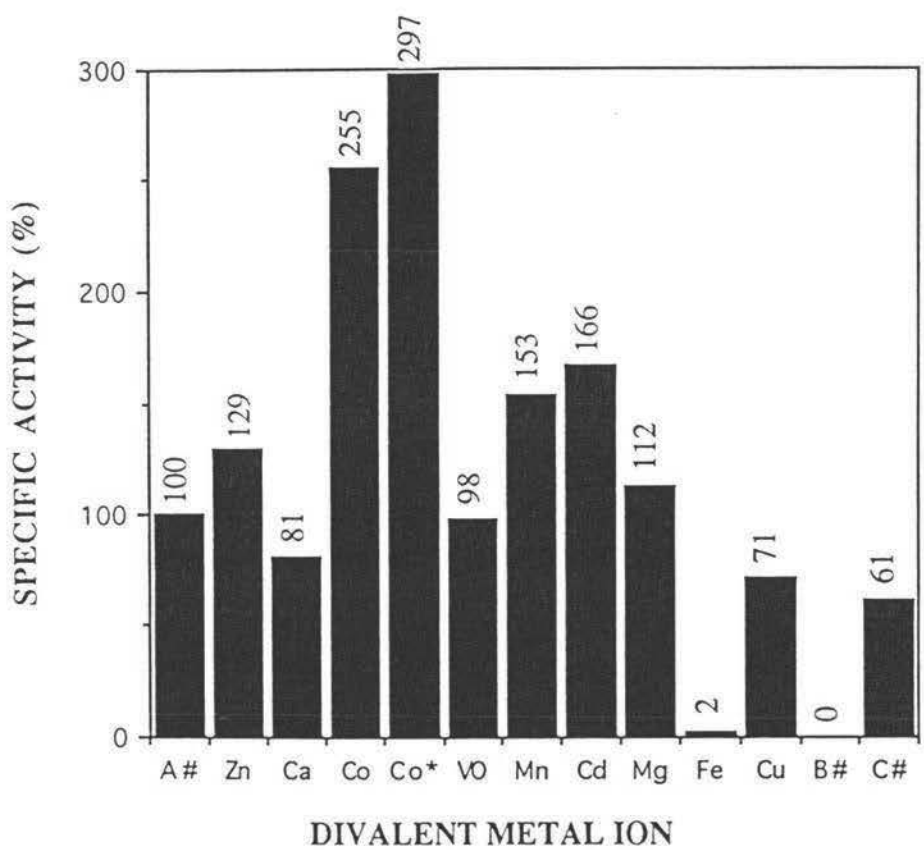

Figure 3.19: Bar graph showing the effect of pre-incubating the recombinant epimerase with selected divalent metal ions on the activity of epimerase. The epimerase was incubated with 1 mM Zn^{2+}, Ca^{2+}, Co^{2+}, VO^{2+}, Mn^{2+}, Cd^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, EDTA ($B^{\#}$) and with no metal ion added ($C^{\#}$). A control sample ($A^{\#}$) with no metal ion added was stored on ice, while the other samples were incubated at 60°C for 5 h. The Co^{2+} treatment represented by Co* was incubated at 60°C for 10 h. At the completion of the incubations the samples were placed on ice and assayed for epimerase activity. The percentage activity of each sample relative to the control ($A^{\#}$) is shown above each bar.

3.1.9.4 *N*-TERMINAL SEQUENCING OF EPIMERASE

The first 14 *N*-terminal residues of the 'wild-type' and the recombinant epimerases were sequenced (Figure 3.20) using the automated Edman degradation method (Hewick *et al.*, 1981). These sequences were consistent with the amino acid sequence deduced from the DNA sequence. About 50% of the recombinant epimerase retained the *N*-terminal methionine residue whereas this residue was absent in the 'wild-type' epimerase. The partial processing of the *N*-terminal methionine may be due to the very high level of epimerase expression resulting in the *E. coli* enzymatic cleavage system for removal of the *N*-terminal methionine not being able to cope.

S N E D L F I C I D H V A Y -	'wild-type' epimerase
M S N E D L F I C I D H V A Y -	recombinant epimerase

Figure 3.20: The *N*-terminal amino acid sequence of the 'wild-type' and recombinant epimerases. About 50% of the recombinant epimerase protein still retains the *N*-terminal methionine (underlined) which has been completely cleaved from the 'wild-type' enzyme.

3.1.10 EPIMERASE CRYSTALLIZATION

An initial screen for optimum conditions for growing epimerase crystals was set-up using the purified 'wild-type' epimerase (see section 2.2.8 for experimental details). 'Wild-type' enzyme was chosen over recombinant enzyme because it had no *N*-terminal heterogeneity. Of the 46 crystallization conditions tested, 3 produced crystals after one week at 4°C. These were conditions 9, 20 and 30 in Table 2.4. The best crystals (i.e. the largest and most regular in appearance) were formed in a buffer containing 0.2 M ammonium acetate, 0.1 M citrate (pH 5.6) and 30% PEG 4000 as a precipitant (condition 9). The best crystals have the appearance of large disordered elongated rectangular flakes or plates. A similar set-up with the same precipitant using the purified recombinant epimerase was found to produce crystals of a similar appearance.

A second screen was carried out, with both the 'wild-type' and recombinant epimerases, using conditions that varied slightly around the best conditions identified in the first

screen. Small spikey clumps of crystals, needle-like in appearance, were produced in 0.2 M ammonium acetate, 0.1 M citrate (pH 5.5) and 30% PEG 4000. These conditions are very similar to those that produced the best crystals in the first screen, but the crystals in this second screen were considered inferior to the initial crystals.

A third screen, in which buffer type and PEG type and concentration were varied was carried out on both recombinant and 'wild-type' epimerase, but this screen did not produce useful crystals.

A final crystallization trial was conducted for recombinant epimerase using the best conditions found in screen 1 for the 'wild-type' epimerase (i.e. 0.2 M ammonium acetate, 0.1 M citrate (pH 5.6) and 30% PEG 4000 as precipitant). Larger hanging drops were set-up by increasing the amount of the buffer/precipitant used to 2 µl and the amount of protein used to 2 µl to produce a hanging drop size of 4 µl. This produced larger crystals, although still poorly ordered, with the appearance described for the initial screen. These crystals are shown in Figure 3.21. The crystals were found to diffract to 4.5 Å.

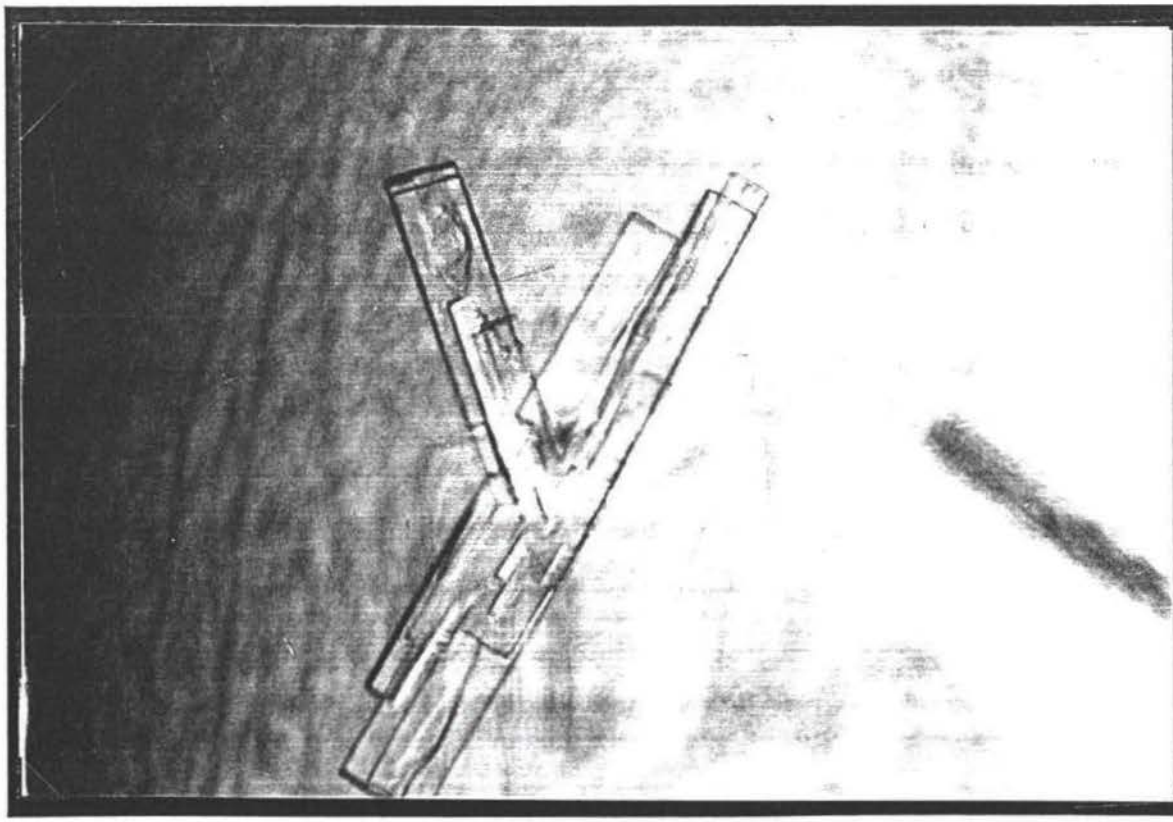

Figure 3.21: Photograph of crystals of the recombinant *P. shermanii* epimerase grown from buffer condition No. 9 of screen 1 (0.2 M ammonium acetate, 0.1 M citrate (pH 5.6) and 30% PEG 4000 as precipitant) in 4 µl hanging drops.

CHAPTER 4: DISCUSSION

4.1 DNA MANIPULATION

4.1.1 THE AMPLIFICATION OF THE EPIMERASE GENE BY PCR

A 454 bp PCR product containing the gene (minus the A nucleotide of the *N*-terminal methionine ATG codon) coding for methylmalonyl-CoA epimerase from *P. shermanii* was amplified by PCR. PCR products were not obtained reproducibly, or in high yield, unless the heating block of the PCR machine was pre-heated to 94°C before loading the reaction tubes. Although purified primers were used in all successful PCR experiments, it seems unlikely that primer purity was a factor in the failure of preliminary PCR trials. Apart from the need to pre-heat the heating block, the main determinants of product yield at the 52°C annealing temperature were [Mg^{2+}], and having enough cycles in the PCR amplification. The values of both these parameters (4 mM Mg^{2+} and 35 cycles) are greater than those typically required in comparable amplifications from bacterial genomic DNA (Saiki, 1990).

4.1.2 CONSTRUCTION OF THE EPIMERASE EXPRESSION PLASMID pTEEX

The 0.454 kbp PCR product, containing virtually all of the epimerase gene, was prepared for ligation into the *E. coli* expression vector pT7-7 by sequential treatment with T4 DNA polymerase and *Eco*RI. The pT7-7 vector was prepared for ligation by sequential treatment with *Nde*I, Klenow, and *Eco*RI. Several ligation experiments were carried out that were unsuccessful in that no insert DNA was found in any of the hundreds of plasmid 'mini-preps' isolated from ampicillin-resistant colonies resulting from the transformation of ligation reaction mixes into *E. coli* XL1-Blue. Because of the difficulty in obtaining the desired ligation product, procedures for producing the treated PCR product and pT7-7 vector were gradually modified until the use of neutral phenol and neutral phenol-chloroform extractions, and precipitation steps, had been eliminated, with the exception of the initial purification of the PCR product from the PCR reaction. Along with elimination of these reagents and procedures, the previous use of gel-purified fragments was discontinued. These changes may have been responsible for the ligation reactions finally bearing fruit in the form of 'mini-preps' that contained insert DNA. Several products now available for molecular biology would have simplified the procedures for the treatment of the PCR product and pT7-7 that were finally adopted

(section 2.2.1.4). For example, the use of StrataClean™ resin (Stratagene) would have eliminated the need for many of the heat inactivation steps.

Although the ligation described in section 2.2.1.4 did produce plasmids with insert DNA, these occurred at the low frequency of approximately 1 in 15 plasmids. Further, not all the plasmids with insert DNA were the desired pTEEX plasmids; at least some produced a recombinant protein that was larger than the recombinant epimerase, e.g. plasmids #23 and #30 produced proteins that were larger than that produced by pTEEX(#4) (see Figure 3.6). It is difficult to attribute this low efficiency of the desired ligation reaction to a particular source, but it is possible that the treatment of the DNA for ligation was not always completely effective in generating the desired ends. In particular, incomplete inactivation of the Klenow polymerase and/or incomplete digestion by *Eco*RI in the preparation of the pT7-7 vector could have been responsible for the high background of insert-free plasmids that was seen. In addition, if a small proportion of the pT7-7 vector had escaped cleavage by *Nde*I, this could also have caused a high background of insert-free ligation products.

With the benefit of hindsight, a more efficient ligation strategy could have been developed. The EPCRB PCR primer could have been used to introduce a recognition site for a restriction endonuclease that is present in the pT7-7 multiple cloning site downstream of the *Eco*RI site and that, like *Eco*RI, does not occur in the epimerase gene, e.g. *Bam*HI or *Hin*dIII. This site would have been used for the ligation of appropriately treated pT7-7 and PCR product, and the ligation reaction digested with *Eco*RI, to linearise circular DNA not containing an insert, before transformation. This strategy would have solved most potential problems of a high insert-free plasmid background in this ligation, with the exception of the incomplete inactivation of Klenow.

4.1.3 CHARACTERIZING THE PUTATIVE pTEEX PLASMIDS

Putative pTEEX plasmids, formed in the ligation of treated PCR product and pT7-7 vector, were identified by agarose gel electrophoresis at a frequency of about 1 in 15 from 'mini-prep' plasmids and transformed into *E. coli* SRP84/pGP1-2 to test if they would support the production of a recombinant protein that had the electrophoretic mobility expected for the 16.72 kDa epimerase protein. The pGP1-2/pT7-7 dual plasmid system of Tabor and Richardson (1985) was used to express the recombinant proteins. In this expression system the cell culture is subjected to a brief heat shock at 42°C. The elevated temperature denatures the temperature-sensitive product of λ repressor *cI857* gene in pGP1-2, which results in the transcription of the T7 RNA polymerase gene from the λ P_L promoter on pGP1-2. Once this transcript as been translated, the T7 RNA polymerase

transcribes any gene under the control of the ϕ10 T7 promoter, in pT7-7, and the large amounts of mRNA made saturate the *E. coli* translation machinery, substantially decreasing the production of host proteins in cases where the foreign gene is produced at high levels (30%+ of total cell protein). A variant of this expression system, developed by Studier *et al.* (1990), in which the T7 RNA polymerase gene is integrated into the *E. coli* chromosome under the control of the *lac*UV5 promoter in *E. coli* strain BL21(DE3), was not tested for pTEEX-based epimerase production because of the relatively high cost of using the gratuitous inducer IPTG (isopropyl β-D-thiogalactopyranoside) on a large scale.

Induction of cultures of SRP84/pGP1-2 transformed with putative pTEEX plasmids resulted in the expression of the recombinant proteins. As expected, the control strain bearing the pT7-7 vector instead of a putative pTEEX plasmid did not show a band corresponding to these proteins when the whole-cell protein of induced cultures was analysed by SDS-PAGE. The recombinant proteins expressed in induced SRP84/pGP1-2/putative-pTEEX cultures all migrated above the 20.1 kDa marker protein (Figure 3.6). At this stage no 'wild-type' epimerase had been purified. Therefore, it was not possible to say if the unexpectedly low electrophoretic mobility of the recombinant proteins was due to an error in the putative pTEEX constructs, resulting in the production of proteins that were larger than the 'wild-type' epimerase, or if the epimerase proteins simply migrate anomalously in discontinuous modified Laemmli Tris-glycine 20% SDS-PAGE gels. When purified 'wild-type' epimerase became available the anomalous migration of epimerase in these gels was confirmed. Prior to the purification of 'wild-type' epimerase, the insert DNA of one of the putative pTEEX constructs (#4) was completely sequenced and shown to be identical to the sequence described by Davis (1987). This indicated that the unexpectedly low mobility of the recombinant epimerase on SDS-PAGE gels was probably due to the anomalous migration of epimerase. One reason for initially doubting this welcome result was the magnitude of the anomalous migration, which predicted a M_r for the recombinant protein of about 21.6 kDa (section 3.1.9.2), ~30% higher than the expected M_r of 16.7 kDa.

If time had permitted, it would have been interesting to fully sequence the plasmid 'mini-preps' #23 or #30 to determine what error(s) was responsible for the higher M_r of the recombinant protein produced by these plasmids.

4.2 EPIMERASE PROTEIN PURIFICATION:

4.2.1 PURIFICATION OF 'WILD-TYPE' EPIMERASE FROM *P. SHERMANII*

The results of the purification of the homodimeric 'wild-type' epimerase from *P. shermanii* were similar to those reported by Leadlay (1981). The current work achieved a 215-fold purification with an overall yield of 63% that gave approximately 11.8 mg of purified epimerase enzyme from 40 g of cell paste. In the earlier study of Leadlay (1981) a 96-fold purification with an overall yield of 38% gave 88 mg of protein from 350 g of cell paste.

As use of a heat treatment step early in the purification of recombinant epimerase, while not without potential drawbacks, greatly facilitated the purification. The similar addition of a heat treatment step in future 'wild-type' epimerase purifications to exploit the enzyme's thermostability could prove useful. However, very little is known about the proteolytic enzymes that might be present in a *P. shermanii* extract that could attack the epimerase at higher temperatures. It is also not known whether the *P. shermanii* proteins might be more stable on average than *E. coli* proteins and so not denature and precipitate as readily. These questions need to be investigated before a heat treatment step could be incorporated into the 'wild-type' epimerase purification scheme.

4.2.2 PURIFICATION OF RECOMBINANT EPIMERASE PRODUCED IN INDUCED *E. COLI* SRP84/pGP1-2/pTEEX

Prior to the large-scale (5 L) induction experiments, it was decided to test the solubility of the recombinant epimerase protein produced *in vivo*, because the proteins of foreign genes expressed to a high level in *E. coli* may form insoluble inclusion bodies (Chaudhuri, 1994). Foreign bacterial proteins expressed in *E. coli* are typically exempt from the risk of inclusion body formation, but this is not always the case. For example, under certain growth conditions the *P. shermanii* methylmalonyl-CoA mutase will form inclusion bodies when expressed in *E. coli* (McKie *et al.*, 1990), as will the pNGase F enzyme from *Flavobacterium meningosepticum* (G.E. Norris, personal communication). From the SDS-PAGE gel shown in Figure 3.7, the recombinant epimerase from *E. coli* was found to be 95-100% soluble on the basis of its distribution in the pellet and supernatant fractions.

The purification of 10 g of the recombinant epimerase induced from the *E. coli* expression strain SRP84/pGP1-2/pTEEX is summarised in Table 3.2 and Figure 3.10.

The purification steps were identical to those used for the 'wild-type' epimerase purification except for the addition of a heat treatment step early in the purification scheme. The advantages of using a simple heat treatment of *E. coli* extracts for the purification of thermostable recombinant proteins expressed in *E. coli* has been noted (Patchett *et al.*, 1989), and the 15 min incubation at 70°C was particularly effective for the recombinant epimerase. The high thermostability of epimerase relative to most of the *E. coli* protein contaminants in the extract resulted in the selective denaturation and precipitation of the majority of the *E. coli* proteins.

The elution profile of the recombinant epimerase from the hydroxyapatite column (Figure 3.9) consists of two major and several smaller peaks. When analysed by SDS-PAGE, the two major peaks, and most of the other A_{280}-absorbing fractions, contained some epimerase protein. That the recombinant epimerase should be spread so widely over the 10-300 mM NaP buffer (pH 6.5) gradient is puzzling. The first major epimerase peak elutes at ~90 mM NaP, and the second at ~150 mM. In the hydroxyapatite purification step for the 'wild-type' epimerase, the enzyme eluted at ~150 mM NaP. This might suggest that there is some difference between the recombinant epimerase protein in the fractions corresponding to the two peaks, but pooling these fractions for the final purification step did not seem to compromise the quality of the purified enzyme with regard to its specific activity or ability to form crystals. Any difference between the epimerase protein in the two peaks from the hydroxyapatite separation may be minor, and further work to understand the elution behaviour of recombinant epimerase from the hydroxyapatite column is not of high priority.

The yield of purified recombinant epimerase per gram of cell paste was at least 20 times greater than the yield of 'wild-type' epimerase from *P. shermanii*. The heat-treated *E. coli* cell lysate supernatant fraction consisted predominantly of recombinant epimerase and was found to be free of NADH oxidase activity making this material suitable for use as a coupling enzyme in methylmalonyl-CoA mutase and transcarboxylase assays.

The use of PEI precipitation of nucleic acids as a purification step should be avoided in future work. The first recombinant epimerase purification using PEI led to the precipitation of both nucleic acids and epimerase with the consequent loss of the epimerase.

4.3 CHARACTERIZATION OF THE EPIMERASE ENZYMES

4.3.1 DETERMINATION OF THE PURIFIED EPIMERASE PROTEIN CONCENTRATIONS

The concentrations of the purified 'wild-type' and recombinant epimerase were determined by measurements using the Biuret, bicinchoninic acid, and UV $A_{205/280}$ methods (Table 3.3). The average of the Biuret and UV $A_{205/280}$ measurements was taken as the final concentration of the 'wild-type' and recombinant epimerase (Table 3.4) because, unlike the bicinchoninic acid method, these two methods are largely independent of the amino acid composition of the protein measured. Given the importance of an accurate determination of protein concentration in the measurement of specific activity, it was disappointing that the protein concentrations of the purified 'wild-type' epimerase determined by the UV $A_{205/280}$ method and the Biuret method were so different. As both these methods are supposed to be largely independent of the amino acid composition of a protein, they should theoretically give similar results. That the values obtained (Biuret 11.8 mg/ml, UV 9.3 mg/ml) differed by 23% was therefore unexpected, especially when considering that the corresponding values for the recombinant protein only differed by 6%. The large discrepancy between the Biuret and UV $A_{205/280}$ methods for the 'wild-type' epimerase could be due to non-protein contaminants, such as lipids, carbohydrate or DNA, present in the purified epimerase, but this is speculative. Similarly, a difference in the conformation of the two purified proteins might be responsible for unusual $A_{205/280}$ measurements for one of the enzymes. In future work it might be possible to resolve this by carrying out UV $A_{205/280}$ measurements of protein concentration in a 5 mM orthophosphoric acid solution.

4.3.2 SPECIFIC ACTIVITY STUDIES

The specific activity of the 'wild-type' and recombinant epimerase was measured as described in the Materials and Methods section 2.2..5.1. The specific activity of the final purified 'wild-type' epimerase was 86 U/mg. The specific activity of the final purified recombinant epimerase was 84 U/mg. Because of the large error inherent in these specific activity measurements, it is not possible to say for certain that the 'wild-type' and recombinant epimerases have exactly the same specific activity, only that the specific activity values for the two enzymes agree within the limits of experimental error.

The results summarised in Figures 3.18 and 3.19 show the effects of incubation of 'wild-type' and recombinant epimerase samples with a number of divalent metal ions. Similar trends were observed for both the 'wild-type' and recombinant epimerases. Both showed

a marked increase in activity of 255-280% compared to the control upon incubation with 1 mM Co^{2+} at 60°C for 5 h. This is very similar to the 283% activation seen by Leadlay (1981) for *P. shermanii* epimerase, even though those incubations were with 20 μM Co^{2+} for 20 min at room temperature. In contrast to the results of Leadlay, Cd^{2+} and Zn^{2+} were also found to stimulate the activity of both 'wild-type' and recombinant epimerase. The epimerase from rat liver is also activated by Co^{2+}, and to a lesser extent by Co^{3+}, Fe^{2+} and Mn^{2+}, but is completely inhibited by Zn^{2+}, Cd^{2+} and Cu^{2+} (Stabler and Allen, 1988). Clearly, while activation by Co^{2+}, and to a lesser extent by Ni^{2+} and Mn^{2+}, and inactivation be incubation with EDTA, seem to be common features of the epimerase enzymes from *P. shermanii* and rat liver, the response of these two enzymes to other metal ions is variable, and may depend on the incubation conditions used. When purified in the presence of EDTA, purified rat liver epimerase binds 1 Co^{2+} ion per subunit, but the kind of metal ion bound *in vivo*, or the degree of saturation of the metal ion binding site, is not known for any methylmalonyl-CoA epimerase.

Epimerase samples incubated for the longer time of 10 h at 60°C showed a further increase in specific activity, but this was small suggesting that the activation is largely complete after 5 h and that the enzyme is fairly stable at 60°C under these conditions. The 'wild-type' epimerase samples incubated with the divalent metal ions Ni^{2+}, Mn^{2+}, Zn^{2+} and Cd^{2+} at 60°C for 5 h all showed an increase in the specific activity of epimerase relative to the control although not as great as for samples incubated with Co^{2+}.

The reversible inactivation of methylmalonyl-CoA epimerases by EDTA, and the increase in activity due to incubations with divalent metal ions, suggests a catalytic requirement for a divalent metal ion. This is strongly supported by the findings of Leadlay (1981), where atomic-absorption spectroscopy showed that the 'wild-type' epimerase contains tightly bound metals in stoichiometric amounts, although a correlation between metal content and enzymatic activity is yet to be demonstrated. However, the involvement of metal ions has been demonstrated, or is at least suspected, in several other enzyme-catalysed reactions where thioester-stabilized carbanions are thought to be enzyme-bound intermediates (Roeder and Kohlhaw, 1980). Interestingly, mandelate racemase from *Pseudomonas putida*, where there is evidence for the formation of a carbanion intermediate (Kenyon and Hegeman, 1970), has an absolute requirement for a divalent metal ion (Fee *et al.*, 1974). By analogy, a mechanistic role can be envisaged for a divalent metal ion in epimerase (Leadlay, 1981), although the mechanism(s) for activation and possible stabilization need further investigation.

4.3.3 EPIMERASE ELECTROPHORETIC MOBILITY DETERMINATION

The 'wild-type' and recombinant epimerase proteins showed anomalously low electrophoretic mobility in SDS-PAGE gels using the modified Laemmli discontinuous Tris-glycine buffer system describe in section 2.2.6.3. An apparent M_r of 21.6 kDa, 30% higher than the expected 16.7 kDa, was obtained. Other proteins have been shown to behave anomalously in Laemmli discontinuous Tris-glycine alkaline buffer systems (Andrews, 1986), although usually only glycoproteins show M_r deviations greater than 30% (Låås, 1989).

The Weber and Osborn continuous phosphate-buffer system gave a much more accurate estimation of the M_r (16.6 kDa). This result is consistent with the M_r of 16.72 kDa (*N*-terminal methionine on) calculated from the amino acid sequence deduced from the known DNA sequence of the *P. shermanii* methylmalonyl-CoA epimerase gene.

Although it is well known that some proteins exhibit anomalous electrophoretic mobilities in SDS-PAGE experiments that employ a Laemmli-type discontinuous alkaline Tris-glycine buffer system, these systems are still very popular. This is probably because the anomalous migration is usually less than 10%, and because the discontinuous buffer systems produce very sharp, well resolved protein bands that are both aesthetically pleasing and effective for samples containing complex mixtures of proteins. However, if any uncertainty develops over the identity of a protein band due to anomalous migration, the Weber and Osborn continuous phosphate buffer system should be considered as an option to confirm the M_r of the protein.

4.3.4 *N*-TERMINAL SEQUENCING

The *N*-terminal sequence data, along with SDS-PAGE analysis using the modified Laemmli and the Weber and Osborn buffer systems, confirms the identity of the recombinant protein as an authentic epimerase. The heterogeneity introduced by 50% of the recombinant epimerase molecules still retaining their *N*-terminal methionine residue, compared to that of the 'wild-type' epimerase in which all *N*-terminal methionine residues had been removed (Figure 3.20) apparently did not affect the specific activity or behaviour of the recombinant epimerase in the crystallization trials.

4.4 EPIMERASE CRYSTALLIZATION

Of the 46 conditions screened in the first crystallization trial with 'wild-type' epimerase, a solution of 0.2 M ammonium acetate, 0.1 M citrate (pH 5.6) containing 30% PEG 4000 as a precipitant (condition number 9, Table 2.4) produced the most promising crystals. It was found that increasing the size of the hanging-drop from 2 µl to 4 µl did improve the size of the crystals produced although the crystals were still poorly ordered. The best crystals produced using the above buffer/precipitant combination were found to diffract to 4.5 Å resolution. The presence of a minor contaminant in the purified 'wild-type' epimerase, and the band-blurring on SDS-PAGE gels and *N*-terminal heterogeneity seen for the purified recombinant epimerase, did not prevent crystallization. Both purified epimerases behaved similarly in crystallization trials.

In an attempt to produce crystals of a more ordered nature that might show higher resolution in their diffraction patterns, the most promising conditions that gave the best crystals were manipulated by varying the pH of the buffer used, buffer components and the percentage and type of precipitant used. Unfortunately, none of the various conditions investigated resulted in improved crystal quality. One important point to remember when examining epimerase crystallization trials is to do all the microscope observations at 4°C because the epimerase crystals seem to melt away at room temperature.

Recently, more extensive crystallization trials by Heather Baker in the Massey University Chemistry and Biochemistry Department (personal communication, November 1994) have produced a major improvement in the epimerase crystals obtained. The most successful crystallization experiments used a 7 mg/ml solution of the recombinant epimerase protein in 0.02 M Tris/HCl buffer, pH 8, containing 0.1 mM EDTA, and a buffer/precipitant solution containing 0.2 M cacodylate (pH 6.8), 0.2 M ammonium sulphate and 18-20% PEG 20000. 2 µl hanging drops were made up of 1 µl of protein solution and 1 µl of buffer/precipitant solution. Single diamond-like cube shaped crystals were formed after 2-4 weeks at 4°C. This crystal shape differs greatly in appearance from the crystals grown in the initial study using 30% PEG 4000 as precipitant. These new crystals diffract to 2.6 Å resolution, and can be grown reproducibly.

4.5 CONCLUSIONS AND FUTURE WORK

Given the yield of 66 mg of highly purified recombinant *P. shermanii* methylmalonyl-CoA epimerase from 10 g of induced *E. coli* SRP84/pGP1-2/pTEEX using a purification scheme that has not yet been optimised for *E. coli*, and also given the good quality crystals that can be grown from this material, it is reasonable to hope that an X-ray crystal

structure might be available for this small, homodimeric, metal ion-activated enzyme in the next 2 years. It may be possible to improve the homogeneity of the recombinant epimerase preparation by adding cobalt an various stages of the purification, or in the crystallization buffers, to saturate all the potential Co^{2+}-binding sites and displace other metals that may be bound at these sites. A high-resolution X-ray crystal structure would open the way for rational site-directed mutagenesis experiments to probe the structure-function relationships of this enzyme, in particular aspects of metal ion-mediated catalysis and stability. In the meantime, with an abundant source of recombinant protein, it will be possible to investigate improvements to the purification scheme (e.g. the inclusion of Co2+ in the heat treatment step for stability and activity, or elimination of the heat treatment and other steps), to examine the metal ion effects on specific activity in more detail, and to further characterize any significant differences that exist between the recombinant and 'wild-type' epimerases. The results of the present study would suggest that the recombinant enzyme is sufficiently similar in most respects to the 'wild-type' epimerase to justify its use in future work on this enzyme.

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