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

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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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# LIST OF ABBREVIATIONS

| | |
|---|---|
| $A_x$ | 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.*,

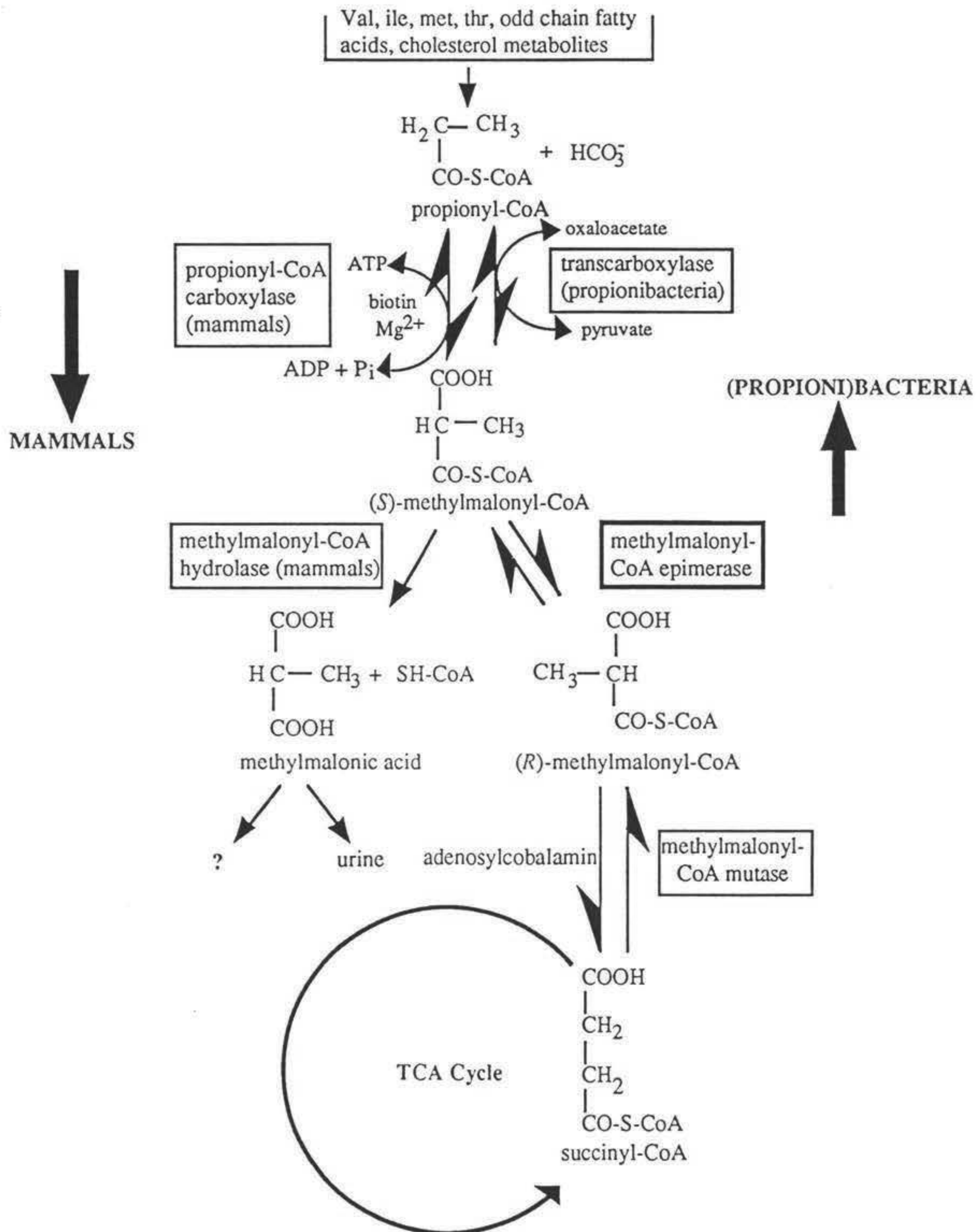


**Figure 1.1**: The reaction pathways involved in the interconversion of succinyl-CoA and propionyl-CoA. In propionibacteria the pathway functions from bottom to top. (i.e. succinyl-CoA is converted to propionyl-CoA, and subsequently propionate). In mammalian tissues the pathway works from the top to 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

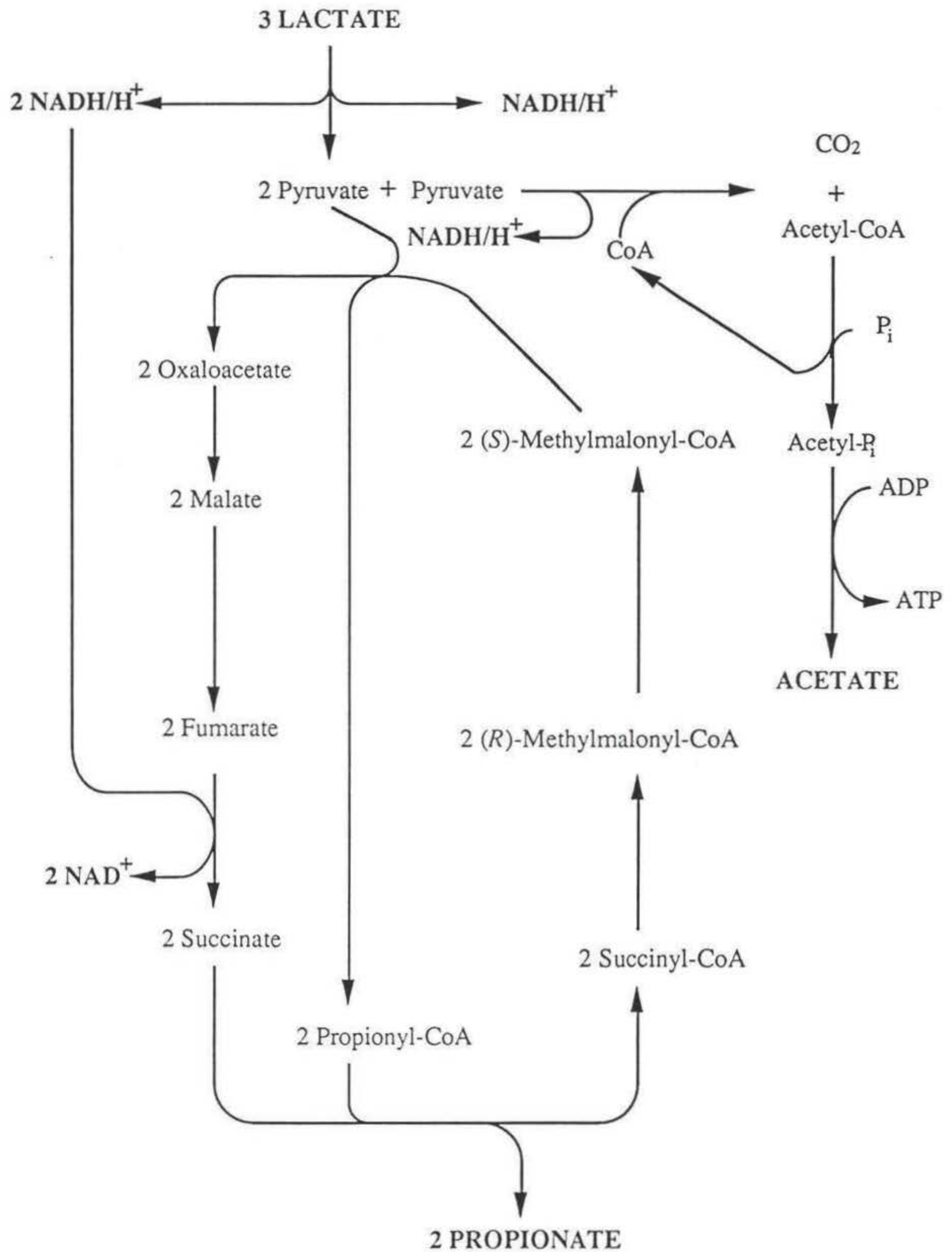


**Figure 1.2**: The metabolism of lactate in propionibacteria (after Asmundson (1982)).

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 $\phi$10 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.