Studies on Methylmalonyl-CoA Mutase from Escherichia coli.

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Suresh Kannan

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Abstract

Methylmalonyl-CoA mutase (MCM, E.C. 5.4.99.2), a coenzyme B_{12}-dependent enzyme, catalyses the inter conversion of succinyl-CoA and methylmalonyl-CoA. The gene (*sbm*) encoding this enzyme is found in *Escherichia coli* (*E. coli*) at 62.3min on the *E. coli* chromosome. However, the metabolic role of this enzyme in the organism is not known. This project involves an investigation into this metabolic obscurity.

The *sbm* gene is part of a four gene operon which also includes *arg*K (or *ygfD*) that codes for a protein kinase catalysing the phosphorylation of two periplasmic binding proteins involved in cationic amino acid transport, *ygfG* that codes for methylmalonyl-CoA decarboxylase and *ygfH* that codes for propionyl-CoA: succinyl-CoA transferase. From existing literature we suspect that this operon, including the *sbm* gene, could be involved in the utilisation of unusual carbon sources such as succinate and propionate. An insertion mutant of the *sbm* gene created by transposon mediated mutagenesis was used for investigating the role of this gene. The wild type *E. coli* K12 strain, *E. coli* TR6524 and the mutant *E. coli* K12 (*sbm*::*MudJ*) were used in this study.

Growth of the two strains (*E. coli* TR6524 and FA1P1) in minimal media with three different concentrations (0.05, 0.5, 5.0μg/mL) of vitamin B_{12} and in the presence succinate, propionate or glucose as the sole source of carbon, was studied. Growth was typical in media with glucose with no major differences in the growth pattern of the wild type and mutant strain. However, the two strains exhibited a differential growth pattern in media containing succinate, with the wild type growing faster than the mutant, indicating the role of the *sbm* gene in the utilisation of this carbon source. Growth in media containing propionate as the sole carbon source indicated only marginal differences in the growth pattern of the wild type and mutant strain. This result possibly suggests that the other pathways for propionate utilisation in *E. coli* compensate for the lack of a functional Sbm protein in the mutant strain.

Promoter analysis indicated the presence of a promoter induced by σS, a transcription factor involved in the expression of proteins under stress or stationary phase growth conditions. Reverse transcription polymerase chain reaction (RT-PCR) studies of the genes of the *sbm* operon (*sbm*-*arg*K-*ygf*G-*ygf*H) under the same growth conditions were carried out. Densitometric analysis of the PCR products suggested that the transcription level of *sbm* was higher in *E. coli* grown in succinate as compared to when grown in glucose and not as much when grown in propionate indicating a transcriptional level control of the *sbm* gene expression during the utilisation of succinate. RT-PCR studies also indicated a higher level of transcription of the gene in the stationary phase of the culture during the utilisation of succinate. Real time reverse transcription PCR (QPCR) analysis was used for the absolute quantification of the transcription of the genes of the *sbm* operon. An increase in the mRNA levels corresponding to the *sbm, arg*K and *ygf*G genes was observed as *E. coli* TR6524 growth reached stationary phase, in the presence of succinate or propionate as the sole source of carbon as compared to glucose, In contrast, the highest mRNA levels corresponding to the *ygf*H gene were observed in the early log-phase of growth. This indicated a differential transcriptional level control of the genes within the operon. This study further established the possible role of this operon in the utilisation of succinate and propionate.

The MCM enzyme activity measurement in the whole cell extracts of the wild type *E. coli* K12, grown under the above mentioned conditions, led to the first ever measurement of MCM activity in wild type *E. coli*. These measurements also revealed a four fold increase of the MCM specific activity in the case of growth in succinate (4.76×10^{-3}U/mg) and a two fold increase for growth in propionate (2.79×10^{-3}U/mg) compared to that observed with growth in glucose (1.37×10^{-3}U/mg), indicating a significant level of involvement of the enzyme in succinate utilisation, and to a lesser extent in propionate utilisation.

The proteomic analysis to understand the gene expression pattern of *E. coli* TR6524 was carried out using cells harvested at the stationary phase. The results showed that growth conditions induced the expression of transport related (HisJ, DppA) and energy generating proteins (PckA, AceF) required by *E. coli* to cope

with the stressful growth conditions. However, Sbm was not identified among the limited protein spots that were analysed.

Finally, *E. coli* K12 *sbm* gene was successfully cloned into *B. cereus* SPV leading to the development of a metabolically engineered polyhydroxyalkanoate producing strain of *B. cereus*. The intention was to provide the bacteria with a natural intracellular source of propionyl-CoA, leading to the production of the P(3HB-*co*-3HV) copolymer from structurally non related carbon sources like glucose.

Hence, this work has initiated investigation into the metabolic role of the *sbm* gene product in *E. coli*. In addition, it has also led to the use of this gene product in metabolic engineering applications.

List of abbreviations

2DE/ 2-DE	Two dimensional electrophoresis
AdoCbl	Adenosyl cobalamin
CAP	Catabolic activator protein
DMB	Dimethylbenzimidazole
GABA	Gamma amino butyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gDNA	Genomic DNA
HPLC	High performance liquid chromatography
IEF	Iso-electric focussing
MCM	Methylmalonyl-CoA mutase
MeCbl	Methyl cobalamin
MMCoA	Methylmalonyl-CoA
ORFs	Open reading frames
P(3HB)	Poly-3-hydroxybutyrate
P(3HB-co-3HV)	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
PHA	Polyhydroxyalkanoates
QPCR	Real time quantitative PCR
RNAP	RNA polymerase
RT-PCR	Reverse transcription PCR
TCA	Tricarboxylic acid

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Chapter 1

Introduction

1.1 Vitamin B_{12}- a brief look into the past

The history of vitamin B_{12} (Figure 1.1) dates back to 1925 with the descriptions by George Whipple, George Minot and William Murphy of the anti-pernicious anaemia factor which won them the Nobel Prize in 1934. This in turn led to the isolation of the cofactor by Smith and Folkers in 1948. This independent discovery changed the therapeutic treatment for pernicious anaemia from several grams of uncooked liver a day to a few micrograms of a red crystalline compound (Banerjee and Ragsdale, 2003).

Figure 1.1: Vitamin B_{12} crystals

1.2 Structure of coenzyme B_{12}

As major attempts were being made to elucidate the identity and structure of the anti-pernicious anaemia factor, Barker (1958) demonstrated that the transformation of glutamate to β-methylaspartate required a biologically active cobalamin. This observation stimulated an interest in the search for coenzyme B_{12}-dependent enzymes that led to the discovery of a biologically active form of cobalamin, methyl cobalamin (MeCbl). Presently, three classes of coenzyme B_{12}-dependent enzymes are recognised, the isomerases, the methyltransferases, and the reductive dehalogenases (Banerjee, 2003 & Banerjee and Ragsdale, 2003). The crystal structure of vitamin B_{12} determined by Hodgkin (1961) showed that it consists of a

low spin Co^{3+} ion that is equatorially ligated to four nitrogens donated by pyrroles A-D of the corrin ring (Figure 1.2). Unlike other tetrapyrroles, vitamin B_{12} has a built-in axial ligand appended from the periphery of ring D of the corrin macrocycle (Hodgkin and Lenhert, 1961). The cobalt ion is coordinated on the "lower" face by a nitrogen from the intramolecular base 5,6-dimethylbenzimidazole (DMB) and on the "upper" face by cyano-, methyl-, and deoxyadenosyl-groups forming vitamin B_{12}, MeCbl and AdoCbl or coenzyme B_{12} respectively (Banerjee and Ragsdale, 2003 & Brooks *et al*., 2004).

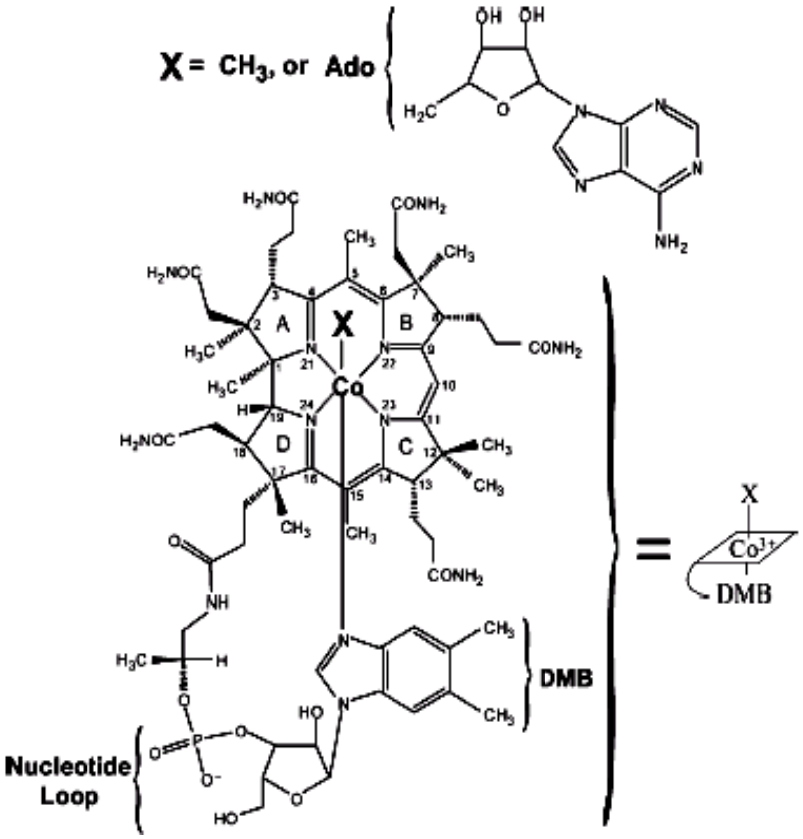

Figure 1.2: Chemical structure and numbering scheme for cobalamin. X indicates the upper axial ligand based on which cobalamin could become MeCbl or AdoCbl (from Brooks *et al*., 2004).

The stability of the oraganometallic cobalt-carbon bond (Co-C) of coenzyme B_{12} plays a key role in its reactivity. Enzymes utilising coenzyme B_{12} catalyse radical-induced rearrangement reactions or ribonucleotide reduction by homolytic cleavage of the Co-C bond (Brooks *et al*., 2004).

1.3 Coenzyme B_{12}-dependent isomerases

Twelve coenzyme B_{12}-dependent enzymes have been identified (Ludwig and Matthews, 1997 & Krautler, 2005). The discovery and isolation of glutamate mutase, the first B_{12} enzyme to be recognised, led to the discovery of the coenzyme forms of B_{12}. The reactive Co-C bond at the core of cobalamin, depending on whether it is in an active site of a methyltransferase or an isomerase, cleaves either heterolytically or homolytically, respectively (Banerjee, 1997). In all of the adenosylcobalamin dependent enzymes except ribonucleotide reductase, homolytic cleavage and generation of a substrate radical is followed by a rearrangement involving the interchange of the adjacent 'H' and 'R' substituents (Figure 1.6). These reactions, that include the inter-conversions of methylmalonyl-CoA and succinyl-CoA, glutamate and methylaspartate, and methyleneglutarate and methyl aconitate are chemically difficult rearrangements in which carbon-carbon bonds are broken and reformed (Ludwig and Matthews, 1997; Banerjee and Ragsdale, 2003 & Krautler, 2005). The interconversion of isobutyryl-CoA and n-butyryl-CoA, a reaction very similar to that catalysed by methylmalonyl-CoA mutase, involves the coenzyme B_{12}-dependent isobutyryl CoA mutase (Zerbe-Burkardt *et al*., 1998). Another group of mutases catalyse rearrangements in which carbon-oxygen or carbon-nitrogen bonds are broken and remade in the conversions of diols to aldehydes and of ethanolamine to ammonia and acetaldehyde. In these reactions, the shifts of 'OH' or 'NH_2' are followed by elimination of water or ammonia. A further group of mutases catalyse the migration of amino groups in lysine, ornithine, or leucine. Hence, the diversity of the migrating group for the reactions catalysed by coenzyme B_{12}-dependent mutases ranges from carbon containing groups (in methylmalonyl-CoA mutase, isobutyryl-CoA mutase, glutamate mutase, and methyleneglutarate mutase) to nitrogen (in ethanolamine ammonia lyase, D-ornithine aminomutase, and β- lysine mutase) and oxygen (in diol dehydratase) (Ludwig and Matthews, 1997 & Banerjee and Ragsdale, 2003).

Ribonucleotide reduction by adenosylcobalamin dependent ribonucleotide triphosphate reductase follows a unique pathway where the reaction is initiated by

formation of the deoxyadenosyl radical like the other mutases, however, the 'R' group (here, OH) does not migrate to the neighbouring carbon but instead gets eliminated (Ludwig and Matthews,1997).

1.3.1 Subclasses of isomerases

Crystal structures and spectroscopic studies have led to the recognition of two subclasses of coenzyme B_{12}-dependent isomerases that differ with respect to their mode of cofactor binding (Figure 1.3) (Ludwig and Matthews, 1997)

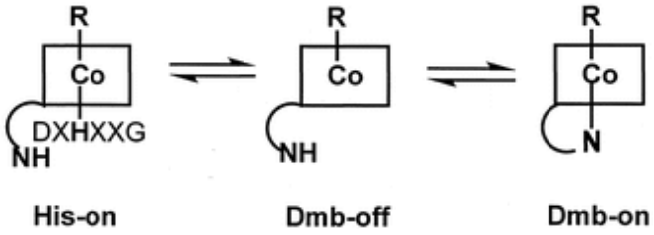

Figure 1.3: Conformation of cobalamin in solution. A schematic representation of the His on, base-off and base-on conformations of the cofactor in solution (Banerjee *et al*., 2003)

In solution and at physiological pH, the lower ligand position in cobalamin is occupied by the endogenous base, dimethylbenzimidazole, in a conformation that is referred to as 'Dmb-on'. In the 'Dmb-on' family of coenzyme B_{12}-dependent isomerases, the solution conformation of the cofactor is retained upon binding to the active site. This mode of coenzyme B_{12} binding has been seen in the crystal structures of diol dehydratase and ribonucleotide reductase (Banerjee and Ragsdale, 2003)

Protonation of the lower base, in AdoCbl, causes the dimethylbenzimidazole ligand in cobalamin to no longer coordinate with the cobalt and instead coordinate with the solvent leading to the 'Dmb-off' conformer. Crystal structures of the Class I or 'His-on' family of coenzyme B_{12}-dependent isomerases have revealed the presence of another cofactor conformation in which the lower axial ligand, dimethylbenzimidazole, is replaced by a histidine residue donated by the protein.

The histidine is embedded in a 'DX**H**XXG' sequence, which is the only primary sequence motif that appears to be conserved in both the coenzyme B_{12}-dependent isomerase and methyltransferase family members that use the same cofactor binding mode. In this mode, the nucleotide tail is bound in an extended conformation that results in dimethylbenzimidazole being >10Å removed from the cobalt to which it is coordinated in the absence of the enzyme. The isomerases in this subfamily include methylmalonyl-CoA mutase, glutamate mutase, methyleneglutarate mutase, isobutyryl-CoA mutase, and lysine 5, 6 aminomutase (Ludwig and Matthews, 1997 & Banerjee and Ragsdale, 2003).

1.3.2 Coenzyme B_{12} -dependent carbon skeleton isomerases

The carbon skeleton isomerases are the largest subfamily of coenzyme B_{12}-dependent enzymes found in bacteria, where they play important roles in fermentation pathways. The only exception is methylmalonyl-CoA mutase, which is found in both bacteria and man. Figure 1.4 shows the reactions catalysed by four of the coenzyme B_{12}-dependent carbon skeleton isomerases. The groups that migrate in the forward reaction are shown within the boxes (Banerjee, 2003).

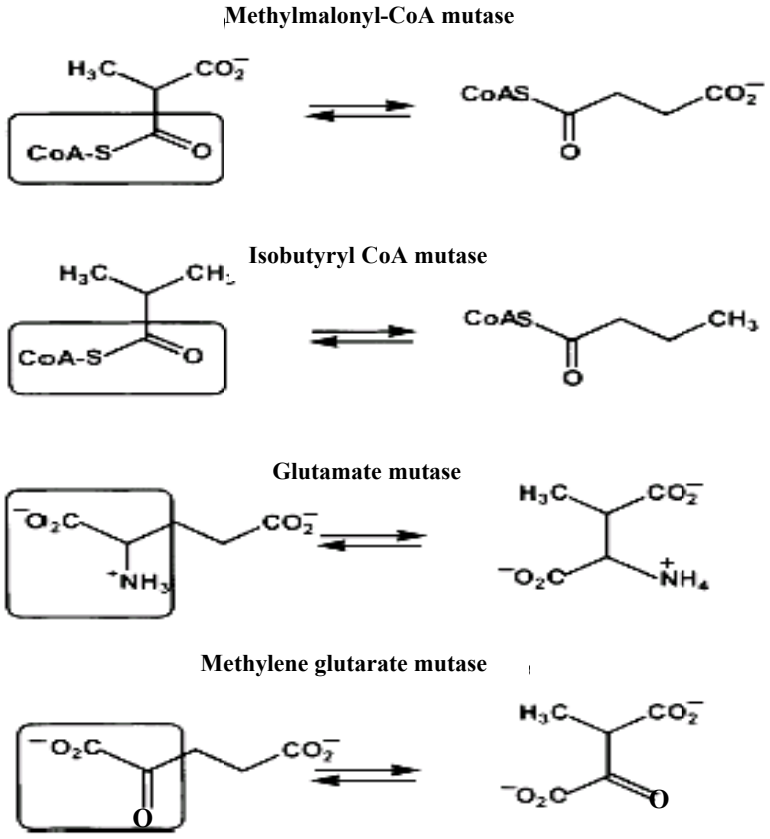

Figure 1.4: Reactions catalysed by carbon skeleton isomerases (Banerjee, 2003)

1.3.3 Rearrangement mechanisms of carbon skeleton isomerases

Crystal structure determinations and computational studies have helped in understanding the mechanisms of rearrangement catalysed by coenzyme B_{12}-dependent carbon skeleton isomerases. Polar radical pathways have been predicted for the carbon skeleton rearrangements catalysed by methylmalonyl-CoA mutase and glutamate mutase. However, the difference between the two reactions is the migration of a sp^2-hybridized carbon in methylmalonyl-CoA mutase and a sp^3 centre in glutamate mutase (Maiti *et al*., 1999 & Thoma *et al*., 2000).

1.4 Methylmalonyl-CoA mutase (MCM, E.C. 5.4.99.2)

Methylmalonyl-CoA mutase, glutamate mutase, isobutyrl CoA mutase and methyleneglutarate mutase all catalyse chemically similar reactions (Figure 1.4). MCM catalyses the reversible and stereospecific equilibration reaction of (2R)-

methylmalonyl-CoA and succinyl-CoA as shown in Figure 1.5 (Gruber and Kratky, 2001).

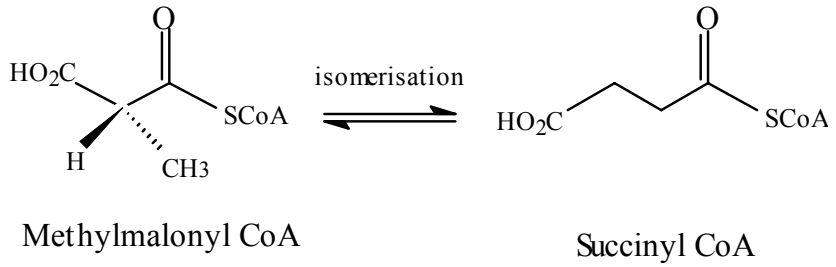

Figure 1.5: **The reaction catalysed by methylmalonyl-CoA mutase**

The rearrangements involving hydrogen abstraction from an aliphatic carbon, catalysed by the carbon skeleton mutases, are particularly difficult reactions that require a powerful catalyst. The reaction catalysed by MCM involves the positional interchange of a COSCoA group and a hydrogen atom on the vicinal carbon. The mechanism is shown in Figure 1.6.

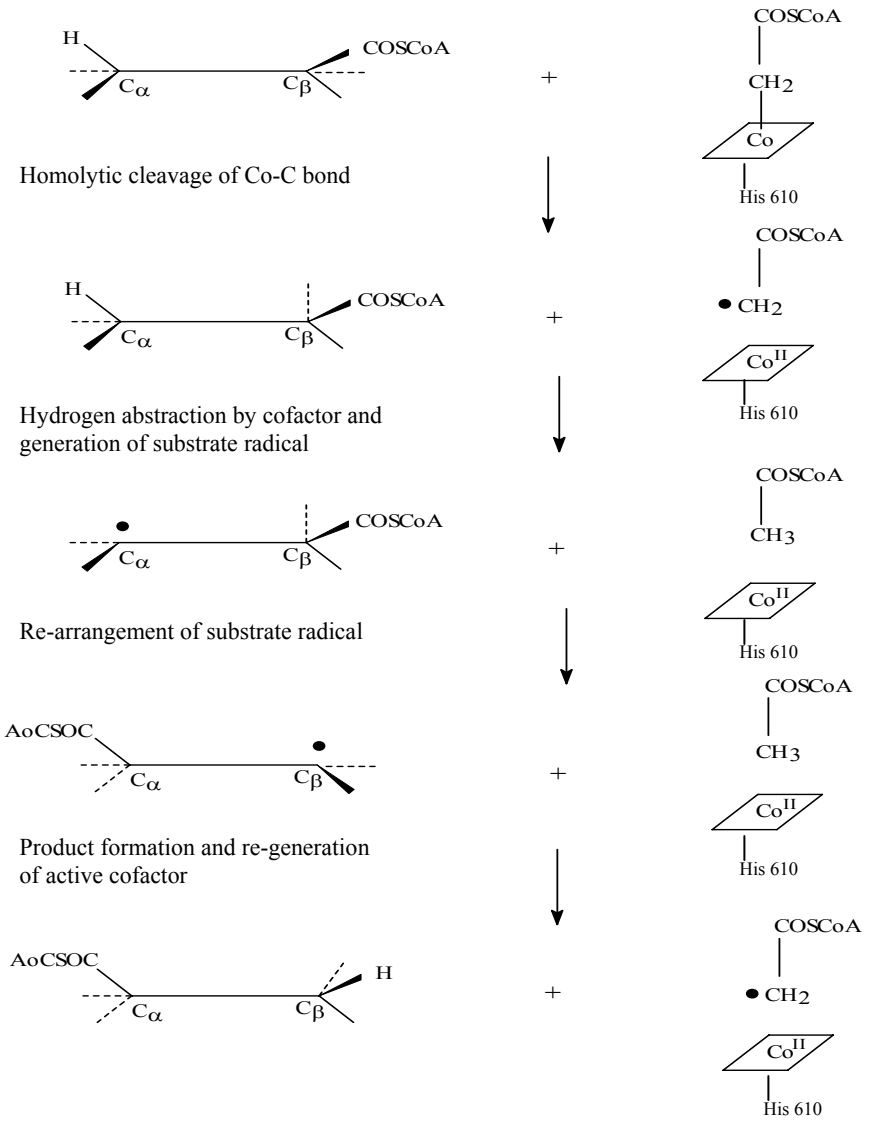

Figure 1.6: Reaction mechanism of the reaction catalysed by MCM

Catalysis is initiated by substrate induced homolysis of the Co-C bond leading to the formation of a 5'-deoxyadenosyl radical and a reduced cofactor with its metal atom in the cobalt (II) oxidation state (referred to as cob(II)alamin). The 5'-deoxyadenosyl radical subsequently abstracts a hydrogen atom from the bound substrate, thus generating a protein bound 5'-deoxyadenosine and a 'substrate-derived' radical. The rearrangement of the latter results in the formation of a 'product-related' radical. Retrieval of hydrogen from the methyl group of 5'-deoxyadenosine yields the product and regenerates the 5'-deoxyadenosyl radical, which can then recombine with the reduced cofactor (Roth *et al*., 1996; Ludwig and Matthews, 1997 &

Krautler, 2005). The enzyme has been reported to have three functions during the rearrangement reaction which include catalysis of the Co-C bond cleavage, protection of the intermediate radical from unwanted side reactions and the catalysis of the rearrangement itself (Mancia *et al*., 1999).

The majority of the bacterial carbon skeleton isomerases including MCM have been identified as heterodimers (or higher order oligomers of a heterodimer) with only one of the two subunits binding to the cofactor (Banerjee, 2003). The *Propionibacterium shermanii* (*P. shermanii*) and *Streptomyces cinnamonensis* (*S. cinnamonensis*) MCM contain two homologous but non-identical subunits:α-subunit (~79 kDa) and β-subunit (~65 kDa) (Korotkova and Lidstrom, 2004). In *Propionibacterium shermanii*, the alpha subunit of MCM binds a molecule of 5'-deoxyadenosylcobalamin and includes the active site while the beta subunit is inactive (Gruber and Kratky, 2001) (Figure 1.7).

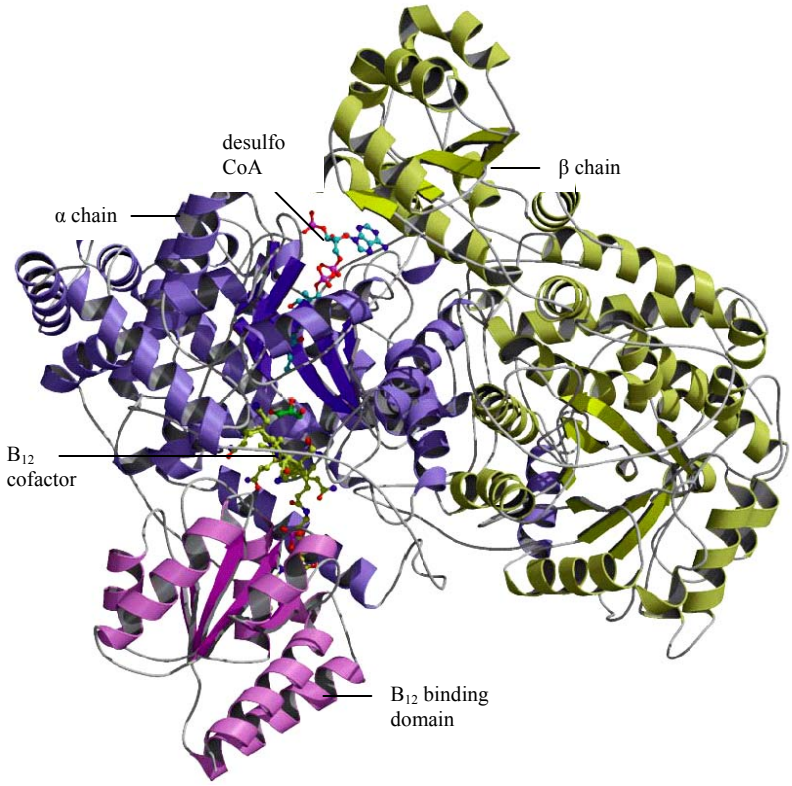

Figure 1.7: Schematic representation of the heterodimer structure of MCM from *Propionibacterium shermanii*. The figure shows the B_{12} cofactor molecule and the partial substrate desulfo-CoA. The β chain is in green, α chain is in blue (barrel domain) and the coenzyme B_{12} binding domain in magenta (Gruber and Kratky, 2001)

Crystal structures of *P. shermanii* MCM revealed that both α and β subunits consist of two principal domains: an eight-stranded α/β triose phosphate isomerase barrel $(\alpha/\beta)_8$ and a flavodoxin-like AdoCbl-binding fold. The cofactor is sandwiched between the $(\alpha/\beta)_8$ barrel and the flavodoxin-like AdoCbl-binding fold. The human, mouse, and *Escherichia coli* (*E. coli*) enzymes, however, are homodimers. (Korotkova and Lidstrom, 2004) and the structure of the homodimer, unlike the heterodimer, is not clear.

1.4.1 The biological function of MCM

1.4.1.1 Bacteria

In prokaryotes such as *P. shermanii,* MCM is involved in the synthesis of propionate from the tricarboxylic acid (TCA) cycle intermediates and serves to maintain the redox balance of the organism (Banerjee, 1997). The reaction catalysed by MCM in *S. cinnamonensis*, is a source of building blocks in polyketide antibiotic biosynthesis by directing the flux of carbon towards the raw materials for antibiotic assembly (Zerbe-Burkardt *et al*., 1998) in addition to its role in the conversion of succinyl-CoA into propionate (Roy, 1996 & Gruber and Kratky, 2001). In the case of polyhydroxyalkanoate-producing *Nocardia corallina* (*N. corallina*), Valentin and Dennis (1996) identified that the supply of the precursor for co-polymer, poly(3-hydroxybutyrate-co-3-hydroxyvalerate), P(3HB-co-3HV) production mainly depended on the availability of propionyl-CoA. The latter was obtained from the TCA cycle intermediate succinyl-CoA *via* MCM. A *N. corallina* mutant for the MCM gene was unable to grow on odd chain fatty acids but exhibited wild type growth on glucose, succinate and even-chain fatty acids. This suggested that propionate is utilised after its conversion to succinyl-CoA principally *via* MCM for its further oxidation or utilisation in biosynthetic pathways, in addition to the accumulation of polymers with high content of 3-hydroxyvalerate (Valentin and Dennis, 1996). These results have been the basis of metabolic engineering studies that have reported an increased P(3HB-co-3HV) production by recombinant *Salmonella typhimurium* (*S. typhimurium*) from non related carbon sources such as glycerol (Aldor *et al*., 2002). The conversion of succinyl-CoA to methylmalonyl-

CoA has been reported to be an important stage in rifamycin SV biosynthesis of *Amycolatopsis mediterranei* (Zhang *et al.*, 1999). In *Saccharopolyspora erythraea* (formerly *Streptomyces erythraeus*), it was observed that succinyl-CoA was isomerised to methylmalonyl-CoA before its incorporation into the macrocyclic lactone of erythromycin. The presence of methylmalonyl-CoA mutase, which catalyses this isomerisation, was demonstrated in cell-free extracts prepared from this organism. It was also found that MCM activity increased over 12-fold at the time of the most rapid antibiotic production, and the activity level dropped when the antibiotic production ceased (Hunaiti *et al*., 1984). In certain bacterial fermentations, succinate is converted to propionate *via* the mutase, rather than being removed from the system (Roth *et al.*, 1996).

1.4.1.2 Parasites

In *Ascaris lumbricoides* (*A. lumbricoides*), one molecule of adenosylcobalamin binds tightly to each of the two identical subunits of the enzyme. The nematode accumulates propionate and volatile fatty acids derived from propionate in the mitochondria. The conversion of succinate to propionate *via* propionyl-CoA carboxylase, MCM and acyl CoA transferase enzyme systems is coupled to a substrate level phosphorylation in the nematode (Han *et al.*, 1984). MCM from the helminths *Fasciola hepatica* and *Spirometra mansonoides* has also been shown to have a function similar to that in *A. lumbricoides* (Pietrzak and Saz, 1981).

1.4.1.3 Mammals

The highest concentration of MCM is found in ruminant kidney and tissues as propionate constitutes the major part of the ruminant diet. In mammals, methylmalonyl-CoA mutase is required for the metabolism of propionate, which may be derived from odd chain fatty acids, branched chain amino acids and cholesterol, to succinyl-CoA (Banerjee *et al*., 2001 & Gruber and Kratky, 2001). The conversion of odd chain fatty acids and branched amino acids to succinyl-CoA by MCM occurs in the mammalian liver and the succinyl-CoA formed can then enter the TCA cycle (Ledley *et al.*, 1990). Propionate is first activated to propionyl-

CoA, then carboxylated to methylmalonyl-CoA and finally isomerised to succinyl-CoA by MCM found in the mitochondrial matrix, as shown in Figure 1.8.

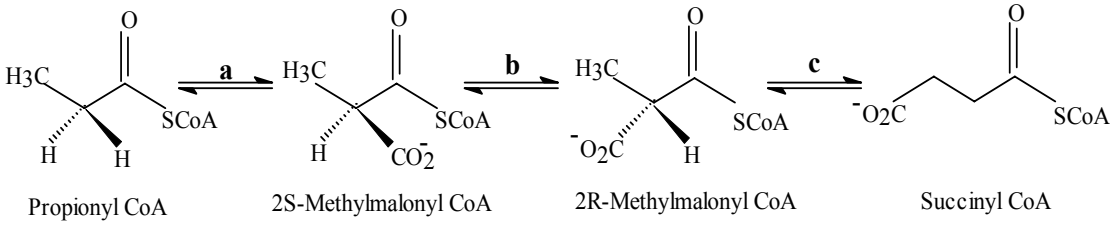

Figure 1.8: Reaction catalysed by MCM in mammals. General route of conversion of propionyl-CoA to succinyl-CoA (a: propionyl CoA carboxylase, b:methylmalonyl-CoA epimerase and c: methylmalonyl-CoA mutase)

1.4.2 Clinical importance of MCM

Impaired functioning of methylmalonyl-CoA mutase either caused by genetic defects or induced by cobalamin deficiency leads to methylmalonic acidemia in which precursors and abnormal metabolites of methylmalonyl-CoA accumulate. Depending on the severity of the condition, the clinical consequences range from benign which can be cured by dietary supplementation with vitamin B_{12} (Ledley *et al*., 1984) to neonatal death (Matsui *et al*., 1983). Renal insufficiency, chronic tubulointerstitial nephropathy and end stage renal failure, are symptoms reported in patients suffering from methylmalonic aciduria due to MCM deficiency (Van Calcar *et al*., 1998). Patients with inborn errors in methylmalonyl-CoA mutase metabolism are classified as being either mut^- (enzyme activity is detectable), or mut^0 (enzyme activity is not detectable). Over 20 mutations related to in-born errors have been reported in the human MCM gene and many of these are clustered in the carboxy-terminal B_{12} binding domain (Banerjee, 1997).

1.5 Coenzyme B_{12} biosynthesis

In constructing adenosylcobalamin, multiple components are synthesized individually and then assembled. The nucleotide loop is assembled by first activating the aminopropanol side chain of cobinamide to form GDP-cobinamide. The Co-axial ligand, dimethylbenzimidazole (Dmb), is synthesized separately and converted to a

nucleotide (DmbMN) by addition of ribose derived from nicotinic acid mononucleotide (NaMN), an intermediate in the synthesis of NAD. Ultimately, Dmb nucleoside is added to the end of the activated isopropanol side chain to form the nucleotide loop and complete the synthesis of coenzyme B_{12} (Roth *et al*., 1996).

1.5.1 *Salmonella typhimurium*

Cobalamin participates as a cofactor in both prokaryotic and eukaryotic metabolism and is derived from uroporphyrinogen III, a precursor in the synthesis of heme, siroheme, cobamides and chlorophylls (in photosynthetic organisms) (Lawrence and Roth, 1996 & Rodionov *et al*., 2003). In *S. typhimurium*, about thirty enzymes, in very low concentrations and with the formation of highly labile intermediates, are involved in the complete synthesis of AdoCbl. The genes coding for the enzymes responsible for cobalamin synthesis in *S. typhimurium* are clustered together and constitute a 20 gene operon located at 44 min on the genetic map (Jeter *et al*., 1984 & Lawrence and Roth, 1995). As seen in Figure 1.2, coenzyme B_{12} consists of three main structural units. The first unit is a central tetrapyrrole-derived corrin ring cobinamide structure. The genes (*cbiABCDETFGHIJKLMNOQP*) that encode enzymes responsible for the reactions leading to the formation of cobinamide from uroporphyrinogen III belong to the pathway referred to as CobI. A mutant for Cob I (with one of the first 17 genes affected) synthesises B_{12} if supplied with cobinamide. The second unit in coenzyme B_{12} is the dimethylbenzimidazole (DMB) nucleotide loop between the aminopropanol side chain and the central cobalt ion. The gene encoding the enzyme for the synthesis of this DMB nucleotide is a part of the pathway referred to as CobII. The attachment of the corrin ring to the DMB and the addition of upper coordinating ligand for the cobalt (adenosyl or methyl group) are catalysed by the enzymes encoded by the *cobUS genes* that belong to the CobIII pathway. A mutant for Cob II has been shown to synthesise B_{12} if Dmb is provided. Mutations in Cob III (affecting *cobU* and *cobS*) have shown to interfere with the ability to join cobinamide and Dmb and make B_{12} even when both intermediates are provided. The CobT protein has multiple activities and acts in both Cob II and III of the cobalamin synthesis.The *cob* genes of the cobinamide operon of *S. typhimurium*

represented by their respective pathways are organised in the order cobI, cobIII and cobII as shown in Figure 1.9 (Lawrence and Roth, 1995; Raux *et al.*, 1996 & Roth *et al.*, 1996).

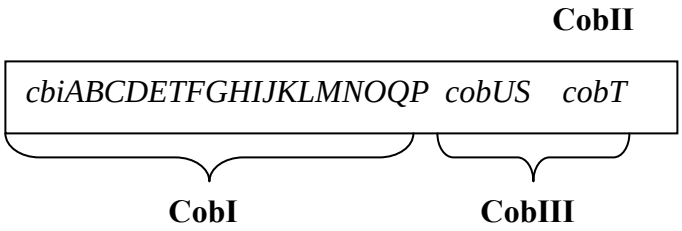

Figure 1.9: Organisation of the genes of CobI, III and II pathways in *S. typhimurium.*

1.5.2 *Escherichia coli*

Although most enteric bacteria can synthesise cobalamin *de novo* under either aerobic or anaerobic conditions, *E. coli* synthesises cobalamin only when provided with the complex intermediate, cobinamide (Lawrence and Roth, 1995). *E. coli* possesses a high affinity active transport system for vitamin B_{12}. It has the genes of the CobII and CobIII pathways but not the genes of the CobI pathway that are essential for cobinamide synthesis (Bassford and Kadner, 1977 & Raux *et al.*, 1996). Thus, *E. coli* cannot synthesise its own cobalamin, but can transport (Figures 1.10 and 1.11) and utilise B_{12} from the environment, if necessary. Genetic analysis of B_{12} uptake by *E. coli* has shown that three gene products are involved in the biphasic process. An outer membrane protein (*btuB* gene product) in *E. coli* binds specifically to B_{12} in an energy-independent process and the second process involves a slower energy-dependent accumulation of B_{12} in the cell catalysed by the *tonB* gene product (Kadner, 1990 & Roth *et al*., 1996). BtuB brings vitamin B_{12} across the outer membrane into the periplasmic space where it is bound by BtuF. Bassford and Kadner (1977) had identified a less efficient *btuCD* coded B_{12} uptake system in the cytoplasmic membrane in *E. coli* that acts on the substrate (vitamin B_{12}) in the periplasm (Bassford and Kadner, 1977). The bound B_{12} is delivered to the ABC transporter BtuCD which uses ATP to transport the vitamin into the cytoplasm (Oloo *et al*., 2006).

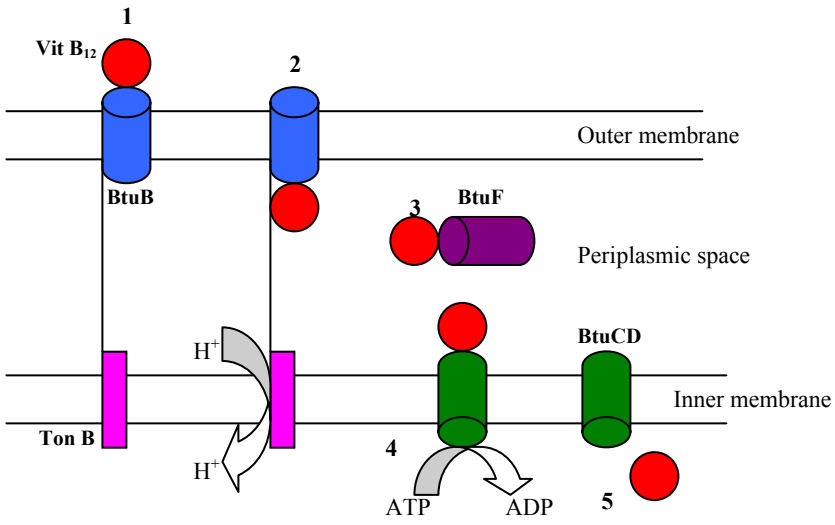

Figure 1.10: Uptake and transportation of vitamin B_{12} in *E. coli*. 1-Binding of B_{12} to BtuB, 2-Energy-dependent translocation, 3-Binding to BtuF, 4-Transfer to BtuCD and 5-Translocation to cytoplasm.

The *btu*B, *ton*B and *btu*C genes are located at 88, 28 and 37.7 min, respectively on the *E. coli* chromosomal map (Bassford and Kadner, 1977 & Postle and Good, 1983). The B_{12}, once transported into the cell, gets converted to the coenzyme form by the gene products of the CobIII (*cobUS*) and CobII (*cobT*) pathways (Raux *et al*., 1996).

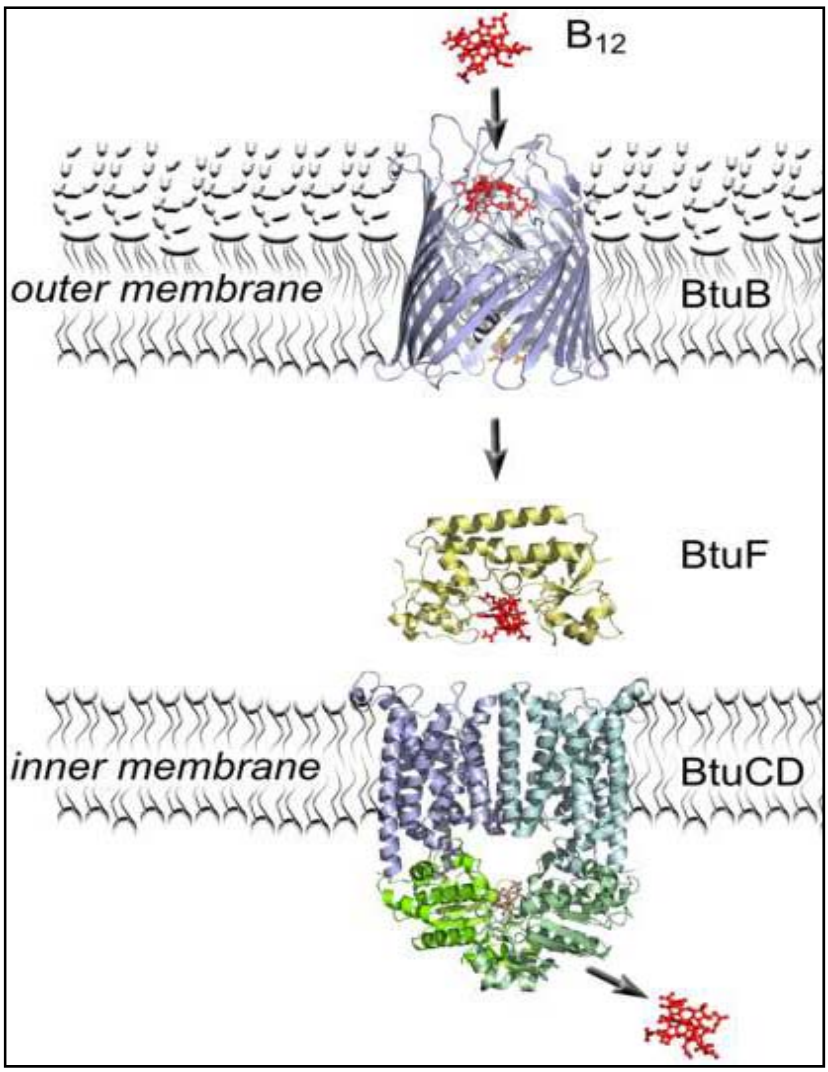

Figure 1.11: 3D schematic representation of vitamin B_{12} uptake and transport in *E. coli* (Oloo *et al*., 2006).

BtuCD consists of four subunits, arranged as two homodimers. BtuC spans the membrane with 10 alpha helices per subunit and contains the translocation pathway. BtuD is bound to BtuC on the cytoplasmic side of the membrane, each subunit containing one ATP binding site. The whole transportation process is powered by ATP-hydrolysis which takes place in these nucleotide binding domains. BtuF is a specialised periplasmic binding protein with high affinity for binding and delivering vitamin B_{12} to BtuC. Once BtuF (with B_{12} bound) has successfully docked to BtuC, the transfer mechanism is initiated (Roth *et al*., 1996 & Oloo *et al*., 2006).

1.5.3 Comparative analysis of the *E. coli* and *S. typhimurium cob* operons

According to Lawrence and Roth (1995) three of the five inferred open reading frames that were found in *E. coli* were homologous to the *cobUST* genes in the *Salmonella typhimurium cob* operon which provide the functions of the genes of the cobII and cobIII pathways. The *E. coli* genes were termed *cobU*, *cobS*, and *cobT* to indicate their similarity to the *Salmonella* homologues. Database searches revealed that the *E. coli cobUST* genes, like the *S. typhimurium cobUST* genes, are also similar to the homologous cobalamin synthetic genes (*cobPVU*) from *Pseudomonas denitrificans* (Lawrence and Roth, 1995).

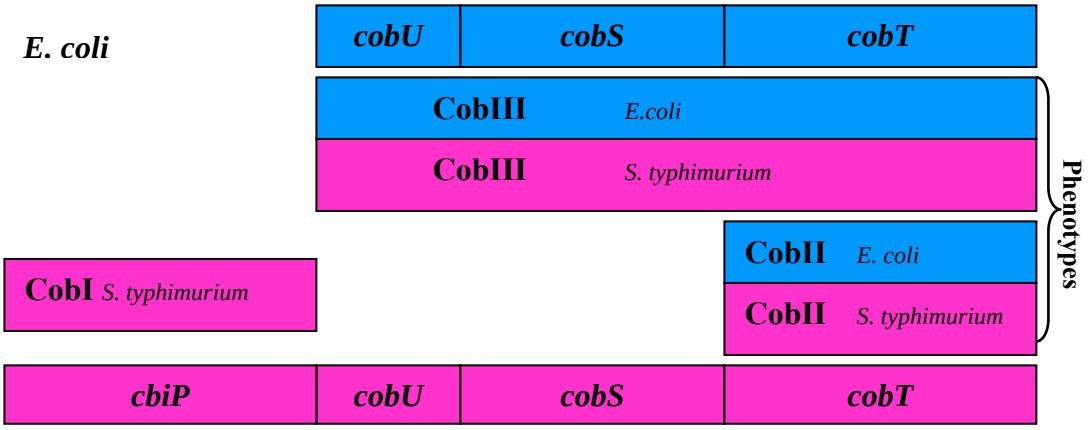

Figure 1.12: Organisation of the *E. coli* and *S. typhimurium cob* operons. The *cobUST* genes shown in blue are present in *E. coli* and the *cbiP* and *cobUST* genes in pink are present in *S. typhimurium*. The overlapping blue and pink boxes indicate the part of the operons that shows sequence similarity in both bacteria. *E. coli* lacks the *cbiP* gene (CobI) responsible for cobinamide synthesis but expresses genes coding CobII and CobIII enzymes that are involved in the conversion of an external source of B_{12} into coenzyme B_{12} required for enzyme catalysis. CobIII and CobII pathways share cobT while cobUS is only a part of the cobIII pathway.

The physical map of the *cob* operon, located at 43.5 min on the *E. coli* genetic map, has shown that the *cobU*, *cobS*, and *cobT* genes all lie close to each other such that the 5' end of the *cobS* gene overlaps the 3' end of the *cobU* gene. The proximity of the genes suggested that they may form an operon expressed by a promoter upstream

of the *cobU* gene (Lawrence and Roth, 1995). A schematic representation of this observation is shown above in Figure 1.12. The three *cob* genes of *E. coli* showed between 69% and 72% nucleotide identity with the *S. typhimurium cobU*, *cobS*, and *cobT* genes and the proteins were between 76 and 82% identical (Lawrence and Roth, 1995), indicating that the *E. coli cob* genes were also involved in cobalamin biosynthesis.

Despite this high degree of homology observed between the *cob* genes, typical pairs of homologous genes in *E. coli* and *S. typhimurium* have been to found to show much higher nucleotide identities (84 to 95%) and encode proteins with more than 91% amino acid identity (Sharp, 1991). This indicated that the *E. coli cobUST* genes and *S. typhimurium cobUST* genes are significantly more different from each other than typical gene pairs. The large differences between the *S. typhimurium cob* genes and the *E. coli cob* genes reflect very weak selection for cobalamin synthesis as genes under strong selection for function show fewer differences between *E. coli* and *S. typhimurium* (Sharp, 1991). This indicates that *E. coli* does not synthesise or need cobalamin for its growth under natural conditions.

1.5.4 Other bacteria

Dependence on coenzyme B_{12} for enzyme function has been reported in many bacteria including *P. shermanii, S. cinnamonensis, Porphyromonas gingivalis* and *Saccharopolyspora erythraea* (Raux *et al*., 1996) . Bacteria such as *Bradyrhizobium japonicum* require coenzyme B_{12} and can synthesise it and *Lactobacillus leishmannii* requires B_{12}, but cannot synthesise it. Genes clustered together in an operon (*cobPVU*) organisation in *Pseudomaonas denitrificans* responsible for B_{12} synthesis have been shown to have a sequence similar to that of the *cobUST* operon in *E. coli* and *S. typhimurium*. *Klebsiella pneumoniae* can synthesise coenzyme B_{12}, but only under anaerobic conditions (Jeter *et al.*, 1984, Lawrence and Roth, 1996) due to the formation of oxygen sensitive intermediates during the synthesis (Raux *et al*., 1996).

1.6 *E. coli*, MCM and the *sbm* gene

Roy and Leadlay (1992) identified the gene, *sbm,* encoding MCM to be located at 62.8min on the *E. coli* chromosome. Analysis of this gene showed a high degree of sequence similarity to the human MCM and to the larger subunit of the *P. shermanii, S. cinnamonensis* and *S. erythraea* methylmalonyl-CoA mutases. A detailed comparative study showed that the substrate binding site, the active site and the cobalamin binding region, indicated in the crystal structure of the MCM from *P. shermanii* were all conserved in the Sbm protein from *E. coli*. This led to the hypothesis that the *sbm* gene codes for MCM in *E. coli* (Roy and Leadlay, 1992). Further research has determined that the *sbm* gene actually encodes MCM in *E. coli,* since, the protein product when expressed as a *His* tagged protein was found to be an active methylmalonyl-CoA mutase (Haller *et al*., 2000). The *sbm* gene forms a part of a four-gene operon (Figure 1.13).

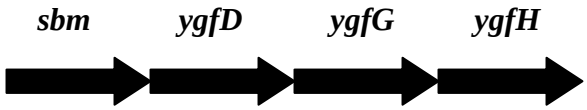

Figure 1.13: The four-gene operon containing the *sbm* gene (Haller *et al.*, 2000), *sbm* codes for methylmalonyl-CoA mutase, *ygf*D (or *arg*K) codes for a protein kinase that catalyses the phosphorylation of two periplasmic binding proteins involved in cationic amino acid transport, *ygfG* codes for methylmalonyl-CoA decarboxylase and *ygf*H codes for propionyl-CoA: succinyl-CoA transferase.

Haller and coworkers (2000) suggested that the proteins encoded by the four-gene operon could be involved in the decarboxylation of succinate to propionate as shown in Figure 1.14. However, this postulated pathway does not involve the *ygf*D gene product.

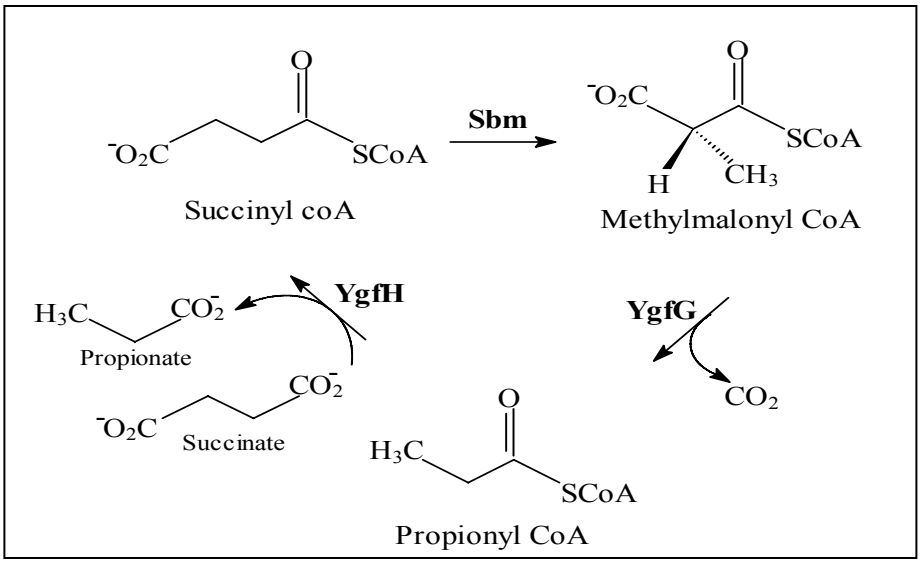

Figure 1.14: Succinate to propionate. The postulated pathway involving the conversion of succinate to propionate using the *sbm* gene in *E. coli* (Haller *et al*., 2000)

The previously known methylmalonyl-CoA mutase pathway involves a biotin dependent propionyl-CoA carboxylase, methylmalonyl-CoA epimerase and methylmalonyl-CoA mutase reactions (Figure 1.8). *E. coli* possess MCM, but it is not known to have methylmalonylCoA epimerase or the biotin dependent propionylCoA carboxylase necessary for the completion of the latter cycle (Haller *et al.*, 2000). However, the operon does consist of a methylmalonyl-CoA decarboxylase encoded by *ygfG* that yields the 2R-methylmalonyl-CoA, circumventing the necessity of the methylmalonyl-CoA epimerase and the propionylCoA carboxylase found in other organisms (Figure 1.14). This isomer is a substrate for methylmalonyl-CoA mutase and hence this pathway shown in Figure 1.8 could be used in the reverse direction for the utilisation of propionate.

1.7 Propionate and succinate metabolism in *E. coli*

1.7.1 MCM and propionate utilisation

Ashworth and Kornberg (1964) suggested a role for MCM in the utilisation of propionate by *E. coli*. In the experimental study, they observed that isocitrate lyase-less mutants of *E. coli* could utilise propionate but were unable to grow on acetate (Ashworth and Kornberg, 1964). The difference in the growth of the bacteria was

postulated to be due to the conversion of propionate to succinate *via* the reaction catalysed by MCM (Figure 1.8). Extensive studies on the growth of *E. coli* E26 using propionate as sole carbon source carried out by Kolodziej *et al*., 1968 showed that *E. coli* E26 grew with an extended lag phase of about 90 hours when it was grown in minimal salts media with propionate as the sole source of carbon. This, they suggested, was a sign of adaptation to the fatty acid and not a mutational process because, when the propionate adapted cultures were sub-cultured in complex media and re-introduced into propionate media, the cells appeared to have lost their capacity to initiate a rapid growth in the substrate. Inclusion of a source of B_{12} in the propionate growth media for *E. coli* reduced the lag phase from 90 hours to 55 hours. This observation suggested that adaptation to propionate is limited by a B_{12}-dependent pathway, possibly involving MCM (Wegener *et al.*, 1968). Further, the lag phase was reduced to 65 hours (from 90 hours) when the *E. coli* was allowed to grow in the propionate media with limiting concentrations (0.5µmoles/ml) of succinate and found that succinate induced the enzyme formation necessary for the utilisation of propionate indicating the use of common enzyme systems for the metabolism of both carbon sources (Kolodziej *et al*., 1968).

Evans *et al*. (1993) proposed that in *E. coli*, propionate is activated to propionyl-CoA which can then follow one of two pathways that leads to the formation of acetyl-CoA (propionate oxidation, Figure 1.15a) or succinyl-CoA (propionate carboxylation, Figure 1.15b) which then enter the tricarboxylic acid (TCA) cycle. In order to understand the relative contribution of the two pathways, Evans *et al*. (1993) analysed the mechanism by which glutamate was formed from [3-^{13}C] propionate in *E. coli.* Using ^{13}C NMR analysis they measured the ^{13}C label distribution in glutamate through the two pathways. It was thought that [3-^{13}C] propionate would be oxidised to [3-^{13}C] pyruvate which would then be transformed to [2-^{13}C] acetyl CoA by pyruvate dehydrogenase before entering the TCA cycle (Figure 1.15a). The ^{13}C label in the glutamate produced would predominantly be on C-4. During the carboxylation reaction [3-^{13}C] propionyl-CoA would be carboxylated to [2-^{13}C] succinyl-CoA by propionyl-CoA:succinyl-CoA

transferase which would then enter the TCA cycle as shown in Figure 1.15b. ^{13}C that entering the TCA cycle *via* the latter path would lead to the production of glutamate labelled at C-2 or C-3.

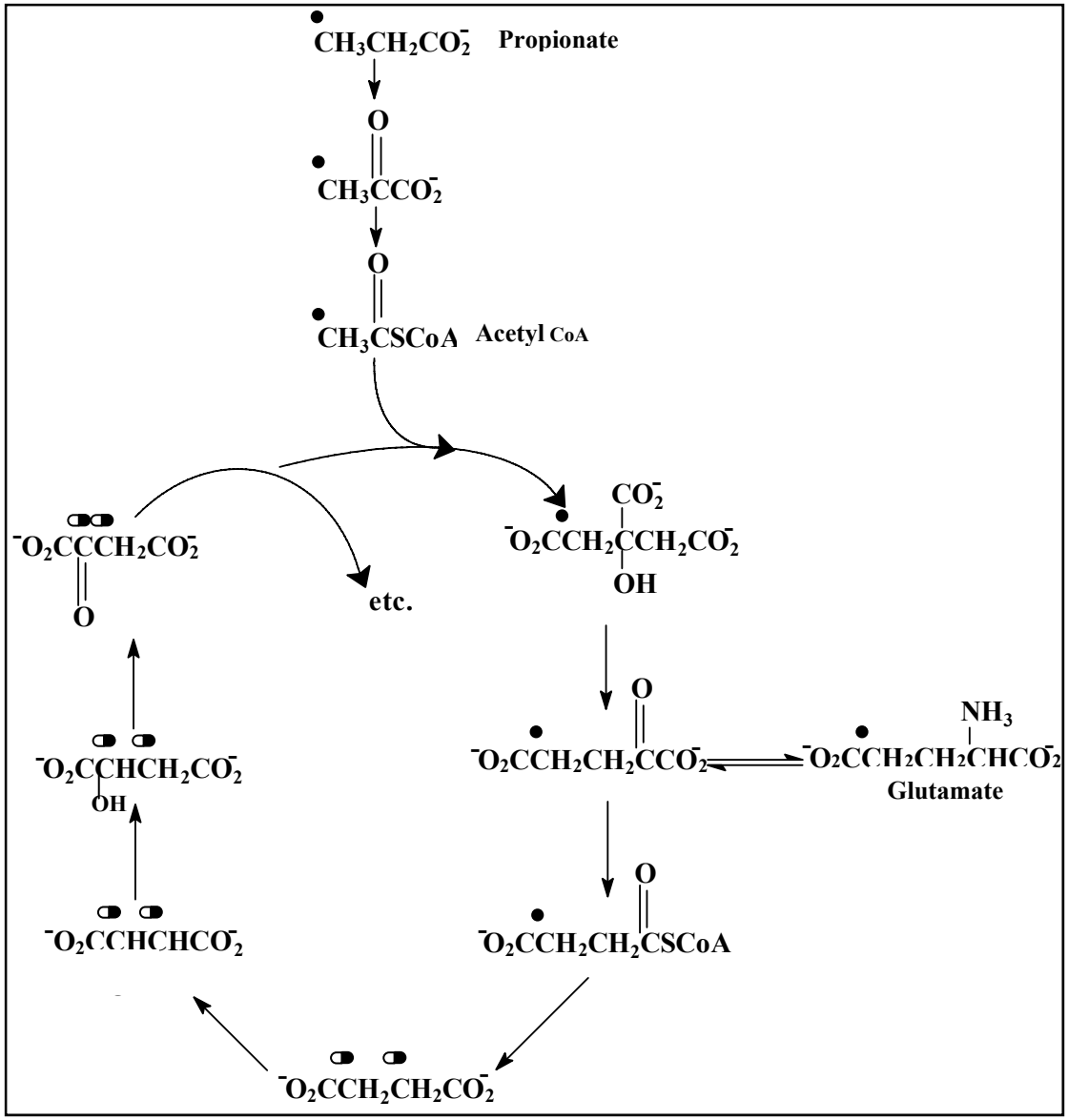

Figure 1.15a: NMR analysis- propionate oxidation. ^{13}C isotope distribution during one turn of the TCA cycle with [3-^{13}C] propionate as the substrate showing the oxidation of propionate to acetyl CoA ●: ^{13}C;◒ : 50:50 chance of ^{12}C or ^{13}C in this site (adapted from Evans *et al.*, 1993).

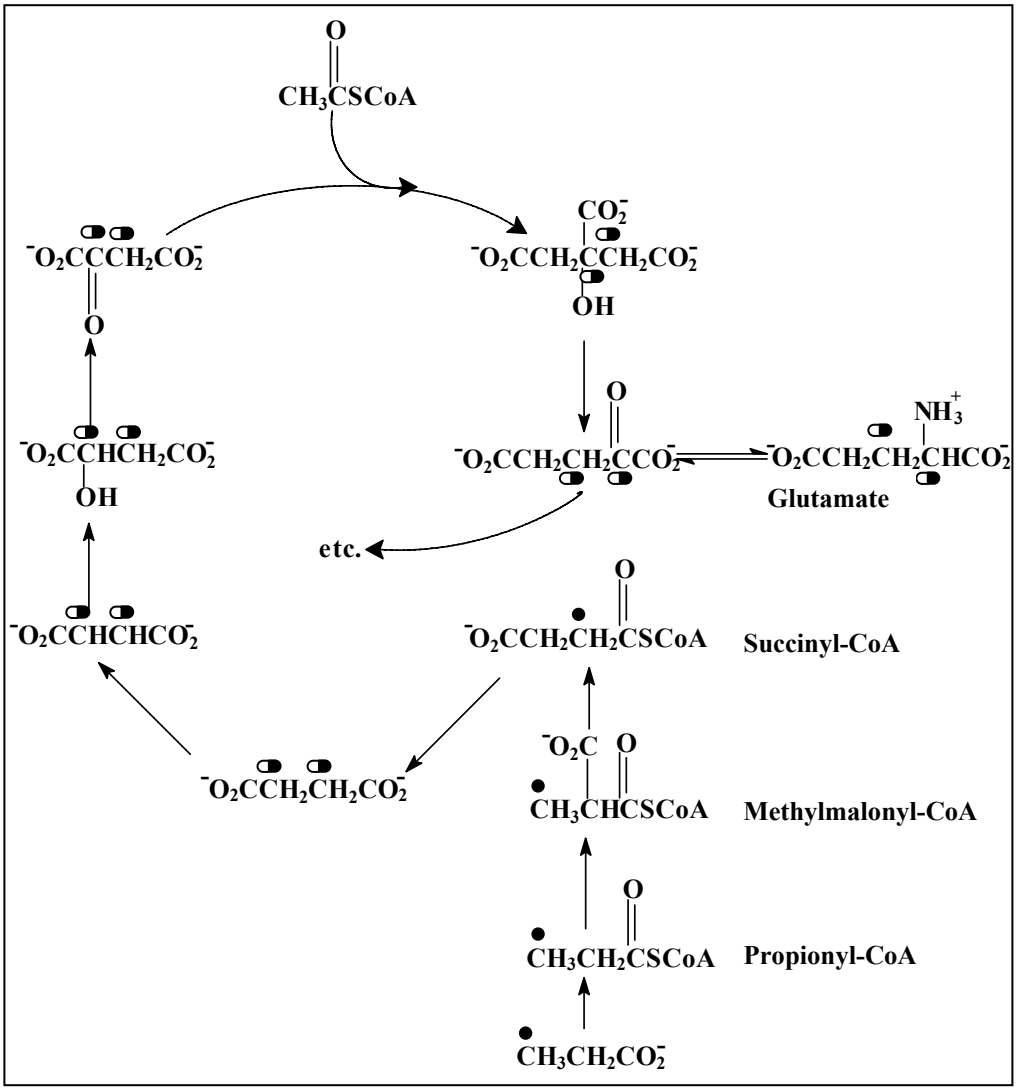

Figure 1.15b: NMR analysis- propionate carboxylation. ^{13}C isotope distribution during one turn of the TCA cycle with [3-^{13}C] propionate as the substrate showing the carboxylation of [3-^{13}C]propionyl-CoA to [2-^{13}C] succinyl-CoA. ● : ^{13}C; ◒: 50:50 chance of ^{12}C or ^{13}C in this site (adapted from Evans *et al.*, 1993)

The results of the NMR analysis of the growth of *E. coli* in minimal media with propionate as the sole source of carbon showed that there was a significant increase in the labelling of glutamate at the C-2 and C-3 positions when the cells were grown in minimal media with B_{12}. This supported the finding that exponential growth on propionate was stimulated by inclusion of B_{12} in the media confirming earlier observations by Kolodziej *et al*. (1968) and Wegener and coworkers (1968). These

results showed that some of the [2-^{13}C] propionate entered the TCA cycle *via* the carboxylation pathway, *i.e.*, through succinyl-CoA derived from propionate. Thus, from the NMR analysis, Evans *et al*. (1993) concluded that the B_{12} dependent MCM reaction pathway was one of the pathways used by *E. coli* when grown on propionate as a sole source of carbon (Kolodziej *et al*., 1968; Wegener *et al*., 1968 & Evans *et al.*, 1993).

Propionate utilisation by microorganisms has been under continuous scrutiny. This could be attributed to the fact that unlike other fatty acids that undergo just oxidation, propionate is thought to be metabolised using multiple pathways which include α-oxidation, β-oxidation, α-carboxylation, reductive carboxylation or Claisen condensation. Textor *et al*. (1997) identified the presence of the metylcitrate pathway (Figure 1.16), which they described as the pathway of choice for the oxidation of propionate in *E. coli* (Textor *et al*., 1997).

This led to the identification of the methylcitrate synthase encoded by the *prp*C gene located at 8min in the *E. coli* genome. This gene shared a 96% sequence homology with the identified *prp*C gene involved in propionate utilisation in *Salmonella typhimurium*. The gene coding for methylcitrate synthase in *E. coli* belongs to a cluster of four genes, the products of which have high similarities to those of the propionate catabolism operon (*prpBCDE*) in *S. typhimurium* (Textor *et al*., 1997).

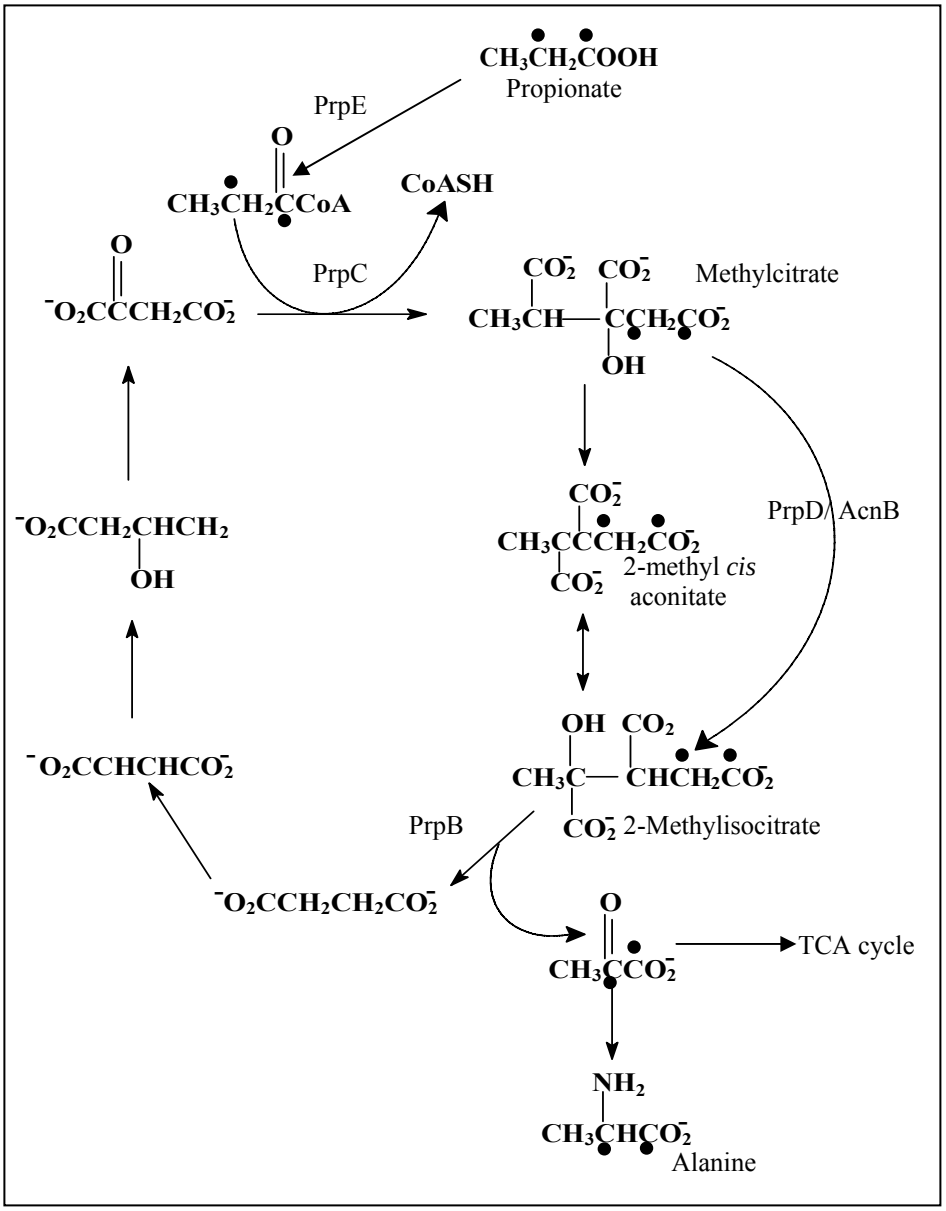

Figure 1.16: Methylcitrate cycle for breakdown of propionate. PrpE- Propionyl-CoA synthetase, PrpC- 2-methylcitrate synthase, PrpD- 2-methylisocitrate hydratase, AcnB- aconitase, PrpB- 2-methylisocitrate lyase.

In the case of the [2-^{13}C] propionate precursor (Evans *et al*., 1993), flux *via* the methylcitrate pathway could lead to [2-^{13}C] pyruvate and to [2-^{13}C] oxaloacetate, which could result in an analogous labelling of glutamate C-2 or C-3. Hence,

London and co-workers (1999) used [1, 2-^{13}C] propionate to determine retention of the ^{13}C-^{13}C-^{12}C carbon skeleton in pyruvate and alanine (Figure 1.16) involving propionate adapted *E. coli*. The pattern of labeling of alanine was mostly retained, even if the [1, 2-^{13}C] propionate precursor was substantially diluted with unlabelled propionate. The retention of the carbon skeleton of the propionate was consistent with flux *via* the methylcitrate pathway but not with flux through the methylmalonyl pathway (London *et al*., 1999). Although this analysis produced convincing argument for propionate utilisation *via* the methylcitrate pathway, it is important to emphasise that *E. coli* MG1655 used in the study was grown in defined media without any source of vitamin B_{12} added to it, unlike that of Evans *et al*. (1993). The absence of vitamin B_{12} would not allow the formation of MCM holoenzyme and hence the carboxylation pathway involving *sbm* (MCM) would not be active in the breakdown of propionate.

1.7.2 MCM and succinate utilisation

The earliest evidence for growth on succinate was provided by Kolodziej *et al*. (1968) when the lag phase of *E. coli* E26 was reduced to 65 hours when the bacteria was allowed to grow in the propionate media with limiting concentrations (0.5μmoles/ml) of succinate and suggested that succinate and propionate metabolism involved the use of common enzyme systems (Kolodziej *et al*., 1968). Limited studies have been carried out on succinate metabolism in *E. coli* (Liu *et al*., 2005 & Paliy and Gunasekara, 2007). Liu and coworkers (2005) noticed an upregulation of 59 genes and down regulation of only 3 genes when *E. coli* was grown in succinate and have noted that as the substrate was used and products were formed, there was an increase in the transcription of new stress related genes, genes required for motility and σS encoding *rpo*S gene (Liu *et al*., 2005). None of the genes of the *sbm* operon were reported by Liu *et al*. (2005) and this could be due to the absence of any vitamin B_{12} in the growth conditions used. Paliy and Gunasekara (2007) reported an increased lag phase of 20 hours when *E. coli* BL21 was grown in minimal media with succinate compared to that of the wild type *E. coli* which did not show any such delay in growth. The lag phase observed with BL21 was attributed to a lower

expression of *fum*A gene, encoding fumarase, through which the utilisation of both fumarate and succinate proceed, *via* the TCA cycle, and an insufficient initial metabolic flux through fumarase might not sustain growth. An insufficient transport capacity for succinate in *E. coli* BL21 was thought to be due to possible mutations in the genes of the *atp* operon that encodes the H^+-ATP complex required for the transport of C4-dicarboxylates such as succinate, resulting in a lag of 20 hours (Paliy and Gunasekara, 2007). Although these studies describe certain aspects of growth of *E. coli* on succinate, there has been no evidence of the involvement of MCM or the other genes of the *sbm* operon. As described previously in Section 1.6, Haller *et al*. (2000), identified the four gene operon (Figure 1.13) and confirmed that *sbm* coded for MCM in *E. coli*. The authors also described the possibility of the involvement of three of the four genes of the operon in the decarboxylation of succinate to propionate. The current study has been the only report so far that has attempted to describe the relation between succinate utilisation and MCM.

Succinate is also an important intermediate in the TCA cycle and is hence involved in large number of complex metabolic networks. Utilisation of propionate by *E. coli* has been well established and many authors (as reviewed above, Section 1.7.1) have researched on the different pathways by which propionate is being used by *E. coli* and other bacteria. Relatively less research has been carried out on the growth of *E. coli* on succinate as the sole carbon source as reviewed in Section 1.7.2. This study attempted to investigate the role of MCM in both propionate and succinate metabolism on the basis of the hypothesis proposed by Haller *et al*., 2000.

1.8 Polyhydroxyalkanoates (PHA)

Polyhydroxyalkanoates (PHAs; Figure 1.17), are polyesters of 3-, 4-, 5-and 6-hydroxyalkanoic acids, produced by a variety of bacterial species under nutrient-limiting conditions with excess carbon. PHAs exhibit a high degree of polymerisation and possess very high molecular masses. They are biodegradable, insoluble in water, non-toxic, biocompatible, piezoelectric, thermoplastic and/or

elastomeric (Anderson and Dawes, 1990; Choi and Lee, 2000; Hazer and Steinbuckel, 2007 & Valappil *et al*., 2007a).

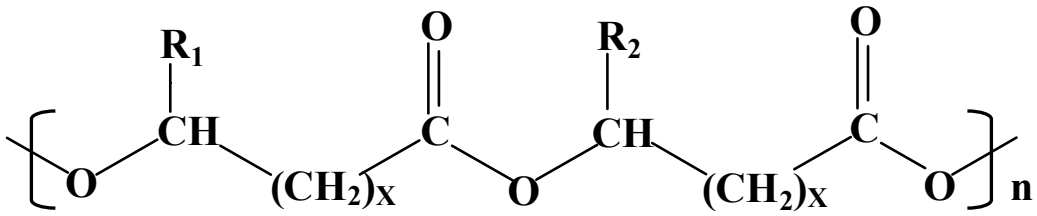

Figure 1.17: General chemical structure of PHAs. R_1 and R_2 = H, alkyl groups C_1-C_{13}, x= 1-4 and n=100-300,000

Polyhydroxyalkanoates from prokaryotes represent the most important group of natural polyoxoesters beside polymalic acid from eukaryotes and cutin and suberin from plants. About 150 different constituents have been observed in PHAs isolated from different bacterial species (Valappil *et al*., 2007a). PHAs have been classified into two types according to the length of the side chain. The short-chain-length (*scl*) polyhydroxyalkanoic acids have an alkyl side chain with up to two carbon atoms (*e.g*. PHAs produced by *Cupriavidus necator, C. necator*) and the medium chain length hydroxyalkanoic acids, *mcl*PHA, have an alkyl side chain consisting of at least three carbon atoms (*e.g*. PHAs accumulated by *Pseudomonas oleovorans*). Members of *scl*PHAs include poly(3-hydroxybutyrate), P(3HB); poly(3-hydroxyvalerate), P(3HV) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), P(3HB-*co*-3HV) copolymer. On the other hand, poly(3-hydroxyoctanoate), P(3HO) and poly(3-hydroxynonanoate), P(3HN), are mostly formed as copolymers of 3-hydroxyoctanoate or 3-hydroxynonanoate together with 3-hydroxyhexanoate, 3HHx; 3-hydroxyheptanoate 3HHp and/or 3-hydroxydecanoate, 3HD are typical examples of *mcl*PHAs. Some *scl*PHAs may be too rigid and brittle and may lack the superior mechanical properties required for biomedical and packaging film applications while *mcl*PHAs may be elastomeric but have very low mechanical strength (Hazer and Steinbuckel, 2007 & Valappil *et al*., 2007a).

1.8.1 PHA producing bacteria

PHAs are produced on an industrial scale using Gram negative bacteria such as *Cupriavidus necator, Methylobacterium organophillum, P. oleovorans* and recombinant *E. coli*. PHA producing Gram positive species include *Corynebacterium matruchotii*, *Rhodococcus ruber*, *Nocardia corollina*, *Bacillus cereus* and *Bacillus megaterium*. The Gram-positive genera *Corynebacterium*, *Nocardia* and *Rhodococcus* are the only wild-type bacteria, which naturally synthesise the commercially important co-polymer P(3HB-co-3HV) from simple carbon sources such as glucose (Valappil *et al*., 2007a).

1.8.2 Polyhydroxybutyrate, P(3HB)

P(3HB) is one of the most abundant PHA that has been studied in detail. P(3HB) occurs as insoluble cytoplasmic inclusions found exclusively in many eubacteria and also in some extremely halophilic archaea as storage compound for carbon and energy. It contributes to up to about 90% (w/w) of the cellular dry mass. It can be produced from renewable resources and are hence considered an alternative to non-biodegradable plastics produced from fossil oils (Steinbuchel and Lutke-Eversloh, 2003).

1.8.2.1 Importance of P(3HB) in bacteria

The possession of P(3HB) in some bacteria slows the degradation of cellular components such as RNA and protein during nutrient starvation (Steinbuchel and Lutke-Eversloh, 2003). P(3HB) enhances the survival of some bacteria by serving as a carbon and energy source for spore formation in some *Bacillus* species and for the encystment of azotobacters (Anderson and Dawes, 1990). It has been hypothesised that P(3HB) in azotobacters and *Rhizobium* sp. strain ORS 571 provides an oxidisable substrate that offers respiratory protection to nitrogenase under environmental conditions when appropriate exogenous substrate(s) is not immediately available for oxidation (Anderson and Dawes, 1990).

1.8.2.2 Metabolic engineering for P(3HB) production

P(3HB) is a crystalline homopolymer that can be produced from a single carbon substrate such as glucose (Choi and Lee, 2000). Metabolically engineered microbes and insects have been used for P(3HB) production.

PHA synthase gene from *C. necator* was introduced into *Saccharomyces cerevisiae* cells and it was observed that P(3HB) granules accumulated to only 0.5% of the cell dry weight. This low level of P(3HB) was thought to be due to the insufficient activity of the endogenous β-ketoacyl-CoA thiolase and acetoacetyl-CoA reductase (Figure 1.18) enzymes (Madison and Huisman, 1999).

In another study, expression of the *C. necator phbC* gene in insect cells was achieved in cabbage looper, *Trichoplusia ni*, cells by using a baculovirus system. Expression of PHA synthase gene was successful and 50% of the total protein was P(3HB) synthase, as observed within 60 hours of viral infection (Madison and Huisman, 1999). In addition, a study with insect cells attempted to create a diverse set of PHA monomers endogenously by transfecting a mutant form of the rat fatty acid synthase into fall armyworm, *Spodoptera frugiperda*, cells by using a baculovirus. This previously characterised fatty acid synthase mutant did not extend fatty acids beyond 3-hydroxybutyrate, which was subsequently converted to P(3HB) by the co-transfected P(3HB) synthase from *C. necator*. The presence of P(3HB) granules in the insect cells was visualised by immunofluorescence (Williams *et al*., 1996).

1.8.3 Polyhydroxybutyrate-co-hydroxyvalerate, P(3HB-co-3HV)

P(3HB-co-3HV) is a copolymer of monomers of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) and can exist with various mole percentages of 3-hydroxyvalerate (3HV). Incorporating HV in the structure of P(3HB) changes the mechanical properties of the polymer giving the co-polymer more flexibility and processability. It has been shown that by varying the hydroxyvalerate content of P(3HB-*co*-3HV), the flexibility, impact strength, and degree of degradation of the

final copolymer can be altered. Hence, P(3HB-co-3HV) is more appropriate for commercial applications as compared to P(3HB). Thus P(3HB-*co*-3HV) is an ideal candidate for *in-vivo* use such as drug delivery or tissue engineering scaffolds for bone regeneration. (Choi and Lee, 2000; Aldor and Keasling, 2003 & Misra *et al*., 2006).

1.8.3.1 Bacteria producing P(3HB-co-3HV)

P(3HB-co-3HV) copolymer is produced by *Alcaligenes latus*, *Pseudomonas pseudoflava*, *Micrococcus halodenitrificans*, *B. cereus*, *P. cepacia*, and *C. necator*. The microorganisms need to be supplied with glucose (or sucrose in case of *A. latus*) and propionic acid or other propionogenic carbon sources under nitrogen-limited conditions. The mixture of the substrates produce either P(3HB) homopolymer or P(3HB-co-3HV) copolymer depending on their relative concentrations (Hazer and Steinbuckel, 2007).

1.8.3.2 Metabolic pathways leading to the production of P(3HB-co-3HV)

Industrial production of P(3HB-co-3HV) involves the addition of glucose and propionate to a fed-batch fermentation processes. Adjusting the ratio of these carbon sources in the feed is used to control the copolymer composition. Propionate which is activated to form the propionyl-CoA precursor of HV production is not only toxic, but also very expensive to produce industrially. Hence, a more economical alternative is to produce propionyl-CoA *via* internal metabolism, from a relatively inexpensive carbon source such as glucose. P(3HBV-co-3HV) is synthesised from its precursors, acetyl-CoA and propionyl-CoA, by three enzymes as shown in Figure 1.18.

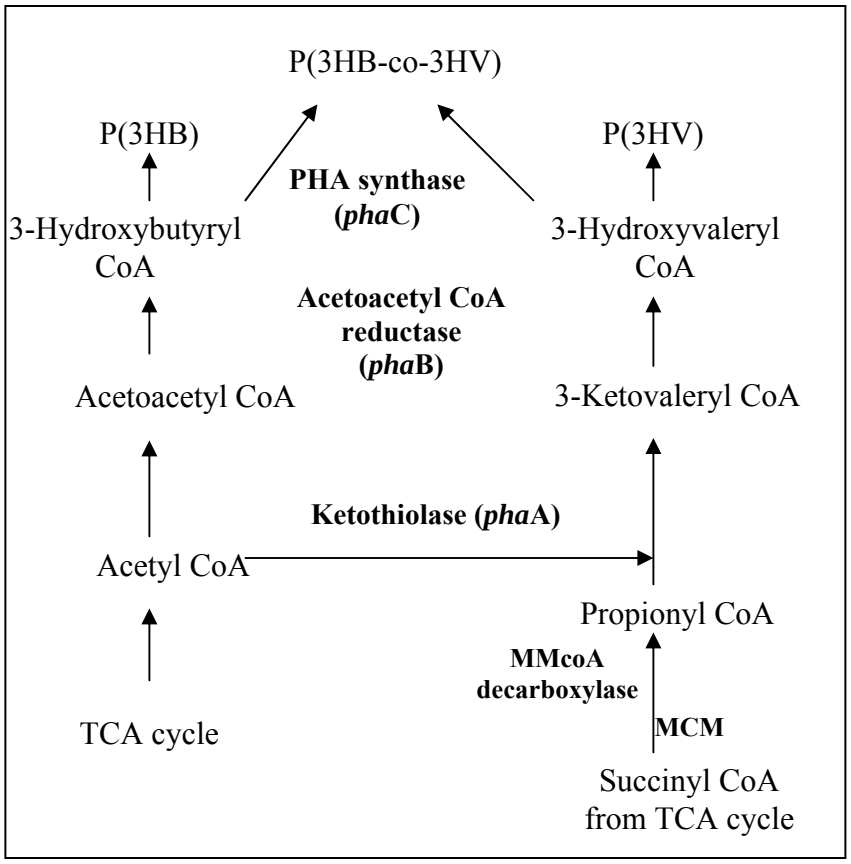

Figure 1.18: Metabolic pathway involved in the production of P(3HB-co-3HV).

The first step involves either a condensation of two molecules of acetyl-CoA or a condensation of acetyl-CoA and propionyl-CoA by a 3-ketothiolase encoded by *phaA*. The resulting intermediate, either acetoacetyl-CoA or 3-ketovaleryl-CoA, is reduced by the *phaB* gene product, an NADPH-dependent acetoacetyl-CoA reductase. This reaction generates 3-hydroxybutyryl-CoA and 3-hydroxyvaleryl-CoA, which are incorporated into the growing polymer chain by PHA synthase or polymerase, encoded by *phaC*, as four- and five-carbon monomers, respectively.

Valentin and Dennis (1996) identified that the supply of the precursor for co-polymer P(3HB-*co*-3HV) production in *N. corallina,* mainly depended on the availability of propionyl-CoA which was obtained from the TCA cycle intermediate succinyl-CoA *via* MCM. This was confirmed by the inability of the MCM mutant of *N. corallina,* to grow on odd chain fatty acids, leading to the conclusion that

propionate is utilised, after its conversion to succinyl-CoA principally *via* MCM (Valentin and Dennis, 1996). Nuclear magnetic resonance spectroscopy (NMR) studies suggested that the 3HV monomer is derived from acetyl-CoA and propionyl-CoA, where the latter is a product of the methylmalonyl-CoA pathway (Madison and Huisman, 1999). Hence, *R. ruber* and *N. corallina* accumulate PHAs containing 3HV even in the absence of typical HV precursors such as propionate or valerate in the medium.

1.8.3.3 Evidence for metabolic engineering for P(3HB-*co*-3HV) production

The price of the P(3HB-co-3HV) will ultimately depend on parameters such as substrate cost, polymer yield on the substrate, and the efficiency of product formulation in the downstream processing. Successful commercial production requires that all conditions for production be optimised. P(3HB-*co*-3HV) producers such as *R. ruber* and *N. corallina* have evolved with the ability to accumulate the co-polymer, naturally. However, they tend have a long generation time and relatively low optimal growth temperature, are often hard to lyse and contain pathways for polymer degradation. Bacteria such as *E. coli* can neither synthesise nor degrade the polymer, but, it grows fast providing a shorter cycle time for the production process and at a higher growth temperature saving the cost associated with the heating and cooling of the bioreactor. It is also easy to lyse, leading to a cost effective purification of the polymer granules. Construction of better P(3HB-*co*-3HV) producers by applying the insights of genetic and metabolic engineering promises the production of higher amounts of the polymer and in organisms suh as *E. coli* that are easy to manipulate.

An interesting pathway for poly(3HB-*co*-3HV) biosynthesis was recently engineered in recombinant *S. enterica* serovar *typhimurium* by Aldor and co-workers (2002). The genes for (2R)-methylmalonyl-CoA mutase (*sbm*) and (2R)-methylmalonyl-CoA decarboxylase (*ygfG*) from *E. coli* were cloned and expressed in this *Salmonella* strain, thus enabling the conversion of succinyl-CoA to propionyl-CoA. In addition, they inserted into the gene for 2-methylcitric acid synthase (*prpC*) of

this bacterium the PHA biosynthesis operon of *Actinobacter* (*phaCAB*) thereby establishing PHA biosynthesis and disrupting propionate utilisation *via* the methylcitrate pathway (Figure 1.16). This recombinant *Salmonella* sp. synthesised P(3HB-*co*-3HV) with up to 31 mol% 3HV *via* succinyl-CoA when the cells were cultivated on glycerol as carbon source (Aldor *et al*., 2002).

Further examples of the use of metabolic engineering for the production of P(3HB-co-3HV) co-polymer have been discussed in Chapter 7.

1.9 Aims of the project

The aim of the study is to understand the metabolic role of methylmalonyl-CoA mutase in *Escherichia coli* and the use of the mutase in metabolic engineering. The aim was intended to be achieved by

1. Comparing the growth of a mutant (*sbm* gene inactivated) and wild type *E. coli* in glucose, succinate or propionate as the sole source of carbon. We will be focussing specifically on these growth conditions because available literature indicates the possible importance of the *E. coli* methylmalonyl-CoA mutase in the utilisation of these carbon sources.
2. Identifying a promoter region to determine the possible conditions under which MCM could be induced.
3. Determining the mRNA copy number corresponding to the four genes of the *sbm* operon under growth conditions with succinate and propionate as sole sources of carbon and growth in minimal media with glucose as the sole source of carbon as a control. This will allow the estimation of the level of transcription of these genes under the specified conditions. The mRNA will be estimated using RT-PCR first and later using the real-time quantitative PCR technique.
4. Assaying for the activity of methylmalonyl-CoA mutase in whole cell extract using the high performance liquid chromatography (HPLC) technique, from cells grown in minimal media with succinate or propionate as sole sources of carbon and comparing with a control assay with extract from cells grown in minimal media with glucose as the sole source of carbon.
5. Expression proteomics to understand the global changes in protein expression, including that of Sbm, under specific growth conditions with glucose, succinate or propionate as sole source of carbon
6. The cloning of the *sbm* gene from *E. coli* into *B. cereus*; this would be the first step forward towards metabolic engineering of *B. cereus* leading to the production of the commercially important biodegradable polymer, P(3HB-co-3HV).

Chapter 2

Comparative growth analyses of wild type *E. coli*, TR6524 and the *sbm* disruption mutant, FA1P1

2.1 Introduction

Bacteria such as *E. coli* have genes that are frequently clustered together and organised in operons that encode enzymes responsible for a particular metabolic pathway. Occasionally, in prokaryotes, the operon organisation of genes may provide clues for the identification of unknown proteins, if the functions of the other proteins encoded by the genes in the operon are known (Haller *et al.*, 2000 & Bobik and Rasche, 2001).

About 40% of the identified open reading frames (ORFs) in bacteria encode proteins of unknown function or whose metabolic role is uncertain (Haller *et al.*, 2000). Energy production and metabolite inter-conversions in cells are mainly being studied *in vitro* using purified enzymes and substrates, under non-physiological conditions, and extrapolated in an attempt to understand whole cell metabolism (Evans *et al.*, 1993). However, understanding a gene's metabolic role *in vitro* should ultimately lead to the understanding of the function of the gene product *in vivo* (Bobik and Rasche, 2001).

Although the genome of *E. coli* has been sequenced (Berlyn, 1998) many of the proteins encoded by various genes are yet to be characterised and among those that have been characterised, the metabolic role of some are unclear. One such protein is the coenzyme B_{12}-dependent enzyme, methylmalonyl-CoA mutase (MCM), encoded by the *sbm* gene.

MCM plays an important role in propionate metabolism in both mammals and bacteria. In mammals the enzyme is involved in the breakdown of odd chain fatty acids. In *P. shermanii*, MCM catalyses the interconversion of methylmalonyl-CoA and succinyl-CoA as a part of the propionate fermentation pathway (Roy, 1996) and in *S. erythraea* the isomerisation of succinyl-CoA to methylmalonyl-CoA is involved in the synthesis of the precursor for erythromycin production (Hunaiti and Kolattukudi, 1984). In *E. coli*, the MCM containing pathway has also been thought to be involved in propionate metabolism (Evans *et al.*, 1993). Further, Haller (2000) determined that the *sbm* gene codes for MCM in *E. coli* and that it is a part of the

four gene operon possibly responsible for the decarboxylation of succinate to propionate (Haller *et al*., 2000). A detailed account of propionate and succinate metabolism in *E. coli* is provided in Section 1.7 of Chapter 1. On the basis of this literature, succinate or propionate were chosen as the sole carbon sources for the current study, with growth on glucose as the control. This was carried out to determine the likely role of the *sbm* gene in the metabolism of these carbon sources.

In order to understand the possible role of MCM in unusual carbon source utilisation, a mutant with the non functional *sbm* gene was created previously (I. Roy, unpublished data). The *sbm* gene was inactivated by a Mu dJ insertion mutation and was created using *S. typhimurium* LT2 and a temperature sensitive Pol A mutant (Pol A^-) of *E. coli*. This was then used for the comparative growth analysis of the wild type TR6524 and mutant FA1P1 in glucose, succinate or propionate as the sole source of carbon in a chemically defined minimal media. Firstly, the mutation was verified and confirmed and then the growth curves for both the wild type *E .coli* strain, TR6524 and the mutant *E. coli* strain, FA1P1 were plotted and the results compared.

The presence of vitamin B_{12} in the growth media has shown to decrease the lag phase from 90 hours to 70 hours when *E. coli* was grown in minimal media with propionate as the sole source of carbon (Kolodziej *et al*., 1968 & Wegener *et al.*, 1968). Vitamin B_{12} is expected to affect the level of expression of the *sbm* gene since MCM is a vitamin B_{12}-dependent enzyme. Hence, comparative growth studies were carried out using different concentrations (0.05, 0.5 or 5.0µg/ml) of vitamin B_{12}. The choice of the concentration of vitamin B_{12} (0.5µg/ml) was based on the conditions used in previous studies (Wegener *et al*., 1968 & Evans *et al*., 1993).

2.2 Results

2.2.1 Verification of the disruption mutation in the *sbm* gene

The disruption mutation in the *sbm* gene is represented as shown in Figure 2.1. Firstly, this mutation was verified using PCR. The primer pairs used in the reaction were *ici*A2-*R*55 and *sbm*11-*F*33 (primer sequences and PCR conditions are described in Tables 9.1 and 9.2, respectively). The primers *ici*A2 and *sbm*11 were based on sequences upstream and downstream of the *sbm* gene in the *E. coli* chromosome. Genomic DNA (gDNA) templates from the mutant strain (FA1P1) and from the wild type strain (TR6524) (as negative control) were used for the reaction.

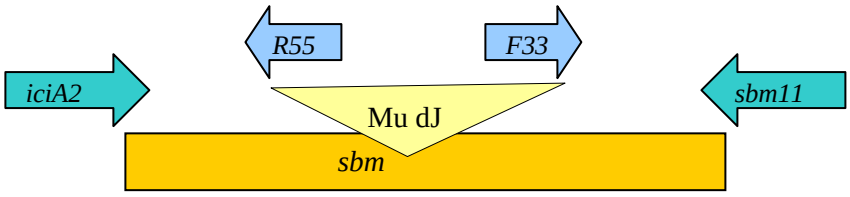

Figure 2.1: *sbm* disruption mutation. The *sbm* gene with the Mu dJ insertion. *ici*A2, *R*55, *F*33 and *sbm*11 show the location of the primers used to verify the disruption mutation.

The PCR conditions were first optimised with a magnesium ion concentration of 1.5mM at three different annealing temperatures 55^{o}C, 60^{o}C and 65^{o}C (Figure 2.2).

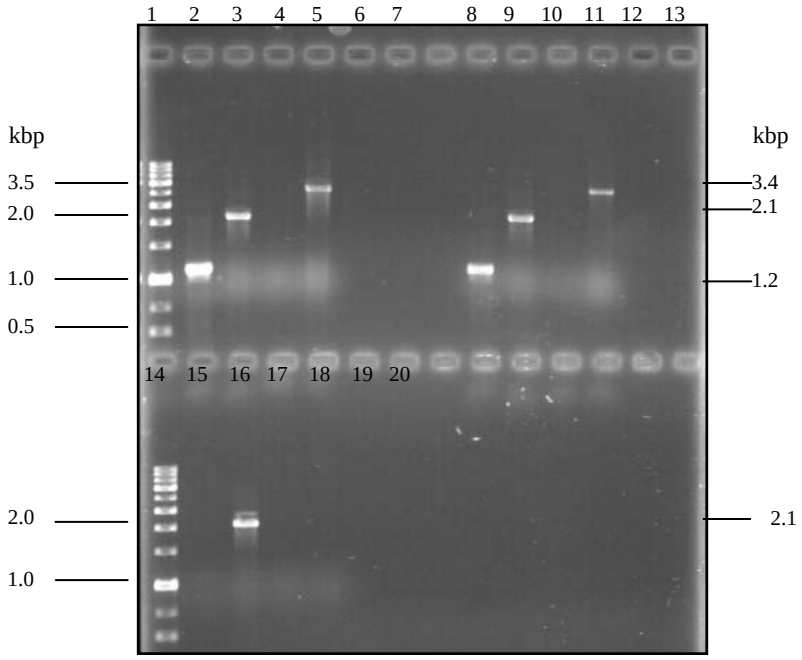

Figure 2.2: Agarose gel showing PCR products of the PCR grid with 3 different temperatures and magnesium ion concentration of 1.5mM, to verify the presence of the Mu dJ insertion. Lanes 1-7 show products from samples annealed at 55^{o}C, lanes 8-13 show products from samples annealed at 60^{o}C and lanes 15-19 show products from samples annealed at 65^{o}C.
Lanes 1 &14 - Hyper ladder III (marker DNA from Bioline, UK)
Lanes 2, 8 &15- PCR products of *iciA2* and *R55*; FA1P1 gDNA template
Lanes 3, 9 &16- PCR products of *sbm11* and *F33*; FA1P1 gDNA template
Lanes 4, 10 &17- samples with *iciA2* and *sbm*11; FA1P1 gDNA template
Lanes 5, 11 &18- products from *sbm* 11 and *iciA2*; TR6524 gDNA template
Lanes 6, 12 & 19- samples with *iciA2* and *R55* as primers; TR6524 gDNA template
Lanes 7, 13 & 20- samples with *F33* and *sbm11* as primers; TR6524 gDNA template

It can be observed from Figure 2.2 that the expected products were formed under two of the three annealing temperatures tested (55^{o}C and 60^{o}C), however, at an annealing temperature of 65^{o}C only one product was formed. In order that the PCR was performed at the most stringent condition possible, 60^{o}C and 1.5mM magnesium chloride were chosen and the PCR was repeated to check for consistency and the products obtained are shown in Figure 2.3.

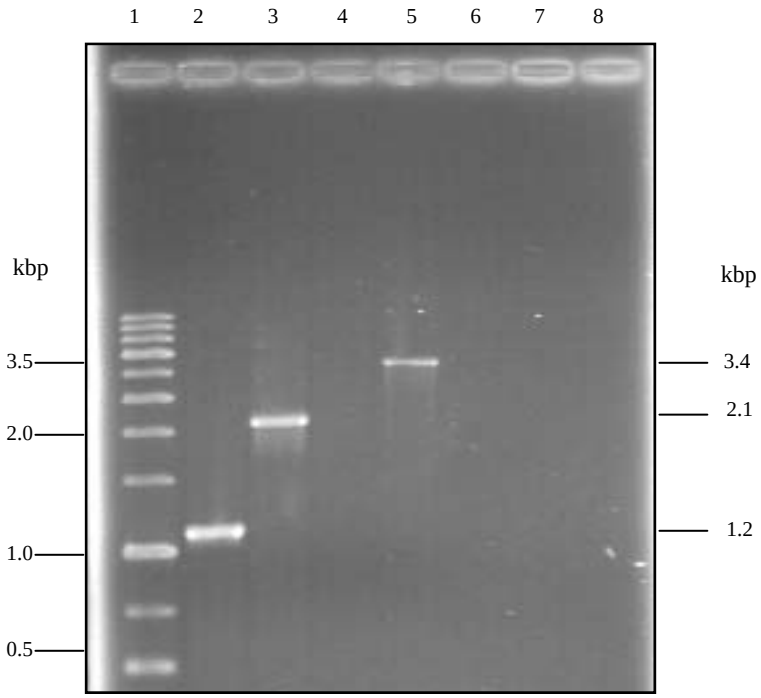

Figure 2.3: Agarose gel showing PCR products verifying the presence of the Mu dJ insertion.
Lane 1- Hyper ladder III (marker DNA from Bioline, UK),
Lane 2- PCR product of *ici*A2 and *R*55; FA1P1 gDNA template
Lane 3- PCR product of *sbm*11 and *F*33; FA1P1 gDNA template
Lane 4- sample with *ici*A2 and *sbm*11; FA1P1 gDNA template
Lane 5- product from *sbm* 11 and *ici*A2; TR6524 gDNA template
Lane 6- sample with *ici*A2 and *R*55 as primers; TR6524 gDNA template
Lane 7- sample with *F*33 and *sbm*11 as primers; TR6524 gDNA template

The PCR reactions with primer pairs *ici*A2-*R*55 and *F*33-*sbm*11 and FA1P1 gDNA as template (Figure 2.1) formed a 1.2kbp product and 2.1kbp product respectively, thus confirming the presence of the Mu dJ insertion within the *sbm* gene (Figure 2.3). Since Mu dJ is known to insert anywhere in the genome there is no specific size expected in this case. There were no products formed with these primers when gDNA from TR6524 was used as the template, as expected, confirming the absence of the Mu dJ insertion in the TR6524 genome. In the reaction with *ici*A2-*sbm*11 primer pair, a 3.4kbp product was formed when gDNA template from TR6524 was used. This is the expected size of the amplified *sbm* gene along with a part of the

iciA gene. The reaction using the primer pair *ici*A2 and *sbm*11 with genomic DNA from FA1P1 as the template did not give a product as the inserted Mu dJ is approximately 11kbp in size and hence the resulting product size would be 15kbp which is too large a product to be formed under the reaction conditions used.

These results confirmed the disruption mutation in the *sbm* gene. Hence the Kan^r (kanamycin resistant) transductant with the Mu dJ insertion in the *sbm* gene, as verified by PCR, was established as the *sbm*-disrupted mutant. The insertion is represented as shown in Figure 2.1. This mutation would allow the comparison of wild type *E. coli* TR6524 and the mutant strain (FA1P1) lacking the functional gene.

2.2.2 Comparative growth analyses

Growth of the two strains (wild type TR6524 and mutant FA1P1) in minimal media with glucose, succinate or propionate as the sole source of carbon and in the presence of 3 different concentrations (0.05, 0.5, 5.0µg/ml) of vitamin B_{12} was studied. Growth in minimal media with glucose as the sole source of carbon was used as a control condition.

2.2.2.1 Growth studies with glucose as the sole source of carbon

The results of the growth of the two strains in minimal media with glucose as the sole source of carbon with the conditions mentioned above are shown in Figures 2.4a, b, c, d, e and f.

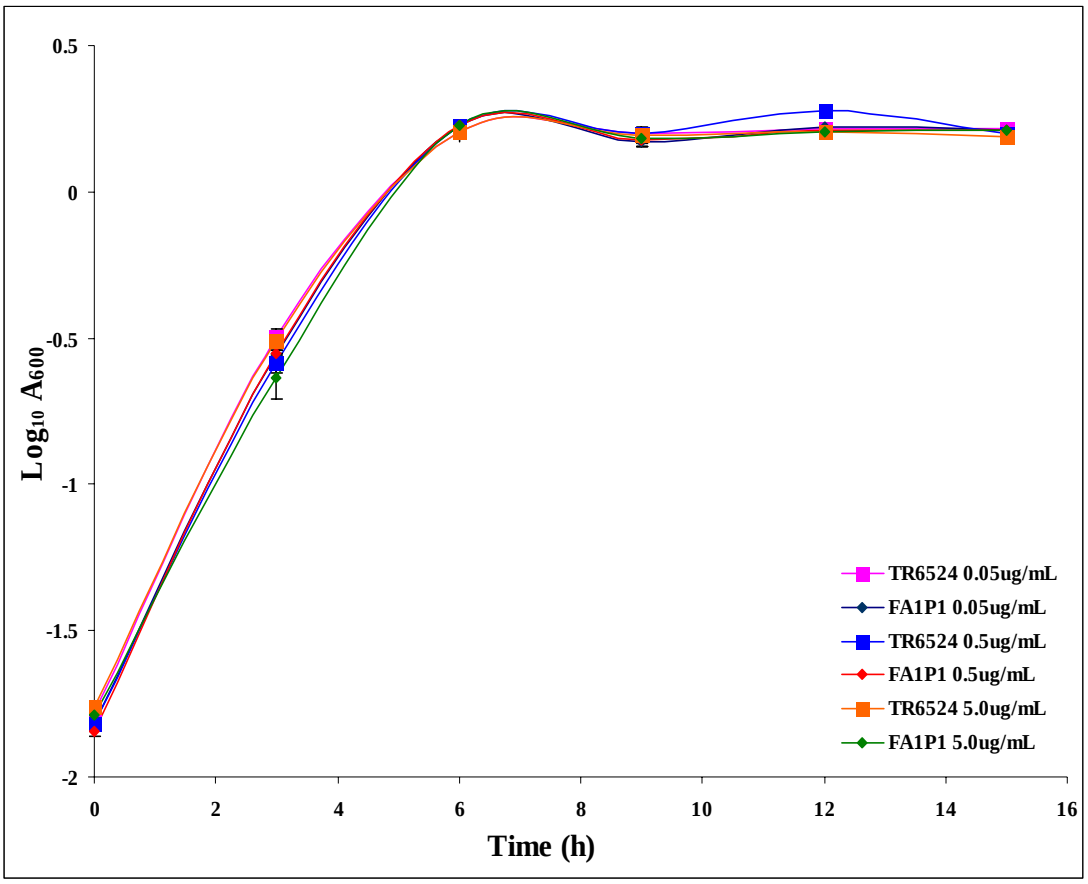

Figure 2.4a: Comparative growth curves for *E. coli* K12 TR6524 and the Mu dJ insertion mutant FA1P1 in minimal media with glucose as the sole source of carbon in the presence of three different concentrations (0.05, 0.5, 5.0µg/mL) of vitamin B_{12} as indicated in the figure. The experiment was done in triplicates.

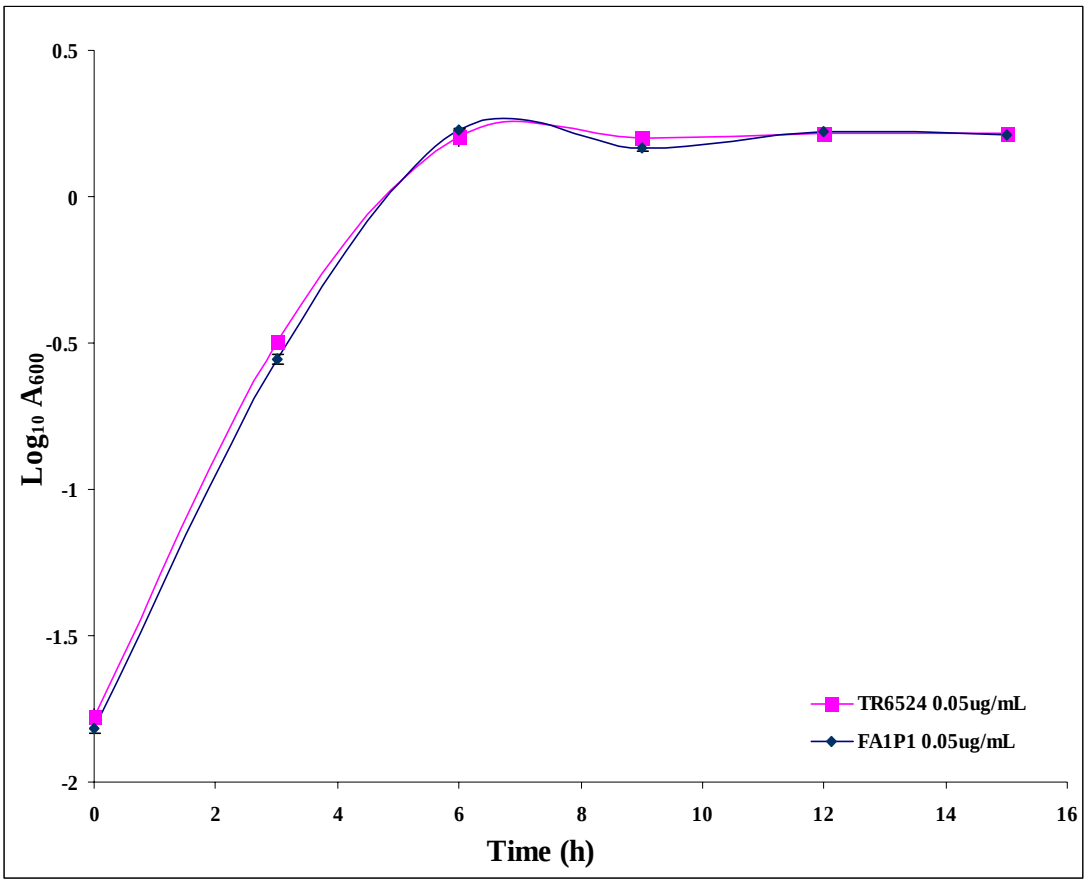

Figure 2.4b: Comparative growth curves for *E. coli* K12 TR6524 and the insertion mutant FA1P1 in minimal media with glucose as the sole source of carbon in the presence of 0.05µg/mL vitamin B_{12} as indicated in the figure. The experiment was done in triplicates.

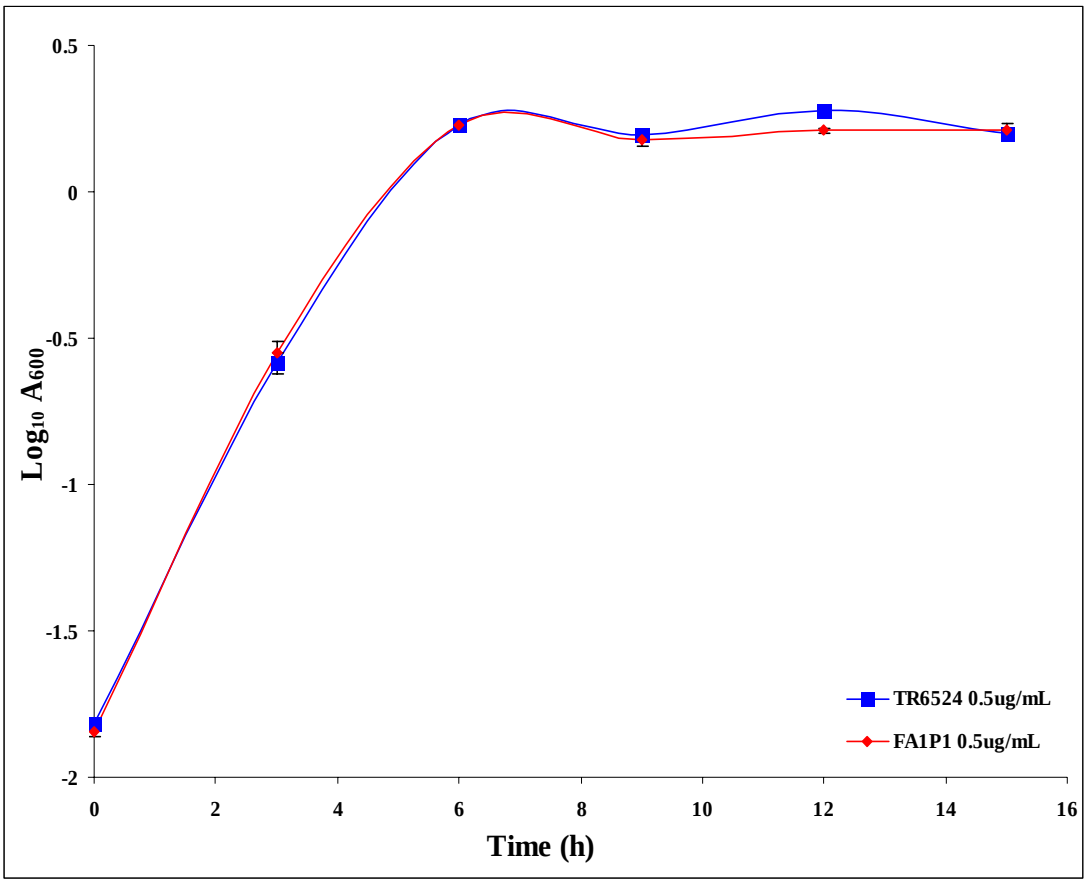

Figure 2.4c: Comparative growth curves for *E. coli* K12 TR6524 and the insertion mutant FA1P1 in minimal media with glucose as the sole source of carbon in the presence of 0.5µg/mL vitamin B_{12} as indicated in the figure. The experiment was done in triplicates.

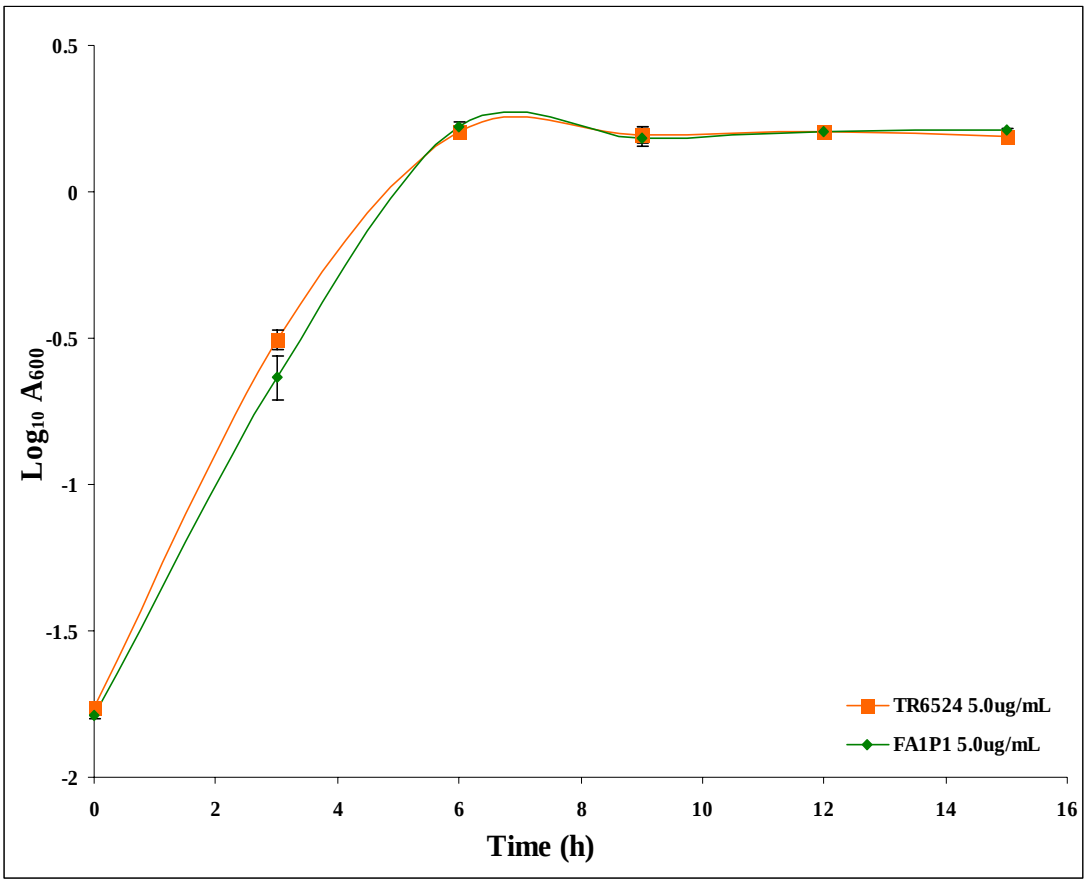

Figure 2.4d: Comparative growth curves for *E. coli* K12 TR6524 and the insertion mutant FA1P1 in minimal media with glucose as the sole source of carbon in the presence of 5.0µg/mL vitamin B_{12} as indicated in the figure. The experiment was done in triplicates.

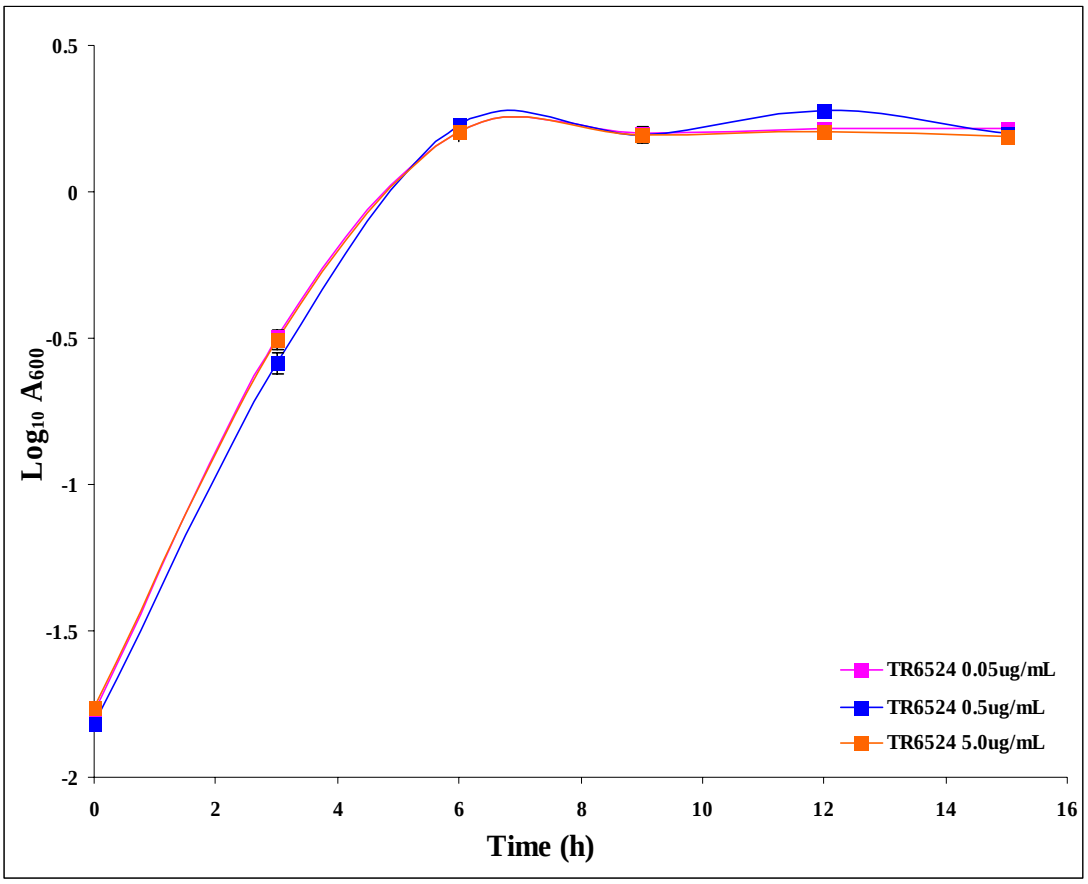

Figure 2.4e: Comparative growth curves for *E. coli* K12 TR6524 in minimal media with glucose as the sole source of carbon in the presence of 0.05, 0.5 and 0.5µg/mL vitamin B_{12} as indicated in the figure. The experiment was done in triplicates.

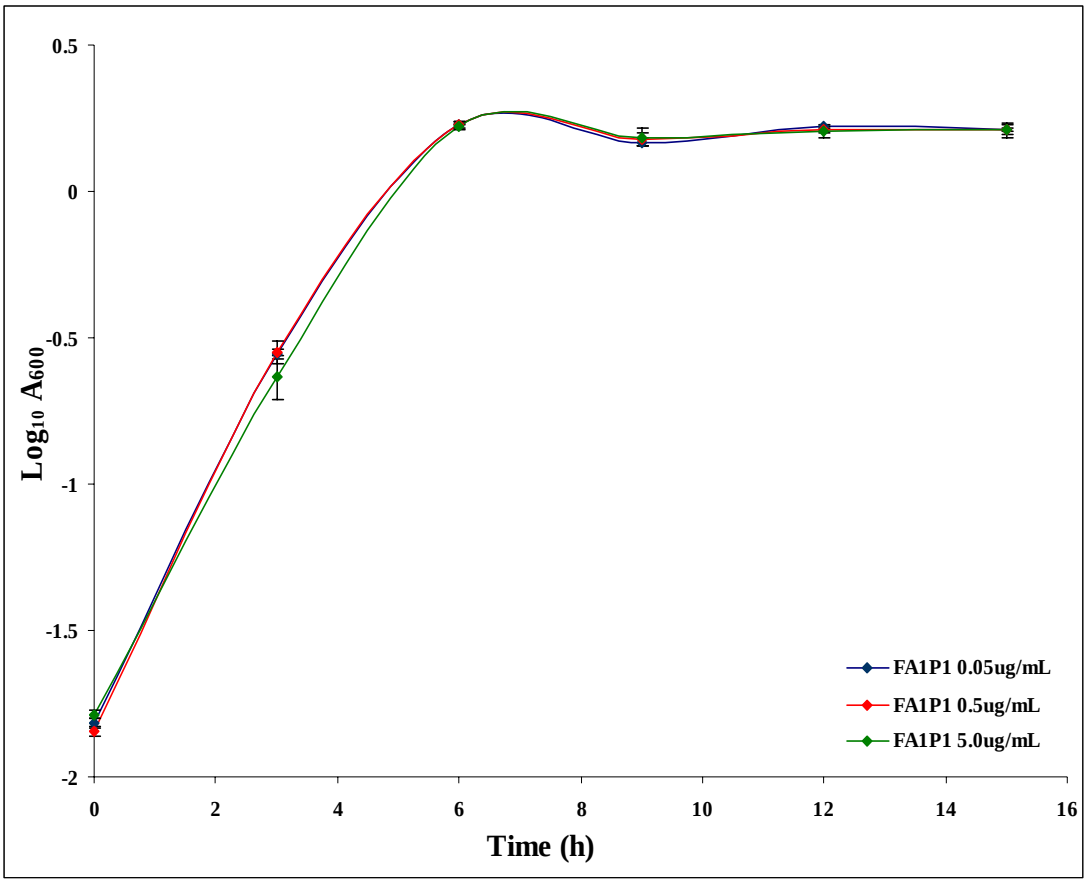

Figure 2.4f: Comparative growth curves for *E. coli* K12 insertion mutant FA1P1 in minimal media with glucose as the sole source of carbon in the presence of 0.05, 0.5 and 0.5µg/mL vitamin B_{12} as indicated in the figure. The experiment was done in triplicates.

Figure 2.4a shows the consolidated growth curves for *E. coli* K12 wild type TR6524 and the FA1P1 mutant in minimal media with glucose and the three different concentrations of vitamin B_{12} (as indicated in the figures). Figures 2.4b, 2.4c and 2.4d represent a comparison of the individual growth curves for the two strains in minimal media with glucose as the sole source of carbon with 0.05µg/ml, 0.5µg/ml and 5.0µg/ml of vitamin B_{12} added to the media, respectively. Figures 2.4e and 2.4f show a comparison of the growth curves for the wild type and the mutant strains, respectively, in minimal media with glucose as the sole source of carbon with the three vitamin B_{12} concentrations, as indicated in the figures.

Growth in minimal media with glucose was almost spontaneous without any noticeable lag phase for the wild type, TR6524 and the mutant, FA1P1 and the stationary phase of growth was observed to have been reached within 7-8 hours of growth, for both strains, in all growth conditions (consolidated curves, Figure 2.4a). There was no significant difference in the growth pattern between the wild type and mutant strains.

2.2.2.2 Growth studies with succinate as sole carbon source

The possible role of the *sbm* gene in succinate utilisation has been proposed (Haller *et al*., 2000). Hence, the wild type and the mutant *E. coli* strains were grown in minimal medium with succinate as the sole source of carbon. The results of the growth of the two strains in minimal media with succinate as the sole source of carbon, in the presence of the three concentrations of vitamin B_{12} are shown in Figures 2.5a, b, c, d, e and f.

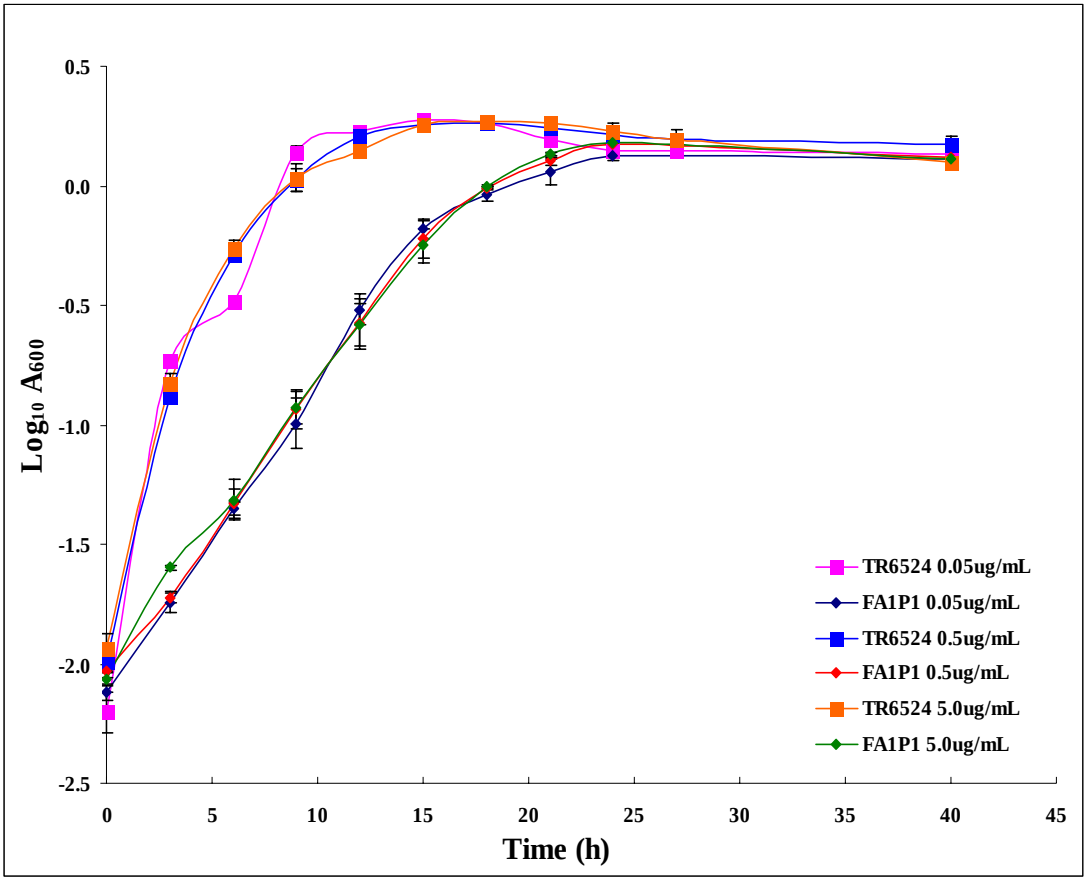

Figure 2.5a: Comparative growth curves for *E. coli* K12 TR6524 and the Mu dJ insertion mutant FA1P1 in minimal media with succinate as the sole source of carbon in the presence of three different concentrations (0.05, 0.5, 5.0µg/ml) of vitamin B_{12} as indicated in the figure. The experiment was done in triplicates.

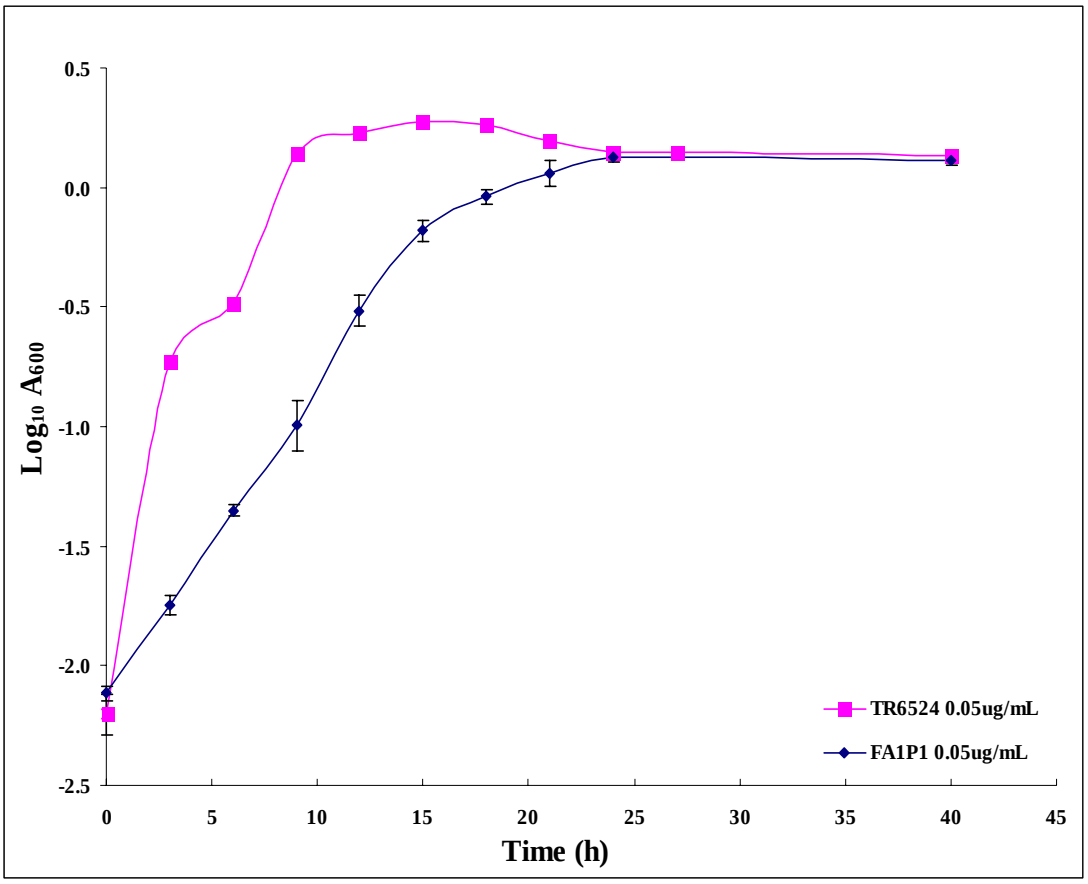

Figure 2.5b: Comparative growth curves for *E. coli* K12 TR6524 and the Mu dJ insertion mutant FA1P1 in minimal media with succinate as the sole source of carbon in the presence of 0.05µg/ml vitamin B_{12} as indicated in the figure. The experiment was done in triplicates.

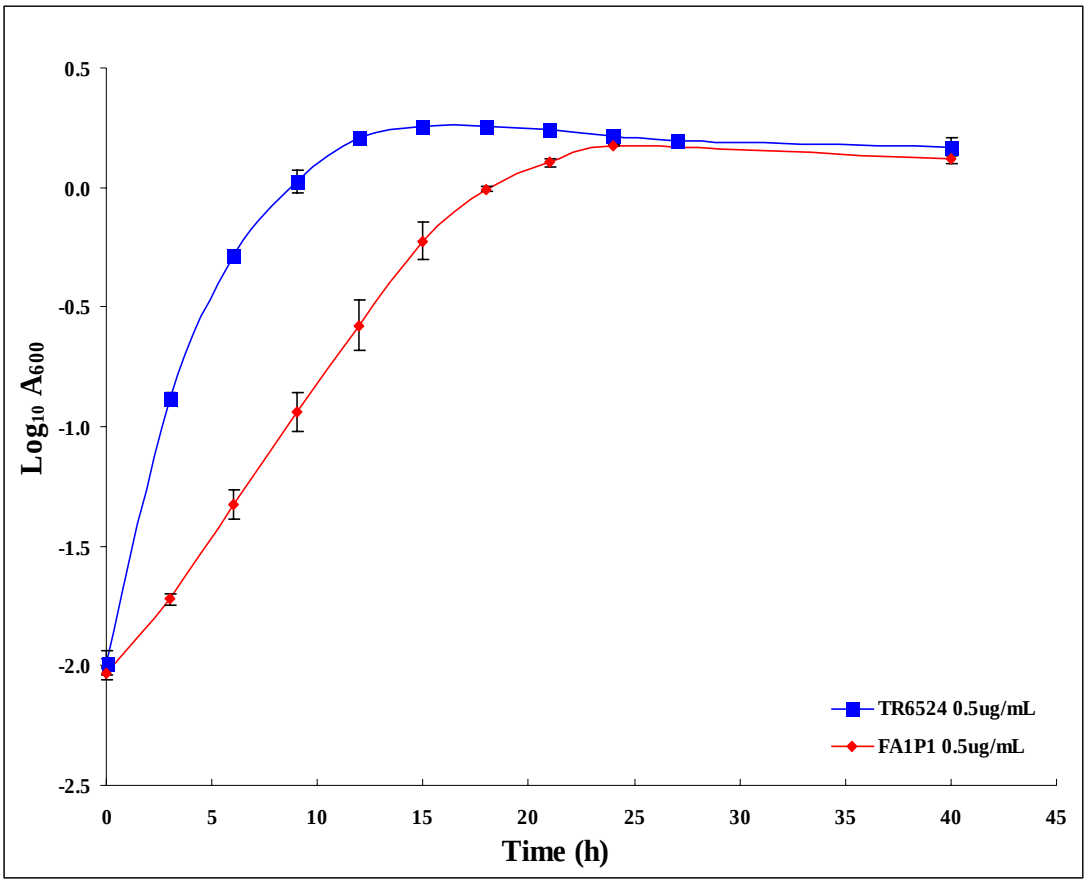

Figure 2.5c: Comparative growth curves for *E. coli* K12 TR6524 and the Mu dJ insertion mutant FA1P1 in minimal media with succinate as the sole source of carbon in the presence of 0.5µg/ml vitamin B_{12} as indicated in the figure. The experiment was done in triplicates.

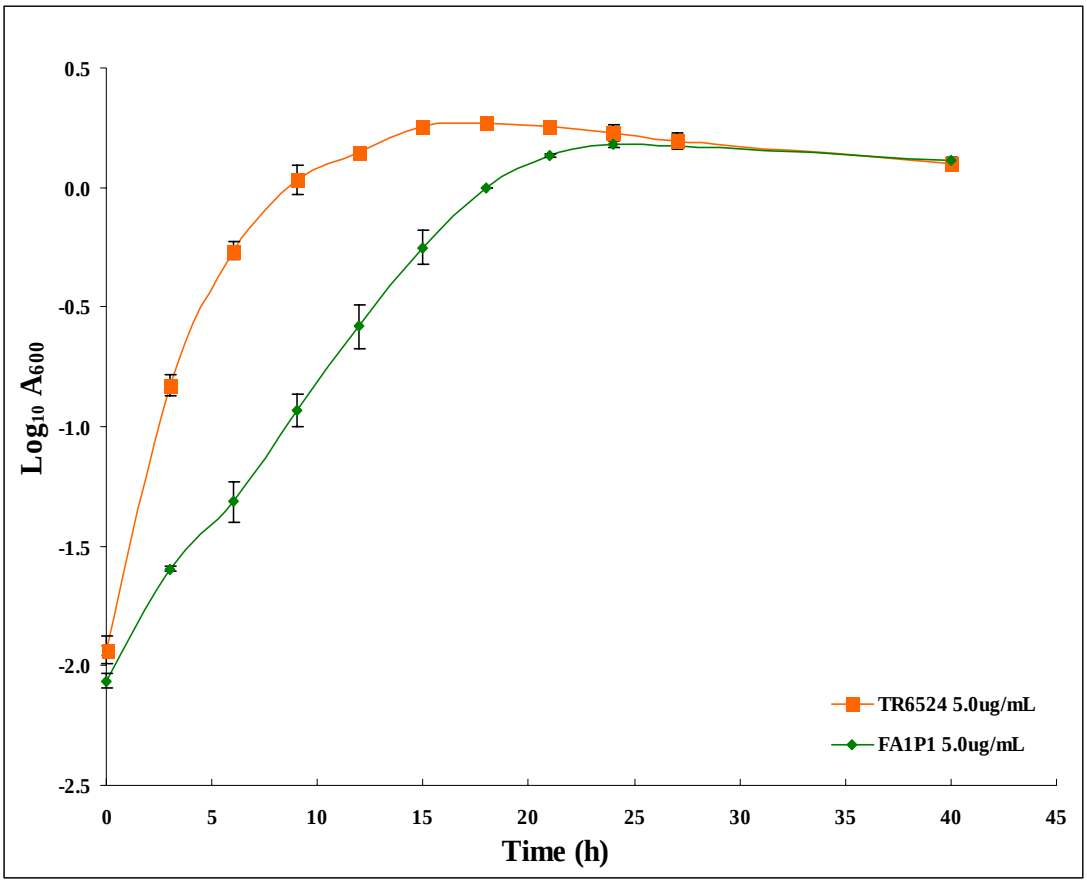

Figure 2.5d: Comparative growth curves for *E. coli* K12 TR6524 and the Mu dJ insertion mutant FA1P1 in minimal media with succinate as the sole source of carbon in the presence of 5.0µg/ml vitamin B_{12} as indicated in the figure. The experiment was done in triplicates.

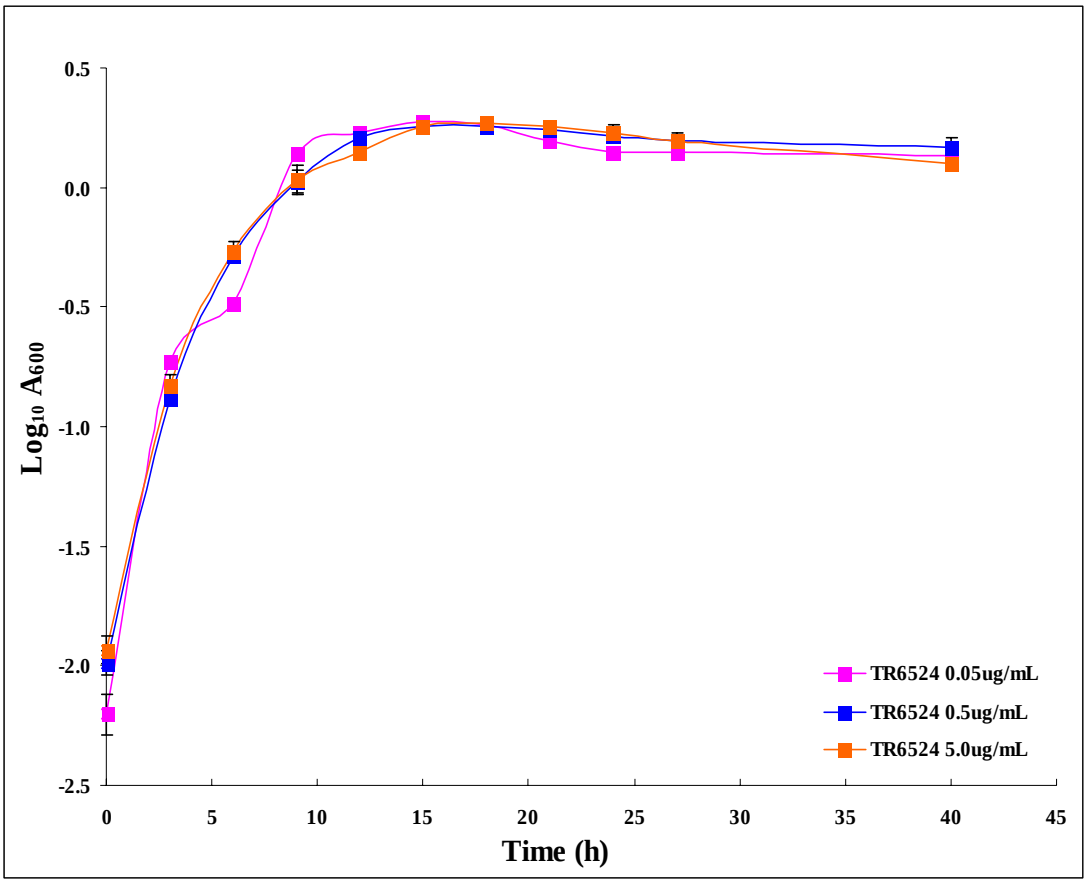

Figure 2.5e: Comparative growth curves for *E. coli* K12 TR6524 in minimal media with succinate as the sole source of carbon in the presence of 0.05, 0.5 and 5.0µg/ml vitamin B_{12} as indicated in the figure. The experiment was done in triplicates.

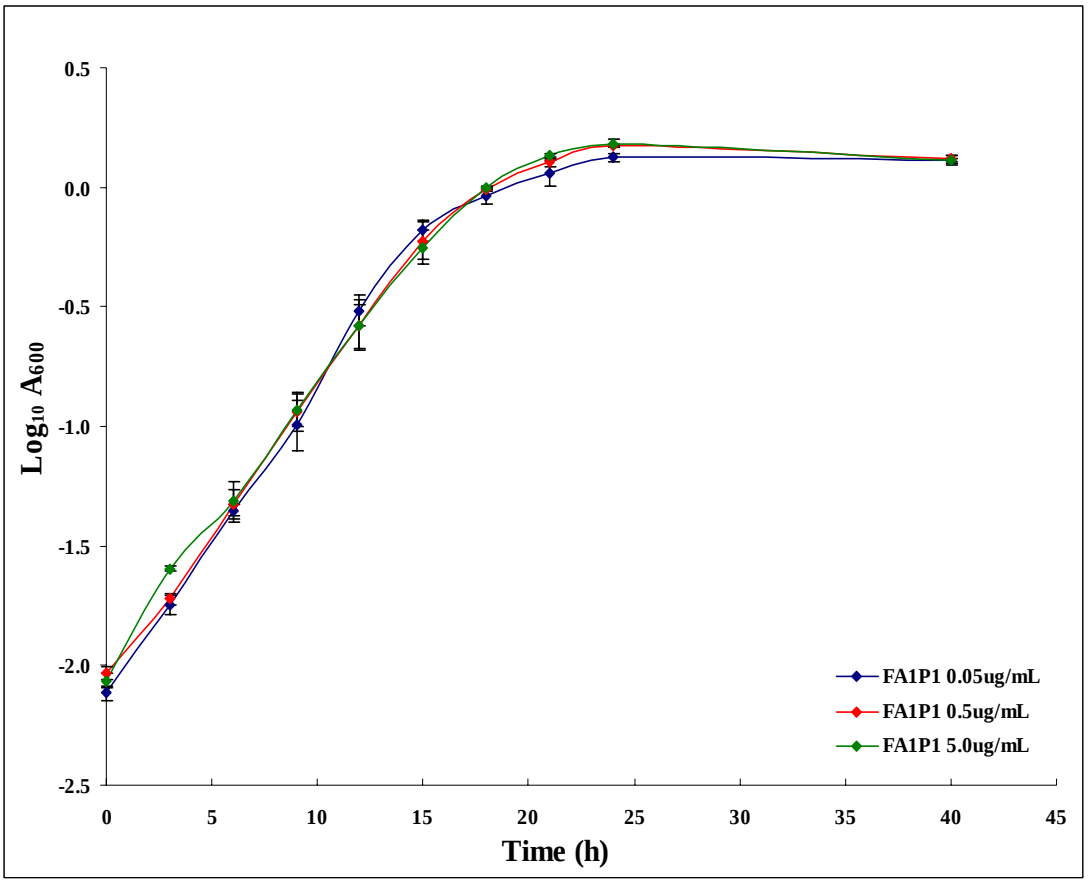

Figure 2.5f: Comparative growth curves for *E. coli* K12 insertion mutant, FA1P1 in minimal media with succinate as the sole source of carbon in the presence of 0.05, 0.5 and 5.0µg/ml vitamin B_{12} as indicated in the figure. The experiment was done in triplicates.

Figure 2.5a shows the consolidated growth curves for *E. coli* K12 wild type TR6524 and the FA1P1 mutant in minimal media with succinate and the three different concentrations of vitamin B_{12} (as indicated in the figure). Figures 2.5b, 2.5c and 2.5d represent a comparison of the individual growth curves for the two strains in minimal media with succinate as the sole source of carbon with 0.05µg/ml, 0.5µg/ml and 5.0µg/ml added to the media, respectively. Figures 2.5e and 2.5f show a comparison of the growth curves for the wild type and the mutant strains, respectively, in minimal media with succinate as the sole source of carbon with the three vitamin B_{12} concentrations, as indicated in the figures.

Figures 2.5b, c, and d show that, under the specified conditions of growth, the wild type TR6524 grows better than the mutant FA1P1 strain under all the conditions tested. The wild type TR6524 reached the stationary phase at about 15 hours of growth while the mutant, FA1P1, on the other hand, reached stationary phase at 24 hours (an appreciable 9 hours later than that observed for the wild type TR6524). These results indicate that the *sbm* gene possibly has a role in the utilisation of succinate in *E. coli*.

A noticeable difference between the growth patterns of the two strains was observed as seen in Figures 2.5b, c and d. The growth curve for the wild type, TR6524, was similar to that observed for glucose (Figures 2.4b, c and d) in all growth conditions while the mutant, FA1P1, was observed to lag behind throughout the period of its growth until about the 25^{th} hour, since the beginning of the growth. The mutant strain (FA1P1) showed a distinctly different growth pattern and was observed to have a slower growth compared to that of the wild type in all growth conditions used. This was a major difference noticed in the growth pattern of the mutant strain indicating the role of the *sbm* gene in the utilisation of succinate, thus confirming the postulate made by Haller *et al*. (2000).

2.2.2.3 Growth studies with propionate as sole carbon source

The methylmalonyl-CoA mutase pathway has been reported to be one of the pathways involved in the utilisation of propionate by *E. coli* (Evans *et al*., 1993; Textor *et al*., 1997 & London *et al*., 1999). Hence, in order to further explore the role of the *sbm* gene in the utilisation of propionate, the wild type and mutant strains of *E. coli* were grown in minimal media with propionate as the sole source of carbon in the presence of the three different concentrations of vitamin B_{12}. The results obtained are shown in Figures 2.6a-f.

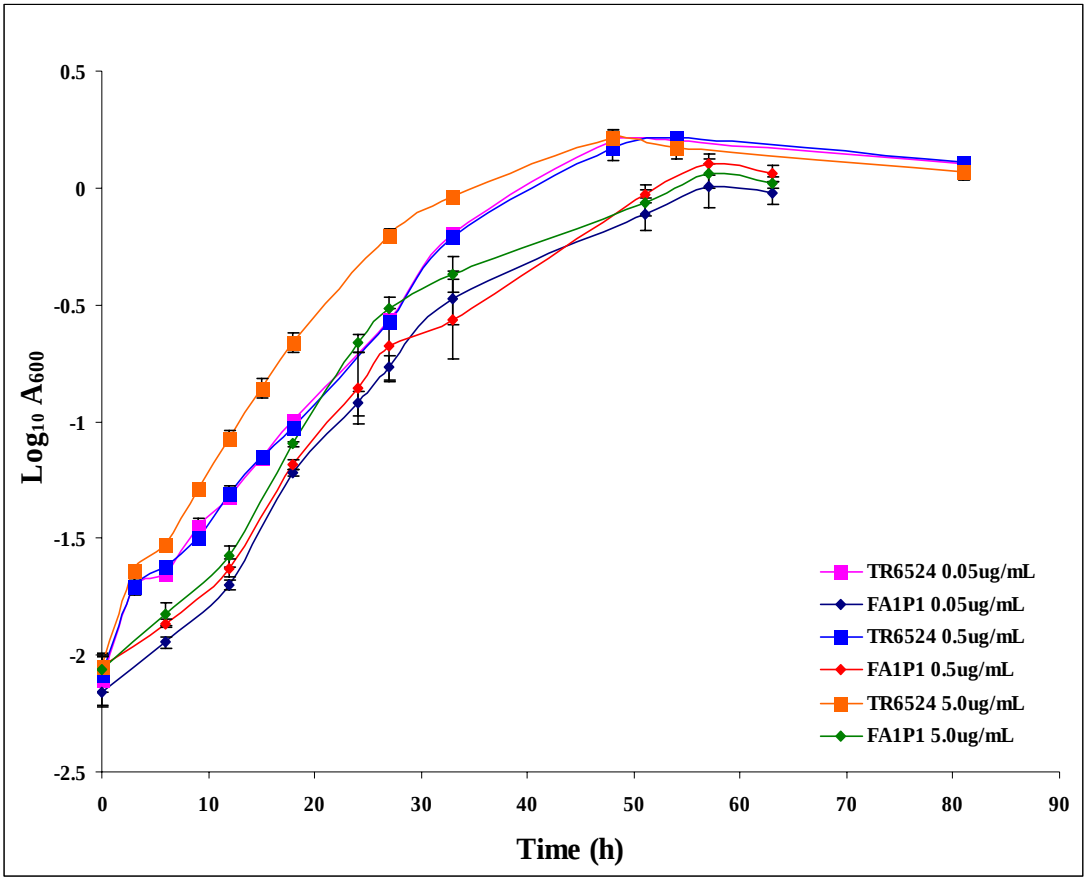

Figure 2.6a: Comparative growth curves for *E. coli* K12 TR6524 and the Mu dJ insertion mutant FA1P1 in minimal media with propionate as the sole source of carbon in the presence of three different concentrations (0.05, 0.5, 5.0µg/ml) of vitamin B_{12} as indicated in the figure. The experiment was done in triplicates.

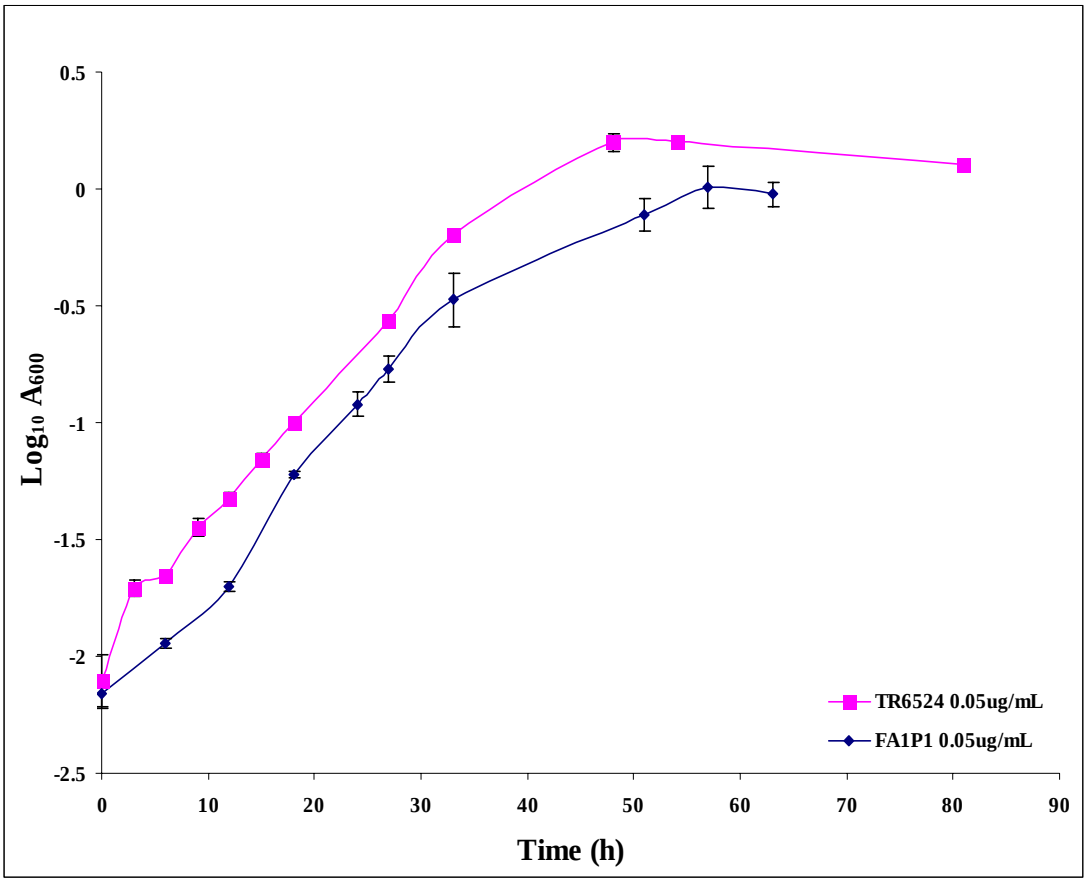

Figure 2.6b: Comparative growth curves for *E. coli* K12 TR6524 and the Mu dJ insertion mutant FA1P1 in minimal media with propionate as the sole source of carbon in the presence of 0.05µg/ml vitamin B_{12} as indicated in the figure. The experiment was done in triplicates.

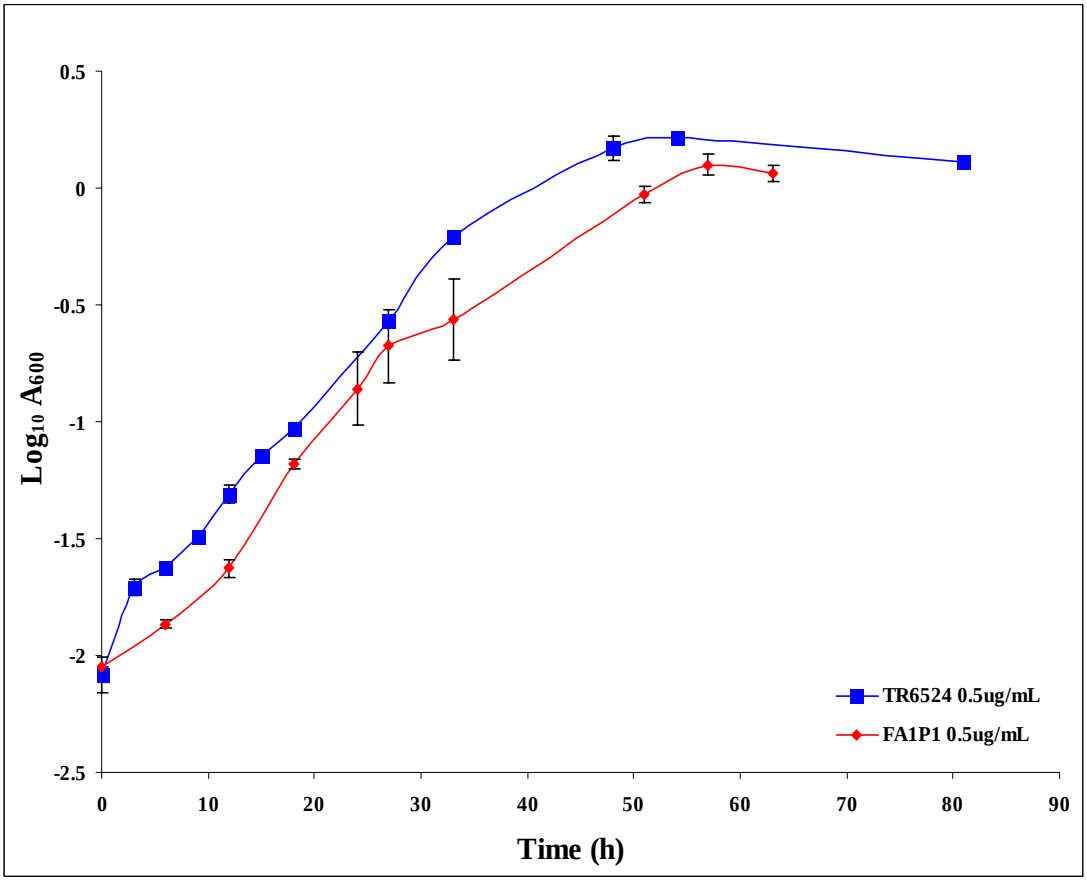

Figure 2.6c: Comparative growth curves for *E. coli* K12 TR6524 and the Mu dJ insertion mutant FA1P1 in minimal media with propionate as the sole source of carbon in the presence of 0.5µg/ml vitamin B_{12} as indicated in the figure. The experiment was done in triplicates.

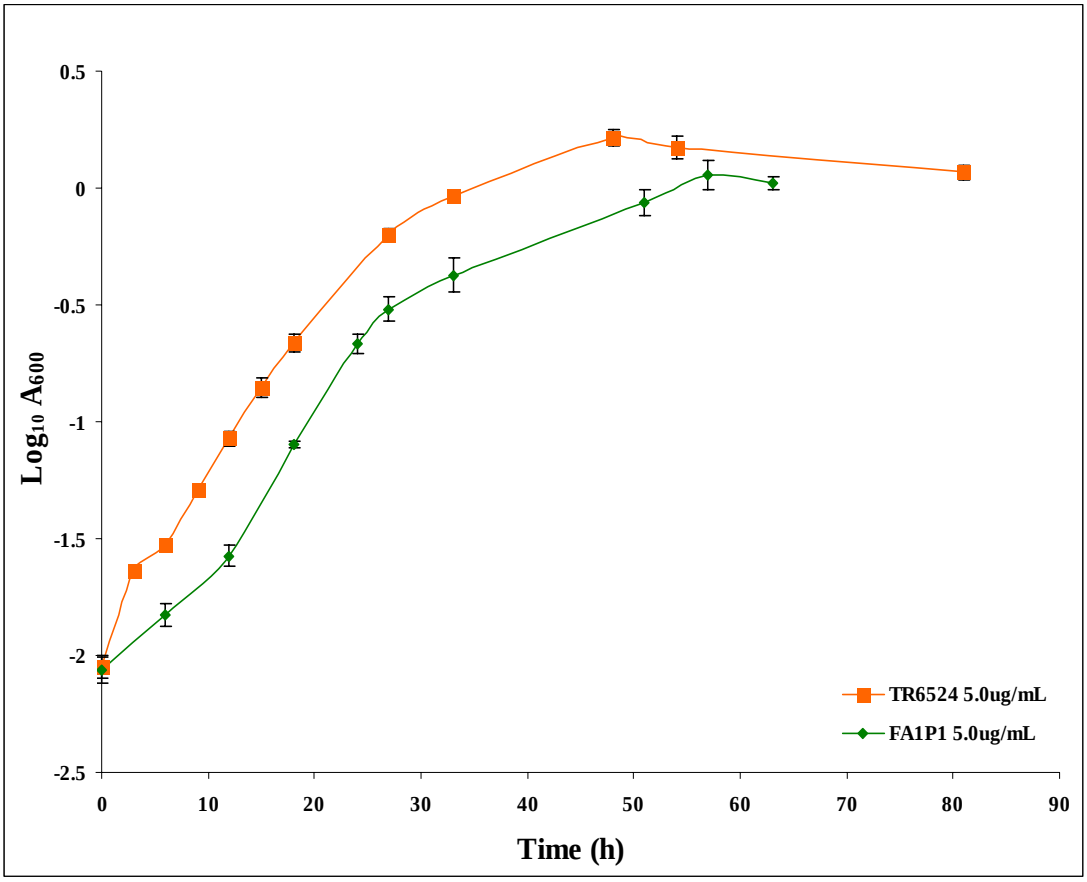

Figure 2.6d: Comparative growth curves for *E. coli* K12 TR6524 and the Mu dJ insertion mutant FA1P1 in minimal media with propionate as the sole source of carbon in the presence of 5.0µg/ml vitamin B_{12} as indicated in the figure. The experiment was done in triplicates.

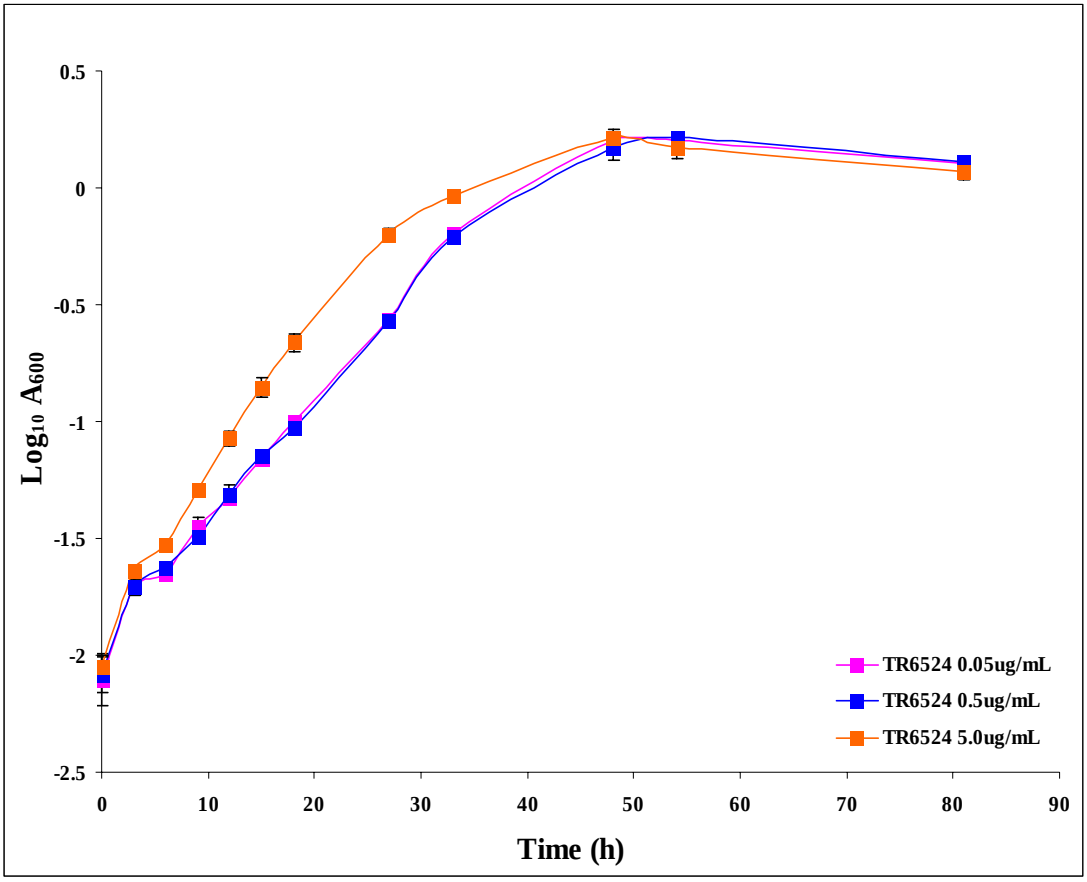

Figure 2.6e: Comparative growth curves for *E. coli* K12 TR6524 in minimal media with propionate as the sole source of carbon in the presence of 0.05, 0.5 and 5.0µg/ml vitamin B_{12} as indicated in the figure. The experiment was done in triplicates.

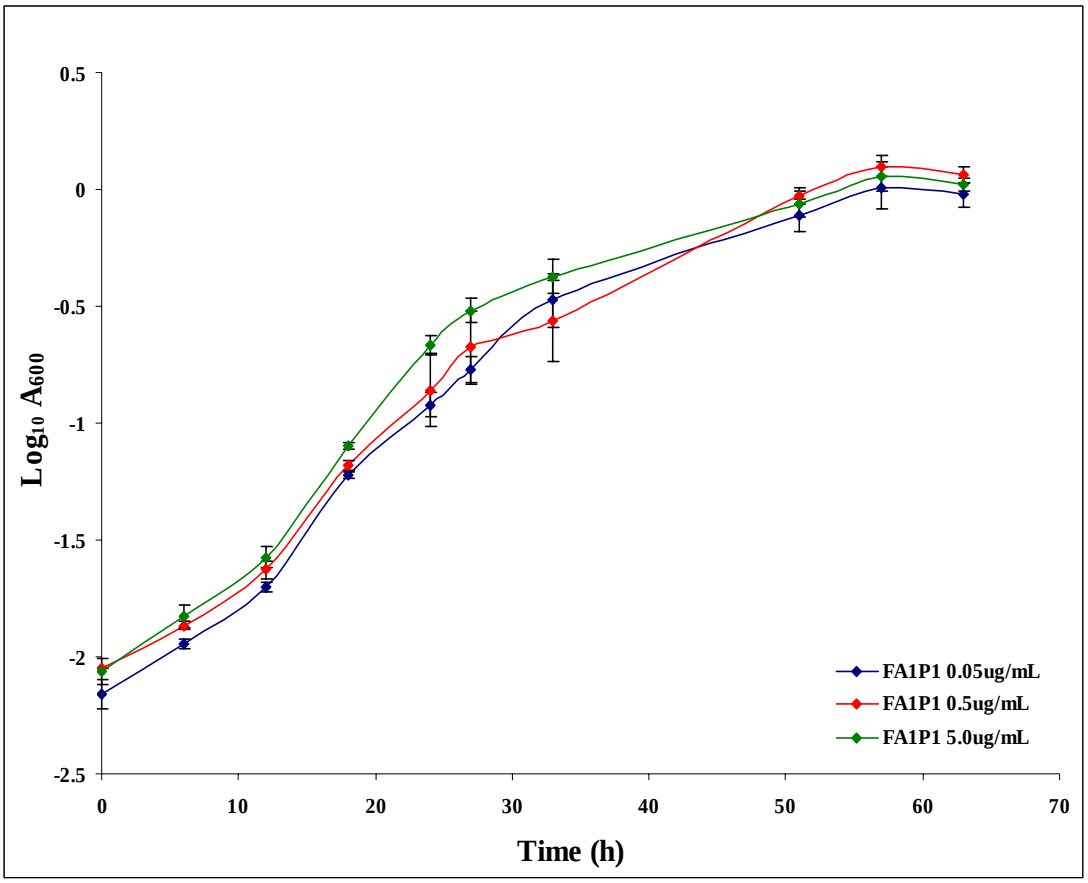

Figure 2.6f: Comparative growth curves for *E. coli* K12 insertion mutant, FA1P1 in minimal media with propionate as the sole source of carbon in the presence of 0.05, 0.5 and 5.0µg/ml vitamin B_{12} as indicated in the figure. The experiment was done in triplicates.

Figure 2.6a shows the consolidated growth curves for *E. coli* K12 wild type TR6524 and the FA1P1 mutant in minimal media with propionate and the three different concentrations of vitamin B_{12} (as indicated in the figures). Figures 2.6b, 2.6c and 2.6d represent a comparison of the individual growth curves for the two strains in minimal media with propionate as the sole source of carbon with 0.05µg/ml, 0.5µg/ml or 5.0µg/ml added to the media, respectively. Figures 2.6e and 2.6f compare the differences in the individual growth pattern of the two strains in the presence of the three different concentrations of vitamin B_{12} in the medium.

The wild type strain TR6524 exhibited marginally better growth than the FA1P1 mutant within all the growth conditions (Figures 2.6 b-d). The growth pattern within

the wild type was slightly better in the presence of 5.0µg/ml vitamin B_{12} than in the presence of 0.05µg/ml or 0.5µg/ml vitamin B_{12} (Figure 2.6e). A similar observation was not made in the case of the mutant (Figure 2.6f). Although, the mutant strain lacked a functional Sbm, its growth was not significantly affected unlike the growth of the mutant in minimal media with succinate as the sole source of carbon (Figure 2.5c, d and e). One of the other pathways (Textor *et al*., 1997) possibly compensated for the inactive, vitamin B_{12} utilising MCM pathway. Overall, the only difference observed with the growth of both strains was that the wild type strain entered the stationary phase slightly earlier than the mutant strain.

2.3 Discussion

The growth of the wild type, TR6524 and the mutant, FA1P1 in minimal media with glucose, succinate or propionate as the sole source of carbon and in the presence of 0.05, 0.5, 5.0µg/ml vitamin B_{12} have been established. It could be observed from these curves that neither of the strains had any lag phase. The absence of a lag phase in minimal media with unusual carbon sources such as succinate and propionate could be due to the use of exponential phase cultures, previously adapted to the media in small scale (Section 9.2.2), to inoculate the appropriate large scale volumes that were subsequently used for the growth analyses, thus, allowing the cells to continue their growth in the exponential phase (Mendelstam *et al*., 1982 & Ingraham *et al*., 1983).

2.3.1 Growth of *E. coli* TR6524 and FA1P1 on glucose

Glucose is the simplest and the most favoured carbon source for *E. coli*. Growth of the two strains TR6524 and FA1P1 on this carbon source was used as a control to measure the possible differences observed in the growth patterns on succinate or propionate when these fatty acids are used as the sole source of carbon in M9 minimal media.

Strain and growth condition	Growth rate, k (h^{-1})	Doubling time, g (h)	Doublings/ hour (μ)
TR6524 0.05μg/ml vitamin B_{12}.	0.762	0.40	2.53
FA1P1 0.05μg/ml vitamin B_{12}.	0.784	0.38	2.60
TR6524 0.5μg/ml vitamin B_{12}.	0.784	0.38	2.60
FA1P1 0.5μg/ml vitamin B_{12}.	0.795	0.38	2.64
TR6524 5.0μg/ml vitamin B_{12}.	0.755	0.40	2.50
FA1P1 5.0μg/ml vitamin B_{12}.	0.772	0.39	2.56

Table 2.1: Measurement of *E. coli* growth in glucose. The growth rate, doubling time and number of doublings/hour were measured for *E. coli* TR6524 and FA1P1 in minimal media with glucose as the sole source of carbon in the presence of 0.05, 0.5 and 5.0μg/mL vitamin B_{12}.

There is no noticeable difference in the pattern of growth observed with the change in the concentration of vitamin B_{12} in the medium. The growth of the two strains in glucose was also quantified and the growth rate, doubling time and doublings per hour measurements are as shown in Table 2.1.

Growth measurement information calculated from the curves shows that both strains, TR6524 and FA1P1, have very similar growth rates with doubling times of 0.38 - 0.40 hour under all conditions of growth, irrespective of the concentration of vitamin B_{12} in the media. This showed that the pathway employing the *sbm* gene product (MCM) was not crucial in the utilisation of glucose. This was also indicative of the fact that MCM was possibly induced only under specific conditions of growth.

2.3.2 Growth of *E. coli* TR6524 and FA1P1 on succinate

Kolodziej *et al*. (1968) showed that the lag phase of *E. coli* E26 was reduced to 65 hours from 90 hours when the bacteria was allowed to grow in the propionate media with limiting concentrations (0.5μmoles/ml) of succinate and suggested that succinate and propioante metabolism involved the use of common enzyme systems (Kolodziej *et al*., 1968). Only a few previous studies have used succinate as a source

of carbon for *E. coli* growth (Liu *et al*., 2005 & Paliy and Gunasekara, 2007). So this study attempted to understand the growth of *E. coli* in succinate and eventually the metabolic context of MCM in succinate metabolism in the bacteria.

The growth study of the wild type and the mutant strains in minimal media with succinate as the sole source of carbon was inspired by the observations of Haller *et al*. (2000). The relatively slower growth and the delay in reaching the stationary phase for the mutant strain during growth in minimal media with succinate as the sole source of carbon confirms, for the first time, the hypothesis made by Haller and co-workers (2000), *i.e*., the *sbm* containing operon was involved in the decarboxylation of succinate to propionate (Haller *et al*., 2000). This is the first experimental verification of the role of the *sbm* gene product in succinate utilisation.

The vitamin B_{12} in the medium would not only compliment the Sbm, which is a B_{12}-dependent enzyme but the use of three different concentrations would help to conclusively see its effect on MCM and ultimately on the growth of the wild type and the mutant *E. coli* strains. Growth rate, doubling time and the number of doublings per hour were calculated and the results are shown in Table 2.2.

Strain and growth condition	**Growth rate, k (h^{-1})**	**Doubling time, g (h)**	**Doublings/ hour(μ)**
TR6524 0.05μg/ml vitamin B_{12}.	0.55	0.54	1.85
FA1P1 0.05μg/ml vitamin B_{12}.	0.25	1.19	0.84
TR6524 0.5μg/ml vitamin B_{12}.	0.50	0.59	1.69
FA1P1 0.5μg/ml vitamin B_{12}.	0.24	1.21	0.82
TR6524 5.0μg/ml vitamin B_{12}.	0.49	0.61	1.65
FA1P1 5.0μg/ml vitamin B_{12}.	0.246	1.22	0.81

Table 2.2: Measurement of *E. coli* growth in succinate. The growth rate, doubling time and number of doublings/hour were measured for *E. coli* TR6524 and FA1P1 in minimal media with succinate as the sole source of carbon in the presence of 0.05, 0.5 and 5.0μg/mL vitamin B_{12}

Unlike the growth curves for the two strains in minimal media with glucose, the wild type (TR6524) and the mutant (FA1P1) showed a significant difference in their growth pattern in media with succinate as the sole carbon source (Figure 2.5a), indicating the possibility of utilisation of succinate *via* the MCM pathway. This was further reiterated by the growth measurement values shown in Table 2.2. The wild type cells were observed to have doubling times of 0.54, 0.59 and 0.61 hours in the presence of 0.05, 0.5, 5.0µg/ml vitamin B_{12}, respectively, while the *sbm* mutant was observed to have 'g' values of 1.19, 1.21 and 1.22 hours in 0.05, 0.5, 5.0µg/ml vitamin B_{12}, respectively. Also, number of doublings/ hour (µ) for the wild type (TR6524) was twice that of the mutant, FA1P1 (Table 2.2). It is obvious that, in the absence of a functional *sbm* gene, the mutant cannot utilise succinate through the MCM pathway and hence shows relatively slower growth than the wild type strain. The absence any effect of the increasing concentration of vitamin B_{12} (Figure 2.5f and Table 2.2) on the growth of the *sbm* mutant (FA1P1) is expected since MCM (encoded by *sbm* gene), which possibly is involved in the utilisation of succinate, is coenzyme B_{12} dependent and this strain has its *sbm* gene inactivated. The absence of any appreciable difference in the growth pattern within TR6524 with change in B_{12} concentration (Figure 2.5e) could be indicative of a saturation of the required B_{12} concentration at 0.05µg/mL. Recent reports have indicated the presence of a B_{12} riboswitch (described in Section 3.1.1.4) upstream of the *btu*B gene responsible for the transportation of external B_{12} into the *E. coli* cell. These RNA elements selectively bind vitamin B_{12} and control *btu*B gene expression in response to high ligand concentrations (Vitreschak *et al*., 2003 & Nahvi *et al*., 2004) in turn affecting the functioning of coenzyme B_{12}-dependent MCM.

The results from the growth of the two strains in minimal media with succinate as the sole carbon source indicate that the *sbm* gene is important for succinate uptake. These results show for the first time that the *sbm* gene product of *E. coli* is important for its growth on succinate.

2.3.3 Growth of *E. coli* TR6524 and FA1P1on propionate

Propionate is a common carbon source encountered by *E. coli* in the colon as a result of the oxidation of odd chain fatty acids (Textor *et al.*, 1997; Polen *et al.*, 2002 & Nelson and Cox, 2005). Previous studies have indicated that vitamin B_{12} in the minimal medium with propionate as the sole source of carbon had reduced lag phase of the wild type *E. coli* to 70 hours from 90 hours (Kolodziej *et al*., 1968 & Wegener *et al*., 1968). Also, cells previously adapted to propionate have been shown to have a reduced lag phase when grown on propionate as the sole source of carbon indicating that growth using propionate is an adaptive process requiring the induction of specific enzyme systems (Kolodziej *et al*., 1968). In this study, similar observations were made and the wild type *E. coli* K12 grew without a lag phase in minimal media with propionate as the sole source of carbon. The difference in the growth of the wild type in the presence of the vitamin B_{12} indicates the possibility of the involvement of the Sbm protein in propionate utilisation. The absence of lag phases observed in this case compared to that reported by Wegener *et al*. (1968) is due to the fact that the cultures of *E. coli* K12 in this work have been previously adapted for growth in propionate minimal media in addition to it being a different strain of *E. coli*.

In all the growth conditions with propionate as the sole source of carbon (Figures 2.6 b-d), it was observed that the growth of the wild type strain TR6524 was marginally better than that of the mutant FA1P1. Table 2.3 shows the growth rate, doubling time and the number of doublings/ hour for the two strains in minimal media with propionate as the sole source of carbon.

Strain and growth condition	Growth rate, k (h-1)	Doubling time, g (h)	Doublings/ hour (μ)
TR6524 0.05μg/ml vitamin B_{12}	0.11	2.85	0.36
FA1P1 0.05μg/ml vitamin B_{12}	0.10	3.11	0.33
TR6524 0.5μg/ml vitamin B_{12}	0.11	2.80	0.36
FA1P1 0.5μg/ml vitamin B_{12}	0.10	3.08	0.32
TR6524 5.0μg/ml vitamin B_{12}	0.11	2.74	0.36
FA1P1 5.0μg/ml vitamin B_{12}	0.10	3.20	0.33

Table 2.3: Measurement of *E. coli* growth in propionate. The growth rate, doubling time and number of doublings/hour were measured for *E. coli* TR6524 and FA1P1 in minimal media with propionate as the sole source of carbon in the presence of 0.05, 0.5 and 5.0μg/mL vitamin B_{12}

The growth curves (Figures 2.6 a-f) and the data shown in Table 2.3 indicate that the growth patterns of the wild type and the mutant *E. coli* strains do not differ significantly (as indicated by the growth rate and the doublings/hour values). Also, with an increase in the vitamin B_{12} concentration only a slightly better growth and marginally decreased doubling time for the wild type strain was observed. This indicates that the coenzyme B_{12}-dependent MCM pathway is not significantly important in propionate utilisation in *E. coli*. In the case of FA1P1, the *sbm* gene is non functional due to the Mu dJ insertion. Hence, the variation in the B_{12} concentration or even the presence of vitamin B_{12} in the growth medium had no effect on the growth of the strain. This ability of the wild type and mutant *E. coli* strains to grow in minimal media with propionate shows that there are other pathways (including the methylcitrate pathway) that compensate allowing the bacteria to grow as observed from the growth curves. Nevertheless, the increase in the concentration of vitamin B_{12} had a slight positive influence on the growth of the wild type *E. coli* strain TR6524. This could be indication of the functioning of B_{12} dependent pathway for propionate utilisation and in turn of the role of B_{12} dependent MCM in propionate utilisation.

From these results, it is evident that *E. coli* uses multiple pathways (possibly MCM pathway and five other) for propionate utilisation, as previously reported (Textor *et al*., 1997). From the comparative growth analyses of the wild type TR6524 and mutant FA1P1, there is an indication that in the presence of vitamin B_{12}, *E. coli* might utilise propionate through the carboxylation pathway (*via* MCM). The minor differences in the growth pattern of the mutant with changes in B_{12} concentration could be due to the presence of utilisable metabolites from other pathways that may possess B_{12} dependent enzymes thus helping the mutant in the media with a higher B_{12} (5.0µg/ml) concentration grow slightly better than the FA1P1 strain growing in media with lesser B_{12} (0.05 or 0.5 µg/ml) concentration. The growth of the *sbm* mutant on propionate possibly utilises the other pathways including the methylcitrate pathway as there is no competition from the MCM pathway for the propionyl CoA. This is in accordance with the findings of Textor *et al*., 1997 and Evans *et al*., 1993.

Thus, overall we can conclude that the difference in growth between the wild type cells and the mutant strain in propionate is not as markedly different as in the case of growth on succinate. This can be attributed to the fact that *E. coli* is known to have numerous pathways for propionate utilisation, hence in the case of the mutant strain the other pathways compensate quite well for the lack of the Sbm pathway (Kolodziej *et al*., 1968 & Textor *et al*., 1997).

Chapter 3

Promoter analysis of sequence upstream of the *sbm* gene-containing operon

3.1 Introduction

Transcription of a number of genes within bacterial cells is induced resulting in protein synthesis required to survive environmental stresses. *E. coli* has been shown to survive environments characterised by nutrient scarcity, starvation, limited oxygen availability, toxic chemicals, and high osmolarity (Wise *et al*., 1996; Alexeeva *et al*., 2003 & Levanon *et al*., 2005). Promoter sequences upstream of a gene can indicate the conditions in which a gene product is expressed. The specific condition required for the initiation of transcription at a promoter sequence is governed by the sigma (σ) factor, which binds to the core RNA polymerase (RNAP) complex to form the RNAP holoenzyme (Burgess, 1969; Hengge-Aronis, 2002a & Hengge-Aronis, 2002c). Table 3.1 provides a list of the different sigma factors, the gene family they specifically induce and the promoter sequences to which they specifically bind (Helmann and Chamberlin, 1988; Hengge-Aronis, 2002a & Hengge-Aronis, 2002c).

Sigma factor	Gene family	- 35 sequence	-10 sequence	Function
70	General	TTGACA	TATAAT	Principal sigma factor
32	Heat shock	TCTCNCCCTTGAA	CCCCATNTA	Heat shock gene transcription
54	Nitrogen stress	CTGGCAC	TTGCA	Nitrogen regulated gene transcription
28	Flagella synthesis	CTAAA	CCGATAT	Expression of flagellar operons
38	Stationary phase genes	CGTCAA	CTNNTATAAT	Transcription of stationary phase genes
20	Iron dicitrate transport	TGGAAA	TGTAAT	Regulates iron citrate transport
24	Extracytoplasmic proteins	GAACTTC	TCTGA	Regulates extracytoplasmic proteins

Table 3.1: Different sigma factors and their recognition sequences

E. coli promoters are categorised into three classes (Figure 3.1) based on the relationship between their expression level and the rate of cell growth (Vicente *et al*., 1991). The housekeeper promoters are constantly active independent of the growth rate, the stringent promoters are dependent on the growth rate, being more active at higher growth rate and gearbox promoters, whose activities are inversely related to the growth rate (Tanaka *et al*., 1993).

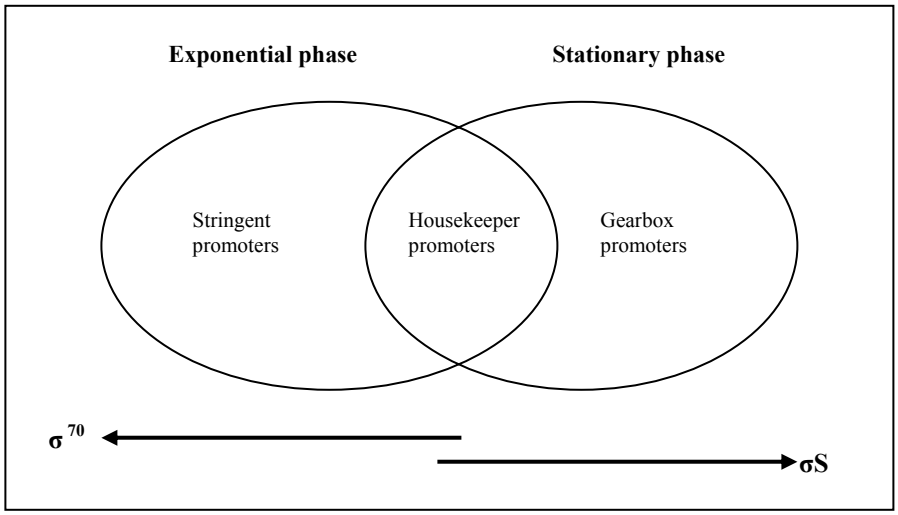

Figure 3.1: An equilibrium model representing the 3 classes of promoters. The two circles represent sets of promoters, each of which is active in the exponential phase and/or in stationary phase. Equilibrium of the two principal sigma factors determines the overall balance of cellular transcription. The predominance of σ^{70} or σS results in a shift of the balance in the direction indicated by the arrow (Tanaka *et al*., 1993).

3.1.1 Regulators of gene expression

The ability of *E. coli* to grow under a wide range of environmental conditions owing to its catabolic flexibility is recognised by its response to differences in the growth environment (Alexeeva *et al*., 2003). *E. coli* cells respond to these fluctuations by altering the expression of a number of membrane associated nutrient uptake or excretion systems and metabolic pathways to modulate the carbon and energy flow (Salmon *et al*., 2003). This regulation is mediated at the transcriptional level by regulatory networks of which the well studied and characterised regulons pertaining to this study are briefly discussed below.

3.1.1.1 Sigma (σ) factors as gene expression regulators

The principal σ factor, σ^{70}, potentiates the transcription of genes controlled by the consensus promoters consisting of two hexanucleotide sequences, TTGACA and TATAAT, in exponentially growing *E. coli*. Other species of σ factors recognise different sets of promoter sequences, each being associated with a limited number of genes that are mostly expressed in response to various stress conditions as listed in Table 3.1 (Helmann and Chamberlin, 1988).

One of the alternate sigma factors, σS (also known as σ^{38}), encoded by the *rpoS* gene, (due to its importance in stationary phase or 'under stress' conditions) is a sigma subunit of RNA polymerase in *E. coli* that is induced and can partially replace the vegetative sigma factor σ^{70} under many stress conditions (Hengge- Aronis, 2002a & Hengge-Aronis, 2002c). The σS subunit of RNA polymerase (or RpoS) is reported as the master regulator of the general stress response in *E. coli* (Fischer *et al*., 1998). σS induces the transcription of many genes whose products help the cell to cope with various environmental stresses. Stationary phase induction is characteristic of σS dependent genes. Many σS dependent genes (*bolA*, *otsBA*, *treA*, *osmB*, *osmY*, and *pexB*) have been reported to be induced by hyperosmotic stress during exponential growth in a defined minimal medium (Wise *et al*., 1996 & Polen *et al*., 2003). While σS levels are low in rapidly growing cells not exposed to any particular stress, it is induced in response to a variety of environmental stress conditions that include starvation for various nutrients and stationary phase in general. The cellular content of the σS protein has been observed to increase during stationary phase, thus correlating with σS dependent gene induction (Fischer *et al*., 1998). Metabolism is also affected by σS controlled genes, consistent with σS being important under conditions where cells switch from maximal growth to maintenance. σS control of genes mediating programmed cell death in stationary phase by sacrificing some of the population to provide nutrients for the remaining surviving cells, increases the survival rate for bacterial population under stressful conditions (Hengge- Aronis, 2002b).

It has been shown that σS and σS dependent genes not only are induced in stationary phase and starvation conditions, but actually respond to many different stress conditions including hyperosmolarity (Muffler *et al*., 1996), heat shock (Muffler *et al*., 1997) and acid tolerance (Lee *et al*., 1995), leading to the popular belief that σS is the master regulator of the general stress response triggered by many different stress signals (Weber *et al*., 2005).

3.1.1.2 FNR regulon

FNR is a catabolic activator protein (CAP) homologue that contains an oxygen labile iron-sulfur centre as a sensor element for anaerobiosis. Mutations in the *fnr* gene affect the synthesis of nitrite, nitrate, and fumarate reductases as well as fermentation pathway genes. Over 70 genes in 31 operons have been recognised as members of the FNR gene regulatory network (Lambden and Guest, 1976 & Salmon *et al*., 2003). The primary role for FNR is recognised to be the coordination of carbon and energy metabolism during growth under anaerobic conditions (Salmon *et al*., 2003 & Levanon *et al*., 2005).

E. coli controls its response to oxygen availability by altering its gene expression pattern. Under oxygen limiting conditions, expression of genes involved in oxygen utilisation are switched off and expression of genes encoding alternative anaerobic electron transport pathways or genes needed for fermentation are switched on. Many of these metabolic transitions are controlled at the transcriptional level by the activities of the global regulatory protein, FNR. (Salmon *et al*., 2003 & Levanon *et al*., 2005)

Many of the genes affected by oxygen availability are involved in aerobic or anaerobic electron transport processes, carbon flow through the Krebs cycle and/or fermentation, small molecule biosynthesis, macromolecular synthesis, and a variety of nutrient uptake or nutrient excretion reactions. In a recent study carried out by Salmon *et al*. (2003), the analysis of microarray data obtained to understand the global gene expression pattern of *E. coli*, in the presence and absence of oxygen, demonstrated that the expression of over a third of the genes during growth under

aerobic conditions were altered when *E. coli* cells made a transition to an anaerobic growth state, and that the expression of 712 of these genes were either directly or indirectly modulated by FNR (Salmon *et al*., 2003).

3.1.1.3 ArcAB regulon

The ArcAB (microaerobic respiratory control) is a two component regulatory system composed of ArcA, the cytosolic response reguator and a membrane associated histidine kinase, ArcB. Genes for the TCA cycle (*sdh-CDAB, icd, fumA, mdh, gltA, acnA*, and *acnB*), for pyruvate metabolism and superoxide dismutase (*pfl* and *sodA*), and genes for the cytochrome *o* oxidase (*cyo*ABCDE) and cytochrome *d* oxidase are some of the genes controlled by ArcA (Lynch and Lin, 1996; Alexeeva *et al*., 2003; Salmon *et al*., 2003 & Levanon *et al*., 2005).

Originally thought to be involved in the control of transcription of genes under aerobic growth conditions (Salmon *et al*., 2003), a recent observation by Levanon *et al*, (2005) showed that the ArcAB regulator was rather involved with the ability of *E. coli* to grow under microaerobic, oxygen restricted growth conditions. Elevated levels of FNR regulated genes in Arc mutants of *E. coli* indicated a complex network formed by these regulatory proteins (Levanon *et al*., 2005).

3.1.1.4 Riboswitches

Recently, a new class of gene expression regulators called riboswitches have been identified (Nahvi *et al*., 2002). Riboswitches serve as ligand-responsive genetic control elements that modulate the expression of certain genes in response to changing concentrations of metabolites. These RNA elements are embedded within the 5' untranslated region (5′-UTR) of specific prokaryotic mRNAs, and are composed of two functional and sometimes distinct structural domains. One domain serves as a natural aptamer that binds the target metabolite with high selectivity while the other domain is an 'expression platform' that harnesses allosteric changes in RNA structure, brought about by aptamer-ligand complex formation, to control expression of the adjacent gene or operon (Vitreschak *et al*., 2003 & Nahvi *et al*., 2004). Examination of a 202-nucleotide fragment of the 5′-UTR of the *btuB* gene of

E. coli and the homologous mRNA fragment for *S. typhimurium* confirmed that an mRNA domain directly binds to a metabolite in the absence of proteins indicating its function as a metabolite-dependent genetic control element (Nahvi *et al*., 2002). Both RNAs bind coenzyme B_{12} with high affinity and specificity, and thus function as natural aptamers. These RNAs, along with the 5′-UTR of the *cob* gene have been shown to carry a short conserved sequence domain termed the B_{12} box. Genes under the control of a coenzyme B_{12} riboswitch are involved in the transport of cobalamin compounds or metals, or associated with the biosynthetic pathway for the coenzyme (as in the case of *S. typhimurium*). Global analysis of the B_{12} elements in available bacterial genomes has shown that this conserved RNA regulatory element, widely distributed in eubacteria, is involved in the control of gene expression by transcription termination in Gram positive bacteria and at the translational level in Gram negative bacteria (Nahvi *et al*., 2002 & Nahvi *et al*., 2004).

Salmon *et al*. (2003) identified a putative FNR recognition sequence upstream of the *sbm* gene. An increase in the *sbm* gene expression (transcription) during anaerobic growth of *E. coli* indicated a possible relation between FNR and Sbm. However, they were unable to discuss the significance or the effect of this in their report. In addition, many genes shown to be regulated by Lrp (leucine responsive protein) were also found to be regulated by FNR (Salmon *et al*., 2003). The global regulatory protein Lrp has been reported to affect σS and/or σ^{70} selectivity of many promoters indicating that certain elements of the σS-dependent general stress response are employed by stress-specific regulons, which are controlled by other stress-responsive regulators that act together with σ^{70} RNAP, suggesting functional interactions between global regulatory networks (Tani *et al*., 2002 & Weber *et al*., 2005).

With these findings and reports as the basis, the current work attempted to locate suitable regulon binding sites in order to determine conditions in which the *sbm* gene might be transcribed and possibly indicate the metabolic role of the gene.

3.2 Results

3.2.1 Regulon recognition sites

σ70 and σS are highly related to one another. Genes transcribed by σS *in vivo* have been found to be transcribed by σ70 *in vitro* (and the reverse being equally efficient). This has made the identification of a σS consensus promoter sequence and the prediction of σS-controlled promoters in upstream regions of genes in the *E. coli* genome difficult (Weber *et al*., 2005).

A search was carried out to locate conserved sigma factor binding sequences upstream of the *sbm*-containing operon. Different spacer lengths from 15 to 19 spaces were explored between the -35 and -10 region. A putative σS binding site (-35: CGTCAA; -10: CTNNTATAAT) that promotes the expression of genes under stress conditions or stationary phase conditions was found 29 base pairs upstream of the *sbm* start codon, as shown in Figure 3.2

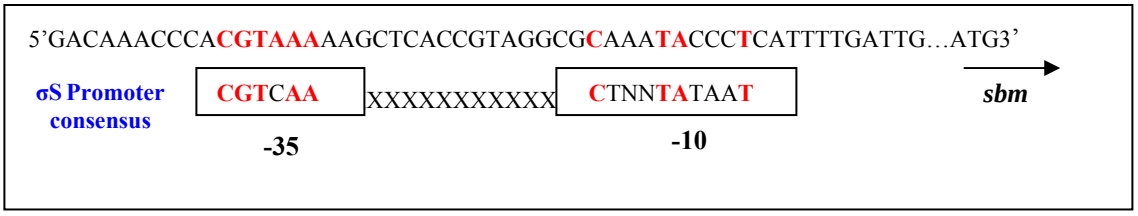

Figure 3.2: Alignment of the σS promoter sequence with sequence upstream of the *sbm* gene. There were 16 spaces between the -35 (CGTAAA) and the -10 (CAAATACCCT) sequences in the alignment. This was achieved using the Multalin program (Corpet, 1988).

The putative -35 region found upstream of the *sbm* gene aligned with 83% sequence identity with the consensus sequence of the σS binding site and the -10 region was 50% similar to the known consensus sequence (Figure 3.2).

A similar search carried out in an attempt to identify putative binding sites corresponding to the proposed ArcA consensus sequence (WAGTTAATTAW) (Shen & Gunsalus, 1997) upstream of the *sbm* gene showed alignments as shown in Figure 3.3.

Figure 3.3: Alignment of the proposed sequence for the ArcA binding site with sequence upstream of the *sbm* gene. The bases that aligned with the bases upstream of *sbm* are highlighted in bold red and the 'W' could be substituted with 'A' or 'T'. This was achieved using the Multalin program (Corpet, 1988).

Figure 3.3 shows the possibility of the presence of an ArcA binding site with a 45% sequence identity located about 104 base pairs upstream of the *sbm* gene. As mentioned in Section 3.1.1.2, the Arc regulon has been associated with the regulation of genes responsible for aerobic and/or microaerobic growth of *Escherichia coli*.

An alignment of the putative binding sites corresponding to the FNR binding site (TTGATxxxxATCAA) (Shen & Gunsalus, 1997) to base pairs upstream of the *sbm* gene is shown in Figure 3.4.

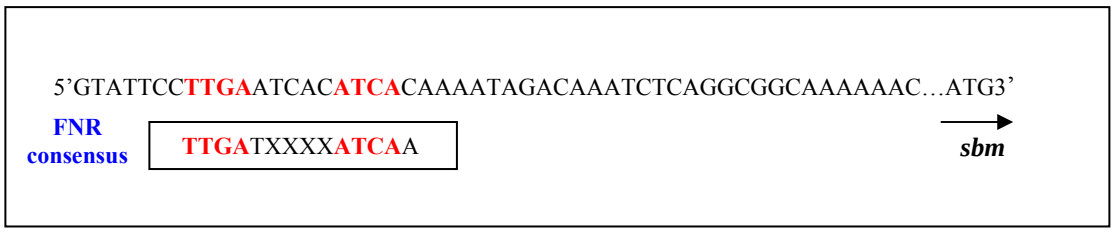

Figure 3.4: Alignment of the sequence for the FNR binding site with sequence upstream of the *sbm* gene. The bases that aligned with the bases upstream of *sbm* are highlighted in bold red and this was achieved using the Multalin program (Corpet, 1988).

The search for a putative binding site upstream of *sbm* was based on the findings of Salmon *et al*. (2003). It was reported that the FNR binding site was present somewhere between 100 and 200 base pairs upstream of the *sbm* gene; however, the

exact location was not indicated (Salmon *et al*., 2003). In this study we managed to align the FNR consensus at about 139 base pairs upstream of the *sbm* gene (Figure 3.4) with 80% sequence identity.

A similar search for a putative binding site for the B_{12} box upstream of the *sbm* gene returned no match indicating the absence of the B_{12} box upstream of the *sbm* gene. However, the B_{12} ribowitches affect the expression of *btu*B gene (Vitreschak *et al*., 2003 & Nahvi *et al*., 2004), the gene product of which is involved in vitamin B_{12} uptake in *E. coli*. This in turn could affect the formation of Sbm holoenzyme and eventually the utilisation of succinate and propionate *via* the MCM pathway.

3.3 Discussion

Since *E. coli* lives in rapidly changing environments, its survival depends on its ability to regulate its gene expression to produce enzymes capable of transporting proteins required for the growth and metabolism of the cell in the new environment (Magasanik, 2000). The general stress sigma factor σS is strongly induced when *E. coli* cells are exposed to various stress conditions including growth on unusual carbon sources such succinate or propionate as the sole sources of carbon. The presence of σS binding site upstream of the *sbm* gene indicates that the genes of the *sbm* operon may be involved in pathways related to stationary phase of growth or glucose starvation, since σS is the key regulator under such conditions. σS has been reported as the inducer of transcription of over 80 genes required to provide resistance and protection against stresses such as starvation or unfavourable growth conditions (Hengge- Aronis, 2002 & Weber *et al*., 2005). Thus, the conditions investigated in the work discussed in this thesis are similar to the conditions of induction regulated by σS.

Owing to its rapid kinetics and transient nature, the induction of σS has been identified to possess the characteristics of a rapid emergency response. The speed and the flexibility of *E. coli*'s response to stress shows the remarkable ability of the bacteria to be able to survive frequent, rapid, and extreme changes in their growth

environments. Fischer *et al*. (1998) found that glucose starvation during diauxic shift, in the presence of lactose, activated many σS dependent genes resulting in significant changes in physiology, including the expression of a strong general stress resistance, and cellular morphology. This led to the conclusion that glucose starvation, even in the presence of another eventually utilisable carbon source (lactose), initially represents a stress condition that the cells respond to by rapidly increasing their σS content and inhibiting the proteolysis of this sigma factor (Fischer *et al*., 1998). However, abrupt cessation of growth, as for example, in response to sudden glucose starvation, only weakly (about two fold) increased σS transcription (Hengge-Aronis, 2002b).

Salmon *et al*. (2003) indicated the expression of the *sbm* gene in the absence of oxygen suggesting the presence of a putative FNR binding site upstream of the *sbm* gene in *E. coli* (Figure 3.4). This suggests that *sbm* is probably induced under anaerobic conditions of growth (Salmon *et al*., 2003). *E. coli* is a facultative anaerobe and is also found in the intestinal tract of higher organisms where there is limited or no oxygen supply. This could lead to metabolism through the fermentative pathway. Under certain circumstances the cell might have to utilise fatty acids such as propionate for energy production. Propionate is initially converted to propionyl CoA which can then undergo oxidation (methylcitrate pathway) or carboxylation (MCM pathway). The carboxylation pathway requires the *sbm* gene product and, based on the finding by Salmon and co-workers (2003), could possibly be induced by FNR under anaerobic conditions.

It has been shown that global regulatory proteins (σ^{70}, σS, FNR, ArcA) function as a network and that two or more of these regulatory proteins, together, control the expression of many genes (Hengge-Aronis *et al*., 2002a; Hengge-Aronis, 2002b; Salmon *et al*., 2003 & Levanon *et al*., 2005). Wise *et al*. (1996) reported that DNA sequences at the -35 site of σS promoter form part of a discriminatory mechanism that confines transcription to conditions that increase the availability of σS. This indicates the significance of the 83% alignment at the -35 site of the putative σS

promoter upstream of the *sbm* gene. The identification of putative binding sites for σS (83% and 50% at the -35 and -10 regions, respectively), ArcA (45% sequence identity) and FNR (80% sequence identity) regulons 104 and 139 bases, respectively, upstream of the *sbm* gene (Figures 3.3 and 3.4) confirms the possibility of a control of expression of this gene, and hence the operon, by a complex regulatory network of genetic control elements. It can be suggested that in the presence of difficult carbon sources and under anaerobic conditions, the *sbm* gene is probably induced by both FNR and σS regulatory proteins.

Previous studies (Salmon *et al*., 2003) used minimal media with glucose as the carbon source aiming only to understand the global gene regulation pattern of *Escherichia coli*. This study has provided an insight into possible conditions leading to the induction of the *sbm* gene expression in *E. coli.* For the first time, the possible involvement of the σS factor has been proposed in addition to the FNR and ArcA regulatory proteins. The findings reported here can be confirmed by the use of microarray analysis or real time PCR quantification of gene expression pattern under specific growth conditions corresponding to the conditions discussed above. In this study aerobic growth in the presence of succinate and propionate as the sole carbon source were investigated within which the stress conditions due to difficult carbon sources (propionate and succinate), glucose starvation and stationary phase conditions have been studied. This study is covered in the next Chapter. In the future, analogous anaerobic conditions will need to be investigated.

Chapter 4

Transcriptional level control of the *sbm* operon

4.1 Introduction

The conventional methods for mRNA quantification such as Northern blotting or Ribonuclease protection assay are not completely sensitive to study low abundance mRNAs. The sensitivity of the RT-PCR in combination with real time recording of the amplification data has proven to be extremely useful in amplifying specific mRNAs (especially those present in low copy number) and has eliminated the variability associated with end point PCR, thus allowing routine and reliable quantitation of the PCR product. Currently**,** reverse transcription based assays are being widely used for characterising gene expression patterns and comparing mRNA levels in different sample populations as these assays represent a powerful tool for the detection and quantification of mRNA (Bustin, 2002).

4.1.1 Real time reverse-transcription PCR (real time RT-PCR)

4.1.1.1 History and chemistry

Originally, Higuchi *et al*. (1993), pioneered the analysis of PCR kinetics by constructing a "real-time" system that included ethidium bromide (to intercalate DNA), an adapted thermal cycler to irradiate the samples with ultraviolet light and computer-controlled cooled camera to detect the resulting fluorescence. Amplification caused an increase in the DNA which bound to the ethidium bromide resulting in increased fluorescence. By plotting the increase in fluorescence versus cycle number, amplification plots that provided a complete picture of the PCR process were produced (Higuchi *et al*., 1993).

The real-time, fluorescence-based reverse transcription polymerase chain reaction (RT-PCR) uses fluorescent reporter molecules to monitor the production of amplification products during each cycle of the PCR reaction by combining the nucleic acid amplification and detection steps into one homogeneous assay thus making it simple, specific and sensitive for the detection and/or comparison of RNA levels (Bustin *et al*., 2005).

The fluorescent small molecules such as SYBR-green I used in real time RT-PCR analysis quantitates amplification by the use of a non-sequence specific fluorescent intercalation and is used as an alternative to classical intercalaters such as ethidium bromide which interfere with the PCR. SYBR-green I is an asymmetric cyanine that has two aromatic systems containing nitrogen, one of which is positively charged, connected by a methine bridge (Figure 4.1).

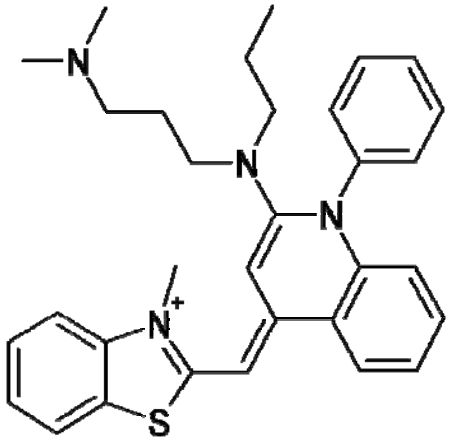

Figure 4.1: Structure of SYBR green I (Kubista *et al*., 2006)

Asymmetric cyanines have virtually no fluorescence when free in solution due to vibrations engaging both aromatic systems, which convert electronic excitation energy into heat that dissipates to the surrounding solvent. However, on binding to the minor groove of the DNA the dyes become brightly fluorescent emitting a strong signal since then their rotation around the methane bond is restricted (Figure 4.2). Thus SYBR green is a fluorogenic minor groove binding dye that does not bind ssDNA (Nygren *et al*., 1998, Kubista *et al*., 2006 & Dorak, 2006). At the melting temperature (T_m), the double stranded DNA separates and the dye comes off resulting in an abrupt drop in the fluorescence. Primer dimer products are shorter than the targeted product and hence, melt at a lower temperature. This melting curve analysis helps in distinguishing the primer dimers from the PCR product (Ririe *et al*., 1997).

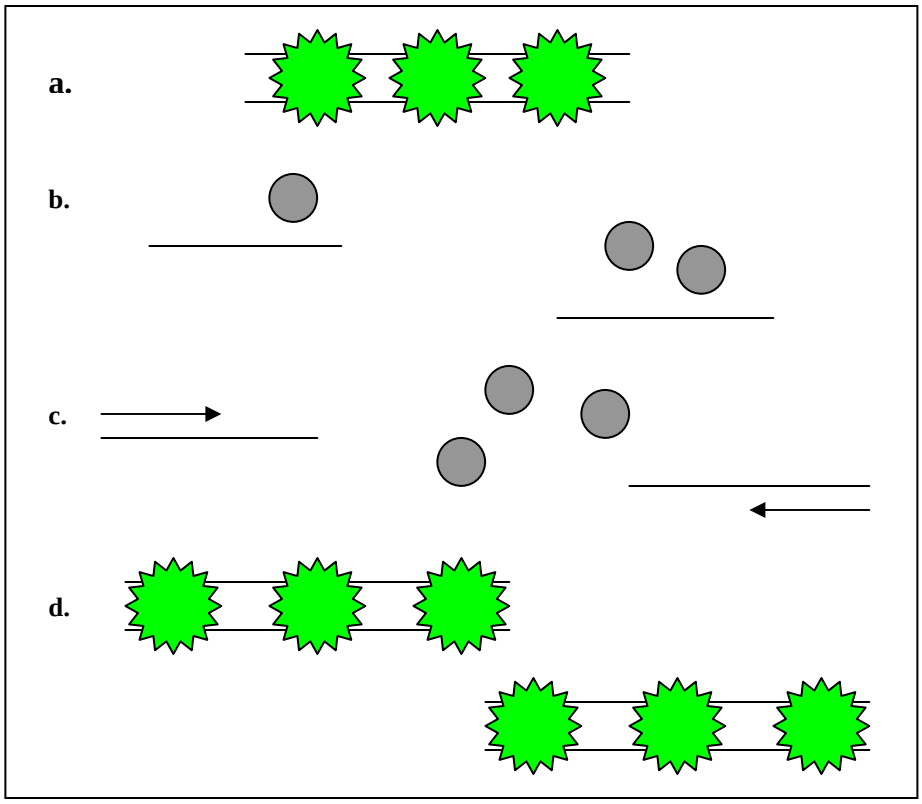

Figure 4.2: A schematic representation of SYBR green I chemistry. a. SYBR green dye fluoresces (represented by green spiky circles) when attached to dsDNA. b. The dye floats free on denaturation of DNA and becomes non fluorescent (represented by grey circles). c. The primers anneal to the strands. d. The dye binds to the double stranded product and fluoresces. The black lines in the figure represent the DNA strands.

4.1.1.2 Reverse transcription and priming strategies

The gene transcriptional level measurement by real time RT-PCR follows the successful reverse transcription (RT) of mRNA to cDNA. The RT step is critical for sensitive and accurate quantification, since it has been found that the RT reaction contributes to most of the variation in the experimental determination of mRNA quantities (Stahlberg *et al*., 2004).

Reverse transcription involves the transcription of RNA molecules to DNA using a reverse transcriptase. It has been reported that primer addition only increases efficiency of the reaction, although the reaction can proceed without added primer

(Stahlberg *et al*., 2004). The three basic priming strategies, in general are based on oligo(dT) primer, random sequence primers (random hexamers), and gene specific primers (Figure 4.3). Random hexamers are short oligomers of all possible sequences, usually 6 bases long, that copy all RNA, including tRNA, rRNA and mRNA. These are recommended as the priming strategy of choice for total reverse transcription of prokaryotic mRNA owing to the simplicity in quantification when used with SYBR green I dye (Morrison *et al*., 1998 & Kubista *et al*., 2006).

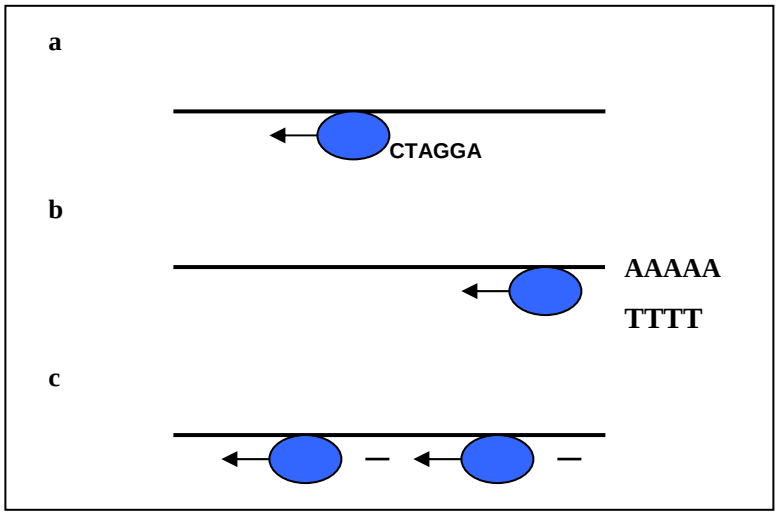

Figure 4.3: Priming strategies. A schematic representation of the three different priming strategies commonly used in reverse transcription (Kubista *et al.*, 2006). a. gene specific priming, b. oligo (dT) priming and c. random hexamer priming.

Random hexamers were used for this study for the synthesis of a cDNA pool from total RNA as this maximises the number of genes that can be assayed from a small RNA sample (Pfaffel, 2004).

Real time PCR is characterised by the detection of amplified product when it is first detected during the cycling rather than the amount of accumulated PCR product. The traditional RT-PCR measures data at the end-point (plateau), while real-time PCR collects data in the exponential phase of amplification. Also, an increase in reporter fluorescent signal is directly proportional to the number of amplicons generated and the system eliminates the need for post PCR processes (electrophoresis of amplified

DNA) since the quantification occurs within a closed system and as a part of the reaction (Kubista *et al*., 2006 & Dorak, 2004). The use of threshold cycle (C_t) values expands the range of quantitation as data is collected for every cycle of PCR. The C_t values are relatively less sensitive to the effects of PCR inhibitors as measurements are from the exponential phase where reaction components are not limiting as compared to end point PCR. The quantification of RNA (and DNA) is much more precise and reproducible as real time PCR relies on the threshold cycle values determined during the exponential phase of PCR rather than endpoint.

4.1.1.3 Methods of transcription quantification- absolute and relative

Absolute quantification of transcription helps in the precise determination of the transcript copy number of the target gene present in the cell (Bustin, 2002). The calibration curves for absolute quantification can be based on known concentrations of DNA, *e.g*., recombinant plasmid DNA (Bustin, 2002, Whelan *et al*., 2003 & Pffafl, 2004), genomic DNA (Eleaume *et al*., 2004), RT-PCR product or commercially synthesised oligonucleotide also referred to as 'artificial' RNA (Pffafl, 2004 & Hugget *et al*., 2005). In this study, absolute quantification of the target gene sequences was based on an external standard curve plotted using known concentrations of real time RT-PCR products of the respective genes. The standard curve was generated by plotting the threshold cycle (C_t) values against the logarithm of initial copy numbers. The transcript copy numbers of the target genes were calculated using this standard curve.

Relative quantification determines the changes in steady state transcription of a gene and expresses it relative to the internal control RNA. The control gene is a housekeeping gene (*e.g*., 18s or 16s RNA depending on the sample species, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin) that is more abundant and remains constant, in proportion to total RNA of the test samples. Therefore, the relative quantification does not require standards with known concentrations and is based on the expression levels of a target gene versus a

reference gene. The ratio of the C_t values of the target to the reference gene is calculated and the relative expression is obtained (Bustin, 2002 & Pfaffl, 2004).

4.1.2 The effect of nutrient conditions on the mRNA population of *E. coli*

A DNA microarray study by Polen and co-workers (2003) aimed to reveal the gene expression changes during the short (50min) and long (20 generations) term growth of *E. coli* MG1655 on LB in the presence of the sodium salt of acetate or propionate at a neutral pH. The adaptive responses to acetate and propionate were similar involving an increase in the expression levels of genes responsible for chemotaxis and motility, a decrease, in both cases, in the expression of genes involved in the uptake and utilisation of carbon sources such as sugars (*e.g*. maltose, galactose, trehalose), sugar alcohols (glycerol and sorbitol) and amino acids (serine, threonine, tryptophan and proline), suggesting catabolite repression by acetate and propionate. Expression of σS dependent stress response genes (such as *osm*Y, *dps*) were observed to increase, however, this was more pronounced after short term exposure to the stress of growing in acetate or propionate. The adaptive response analysis was carried out following 20 generations of growth of *E. coli* MG1655 on propionate or acetate. Cells obtained following the growth on propionate showed an increase in the expression of threonine and isoleucine biosynthetic genes and genes that confer multiple antibiotic resistance (*mar*RAB operon), however, none of the genes of the *sbm* operon were reported. Adaptation to propionate and acetate resulted in changes in the expression of the genes of the sorbitol operon, but, the changes were extreme for acetate adapted cells compared to the control cells grown in its absence (Polen *et al*., 2003).

Franchini and Egli (2006) also used microarray technology to understand the cellular events at the transcription level during short-term and long-term adaptation of *E. coli* K12 under glucose limited conditions (possibly similar to growth on succinate or propionate, since glucose is completely missing from the medium used). Short-term and long-term adaptation were assessed by comparing the mRNA levels isolated after 40 or 500 hours of glucose limited continuous culture with those from batch

culture with excess of glucose. A large number of genes encoding periplasmic binding proteins and proteins encoded by the genes of the maltose (*mal/lam*B) and galactose (*mgl/gal*) operons were upregulated, indicating that the cells are prepared for high-affinity uptake of different types of carbon sources during glucose limited growth in continuous culture. A similar transcription pattern was observed for long-term cultures but with lower expression levels than in the short-term adaptation. The patterns of upregulation were confirmed by real time RT-PCR (Franchini and Egli, 2006).

In this study, the specific role of the *sbm, ygf*G, *ygf*H and *arg*K genes in the utilisation of succinate or propionate was investigated using real time RT-PCR. *E. coli* TR6524 cells grown in minimal media with 0.5µg of vitamin B_{12} and with glucose, succinate or propionate as the sole source of carbon were chosen for all transcription quantification and later for proteomics (Chapter 6) analyses. The RNA from the mutant was also isolated from cells grown under these specific conditions to show the absence of the formation of PCR products corresponding to the *sbm* gene and hence all the genes of the operon, confirming their lack of transcription in the disruption mutant. This study enabled us to understand the control on the levels of transcription of the genes of the *sbm* operon and relate that to the results obtained from growth on glucose, succinate and propionate (Chapter 2) and with previous studies (Kolodziej *et al*., 1968; Wegener *et al*., 1968 & Evans *et al*., 1993). It was observed from Chapter 2, that, growth on glucose as the sole carbon source was not dependent on the *sbm* pathway and hence it was concluded that MCM was not important for glucose utilisation. Therefore RNA isolated from glucose grown cells was used as the control in this study.

4.2 Results

4.2.1 Total RNA isolation

Total RNA was isolated from both wild type and mutant *E. coli* grown in minimal media with glucose, succinate or propionate as the sole sources of carbon. The cells used were harvested from different phases of growth. Figure 4.4 shows an agarose gel with isolated total RNA from both wild type and mutant *E. coli*. The total RNA preparation includes the mRNA corresponding to the streaks (Figure 4.4) on the gel and the ribosomal RNA corresponding to the two thick bands, which can be clearly seen in all the samples. The bright spots at the bottom of the gel are indicative of degradation during the isolation process.

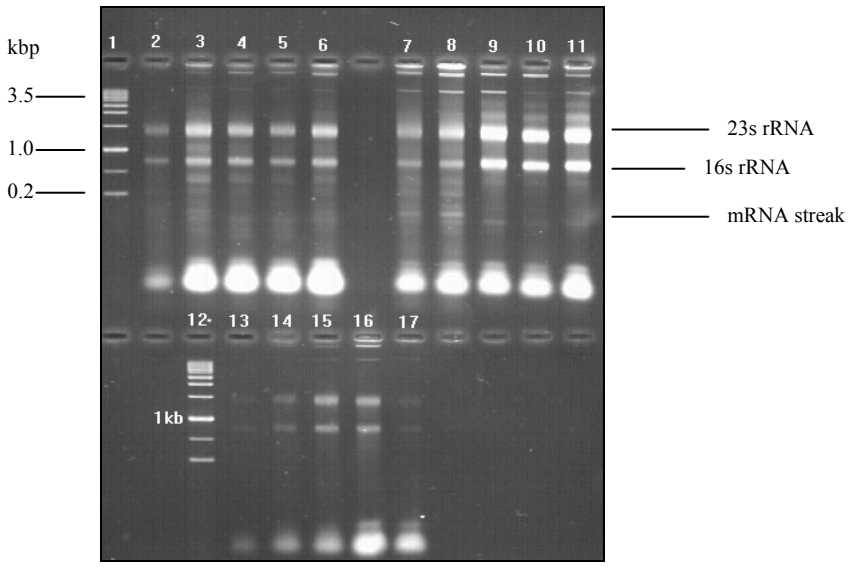

Figure 4.4: Total RNA isolation. A 1.5% agarose gel showing the 16S & 23S (ribosomal RNA) and mRNA of TR6524 and FA1P1 strains *E. coli* grown in minimal media with glucose, succinate or propionate as the sole source of carbon. Lanes 1& 12- 1 kbp DNA ladder. Lanes 2-17 (except 12) were loaded with RNA samples isolated from either TR6524 or FA1P1 cells grown in minimal media with glucose, succinate or propionate as the sole source of carbon.

The presence of intact well defined bands for the ribosomal RNA and an $A_{260/280}$ ratio of 1.8 -1.95 showed that the total RNA preparation was of good quality.

4.2.2 Reverse transcription and end point PCR- preliminary studies

The isolated RNA was reverse transcribed to obtain cDNA which was amplified using PCR. Two sets of primers (*sbm*1 and *sbm*11) and (*his*G1 and *his*G2) (Table 9.1) were used in the same PCR reaction. The *his*G primers were targeted to amplify a part of the *his*G gene encoding the constitutive enzyme, ATP phosphoribosyl transferase of the histidine biosynthetic pathway, while the *sbm* primers were used to amplify part of the *sbm* gene. This estimation was used to obtain preliminary results and hence no duplicates were carried out. The *sbm* gene product was formed only in the samples from the wild type TR6524 as the mutants have had their *sbm* gene inactivated by the Mu dJ insertion mutation and hence eliminates the corresponding mRNA formation (Figure 4.5).

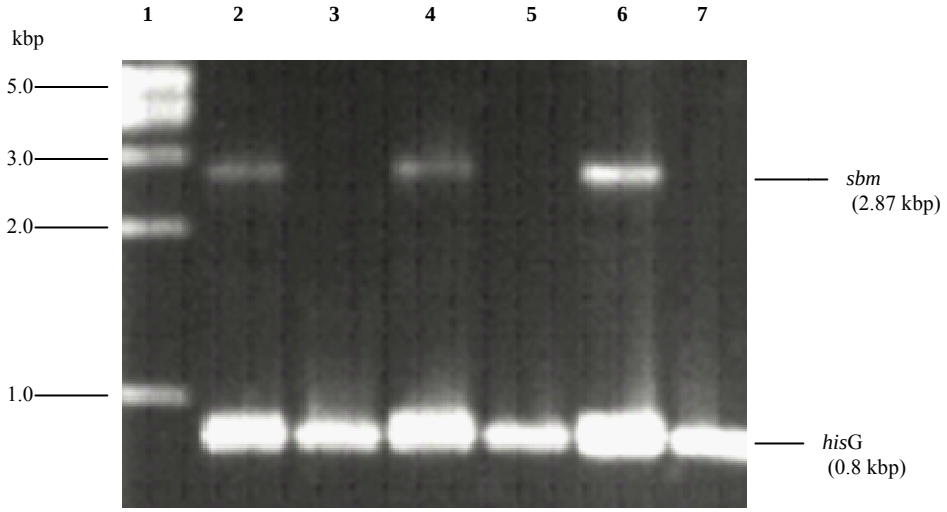

Figure 4.5: Agarose gel showing reverse transcription end point PCR products. The cDNA templates used in the reaction were obtained from cells grown in minimal media with glucose, succinate or propionate as the sole source of carbon with 0.5 µg/ml vitamin B_{12}. Lane1-1 kb DNA step ladder, Lane 2-TR6524 in glucose, Lane 3-FA1P1 in glucose, Lane 4-TR6524 in succinate, Lane 5-FA1P1 in succinate, Lane 6-TR6524 in propionate, Lane 7-FA1P1 in propionate.

The amount of mRNA produced corresponding to the *sbm* gene (from cells harvested at the stationary phase of growth) was deduced under each of these growth conditions using a densitometric quantification of the PCR product seen on the

agarose gel. The analysis was carried out on the PCR products observed as bands on the agarose gels. The ratio of the amount of mRNA related to the *sbm* gene and the mRNA related to the internal standard *his*G was compared (a graphical representation is shown in Figure 4.6) under the three different growth conditions (glucose, succinate or propionate as the sole source of carbon). As expected, the FA1P1 strain, being a *sbm* insertion mutant, did not form any PCR product as there would have been no mRNA produced corresponding to the *sbm* gene.

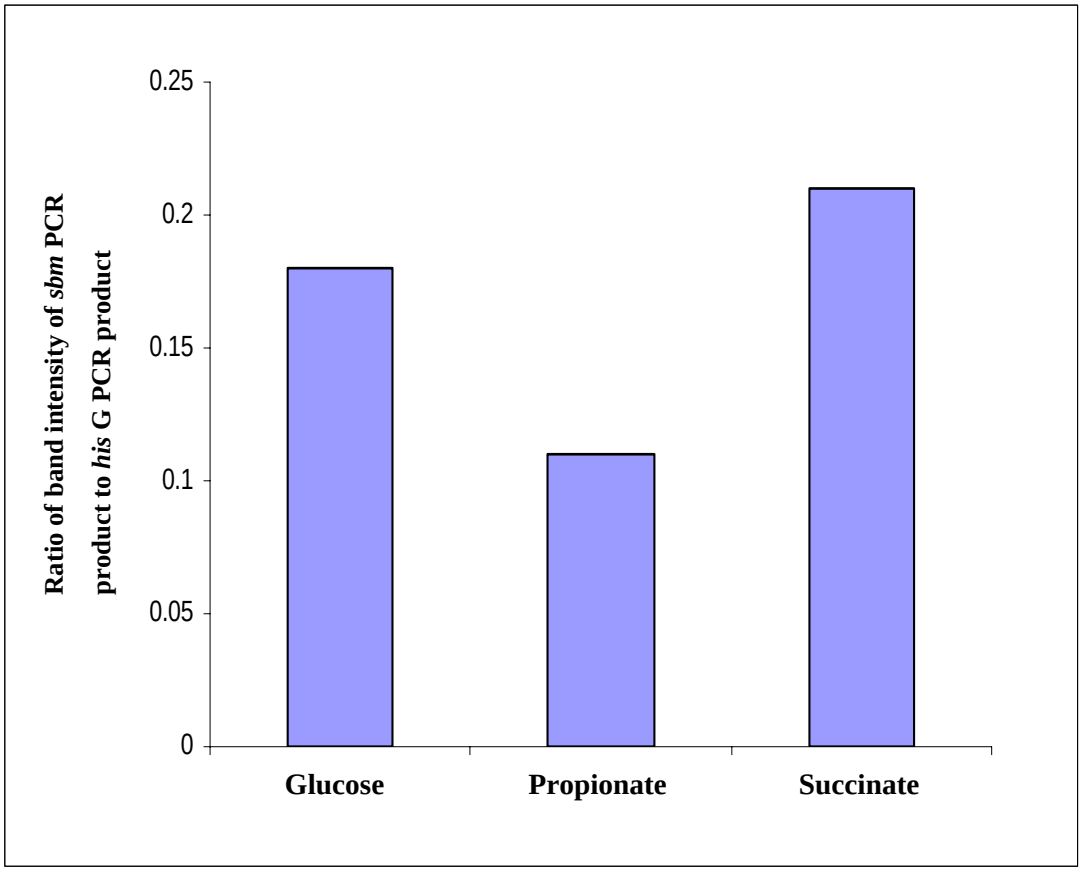

Figure 4.6: Densitometric analysis of the relative concentrations of the *sbm* and *his*G mRNA using RT-PCR. The ratio of the concentration of the *sbm* specific mRNA to the *his*G specific mRNA, when TR6524 was grown in 1-glucose, 2- propionate and 3- succinate.

It can be seen from Figure 4.6 that the ratio of intensity (as deduced from the band volume corresponding to the PCR product) of *sbm* to *his*G related PCR products and hence their mRNA levels is highest when *E. coli* is grown in succinate (0.21) as the sole carbon source, intermediate when grown on glucose (0.18) and the least when

grown in propionate (0.11). This result shows higher level of mRNA is produced corresponding to the *sbm* gene when *E. coli* is grown in succinate rather than propionate or glucose. This preliminary result indicates that Sbm has a key role in the utilisation of succinate in *E. coli* and is controlled at the transcriptional level.

This end point PCR reaction was next repeated with a new set of primers, redesigned to give a smaller sized product (229bp, for use later in real time PCR analysis), for the *sbm* gene and the cDNA template (from reverse transcription of the RNA) from cells grown in minimal media with succinate as the sole source of carbon. Growth in succinate was chosen based on the previous result (Figure 4.6). The cells were harvested at 3 different points (early-log, mid-log and stationary phases) of growth. This experiment was also aimed at obtaining preliminary results; hence no duplicates were carried out. The results obtained are shown in Figures 4.7 and 4.8.

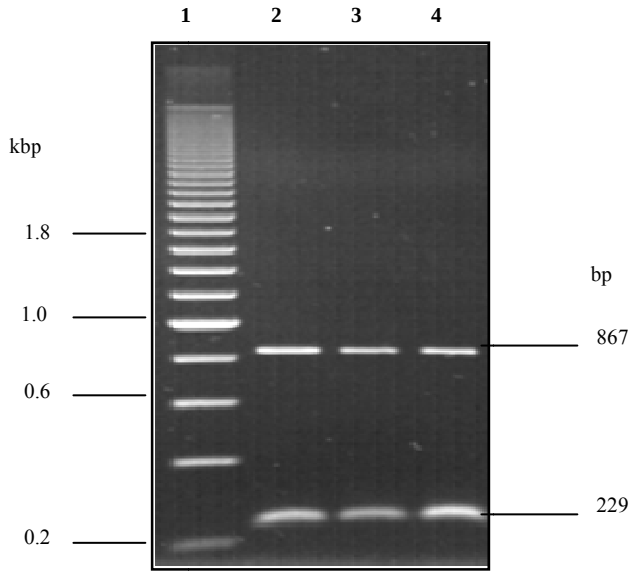

Figure 4.7: Agarose gel showing PCR products from *E. coli* TR6524 grown in succinate as the sole source of carbon. Lane 1- 200bp ladder. Lanes 2, 3, 4- PCR products obtained after amplification of the reverse transcribed RNA (cDNA template) from TR6524 in minimal media with 0.5µg/ml B_{12} and with succinate as the sole source of carbon. The RNA used here for the reaction was obtained from cells harvested at the early-log, mid-log and stationary phases of growth.

The gel (Figure 4.7) showed a good amplification of the *sbm* gene product of appropriate size. However, several attempts had to be made to optimise the PCR conditions in order to obtain these results. The ratio of the intensities of the *sbm* gene product to that of *his*G gene product obtained following the densitometric analysis are represented as shown in Figure 4.8.

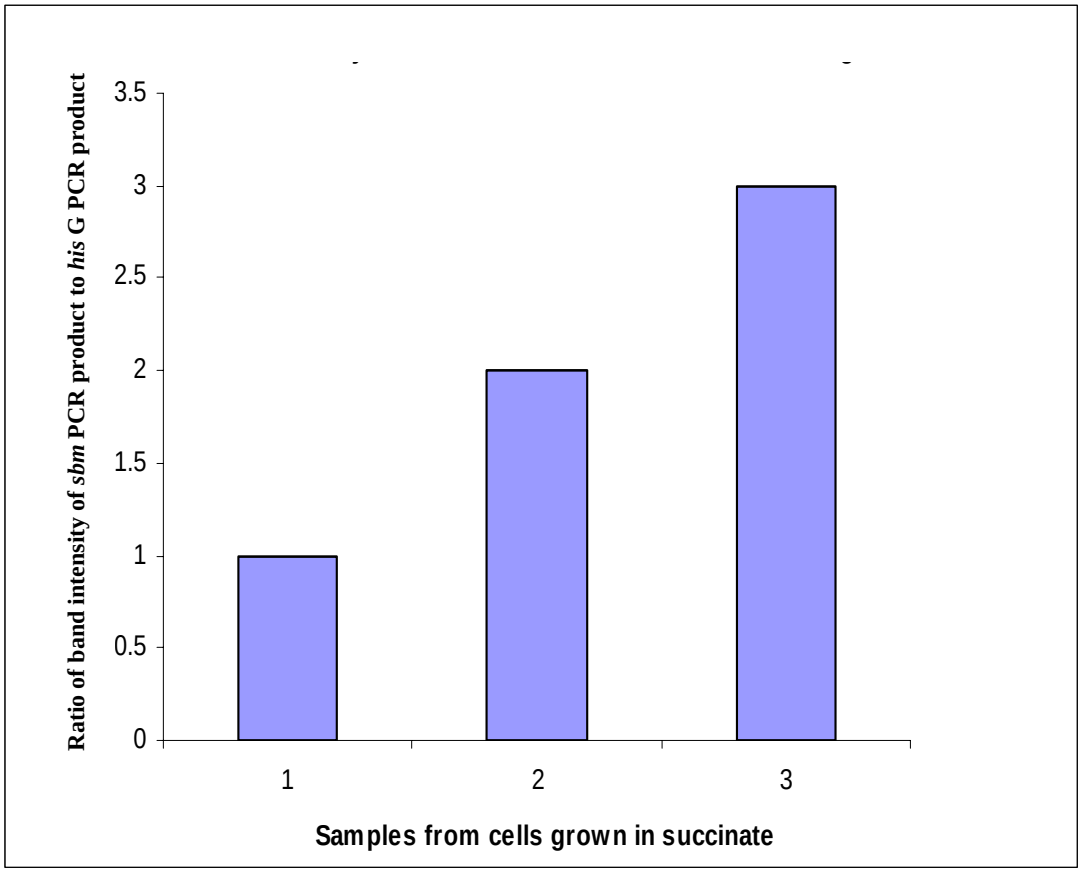

Figure 4.8: Densitometric analysis of the ratio of the *sbm* and the *his*G mRNAs. Graphical representation of the ratio of *sbm* specific mRNA to *hisG* specific mRNA when TR6524 was grown in succinate with 0.5 µg/ml vitamin B_{12} at early-log (1), mid-log (2) and stationary (3) phases of growth.

It can be seen from Figure 4.8 that the ratio of intensities of *sbm* to *his*G product, and hence mRNA levels related to the *sbm* gene, increases as the growth progresses. The cells were harvested at early-log, mid-log and stationary phases of growth and the ratios of the mRNA corresponding to the *sbm* gene and the mRNA

corresponding to the *hisG* gene were observed to be 0.59, 1.08 and 1.69 respectively indicating an increase in the mRNA formation at the stationary phase.

4.2.3 Real time quantification (QPCR) of the genes within the *sbm* operon

Absolute quantification of the mRNA levels of the genes corresponding to the *sbm* operon was carried out in order to investigate the transcriptional level control on the function of this operon.

Initially, dilutions with known copy numbers (10^5, 10^4, 10^3 and 10^2) of the respective RT-PCR products of the four genes (*sbm*, *arg*K, *ygf*G and *ygf*H) were prepared and used for plotting the standard curve (Pffafl, 2004 & Huggett *et al*., 2005). The amplification plot for one of the four genes, *sbm,* from which the standard curve(s) was derived, is shown in Figure 4.9.

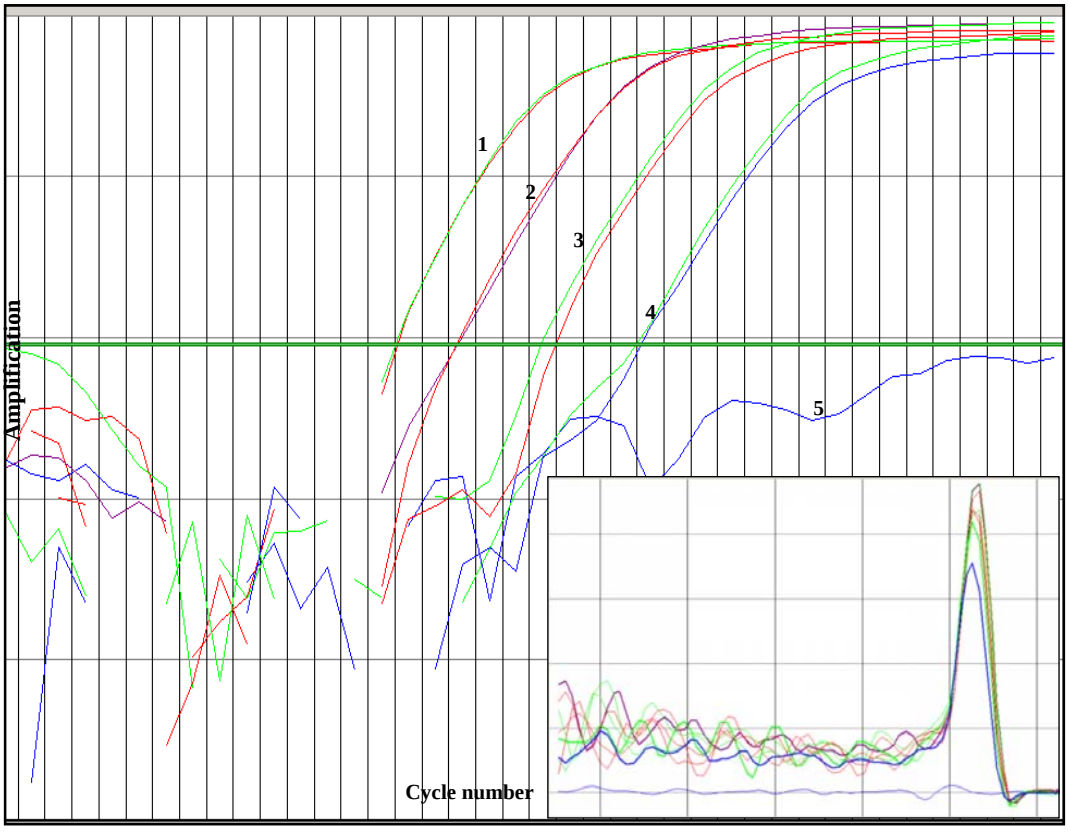

Figure 4.9: Amplification of *sbm* gene observed during real time PCR.

1, 2, 3 and 4 show the amplification with 10^5, 10^4, 10^3 and 10^2 copies of the mRNA corresponding to the *sbm* gene, respectively. 5-represents the negative control above which the threshold (bold green line) was set for C_t value calculations for the quantitative analyses. And inset, the dissociation curve confirming the formation of the desired product in all dilutions. The horizontal blue line at the bottom of the dissociation curve corresponds to the negative control with the RNA template and hence does not show any product formation.

Figure 4.9 shows the amplification plot and the dissociation curve (inset, Figure 4.9) as observed at the end of the amplification during a real time PCR reaction. It can be seen, as expected, that the copy numbers are inversely proportional to the C_t values (Gibson *et al*., 1996). The dissociation curve confirmed the formation of one specific product as all the peaks merged into one at a specific temperature. The dissociation curve for the negative control (reaction with RNA template) was observed as a horizontal line at the bottom of the curves (Figure 4.9) confirming the absence of any product formation and hence lack of any gDNA contamination in the sample. The dissociation curve analysis also helped in distinguishing the primer dimers from the individual PCR products (Ririe *et al*., 1997). The amplification plots and the dissociation curves for the remainder of the three genes (not shown) of the *sbm* operon were similar to that of the *sbm* gene (Figure 4.9). The threshold was set above the negative control and the C_t values were noted for each of the dilutions and a standard curve (Figure 4.10) was plotted for each of the four genes of the operon. The standard curves, used to determine the mRNA copy numbers corresponding to the four genes, had R^2 values ranging between 0.98-0.99.

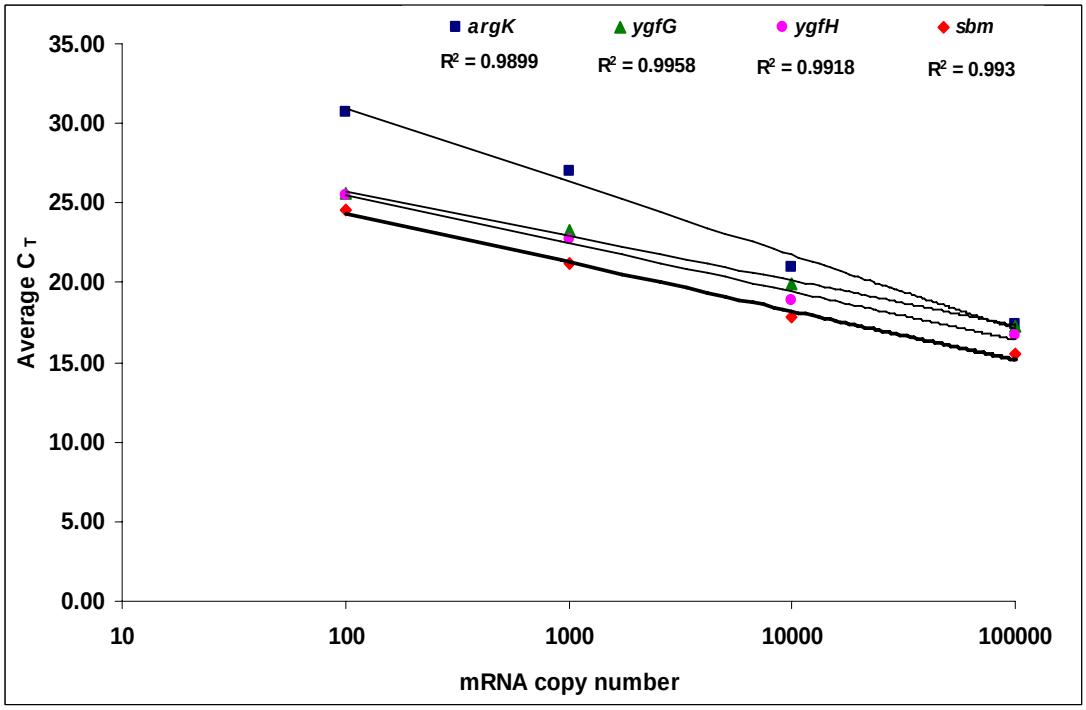

Figure 4.10: Standard curves obtained for the four genes of the *sbm* operon. The genes and the corresponding R^2 values are as shown on the graph.

Based on the equations from the respective standard curves and using the C_t values obtained previously from the actual samples, the mRNA copy number of each of the four genes of the *sbm* operon was calculated. The results obtained have been graphically represented in Figures 4.11, 4.12, 4.13 and 4.14. The calculation of the transcript copy numbers of the target genes was based on the following formula adapted from Ritahlati *et al*. (2006),

$$\text{Molecular weight of the product} = \text{Base pairs} \times 665 \text{ Da}$$

$$\text{No of moles} = \left(\frac{\text{Concentration of the product } (\mu g)}{\text{Mol. Weight of the product}} \right) \times 10^6$$

$$\text{Copy number} = \text{No. of moles} \times 6.023 \times 10^{23} \text{ (Avagadro's number)}$$

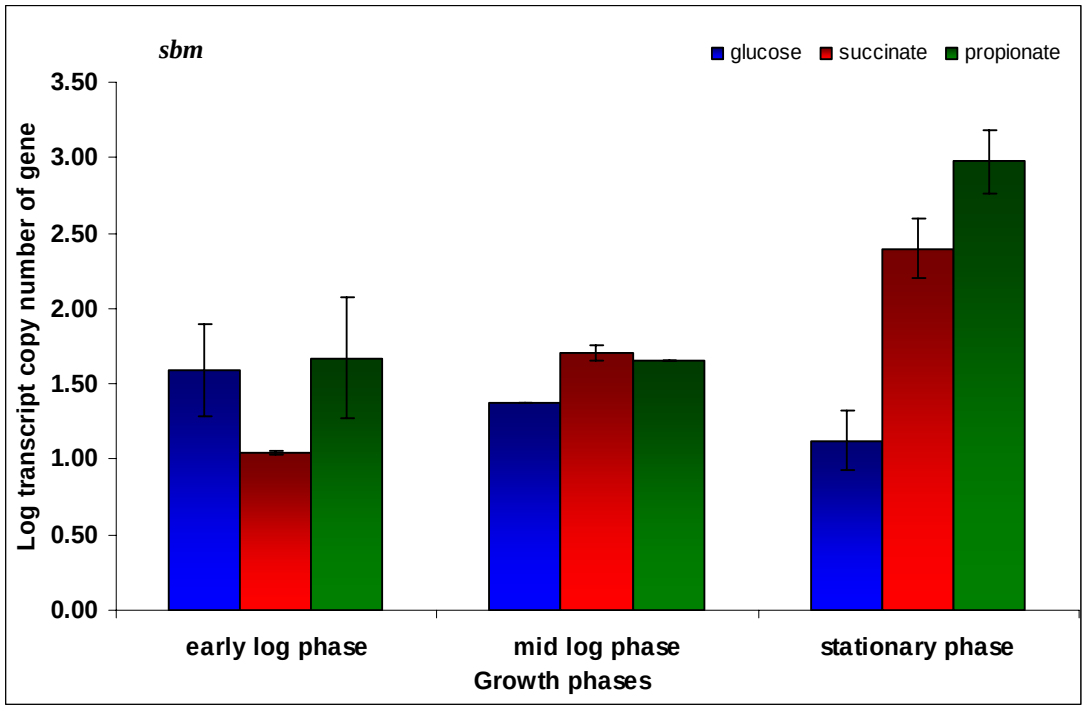

Figure 4.11: Graphical representation of QPCR results showing the absolute copy number of mRNA corresponding to the *sbm* gene from TR6524 cells, during the different growth phases, in the presence of glucose, succinate or propionate in the minimal media. The experiment was carried out in triplicates

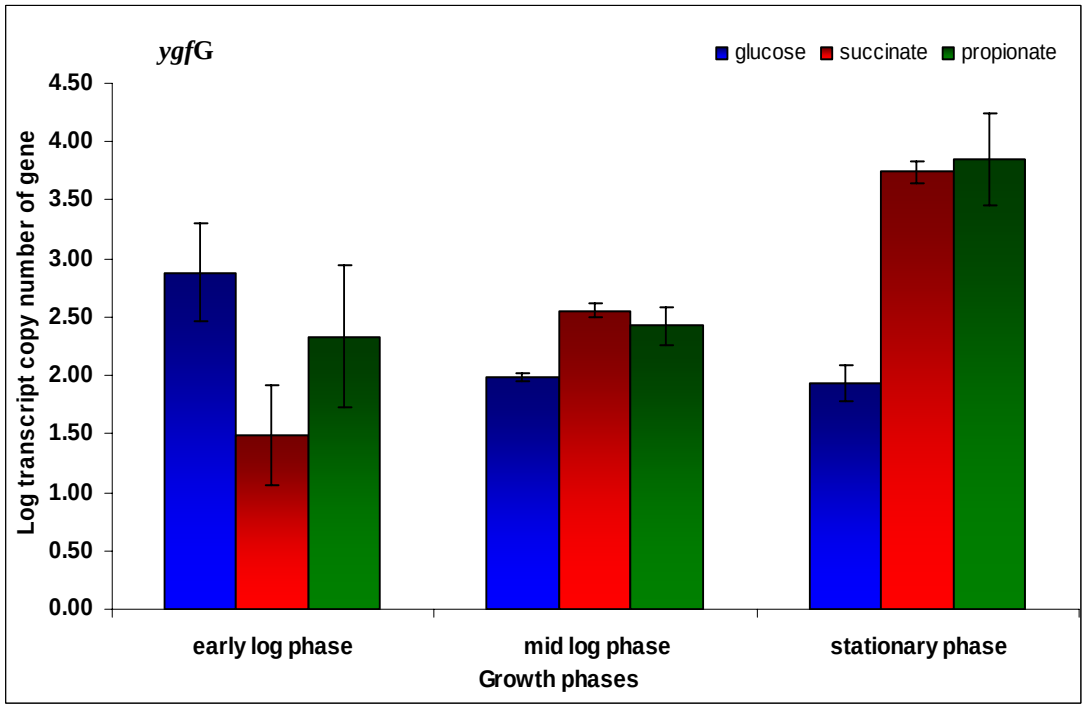

Figure 4.12: Graphical representation of QPCR results showing the absolute copy number of

the *ygf*G gene from TR6524 cells during the different growth phases in the presence of glucose, succinate or propionate in the minimal media. The experiment was carried out in triplicates

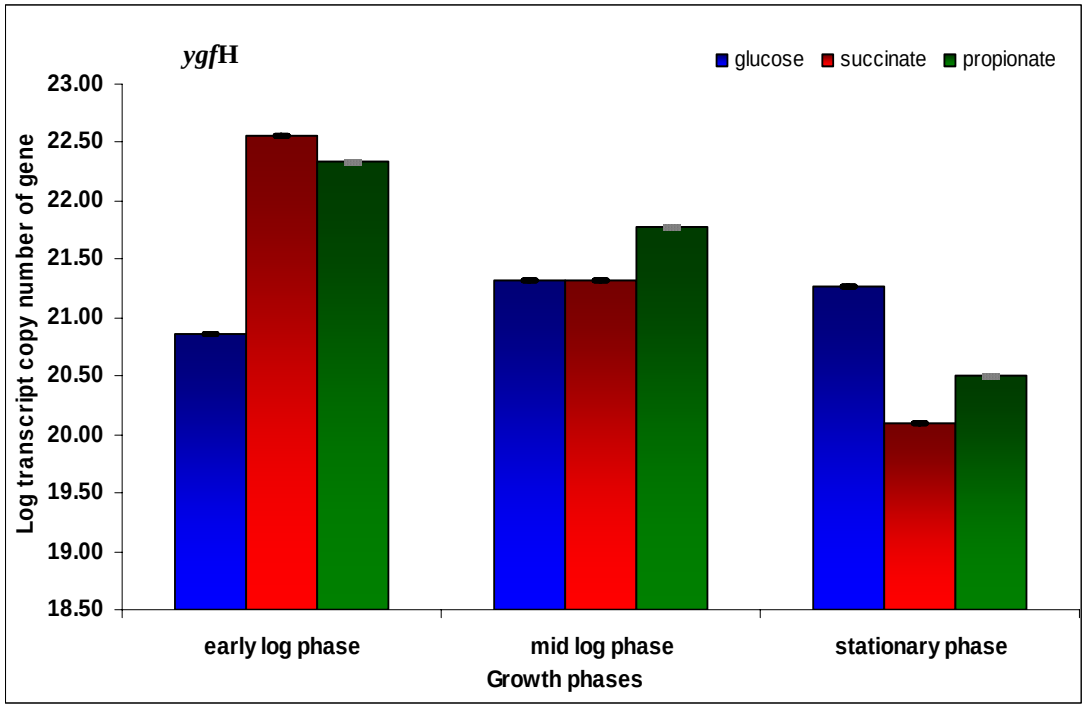

Figure 4.13: Graphical representation of QPCR results showing the absolute copy number of the *ygf*H gene from TR6524 cells during the different growth phases in the presence of glucose, succinate or propionate in the minimal media. The experiment was carried out in triplicates

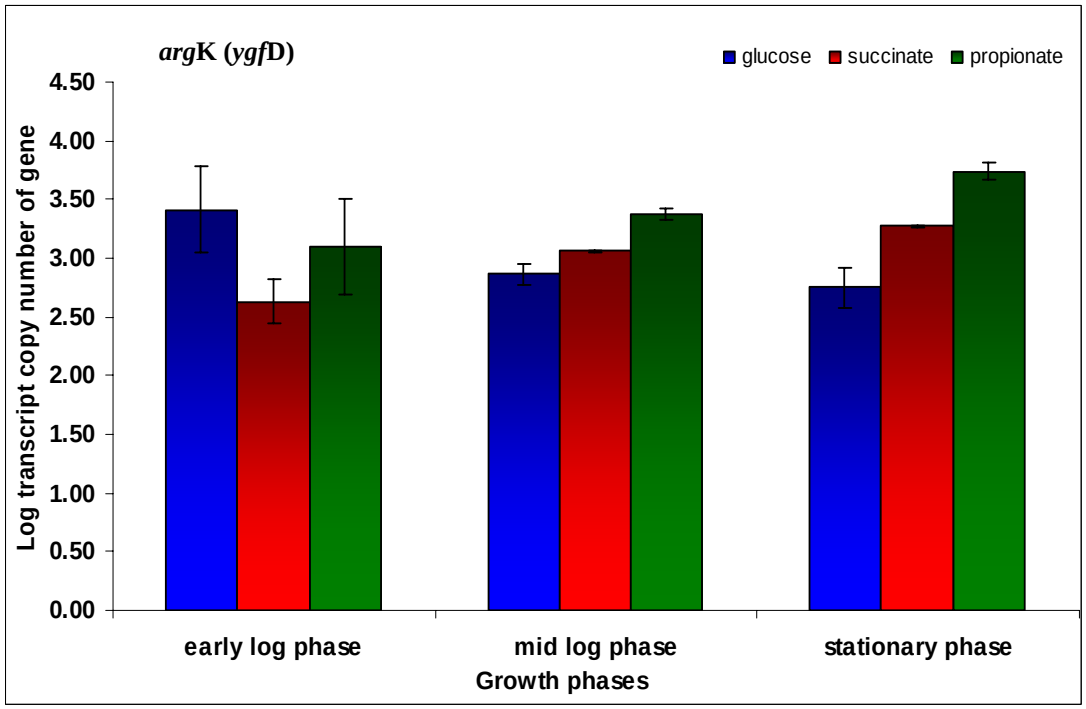

Figure 4.14: Graphical representation of QPCR results showing the absolute copy number of the *arg*K gene from TR6524 cells during the different growth phases in the presence of glucose, succinate or propionate in the minimal media. The experiment was carried out in triplicates

In the case of the *sbm* gene (Figure 4.11), the corresponding mRNA levels decrease as the culture progresses from early-log phase, through to the mid-log phase, into the stationary phase, when glucose is used as the sole carbon source. In contrast, the mRNA levels corresponding to *sbm* gene increases as the culture progresses from the early-log phase, through to the mid-log phase and finally into the stationary phase, when succinate or propionate is used as the sole carbon source. In the stationary phase there is a 2.5 and 3 fold higher level of mRNA corresponding to the *sbm* gene in cultures with succinate and propionate as the sole carbon source, respectively, as compared to that with glucose as the sole carbon source. This observation confirms the role of the *sbm* gene product in the utilisation of succinate and propionate specifically in the stationary phase of growth. A similar trend was observed in the case of the *ygf*G (Figure 4.12) and *arg*K (Figure 4.14) genes. The mRNA levels were 2.0 and 2.2 fold for the *ygf*G gene and 1.2 and 1.4 fold higher for the *arg*K gene, in the presence of succinate and propionate, respectively, as the sole carbon source, as compared to glucose. These observations confirm the role of the *ygf*G and *arg*K genes in the utilisation of succinate and propionate during the stationary phase of growth. However, the mRNA levels corresponding to the *ygf*H gene (Figure 4.13) showed a very different trend to all the other genes in the operon, with a maximum concentration at the beginning of the growth, i.e. the early-log phase, with a concomitant decrease in the mid-log and stationary phases of the culture. In the early-log phase there was a 1.2-1.3 fold higher level of mRNA corresponding to this gene when succinate and propionate were used as the sole carbon sources, respectively, as compared to that from cells grown in minimal media with glucose as the sole carbon source.

4.3 Discussion

4.3.1 Reverse transcription end point PCR

The RT-PCR results (Figures 4.6 and 4.8) showed that the level of the *sbm* gene transcription was greater when *E. coli* was grown in succinate as compared to the levels of mRNA corresponding to the *sbm* gene when grown in glucose or propionate in the presence of 0.5μg/ml vitamin B_{12} in the medium. Further, the mRNA formation was observed to increase steadily and reached the highest observed value at the stationary phase. This confirmed the results obtained during the growth analyses (Chapter 2) and indicated the involvement of the *sbm* gene in succinate utilisation. This also showed that possibly, one of the levels of control related to the *sbm* gene expression was at the transcriptional level, during the utilisation of succinate. However, these were preliminary experiments with no duplicates carried out for verification.

The results of the densitometric analysis from the end point RT-PCR indicated an active role of the *sbm* gene in the growth of the wild type TR6524 in minimal media with succinate as the sole source of carbon. However, the relatively lower level of transcription of the *sbm* gene, in the presence propionate, as compared to glucose, indicated a lack of any role for the *sbm* gene in propionate utilisation. These preliminary studies were obviously not conclusive and the results did not provide a clear understanding of the number of copies of the *sbm* gene present in the sample. Thus, the more accurate QPCR studies were carried out. Primers were designed for the amplification of all the genes within the *sbm* operon and the RNA from cells harvested at the early log, mid log and stationary phases of growth from all the above mentioned conditions were used for the study.

4.3.2 QPCR- absolute quantification of the genes of the *sbm* operon

QPCR was carried out with cDNA template following the reverse transcription of RNA isolated from cells harvested at the early-log, mid-log and stationary phases of growth of wild type *E. coli* TR6524 in minimal media with glucose, succinate or propionate as the sole source of carbon. Since it was an obvious and a confirmed fact (Figure 4.5) that there was no transcription of the *sbm* gene in the mutant FA1P1, these cells were not used for the real time analyses. Sequence specific primers for

the individual genes (*sbm*, *ygf*G, *ygf*H and *arg*K) were designed (Table 9.1) for the PCR and the resulting threshold cycle values were noted for determining the mRNA copy numbers present in the samples. The reporter signal was normalised to the fluorescence of an internal reference dye (ROX), to allow for corrections in fluorescent fluctuations caused by changes in concentration or volume (Gibson *et al*., 1996 & Bustin, 2002).

This study provided us with an understanding of the transcription of the corresponding genes during the growth of the wild type *E. coli* in minimal media with glucose, succinate or propionate as the sole source of carbon. An increase in the mRNA levels corresponding to the *sbm* (2.5 and 3 fold), *ygf*G (2.0 and 2.2 fold), and *arg*K (1.2 and 1.4 fold) genes in the stationary phase of growth, in the presence of succinate and propionate as the sole source of carbon, respectively, was observed. A similar increase was noted for the *ygf*H (~1.2-1.3 fold) mRNA level in the early-log phase of growth in succinate and propionate as the sole source of carbon. The elevated mRNA levels for the 4 genes of the *sbm* operon (during the different phases of growth), in the presence of succinate or propionate as the sole source of carbon as compared to when grown in the presence of glucose, indicates the role of this pathway in the utilisation of these substrates.

Thus, the stage of growth at which maximum mRNA level was observed was found to be the stationary phase of growth in three out of the four genes with the *ygf*H gene being the only exception where maximal mRNA levels were actually found during the early-log phase of growth. This observation possibly indicates that the *ygf*H gene product, *i.e.* the propionyl-CoA: succinyl-CoA transferase, plays a major role in the adaptation of *E. coli* to the utilisation of succinate and propionate and is possibly the rate limiting step in the pathway proposed by Haller *et al*., 2000. Hence, it plays a major role during the early-log phase of growth of the organism. The three other genes of the operon, on the other hand, are transcribed at higher levels during the stationary phase possibly due to the effect of the σS transcription factor. The results indicate that the genes of the *sbm* operon are differentially controlled at the

transcriptional level with an increased level of transcription observed for the first three genes-*sbm*, *arg*K and *ygf*G. *ygf*H, being the last gene of the operon (Figure 1.13), was observed to have a higher transcription level during the early stage of growth. This result indicated that *ygf*H might not be a part of the *sbm* operon and might be under the control of a different promoter. Also, the change in the mRNA levels corresponding to the *arg*K gene is not as much as seen in the case of the other genes. As shown earlier (Figure 1.14) the proposed pathway of Haller *et al*. (2000) does not include the product of this gene (Haller *et al*., 2000). This gene product is however homologous to the MeaB protein found in *Methylobacter extorquens* AM1, known to function as a chaperone protein required for the stability of Mea A protein, a known mutase involved in the conversion of butyryl CoA to propionyl CoA. Thus one can postulate that *arg*K, in addition to its known role in lysine-arginine-ornithine transport (Korotkova and Lidstrom, 2004), is also a chaperone protein required for the stability of the Sbm protein. This postulate is reinforced by the fact that the Sbm protein from *E. coli*, when expressed on its own in *E. coli*, without a histidine tag, was found to be inactive (Dr I. Roy, personal communication). This role as a chaperone does not possibly require a marked variation in the mRNA levels of this gene.

These results, following the quantification of transcription, for the first time, indicate a role of *sbm* and the related enzymes of the operon in succinate utilisation, and to a lesser extent in propionate utilisation during the stationary phase of growth. The results correlate with the data from the promoter analysis (Chapter 3) which indicated that the *sbm* gene and hence the operon expression is induced as cells enter the stationary phase and under conditions of stress *i.e.* growth in succinate and propionate.

Chapter 5

Methylmalonyl-CoA mutase activity in *E. coli*, TR6524

5.1 Introduction

Sbm showed a 59% similarity to the structurally characterised methylmalonyl-CoA mutase from *P. shermanii*. Despite this high level of sequence identity, it was thought that Sbm might not catalyse the MCM reaction since the activity of the protein could not be demonstrated (Roy and Leadlay, 1992). Multiple unsuccessful attempts were made to establish the activity of the over-expressed Sbm protein. Another possible reason for the lack of detectable activity was thought to be due to the higher number of cysteine residues (compared to the mutase from *P. shermanii*) which might get oxidised under the assay conditions that were used. Hence, the use of higher amounts of reducing agents (such as DTT) in future assay reactions was recommended (Dr. I. Roy, PhD thesis). Later, Haller and co-workers (2000) showed that *sbm* did encode an active MCM when expressed in the form of a recombinant protein with a His-tag (Haller *et al*., 2000). It was suggested that the activity measured was probably due to the presence of the N-terminal His-tag that was used to isolate the protein as they found that the activity, after cleavage of the His-tag, dropped to <7% of that measured with the His-tag present. It was speculated that the N-terminal His-tag used for purification may favour an oligomerisation necessary for catalytic activity (Haller *et al*., 2000). The lack of activity in the previous conditions could possibly be due to the instability of the non-His tagged recombinant protein. A possible chaperone-like protein required for the stability of the Sbm protein in *E. coli* would be YgfD (or ArgK), encoded by the gene downstream of the *sbm* gene. This protein is homologous to the MeaB protein found in *M. extorquens* AM1, known to function as a chaperone protein required for the stability of Mea A protein. MeaA is a known mutase involved in the conversion of butyryl CoA to propionyl CoA (Korotkova and Lidstrom, 2004). Thus it can be postulated that YgfD (or ArgK), in addition to its known role in lysine-arginine-ornithine transport (Korotkova and Lidstrom, 2004), is a chaperone protein required for the stability of the Sbm protein (discussed in detail in Chapter 4). Hence, in the absence of this chaperone protein, the recombinant Sbm protein lost activity during purification. This loss of activity was possibly prevented in the case of the His tagged protein due to the drastically reduced time required for the purification. Attempts to show the

MCM activity in wild type *E. coli* whole cell extracts have been unsuccessful (Vlasie *et al*., 2002 & Bobik and Rasche, 2003).

Previously, several methods have been described and employed to measure methylmalonyl-CoA mutase activity. These include radiometric methods using DL[CH_3-^{14}C] methylmalonyl-CoA by paper chromatography (Whitaker and Giorgio, 1973), spectrophotometric assays involving enzyme coupling to NADH dependent dehydrogenases (Watanabe *et al*., 1993), a gas chromatographic radiometric assay (Goodey & Gompertz, 1972) and a non radioactive HPLC assay (Kikuchi *et al*., 1989 & Riedel *et al*., 1995). The HPLC assay has been found to be simple, sensitive, rapid and highly reproducible when compared with the radiometric assay as reported by a comparative study conducted by Gaire and coworkers (Gaire *et al*., 1999).

The current study attempted the measurement of MCM activity in whole cell extracts from wild type *E. coli* in the presence of succinate and propionate as the sole carbon source, the conditions thought to induce Sbm activity. The promoter analysis (Chapter 3) showing the presence of a sequence closely similar to the consensus sequence for σS (sigma factor associated with stress relief and stationary phase maintenance in *E. coli* and other enterobacteria) was found 29 bases up-stream of the *sbm* gene. This supported the findings of the transcription analyses (Chapter 4) that this gene was possibly induced and expressed during the stationary phase of growth. Results from the quantitation of transcription (Chapter 4) indicated a strong possibility of increasing involvement of the *sbm* gene, in succinate and propionate utilisation, as growth progressed from exponential to the stationary phase. Hence, for enzyme activity measurement, total protein from cells harvested at the stationary phase was chosen.

5.2 Results

5.2.1 Expression of MCM from *P. shermanii* in *E. coli* BL21-DE3 - the positive control

The gene coding for the methylmalonyl-CoA mutase from *P. shermanii* had been cloned earlier and the clone was named pMEX2 (McKie *et al*., 1990). This pMEX2 plasmid (kind gift from Dr N. H. Keep, Birkbeck College University of London, UK) was used to transform *E. coli* BL21-DE3. Growth and induction conditions for the over-expression of MCM in BL21-DE3 were optimised following repeated trials. The optimal expression conditions are described in Section 9.2.8.3. The results of the expression analysis are shown in Figure 5.1.

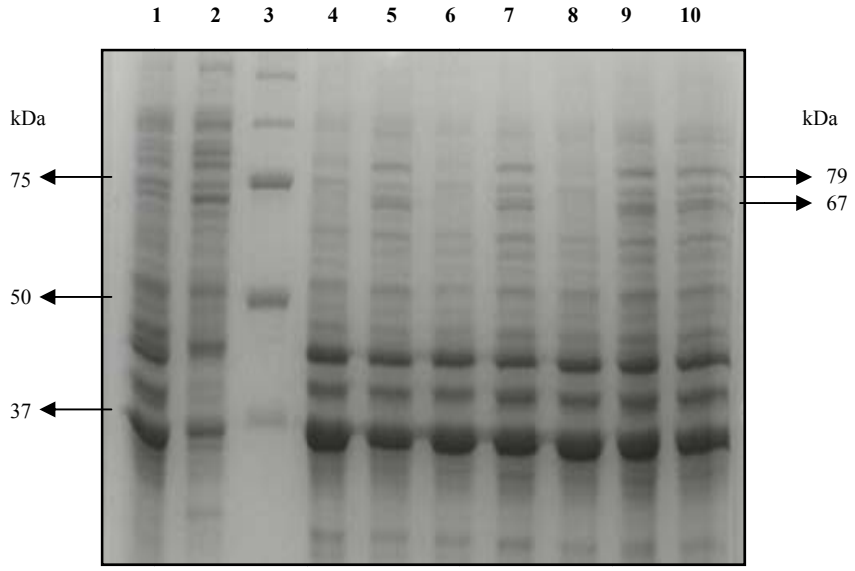

Figure 5.1: Expression of MCM in *E. coli* BL21-DE3. A 10% SDS-PAGE gel showing the over-expression of *P. shermanii* MCM in *E. coli* BL21-DE3. The α and β subunits of *P. shermanii* MCM are 79 and 67kDa respectively. Lane 1-*E. coli* BL21-DE3 whole cell extract before transformation, lanes 2, 4, 6 and 8- un-induced *E. coli* BL21-DE3 cells following transformation with pMEX2, lane 3- Broad range (9-225kDa) protein marker from Promega, lane 5-*E. coli* BL21-DE3 with pMEX2 induced with 0.5mM IPTG, lane 7-*E. coli* BL21-DE3 with pMEX2 induced with 0.7mM IPTG, lane 9-*E. coli* BL21-DE3 with pMEX2 induced with 1.0mM IPTG, lane 10-*E. coli* BL21-DE3 with pMEX2 induced with 1.5mM IPTG.

Since the expression of the MCM was under the influence of a T7 promoter, a range of IPTG concentrations and induction conditions were tested in order to maximise the expression of the MCM. The best expression was observed in the presence of

1mM IPTG (lane 9) in Figure 5.1. The figure shows bands corresponding to proteins from samples following growth at 28^{o}C before induction and incubation at 18^{o}C following induction with different IPTG concentration as mentioned in the figure legend. Control samples (non transformed *E. coli* BL21 DE3) and un-induced transformed cell samples were also prepared and run along side to confirm the over-expression. The whole cell extract of this *E. coli* BL21-DE3 transformed with pMEX2 that contains the gene encoding MCM from *P. shermanii* (McKie *et al*., 1990) was used in the positive control reaction (Section 9.2.8.6), since the pure form of the enzyme was not commercially available.

5.2.2 HPLC assay of the MCM protein from *P. shermanii*

The assay was carried out as described in Section 9.2.8.6.2. The positive control reaction using the *E. coli* BL21-DE3 cell extract helped to standardise assay conditions suitable for the test samples. This positive control reaction was carried out at two different conditions. One set of the reaction mixture was incubated at 4^{o}C (Haller *et al*., 2000) and the other at 37^{o}C (Riedel *et al*., 1995). The reactions were stopped at 5, 10, 20 and 40 minute intervals in both cases and the peaks corresponding to the disappearance of the substrate and appearance of the product were observed using the HPLC assay.

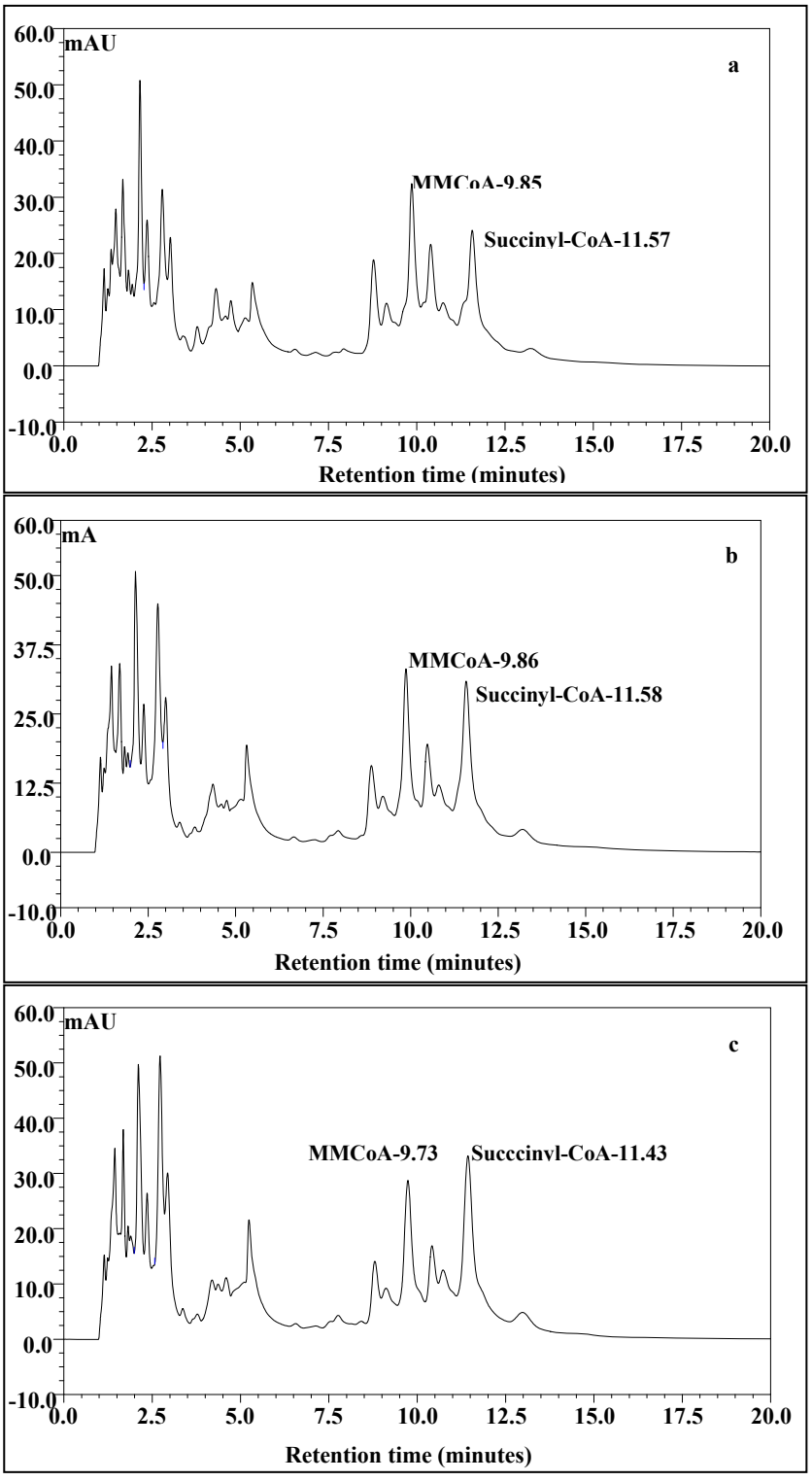

Figure 5.2: HPLC peak data for over-expressed *P. shermanii* MCM. MCM from *P. shermanii* was expressed in *E. coli* BL21-DE3 and the total protein was used for the reaction which was incubated at 37^{o}C for 5, 10 and 20min. 5.2 a, b and c show the results of the assay for MCM activity from reaction mixtures incubated at 5, 10 and 20min, respectively. The decrease in peak height for MMCoA and the corresponding increase for succinyl-CoA can be clearly seen as incubation progresses.

The results for the positive control as observed using HPLC showed that reactions incubated at 37^{o}C produced peaks of decreasing intensities for the substrate methylmalonyl-CoA (MMCoA), while the peak intensities for the product succinyl CoA increased (Figures 5.2 a, b, c). This observation was expected in reaction mixtures with cell extract that contained an over-expressed MCM protein. However, this was not the case with reaction mixtures incubated at 4^{o}C. The peak intensities were inconsistent and unreliable. Hence reactions with test samples were carried out at 37^{o}C. Since the volume of the extract used in the reactions was kept constant, the amount of protein in the reactions was not the same. However, calculation of specific activity normalised this difference.

5.2.3 HPLC assay of the reaction of the protein extract from cells grown in glucose, succinate or propionate

As described in Section 9.2.8.6.1, solutions of commercially available lithium salt of MMCoA and sodium salt of succinyl-CoA were prepared in order to produce a standard calibration curve. The solutions of the standards were manually injected into the C-18 column connected to the HPLC detection system and peak areas were analysed and used for the calculation of MCM specific activity. The generation of MMCoA and succinyl-CoA standard curves was then followed by HPLC assay carried out with reaction mixtures containing the whole cell extract from cells grown in minimal media with glucose, succinate or propionate as the sole source of carbon and harvested at the stationary phase of growth. Based on the results of the positive control mentioned above (5.2.2), the test reactions were incubated at 37^{o}C and the reactions were stopped at 5, 10, 20 and 40 minute intervals. The total protein extracts from *E. coli* cells grown in the presence of glucose, succinate and propionate that were used in the reactions were first run through the assay column to exclude any peak that could interfere with the interpretation of the results.

The multiple peaks that were observed in these protein samples appeared within five minutes of sample injection, much ahead of the peaks corresponding to MMCoA (about 9min) or succinyl-CoA (about 11min). This allowed an easier analysis of the

peaks that appeared due the action of MCM on MMCoA to form succinyl-CoA. The total protein measured, in each case, using the Bradford assay was found be to be 0.13mg/ml (glucose), 0.14mg/ml (succinate) and 1.29mg/ml (propionate).

The peak data obtained from the HPLC assay for reactions with protein extract from cells grown in minimal media with glucose, succinate or propionate as the sole source of carbon (5min incubation) and the relevant negative control reactions with only the protein sample are shown in Figures 5.3, 5.4 and 5.5, respectively.

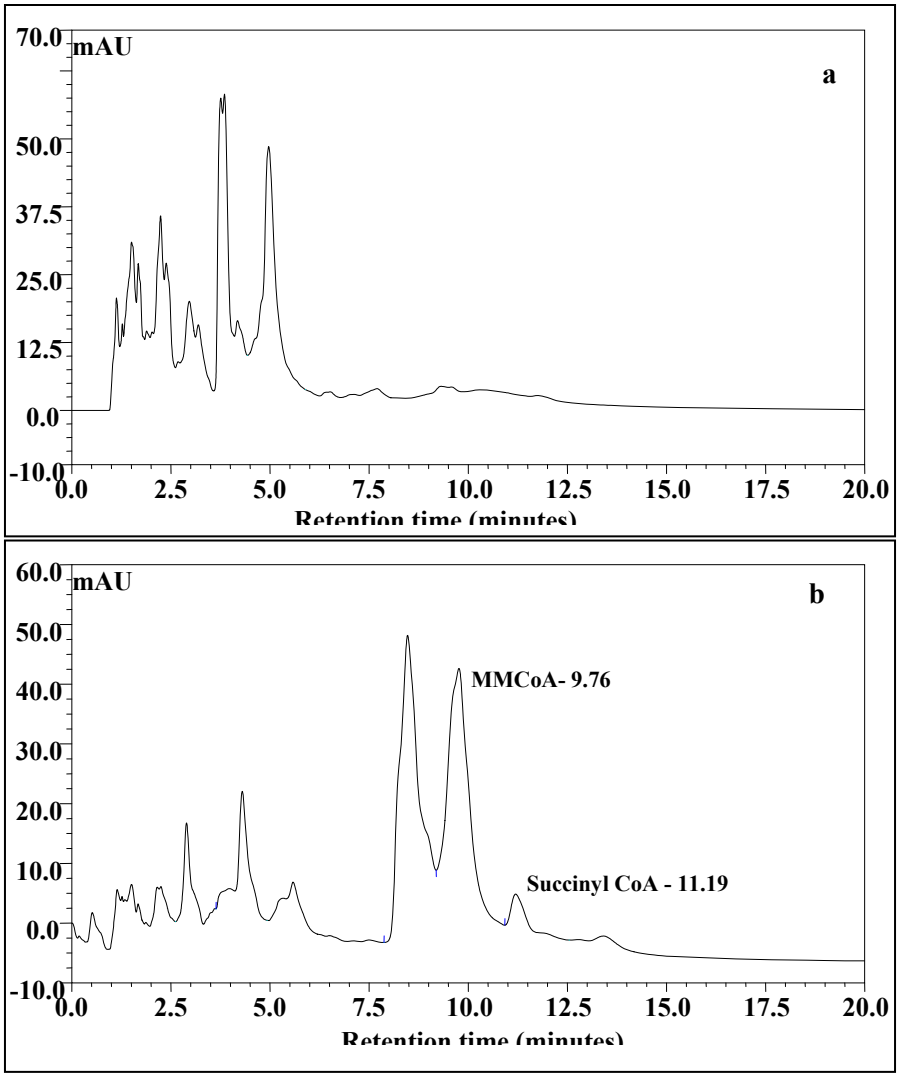

Figure 5.3: HPLC peak data for cells grown in minimal media with glucose as the sole source of carbon. Chromatogram showing peaks observed for (a) whole cell extract (total protein) from glucose grown cells and (b) MMCoA and succinyl-CoA in an HPLC assay following 5min incubation reactions.

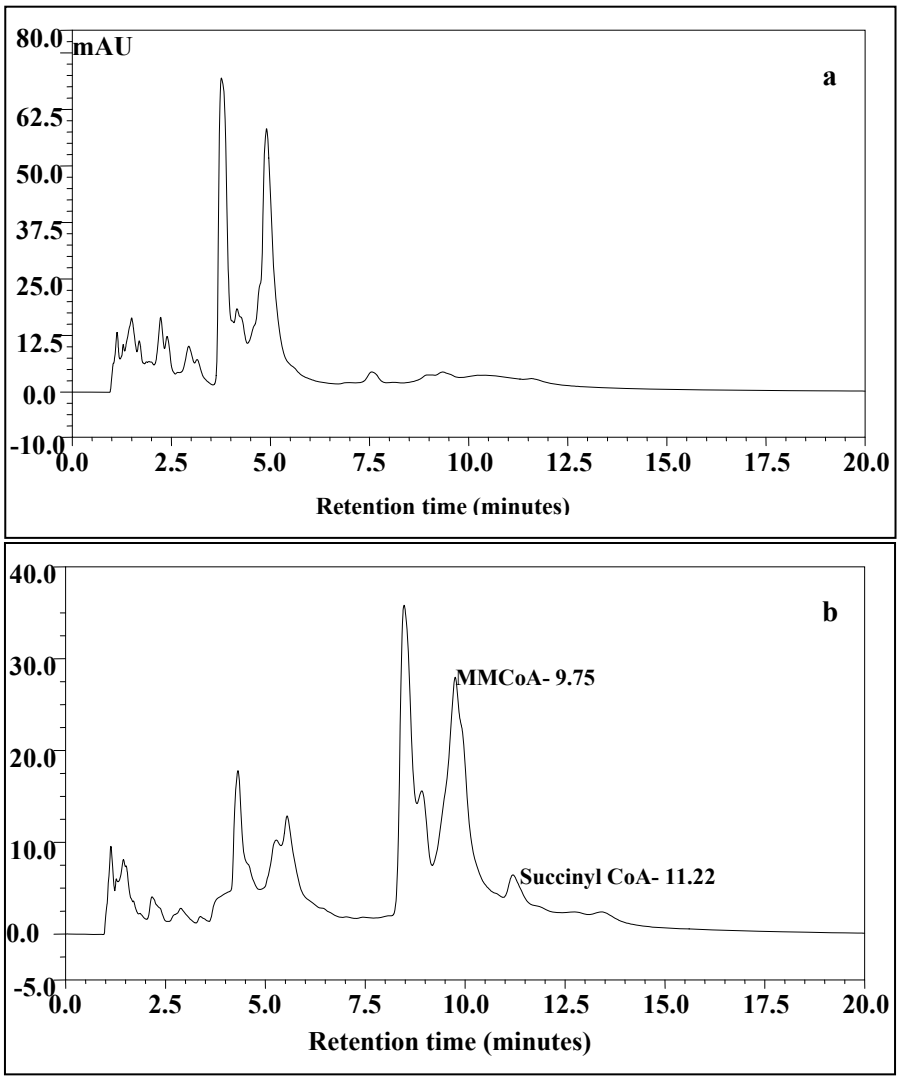

Figure 5.4: HPLC peak data for cells grown in minimal media with succinate as the sole source of carbon. Chromatogram showing peaks observed for (a) whole cell extract (total protein) from succinate grown cells and (b) MMCoA and succinyl-CoA in an HPLC assay following 5min incubation reactions.

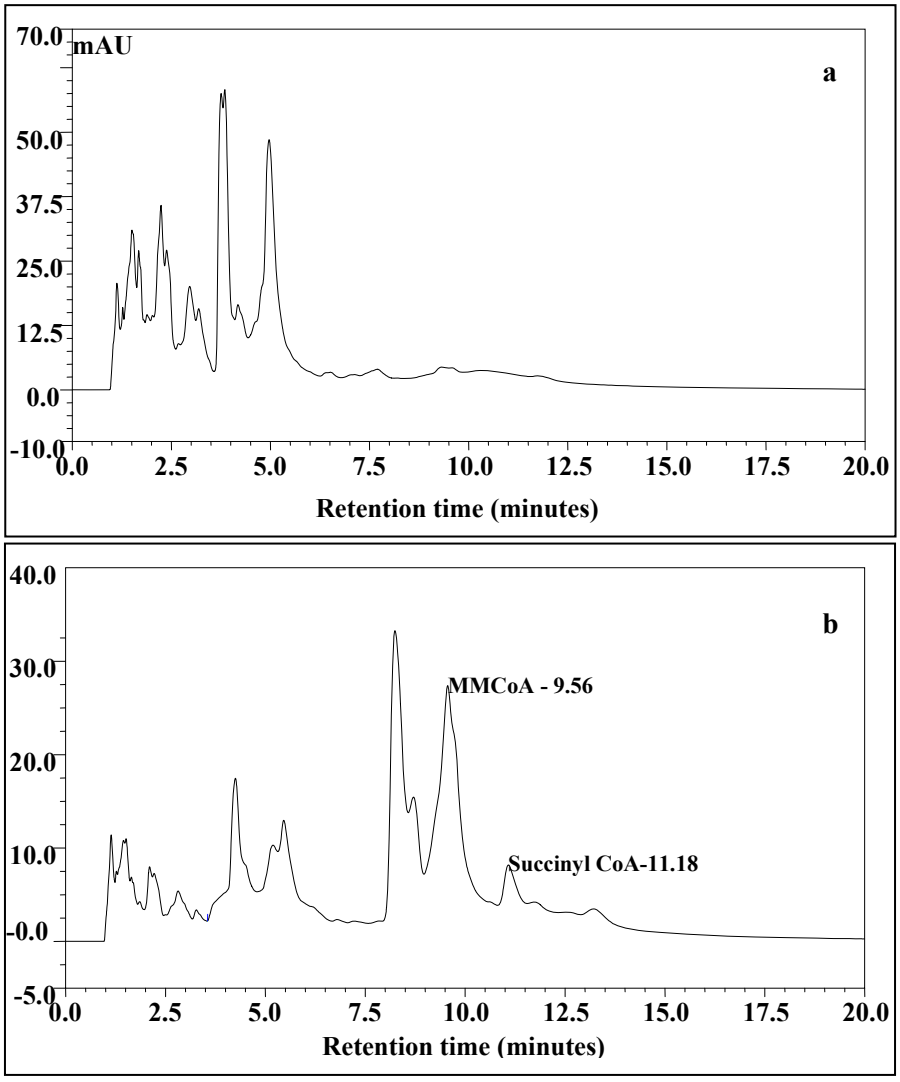

Figure 5.5: HPLC peak data for cells grown in minimal media with propionate as the sole source of carbon. Chromatogram showing peaks observed for (a) whole cell extract (total protein) from propionate grown cells and (b) MMCoA and succinyl-CoA in an HPLC assay following 5min incubation reactions.

The retention times for MMCoA (9-9.99min) and succinyl-CoA (11-11.25min) varied slightly in all three conditions as shown in the figures above. This was due to the differences introduced as a result of manual injection. The incubation time of up to 40min was chosen in this study in order to identify the time required for complete conversion MMCoA to Succinyl CoA by MCM since this is the first time ever that an attempt has been made to assay MCM activity using the HPLC method and the whole cell extract from wild type *E. coli* grown under the specified conditions.

5.2.4 MCM activity of total protein extract from wild type *E. coli* TR6524 grown in minimal media with glucose, succinate or propionate as the sole source of carbon

The specific activity for MCM in cells grown in minimal media with glucose, propionate or succinate as the sole source of carbon was measured based on the disappearance of the MMCoA substrate during the course of the 40min incubation and the values were tabulated as shown in Table 5.1. The specific activity of MCM obtained is also graphically represented in Figure 5.6. As the incubation time for the reactions was increased, the activity was observed to decrease rather than an expected increase, in all cases. The only exception was the 10 minute reaction with total protein from cells grown in glucose that showed an increase as compared to the 5min reaction (Table 5.1).

Incubation time (minutes)	**Specific activity of Sbm protein (U/mg) from cells grown in minimal media with**		
	Glucose	**Succinate**	**Propionate**
5	1.37×10^{-3}	4.76×10^{-3}	2.79×10^{-3}
10	2.69×10^{-3}	3.19×10^{-3}	1.58×10^{-3}
20	1.57×10^{-3}	1.60×10^{-3}	0.72×10^{-3}
40	0.78×10^{-3}	0.88×10^{-3}	0.41×10^{-3}

Table 5.1: Specific activity of MCM with respect to the disappearance of MMCoA substrate

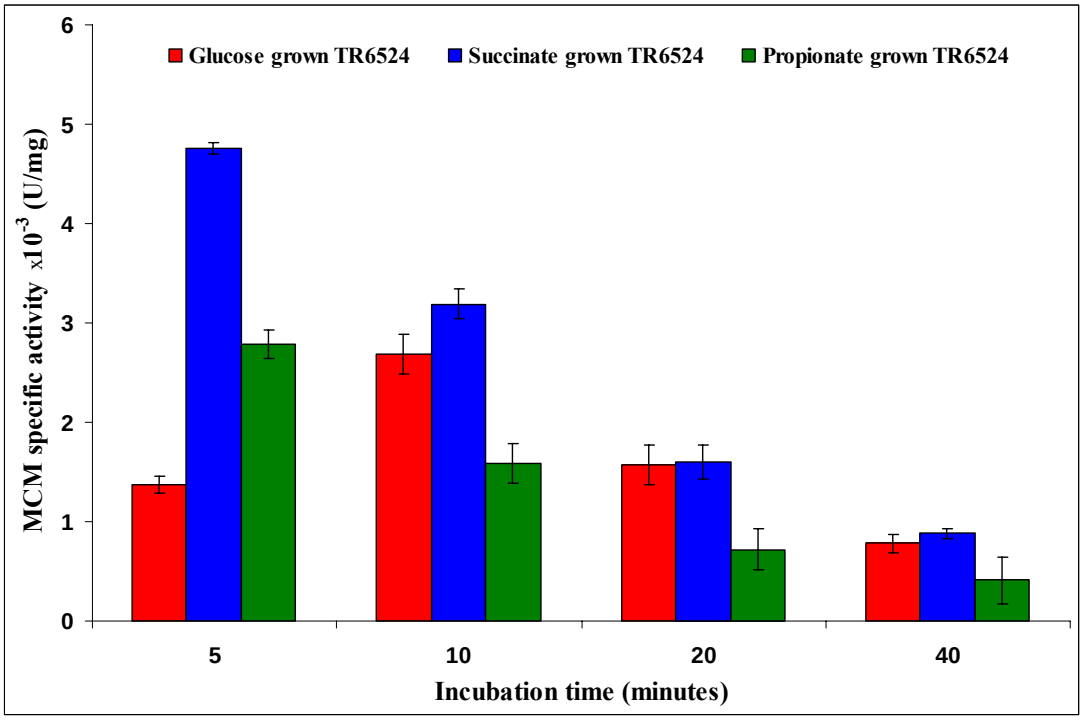

Figure 5.6: Graphical representation of the specific activity of MCM (Table 5.1) across the test conditions.

The MCM specific activity observed for the reaction stopped at the end of 5min incubation (Table 5.1) was found to be 1.37×10^{-3} U/mg in the cells grown in minimal media with glucose as the sole source of carbon. In contrast, it was found to be four times higher (4.76×10^{-3} U/mg) in the whole cell extract of cells grown in minimal media with succinate and two times higher (2.79×10^{-3} U/mg) in the whole cell extract of cells grown in minimal media with propionate as the sole source of carbon.

This is the first ever report of the detection of MCM activity in the whole cell extracts of wild type *E. coli*. The presence of higher MCM activity in the presence of succinate and propionate as compared to the activity observed in the presence of glucose strongly indicates the role of the enzyme in the utilisation of these carbon sources in the stationary phase. This is the first time such a direct correlation has been made between MCM activity and the utilisation of substrates such as succinate

or propionate. Also, the results correlated well with the growth analysis and the transcription analysis.

5.3 Discussion

Previous studies (Haller *et al*., 2000 & Bobik & Rasche, 2003) have assayed the activity of the purified recombinant Sbm protein. This is the first report of methylmalonyl-CoA mutase activity in wild type *E. coli*. Higher specific activities were found in the presence of succinate and propionate, as compared to the specific activity observed in the presence of glucose. Specific activities of $4.76x10^{-3}$ U/mg and $2.79x10^{-3}$ U/mg were measured in cells from succinate and propionate, respectively, compared to $1.37x10^{-3}$ U/mg that was observed with cells from glucose (Table 5.1). This strongly indicates the role of the enzyme in the utilisation of these carbon sources in the stationary phase.

Riedel (1995) studied the stability of methylmalonyl-CoA mutase in crude cell (human fibroblasts, human glioma and rat liver) homogenates and found that the enzyme activity declined at a rate of 0.08 h^{-1} (Reidel *et al*.,1995). Bobik and Rasche (2003) used *E. coli* extracts in unsuccessful attempts in their study and suggested that partial purification of the enzymes as recombinant proteins with affinity tags was required to demonstrate activity *in-vitro* (Bobik and Rasche, 2003). The *His* tag used in the purification of recombinant Sbm was thought to be required for the catalytic activity of the enzyme *in-vitro* (Haller *et al*., 2000). The current study did not involve the use of any stabilising agents (Reidel *et al.,* 1995) and did not employ recombinant or purification methods using affinity tags. The decreasing activity of MCM (Table 5.1) with increasing incubation time could be due to the absence of a suitable stabilising agent (such as glycerol) that maintains the enzyme intact (Riedel *et al*., 1995).

The specific activity of MCM from total cell extract for wild type *E. coli* grown in minimal media with glucose, succinate or propionate as the sole carbon source is as shown in Table 5.1. The decrease in specific activity of the mutase, with the

exception of that observed for glucose (10min), with an increase in the incubation time (Figure 5.6) indicates the need for shorter incubation times for the reaction to obtain kinetic parameters. In fact, the specific activity, in the case of succinate and propionate, was observed to decrease by almost half as the reaction incubation time doubled (Table 5.1). This trend was observed for glucose grown cells for the 20min and 40min incubation periods.

The specific activity measured in this work is relatively low compared to the mammalian MCM activity of 0.39 U/g (Riedel *et al*., 1995) and 26.4 U/mg (Carlucci *et al*., 2007). Both these activities were measured in crude protein extract from rat liver cells, indicating the occurrence of a high concentration of MCM for metabolism of propionate, proving the evolutionary importance of methylmalonyl-CoA mutase in mammals. Also, in this work, the peak data (Figure 5.2) suggested that the specific activity of *P. shermanii* MCM was higher as the *P. shermanii* MCM was over expressed using a strong promoter in *E. coli,* hence more active enzyme would be present. *E. coli* would be expected to produce MCM at a relatively lower concentration when grown in the presence of vitamin B_{12} and the appropriate carbon source such as succinate or propionate. This would explain the relatively low specific activity observed in the wild type *E. coli* extract.

In conclusion thus, this work resulted in the first successful measurement of MCM activity in wild type *E. coli*. MCM specific activity analysis revealed a four fold increase of the specific activity in the case of growth in succinate compared to that observed with growth in glucose and two fold increase in the case of growth in propionate. These results indicate a direct involvement of MCM in succinate and propionate utilisation, in the stationary phase of growth. The specific activity data confirm the findings of the growth and transcription analyses (Chapters 2 and 4) where the *sbm* gene encoding MCM was thought to be of importance in succinate and propionate uptake and utilisation. The transcription quantitation data showed that the *sbm* gene transcript copy number was higher in the cells grown in succinate compared to that seen in the case of cells from propionate or glucose and the MCM

specific activity measured using the HPLC analysis reconfirm these findings. The results from the HPLC analysis also verify the possibility of *sbm* gene expression in the stationary phase of growth as the total protein used in the reactions for the assay were obtained from *E. coli* TR6524 cells harvested at the early stationary phase of growth thus correlating well with the data from the promoter analysis which suggested that *sbm* could be one of the genes under σS regulation.

Chapter 6

A proteomic analysis of *E. coli*, TR6524 grown in glucose, succinate or propionate

6.1 Introduction

Proteomic approaches have been employed in the systematic identification and characterisation of proteins (Gerber *et al.*, 2003 & Vollmer *et al*., 2003). The complexity of the proteome and the inability to amplify proteins, unlike the nucleic acids, has led to the development of this technique that enables the characterisation of proteins in microgram quantities (Minden, 2007).

The proteome is a highly dynamic system where synthesis, degradation, and modification of proteins are subject to change in response to internal and external stimuli (Vollmer *et al*., 2003). Proteomic studies have helped elucidate the complex cellular responses of bacteria such as *E. coli* (which has been frequently adopted as a model organism for structural and functional studies). For example, two dimensional electrophoresis (2DE) has been used to identify the channel-forming transmembrane proteins responsible for the passage of phosphate (PhoE), maltose; maltodextrins (LamB) and nucleosides (Tsx). In addition, high affinity receptors for the transport of ferric iron (FepA, FhuA), vitamin B_{12} (BtuB), and fatty acids (FadL) have also been elucidated using 2DE in *E. coli* (Molloy *et al*., 2000).

2DE is a high resolution technique for protein separation and identification. It comprises the fractionation of complex protein mixtures according to their charge in the first dimension (iso-electric focusing, IEF) and to the independent parameter of molecular mass in the second dimension. The reproducibility of 2DE is highly dependent on the precision of the focusing in the first dimension (Westermeier *et al*., 1983). The use of immobilised pH gradient (IPG) strips has made IEF more consistent; allowing easier comparison among different gel samples (Lee and Lee, 2003) and ensures highly reproducible pH gradients which show less sensitivity to sample components. The stability of the IPG strips permit focusing times of sufficient duration for polypeptides to attain their isoelectric point thus improving focusing reproducibility and simplifying spot identification (Bjellqvist *et al*., 1993).

Oh (2002) carried out a global gene expression analysis of *E. coli* grown on acetate and used growth on glucose as a reference. The study not only identified genes (glyoxylate, TCA cycle and gluconeogenic) known to be associated with the metabolic pathways associated with acetate utilisation but also genes that had not been linked previously (such as malic enzymes and PEP synthase) with the metabolic pathway under study. Peng and Shimizu (2003) observed the global *E. coli* responses to growth under different carbon sources (gluconate, glycerol and acetate) and showed that glycolytic enzymes were up-regulated in *E. coli* during growth on all these carbon sources and that the flux in the gluconeogenic direction is smaller than the glycolytic flux when acetate was metabolised. In addition, up-regulation of TCA cycle enzymes was observed in all growth conditions (Peng and Shimizu, 2003). A proteomic approach (using nano-LC/MS) was used to investigate the gene expression of *E. coli* in lactose and glucose as sole sources of carbon. This revealed that, although the glycolysis and TCA cycle genes were common for the metabolism of both the sugars, proteins coded by genes specific for lactose transport and metabolism were identified only from cells harvested from media containing lactose (Vollmer *et al*., 2003).

A more recent LC/MS study by Silva *et al*., 2006 aimed to quantify the *E. coli* proteome when the bacteria were cultured in glucose, lactose or acetate. A slower growth observed in acetate was attributed to reduced protein synthesis as evident from the down regulation of ribosomal proteins and associated chaperonins (such as GroEL). The gene expression pattern for cells grown in lactose and glucose was similar to that observed by Vollmer (2003) (Silva *et al*., 2006). Raman *et al*. (2005) evaluated the differences in the response of *E. coli* W3110 to glucose limitation under fed-batch and exponential culture conditions, using proteomic analysis. Proteins involved in high affinity glucose uptake, the transportation and degradation of alternate carbon sources and TCA cycle were observed to be up-regulated under fed-batch conditions indicating the organism's ability to transport and utilise alternate carbon sources as glucose was depleted. Ribonucleotide synthesis proteins and ribosomal recycling factors were some of the proteins that were found to be up-

regulated in continuous culture, thus, equipping the organism with increased nucleotide and protein biosynthesis required for the exponential growth (Raman *et al*., 2005). A recent study by Han *et al*. (2007) revealed 52 significantly altered proteins in *E. coli* grown with long chain fatty acid, oleic acid, as the sole source of carbon compared to growth on glucose. The authors noted increased levels of long chain fatty acid degradation proteins, FadA and FadB, proteins involved in fatty acid transport (FadL), glyoxylate shunt (AceA) and TCA cycle (Mdh, SdhA, SucC and SucD) among others (Han *et al*., 2007).

2D gel electrophoresis carried out by Brock (2002) to monitor differences in the protein pattern of *E. coli* W3350 cells grown on acetate or propionate showed that proteins (except propionyl CoA synthetase, PrpE) encoded by the *prp* operon were exclusively produced during growth on propionate along with high amounts of acetyl CoA synthase (*acs*). The analysis also reported increased levels of malate synthase (*ace*B) during growth on propionate. Reduced amounts of phosphoglycerate mutase-I (*gpm*A), propanol-preferring alcohol dehydrogenase (*adh*P), and pyruvate kinase (*pyk*F) were observed in propionate-grown cells compared with cultures grown in the presence of acetate. The authors aimed at a better understanding of propionate oxidation and it is important to note that their media did not incorporate any source of vitamin B12 and hence, would not have allowed the expression of active Sbm (Brock *et al*., 2002).

This study sought to evaluate changes in the *E. coli* proteome when the cells were cultured in minimal media with glucose, succinate or propionate as the sole source of carbon. Such an analysis would reveal the change in protein expression under these conditions of growth. However, in this study only a few protein spots from different areas of the gels from all conditions were analysed due to financial constraints.

6.2 Results

The promoter analysis (Chapter 3) indicated that the onset of stationary phase was one possible condition when the *sbm* gene containing operon might be induced. Further, the QPCR data (Chapter 4) supported this hypothesis. An increased mRNA transcript copy number was observed in cells harvested at the beginning of the stationary phase when *E. coli* was grown in the presence of succinate and propionate as the sole carbon source. Hence, whole cell extracts from cells harvested at the beginning of the stationary phase was used for the proteomic analysis. The amount of protein loaded onto the 2DE gel was controlled to ensure that an equal amount of protein from each condition was applied onto the IPG strip. This study used the Progenesis Same Spots software (Non-Linear Dynamics, UK) to match and compare 2DE separations of proteins from *E. coli* TR6524 grown in glucose, succinate or propionate as the sole carbon source. In this approach, an average of 980 protein spots across all the conditions was identified using the software. Based on the estimations of Raman and co-workers (2005) this number of spots would represent about 25% of the entire *E. coli* proteome (Raman *et al*., 2005). Owing to the cost of spot identification and analyses only twenty six spots were chosen for analysis in total from the three different growth conditions. As the spots were hand picked, it was important they were reasonably separated and well stained to minimise errors.

The results of 2DE analysis of the *E. coli* extracts are as shown in Figure 6.1

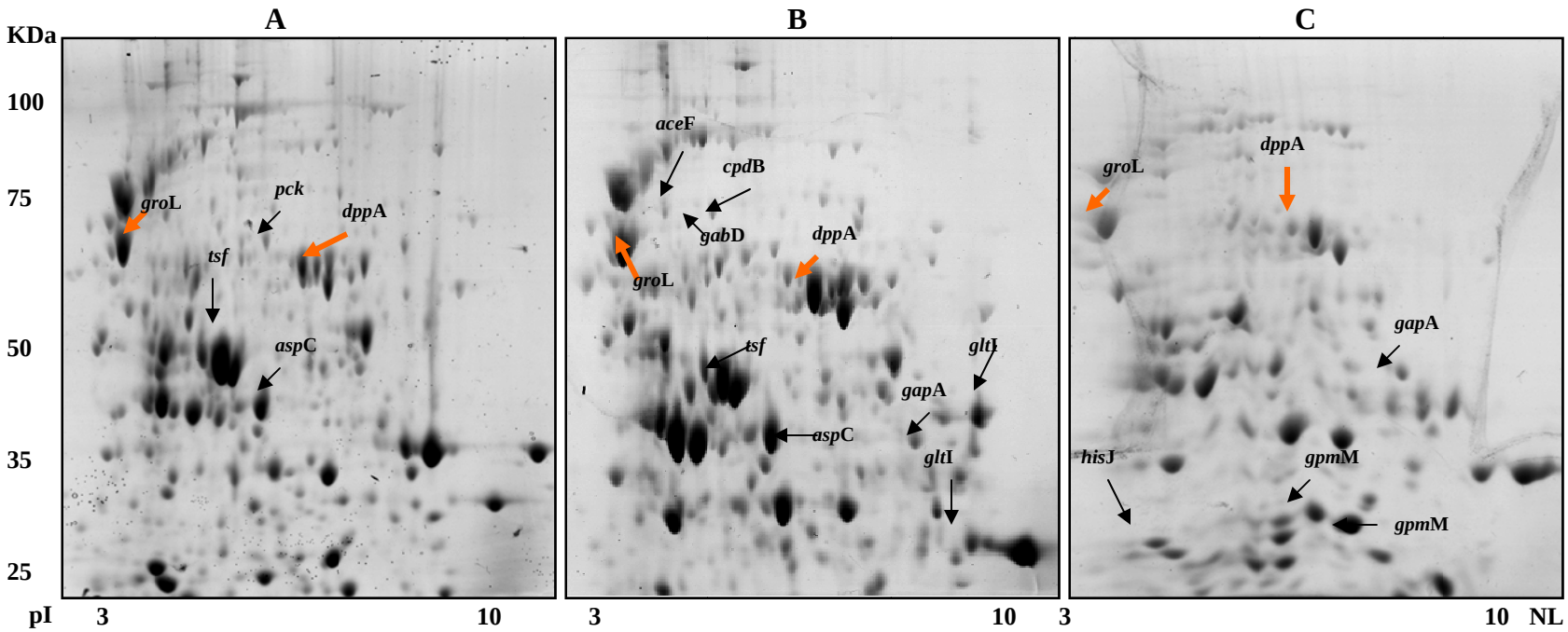

Figure 6.1: Comparative 2DE analysis. 2DE gel showing proteins from wild type *E. coli* TR6524 cells grown in minimal media with glucose (A), succinate (B) or propionate (C) as the sole source of carbon. The samples were focused on a 3-10 non linear IPG strip (GE-health care). The protein spots that were analysed using MS are labeled and the land mark proteins are shown using orange arrows.

Figure 6.1 compares the protein profile of wild type *E. coli* TR6524 grown in minimal media with glucose, succinate or propionate as the sole source of carbon and harvested at the onset of the stationary phase. There were obvious recognisable differences in the spot pattern observed in the different conditions of growth. Good spot resolution was obtained with only minimal streaking indicating high protein solubility and with only a few protein spots merging into one another.

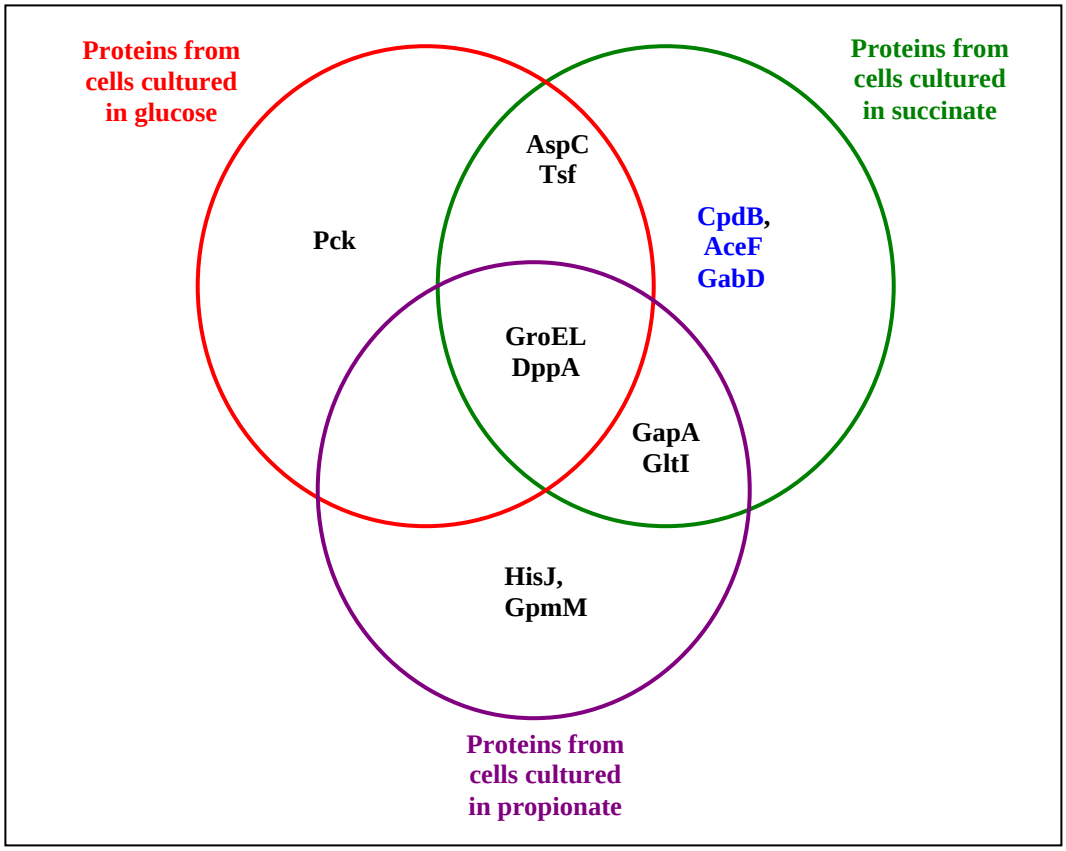

Figure 6.2: Venn diagram of proteins identified in this study. A schematic representation of the overlap among the proteins identified in the current study; the proteins with M_w and pI values similar to that of the Sbm are highlighted in blue.

The proteins identified under different growth conditions are summarised in Figure 6.2. Among these, two land mark protein spots (DppA and GroEL, indicated by the orange arrows in Figure 6.1) from each condition were chosen to show the reproducibility of the 2DE method. Since growth on glucose was a control condition, 5 spots, including the two landmark protein spots, were chosen for

analysis from the representative gel. In order to maximise the spots picked for analysis from succinate and propionate, only three other spots (apart from the landmark proteins), from various parts of the glucose gel were analysed. Four protein spots with M_w and pI values similar to Sbm were analysed from succinate grown cells while no such clear spots could be found in the same region of the gel with sample from propionate grown cells. The remaining spots that were analysed were picked from different parts of the gels, as shown in Figure 6.1, in an attempt to obtain an overview of proteins expressed when *E. coli* is grown in the presence of succinate or propionate. The identified proteins, the genes that code for them and the corresponding gene 'b' numbers (locus tags) are listed in Table 6.1.

Protein as identified by mass spectrometry (MS)	**Gene**	**'b' number**	**Function**	**Sequence coverage (%)**	**Mascot score**	**Carbon source**
Chaperonin GroEL	*gro*EL	b4143	Factor; chaperones involved in protein folding	29	718	glucose
PEP carboxykinase	*pck*	b3403	Central intermediary metabolism	27	624	glucose
ABC dipeptide transport protein	*dpp*A	b3544	Transport; Protein, peptide secretion	9	169	glucose
Protein chain elongation factor EF-Ts	*Tsf*	b0170	Factor; protein translation and modification	34	498	glucose
Aspartate aminotransferase	*asp*C	b0928	Amino acid biosynthesis:aspartate	24	305	glucose
Chaperonin GroEL	*gro*EL	b4143	Factor; chaperones involved in protein folding	26	614	succinate
2':3'-cyclic-nucleotide 2'-phosphodiesterase	*cpd*B	b4213	Salvage of nucleotides and nucleosides	2	24	succinate
ABC dipeptide transport protein	*dpp*A	b3544	Transport; protein or peptide secretion	24	381	succinate
Aspartate aminotransferase	*asp*C	b0928	Amino acid biosynthesis:aspartate	12	143	succinate

Protein as identified by mass spectrometry (MS)	Gene	'b' number	Function	Sequence coverage (%)	Mascot score	Carbon source
Glyceraldehyde-3-phosphate dehydrogenase	*gap*A	b1779	Energy metabolism (glycolysis)	23	301	succinate
Glutamate and aspartate transporter subunit	*glt*I	b0655	Putative transport, componenet of ABC superfamily	36	492	succinate
Glutamate and aspartate transporter subunit	*glt*I	b0655	Putative transport, componenet of ABC superfamily	37	629	propionate
Chaperonin GroEL	*gro*EL	b4143	Factor; chaperones involved in protein folding	26	422	propionate
ABC dipeptide transport protein	*dpp*A	b3544	Transport; protein, peptide secretion	19	474	propionate
Glyceraldehyde-3-phosphate dehydrogenase	*gap*A	b1779	Energy metabolism (glycolysis)	22	300	propionate
Phosphoglycerate mutase	*gpm*M	b3612	Putative enzyme; carbohydrate catabolism	36	492	propionate
Histidine-binding periplasmic protein	*his*J	b2309	Hitidine transport	54	677	propionate
Phosphoglycerate mutase	*gpm*M	b3612	Putative enzyme; carbohydrate catabolism	50	866	propionate
Dihydrolipoamide acetyltransferase	*ace*F	b0115	Energy metabolism	16	431	succinate
NAD linked succinate semi-aldehyde dehydrogenase	*gab*D	b2661	Central intermediary metabolism	34	756	succinate

Table 6.1: Proteins identified in this study, their function and the corresponding genes that code for them. The sequence coverage information and the MASCOT scores have also been provided. The proteins and the growth conditions in which they were expressed are as shown in Figure 6.2. Mascot is a peptide identification search engine which uses a probability-based scoring algorithm to analyse mass spectrometry data against primary sequence databases.

Of the 26 protein spots that were picked for identification 20 spots returned a positive match with known proteins and good confidence interval (CI) scores (95-100%). The remaining 6 protein spots were identified as hypothetical proteins with poor CI scores (zero) and hence have not been reported here. The proteins identified in the current study had a sequence coverage (fraction of the complete protein sequence analysed) that ranged from 2-54% and these identified proteins represent a small fraction of the overall protein spots that were observed. Among these, proteins responsible for the transport (DppA, HisJ, GltI) of peptides and amino acids and proteins that function in energy metabolism (AceF, GabD, GapA) including GpmM involved in carbohydrate catabolism were identified (Table 6.1). In addition, the elongation factor, Tsf, involved in protein translation and the chaperonin, GroEL, (generated under stress conditions) that prevents protein misfolding and promotes the refolding and proper assembly of unfolded polypeptides were also identified (Han & Lee, 2006). The dipeptide transport protein, DppA (Figure 6.3b) and the GroEL chaperonin protein (Figure 6.3a) were identified in all the three growth conditions. GapA (glyceralsehyde-3-phosphate dehydrogenase) was common to gels with samples of *E. coli* whole cell extracts grown with succinate and propionate as the sole carbon source. AspC, aspartate aminotransferase (amino acid biosynthesis) and Tsf were found to be the common proteins found in *E. coli* whole cell extracts grown with glucose and succinate as the sole carbon source (Figure 6.2).

The ‘mountain’ plots for three of the proteins that showed differential levels when the *E. coli* was grown in glucose, succinate or propionate are shown in Figures 6.3 a-c as a representation of the results obtained in the analysis.

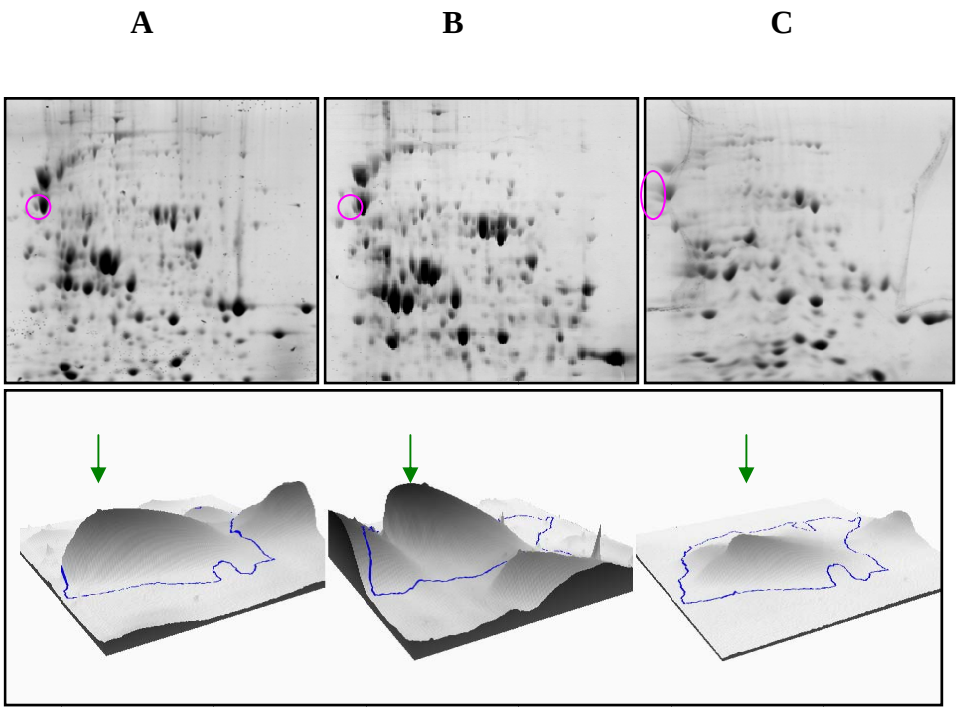

Figure 6.3a: The 'mountain plot' generated by Progenesis. Spots corresponding to the chaperonin (GroEL) protein observed from samples from *E. coli* TR6524 cells cultured in minimal media with A. glucose, B. succinate or C. propionate as the sole source of carbon. The protein spots represented by the 3D plots are highlighted in the 2DE gel image.

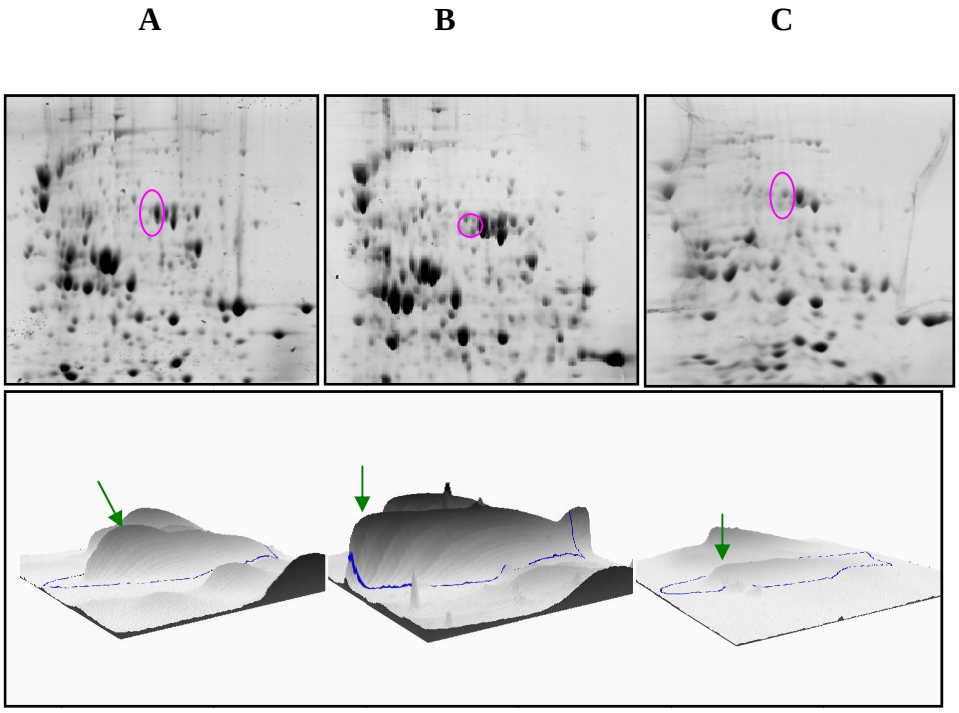

Figure 6.3b: The 'mountain plot' generated by Progenesis. Spots corresponding to the ABC transporter (*dpp*A) protein observed from samples from *E. coli* TR6524 cells cultured in minimal media with A. glucose, B. succinate or C. propionate as the sole source of carbon. The protein spots represented by the 3D plots are highlighted in the 2DE gel image.

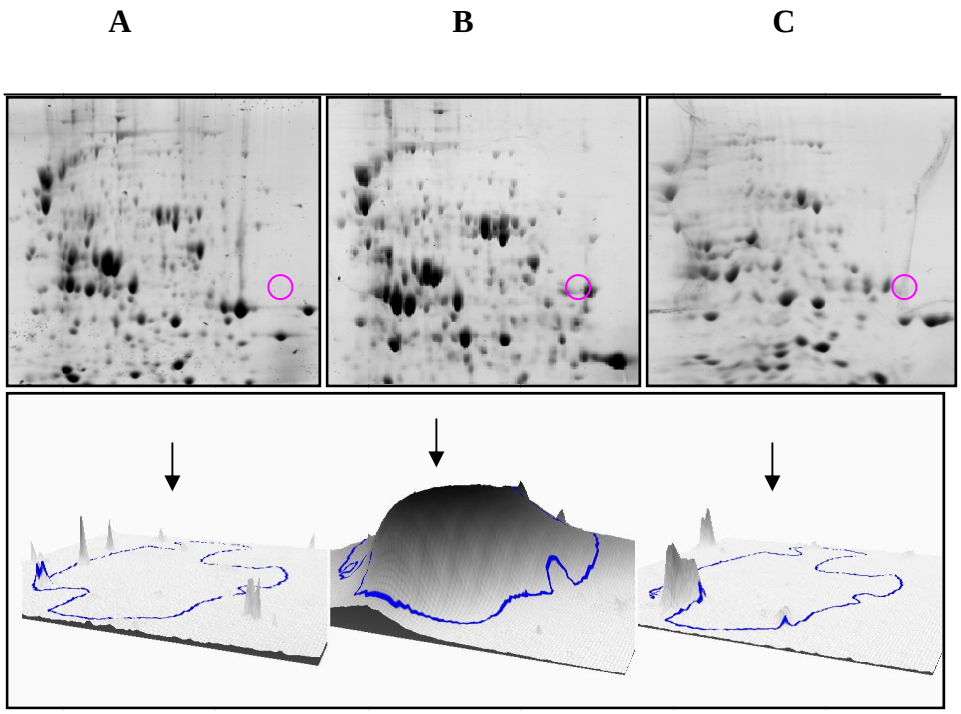

Figure 6.3c: The 'mountain plot' generated by Progenesis. Spots corresponding to the GltI protein observed from samples from *E. coli* TR6524 cells cultured in minimal media with A. glucose, B. succinate or C. propionate as the sole source of carbon. The protein spots represented by the 3D plots are highlighted in the 2DE gel image. While the protein spot is clearly visible in gel B, no such spot can be seen in the corresponding area in gels A and C.

Figures 6.3a and 6.3b show the 'mountain' plots for GroEL chaperonin and DppA proteins induced in all three test conditions; however, the intensity of the peaks generated in each case is different. The peak intensities observed in the images, corresponding to the two proteins, from TR6524 cells grown in glucose and succinate are higher to that presented from growth in propionate. Figure 6.3c compares the 'mountain' plots of a protein thought to be induced only during growth in succinate. The GltI protein, the glutamate and aspartate transporter subunit, was seen to have a very clear peak in gel with samples from succinate grown cells while no corresponding peaks were observed in the 2DE maps of samples from other growth conditions. Although the 'mountain' plots are not shown here, CpdB (2':3'-cyclic nucleotide 2'-phosphodiesterase that catalyses two consecutive reactions converting 2-, 3- cyclic nucleotide to 3-nucleotide and then 3-nucleotide to nucleic

acid and phosphate) is another protein that was found only in the proteome of samples from *E. coli* TR6524 growth on succinate. AceF and GabD (Table 6.1) were also observed in the samples from succinate grown cells. CpdB, AceF and GabD were among the protein spots that had pI values and M_ws similar to that of Sbm. AspC was observed in whole cell extracts grown in glucose and succinate as shown in Figure 6.2. GpmM (involved in carbohydrate catabolism) was found in cells grown in minimal media with propionate as the sole source of carbon.

6.3 Discussion

All organisms respond to environmental variations such as nutrient availability, stress or oxygen limitation. Physiological changes are accompanied by corresponding alterations in gene and protein expression and a better understanding of these changes in cells is of fundamental importance.

This proteomic study has been carried out with wild type *E. coli* TR6524 cells to understand the protein expression pattern when the strain is grown in minimal media with succinate or propionate as the sole source of carbon. A colour coded summary of the proteins identified in this study is shown in Figure 6.2.

Figure 6.4 shows the proteins that were expressed under the specific growth conditions. Growth of *E. coli* in the minimal media with glucose, succinate or propionate obviously involves the induction of a multitude of pathways and this preliminary proteomic study has been able to identify a few important proteins that are an integral part to these pathways.

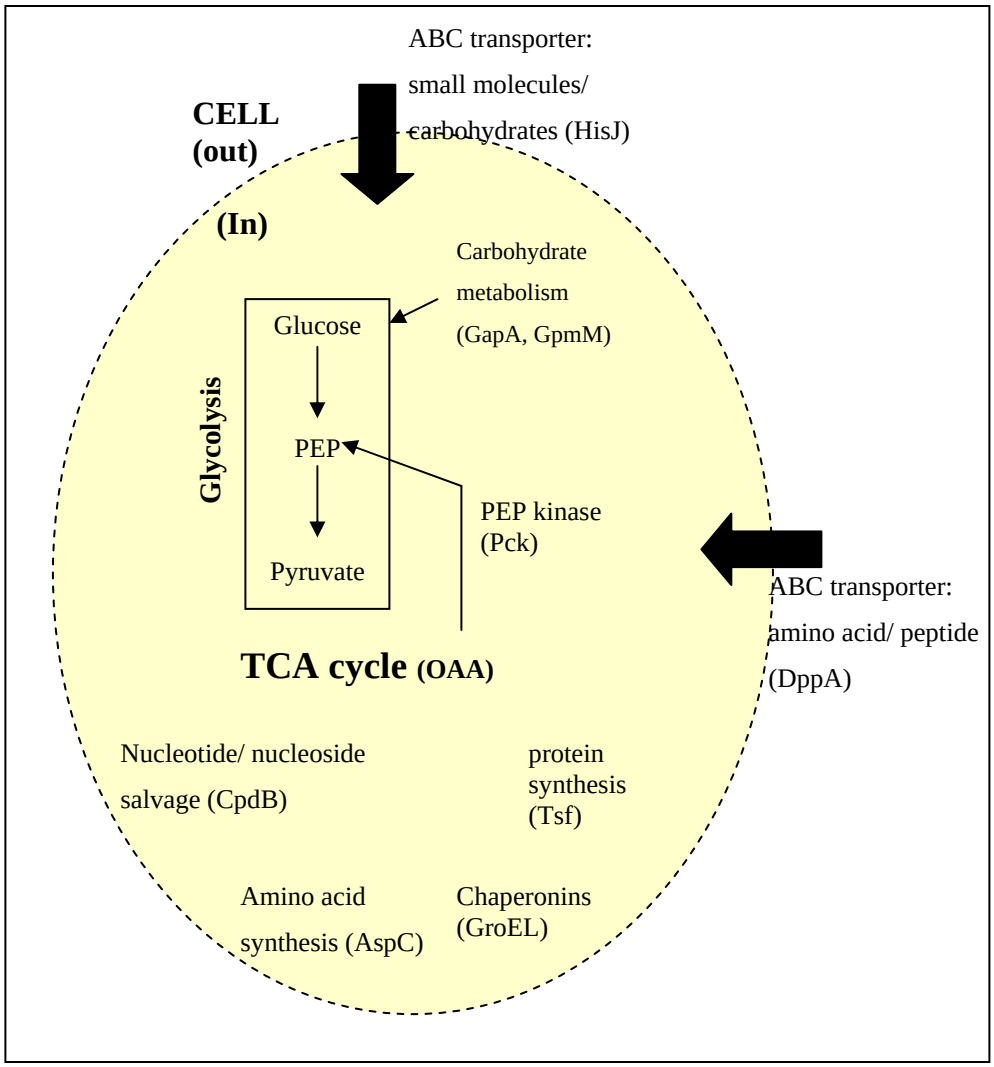

Figure 6.4: A schematic representation of *E. coli* cell showing the proteins identified in the present work and their functions.

The ABC dipeptide transporter protein (DppA) is a dipeptide-binding protein of a transport system that is required for peptide chemotaxis and increases in response to the physiological short-term adaptation to glucose limitation. DppA is a periplasmic binding protein of the ABC transporters involved in the carbohydrate and amino acid uptake that is important during glucose limitation (Han & Lee, 2006). The chaperonin protein (GroEL) occurs in all growth conditions along with DppA. The

'mountain' plots for both these proteins (Figures 6.3a and 6.3b) showed higher peak intensity in succinate and glucose grown cells than that observed in propionate grown cells. The higher peak intensity (an indicator of the amount of the protein produced) for DppA in gels representing growth on succinate suggests the need for enhanced transport of nutrients to facilitate the growth of *E. coli* TR6524 in the presence of difficult carbon sources (in minimal media with succinate and also be the case for propionate). *E. coli* is known to release cell wall murein peptides into the culture medium during exponential growth (Goodell and Schwartz, 1985). Also, it is known that cells at the stationary phase degrade membrane phospholipids, transport and utilise the derived fatty acids as a source of nutrition and energy (DiRusso and Nystrom, 1998). Thus, the up-regulated peptide transport protein, DppA, may play a role in recycling these excreted peptides, and possibly in utilisation of peptides from lysed cells, under the nutrient limited conditions experienced in exponential conditions.

The GroEL chaperonin protein is involved in protein folding and post translational modifications and is thus required for the synthesis of complete functional proteins. The presence of this protein in all three growth conditions (minimal media with glucose, succinate or propionate) shows the general relevance of this protein under any condition of growth. Chaperones such as GroEL occur at different locations on 2DE gels indicating multiple forms of their existence within the *E. coli* cell (Han & Lee, 2006). GroEL expression on the gel with samples from propionate could correspond to a form of the protein that is less abundant compared to those observed in succinate or glucose. Studies concerned with the 2DE protein map of *E. coli* under glucose excess (Wick *et al*., 2001) and glucose limited (Ferenci, 1999; Peng and Shimizu, 2003 & Raman *et al*., 2005) conditions and the *E. coli* proteome under these conditions have been extensively analysed. Reports have also been published on fed batch and continuous cultures (Raman *et al*., 2005 & Franchini *et al*., 2006) of *E. coli* grown in the presence of glucose. Silva and co-workers (2006) reported a lower growth rate when *E. coli* was grown on acetate and correlated this slow growth to a lower rate of protein synthesis and decreased levels of ribosomal

proteins and associated chaperones. Among many other proteins, GroEL (encoded by *gro*L) was observed to be up-regulated in glucose. The authors concluded that the increased protein production was due to higher growth rate supported by glucose providing the need for these chaperones to facilitate protein folding and post-translational modification. Growth on acetate, unlike glucose, required the cell to redirect the carbon flux through different metabolic pathways to sustain growth (Oh *et al*., 2002 & Silva *et al*., 2006). This observation is consistent with that of ours (Figure 6.3a) where the mountain plots reveal higher peaks for GroEL in succinate and glucose and a relatively low peak in propionate. Howe and Hershey (1983) showed that initiation factors, elongation factors, and ribosomes are regulated coordinately with growth rate of cells, using various media (Howe and Hershey, 1983) which correlates well with the findings of the present study.

CpdB, as mentioned earlier, is responsible for catalysing the conversion of 2,3-cyclic nucleotide to nucleic acid and phosphate *via* 3-nucleotide (Han & Lee, 2006) thus being a part of the nucleotide and nucleoside salvage pathways and plays a role in the recovery of these compounds to compensate for the nutrient limiting conditions in the stationary phase.

Two protein spots from growth on succinate were identified as the glutamate/ aspartate transporter subunit (GltI). GltI is a transporter protein that is involved in the transport of glutamate and aspartate under nutrient limiting conditions. This indicates that in the stationary phase the cells are recruiting other carbon sources such as peptides and amino acids for their survival.

Two spots from growth on propionate were identified as phosphoglycerate mutase (GpmM). Phosphoglycerate mutase is an enzyme involved in glycolysis and hence in energy production. The identification of the same protein as two different spots is not uncommon. A similar observation reported by Lee and Lee (2003) showed seventeen *E. coli* proteins including Tsf, DnaK and GapA to appear as multiple spots

in the 2DE system. They attributed the finding to isoforms or post translational modifications in the case of DnaK and protein degradation in the case of Tsf.

The physiological adaptation of *E. coli* during carbon limited growth is characterised primarily by an over expression of high-affinity transporter genes. Generally, an increased expression of alternative carbon/ energy and nitrogen source transport systems and increased expression of components of stress response regulons are observed under such conditions of growth. Studies of glucose limitation (which is analogous to conditions with only succinate or propionate as carbon sources) with *E. coli* also showed that TCA cycle enzymes were repressed, while enzymes involved in acetate and formate production and the Embden-Meyerhof-Parnas pathways were induced. More recent studies have indicated that, in addition to the central metabolism proteins, periplasmic binding proteins involved in uptake of carbohydrates and amino acids are also upregulated with glucose limitation (Wick *et al*., 2001). Among them many genes encoding substrate-binding proteins and transport proteins for sugars were detected at increased expression levels. Amino acid transporters such as those for histidine (HisP), glutamate/aspartate (GltL) were also highly upregulated. Wick and co-workers (2001) and Fischer and Sauer (2003), from their proteome and carbon flux study, respectively, indicated the presence of the PEP glyoxylate cycle that increases growth efficiency under glucose-limited conditions (Wick *et al*., 2001 & Fischer and Sauer, 2003). The PEP-glyoxylate cycle, which is a part of the anapleurotic shunt, is known to work only in the presence of acetate or fatty acids (succinate or propionate in this study) and confer more metabolic flexibility to the cells by synthesing or upregulating the necessary proteins thus allowing the cells to cope with the growth conditions (Cronan and Laporte, 1996; Raman *et al*., 2005 & Franchini *et al*., 2006). The induction of HisJ and GltI (Table 6.1 and Figure 6.4) involved in transport and uptake of amino acids and identification of proteins involved in carbohydrate metabolism (such as *gap*A and *pck*) in this study adds to and confirms these findings.

Succinate semialdehyde dehydrogenase, encoded by *gab*D located at 60.14 min on the *E. coli* chromosome is a part of the *gab*DTPC operon involved in the catabolism

of gamma aminobutyric acid (GABA) and arginine (Bartsch *et al*., 1990 & Fuhrer *et al*., 2007). AceF (dihydrolipoamide acetyltransferase) is a part of the multienzyme complex involved in the conversion of pyruvate to acetyl CoA and carbon dioxide following glycolysis and is reported to increase during aerobic growth (Han & Lee, 2006). The increased incidence of amino acid transport proteins (DppA) and proteins involved in amino acid metabolism (*gab*D) shows the interaction between multiple pathways to help *E. coli* cope with difficult growth conditions.

In conclusion, this proteomic approach was adopted to understand the global protein expression pattern when wild type *E. coli* is grown in minimal media with succinate or propionate as the sole source of carbon. As observed these growth conditions have induced the expression of transporter proteins and energy generating proteins (Table 6.1) required by *E. coli* to cope with the growth conditions. Also, interestingly there is a noted rise in the expression of amino acid transporters specifically and the *sbm* operon comprises a membrane ATPase protein (ArgK) required for the active incorporation of arginine, ornithine, and lysine into cells. Although a transporter, *arg*K, is not an ABC transporter possibly owing to the restrictions imposed by its ATPase nature (Celis *et al*., 1998). Having a transport protein within the operon leads to the possible speculation that Sbm could be part of a pathway that gets switched on under stressful growth conditions allowing lysine and arginine to be transported within the cell to support the growth of the bacteria. Sbm possibly allows metabolism of propionate or succinate as examples of stressful growth conditions. Hence, this operon might be a stress induced operon with multiple roles. Previously, we have also discussed the possibility of ArgK being a chaperone for Sbm (Chapter 4).

However, the lack of detection of Sbm under the current test conditions could be attributed to the facts, that only a few spots were analysed and the low sensitivity of gel based assays with coomassie blue based detection methods. In addition, 2D PAGE has been reported to be quite insensitive to proteins that are not soluble during the isoelectric focusing stage of the separation (Silva *et al*., 2006). The Sbm

protein might have been produed in very low amounts and hence, was not detected under the experimental conditions that were used. Examination of codon bias values (which is a measure of abundance of proteins) detected in 2D gels, has shown that Sbm is a low abundance protein. Hence, pre-gel enrichment (Gygi *et al*., 2000) would possibly be required in order to detect Sbm.

Although the results presented here are preliminary and in their early stages, it is important to note that the data obtained from the protein spot analysis fit in quite well with previous research. The information obtained provides a better understanding of how the *E. coli* is able to adapt its metabolism to environmental alterations. These results are encouraging and with the appropriate experimental design and large-scale analysis offered by proteomics this could provide the basis for further investigations into the complex biological networks that are altered when an organism adapts to changes in environmental conditions.

Chapter 7

Cloning of methylmalonyl-CoA mutase in *B. cereus* SPV- a step towards metabolic engineering

7.1 Introduction

The composition of polyhydroxyalkanoates (PHAs) is governed by the bacterial strain used and the carbon source utilised to grow the bacteria (Valappil *et al*., 2007a & Valappil *et al*., 2007b). Currently, Gram-negative bacteria such as *Cupriavidus* (previously *Wautersia*) *necator, Methylobacterium organophillum, Pseudomonas oleovorans* and recombinant *E. coli* are employed in the production of PHAs on an industrial scale. However, PHAs isolated from Gram-negative organisms contain the pyrogenic outer membrane lipopolysaccharide (LPS) endotoxins, which are known to co-purify with the PHAs. The presence of LPS induces a strong immunogenic reaction and is therefore undesirable for the biomedical application of the PHAs (Chen and Wu, 2005). The absence of LPS in Gram-positive bacteria has made them potentially a better source of PHAs that can be used for biomedical applications (Lee *et al*., 1999).

In addition to being Gram positive, the genus *Bacillus* has been recognised as a potential host for gene expression due to the availability of a great deal of information on its transcriptional and translational regulation. Moreover, members of the genus can be easily grown to very high cell density using inexpensive carbon and nitrogen sources. These factors have made the idea of metabolic engineering of PHA biosynthetic pathways in *Bacillus* an attractive proposition (Law *et al*., 2003 & Valappil *et al*., 2007a). Hori (2002) described the construction of a self-disruptive *B. megaterium* strain that lyses as a response to substrate exhaustion during P(3HB) production (Hori *et al*., 2002) and similarly Law (2003) and Wang (2006) used recombinant *B. subtilis* to clone and express *pha* genes from *B. megaterium* (Law *et al*., 2003) and to produce *mcl*-PHA (Wang *et al*., 2006), respectively. The results from these studies have identified the importance of members of the genus *Bacillus* in PHA production.

This study aimed at the modification of the PHA producing strain *Bacillus cereus* SPV (Valappil *et al*., 2007b) by incorporating the MCM encoding *sbm* gene from *E. coli* K12 into *B. cereus* SPV. This modified *B. cereus* strain would then be able to

use the methylmalonyl-CoA pathway for the production of the commercially important polymer, polyhydroxybutyrate-co-hydroxyvalerate [P(3HB-co-3HV)] as shown in Figure 7.1 from structurally unrelated and relatively cheap carbon source such as glucose, rather than the routinely used propionate.

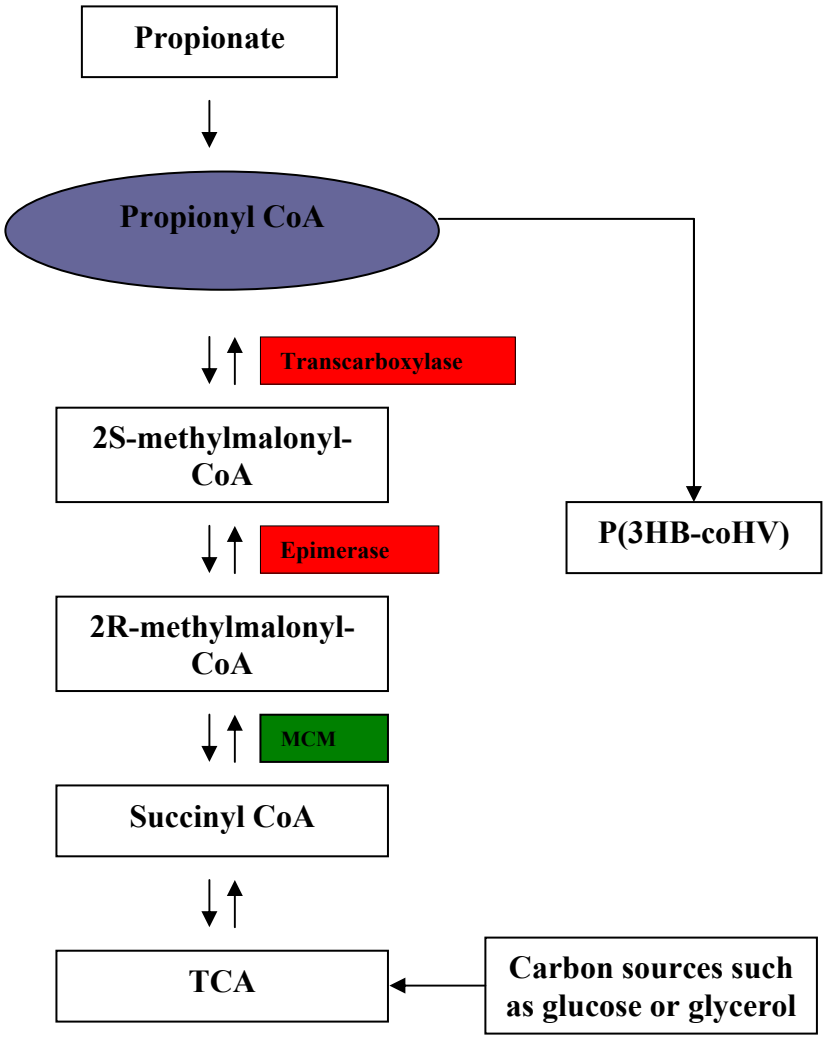

Figure 7.1: PHB-co-HV biosynthesis using MCM pathway. A schematic representation of the reactions that would be involved in P(3HB-co-3HV) production using the MCM pathway. MCM shown in green box shows the incorporation of the *sbm* gene (carried out in the current study) and the transcarboxylase and epimerase shown in the red boxes indicate the identification of the existing sequences within the *B. cereus* genome.

Valappil *et al*. (2007a) indicated the presence of a methylmalonyl-CoA epimerase in *B. cereus* (Genbank accession number AAP08811 assigned to a *B. cereus* 14579)

that catalyses the conversion of the (2R)-methylmalonyl-CoA to the (2S)- methylmalonyl-CoA. A gene encoding a transcarboxylase (Genbank accession number NP_978861 assigned to *B. cereus* ATCC 10987), involved in the decarboxylation of (2S)-methylmalonyl-CoA to propionyl-CoA, was also identified.

This chapter describes the cloning of the *sbm* gene in an attempt towards the metabolic engineering of *B. cereus* SPV in order to study, in the future, the effect of MCM in the accumulation of P(3HB-co-3HV) from non related simple carbon sources such as glucose. However, the uptake of vitamin B_{12} is essential for the functioning of coenzyme B_{12}-dependent MCM, since *B. cereus* SPV is not known to synthesise endogenous vitamin B_{12}. Thus the *B. cereus* genome was also searched for genes which would allow vitamin B_{12} uptake. A putative cob(I)alamin adenosyl transferase (Genbank accession number NP_977924) was identified in the in *B. cereus* 10987 sequence with a 64% sequence identity to a hypothetical protein, encoded by the gene *yvq*K, considered to catalyse coenzyme transport and processing in *B. subtilis*.

Thus it was thought that the cloning of the sbm gene would allow *B. cereus* SPV to produce P(3HB-co-3HV) using the pathway shown in Figure 7.1.

7.2 Results

7.2.1 Cloning of the *sbm* gene into pHCHM05, a *Bacillus* expression vector

The first step in the process was the cloning of the *sbm* gene into a vector compatible with *B. cereus* SPV. This involved the isolation of pEX3, a construct containing the *sbm* gene (Figure 7.2a) cloned into pT7-18 constructed previously (Dr. Ipsita Roy, unpublished data). This was followed by the PCR amplification of the *sbm* gene (Figure 7.2b) using suitably designed primers (Table 9.1) with recognition sites for the restriction endonucleases *Xba*1 and *Xma*1 required for cloning into pHCHM05 (Figure 9.1).

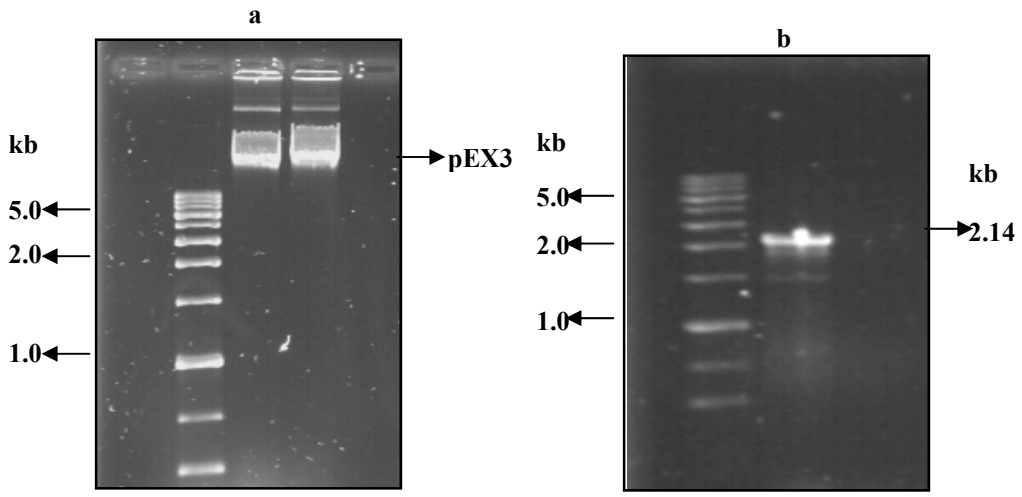

Figure 7.2: Plasmid preparation and PCR. Agarose gel run with a 1 kb step ladder showing (a) pEX3 plasmid and (b) PCR amplified *sbm* gene.

The gel purified PCR product and the *E. coli*- Gram positive shuttle vector, pHCMC05, were then restricted and ligated leading to the production of the construct pHCMC05*sbm*. The steps involved in the cloning of the *sbm* gene into pHCMC05 are represented in Figure 7.3.

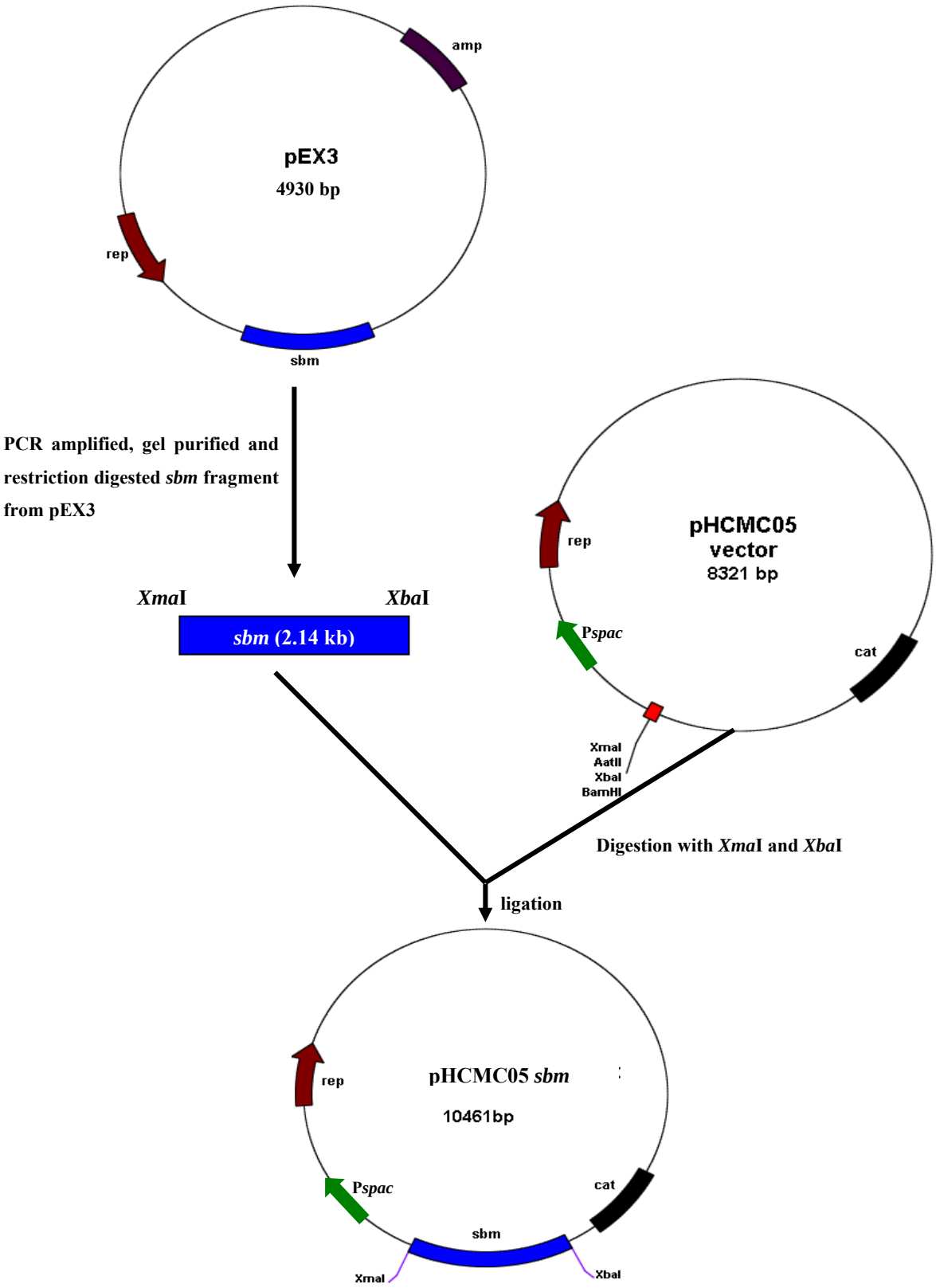

Figure 7.3: Construction of pHCMC05*sbm*. Representation of the steps involved in the construction of the pHCMC05*sbm* construct ('cat' and 'amp' refer to the chloramphenicol and ampicillin resistance genes, respectively, P*spac* refers to the promoter and 'rep' denotes the origin of replication).

The ligation mixture was then used to transform *E. coli* DH5α and *B. subtilis* 1604 strains as described in the methods section (Xue *et al*., 1999). This confirmed the shuttling ability of the vector and allowed the generation of two bacterial strains with the recombinant construct. *E. coli* DH5α is a commonly used *E. coli* strain for the maintenance of plasmid constructs. The construct in *B. subtilis* would allow the preparation of the plasmid DNA providing the correct conformation for easier transformation into *B. cereus*. The transformation was followed by overnight growth of the recombinant *E. coli* and *B. subtilis* on selective agar media. This was followed by overnight liquid culture of possible clones and plasmid preparation and a restriction enzyme digest to verify the presence of the *sbm* insert. The insert release observed following the restriction digestion of pHCM05*sbm* isolated from *B. subtilis* 1604 is shown in Figure 7.4.

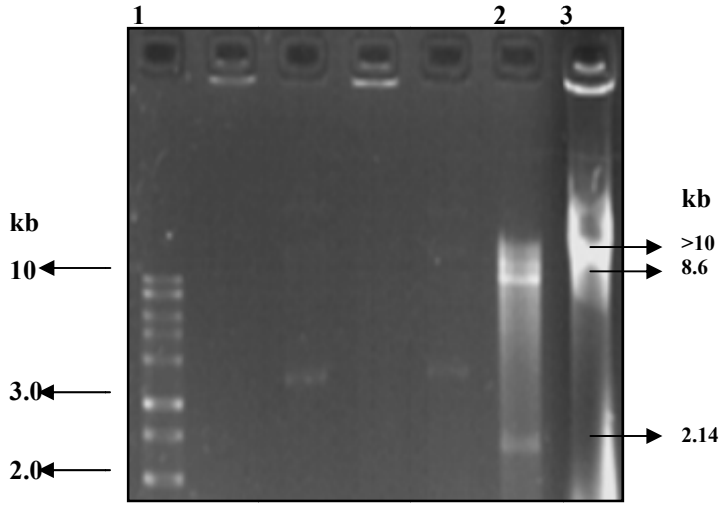

Figure 7.4: Insert release I. Agarose gel showing the restriction digestion of the plasmid isolated from *B. subtilis* 1604 and the release of the *sbm* gene insert from pHCMC05*sbm*. Lane 1- 1kb step ladder, lane 2- cut plasmid DNA isolated from transformed *B. subtilis* 1604 colonies, lane 3- the corresponding uncut plasmid (>10 kb)

As observed in Figure 7.4, the restriction digestion of pHCM05*sbm* isolated from *B. subtilis* 1604 produced 2 bands- one corresponding to the 2.14 kb *sbm* gene and a second band representing the 8.6 kb pHCMC05 *E. coli*-Gram positive shuttle vector. This confirmed the incorporation of the *sbm* gene into the shuttle vector and the

successful transformation of *B. subtilis* 1604. A similar result (Figure 7.5) was observed when the insert release step was repeated with the plasmid isolated from the *E. coli* colonies.

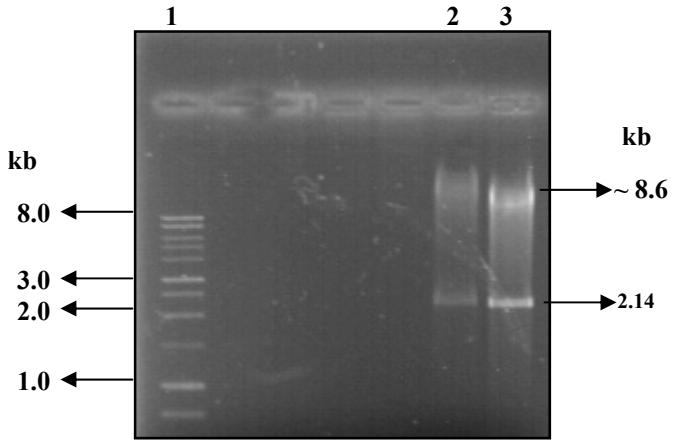

Figure 7.5: Insert release II. Agarose gel showing the restriction digestion of the plasmid isolated from *E. coli* DH5α and the release of the *sbm* gene insert from pHCMC05*sbm*. Lane 1- 1kb ladder, lanes 2 and 3- pHCMCO5*sbm* isolated from 2 different *E. coli* DH5α colonies picked and cut using *Xma*I and *Xba*I

The pHCM05*sbm* construct isolated from *B. subtilis* was used for the transformation of *B. cereus* SPV.

7.2.2 Transformation of *B. cereus* SPV

The electroporation method (Xue *et al*., 1999) used for the transformation of *B. subtilis* 1604 was found to be unsuitable for the transformation of *B. cereus* SPV and protocols that were specifically developed for the transformation of *B. cereus* using electroporation ((Belliveau and Trevors, 1999 & Turgeon *et al*., 2006) were tried unsuccessfully. The challenge of transforming *B. cereus* SPV with the pHCM05*sbm* construct was finally overcome with a protoplast preparation and a polyethylene glycol (PEG) induced transformation protocol developed for *Bacillus licheniformis* (Pragai *et al*., 1994).

Growth of colonies of possible recombinant strains of *B. cereus* SPV harbouring the pHCMC05*sbm* construct were observed on ART media plates (Pragai *et al*., 1994) two days after the transformation. Unlike the results observed for the insert release for *B. subtilis* and *E. coli* DH5α (Figures 7.4a and 7.4b), the restriction of the pHCMC05*sbm* isolated from *B. cereus* SPV did not result in the release of the *sbm* insert. This was possibly due to the inability of the restriction endonucleases to digest the DNA as *B. cereus* is known to protect its DNA by methylation. Site-specific DNA methylation interferes with sequence specific DNA binding proteins such as restriction endonucleases (Nelson and McClelland, 1991 & Roberts *et al*., 2007). *B. subtilis* 1604 used previously in this study, on the other hand, is not known to have any modification on its DNA (Dr. Mark Clements, personal communication) thus allowing the restriction digest to proceed as normal.

Finally, a PCR protocol, where the plasmid DNA isolated from the regenerated *Bacillus cereus* SPV protoplasts was used as the template to amplify the *sbm* gene using specific primers. The results are shown in Figure 7.6.

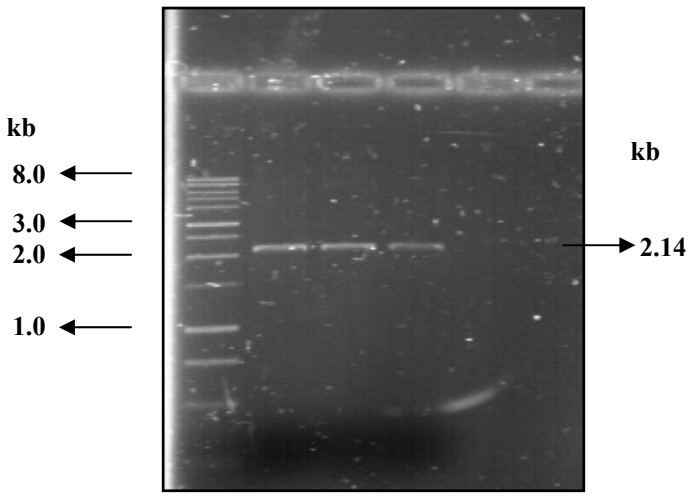

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Figure 7.6: PCR using plasmid DNA from regenerated *B. cereus* SPV protoplasts. Agarose gel showing the PCR product corresponding to the *sbm* gene obtained using specific primers specific to the pHCM05*sbm* construct.

As observed in Figure 7.6, the PCR method yielded a positive result confirming the successful transformation of pHCM05*sbm* into *B. cereus* SPV. The amplification produced bands of the appropriate size (2.14 kb) when plasmid DNA isolated from 3 different protoplast colonies were used as templates in the PCR.

7.3 Discussion

P(3HB-*co*-3HV) is a biodegradable PHA thermoplastic that has more flexibility and reduced strength making it easier to process this co-polymer than P(3HB). Production of P(3HB-*co*-3HV) has been dependent on the use of many exogenous and endogenous propionigenic substrates such as propionic acid which has been the most widely used 'precursor substrate' for P(3HB-*co*-3HV) biosynthesis. P(3HB) homopolymers can be produced from a single carbon substrate such as glucose, however, a co-substrate such as propionate or valerate (precursors of 3HV) is required for the production of copolymers such as P(3HB-co-3HV).

Host cell genome manipulation has been demonstrated to eliminate competing pathways or modify native regulation for improved function of desirable pathways (Aldor and Keasling, 2003). The significant achievements and breakthroughs made for novel processes for biotechnological production of PHAs have shown that any production system will most probably rely on genetically engineered organisms and bacteria have been the best and almost only source for PHAs owing to their high density cultivation and relatively easier recovery of the resulting polymer. An efficient production of P(3HB-co-3HV) by fed-batch culture of a recombinant *E. coli* XL1-Blue harbouring a plasmid containing the *Alcaligenes latus* PHA biosynthesis genes, in minimal media using glucose and propionic acid as co-substrates was reported recently (Choi and Lee, 2000). However, propionic acid is more expensive than simple carbon sources such as glucose and thus significantly affects the cost of production. In addition, propionic acid is also a highly toxic compound, making the growth of the bacteria quite difficult (Steinbuchel & Lutke-Eversloh, 2003).

The alternative solution to reduce this expense would be to use organisms that are able to use structurally unrelated carbon sources. The only wild-type bacteria, which naturally synthesise P(3HB-co-3HV) with very high contents of 3HV (>75mol%) from unrelated carbon sources like glucose are species belonging to the genus *Nocardia* or *Rhodococcus.* These bacteria degrade glucose to pyruvate which is then carboxylated to oxaloacetate and subsequently converted to succinyl-CoA by reverse reactions of the TCA cycle. Succinyl-CoA is then converted to methylmalonyl-CoA using methylmalonyl-CoA mutase, which is then decarboxylated to propionyl-CoA. Thus there is endogenous generation of the propionyl-CoA from simple carbon sources (Steinbuchel & Lutke-Eversloh, 2003). This propionyl-CoA is then used as a precursor for the 3HV monomer in P(3HB-co-3HV) biosynthesis. The pathway described above is schematically represented in Figure 7.1. However, large scale production of the copolymer using these natural producers has been difficult owing to the growth and recovery processes (Steinbuchel & Lutke-Eversloh, 2003).

The other approach to the economical production of P(3HB-co-3HV) is the use of metabolic engineering. For example, the introduction of the methylmalonyl-CoA pathway into other organisms can lead to the introduction of an endogenous source of propionyl-CoA. In this work, cloning of the *sbm* gene into *B. cereus* is the first step towards such metabolic engineering. A similar strategy was involved in a study describing the construction of a recombinant *Salmonella enterica* Serovar *typhimurium* for the production of P(3HB-co-3HV). The genes for (2R)-methylmalonyl-CoA mutase (*sbm*) and a (2R)-methylmalonyl-CoA decarboxylase (*ygfG*) from *E. coli* were cloned into *S. enterica* Serovar *typhimurium*, thus enabling the conversion of succinyl-CoA to propionyl-CoA. In addition, the PHA biosynthesis operon of *Actinobacter* was inserted into the gene encoding 2-methylcitrate synthase (involved in propionate uptake) of this bacterium, thereby establishing PHA biosynthesis *via* the MCM pathway and disrupting propionate utilisation. This recombinant *Salmonella* sp. synthesised P(3HB-*co*-3HV) with up to 31 mol% 3HV *via* succinyl-CoA when cultivated on glycerol (unrelated carbon source) as shown in Figure 1.18 (Aldor *et al*., 2002 & Aldor *et al*., 2003).

Poly(3HB-*co*-4HB) is another useful PHA copolymer used in drug delivery, tissue scaffolds and composite development among other uses. In another metabolic engineering approach, succinyl-CoA, derived from the breakdown of glucose (*via* the TCA cycle), was converted to 4-hydroxybutyrate for Poly(3HB-*co*-4HB) production in recombinant *E. coli* harbouring the *C. necator* PHA operon and the *Clostridium kluyveri* succinate degradation genes thus circumventing the need for the addition of immediate precursors such as 4-hydroxybutyrate, 1, 4- butanediol or gamma butyrolactone for the copolymer synthesis (Valentin and Dennis, 1997). This is illustrated using a schematic as shown in Figure 7.7.

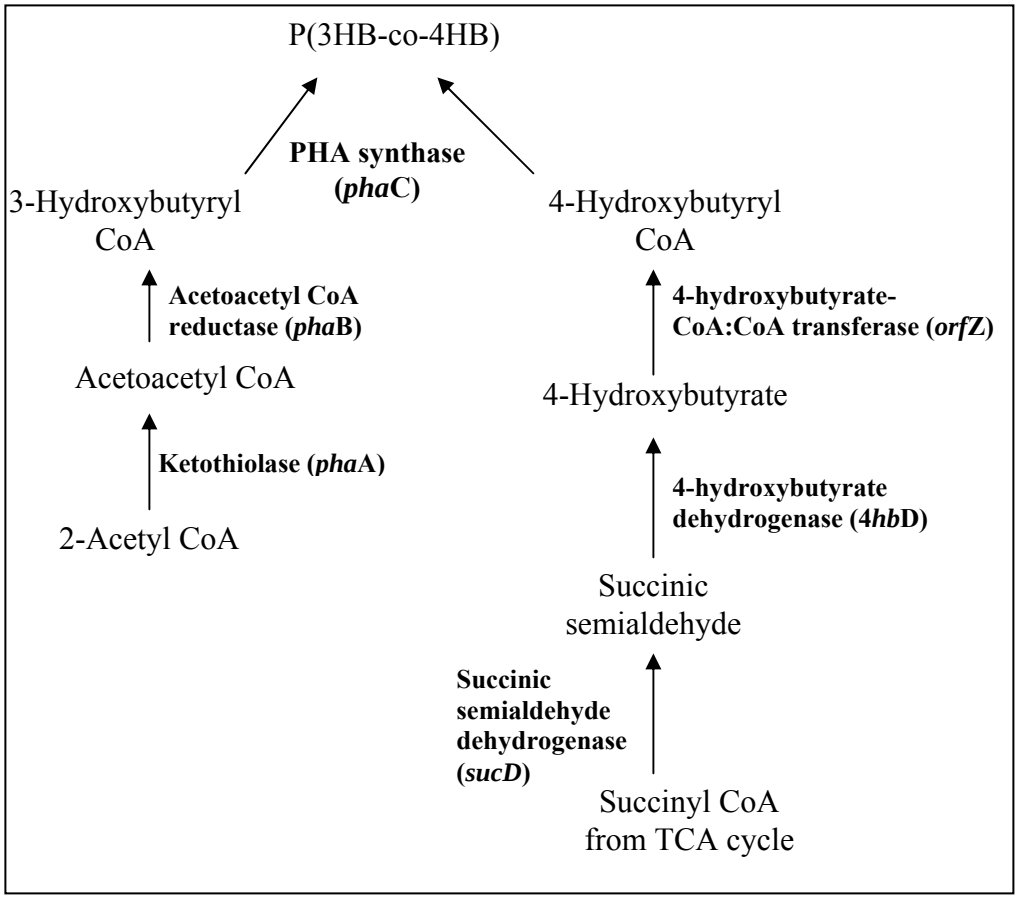

Figure 7.7: Biosynthesis of P(3HB-co-4HB). Pathway for P(3HB-*co*-4HB) synthesis in recombinant *E. coli* harbouring the PHA synthesis operon from *C. necator* and the succinate utilisation pathway from *C. kluyveri* (adapted from Valentin and Dennis, 1997)

In conclusion, the recombinant *B. cereus* SPV that has been developed in the current study should possess all the necessary genes for the accumulation of 3HV containing copolymer, when grown in a simple inexpensive carbon source such as glucose, through the MCM pathway. Unfortunately, due to the time constraints, further work related to the production of P(3HB-co-3HV) using the recombinant *B. cereus* SPV strain and its optimisation could not be undertaken. However, this work marks the beginning of a new approach, using metabolic pathway engineering methods, in the production of the copolymer P(3HB-*co*-3HV) using *B. cereus* SPV. Such novel processes for the production of P(3HB-*co*-3HV) and other useful copolymers using genetically engineered organisms would be an industrially useful step forward.

Chapter 8

Conclusions and future work

8.1 Conclusions

The *sbm* gene encoding methylmalonyl-CoA mutase (MCM) was identified and reported to be located at 62.3min on the *E. coli* chromosome (Roy and Leadlay, 1992). This discovery opened up an interesting area of research involving the new and unexplored metabolic pathway in the organism. This study is based on earlier growth studies of *E. coli* (Kolodziej *et al*., 1968 & Wegener *et al*., 1968) and ^{13}C-NMR analyses (Evans *et al*., 1993; Textor *et al*., 1997 & London *et al*., 1999) of the growth of *E. coli* on fatty acids such as propionate, and the recognition of the *sbm* gene as part of a four gene operon possibly involved in succinate utilisation (Haller *et al*., 2000). A detailed examination of various aspects of the expression of the gene has been investigated in an attempt to elucidate the metabolic role of MCM in *E. coli*. In addition to this basic research, this study has also laid the foundation of utilising the *sbm* gene product in metabolic engineering experiments.

A comparative growth study was carried out using wild type *E. coli* K12, TR6524 and the *sbm* insertion mutant, FA1P1, in minimal media with glucose, succinate or propionate as the sole source of carbon. Growth on glucose showed no particular difference in the growth patterns of the two strains leading to the conclusion that the pathway employing the *sbm* gene product (MCM) was not crucial in the utilisation of glucose. An appreciable difference between the growth of the wild type strain and the mutant strain was observed in minimal media with succinate as the sole carbon source. This showed that the *sbm* pathway had a pivotal role in the metabolism of this dicarboxylic acid, possibly involving the decarboxylation of succinate to propionate as proposed by Haller *et al*., 2000. However, a change in the concentration of vitamin B_{12} (MCM being a coenzyme B_{12} dependent enzyme) did not seem to have an effect on the growth of the wild type strain, as it should have. This was probably because the concentration of vitamin B_{12} being used was saturating at the lowest concentration used. The growth of the wild type *E. coli* and the *sbm* disruption mutant in minimal media with propionate as the sole carbon source was also compared. A minor difference in the growth pattern was observed between the two strains during the mid-log phase, however, growth parameters such

as doubling time and doubling rate were almost identical (Table 2.3). Unlike in succinate, the increase in vitamin B_{12} concentration had a slight positive effect on the growth of *E. coli* TR6524. These results indicate that the mutation in the *sbm* gene in FA1P1 was compensated for, by other pathways (such as the methylcitrate pathway) providing it the ability to cope with propionate as the sole carbon source.

The identification of a σS controlled promoter (50% and 83% sequence identity at the -10 and -35 regions, respectively) 29 bases upstream of the *sbm* operon, indicated that *sbm* was induced under stress conditions, such as growth in minimal media with difficult carbon sources (such as succinate or propionate), nutrient depletion, or stationary phase. A search for consensus sequences with genes encoding other regulatory proteins, such as the FNR and ArcA, led to the identification of sequences with 45% and 80% homology, respectively, about 100-200 bases upstream of *sbm*, suggesting the possibility of the involvement of a network of regulatory proteins in *sbm* gene expression.

Further, an increase in the mRNA levels corresponding to the *sbm, arg*K and *ygf*G genes was observed as growth progressed through the three phases when *E. coli* TR6524 cells were grown in the presence of succinate or propionate as the sole source of carbon as compared to when grown in the presence of glucose. This was in agreement with the finding that the *sbm* operon was under the control of σS, (*rpo*S gene product), responsible for the induction of genes required to cope with survival during stress conditions and stationary phase. In contrast, a relative increase in mRNA levels corresponding to the *ygf*H (which codes for propionyl-CoA: succinyl-CoA transferase) gene was observed in the early log phase of growth. This indicated that this gene product possibly plays a role in the adaptation of *E. coli* for the utilisation of succinate or propionate and is possibly the rate limiting step in the pathway proposed by Haller *et al*., 2000. Hence, the increased level of transcription indicates a major role during the early-log phase of growth of the organism. Amongst the four genes, the change in the mRNA levels corresponding to the *ygf*D (or *arg*K) gene is not as much as seen in the case of the other genes. This is

consistent with the proposed pathway of Haller *et al*., 2000, which did not include the product of this gene (Figure 1.14). The *ygf*D (or *arg*K) gene product is homologous to the MeaB protein found in *Methylobacter extorquens* AM1. It is known to function as a chaperone protein required for the stability of Mea A protein, a known mutase involved in the conversion of butyryl CoA to propionyl CoA. Hence, it can be assumed that *ygf*D, in addition to its known role in lysine-arginine-ornithine transport is also a possible chaperone protein required for the stability of the Sbm protein. This postulate is reinforced by the fact that Sbm protein when expressed on its own in *E. coli*, without a histidine tag, was found to be inactive (Haller *et al*., 2000; Bobik and Rasche, 2003 & I. Roy, PhD thesis). This role as a chaperone does not possibly require a marked variation in the mRNA levels of this gene.

The MCM enzyme activity measurement in the whole cell extracts of the wild type *E. coli* K12 grown under the above mentioned conditions led to the first ever measurement of MCM activity in wild type *E. coli* These measurements also revealed a four fold increase of the MCM specific activity in the case of growth in succinate ($4.76x10^{-3}$U/mg) and a two fold increase for growth in propionate ($2.79x10^{-3}$U/mg) compared to that observed with growth in glucose ($1.37x10^{-3}$U/mg). These results for the first time indicate a strong involvement of the enzyme in succinate utilisation, and to a lesser extent in propionate utilisation, owing to the multiple pathways that could be in operation (Textor *et al*., 1997; London *et al*., 1999). These results also correlate well with the data from the comparative growth study (where a significant difference was observed in the growth pattern of the two strains in succinate and less so in propionate), the promoter analysis (identification of sequence consensus with the stress related, stationary phase induced sigma factor, σS) and the transcription analysis (increased level of mRNA corresponding to *sbm* from cells grown in succinate).

The proteomic analysis to understand the gene expression pattern of *E. coli* TR6524 was carried out using cells harvested at the stationary phase of growth from all the

above mentioned growth conditions (minimal media with glucose, succinate or propionate as the sole sources of carbon). The Sbm protein was not identified among the protein spots analysed in this study. However, the growth conditions led to induction of the expression of transport (HisJ, DppA) and energy generating proteins (PckA, AceF) required by *E. coli* to cope with the difficult growth conditions. Analysis of only a limited number of protein spots due to financial constraints and the low sensitivity of gel based assays with coomassie blue based detection methods could have led to the lack of detection of Sbm. The results corresponded well with previous studies that have reported the identification of proteins involved in coping with difficult carbon sources (long-chain fatty acids) and nutrient depletion (fed batch cultures and glucose limitation) (discussed in detail in Chapter 6) providing a better understanding of the ability of *E. coli* to adapt its metabolism to environmental alterations.

Finally, as a first step in the process to study the effect of MCM on P(3HB-co-3HV) production, the *E. coli* K12 *sbm* gene was successfully cloned into *B. cereus* SPV leading to the development of a metabolically engineered polyhydroxyalkanoate producing strain of *B. cereus*. The introduction of the *sbm* gene into *B. cereus* should result in a natural intracellular source of propionyl-CoA leading to the production of the P(3HB-co-3HV) copolymer from structurally non related carbon sources like glucose. Hence, the recombinant strain should allow production of P(3HB-co-3HV) without the need to supply propionate externally. This would lead to a relatively cheaper production of this industrially useful copolymer.

This study thus showed, for the first time, direct experimental evidence of the role of the *sbm* gene and the other genes in the *sbm* operon, in the utilisation of propionate and succinate in *E. coli* K12. Hence, one of the metabolic roles of methylmalonyl-CoA mutase in *E. coli* is to facilitate the utilisation of unusual carbon sources. This study marks the beginning of the recognition of a new metabolic pathway involving methylmalonyl-CoA mutase in *E. coli*.

8.2 Future work

This study on MCM from *E. coli* has provided an interesting insight into the role of the *sbm* gene containing operon in the metabolic networks involved in *E. coli*. The metabolic role of these new reactions, need further detailed probing in order to obtain a clear idea of the metabolic pathways involved. Some possible experiments to be carried out in the future could include,

8.2.1 Comparative growth analyses

^{13}C-NMR analyses have shown wild type *E. coli* to utilise propionate *via* the methylmalonyl-CoA mutase and the methylcitrate pathway (Evans *et al*., 1993; Textor *et al*., 1997 & London *et al*., 1999). The experimental data from this study has revealed that, in the presence of vitamin B_{12} in the media, propionate is possibly utilised *via* the carboxylation pathway that involves a reaction catalysed by MCM confirming the findings of Evans *et al*., 1993. In order to provide conclusive evidence of propionate utilisation by the MCM pathway, the *prp*C gene (encoding methylcitrate synthase) in the *sbm* mutant FA1P1 and the wild type *E. coli*, could be inactivated. Comparative growth analyses in minimal media with added vitamin B_{12} and propionate as the sole source of carbon could be repeated as described in Chapter 2 of this study. The interpretation of the results would be based on the ability of the *sbm-prp*C mutant (referred to as double mutant) and the *prp*C mutant to grow in minimal media with propionate as the sole source of carbon. If the double mutant is unable to grow or exhibits a significant lag phase during its growth, it can be concluded that both the *prp*C and *sbm* genes are important for propionate metabolism in *E. coli*. Similarly, if the *prp*C mutant is able to grow with a growth pattern analogous to that of the wild type, it can be concluded that the MCM pathway compensates for the loss of the methylcitrate pathway. This can be further confirmed using the ^{13}C NMR analysis similar to that carried out in previous research (London *et al*., 1999) to confirm and provide conclusive evidence for the utilisation of propionate through the MCM pathway.

8.2.2 Growth analyses under anaerobic conditions

Salmon *et al*. (2004) used microarray analysis and indicated that the *E. coli sbm* gene could be under the regulation of the FNR regulon which controls expression of genes under anaerobic conditions (Salmon *et al*., 2004). Also, literature suggests that a single gene can be regulated by a network of regulons (Hengge-Aronis, 2002 & Salmon *et al*., 2004). The promoter search approach used in the present study showed a sequence consensus with that of FNR about 100-200 bases upstream of the *sbm* operon. Hence, it would be interesting to design experiments to culture the bacteria under anaerobic conditions using minimal media with electron acceptors such as fumarate in an anaerobic chamber followed by transcription analyses and proteomics to establish the relationship between the FNR regulon and *sbm*. This would provide a complete picture of the role of *sbm* in *E. coli* metabolism under anaerobic conditions.

8.2.3 Microarray analysis

A microarray analysis (Gygi *et al*., 2003 & Salmon *et al*., 2003) following the growth of *E. coli* on minimal media with succinate or propionate as the sole source of carbon and under both aerobic and anaerobic conditions, would reveal the possible regulatory networks involved in the regulation of the expression of *sbm* and other genes involved in the utilisation of these carbon sources, in addition to the already existing data obtained through such studies using the bacteria. There is a strong possibility that the microarray approach will indicate the *sbm* induction condition(s).

8.2.4 Expression proteomics

The proteomics approach used in this project revealed that growth in minimal media with unusual carbon sources such as succinate or propionate induced transporter and energy generating proteins that helped cope with the growth conditions used. However, only some protein spots could be analysed and in the stationary phase. This could be further extended to protein extract from cells harvested at log phase and an analysis of all the spots that appear on the gels. In addition, protein samples

can be isoelectrically focused in narrow range (*e.g*. 4.0-6.0) IPG strips also known as ultra zoom gels (Hoving *et al*., 2002) targeting protein(s) of interest (here, Sbm) followed by routine MS analysis.

8.2.5 Expression of sbm and P(3HB-co-3HV) production using the recombinant B. cereus SPV strain

Finally, the metabolic engineering of the polyhydroxyalkanoate producing *Bacillus cereus* SPV, which had only been started needs follow through and completion. The appropriate conditions for the over-expression of Sbm have to be determined and its effect verified. This would require comparison of P(3HB-co-3HV) accumulation by the genetically engineered *B. cereus* SPV and wild type *B. cereus* SPV strains grown in polymer production media (Valappil *et al*., 2007b) with the simple, inexpensive and structurally unrelated carbon source such as glucose. NMR analysis of the purified polymer would provide the percentage of the copolymer recovered. Once successful production of P(3HB-*co*-3HV) is achieved, this strain can be used for the large scale production of the copolymer.

Chapter 9

Materials and methods

9.1 Materials

9.1.1 Water

Distilled and HPLC grade water were used where appropriate. The two types of water were obtained from Elga Purelab Options distillation units.

9.1.2 Chemicals

Most of the chemicals used were purchased from Sigma-Aldrich, UK. Those chemicals that were not obtained from Sigma are listed below.

Urea and iodoacetamide used for proteomics studies were of the Plusone grade purchased from GE-Healthcare Ltd, UK. The immobilised pH gradient (IPG) strips used for isoelectric focussing were ordered from Amersham-Pharmacia, UK (now GE-Healthcare Ltd, UK).

The buffers for the HPLC assay were prepared using chemicals purchased from BDH Ltd.

Care was taken to make sure that the chemicals used were of the best grade available (analytical or HPLC where appropriate).

9.1.3 Instruments

Perkin-Elmer Lambda 35 UV/VIS spectrophotometer was used for quantifying DNA and RNA. The Eppendorf gradient thermal cycler was used for conventional polymerase chain reactions. ABI Prism 7000 Real Time thermal cycler was used for the real time PCR and the amplification was carried out in 96 well plates purchased from Applied Biosystems, UK. The bacterial cells were lysed using Status 200, MS73 sonicator probe wherever necessary. Sorvall Discovery 90SE with rotor T-865 was used for protein sample ultracentrifugation for 2DE analysis of samples. Sorvall high speed centrifuge with SL-50T rotor was used for protein sample preparation for the HPLC assay. Dionex Summit HPLC system connected to a Dionex Wavelength Detector (UVD170U) was used to assay methylmalonyl-CoA

mutase activity. The assay was carried out using a 120A C-18 reverse phase column (5µm, 4.6 x 250mm) from Dionex. A Sorvall Legend RT centrifuge with a head for multiple rotors and an Eppendorf micro-centrifuge model 5415D were used for all other sample preparations. A Gene Pulser Xcell electroporator purchased from BioRad was used for electroporation.

9.1.4 Proteins and enzymes

The broad range protein molecular weight marker and ribonuclease A (pancreatic Rnase A) were purchased from Promega, UK. Bovine serum albumin fraction V (BSA) and lysozyme used in Bradford assay and plasmid preparations were from Sigma-Aldrich, UK. Restriction endonucleases- *Xma*I, *Xba*I and T4 DNA ligase- used in bacterial cloning were purchased from New England Biolabs, UK.

9.1.5 Genetic analysis

The kits used for genomic DNA isolation (DNA Wizard), cDNA preparation (ImpromII reverse transcription system) and polymerase chain reaction (PCR master mix) were purchased from Promega, UK. SYBR green jump start kit for real time quantitative PCR was purchased from Sigma, UK. Molecular biology grade reagents were used wherever required. The plasmid purification midi kit was purchased from Qiagen, UK.

The oligonucleotide primers used for PCR were purchased from Life technologies, Invitrogen, UK and are listed as follows

Primer no.	Forward primer sequence and name	Reverse primer sequence and name	Expected product size
1	*iciA2*: **5'**TTTGATCATCTKGATGATATGCA**3'**	*R55*: **5'**CCCGAATAATCCAATGTCCTCC**3'**	1.2 kbp
2	*F33*: **5'**GAAACGCTTTCGCGTTTTTCGTG**3'**	*sbm11*: **5'**GACGCCAACCGTTTCGACAATCA**3'**	2.1 kbp
3	*sbm1*: **5'**ATGTCTAACGTGCAGGAGTGGCAAC**3'**	*sbm11*: **5'**GACGCCAACCGTTTCGACAATCA**3'**	2.87 kbp
4	*sbm*F: **5'**AAAACTGTCGACTCGCTGGT**3'**	*sbm*R: **5'**CCAGGTTACGGCGATAAAAA**3'**	229 bp
5	*his*G1: **5'**ATGACAGACAACACTCGTTTACGCA**3'**	*his*G2: **5'**CAGCGCTTTCAGGTTTCCCAACA**3'**	867 bp
6	*iciA2*: **5'**TTTGATCATCTKGATGATATGCA**3'**	*sbm11*: **5'**GACGCCAACCGTTTCGACAATCA**3'**	3.4 kbp
7	*argK F:* **5'**TTTGACCGCTAAAAGCGTCT**3'**	*argK R:* **5'**GCACTGGAAAAACGTGGAAT**3'**	207 bp
8	*ygfG F:* **5'**CCGGAAATTCGCTGTATCAT**3'**	*ygfG R:* **5'** GGTTTCGGGAATTTTTGGAT**3'**	158 bp
9	*ygfH F:* **5'**CTGCGCAAGATTTACGACAA**3'**	*ygfH R:* **5'**GGGCCATAAAGATCGACAGA**3'**	241 bp
10	*Sbmpla F:* **5'**CGAATTCTTCTAGAATGTCTAACGTGC AGGAGTGG**3'**	*Sbmpla R:* **5'**TTAATCATGATGCTGGCTTATCA CCCGGGCCAGCGTG**3'**	2.14kbp

Table 9.1: Primers used for the different polymerase chain reactions

Two sets of forward and reverse primers were designed for PCR amplification of part or whole of the *sbm* gene. The expected sizes of the PCR products are listed in Table 9.1. The second set of primers (*sbm* F and *sbm* R, Table 9.1) was designed for the real time PCR studies. These were then subsequently used for both RT- PCR and

real time PCR reactions. *his* G was chosen as the internal standard as it is a constitutive gene and is expressed irrespective of the growth conditions.

9.1.6 Bacterial cultures

TR6524 *E. coli* K12 wild type (lambda+) was a kind gift from Prof. John Roth, University of California, Davis, California, USA. The *sbm* mutant *E. coli* K12 *sbm*::MudJ in TR6524 (named FA1P1) was created by a MudJ insertion inactivation of the *sbm* gene by Dr. Ipsita Roy. *Bacilus cereus* SPV and electro-competent *E. coli* DH5α and BL21DE3 cells were used for the cloning studies. *Bacillus subtilis* 1604 used in the cloning studies was kind gift from Dr. Mark Clements, University of Westminster, London, UK. The Gram positive- *E. coli* shuttle expression vector pHCMC05 (Nguyen *et al*., 2005) was obtained from the Bacillus Genetic Stock Centre (BGSC), Department of Biochemistry, Ohio State University, Columbus, Ohio, USA. The strains used and their genetic constitution are listed in Table 9.2.

Strain	Genotype	Source	Media
E. coli K12 (TR6524)	wild type (lambda+)	Kind gift from Prof. J. R. Roth, University of California, USA	2TY
E. coli (FA1P1)	*sbm* mutant *sbm*::Mu dJ	Dr. Ipsita Roy, University of Westminster	2TY
B. cereus SPV	wild type polymer producing strain	Dr I. Roy's lab culture collection	Nutrient broth
B. subtilis 1604	wild type	Kind gift from Dr. Mark Clements, University of Westminster	Nutrient broth

Strain	Genotype	Source	Media
E. coli DH5α (derived from *E. coli* K12)	F- φ80dlacZM15 (*lac*ZYA-*arg*F)U169 deoR *rec*A1 *end*A1 *hsd*R17(r k^-, m k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ- (www.invitrogen.com)	Lab culture collection	LB
E. coli BL21-DE3 (derived from *E. coli* B)	F- *omp*T *hsd*SB (rB^- mB^-) *gal* dcm (DE3). (www.invitrogen.com)	Lab culture collection	LB

Table 9.2: The strains used in this study and their respective genotypes

The bacterial cultures were grown in Luria-Bertani (LB) broth and were stored as frozen glycerol (20% v/v) deeps at -80^{o}C. All antibiotics were filter sterilised and the media were sterilised by autoclaving before use. The bacteria, *E. coli*, *Bacillus subtilis* and *B. cereus* SPV were grown at 37^{o}C unless otherwise stated.

Plasmids pEX3, pMEX2 and pHCMC05 used in this study were obtained from Dr. I. Roy (University of Westminster), Dr. N.H. Keep (Birkbeck College, University of London) and (Bacillus Genetic Stock Centre, Ohio, USA), respectively.

9.1.7 Growth media

The LB broth and the nutrient broth used in the experiments were purchased as readymade powder from Oxoid, UK. The tryptone and the yeast extract used were obtained from Difco, UK and the bacteriological agar was also obtained from Oxoid, UK.

9.1.7.1 2TY medium

Components:	**g/L**
Tryptone	16.0
Yeast extract	10.0

Sodium chloride (NaCl)	5.0
pH	7.0
Autoclaved at 121^{o}C for 15 minutes	

9.1.7.2 ART medium

Components	**g/L**
Sodium chloride	5.0
Yeast extract	5.0
Tryptone	5.0
Tris	5.0
Sucrose	171.15
1mL 0.5M Calcium chloride	
1mL 0.5M Manganese suphate	
pH	8.4
Autoclaved at 121^{o}C for 15 minutes	

9.1.7.3 M9 minimal medium with carbon sources

For a total volume of 1 litre of the medium,

Components	**g/L**
5X M9 salts solution	200.0mL
1 M magnesium sulphate	0.5mL
1M calcium chloride	0.025mL
20% carbon source (glucose, succinate or propionate)	20.0mL
pH	7.0

9.1.7.4 M9 salts

Components	**g/L**
Disodium hydrogen phosphate	64.0
Potassium dihydrogen phosphate	15.0
Sodium chloride	2.5
Ammonium chloride	5.0

The solution was prepared by dissolving the salts in one litre of de-ionised water. The solution was divided into 200mL aliquots and sterilised at 121^{o}C for 15min. The carbon sources were sterilised separately on a sugar run (110^{o}C for 10min) and then added to the medium aseptically under sterile conditions. A molar solution of both magnesium sulphate and calcium chloride were also autoclaved separately and added aseptically to the final media.

LB and Nutrient broths were prepared as specified by the manufacturer. In order to make plates, bacteriological agar (1.2%) was added to nutrient and LB broths before autoclaving.

9.1.7.5 SOC medium (for cloning experiments), pH 7.0

2% Bacto tryptone
0.5% Bacto yeast extract
10mM Sodium chloride
2.5mM Potassium chloride
10mM Magnesium chloride
10mM Magnesium sulphate
20mM Glucose

9.1.8 Buffers and reagents

9.1.8.1 2-Dimensional electrophoresis (2DE) and SDS-PAGE

Lysis buffer (for sonication of cells in 2DE)

Urea 9M
4% w/v CHAPS
2% carrier ampholytes 3-10
1% Dithiothreitol (DTT)

2x sample buffer (for SDS-PAGE)

0.125 M Tris-HCl, pH 6.8

15% glycerol (v/v)

6% SDS

2% DTT

0.005% bromophenol blue

5x SDS-PAGE gel running buffer

Components	**g/L**
Tris base	15.0
Glycine	72.0
SDS	5.0

Rehydration buffer (for IEF)

Urea 9M

4% CHAPS

2% carrier ampholytes

1% DTT

Trace amounts of bromophenol blue to add colour

Equilibration buffer (for equilibration of IPG strips following IEF)

Solution 1

50mM Tris-HCl, pH 8.8

6M urea

30% glycerol

1% SDS

1.25% DTT

Solution 2

50mM Tris-HCl, pH 8.8

6M urea

30% glycerol

0.25% DTT

1.0% SDS

2.5% w/v iodoacetamide added just before use

Agarose sealing buffer (for sealing of IPG strips following IEF prior to SDS-PAGE)

0.5% agarose

Tris base 25mM

Glycine 192mM

0.1% SDS

Trace amounts of bromophenol blue to add colour to the solution

Sonication buffer (for sonication of cells excepting 2DE)

100mM Tris-Cl (pH 7.5)

5mM EDTA

5mM Benzamidine hydrochloride

0.5mM PMSF

2mM DTT

The lysis solution, rehydration buffer, equilibration buffer, agarose sealing buffer and the sonication buffer were prepared and stored as aliquots at -80^{o}C.

Protein fixing solution (for fixing proteins following SDS-PAGE in 2DE)

Methanol (HPLC grade), acetic acid (100%) and water mixed in the ratio 5:1:4 respectively.

Coomassie brilliant blue stain (for staining SDS-PAGE gels)

0.025% Servablue R-250 stain dissolved in 10 mL acetic acid (100%) and 90mL water.

De-staining solution (for destaining SDS-PAGE gels)

10% acetic acid (100%) in water

9.1.8.2 Plasmid extraction and purification

Solution I

50mM glucose

25mM Tris-Cl (pH 8.0)

10mM EDTA (pH 8.0)

Solution II

0.2M Sodium hydroxide

1% SDS

Solution III

5M potassium acetate	60.0 mL
Glacial acetic acid	11.5 mL
Water	28.5 mL

Solution I was prepared and autoclaved at 115^{o}C for 10min. Solution II was always prepared fresh before use. Solution I and Solution III were stored at 4^{o}C until further use.

9.1.8.3 TE pH 8.0

10mM Tris-HCl

0.1mM EDTA

9.1.8.4 5X TBE, pH 8.0

Components	**g/L**
Tris base	54.0
Boric acid	27.5
EDTA	4.5

9.1.8.5 Lysozyme

A 50mg/mL stock solution of lysozyme was prepared in TE (pH 8.0), filter sterilised and stored as 100µL aliquots at -20^{o}C.

9.1.8.6 Antibiotics

A 100mg/mL stock of ampicillin and a 20mg/mL stock of kanamycin were prepared in sterile HPLC grade water. A 20mg/mL stock of chloramphenicol (CHPC) was made using 100% molecular biology grade ethanol. The antibiotic stock solutions were prepared individually, filter sterilised using 0.2µm ethanol tolerant filters and stored as 200µL aliquots at -20^{o}C.

9.2 Methods

9.2.1 Genomic DNA (gDNA) isolation and PCR amplification for verification of the deletion mutation

Genomic DNA was isolated from *E. coli* TR6524 and the *sbm* insertion mutant FA1P1 using the Promega DNA Wizard kit following the manufacturer's protocol. The genomic DNA isolated from the wild type and mutant *E. coli* K12 was quantified using A_{260nm}. 1.0µg of the DNA was used for subsequent PCR reactions. The primers used (*iciA2, R55, F33* and *sbm11*) are listed in Table 9.1 (genetic analysis, materials Section, 9.1.5) and their position on the *E. coli* FA1P1 chromosome is shown (Chapter 2, Section 2.2.1). The PCR reactions were carried out at 3 different annealing temperatures namely 50°C, 55°C and 60°C in order to optimise the conditions for the reaction. The final PCR amplification cycle used is as shown below in Table 9.3.

PCR step	Temperature °C	Time (minutes)	
Initial denaturation	94.0	2.0	
Denaturation	94.0	1.0	30 cycles
Annealing	60.0	1.0	
Extension	72.0	2.0	
Final extension	72.0	5.0	

Table 9.3: Amplification cycle used for the PCR reaction used to confirm the deletion mutation in FA1P1

The products obtained were separated on a 0.8% agarose gel in TBE with a suitable marker DNA as described by Sambrook *et al*., 1989.

9.2.2 Comparative growth analyses

Each strain of *E. coli* K12 (wild type TR6524 and the *sbm* mutant FA1P1) was plated on to 2TY medium. The mutants were grown on plates with kanamycin (20µg/mL, final concentration) in order to maintain the Mu dJ insertion in the *sbm* gene. Routinely, overnight cultures of the bacteria were grown in 2TY media at

37°C. Following this, 100mL of M9 minimal salts media containing glucose, succinate or propionate as sole sources of carbon, was inoculated with the overnight culture of either strain. The mutant was always grown in media containing kanamycin. The final concentration of vitamin B_{12} in the minimal media was maintained at 0.05µg/mL, 0.5µg/mL (Evans *et al*., 1993) or 5µg/mL and the final concentration of bicarbonate was maintained at 5mM (Evans *et al*., 1993). The three different concentrations of vitamin B_{12} were chosen in order to observe the effect of vitamin B_{12} concentration on the growth pattern of the wild type and the mutant *E. coli* strains. The inoculum, cultured individually in three different vitamin B_{12} concentrations, was maintained at 1% of the culture volume in all cases and the cells were allowed to adapt to the media at 37°C and reach an OD_{600nm} of 0.5. Once an actively growing culture was obtained, the adapted culture was further used to inoculate 600mL of the minimal media and incubated at 37°C. This inoculation was approximately 1% of the culture volume (*i.e.* 6mL), however, it was also ensured that the same number of cells was used in each experiment. This made the growth curves comparable. The growth pattern was followed using A_{600} readings and a growth curve plotted. The experiment was carried out in triplicates.

Stock solution of vitamin B_{12} (5mg/mL) was prepared, sterilised and stored in the dark at -20°C for use in the growth experiments.

9.2.3 RNA isolation

The cells grown in large scale (600mL) minimal media with the three carbon sources (glucose, succinate or propionate) were harvested at different time points (early log, mid log and stationary phases) of growth. These cells were then frozen at -80°C for RNA isolation. RNA isolation was carried out using TRI Reagent (Sigma, UK). The manufacturer's protocol was followed with some modifications. The volume of TRI Reagent used to resuspend the cell pellet obtained from 5mL of cell culture was reduced to 150µL as this prevented the dilution of the RNA sample. The volume of all the reagents used in the isolation procedure was altered accordingly. RNA precipitation was carried out at -80°C overnight, and the total RNA obtained was

suspended in 0.1% diethylpyrocarbonate (DEPC) treated water, quantified using A_{260nm} measurements and frozen at -80^{o}C for future use or was reverse transcribed to complimentary DNA (cDNA) as soon as possible.

9.2.4 cDNA preparation

1.0µg of RNA was used for the reverse transcription reaction (ImProm II Reverse Transcription System). The cDNA was synthesised following the manufacturer's technical manual (Technical manual 236). The random hexamers provided in the kit were used for the cDNA preparation.

9.2.5 PCR for RT-PCR

The cDNA prepared was amplified by conventional PCR using primer sequences 3, 4 and 5 (Table 9.1, Section 9.1.5). The magnesium ion concentration in the reaction was increased to 3.0mM final concentration as this was found to produce discrete bright bands. The amplification program used is shown in Table 9.3, Section 9.2.1. The products were run on a 0.8% agarose gel in TBE with a suitable DNA marker.

9.2.6 Real-time quantitative PCR (QPCR)

The QPCR study was aimed to verify and extend the results obtained by the conventional PCR techniques. This method allows quantification of the gene expression and helps to observe the product formation in real time. The SYBR green jump start ready mix kit from Sigma (UK) was used for the amplification reaction. The reaction mix for each reaction comprised 12.5µL of the master mix, 0.25µL of ROX (reference dye), 2µL of the cDNA template, 1µL each of the forward and the reverse primers (0.4µM final concentration, for the corresponding genes of the *sbm* operon) and nuclease free water to a final volume of 25µL. The primers used for the amplification of the four genes in the *sbm* containing operon were 4, 5, 7, 8 and 9 shown in Table 9.1 (Section 9.1.5). The amplification was performed in an ABI Prism 7000 Real-time thermal cycler on a 96 well optical plate (ABI Biosystems, UK) and the thermal cycling parameters used were as follows: 2min at 50^{o}C and 10min at 95^{o}C, followed by 40 cycles consisting of 15s at 95^{o}C and 1min at 60^{o}C. A

10-fold serial dilution series of the gel purified PCR amplification products of the four genes *sbm*, *ygf*G, *ygf*H and *arg*K, each ranging from $1x10^2$ to $1x10^5$ copies/µL was used to construct the standard curves for absolute quantification of mRNA concentration.

9.2.7 Protein studies

9.2.7.1 Protein sample preparation

Protein sample preparations for 2 dimensional electrophoresis (2DE) were made as described earlier (Peng *et al*., 2003) with some modifications. Cell pellets obtained from the stationary phase of growth in large scale M9 minimal media containing glucose, succinate or propionate as the sole source of carbon were washed twice in phosphate buffered saline (PBS) at neutral pH and re-suspended in 3mL of the same. The cells were then lysed by sonication with a sonication cycle of 10 x 20 seconds. The cell lysate was centrifuged at 20000g for 30min at 4°C in an ultracentrifuge. The supernatant was then dialysed using a dialysis bag with 10KDa cut off range, against distilled water, at 4°C overnight. The distilled water was changed at least thrice during the dialysis. The dialysate was lyophilised and the resulting protein powder was solubilised in 1mL of lysis buffer, stored as aliquots at -80^{o}C until further use. The Bradford protein assay (Bradford, 1976) was carried out on the lysate to quantify the total protein content.

9.2.7.2 SDS-PAGE

Protein samples (20µg) were diluted in reducing 2X sample buffer (Section 9.1.8.4), boiled for 5min and allowed to cool down to room temperature. Electrophoresis was carried out using a 10% polyacrylamide gel, initially at 80V for 15min till the samples migrated into the separating gel and then at 120V for 60min, in running buffer.

9.2.7.3 Two dimensional electrophoresis (2-DE/ 2DE)

2-DE experiments were carried out on a 7cm IPG strip pH 3-10, non linear, according to Gorg *et al*., 2000, with slight modifications. 100µg of protein was

diluted in rehydration buffer and gel rehydration was performed at room temperature overnight. The IPG strip was overlaid with molecular biology grade mineral oil (Sigma, UK) to prevent drying during rehydration. First dimensional IEF was performed at 18°C under the following conditions: 300V for 30min; 600V for 30min; 3500V for 2 hours and 44min. The multiphor tray was overlaid with molecular biology grade mineral oil during IEF in order to prevent the gel from drying or burning at the high voltage. The focused IPG strip was stored at -80^{o}C until further use.

In the second dimension, the focused IPG strips were equilibrated for 15 minutes in equilibration Solution I and for another 15min in equilibration Solution II described in Section 9.1.8.1.5 (Peng *et al*., 2003). Equilibrated IPG strips were loaded onto 10% acrylamide gel and covered with sealing buffer. The gels were run initially at 80V for 15min to let the protein samples to migrate into the gel and then at 150V for 1 hour and 45min, in running buffer.

9.2.7.4 Fixing of proteins

Following the separation using electrophoresis, the proteins were subject to a fixing protocol that allowed the proteins to be fixed. The gels were flooded with the protein fixing solution for 30min.

9.2.7.5 Coomassie brilliant blue staining

Protein gels were then stained for 60min in the coomassie brilliant blue staining solution and then de-stained for three hours in the de-staining solution. The gels were then stored in HPLC grade water at 4^{o}C until further analyses. The protein spots were analysed using the Progenesis Samespots software (Non-Linear Dynamics, UK) and the gel images were captured using BioRad densitometer.

9.2.8 HPLC assay for MCM activity

9.2.8.1 Plasmid DNA extraction and purification

The genes coding for methylmalonyl CoA mutase from P*ropionibacterium shermanii* were originally cloned into a pMEX2 plasmid (Marsh *et al*., 1989). The protein extract obtained following the expression of the genes under suitable conditions was used for the positive control reactions to observe MCM activity. Previously, Haller *et al*. (2000) and Bobik and Rasche (2003) have observed the activity of His-tagged purified Sbm from *E. coli* in their studies. The present study aimed, for the first time, to assay the activity of the enzyme as a part of the total protein from wild type *E. coli* TR6524 grown in minimal media with glucose, succinate or propionate as the sole source of carbon. The pMEX2 plasmid was isolated by following a modified protocol described previously (Sambrook *et al*., 1989). A 10mL overnight culture of *E. coli* DH5α containing plasmid pMEX2, grown in LB broth with a suitable antibiotic (here, ampicillin 100µg/mL final concentration) was harvested by centrifuging at 6000g for 10min in a Sorvall Legend RT centrifuge. All the centrifugation steps were carried out at 4^{o}C. The cells were then re-suspended in 250µL of Solution I, 250µL of Solution II and 300µL of Solution III (section 9.1.8.2). The cells were mixed gently by inversion and centrifuged at 16000g for 12min. The supernatant was transferred into a fresh eppendorf tube and gently mixed with equal volumes of phenol: chloroform: isoamylalcohol solution (Invitrogen, UK) by inverting the tube. Following centrifugation at 16000g for 12min the supernatant was then transferred into a fresh eppendorf tube and mixed with equal volumes of isopropanol and the mixture was centrifuged at 16000g for 20min to precipitate the plasmid DNA. The supernatant was discarded and the precipitated DNA was washed with 70% ethanol by repeated pipetting and centrifuged at 16000g for 5min. The ethanol was removed by aspiration and another centrifugation step (16000g, 2min) followed. This step was carried out to remove any remaining ethanol in the tube. The plasmid DNA was allowed to dry at 37^{o}C for 10-15min and re-suspended in 30µL of sterile HPLC grade water and frozen at -20^{o}C for further use. About 1µL of the isolated plasmid

DNA was separated on a 0.8% agarose gel (Sambrook *et al*., 1989) for confirmation of size and purity with a suitable molecular weight marker.

The plasmid DNA extraction procedure was modified with one additional step when carried out with *B. subtilis* and *B. cereus* cells. The cells were treated with 100µL of 30mg/mL lysozyme following the re-suspension of the cell pellet in Solution I and incubated at 37^{o}C for 30 minutes with frequent mixing to prevent them from settling down. This facilitated the breakage of the intact Gram positive cell wall. This was followed by the addition of Solutions II and III and the routine protocol was followed thereafter.

9.2.8.2 Transformation of *E. coli* BL21-DE3 cells

9.2.8.2.1 Preparation of electro-competent *E. coli* BL21-DE3 cells

E. coli BL21-DE3 electro-competent cells were prepared and transformed using the method described by Dower *et al*., (1988). 100µL of an overnight culture of BL21-DE3 cells (originally frozen as glycerol deeps) grown in LB broth at 37^{o}C shaken at 200rpm was used to inoculate 100mL of fresh LB broth that was incubated at the same growth conditions. An optical density between 0.7-0.8 measured at A_{600nm} using a Perkin Elmer spectrophotometer was used as an indication to harvest the cells for competent cells preparation. The cells were washed in cold 1mM HEPES (pH 6.8) and 20% glycerol in alternating steps. The harvested cells were suspended in 25mL of 1mM HEPES and centrifuged at 6000g for 10min. The cells were then washed in 20% glycerol and centrifuged as above. This procedure was repeated three times and the cells were finally suspended in 40µL of 20% glycerol and placed in dry ice. The frozen cells were then stored at -80^{o}C until further use. The cells were treated with great care and handled very gently as they were fragile due to a weakened cell wall.

9.2.8.2.2 Transformation by electroporation

A BioRad Gene Pulser Xcell electroporation unit was used for the transformation. Two tubes containing 40µL of electrocompetent *E. coli* BL21-DE3 (one for the test, another for the control reactions) were placed on ice 5min before the pulse was

provided. One micro litre of the pMEX2 plasmid DNA was added to the test reaction tube and mixed by gently tapping on the side of the tube. The contents were left on ice for 5min and then gently transferred to a previously chilled electroporation cuvette (BioRad, UK) with a 0.2cm electrode gap. Care was taken to avoid introducing any air bubbles as this would affect the electroporation. A voltage of 2500V, a capacitance of 25μF and a resistance of 200Ω were used for providing the single optimal pulse (of 4.8-5.2ms or 12.5kV/cm) required for the transformation. About 200μL of the recovery SOC medium (Section 9.1.7.4) was added immediately to the cuvette with the possible transformants and the contents were gently transferred to a fresh eppendorf tube and incubated at 37^{o}C and shaken at 200rpm for about 60-90min to allow the cells to recover. The procedure was repeated with the cells in the tube marked 'control reaction' except that sterile HPLC grade water was used instead of the plasmid DNA. Following incubation in SOC medium the cells were then spread onto LB agar plates containing 100μg/mL ampicillin and incubated at 37^{o}C overnight and checked for the growth of the transformants.

9.2.8.3 Expression of the gene encoding MCM from pMEX2

The transformants from the ampicillin containing LB plates were grown in LB broth containing antibiotic and colonies obtained were grown in liquid LB and stored as frozen glycerol deeps for future reference and use. In *E. coli* BL21 DE3, the expression of the MCM protein was under the influence of a T7 promoter controlled by the concentration of the T7 polymerase which in turn was under the control of a lac promoter, induced by the presence of IPTG in the growth medium. In order to maximise the expression of the MCM protein, a range of IPTG concentrations and induction conditions were tested. The best expression was observed in the presence of 1mM IPTG in the medium and when the cells were grown to an optical density (OD_{600nm}) of 0.6 at 28^{o}C and incubated at 18^{o}C overnight. The cells were aerated by shaking them at 200rpm. The primary inoculum was grown in LB broth at 37^{o}C overnight and 1mL of this was transferred into 100mL of fresh LB broth and incubated at 28^{o}C before induction and at 18^{o}C following induction with IPTG and

shaken at 200rpm. The cells were harvested and re-suspended in the sonication buffer (Section 9.1.8.1.7). 500µL of the cell suspension was boiled with equal amounts of 2x sample buffer (Section 9.1.8.1.2) for 5-10min and respective protein bands indicative of the expression was checked by running the samples in a 10% polyacrylamide gel. The gel was stained as described in Section 9.2.7.5. The confirmation of over-expression was carried out using controls (non transformed *Escherichia coli* BL21-DE3) and un-induced transformed cell samples that were prepared and separated alongside suitable molecular weight marker.

9.2.8.4 Preparation of whole cell extract for positive control reaction for HPLC assay

The conditions for the over-expression of the genes coding for MCM from *P. shermanii* were optimised, and the over-expressed protein was used in the positive control reaction with an active MCM. The transformed *E. coli* BL21-DE3 cells, containing the plasmid pMEX2, were cultured in 100mL of the broth (in order to produce sufficient protein) under the optimised expression conditions in the presence of 100µg/mL (final concentration) ampicillin. The cells were harvested and re-suspended in 3-5mL of freshly made sonication buffer (9.1.8.1.7) and sonicated using the MS73 probe as described Section 9.2.7.1. The lysed cells were then centrifuged in a Sorvall SL-50T rotor at 18000g for 30min at 4^{o}C. The supernatant containing the total cell extract was then used for the reaction that would eventually be detected using a HPLC assay based on the method developed by Riedel *et al*., 1995. The mobile phase used was a phosphate buffer and methanol gradient and the assay was carried out at 39^{o}C with a flow rate of 2mL/min.

9.2.8.5 Preparation of the total cell extract from minimal media for reactions prior to the HPLC assay

Cell pellets obtained from the early stationary phase growth in large scale M9 minimal media containing glucose, succinate or propionate as the sole source of carbon were washed twice in phosphate buffered saline (PBS) at neutral pH and re-suspended in 3mL of the sonication buffer. The cells were then lysed by sonication

with a sonication cycle of 10 x 20 seconds. The cell lysate was centrifuged at 18000g for 30min at 4°C in a Sorvall centrifuge with a SL-50T rotor.

9.2.8.6 HPLC assay for the detection of the activity of MCM in positive control and test samples

9.2.8.6.1 HPLC assay- standard curve

Commercially available lithium salt of the substrate methylmalonyl-CoA (MMCoA) and sodium salt of the product succinyl CoA were purchased from Sigma, UK and stock solutions of 600µM concentrations were prepared by dissolving the salt in the eluting phosphate buffer (pH 4.6) prepared as described by Riedel *et al*., 1995. The stock solutions were then serially diluted to obtain a series of decreasing concentrations (500µM, 400µM, 300µM, 200µM and a 100µM) in order to produce a standard calibration curve. The salts were dissolved just before they were ready to be used in the reaction or before injection into the column in the case of the standard curve.

20µL of the standards or the samples (protein extract from cells used as positive control and protein extract from the cells grown in minimal media with glucose, succinate or propionate as the sole source of carbon, described in Section 9.2.8.5) were manually injected into the reverse phase C-18 Dionex column connected to the Dionex Summit HPLC detection system. The elution buffer preparation and the detection protocol were followed according to that described by Riedel *et al*., 1995.

9.2.8.6.2 HPLC assay of the positive control MCM protein from *P. shermanii* and the protein extract from *E. coli* K12 cells grown under different conditions of growth

The MCM assay was performed according to Riedel *et al*., 1995 with modifications where the final volume of the reaction was reduced to half of that specified. For the enzyme assay, 25µL of the total protein extract was incubated in the dark with 10µL of the 1mM coenzyme B_{12} for 2 hours at 4°C (Dr. Ipsita Roy, personal communication). The reaction was started with the addition of 60µL of 600µM methylmalonyl CoA to the protein-coenzyme B_{12} mix and incubated at 37°C. The

reaction was stopped following incubation (at 5, 10, 20, 40 minute intervals) using 35µL of 10% trichloroacetic acid. This time range facilitated the observation of decreasing and increasing peak intensities of the substrate (MMCoA) and the product (succinyl CoA) respectively, during the assay. The mixture was centrifuged at 13000g for 3min and the supernatant was transferred to a fresh eppendorf tube and frozen at -80^{o}C until the HPLC analysis. Whole cell extracts of the homogenates without methylmalonyl-CoA were also analysed in order to detect peaks of the metabolites present in the extracts prior to the MCM catalysed reaction. A negative control reaction was also carried out with water as the sample instead of any total protein extract.

The reactions and the assay were carried out for the over-expressed positive control protein extract from *E. coli* BL21 DE3 transformed with pMEX2 and repeated with the protein samples from the wild type *E. coli* (TR6524) cell pellets obtained from the early stationary phase growth in large scale M9 minimal media containing glucose, succinate or propionate as the sole source of carbon. The protein sample preparation, the reaction and the assay were carried out on the same day and storage of any of the sample or reaction mixture were avoided as the salts of the coenzyme A have a limited life in solution (Riedel *et al*., 1995)

9.2.9 Cloning of the *sbm* gene into *Bacillus cereus* SPV

It has been shown that some bacteria (such as *Nocardia* and *Rhodobacter*) produce polyhydroxyalkanoate co-polymers by using the MCM pathway. In order to observe the influence of the *sbm* gene in the production of P(3HB-co-3HV) in *B. cereus*, the *sbm* gene from wild type *E. coli* TR6524 was cloned into *B. cereus* and an attempt was made to study the effect of the inclusion of this gene.

9.2.9.1 Amplification of *sbm* from pEX3

The plasmid construct pEX3 contains the *E. coli sbm* gene cloned into pT7-18 (obtained from Dr. Ipsita Roy). PCR primers were designed to amplify the *sbm* gene from pEX3 with restriction endonuclease sites incorporated into them so as to

produce sticky ends or overhangs that help in routine cloning experiments. The choice of these restriction sites was based on the sites present in the multiple cloning site (MCS) of the *E. coli*- Gram positive shuttle vector (ECE 190 or pHCMC05, Nguyen *et al*., 2005) obtained from BGSC, Ohio, USA. Care was taken to exclude all restriction enzymes which may have sites within the *sbm* gene. This resulted in designing the forward and reverse primers with restriction sites for *Xba*I and *Xma*I respectively (Table 9.1, section 9.1.5). The plasmid DNA was extracted and purified as described earlier in Section 8.2.8.1. One microgram of the plasmid DNA was then used in a routine polymerase chain reaction to amplify the gene using *Taq* polymerase from Promega, UK. The PCR Master mix (Promega, UK) was used in a 100µL reaction as specified by the manufacturer. The reaction cycle used was as described in Table 9.3, Section 9.2.1. The amplified product was then separated on a 0.8% agarose gel and the product subjected to purification and used in subsequent cloning.

9.2.9.2 Gel purification of PCR amplified *sbm* gene

The PCR product that was cut out of the gel was purified using the QIAquick gel extraction kit from Qiagen, UK. The manufacturer's protocol (page 23-24, QIAquick spin handbook) was followed for the purification steps. 30µL of sterile HPLC grade water was used for eluting the purified DNA instead of the elution buffer provided in the kit. The eluted PCR product was stored at -20^{o}C until further use.

9.2.9.3 Preparation of vector DNA

The *E. coli*- Gram positive shuttle vector (pHCMC05, Figure 9.1) was received as a plasmid suspension from BGSC, Ohio State University, Ohio, USA. In order to produce adequate quantities of the vector for future cloning experiments, it was cloned into *E. coli* DH5α cells by electroporation. The electrocompetent cell preparation and the transformation procedure used were as described earlier in Sections 9.2.8.2.1 and 9.2.8.2.2 respectively. The transformants were then grown in 10mL LB broth containing the appropriate antibiotic and incubated at 37^{o}C overnight at 200rpm. Following overnight growth, glycerol deeps of the

transformants were made and stored at -80^{o}C. The remainder of the broth culture was used for plasmid isolation and purification as described in section 9.2.8.1. The vector was then stored at -20^{o}C till further use.

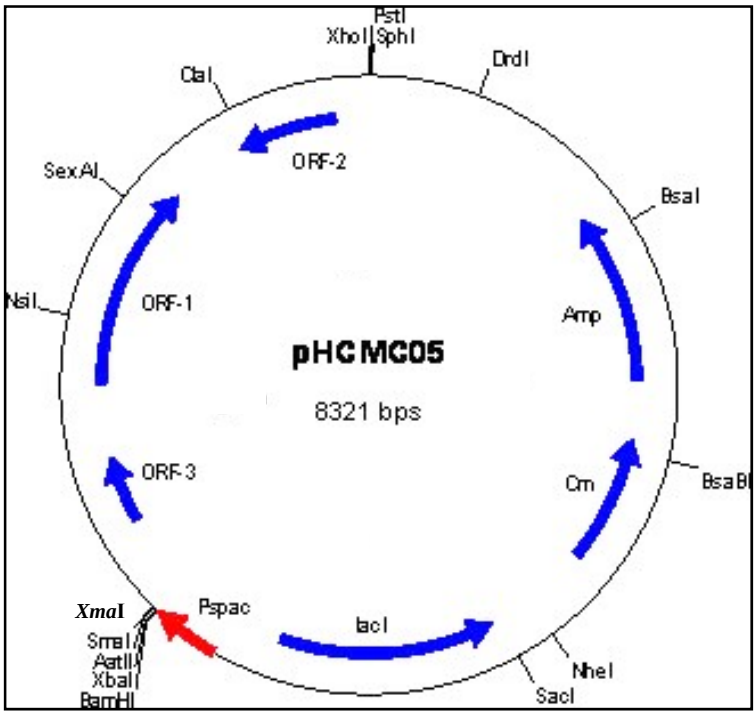

Figure 9.1: Restriction map of the *E. coli*- Gram positive shuttle vector, pHCMC05 (Nguyen *et al*., 2005).

9.2.9.4 Restriction digestion of the PCR product and the vector DNA

The gel purified PCR product and the vector DNA, pHCMC05, were subject to a double restriction digestion reaction using the same pair of enzymes, *Xba*I and *Xma*I. The reaction mix contained 30µL of the gel purified PCR product or the vector DNA, 5µL of the reaction buffer supplied with the restriction enzymes, 0.5µL of BSA supplied with the restriction endoculeases, 2µL of bovine pancreatic RNAse A, 5µL of each of the restriction enzymes and water to make up a total reaction volume of 50µL. The reaction mix was gently mixed by repeated pipetting and the tubes were incubated at 37^{o}C for 4 hours. At the end of the third hour of incubation, 1µL of calf intestinal alkaline phosphatase (CIP) was added to the reaction tubes to prevent the religation of the restricted ends of the DNA. At the end of the

incubation, CIP was inactivated by incubating the reaction tubes at 60^{o}C for 5min. The digested samples were separated on a 0.8% agarose gel with a suitable marker DNA and purified as elaborated above (Section 9.2.9.2) using the QIAquick gel extraction kit.

9.2.9.5 Ligation

The restriction digestion and gel elution of both the *sbm* PCR product and the shuttle vector was followed by the ligation of the two restricted products in order to incorporate the *sbm* gene into the vector which in turn will be used to transfer the *sbm* gene in to *B. cereus* SPV. The ligation reaction mix comprised 0.5µL- vector (1µg) (following restriction digestion and gel purification), 7µL- insert (1µg) (here, the PCR amplified and gel purified *sbm* gene following restriction digestion) 1µL of the ligation buffer (provided with the T4 DNA ligase) and 1.5µL of T4 DNA ligase (NEB, UK) thus making the total reaction volume up to 10µL/tube. A control ligation reaction was carried out using sterile water instead of the insert DNA and incubated along with the above reaction at 4^{o}C overnight.

9.2.9.6 Transformation of *E. coli* DH5α and *B. subtilis* 1604 with pCHMC05*sbm* (pCHMC05 plasmid with the *sbm* insert)

Electrocompetent *E. coli* DH5α and *B. subtilis* 1604 (prepared using the protocol used for that of *E. coli* DH5α) cells were transformed by electroporation (Section 9.2.8.2.2) with the ligation mix. The water control ligation mix was used as the control sample and the ligation reaction mix with the insert was used as the test during the electroporation. Following overnight incubation at 37^{o}C on LB agar plates with the suitable antibiotic (100µg/mL ampicillin for *E. coli* and 5µg/mL chloramphenicol for *Bacillus subtilis* 1604), all the putative transformants from the test plates were inoculated into 10mL of fresh LB broth with the appropriate antibiotic and incubated at the same conditions as the plates and were shaken at 200rpm. It was noted that the control plates (with water in place of the insert during ligation) had no growth at all. Following overnight growth, 1-2mL of the culture was used to prepare glycerol deeps and stored at -80^{o}C while the rest of culture was subject to the routine plasmid isolation and purification method in order to check for

the presence of the desired insert. *B. subtilis* 1604 was used for the transformation in order to produce plasmid DNA suitable for transformation into *B. cereus* SPV as this would enhance the chances of successful cloning of the *sbm* gene into *B. cereus* SPV

9.2.9.7 Insert release

The plasmid isolated from successfully transformed *E. coli* DH5α and *B. subtilis* 1604 cells were subjected to another set restriction digestion reactions (Section 9.2.9.4). The digested samples were run on a 0.8% agarose gel (with a suitable DNA ladder) alongside uncut plasmid to observe the release of the appropriate sized insert.

9.2.9.8 Protoplast preparation and polyethyleneglycol (PEG) induced transformation of *B. cereus* SPV cells

Protoplasts of *B. cereus* SPV cells were prepared and transformed with the shuttle vector- *sbm* insert complex by following the method described by Pragai (1994). Transformants were observed after 48 hours of incubation at 30^{o}C. The colonies obtained were then transferred to LB broth with chloramphenicol (20µg/mL final concentration) and incubated at 30^{o}C and 200rpm.

9.2.9.9 Colony PCR of transformed *B. cereus* SPV cells

The transformed protoplasts were allowed to regenerate in LB in the presence of chloramphenicol. The cells were then used for plasmid purification and the plasmid DNA was used in PCR amplification with the primers specific for *sbm* gene. The amplification conditions used were the same as that used for earlier reactions (Table 9.3). The PCR product was separated on a 0.8% agarose gel with a suitable marker DNA.

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