STUDIES ON LIPOIC ACID METABOLISM

BY

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DISSERTATION

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Abstract

The covalent attachment of lipoic acid to the lipoyl domains (LDs) of the central metabolism enzymes pyruvate dehydrogenase (PDH) and oxoglutarate dehydrogenase (OGDH) is essential for their activation and thus for respiratory growth in the bacterium *Escherichia coli* and the yeast *Saccharomyces cerevisiae*. A third lipoic acid-dependent enzyme system, the glycine cleavage system (GCV), is required for utilization of glycine as a nitrogen source.

In *E. coli*, lipoic acid is assembled on the LDs from the eight-carbon fatty acid, octanoate, in two steps. First, an octanoyltransferase (LipB) transfers an octanoyl moiety from the acyl carrier protein (ACP) of fatty acid biosynthesis to the LDs. A thioester-bound acyl-enzyme intermediate is formed in the process. Then lipoyl synthase (LipA) catalyzes replacement of single hydrogen atoms at carbons 6 and 8 with sulfur atoms using radical SAM chemistry. Alternatively, either exogenous lipoic acid or octanoate can be directly attached to the LDs by lipoate-protein ligase (LplA) via an acyl–AMP intermediate.

E. coli strains containing null mutations in *lipB* are auxotrophic for either lipoic acid (or octanoate), or acetate plus succinate which respectively bypass the PDH- and OGDH-catalyzed steps required for aerobic growth on glucose minimal media. Spontaneously-arising mutant strains that retained the *lipB* mutation, yet did not require supplementation for aerobic growth were isolated. Initial characterization distinguished two types of suppressor strains. In chapter 2 I describe one type in which suppression was caused by single missense mutations within the coding sequence of the *lplA* gene. The LplA proteins encoded by the mutant genes had reduced K_m values for free octanoate, which was detected in the cytoplasm at a concentration of about 28.2 μM, well above the K_m values for the mutant LplA proteins. Thus in these suppressor strains, the

mutant LplA proteins utilize the cytoplasmic octanoate pool to activate PDH and OGDH enabling growth.

In the second type of *lipB* suppressor strains, the causative mutation was a stop codon in the *sdhB* gene, which encodes a subunit of succinate dehydrogenase (SDH). In chapter 3, these *lipB sdhB* strains are further characterized. I show that these strains contain active PDH and require a functional *lplA* gene. Succinate in this strain is produced by three enzymes, any one of which will suffice in the absence of SDH. These three enzymes are: trace levels of OGDH, the isocitrate lyase of the glyoxylate shunt, and aspartate oxidase, the enzyme catalyzing the first step of nicotinamide biosynthesis.

In chapter 4, I characterize the lipoate-protein ligase of the yeast *S. cerevisiae.* The *E. coli* LplA was the first lipoate-protein ligase (Lpl) to be characterized. It catalyzes two partial reactions: activation of the acyl chain by formation of acyl-AMP, followed by transfer of the acyl chain to the LDs. It turns out that there is a surprising diversity within the Lpl family of enzymes, several of which catalyze reactions other than ligation reactions. For example, the *Bacillus subtilis* Lpl homologue LipM is an octanoyltransferase that transfers the octanoyl moiety from octanoyl-ACP specifically to GCV. Another *B. subtilis* Lpl homologue, LipL, transfers octanoate from octanoyl-GCV to other LDs in an amido-transfer reaction. In chapter 4, I report that the Lip3 Lpl homologue of *S. cerevisiae* has octanoyl-CoA:protein transferase activity, and discuss implications of this activity on the physiological role of Lip3 in lipoic acid synthesis.

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Chapter 1: Introduction

Discovery and Structural Characterization of Lipoic Acid

In the fourth and fifth decades of the 20th century, several publications appeared describing "factors" that stimulated microbial growth when added in small amounts to growth media. For example, Esmond Snell reported on a factor that replaced acetate in the growth medium of certain lactic acid bacteria presumably because it enabled the bacteria to produce their own acetate (Guirard et al., 1946). O'Kane and Gunsalus characterized the "pyruvate oxidation factor" which was essential for the conversion of pyruvate to acetate and CO_2 by *Enterococcus faecalis* (O'Kane D and Gunsalus, 1948). Finally the "B. R. factor" described by Kline and Barker was required by *Butyribacterium rettgeri* for growth on lactate (Kline and Barker, 1950)[1]. It became apparent later that all these investigators were studying the same enzyme cofactor, lipoic acid.

Several years later in the fall of 1950, through a collaboration between Gunsalus, Reed and the Eli Lilly Research Laboratories, 30 mg of a yellow crystalline compound were extracted from approximately 10 tons of liver residue and named alpha lipoic acid (Reed, 2001). At the time it had been observed that the lipoic acid-catalyzed pyruvate oxidation by pigeon brain tissue was inhibited by trivalent arsenicals (such as Lewisite), which are known to bind sulfhydryl groups. Inhibition was reversible by dithiols such as 2,3-dimercaptopropanol (British anti-Lewisite or BAL) but not by monothiols (Peters and Wakelin, 1949). It was thus postulated that lipoic acid would contain a dithiol. This was confirmed when the correct structure of the molecule was established as 6,8-dithiooctanoic acid by chemical synthesis (Reed, 1998) (Fig. 1.1).

[1] The exact role of lipoic acid in *B. rettgeri* metabolism remains elusive because lipoic acid supplementation is not required for growth on either glucose or pyruvate, but is required for (anaerobic) growth on lactate. KLINE, L., PINE, L. & BARKER, H. A. 1963. Metabolic Role of the BR Factor in Butyribacterium rettgeri. *J Bacteriol,* 85**,** 967-75, WITTENBERGER, C. & FLAVIN, M. 1963. Butyribacterium rettgeri: a Role of Lipoic Acid in Anaerobic Electron Transport. *Journal of Biological Chemistry,* 238**,** 2529-2535.

Physiological Role of Lipoic Acid-Dependent Enzyme Systems

There are five enzyme systems known to require lipoic acid for function. These are pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase (OGDH), glycine cleavage (GCV), branched-chain 2-oxoacid dehydrogenase (BCDH), and acetoin dehydrogenase (ADH). The overall reactions catalyzed by these enzyme systems take place in multiple steps each occurring at a different subunit. In each system lipoic acid functions to carry reaction intermediates between the various subunits (Perham, 2000). Reduced NADH, which can be used by the cell to fuel biosynthesis reactions, is produced in the final step.

PDH plays a central role in aerobic metabolism. It catalyzes the oxidative decarboxylation of pyruvate, the end product of glycolysis, to acetyl-CoA. Energy is conserved in the final product in the form of a thioester bond between acetate and coenzyme A (CoA). The acetate moiety of acetyl-CoA can then be condensed with oxaloacetate to form the first intermediate of the tricarboxylic acid (TCA) cycle, namely citrate. Additionally, acetyl-CoA is the building block for fatty acid biosynthesis. PDH is important for aerobic growth of *Escherichia coli* because the other major pyruvate- disassimilating enzyme, pyruvate:formate lyase (Pfl), has an oxygen labile active site and is thus inactive in the presence of air (Böck, 1996). *E. coli* contains a third pyruvate-metabolizing enzyme, pyruvate oxidase (PoxB), however it is mainly expressed during early stationary phase (Abdel-Hamid et al., 2001). Additionally PoxB is less efficient at energy conservation because it produces free acetate, which must be activated to acetyl-CoA at the expense of an ATP.

After a few reactions of the TCA cycle, citrate is broken down to 2-oxoglutarate, which undergoes oxidative decarboxylation to succinyl-CoA by OGDH, a second lipoic acid-dependent enzyme system. Succinyl-CoA is required for the synthesis of the amino acids lysine and methionine, and the peptidoglycan component diaminopimelic acid. Additionally succinly-CoA is the source of succinate, which is the entry point to respiration. Succinate is also a precursor for heme synthesis in some organisms, but not in *E. coli* (Beale, 1996). OGDH activity is dispensable

under conditions where the TCA cycle operates as a branch. In such instances, fumarate is the source of succinate and succinyl-CoA.

The third lipoic acid-dependent enzyme system, GCV, catalyzes cleavage of glycine to ammonia, and C1 units which are carried on tetrahydrofolate (THF). GCV is essential for utilization of glycine as sole source of nitrogen. The C1 units are used in various cellular processes including purine biosynthesis, methionine biosynthesis, formylation of Met-tRNA (to be used in translation initiation), and methylation of DNA, RNA and protein. GCV is an important enzyme for C3 plants (Douce et al., 2001). In these plants, the fortuitous oxidation of ribulose 1,5-bisphosphate (termed photorespiration) produces the byproduct 2-phosphoglycolate (2PG) which cannot be further metabolized by the Calvin Cycle. 2PG is converted to glycine, broken down by GCV and used to make serine via serine hydroxymethyltransferase. Serine then feeds into central metabolism at pyruvate.

The lipoic acid-dependent BCDH is involved in breakdown of the branched-chain amino acids, valine, leucine and isoleucine, to their respective branched-chain acyl-CoAs which are used to make branched-chain fatty acids. Initially the amino acids undergo transamination of their amino groups, giving rise to 2-oxoacids which are decarboxylated by BCDH. Branched-chain fatty acids are produced by a number of bacteria in order to regulate membrane fluidity.(Martin et al., 2009).

Finally, ADH catalyzes the breakdown of acetoin to acetyl-CoA. A number of microorganisms produce acetoin instead of acetate as an end product of fermentation because acetoin is pH neutral and is thus better tolerated at the higher concentrations produced by dense cultures (for example at stationary phase). When more favorable carbon sources are depleted, acetoin can be utilized to form acetyl-CoA (Xiao and Xu, 2007).

In addition to the five enzyme systems described above, two proteins were shown to contain the lipoic acid modification *in vivo*. The X protein is a constituent of the yeast and mammalian PDH and is essential for the assembly of

the enzyme complex (De Marcucci and Lindsay, 1985). However, lipoylation of the X protein does not seem to be essential for efficient PDH activity. Cui *et al.* reported that a small protein of unknown function in *Bacillus subtilis* was either lipoylated or biotinylated at the same site when expressed in *E. coli* (Cui et al., 2006). They referred to this protein as BLAP for biotin/lipoyl acceptor protein. *In vitro* however, BLAP is not lipoylated by any of the *Bacillus* lipoate-protein ligases (Christensen et al., 2011b), and the genomic context of the gene expressing BLAP suggests its involvement in a carboxylation reaction and thus favors the biotinylation hypothesis (Hanke and Cronan, personal communication).

PDH, OGDH and GCV are present in *E. coli* and *Sccharomyces cerevisiae,* the two organisms that are the subject of this thesis. Whether *S. cerevisiae* also has a BCDH is unclear (Dickinson and Dawes, 1992, Hazelwood et al., 2008), although the *S. cerevisiae* membrane does not contain branched-chain fatty acids. In yeast all the subunits of PDH, OGDH and GCV are encoded by nuclear genes and translocated into the mitochondria.

Catalytic Function of Lipoic Acid

With the exception of GCV, the lipoic acid-dependent enzyme systems form intricate complexes. The central cores of the complexes are made up of multimers of the liopoic acid-containing subunits (E2 subunits) arranged in octahedral or dodecahedral formation depending on the complex and the organism (Cronan et al., 2005). The cores are surrounded by homodimers of the two other subunits: the thiamine pyrophosphate (TPP)-containing E1 subunits which carry out the decarboxylation reactions (except for ADH where an acetaldehyde group is removed) and the NAD^+-containing E3 subunits, the dihydrolipoyl dehydrogenases. In most cases the E3 subunits of all the types of complexes in the cell are encoded by the same gene (*lpd*).

Lipoic acid is covalently attached in amide linkage to the ε amino group of a conserved lysine of the lipoyl domains (LDs) which protrude from the E2 subunits on flexible proline-rich linkers. E2 subunits may contain up to three of these LDs. The length and flexible linker of the LDs and their lipoate attachments create an

arm that enables translocation of reaction intermediates between the complex subunits (Perham, 2000).

Catalysis at the E1 subunits is initiated when C2 of the thiazole ring of TPP donates a proton to solvent forming a carbanion which nucleophilically attacks the carbonyl carbon of the incoming 2-oxoacid. This results in breakage of the carbon-carbon bond between the carbonyl and the carboxyl carbons of the substrate, releasing CO_2[2]. A double bond now links the substrate to TPP. The oxidized sulfur atom (at C8) of lipoic acid electrophilically attacks the double bond of the intermediate, carrying away the acyl chain (now one carbon shorter). The resulting thioester is finally attacked by the thiol of CoA in the E2 subunit, and lipoic acid is reoxidized by Lpd.

GCV is different from the dehydrogenases in that its subunits do not form a stable complex. Lipoate is attached to the GcvH subunit. Decarboxylation is carried out at the GcvP subunit which uses pyridoxal phosphate (PLP) as cofactor instead of TPP. The carbonyl carbon of PLP forms a Schiff base with the glycine amine nitrogen. The sulfur group of lipoic acid attacks the alpha carbon of glycine, CO_2 is released and the aminomethyl intermediate is carried to the GcvT subunit. The aminomethyl is then split into ammonia and a methyl group by two consecutive nucleophilic attacks by N5 and N10 of THF. Finally lipoate is reoxidized by Lpd.

Lipoic Acid as a Reductant

In the complexes described above, lipoic acid functions in an oxidative capacity. The two sulfur atoms in the active form of the cofactor are in disulfide linkage. Upon completion of a catalytic cycle, the resulting dihydrolipoate (“used” lipoate) is reoxidized for a new round of catalysis. The electrons are passed to an FAD^+ moiety in Lpd and ultimately to NAD^+. It is conceivable that the redox cycle would run in reverse (Fig. 1.1). In this case dihydrolipoate would act as an electron donor in reduction reactions, itself being reduced by Lpd with electrons from

[2] In the case of ADH, the carbon-carbon bond between the carbonyl and secondary alcohol carbon is broken, releasing acetaldehyde.

NADH. Indeed, in 2002 Bryk *et al.* characterized a peroxiredoxin from *Mycobacterium tuberculosis*, AhpC, which protected the bacterium from host peroxides and peroxynitrite. The AhpC reductase was shown to be encoded by a gene directly downstream of and likely cotranscribed with *ahpC* named *ahpD.* By fractionation of *M. tuberculosis* lysates, Bryk *et al.* identified Lpd as the reductant of AhpD. Purified Lpd required lipoamide (6,8-dithiooctanoic amide; Fig. 1.1) to sustain NADH-dependent AhpD reduction. Thus Bryk *et al.* proposed the formation of a peroxidase complex consisting of AhpC, AhpD, Lpd and SucB (the E2 subunit of OGDH and the organism's sole lipoylated protein) (Bryk et al., 2002).

Along similar lines, Cussiol *et al.* demonstrated that the Ohr (organic hydroperoxide resistance protein) of the plant pathogen, *Xylella fastidiosa,* was reduced by lipoate and Lpd as well as LpdA, but not by the thioredoxin/thioredoxin reductase (Cussiol et al., 2010). The *X. fastidiosa* LpdA protein is interesting in that it is an Lpd containing its own LD. If indeed lipoic acid and Lpd can serve in reductive chemistry, this might enable us to assign function to "orphan" Lpd proteins, i.e. *lpd*-encoding genes in genomes which don't encode any lipoate-dependent enzymes; and/or for the growing class of LpdA proteins some of which are also "orphan" (Hakansson and Smith, 2007, Hudson et al., 2006, Li de la Sierra et al., 1997, Smith et al., 2002).

The idea of lipoic acid acting as a reductant is corroborated by a recent study by Feeney *et al*. These workers constructed an *E. coli* strain that is defective in both thioredoxin reductase and glutaredoxin reductase (*trxB* and *gor,* respectively). This strain is unable to grow because the essential enzyme ribonucleotide reductase (RNR) is in the nonfunctional oxidized state. Suppressors were isolated and mapped to the *lpd* gene where they resulted in reduced enzyme activity. Lpd-defective strains should not be able to grow on glucose minimal media because lipoic acid would accumulate in the reduced form, bringing PDH and OGDH activities to a halt. However *lpd*-inactivating mutations in the strain background of Feeney *et al.* were able to grow on glucose minimal. Feeney *et al.*

propose that in the *trxB gor lpd* strains they studied two pathways were rewired into one whereby electrons from reduced lipoate were channeled to the reduction of RNR. Using bioinformatics, they further identified a list of organisms whose genomes lacked the capacity for glutathione biosynthesis, but did encoded genes for glutaredoxins and lipoic acid biosynthesis (Feeney et al., 2011).

Further support for the role of lipoic acid in reduction chemistry comes from a study of ancient metabolism, which concluded that the original role of lipoic acid was to function as a reductant in the CO_2 fixation reaction carried out by GCV (i.e. the reverse of the process described above) (Braakman and Smith, 2014). Braakam and Smith created a metabolic reconstruction of *Aquifex aeolicus,* one of the oldest living species (and an autotroph), based on genome sequence. Braakam and Smith found that the genome of the bacterium lacked genes for PDH and OGDH, but contained those of GCV and lipoic acid biosynthesis. Interestingly the lipoate ligase homologue of *A. aeolicus* was more similar to the *Bacillus* LipM*,* which specifically modifies GCV, than to the *E. coli* LplA which modifies the LDs all three enzyme systems (see below). Furthermore, the *A. aeolicus* genome lacks an LipL homologue, which in the case of *Bacillus* would be responsible for modifying PDH, OGDH and BCDH.

Lipoic Acid Synthesis and Attachment

Since the initial demonstration, purification and synthesis of lipoic acid (lipoate), much has been learned about the synthesis of this essential respiratory co-factor in prokaryotes (Cronan, 2008, Cronan et al., 2005) (Fig. 1.2). In *E. coli*, lipoate is assembled on the LD from the eight-carbon fatty acid, octanoate, in two steps. First, an octanoyltransferase (LipB) transfers an octanoyl moiety from the acyl carrier protein (ACP) of fatty acid synthesis to the LDs (Jordan and Cronan, 2003, Zhao et al., 2005). A thioester-bound acyl-enzyme intermediate is formed in the process (Zhao et al., 2005). Then lipoyl synthase (LipA) catalyzes replacement of single hydrogen atoms at carbons 6 and 8 with sulfur atoms, using radical SAM chemistry (Booker et al., 2007, Miller et al., 2000).

Alternatively, exogenous lipoate or octanoate can be directly attached to LDs by lipoate protein ligase (LplA) via an acyl–AMP intermediate.

Although the LipA sulfur insertion reaction is conserved in all characterized lipoate synthesis pathways, there are three well-characterized variations to the *E. coli* pathway in the other reactions. In the lipoate auxotrophic archeon *Thermoplasma acidophilum*, the lipoate protein ligase is composed of two separately encoded subunits, LplA and LplB, that together catalyze formation of the acyl–AMP intermediate (Posner et al., 2009, Christensen and Cronan, 2009). LplB was found to be dispensable for the second half of the reaction, viz. ligation of the acyl moiety to the acceptor lysine on the LDs (Christensen and Cronan, 2009). The Gram-positive bacterium *Bacillus subtilis* lacks a LipB homologue but encodes three LplA homologues, although only LplJ has the ligase function of *E. coli* LplA (Martin et al., 2011). One of the two remaining homologues (LipM) is an octanoyltransferase that specifically transfers octanoate from ACP to GcvH (Christensen and Cronan, 2010), whereas LipL, the third homologue, is an amidotransferase that transfers the octanoyl moiety from GcvH to the LDs of PDH, OGDH and BCDH (Christensen et al., 2011b, Martin et al., 2011). LipL is also present in *Listeria monocytogenes*, along with two other LplA homologues that are ligases. LplA1 specifically modifies GcvH, whereas the LD specificity of LplA2 is undetermined (Christensen et al., 2011a). The lipoate scavenged by *L. monocytogenes* LplAs might come directly from the host environment or might be released from host-lipoylated peptides by the action of the lipoamidase(s) encoded by this bacterium.

Octanoyltransferases, amidotransferases and lipoate ligases are difficult to distinguish from one another by examination of their amino acid sequences. Members of the three groups share minimal amino acid sequence identity yet have similar structural folds (Ma et al., 2006, Reche, 2000, Christensen et al., 2011a). A case in point is LipM, which has the sequence of a lipoate ligase but is an octanoyltransferase rather than a ligase (Christensen and Cronan, 2010). Whereas octanoyltransferases lack an accessory domain, ligases may contain

the accessory domain at either the C-terminus of the same protein such as the case with *E. coli* LplA (Fujiwara et al., 2010, Fujiwara et al., 2005), or at the N-terminus, such as the case for *Streptomyces coelicolor* LplA (Cao and Cronan, personal communication) (Christensen and Cronan, 2009), or the accessory domain may be encoded by a completely separate gene, such as the *T. acidophilum* LplB (McManus et al., 2006, Posner et al., 2009, Christensen and Cronan, 2009).

The diversity in prokaryotic lipoate attachment mechanisms is surprising given the simple structure of the coenzyme. The situation in eukaryotes is not well understood, and may contribute further enzymatic diversity. In yeast, fungi, plant and animal cells, lipoate-dependent enzyme systems as wells as lipoate synthesis enzymes are targeted to the mitochondria. Additionally plant chloroplasts contain PDH. Work from the Wada lab has shown that *Arabidopsis thaliana* contains two LipA enzymes, one targeted to mitochondria, the other to chloroplasts (Yasuno and Wada, 2002, Wada et al., 2001). The *A. thaliana* genome encodes three LipB/LplA-type enzymes; one for each of the mitochondria and the chloroplasts and the third is still under study (Yasuno and Wada, 2002). Since the plant cellular compartments are expected to contain acyl-ACPs (and thus octanoyl-ACP), but no free fatty acids or lipoate, it may be postulated that the LipB/LplA enzymes characterized thus far are octanoyltransferases, although no enzymology has been conducted to confirm this. On another front, Kang *et al.* cloned a gene from *Oryza sativa* which complemented an *E. coli lipB lplA* strain both in the presence and absence of exogenous lipoate (although in liquid culture complementation was better in the absence of lipoate) (Kang et al., 2007). This enzyme merits further study.

In apicomplexan parasites, PDH is located in the plastid-derived organelle called the apicoplast, while all other lipoate-depended systems localize to the singular mitochondrion of the cell (Thomsen-Zieger et al., 2003). Additionally the apicoplast contains a type II fatty acid biosynthetic pathway complete with an ACP (Thomsen-Zieger et al., 2003). It is therefore not surprising that enzymes of

endogenous lipoate synthesis, LipA and LipB, are found exclusively in apicoplasts (Wrenger and Muller, 2004). Inhibiting apicoplast fatty acid biosynthesis or knocking out the apicoplast ACP encoding gene eliminates lipoate biosynthesis in the organelle (Crawford et al., 2006). One of the two LplAs found in these organisms, LplA1, is targeted to the mitochondria (Allary et al., 2007). Gunther *et al.* showed that LplA1 is essential for *Plasmodium falciparum,* whereas LipB is not (Gunther et al., 2007). LipB is probably dispensable because of the presence of a second LplA, LplA2, which is targeted to both apicoplasts and mitochondria (Gunther et al., 2007). However the exact enzymatic nature of LplA2 remains unclear. It seems that LplA2 is an amidotransferase since labeling studies conducted by Allary *et al.* with radioactive lipoate showed labeling of mitochondrial proteins but not the apicoplast PDH (Allary et al., 2007); i.e. LplA2 was not able to utilize free lipoate. LplA2 merits more study, especially since the LplA2 of *Toxoplasma gondii* seems strikingly different from that of *P. falciparum* at least at the amino acid sequence level (Gunther et al., 2009).

Early studies of the bovine LplA homologue (LipT; for lipoyltransferase) demonstrated that the enzyme was unable to form the acyl–AMP intermediate (Fujiwara et al., 1994). However, LipT was able to transfer the acyl chain from exogenously provided acyl–AMP to LDs *in vitro*, giving rise to the proposal that mammalian lipoate attachment requires a 'lipoate-activating enzyme'. Accordingly, Fujiwara *et al*. purified an enzyme from bovine liver that synthesized lipoyl-GMP *in vitro*, which in turn could be utilized by LipT to modify LDs (Fujiwara et al., 2001). However, this putative lipoate-activating enzyme is one of many poorly characterized medium-chain fatty acyl–co-enzyme A (CoA) ligases that have extremely broad substrate specificities (Kasuya et al., 2006, Vessey et al., 2000) and thus the physiological relevance of its *in vitro* lipoyl-GMP-forming activity is unclear.

Studies by Schonauer *et al.* (2009) on the yeast *S. cerevisiae* showed that Lip2, Lip5, Lip3 (the LipB, LipA and LplA homologues respectively) as well as Gcv3 (the GcvH homologue) are all required for endogenous lipoate synthesis and

attachment (Schonauer et al., 2009). Respiratory growth of a *lip2* strain, which contains a wild type copy of the *lip3* gene, is not supported by exogenous lipoate supplementation (Chen, 1997, Marvin et al., 2001), suggesting that *S. cerevisiae* lacks a lipoate scavenging pathway. On the other hand, early studies by Sulo and Martin (Sulo and Martin, 1993) showed that even though lipoate did not support the respiratory growth of *lip5* strains, the cells contained a substantial level of protein-bound lipoate when grown on lipoate-supplemented media, suggesting the presence of a scavenging mechanism in *S. cerevisiae.*

Genetic Diseases Caused by Lipoic Acid Synthesis Defects

The number of genetic disorders with recognized causation related to mitochondrial function and specifically PDH, OGDH and GVC function is growing. Only one human case of a genetic defect in a lipoic acid synthesis gene has been reported to date. A boy with a homozygous point mutation in the *lipA* gene suffered epileptic seizures starting at two days of age. His condition progressively worsened as he developed muscular hypotonia, lactic acidosis, elevated glycine concentrations in blood and urine, respiratory deficiency, and lung inflammation. The boy eventually died of a severe respiratory tract infection at age four (Mayr et al., 2011).

Genetic manipulations of mice have offered more insight into the central role played by lipoic acid in health. In 2005 Yi and Maeda were able to inactivate the *lipA* gene in mice embryonic stem cells. Unlike in the human case reported above, none of the homozygous null mutants survived to birth. Supplementing the mothers' diets during pregnancy with up to 1.65 g/kg body weight of lipoic acid failed to prevent prenatal death, suggesting that endogenous synthesis of lipoic acid is essential at least during development in mice (Yi and Maeda, 2005).

A similar yet more extensive study was conducted by Smith *el al.* (2012) who knocked out the gene encoding mitochondrial malonyl CoA:ACP transacylase (*Mcat*) in mice. The reaction catalyzed by Mcat initiates mitochondrial fatty acid biosynthesis which produces the octanoyl moiety from which lipoate is synthesized. Smith *et al.* observed a plethora of phenotypic changes in the

knockout mice compared to wild type controls amongst which were weight loss despite higher food consumption, hypothermia, anemia, lactic acidosis and reduced stamina. Closer examination revealed compromised mitochondrial function probably due to the observed reduction in lipoylated proteins. Finally by Western blot analysis Smith *et al.* also found that LipT is primarily expressed in liver tissue (Smith et al., 2012), which could explain why lipoic acid diet supplements were not useful in the Yi and Maeda study

Specific Aims of This Work

There are two parts to this project. In the first part, I studied an *E. coli* Δ*lipB* mutant strain. This strain is defective in endogenous lipoic acid synthesis and thus its aerobic growth on glucose minimal media is strictly dependent on supplementation with either lipoate (or octanoate), or acetate plus succinate which respectively bypass the PDH- and OGDH-catalyzed steps required for TCA cycle function (Herbert and Guest, 1968). I isolated Δ*lipB* suppressor strains. Initial characterization distinguished two types. In one type (discussed in Chapter 2) the mutations causing suppression of the Δ*lipB* defect were mapped to the *lplA* locus. In the second type (discussed in Chapter 3), the causative mutation mapped to the *sdhB* gene, which encodes a subunit of succinate dehydrogenase.

In the second part of this project I sought to further investigate the role of the lipoate-protein ligase homologue, Lip3, in lipoic acid synthesis in *S. cerevisiae*. In Chapter 4, I show that Lip3 has octanoyl-CoA: protein transferase activity and present a model for lipoic acid synthesis in *S. cerevisiae*.

Figures

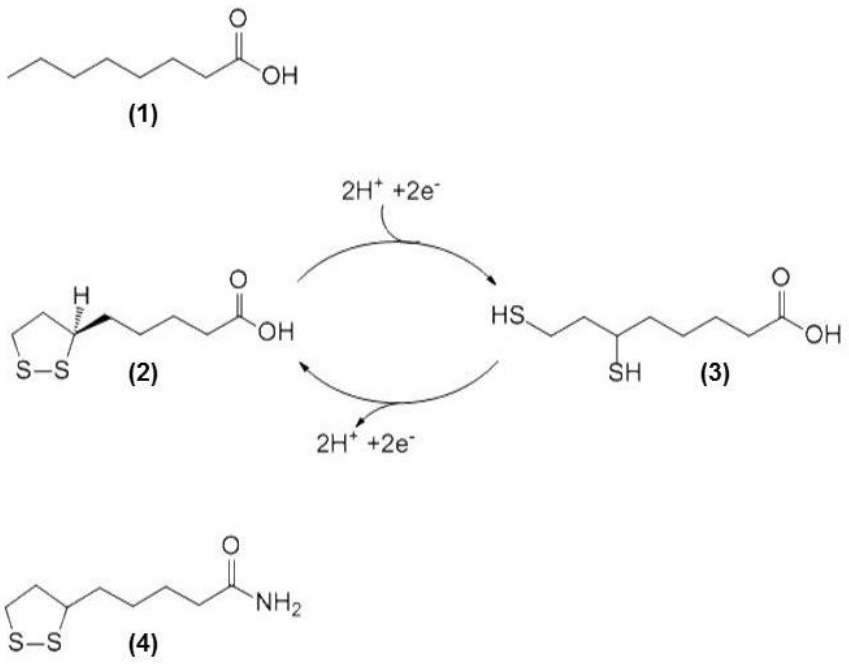


Figure 1.1: Structure of lipoic acid and its precursor. The eight-carbon fatty acid, octanoate (1), is the precursor of lipoic acid (2). Lipoic acid undergoes redox cycling between the oxidized (2) and reduced dihydrolipoate (3) forms. Lipoamide (4) mimics the structure of lipoic acid in amid linkage.

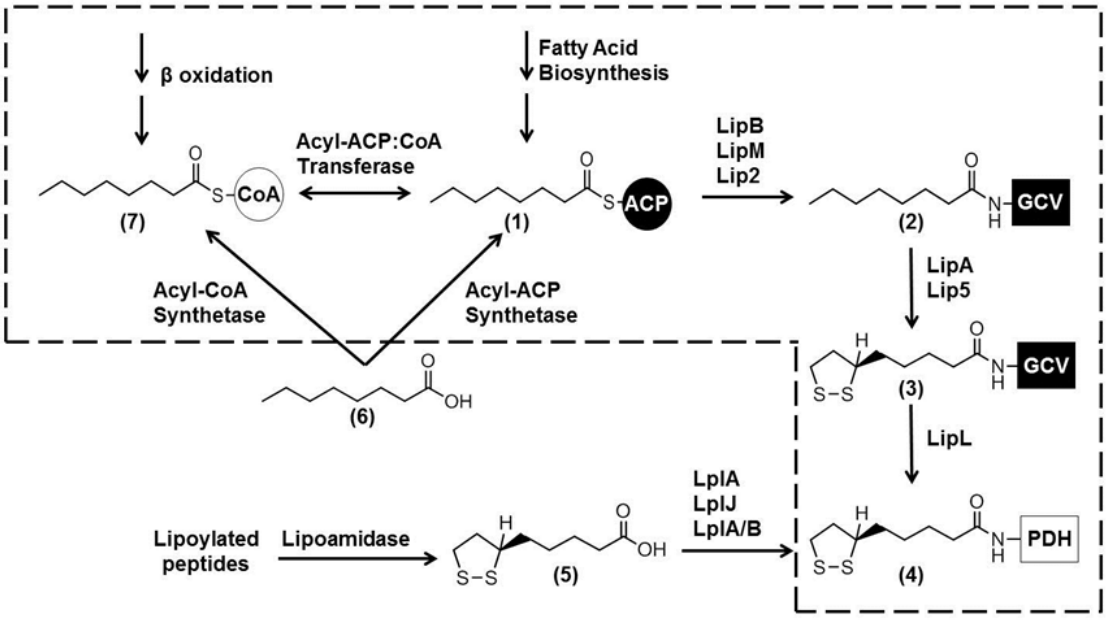


Figure 1.2: Diversity of lipoate attachment mechanisms. In the biosynthetic pathways (dashed box), octanoyl-ACP (1) is extracted from the pool of fatty acid biosynthesis intermediates by an octanoyltransferase (LipB in *E. coli*, LipM in *B. subtilis*, or Lip2 in *S. cerevisae*) which transfers the octanoyl moiety to the LDs (e.g., that of the glycine cleavage system; black box). This is followed by sulfur insertion by a lipoate synthase (designated LipA in prokaryotes and Lip5 in *S. cerevisiae*). The LipL amidotransferases transfer the lipoyl moiety from lipoyl-GCV (3) to pyruvate dehydrogenase (white box), 2-oxoglutarate dehydrogenase and the branched chain oxoacid dehydrogenase (not shown). In the scavenging pathways, free lipoic acid (5) is directly ligated onto the LDs by a lipoate-protein ligase (LplA of *E. coli,* LplJ of *B. subtilis* or the LplA/B complex of *T. acidophilum)*. This figure is from (Hermes and Cronan, 2013).

Chapter 2: Scavenging of Cytosolic Octanoic Acid by Mutant LplA Lipoate Ligases Allows Growth of *Escherichia coli* Strains Lacking the LipB Octanoyltransferase of Lipoic Acid Synthesis[3]

Introduction

Escherichia coli has three lipoic acid-dependent enzyme systems; pyruvate dehydrogenase (DPH), 2-oxoglutarate dehydrogenase (OGDH), and the glycine cleavage system (GCV) (Cronan et al., 2005). PDH catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA, the tricarboxylic acid (TCA) cycle substrate and fatty acid building block. OGDH functions in the TCA cycle where it catalyzes the decarboxylation of 2-oxoglutarate to succinyl-CoA, the precursor of several amino acids. GCV is involved in the breakdown of glycine into ammonia and C1 units. Whereas GCV is expressed only in the presence of glycine, PDH and OGDH are required for aerobic growth. (During anaerobic growth acetyl-CoA is synthesized by other enzymes and an OGDH-independent branched form of the TCA cycle forms succinyl-CoA from succinate.) The three enzyme systems contain subunits (the E2 subunits of PDH and OGDH, and the H protein of GCV) which contain at least one lipoyl domain, a conserved structure of *ca*. 80 residues (Cronan et al., 2005). Lipoic acid is attached in an amide bond to a specific lysine residue of these domains where it functions as a classical “swinging arm”, carrying reaction intermediates between the active sites of the lipoate-dependent systems (Perham, 2000).

Lipoic acid (*R*-5-(1,2-dithiolan-3-yl)pentanoic acid, also called 6,8-dithiooctanoic acid and thioctic acid) is composed of an eight carbon fatty acid backbone to which two sulfur atoms are attached at carbons 6 and 8 (Fig. 2.1). In the oxidized state, the sulfur atoms are in disulfide linkage forming a five-membered ring with

[3] Results presented in this chapter are published in the *Journal of Bacteriology* (2009) 191(22):6796-803.

three backbone carbons. The disulfide bond is reduced upon binding of the intermediates (an acetyl moiety in the case of PDH, a succinyl moiety in the case of OGDH and an aminomethyl moiety in the case of GCV). Following release of the intermediates to form the products of the enzyme complexes, the reduced lipoyl moiety must be reoxidized before entering another catalytic cycle. Oxidation is catalyzed by lipoamide dehydrogenase, a subunit component of the three lipoic acid-dependent enzyme systems (Cronan et al., 2005). *E. coli* strains defective in lipoic acid biosynthesis are unable to grow on aerobic glucose minimal media unless the media are supplemented with acetate and succinate to bypass the need for the two lipoic acid-dependent dehydrogenases (Herbert and Guest, 1968, Perham, 2000).

Studies in our laboratory and others' have elucidated the lipoic acid synthesis pathway of *E. coli* (Fig. 2.1). The LipB octanoyl-[acyl carrier protein]:protein *N*-octanoyltransferase (Jordan and Cronan, 2003, Zhao et al., 2003, Zhao et al., 2005) transfers the octanoyl moiety from octanoyl-acyl carrier protein (ACP), a fatty acid biosynthetic intermediate to lipoyl domains. This reaction proceeds through an acyl enzyme intermediate in which the octanoyl moiety is in thioester linkage to a conserved cysteine residue in the enzyme active site (Ma et al., 2006, Zhao et al., 2005). The thioester bond is then attacked by the ε-amino group of the target lipoyl domain lysine residue to give the amide linked lipoate moiety. The product of this catalysis, octanoyl-domain, is the substrate of the LipA lipoate synthase, an S-adenosylmethionine radical enzyme which inserts sulfur atoms at carbons 6 and 8. In addition to the LipB-LipA pathway of lipoic acid synthesis, *E. coli* also contains an enzyme that scavenges lipoic acid from the growth medium, the LplA lipoate:protein ligase. LplA uses ATP to activate lipoic acid to lipoyl-adenylate, the mixed anhydride of which is attacked by the lipoyl domain lysine reside to give the lipoylated domain (Fig. 2.1). LplA is also active with octanoic acid and efficiently attaches exogenous octanoate to lipoyl domains both *in vivo* and *in vitro* (Green et al., 1995, Morris et al., 1995, Zhao et al., 2003, Morris et al., 1994). Null mutants in *lplA* have no phenotype in strains having an intact lipoic acid synthesis pathway (Morris et al., 1995)

The subject of this report is the behavior of *lipB* null mutants which (as expected from the above discussion) are lipoic acid auxotrophs (Morris et al., 1995, Vanden Boom et al., 1991). Growth of *lipB* strains can also be supported by octanoate supplementation of the medium (Zhao et al., 2003). Upon plating of *lipB* null mutants on minimal glucose medium, colonies arise that no longer require lipoic acid (Morris et al., 1995). These are suppressor mutations because the block in lipoic acid synthesis remains. Suppression in the strains studied in this work maps to the *lplA* gene.The LplA proteins encoded by these suppressor mutants are point mutations that greatly decrease the Michaelis constant for free octanoic acid and allow efficient scavenging of cytosolic octanoate.

Materials and Methods

Bacterial strains and plasmids:

Strains used in this study (Table 2.1) are derivatives of *E. coli* strains MG1655 and W3110. Transduction using phage P1vir and transformation were conducted following conventional methods (Miller, 1992). Strain FH6 was made by transducing strain MG1655 to kanamycin resistance with a P1 phage stock grown on strain KER184. Strains FH26 and FH27 were isolated by plating strain FH6 on glucose minimal plates followed by incubation of the plates for 2-3 days at 37^0C. The tetracycline resistance marker of transposon Tn10 was introduced upstream of the *lplA* gene in strains FH26 and FH27 by transduction with a P1 phage stock grown on strain CAG18430 (Singer et al., 1989) to give strains FH66 and FH35 respectively. The *lplA* genes of FH66 and FH35 were transferred into strain ZX221 by P1 transduction followed by screening for growth on glucose minimal plates in the absence of supplementation. The resulting strains were designated strains FH145 and FH146, respectively. Strains FH46 and FH47 were made by transducing the tetracycline resistance marker from TM135 into strains FH26 and FH27, respectively, with selection on LB plates supplemented with succinate and acetate. Strains FH57 and FH58 were obtained by transducing FH26 and FH27 with a P1 phage stock grown on KER310 with selection on LB plates supplemented with lipoic acid, kanamycin and tetracycline.

Plasmids pFH1 and pFH2 which, respectively, express the S221P and V19L LplAs under control of an IPTG inducible promoter were made by site directed mutagenesis of plasmid pYFJ16 by the QuikChange procedure (Stratagene).

Media and growth conditions:

Luria-Bertani and minimal E media were prepared as described (Miller, 1992). Supplements were added at the following concentrations: glucose, 0.4%; glycerol, 0.4%; succinate, 5 mM; acetate, 5 mM; and lipoic acid, 7.5 ng/ml. Antibiotics were used at the following concentrations (in µg/ml) ampicillin, 100; spectinomycin, 100; tetracycline, 15; kanamycin, 50 and chloramphenicol, 20. The growth temperature was 37^0C.

DNA extraction, PCR and sequencing:

DNA was extracted using the Promega Wizard Genomic DNA Purification Kit. PCR was conducted using either Taq (New England Biolabs) or Pfu (Invitrogene) DNA polymerases and appropriately designed primers per manufacturers' recommendations. DNA sequencing was conducted by the Core Sequencing Facility of the Carver Biotechnology Center of the University of Illinois, Urbana Champaign.

Assays of PDH and OGDH activities:

A method similar to that of (Guest and Creaghan, 1973) was used. Cultures were grown to late exponential phase in 100 ml of glucose minimal media supplemented with succinate and acetate. The cells were then collected by centrifugation, washed twice with 10 ml of 150 mM Tris-HCl buffer (pH 8.5), frozen in a dry ice-ethanol bath and stored overnight at -20 ^{0}C. The next day the cell pellet was thawed on ice. The cells were suspended in 1 ml of 150 mM Tris-HCl buffer (pH 8.5) per mg wet cell weight and lysed by passage twice through a French Press cell at 1000 psi. The crude extract was collected on ice, cleared by centrifugation and the protein concentration determined by the BioRad Bradford assay (Bradford, 1976) using a standard curve of bovine serum albumen. Crude

extract protein preparations (100-300 µg) were added directly to cuvettes containing the assay reaction mixture consisting of 150 mM Tris-HCl buffer (pH8.5), 3 mM L-cysteine hydrochloride as reducing agent, 0.1 mM CoA and 5 mM thiamine pyrophosphate in a 500 µl volume. PDH assay mixtures also contained 5 mM $MgCl_2$. 3-Acetylpyridine adenine dinucleotide (APAD; Sigma-Aldrich) was then added to a final concentration of 2 mM, and the mixture allowed to equilibrate at room temperature for 15 min. The reaction was started by adding either 5 mM pyruvate (PDH activity) or 5 mM 2-oxoglutarate (OGDH activity). Increased absorption at 366 nM corresponding to reduction of APAD was monitored for 5 min in a Beckman DU640 spectrophotometer. The activities of the complexes were calculated in the range of linearity between initial reaction velocity and protein concentration. The extinction coefficient of the reduced form of the cofactor used in the calculation was 7.0 nM^{-1} cm^{-1} (Christensen and Cronan, 2009).

Detection of *in vivo* lipoation by western blotting:

Strains carrying plasmid pGS331 which encodes a hybrid PDH lipoyl domain under control of the tac promoter (Ali and Guest, 1990) were cultured to late exponential phase in 15 ml of glucose minimal media supplemented with succinate, acetate, ampicillin and IPTG (1 mM), with or without octanoic acid (50 µM). The cells were collected, washed twice with 1 ml of 20 mM Tris-HCl (pH 7.5) and stored at -20 ^{0}C overnight. The following day the pellet was thawed on ice and resuspended in 500 µl of the same buffer. The cells were lysed in a Misonix sonicator (two 5 min intervals with 1 min cooling in between). The extract was cleared by centrifugation and protein concentration determined as above. A 20% native polyacrylamide gel was loaded with 100 µg of extract protein and following electrophoresis the proteins were then transferred to a PVDF membrane using a standard protocol (Ausubel et al., 2007). Lipoylated domain was detected using rabbit anti lipoyl protein primary antibodies from Calbiochem and goat anti rabbit antibody from Roche.

Protein expression and purification:

Hexahistidine-tagged (N-terminal) versions of the wild type, V19L and S221P LplAs were purified using Qiagen nickel nitrilotriacetic acid columns as recommended by the manufacturer. Contaminants which copurified with the proteins were removed using a modification of a prior procedure (Green et al., 1995). The proteins were adsorbed to a Vivapure Maxi H type Q columns equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 10% glycerol and 0.1 mM phenylmethanesulfonylfluoride. The LplA proteins were eluted with the same buffer containing 120 mM ammonium sulfate. Protein concentrations were measured by absorbance at 280 nm using a molar extinction coefficient of 46250 M^{-1} cm^{-1} (Green et al., 1995)

Apo-lipoyl domain was expressed from pGS331 in strain QC146 and purified by a modification of the method of Ali and Guest (Ali and Guest, 1990). After titration of the pH of the crude extract to 4 and centrifugation, the pH of the supernatant was increased to 7 with 1 M NaOH. The extract was dialyzed overnight against 10 mM ammonium acetate buffer (pH 5) and then subjected to anion exchange chromatography on a POROS HQ column eluted with a 10-600 mM ammonium acetate gradient at pH 5. Protein concentrations were determined at 280 nm using a molar extinction coefficient of 2020 M^{-1} cm^{-1} (Ali and Guest, 1990).

Holo-acyl carrier protein (ACP) was expressed and purified from strain DK574 carrying plasmid pJT93 as described by Cronan and Thomas (2009). Histidine tagged *V. harveyi* acyl-ACP synthase was expressed from strain YFJ239 and purified using Qiagen nickel nitrilotriacetic acid columns as recommended by the manufacture. Octanoyl-ACP was synthesized as described by Cronan and Thomas (Cronan and Thomas, 2009).

Gel shift assay of LplA activity:

A 20 µl reaction mixture contained 10 mM sodium phosphate buffer (pH 7), 0.3 mM dithiothreitol, 30 µM apo-lipoyl domain and 5 µM enzyme. When checking for ligase activity, 5 mM $MgCl_2$, 5 mM ATP and various concentrations of lipoic acid

or octanoic acid were added. Alternatively, to assay octanoyltransferase activity octanoyl-ACP was added in place of ATP and octanoate/lipoate. The reactions were run for 2 h at 37 ^{0}C after which 10 µl was loaded on a 20% polyacrylamide gel containing 2.5 M urea and separated by electrophoresis.

Filter disk shift assay of LplA activity:

The reaction mixture was the same as that used in the gel shift assay except that the assay contained varying concentration of [1-^{14}C]octanoic acid. After equilibration at 37^{0}C, a sample was removed as a no enzyme control and enzyme added to a final concentration of 100 nM, which was determined to be within the range of linear activity with respect to enzyme concentration under these conditions (data not shown). Samples (15µl) were removed every 3 min for 15 min and spotted onto 2.3 mm diameter Whatman filter disks which had been presoaked in 5% trichloroacetic acid and dried. The disks were then dried and washed three times (10 min each wash) with a solvent mixture containing chloroform-methanol-acetic acid (3:6:1). After a final wash with absolute ethanol the disks were dried, added to vials containing 4 ml of scintillation cocktail, and counted in a Beckman Coulter LS 6500 scintillation counter (Jiang and Cronan, 2005). The data were analyzed using the Prism analysis program and K_m and V_{max} values were determined by the Edie-Hofstee method (Hofstee, 1952).

Fatty acid extraction and butyl ester synthesis:

Strain FH213 was grown in one liter of glycerol minimal media containing spectinomycin and ampicillin. When the culture reached stationary phase, IPTG was added to a final concentration of 1 mM and induction was allowed to proceed for 3 h. The cells were then collected and lysed by freezing in a dry ice-ethanol bath then thawed at room temperature. Fatty acids were converted to their sodium salts by mixing the cell extract with 0.1 M sodium bicarbonate overnight at 4^{0}C, after which the solution was acidified to pH 3 with glacial acetic acid and 3 ml hexane was added. After 1 h of stirring at 4^{0}C, the fatty acid-containing organic phase was collected and transferred to a 5 ml glass vial with a

plastic stopper. Heptanoic acid (1µg) was added to serve as internal standard. Butyl ester synthesis was conducted in a manner similar to Hallmann *et al.* (Hallmann et al., 2008). Butanol/BF_3 (0.1 ml) and 1 gm of anhydrous sodium sulfate were added, the vial was sealed with Parafilm followed by incubated in a 65^0C heating block for 2 h. The reaction was then cooled to room temperature. The solution was washed three times with 3 ml distilled water and concentrated under nitrogen gas to 100 µl of which 5 µl was analyzed by gas chromatography-mass spectrometry (GC-MS). Samples (5 µl) were injected in splitless mode to the GC/MS system consisting of an Agilent 6890N gas chromatograph, an Agilent 5973 mass selective detector and HP 7683B (Agilent Inc, Palo Alto, CA, USA) autosampler. Injections were performed on a 30 m HP-5MS column with 0.25 mm I.D. and 0.25 µm film thickness (Agilent Inc, Palo Alto, CA, USA) with an injection port temperature of 250^0C, the interface set to 250^0C, and the ion source adjusted to 230^0C. The helium carrier gas was set at a constant flow rate of 1 ml min^{-1}. The temperature program was 1 min isothermal heating at 40^0C, followed by an oven temperature increase of 5^0C min^{-1} to 180^0C for 1 min. The mass spectrometer was operated in positive electron impact mode (EI) at 69.9 eV ionization energy in m/z 50-550 scan range. The spectra of all chromatogram peaks were evaluated using the HP Chemstation (Agilent, Palo Alto, CA, USA) and AMDIS (NIST, Gaithersburg, MD, USA) programs.

Random mutagenesis of *lplA*:

The *lplA* gene was PCR amplified from FH6 with primers lplAfor and lplArev (Table 2.1) that are complementary to regions about 150 nucleotides upstream and downstream of the coding region, respectively. The PCR product was purified using the Qiagene PCR cleanup kit then subjected to either 12 or 35 cycles of error prone PCR (Ausubel et al., 2007). The mutagenized products were electroporated into FH6 expressing the λ Red recombinase system (Datsenko and Wanner, 2000). The cells were allowed to recover in glucose minimal medium supplemented with succinate and acetate for 90 min at 37 ^{0}C.

The cells were collected, washed three times with minimal medium and plated on glucose minimal plates.

Results

Suppression in strains FH26 and FH27 requires *lipA* and is due to point mutations in the *lplA* coding region.

Strain FH6 (a *lipB* knockout) is blocked in the first step in lipoyl protein synthesis and thus it lacks active PDH and OGDH complexes (Morris et al., 1995). Consequently, the strain is unable to grow aerobically on glucose minimal medium unless supplemented with either lipoic or octanoic acid which LplA attaches to PDH and OGDH (Morris et al., 1995). Growth also occurs when the medium is supplemented with acetate and succinate which bypass the enzymatic steps catalyzed by PDH and OGDH, respectively. Strains FH26 and FH27 were isolated as suppressors of the *lipB* null mutation in strain FH6 that grew on unsupplemented glucose minimal media. Introduction of a *lipA* null mutation into strains FH26 and FH27 (to give strains FH57 and FH58, respectively) by transduction resulted in loss of the ability to grow on glucose minimal media. These results indicated that suppression was caused by a pathway that resulted in octanoylation of PDH and OGDH. Previous work suggested that *in vitro* LplA has weak octanoyl transferase activity (Jordan and Cronan, 2003) and showed that overproduction of LplA alleviates the growth defect of *lipB* null mutants (Morris et al., 1995). We therefore asked if the growth of strains FH26 and FH27 was mediated by LplA. Two observations supported this hypothesis. First, transductional introduction of an *lplA* null mutation into strains FH26 and FH27 (to give strain FH46 and FH47, respectively) resulted in lack of growth on unsupplemented glucose minimal medium. Second, transductional backcrossing of the *lplA* genes (and linked regions) from strains FH26 and FH27 into a *lipB* knockout strain (to give strains FH145 and FH146, respectively) conferred the ability to grow on glucose minimal medium.

These data suggested two straightforward hypotheses for the observed octanoylation mediated by the mutant *lplA* alleles of strains FH26 and FH27.

First, the *lplA*-linked mutations may result in overexpression of LplA which is known to suppress the *lipB* phenotype (Morris et al., 1995). The second hypothesis was that the mutations were actually in the *lplA* coding region and resulted in mutant LplA proteins which catalyze octanylation more efficiently than does the wild type protein. This could be due to an increase in the putative octanoyltransferase activity of LplA or to a heretofore unrecognized pathway. To test these hypotheses the *lplA* genes and upstream regions of the strains FH26 and FH27 chromosomes were PCR amplified and the amplification products were sequenced. Both strains were found to carry a missense mutation within the *lplA* coding sequence. The *lplA* gene of strain FH26 contained a G58C point mutation which results in a V19L amino acid substitution (referred to here as *lplA10*) whereas strain FH27 contained a T664C point mutation that gives rise to a S221P amino acid substitution (referred to as *lplA11*).

The suppressor strains contain active lipoylated PDH and OGDH complexes.

The PDH and OGDH complexes contain essential lipoate-dependent subunits and thus their activities indicate *in vivo* lipoylation. We assayed the strains carrying either the *lplA10* (strain FH145) or the *lplA11* (strain FH146) alleles for PDH and OGDH activities in cells from cultures grown on glucose minimal medium supplemented with succinate and acetate. Both mutant strains had levels of PDH and OGDH activities similar to those of strain JK1, the wild type parental strain, whereas the activities of the isogenic *lipB* null mutant strain ZX221 were near background (Fig. 2.2). These data indicated that the LplA proteins encoded by strains FH145 and FH146 were able to activate the PDH and OGDH complexes.

In a second approach we expressed a PDH lipoyl domain in various strains grown on glucose minimal medium supplemented with succinate and acetate and asked whether or not the domain became lipoylated. Since all of the strains contained a functional LipA lipoyl synthase, any octanoyl-domain formed would be converted to lipoyl-domain which could be detected by western blotting with

anti-lipoate antibody (the antibody does not recognize octanoyl-domains, Q. Christensen and J. E. Cronan, unpublished data). When the medium was supplemented with octanoic acid (Fig. 2.3B), the wild type strain, a *lipB* null strain and the two strains with mutant *lplA* genes (FH145 and FH146, lanes 5 and 6) accumulated lipoylated domain because these strains contained active LplA which catalyzed attachment of exogenous octanoic acid to the unmodified domain where it was converted to lipoate by LipA. Likewise an *lplA* strain also accumulated lipoylated domain due to LipB function. In contrast strains FH145 and FH146 grown in the absence of octanoic acid supplementation contained detectable levels of domain modified with lipoate whereas the *lipB* strain did not (Fig. 2.3A). These data indicated that the suppressor strains were able to attach octanoate to the lipoyl domain in the absence of exogenous octanoic acid. (The modified domains of the wild type and *lplA* strains resulted from LipB activity).

The mutant LplA proteins efficiently scavenge octanoic acid.

As stated earlier, previous work has suggested that LplA utilizes octanoyl-ACP (Jordan and Cronan, 2003, Zhao et al., 2003). To test the utilization of octanoyl-ACP we purified histidine-tagged versions of wild type, V19L and S221P LplA proteins and tested their octanoyltransferase activity using the gel shift assay. In this assay an unmodified hybrid lipoyl domain is provided as substrate along with octanoyl-ACP (Zhao et al., 2005). Attachment of octanoic acid to the appropriate lysine residue on the lipoyl domain results in loss of a positive charge which causes the domain to migrate more rapidly than the unmodified protein in native gel electrophoresis. In our initial experiments, we observed activity with V19L and S221P LplAs, but not with wild type LplA using as substrate octanoyl-ACP preparations purified by precipitation and washing of the precipitate. However, although octanoylated domain was synthesized, we did not see the expected concomitant release of holo-ACP. We subsequently found that use of preparations of octanoyl-ACP that were extensively dialyzed following acyl-ACP synthetase-catalyzed synthesis of octanoyl-ACP to remove excess octanoic acid, ATP and $MgCl_2$ gave no activity with the LplA proteins whereas the preparations

were active with LipB (Fig. 2.4). These results indicated that the mutant LplAs did not utilize octanoyl-ACP, but rather used the traces of free octanoic acid, ATP and $MgCl_2$ that remained in the octanoyl-ACP preparations purified by precipitation and washing. The fact that we observed a reaction with the mutant LplA proteins, but not with the wild type LplA was the first indication that the mutant LplA proteins may have reduced K_m values for one of the substrates, probably octanoate. This was confirmed by assaying [1-14C]octanoic acid attachment to lipoyl domains by the filter disk assay (Materials and Methods) (Table 2.2). The two mutant LplAs had K_m values for octanoate that were 50 (the S221P mutant) or 20-fold (the V19L mutant) lower than that of the wild type enzyme. The mutations also decreased the V_{max} values of the two enzymes with the S221P protein showing a decrease of 10-fold.

***E. coli* contains free octanoic acid.**

The multiple lines of evidence obtained both *in vivo* and *in vitro* indicated that the mutant LplA proteins must suppress *lipB* null mutants by utilizing cytosolic octanoic acid. This argued that there must be an intracellular pool of this fatty acid, although there were no data in the literature to support this premise. Intracellular pools of short chain free fatty acids, such as octanoate had not been reported in *E. coli*, but this could readily be explained by the slight solubility of octanoic acid in water (ca. 0.7 g/L) plus the volatility of the acid and especially of its methyl ester, the most commonly analyzed derivative. To test for the presence of octanoate we first trapped the short chain acids as their non-volatile sodium salts and then converted the salts to the butyl esters which are much less volatile than the methyl esters.

Free fatty acids were extracted from wild type cells grown in glycerol minimal medium carrying the lysis plasmid pJH2 used in order to allow gentle disruption of the cellular membrane (Crabtree and Cronan, 1984). After lysis the fatty acids were converted to their sodium salts and subsequently acidified to break down the salts and allow extraction of the acids into hexane. Heptanoic acid (1µg) was added as an internal standard and the fatty acids were esterified with butanol in

the presence of acid (BF_3). The butyl esters were detected by GC-MS (Fig. 2.5). A total of 712 ng of octanoic acid was extracted from one liter of cells (about 1.18 x 10^{12} cells). Interestingly, decanoic acid was also detected (Fig. 2.5). Since quantitative extraction and derivatization could not be assumed we determined the efficiency of our extraction by spiking an extract with [1-14C]octanoic acid. One liter of cells was grown, collected and lysed as above. [1-14C]Octanoic acid was added to the extract and the usual extraction procedure was followed as described in Materials and Methods. After the final step (concentration under N_2), the extracted [1-14C]octanoic acid radioactivity was determined by a scintillation counting. Only 17% of the initial radioactivity was recovered. In a separate experiment we determined the efficiency of the butyl ester formation and found it to be quantitative.

Other mutant LplA proteins that suppress a *lipB* null allele.

The *lplA* gene was randomly mutagenized by error prone PCR. The PCR products were transformed into strain FH6 expressing the λ Red recombinase system enzymes (Datsenko and Wanner, 2000). After recovery the cells were plated on glucose minimal media without supplementation. A total of 13 colonies were isolated and the *lplA* gene from each of these colonies was PCR amplified cloned into pCR2.1 TOPO cloning vector and sequenced. Four isolates had the same point mutation as the V19L LplA. Another had the same point mutation as the S221P mutation and two isolates had no mutations within the coding sequence. The remaining six isolates had *lplA* genes containing multiple point mutations (Table 2.3).

Discussion

In this work we isolated and studied two spontaneously arising *lipB* suppressor strains and determined that suppression was caused by single point mutations in the LplA protein which reduce the K_m for free octanoic acid. The V19L mutation resulted in a 20-fold decrease in the K_m for octanoic acid whereas the S221P mutation resulted in a 50-fold decrease. Since the suppressor strains grew and formed lipoyalated proteins in the absence of octanoic acid supplementation, an

in vivo synthesized pool of free octanoic acid must be present in *E. coli*. Indeed we were able to extract 712 ng of free octanoic acid from the cells of a 1 L culture. Taking the cell volume as 0.88 x 10^{-15}L (www.ecocyc.org) and correcting for the low efficiency with which we extracted octanoic acid from cell extracts (17%), this indicates an intracellular octanoate concentration of about 28.2 µM. This intracellular concentration is well above the K_m values of the V19L and S221P LplA enzymes, but 7.6-fold lower than the K_m value of wild type enzyme. Hence, *lipB* strains encoding mutant LplAs (either the V19L or the S221P proteins) are able to synthesize lipoylated proteins (Fig. 2.3) and thus active PDH and OGDH complexes (Fig. 2.2). It is important to note however that despite the benefit gained in these mutant LplAs in terms of reducing the K_m for octanoate, this was not without consequence; both enzymes had decreased V_{max} values. This could explain why these point mutations do not naturally occur in wild type strains. Interestingly, these two residues are far removed from the active site predicted from the *E. coli* LplA-lipoic acid cocrystal structure of Fujiwara *et al.* (Fujiwara et al., 2005). However, we believe the *E. coli* LplA-lipoic acid complex is an experimental artifact because the lipoic acid molecules were heterogeneously bound and were poorly resolved (Fujiwara et al., 2005). In one case the lipoic acid carboxyl was hydrogen bonded to Ser-72 whereas in another case Arg-140 was the hydrogen bond donor (Fujiwara et al., 2005). Since enzymes rarely show such plasticity and lipoic acid is a hydrophobic molecule, it seemed possible that the observed association of the cofactor with a hydrophobic LplA surface in the interdomain cavity was artifactual. Moreover, in prior work Reed and coworkers had isolated LplA mutants resistant to inhibition by an analogue of lipoic acid in which the sulfur atoms had been replaced with selenium (Reed et al., 1994) Since this is a very discrete modification of the LplA substrate, the mutant protein would be expected to have an alteration close to the pocket that binds the lipoic acid thiolane ring. However, the site of this mutation (Gly-76 to serine, (Morris et al., 1995)) was distal from the reported *E. coli* lipoate-binding sites. This dilemma was resolved by two lipoic acid-containing structures of an LplA homologue from the Archaeon, *Thermoplasma acidophilum*

(Kim et al., 2005) that can be readily superimposed on the *E. coli* LplA structure except that the *T. acidophilum* protein lacks the *E. coli* LplA C-terminal domain. In both *T. acidophilum* structures the lipoate thiolane ring was adjacent to the glycine residue that corresponds to *E. coli* Gly-76, the residue giving resistance to the selenium analogue and a plausible reorganization of the molecule to prevent binding of the slightly larger analogue was proposed (McManus et al., 2006). Moreover, addition of lipoic acid to a complex of the *T. acidophilum* LplA with ATP gave lipoyl-AMP which demonstrated that the lipoic acid was bound in a physiologically meaningful manner (Kim et al., 2005). Residue 19, the LplA residue altered in suppressor strain FH145, is close to the lipoic acid binding pocket in the *Thermoplasma acidophilum* LplA/lipoyl-AMP cocrystal structure, although the side chain faces away from the lipoyl moiety. Given the importance of the two suppressor mutations we studied (as demonstrated by their reisolation by random mutagenesis), it is noteworthy that *E. coli* LplA residues 19 and 221 are not conserved in the LplA proteins of other organisms and neither are the residue substitutions found in the V19L and S221P mutants. The V19L mutation replaces a hydrophobic residue with one having a longer side chain. This could increase the affinity of the protein for octanoic acid by creating a more hydrophobic environment. The S221P mutation might open up the lipoic acid/octanoic acid binding pocket and thereby increase substrate access. Except for the F78Y and H79N substitutions, the residue changes of the other mutant LplA proteins isolated by random mutagenesis are not readily interpretable. The altered residues either lie far away from the active site or seem rather modest substitutions. However, a caveat is that although the V19L change is very modest in character (one branched chain residue for another), the mutation has very marked effects on enzyme activity. Judging from the *T. acidophilum* crystal structures residue F78 is located in the ATP binding pocket whereas H79 is located in the lipoic acid binding pocket. Both residues make direct interactions with substrate. However, residue 78 is a threonine in the *T. acidophilum* protein.

The Regulon Data Base (http://regulondb.ccg.unam.mx/) predicts that *lplA* is cotranscribed with the upstream gene, *ytjB.* This prediction is consistent with the

data of Morris e*t al.* (1995) who showed that insertion of a strong transcription terminator into *ytjB* results in deficiency in lipoic acid utilization in a *lipA* background that is alleviated by *trans* expression of LplA, but not of YtjB (then called Smp) (Morris et al., 1995). This is relevant to two puzzling *lplA* alleles that were isolated in our random mutagenesis of l*plA* with selection for *lipB* suppression (Table 2.3). Isolates 35R3 and 35R5 have point mutations in the *ytjB* termination codon. In both strains the termination codon was changed from TGA to the more stringent TAA stop codon. Read-through of TGA codons has often been reported and the nucleotide immediately downstream of the termination codon is known to have a major influence on the efficiency of termination (Tate and Mannering, 1996). It seems noteworthy that TGAC the sequence found at the end of *ytjB* is the weakest of the four base translational stop signals (Tate and Mannering, 1996). The *ytjB* termination codon is 27 nucleotides upstream of the *lplA* initiation codon and thus any read-through would add the *ytjB* coding sequence plus the nine codons of the intergenic region to the N-terminus of LplA with possible deleterious effects on LplA activity. Indeed, if, as annotated, YtjB is a membrane protein, the fusion protein could also mislocalize LplA activity. Another possibility is that ribosomes transversing the intergenic region might interfere with *lplA* translational initiation, resulting in decreased LplA levels. The more stringent TAA codon would more efficiently block translation of the intergenic region (Tate and Mannering, 1996) and perhaps raise LplA expression to a level sufficient to allow effective utilization of the cytosolic octanoic acid pool. It could be argued that decreased read-through would be a minor effect. However, it should be noted that the physiological requirement for lipoic acid is very low (only hundreds of molecules/cell) and thus slight overproduction of wild type LplA (e.g., by vector copy number) is sufficient to allow growth of *lipB* null strains on glucose minimal medium. Moreover, not all of the available lipoyl domains need be lipoylated for optimal PDH and OGDH complex activities (Guest et al., 1985, Jordan and Cronan, 2002)

It has long been thought that there are no cytosolic free fatty acids in *E. coli* because fatty acid biosynthesis is tightly coupled to phospholipid biosynthesis.

However free fatty acids are detected when phospholipid synthesis is blocked (Cronan et al., 1975) and in strains overexpressing a cytosol-entrapped TesA thioesterase (Cho and Cronan, 1995). In both cases detection required that the strains were defective in β-oxidation. Cho and Cronan (Cho and Cronan, 1995) detected traces of octanoic acid. The difficulties in detecting short chain fatty acids such as octanoate comes from their partial solubility in water and their increased volatility relative to the long chain fatty acids found in membrane lipids. In addition, free fatty acids are generally converted to their methyl esters for analysis and such short chain methyl esters are even more volatile than the parent acids. For these reasons we employed a different strategy. The free fatty acids were first converted to their nonvolatile salts then extracted into an organic solvent following acidification. We then made the butyl esters which are less volatile than the methyl esters (and therefore less likely to be lost), but sufficiently volatile to allow analysis by GC-MS.

The question remains as to the source of this intracellular pool of free fatty acids. We constructed strains lacking the TesA and TesB thioesterases in our suppressor backgrounds but these strains retained the ability to grow on glucose minimal medium (data not shown). However, there are five other thioesterases in *E. coli* of unassigned function which may contribute to the pool. Alternatively, the pool may be formed by spontaneous hydrolysis of acyl-ACPs or of intermediates bound to the fatty acid elongation enzymes (e.g., the 3-ketoacyl-ACP synthases). A final possibility is that the pool is generated by hydrolytic removal of acyl chains inappropriately incorporated by an acyltransferase. Evidence for such an editing reaction by the phospholipid acyltransferases has been reported (Rock et al., 1981).

Tables

Table 2.1: Bacterial strains, plasmids and primers used in this work.

Strain	Relevant Characteristics	Reference
CAG18430	*mdoB202::Tn10*	(Singer et al., 1989)
DK574	SJ16 carrying plasmids pMS421 and pMR19	(Cronan and Thomas, 2009)
FH6	*lipB::*Tn*1000*Km	This study
FH26	*lipB::*Tn*1000*Km *lplA10*	This study
FH27	*lipB::*Tn*1000*Km *lplA11*	This study
FH46	FH26 *lplA*::Tn10*tet*	This study
FH47	FH27 *lplA*::Tn10*tet*	This study
FH57	*lipA::*Tn*1000*Km *lipB::*Tn*10*Tet *lplA10*	This study
FH58	*lipA::*Tn*1000*Km *lipB::*Tn*10*Tet *lplA11*	This study
FH35	FH27 *mdoB202::Tn10*	This study
FH66	FH26 *mdoB202::Tn10*	This study
FH145	*lipB::Cm mdoB202::Tn10 lplA10*	This study
FH146	*lipB::Cm mdoB202::Tn10 lplA11*	This study
FH213	MG1655 carrying plasmids pMS421 and pJH2	This study
JK1	*rpsL*	(Morris et al., 1995)
KER184	*rpsL lipB::*Tn*1000*Km	(Morris et al., 1995)
KER310	*rpsL lipA::*Tn*1000*Km *lipB::*Tn*10*Tet	(Morris et al., 1995)
QC146	*lipB lplA*	(Christensen and Cronan, 2009)
TM135	*rpsL lplA::*Tn*10*Tet	(Morris et al., 1995)
YFJ239	BL21 carrying plasmid pYFJ84	(Jiang et al., 2006)
ZX221	*rpsL lipB::Cm*	(Christensen and Cronan, 2009)
Plasmids		
pFH1	*lplA11* on pQE2	This study
pFH5	*lplA10* on pQE2	This study
pJH2	Lysis plasmid	(Crabtree and Cronan, 1984)
pMR19	Encodes *acpP*	(Cronan and Thomas, 2009)
pMS421	Expresses $LacI^q$	(Cronan and Thomas, 2009)
pGS331	hybrid lipoyl domain on p*tac*	(Ali and Guest, 1990)
pTJ93	*acpS*	(Cronan and Thomas, 2009)
pYFJ16	*lplA* on pQE2	(Jiang and Cronan, 2005)
pYFJ84	*Vibrio harveyi aasS* on pET16b	(Jiang et al., 2006)
Primers		
lplAfor	TGGCAATCGGTGTAGTGCTG	This study
lplArev	GCGCTTGGTTAACGGCGATC	This study

Table 2.2: Kinetic parameters for wild type, V19L and S221P LplA proteins for octanoate.

	K_m (μM)	V_{max} (nmole/min)	K_{cat} (mole/min/mole)	K_{cat}/K_m
Wildtype	214.3±37.8	$2.4\ X10^{-2}$±$0.34X10^{-2}$	2.4±0.34	0.011
V19L	10.4 ±3.0	$1.6X10^{-2}$ ±$0.18X10^{-2}$	1.6±0.18	0.058
S221P	4.0 ±1.6	$0.25X10^{-2}$ ±$0.79X10^{-4}$	0.25±$0.79X10^{-2}$	0.063

Table 2.3: Results of random mutagenesis of *lplA* and selection for growth of a *lipB* null mutant. US: mutations upstream of the coding sequence; DS: mutations downstream of the coding sequence.

Isolate	LplA Mutations
12R3	E116A, E312K, L328F, US, DS
12R4	S8T, N63K, F78Y, A110T
12R7	R58L, H79N
35R3/ 35R5	F35L, V113I, 4US
35R4	F15S, T101A, S114I, 3US

Figures

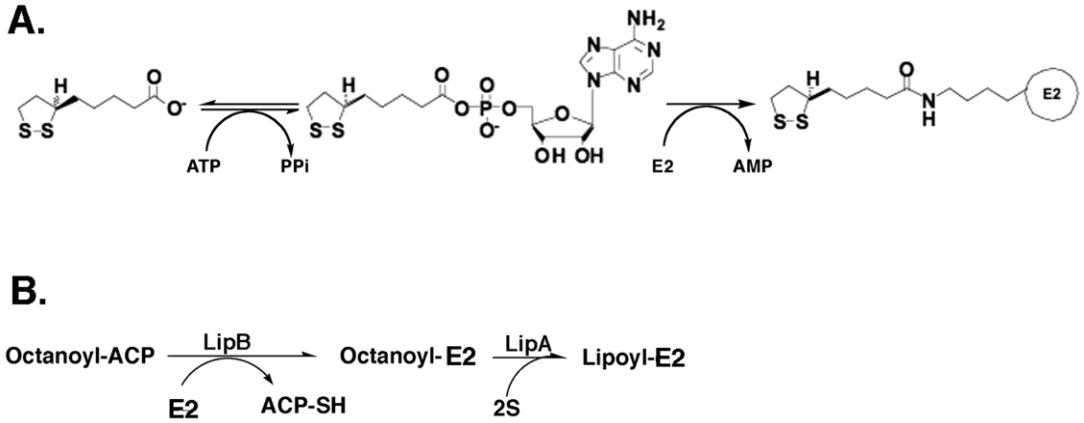


Figure 2.1: Lipoic acid metabolism in *E. coli*.
A: The LplA lipoate ligase reaction. Lipoate reacts with ATP to form the activated intermediate, lipoyl-adenylate (lipoyl-AMP) which remains firmly bound in the active site. The lipoyl-adenylate mixed anhydride bond is then attacked by the ε-amino group of the target lysine residue of the acceptor lipoyl domain to form lipoylated protein. LplA also utilizes octanoic acid.
B: Lipoic acid synthesis in *E. coli*. LipB transfers an octanoyl moiety from the fatty acid biosynthetic intermediate, octanoyl-ACP, to the lipoyl domain of a lipoate accepting protein (in this case the E2 subunit of a 2-oxoacid dehydrogenase). The octanoylated-domain is the substrate of LipA, an S-adenosylmethionine radical enzyme that replaces one hydrogen atom on each of octanoate carbons 6 and 8 with sulfur atoms.

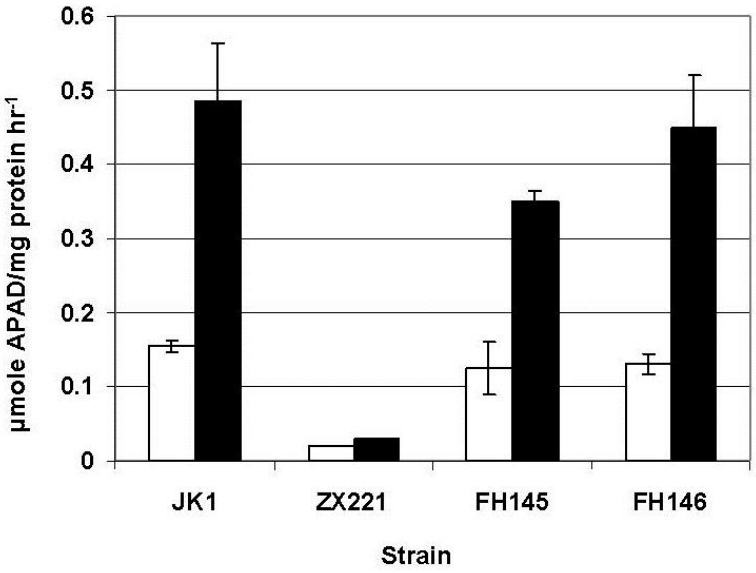


Figure 2.2: PDH (solid bars) and OGDH (open bars) activities of various isogenic strains grown on glucose minimal medium supplemented with succinate and acetate. The values reported are the averages of three measurements. Strain JK1, wild type: strain ZX221, *lipB* FH145: *lipB lplA10* and FH146: *lipB lplA11.*

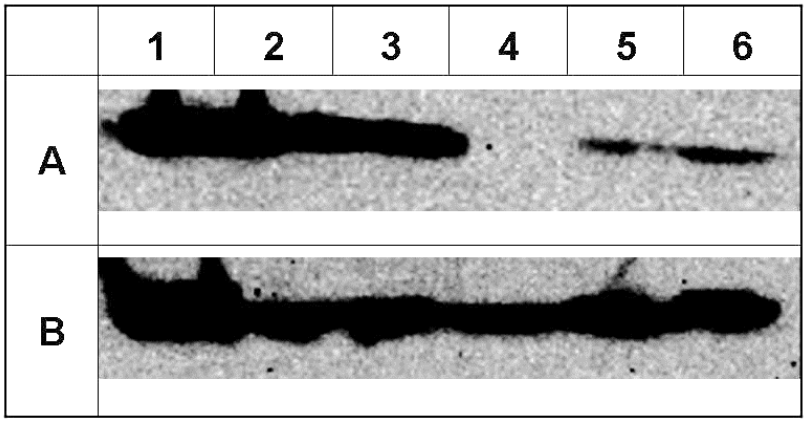


Figure 2.3: Western blot analysis of protein lipoylation. All strains were isogenic and produced a hybrid lipoyl domain encoded by plasmid pGS331 (Ali and Guest, 1990). Equal amounts of total extract protein were loaded in each lane. In panel A the cells were from cultures grown without octanoic acid supplementation whereas in panel B the cultures were grown with octanoic acid supplementation. Lane 1: lipoyl domain standard; lane 2: strain JK1 (wild type); lane 3: strain TM135 (*lplA*); lane 4: strain ZX221 (*lipB*); lane 5: strain FH145 (*lipBlplA10*); and lane 6: strain FH146 (*lipBlplA11*).

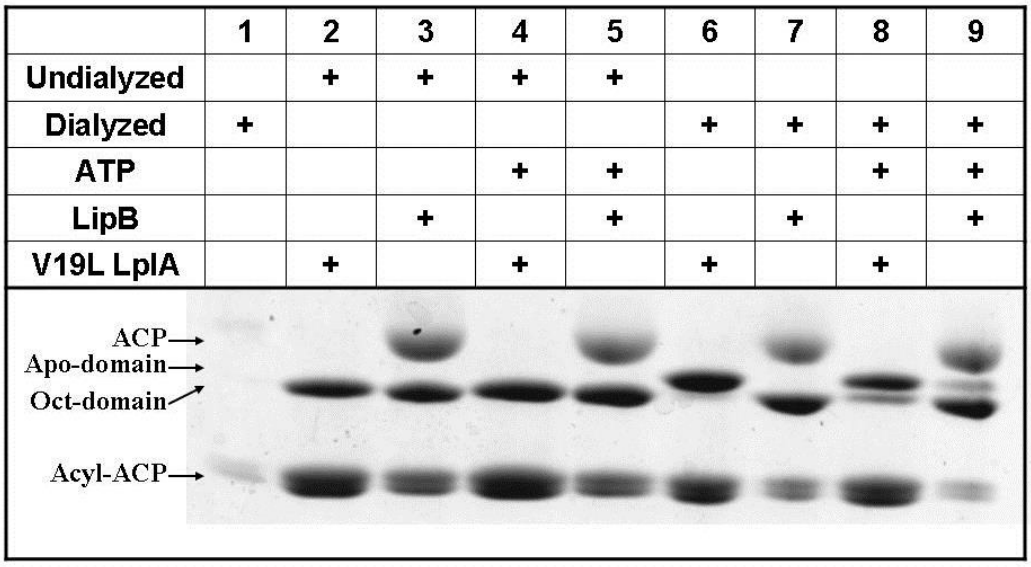


Figure 2.4: Gel shift assay with V19L LplA and octanoyl-ACP as substrate. Two preparations of octanoyl-ACP (undialyzed and dialyzed) were tested. Lanes 3, 5, 7 and 9 were controls for the integrity of the octanoyl-ACP thioester bond in both preparations because LipB cannot utilize free octanoic acid. In these four lanes the modified domain migrates more rapidly than the unmodified domain. In the LipB reactions a holo-ACP band is seen at the top of the gel. In lanes 2 and 4 V19L LplA catalyzed modification of the domain in presence of an undialyzed octanoyl-ACP preparation. However, there was no accumulation of holo-ACP indicating that octanoyl-ACP was not the source of octanoate. In lane 6, an extensively dialyzed octanoyl-ACP preparation was the substrate and no modification of the domain was seen. In lane 8 ATP was added which resulted in some modification of the domain which may be due to hydrolysis of octanoyl-ACP to give free octanoate. The S221P LplA protein gave essentially identical results.

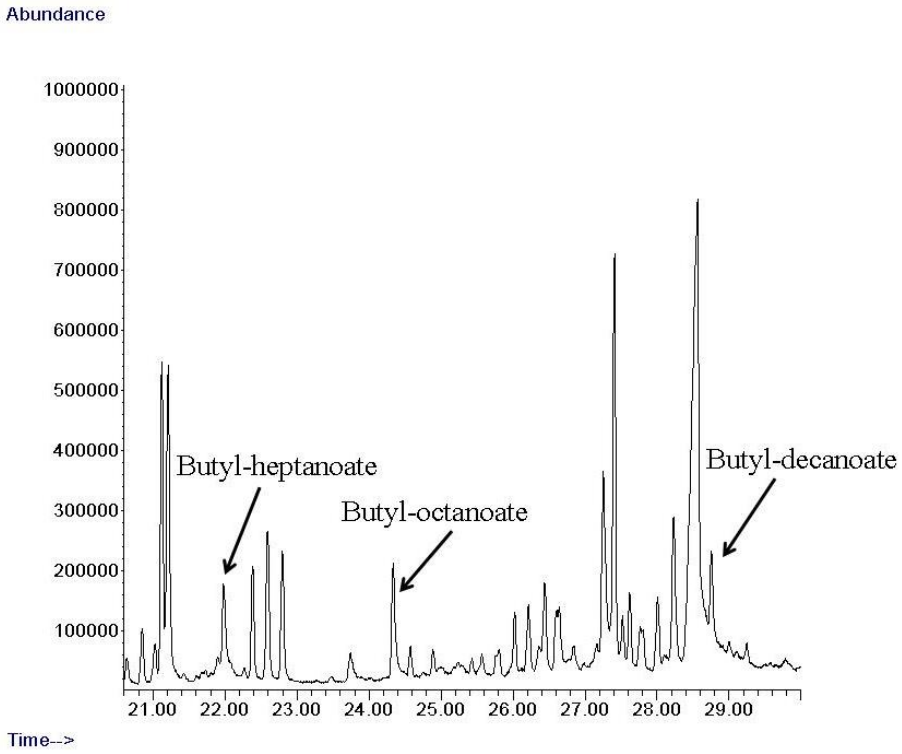


Figure 2.5: Gas chromatogram of the butyl esters of free fatty acid from cell extracts. The internal standard butyl-heptanoate peak represents 50 ng. The mass spectra of the butyl esters of heptanoate, octanoate and decanoate were identical to those of authentic standards and the data bases.

Chapter 3: An NAD Synthetic Reaction Bypasses the Lipoate Requirement for Aerobic Growth of *Escherichia coli* Strains Blocked in Succinate Catabolism[4]

Introduction

The enzyme cofactor lipoic acid ((*R*)-5-(1,2-dithiolan-3-yl)pentanoic acid; 6,8-dithiooctanoic acid) is essential for the function of two enzyme systems of aerobic metabolism in *Escherichia coil:* pyruvate dehydrogenase (PDH) and 2-oxoglutarate dehydrogenase (OGDH). A third lipoate-dependent enzyme system, the glycine cleavage system (GCV), which functions in the metabolism of glycine to a C1 unite and ammonia, is also present in *E. coli.* However, since GCV expression is dependent on the presence of glycine and is not required for aerobic growth, it will not be further discussed (Stauffer, 2004, Douce et al., 2001).

PDH catalyzes the oxidative decarboxylation of pyruvate, the end product of glycolysis, to acetyl-CoA (Fig. 3.1). The acetate moiety of acetyl-CoA is condensed with oxaloacetate to form citrate, the first intermediate of the tricarboxylic acid (TCA) cycle. Additionally, acetyl-CoA is the building block for fatty acids and the amino acid, leucine. PDH is required for aerobic growth of *E. coli* because the other major pyruvate-dissimilating enzyme, pyruvate:formate lyase (Pfl), has an oxygen labile active site and is inactive in the presence of air (Sawers and Watson, 1998). *E. coli* contains a third pyruvate-metabolizing enzyme, pyruvate oxidase (PoxB). However, PoxB is mainly expressed during early stationary phase (Abdel-Hamid et al., 2001, Chang et al., 1994) and is less efficient in energy conservation because it produces free acetate, which must be activated to acetyl-CoA at the expense of ATP.

[4] Results presented in this chapter have been accepted for publication in the Journal *Molecular Microbiology*.

Halfway through the TCA cycle, citrate is broken down to 2-oxoglutarate, which undergoes oxidative decarboxylation to succinyl-CoA by the second lipoate-dependent enzyme system, OGDH (Fig. 3.1). Succinyl-CoA is used in the biosynthesis of the amino acids lysine and methionine, and the peptidoglycan component, diaminopimelic acid. Succinyl-CoA is also the source of succinate, the entry point to respiration (Cronan and Laporte, 2006). OGDH activity is dispensable under conditions where the TCA cycle operates as a branched pathway, such as during anaerobic growth (Creaghan and Guest, 1972). In such instances, fumarate is the source of succinate and hence succinyl-CoA.

The major pathways that utilize succinyl-CoA (methionine and lysine synthesis) do not consume the succinate moiety. Rather succinate is released intact into the cytoplasm and can be recycled by succinyl-CoA ligase (Fig. 3.1). Hence succinate is said to function “catalytically” in these biosynthetic pathways. In wild type cells however, free succinate is consumed by oxidation to fumarate by succinate dehydrogenase (SDH; Fig. 3.1), requiring succinyl-CoA to be continuously supplied by OGDH. Thus mutations in SDH greatly lower the demand for production of new succinyl-CoA by OGDH. In SHD mutants very small amounts of succinate suffice because of succinate recycling by succinyl-CoA ligase (Fig. 3.1).

However, the exact cellular requirement for succinate in strains defective in succinate catabolism (SDH) has been difficult to determine because disruption of OGDH function does not result in a succinate requirement for growth, a finding that suggests that *E. coli* has other pathways of succinate production (Creaghan and Guest, 1977).

PDH and OGDH are very large complexes made up of multiple copies of three different subunits named E1, E2 and E3. The E2 subunits each contain at least one lipoyl domain (LD), a highly conserved structure of about 80 residues (Cronan, 2008, Cronan et al., 2005). Lipoate is attached by an amide bond to a specific lysine residue of each of these domains and functions as a “swinging arm”, carrying reaction intermediates between the active sites of the three

subunits (Perham, 2000). Whereas the E1 and E2 subunits are encoded by genes which are distinct for each complex, the *lpd* gene encodes the E3 subunits of both PDH and OGDH.

In a markedly atypical biosynthetic pathway, lipoate is assembled from the eight-carbon fatty acid, octanoate, following octanoate attachment to the LDs. In *E. coli* the assembly proceeds in two steps. First, octanoyltransferase (LipB) transfers an octanoyl moiety from the octanoyl-acyl carrier protein (ACP) of fatty acid biosynthesis to the specific lysine residues of the LDs (Jordan and Cronan, 2003, Hassan and Cronan, 2011, Zhao et al., 2005, Zhao et al., 2003). Lipoyl synthase (LipA) then catalyzes replacement of single hydrogen atoms at carbons 6 and 8 with sulfur atoms, by use of radical SAM chemistry (Booker et al., 2007, Miller et al., 2000). In an alternative scavenging pathway, exogenous lipoate (or octanoate) can be directly attached to the LDs by lipoate protein ligase (LplA). LplA uses ATP to activate the acyl chain by forming acyl-AMP, the mixed anhydride of which is then attacked by the LD lysine residue (Green et al., 1995, Morris et al., 1994, Morris et al., 1995, Zhao et al., 2003).

Since Δ*lipB* strains are defective in lipoate synthesis, aerobic growth of these strains on glucose minimal media should strictly depend on supplementation with either lipoate (or octanoate) or acetate plus succinate. The latter combination of supplements, respectively, bypass the PDH- and OGDH-catalyzed steps required for TCA cycle function (Herbert and Guest, 1968). (However, see the Results section below.) We previously isolated and characterized Δ*lipB* suppressor strains which grew on glucose minimal media lacking any supplements (Hermes and Cronan, 2009). In these strains, the mutations causing suppression mapped to the *lplA* gene and resulted in amino acid residue changes in the LplA proteins which reduced the enzyme K_m values for free octanoate. This led us to search for intracellular free octanoate, which was detected at a concentration above that of the K_m values for the mutant LplA proteins. Thus suppression of the Δ*lipB* defect in these strains was caused by activation of PDH and OGDH by the mutant LplA enzymes using cytosolic

octanoate (Hermes and Cronan, 2009). This screen also gave rise to a single Δ*lipB* suppressor strain (strain FH34) which retained the wild type *lplA* sequence indicating that suppression in this strain was due to a different mechanism. We report the deciphering of this distinct and rather intricate mode of suppression.

Materials and Methods

Bacterial strains and growth conditions:

The strains used in this study (Table 3.1) were derivatives of *E. coli* wild type K-12 strain MG1655. Strains with the W3110 background were used only as marker donors for the construction of strains in the MG1655 background. P1 transductions and transformations were carried out using conventional methods (Ausubel et al., 2007, Miller, 1992). The Dasenko and Wanner method (Datsenko and Wanner, 2000) was applied to the construction of several strains. Primers used are indicated in table 3.1 and their sequences in table 3.2.

Strain FH34 was isolated in a previous screen by plating strain FH6 on unsupplemented glucose minimal plates (Hermes and Cronan, 2009).

Strains were maintained on Luria-Bertani agar plates (Miller, 1992). Phenotypic analyses were conducted in either liquid culture or on plates using minimal E medium (Vogel and Bonner, 1956) with 0.4% glucose as carbon source. Supplements were added at the following concentrations unless otherwise specified: acetate, 5 mM; succinate, 5 mM; octanoate, 50 µM; nicotinic acid, 100 µM; diaminopimelic acid, 100 µg ml^{-1}; lysine 10 µg ml^{-1}; and methionine, 50 µg ml^{-1}. Antibiotics were used at the following concentration (in µg ml^{-1}): sodium ampicillin, 100; tetracycline HCl, 15; kanamycin sulfate, 50 and chloramphenicol, 20. The growth temperature was 37^0C.

Growth curve experiments were done in a Bioscreen C instrument. Colonies were inoculated into 1 ml glucose minimal medium containing the appropriate supplements and grown overnight. The cells were pelleted, washed three times with 200 µl minimal medium and resuspended in the same medium to a final OD_{600} of 0.15. Each well contained 290 µl of medium to which 10 µl of cell

suspension was added to give a final starting OD_{600} of 0.005 (about 3 X 10^8 cells ml^{-1}). The Bioscreen instrument was set to constant very strong intensity shaking to give maximal aeration. The means of three biologically independent growth curves are presented. When necessary, a succinate starvation step was performed before initiating the growth curves. After the wash and resuspension steps described above, cells were subcultured into fresh media lacking succinate to an initial OD_{600} of 0.02 and allowed to grow for 5 to 6 hours.

Three plasmids were used in this study. pGS133 is a low copy vector carrying the *sdhCDAB* operon with its native promoter (Kita et al., 1989), and pBH146 contains the *sucB* gene under control of a *tac* promoter (Hassan and Cronan, 2011). Plasmid FH112 was constructed by PCR amplification of the *nadB* from genomic DNA using primers NcoI-nadB and HindIII-nadB (Table 3.2) and cloning the PCR product into NcoI/HindIII treated pKK233-2 (Amann and Brosius, 1985), thus placing *nadB* under *trc* expression.

PDH and OGDH activity assays:

The method described in (Hermes and Cronan, 2009) was followed with the exception that supplements were added as indicated here, and cell lysis was performed using the BugBuster Master Mix (Novagen) per the manufacturer's instructions. The means and standard deviations of three repeat measurements are presented.

Mapping the suppressor mutation of strain FH34:

The first step was to link the mutation causing lipoate-independent growth in strain FH34 (referred to here as the lip+ marker) to a tetracycline resistance marker and move it into strain FH160 (Δ*lipB*::FRT::cm^R::FRT). P1 phage was grown on a pool of about 3000 colonies of strain W1458 containing random Tn10 insertions (lab collection) and used to transduce strain FH34 to tetracycline resistance. About 19,000 colonies were pooled and used to prepare a new P1 stock which was used to transduce strain FH160 to tetracycline resistance. The resulting isolates were screened for retention of chloramphenicol resistance and

gain of the lip+ phenotype. In one such isolate, named FH272, tetracycline resistance and the lip+ phenotype were 34% linked. FH272 was used for further mapping.

The first step was necessary in order to separate the suppressor mutation from the kanamycin resistant element used to disrupt *lipB* in the original strain and thereby enable the use of the mini-Tn5 kanamycin resistant transposon of plasmid pRL27 for mapping (Larsen et al., 2002). Strain FH272 was transformed with a preparation of pRL27 by electroporation, followed by selection for kanamycin resistance. (At this point we had reason to suspect that suppression was caused by a recessive mutation.) A P1 phage stock was prepared on a pool of the resulting transductants and used to transduce FH160 to kanamycin resistance. Isolates were screened for retention of chloramphenicol resistance and gain of the lip+ phenotype. One isolate was obtained (FH278) in which kanamycin resistance and lip+ were 26.3% linked. Sequencing out from the mini-Tn5 transposon, followed by a BLAST search revealed that the transposon had inserted into the *ybfC* gene.

Next using P1 lysates of strains from the Singer (Singer et al., 1989) and Keio (Baba et al., 2006) collections, selecting for the antibiotic resistance marker of the donor and screening for the lip+ marker and antibiotic resistance marker of the recipient (or selecting for both antibiotic resistance markers), the lip+ marker was localized to the region between *ybgO* and *mngA.* Finally, using the primers P1-P24 (Table 3.2), the region between these two genes was sequenced and compared to the sequence of the same region in GenBank (accession number U00096.2).

Results

The Δ*lipB* strain FH160 contains functional PDH and requires only succinate for aerobic growth on glucose minimal media.

As stated in the Introduction, it is expected that the PDH and OGDH complexes of Δ*lipB* strains would be inactive as a result of an inability to synthesize

endogenous lipoate. Thus Δ*lipB* strains should be incapable of aerobic growth on glucose minimal medium without supplementation with both acetate and succinate which, respectively, bypass the PDH- and OGDH-dependent steps required for TCA cycle function. We will refer to glucose minimal medium supplemented with acetate and succinate as “bypass medium”. We reported the above growth phenotype and the lack of detectable PDH and OGDH function in Δ*lipB* strains grown on bypass medium (Hermes and Cronan, 2009). A somewhat conflicting observation was previously reported by our laboratory (Reed and Cronan, 1993). The Δ*lipB* strain used in that study grew on minimal medium supplemented only with the products of the OGDH reaction; that is, acetate supplementation was not required. Additionally extracts of the Reed and Cronan Δ*lipB* strain grown on bypass medium contained about 20% of the wild type PDH activity and about 10% of the wild type level of lipoate (Reed and Cronan, 1993).

We observed that the Δ*lipB* strains FH6 and FH160 behaved similarly to the Δ*lipB* strain of Reed and Cronan (Reed and Cronan, 1993). Strains FH6 and FH160 required only succinate for growth on glucose minimal medium (Fig. 3.2A and data not shown). This growth phenotype suggested functional PDH was present and this was indeed detected in extracts of strain FH160 (Fig. 3.2B) and was significantly increased upon supplementation with the LplA substrate and lipoate precursor, octanoate. In contrast OGDH activity was only detected when exogenous octanoate was provided in the growth medium. Thus the lack of detectable OGDH activity explains the requirement of the strain for succinate supplementation. We determined that the Δ*lipB* strain FH160 requires 12 μM succinate for half maximal growth in aerobic glucose minimal medium (Fig. 3.2A). Acetate-independent growth was not observed for Δ*lipB* Δ*lplA* and Δ*lipB* Δ*lipA* strains (Table 3.4) which indicated that the LplA/LipA pathway was responsible for the residual PDH activity of Δ*lipB* strains (Hermes and Cronan, 2009).

We have chosen to measure growth in liquid media rather than on solid media to avoid the possibility of acetate and/or succinate production by anaerobic pathways active in the anoxic cells present in the interior of colonies. Indeed,

Creaghen and Guest (1977) encountered this problem in their studies of the phenotypes of *E. coli lpd* strains.

Lipoate synthesis in the Δ*lipB* suppressor strain FH34 is required for PDH activity, but not OGDH activity.

Strain FH34 was isolated as a suppressor of the Δ*lipB* mutation of strain FH6, which had acquired the ability to grow on unsupplemented glucose minimal medium (Fig. 3.3A). FH34 also grew on glycerol minimal medium although at a reduced rate, but did not grow on acetate or succinate as sole carbon sources. Since lipoate biosynthesis is dispensable for anaerobic growth (Vanden Boom et al., 1991), one possible explanation for the growth phenotype of strain FH34 was a cellular switch to anaerobic metabolism. During anaerobic growth acetyl-CoA is produced by pyruvate formate lyase (*pflB*) whereas succinate is produced from fumarate by fumarate reductase (*frdABCD*). Alternatively, acetate can be produced aerobically, albeit less efficiently, by pyruvate oxidase (*poxB*), expression of which is under RpoS control (Abdel-Hamid et al., 2001, Chang et al., 1994). In order to test if any of these genes played a role in the growth of strain FH34, we constructed strains in the FH34 background in which these genes were deleted. The resulting strains retained acetate- and succinate-independent growth on glucose minimal medium (Table 3.4) and thus these genes were not involved in the suppression phenotype.

Having excluded the hypothesis that anaerobic metabolism and/or PoxB provide acetate and succinate, the most straightforward explanation for the phenotype of strain FH34 was that the strain retained PDH activity and that the mutation giving suppression of the Δ*lipB* mutation somehow resulted in activation of OGDH. In Δ*lipB* strains the LplA/LipA pathway is the only source of endogenous lipoate needed to activate the PDH and OGDH complexes. LplA attaches the free fatty acid octanoate to the complexes and LipA converts the octanoyl modification to the lipoyl cofactor to give the active dehydrogenases (Zhao et al., 2003). In order to test if OGDH was being activated in strain FH34 via the known LplA/LipA route, Δ*lplA* and Δ*lipA* gene deletions were individually constructed in strain

FH34. The resulting two strains acquired an acetate requirement for growth on glucose minimal medium similar to the Δ*lipB* parental strain (Table 3.4). The Δ*lplA* and Δ*lipA* deletion derivatives of strain FH34, however, remained succinate-independent. This indicated that either OGDH was activated by a mechanism independent of lipoate or that OGDH activity was not essential for growth of strain FH34 due to the presence of other succinate production pathways.

We had previously reported that Δ*lipB* strains are suppressed by point mutations in the *lplA* gene which decrease the enzyme K_m for octanoate thus enabling these enzymes to utilize the cytoplasmic pool to modify PDH and OGDH (Hermes and Cronan, 2009). We therefore sequenced the *lplA* gene of strain FH34 and found it to have the wild type sequence. The Δ*lipB* deletion phenotype is known to be suppressed by overexpression of *lplA* (Morris et al., 1995). Therefore, Southern blot analyses were performed using probes specific to the *lipB* and *lplA* genes in order to eliminate the possibility that gene duplication caused the suppression (prior work from this laboratory had demonstrated duplication of the *lipB* locus (Jordan and Cronan, 2002)). The genomic DNA blot patterns of strains FH6 and FH34 were identical (data not shown). Moreover, the nucleotide sequence upstream of the *lplA* gene in FH34 had the wild type sequence, thus eliminating the possibility of a promoter mutation that altered *lplA* expression.

The Δ*lipB* suppressor strain FH34 lacks detectable OGDH activity.

As stated above a possible explanation for the succinate-independent growth of strain FH34 was the presence of active OGDH. Thus crude extracts of strain FH34 grown on bypass medium were assayed for PDH and OGDH activities. As was the case with the Δ*lipB* parent (Fig. 3.2B), FH34 extracts contained functional PDH, but no detectable OGDH activity (Fig. 3.3B). In a further test a Δ*lpd* derivative of strain FH34 was constructed. Since *lpd* encodes a subunit common to both PDH and OGDH, strains defective in Lpd activity require both acetate and succinate supplementation for aerobic growth on glucose (Langley

and Guest, 1978). However the Δ*lpd* derivative of strain FH34 required acetate, but not succinate for growth (Table 3.4). Taken together these two observations indicated that detectable levels of OGDH activity were not essential for growth of strain FH34 and that there must be other sources of cellular succinate.

The mutation causing suppression of the Δ*lipB* gene deletion of strain FH34 maps to the *sdhB* gene and inactivates succinate dehydrogenase.

Using two and three factor transductional crosses, the mutation causing suppression of the Δ*lipB* gene deletion in strain FH34 was localized to the 18 Kbp region between the genes *ybgO* and *mngA* (Table 3.3). The diverse phenotypes and polarity of the metabolic gene cluster *gltA-sdhCDAB-sucAB-sucCD* complicated analysis of results of transductional crosses within this region. We therefore sequenced the entire region and found a single nucleotide change in the *sdhB* gene, which encodes the iron-sulfur subunit of the succinate dehydrogenase (SDH) complex. The mutation changed *sdhB* codon 51 from a glutamine codon to a termination codon (CAG → TAG). Sequencing showed this mutation was not present in the MG1655 wild type and FH6 Δ*lipB* strains.

To confirm that the termination codon caused the growth phenotype of strain FH34, a Δ*lipB* Δ*sdhB* strain was constructed in the MG1655 (wild type) background. The resulting strain FH420 grew on unsupplemented glucose minimal medium thus recapitulating strain FH34 (Table 3.4). Moreover, when a low-copy number plasmid encoding the complete *sdhCDAB* operon was introduced into a Δ*lipB* Δ*sdhB* strain, the resulting derivative was unable to grow without succinate supplementation (Table 3.4).

Strains containing Δ*lipB* Δ*sdhA* and Δ*lipB* Δ*sucA* deletions were also able to grow on unsupplemented glucose minimal medium (Table 3.4). The *sdhA* gene is located upstream of *sdhB* and encodes the flavoprotein subunit of the SDH complex. Hence, any deletion that inactivates SDH results in suppression of the Δ*lipB* mutation. As seen above in the Δ*lipB* Δ*sdhB* strain, succinate-independent growth of the Δ*lipB* Δ*sucA* strain was lost upon introduction of a plasmid

encoding the *sdhCDAB* operon (Table 3.4). This suggested that the Δ*sucA* mutation caused disruption of *sdhB* expression, possibly by destabilizing the polycistronic mRNA (Cunningham and Guest, 1998). Since *sucA* encodes a subunit of OGDH, the phenotype of the Δ*lipB* Δ*sucA* strain further supports the observation that OGDH function is not essential for succinate production in Δ*lipB* strains that lack SDH activity, such as strain FH34 (Δ*lipB* Δ*sdhB).* A Δ*lipB* Δ*sucB* strain however failed to grow on unsupplemented glucose minimal medium (Table 3.4) indicating that the Δ*sucB* deletion does not exert the effect on the expression of the *sdhCDAB* operon seen for the Δ*sucA* deletion. We note that deletion strains of the Keio collection (Baba et al., 2006) were used to construct the Δ*lipB* Δ*sucA* and Δ*lipB* Δ*sucB* strains. These gene deletions are in frame only upon excision of the antibiotic resistance marker, and the markerless Δ*lipB* Δ*sucA* and Δ*lipB* Δ*sucB* versions of our strains retained the phenotypes described here.

Succinate is produced and must be recycled to succinyl-CoA in Δ*lipB* Δ*sdhB* strains.

The biosynthetic pathways that utilize succinyl-CoA do not consume the succinate moiety. In methionine biosynthesis, the succinate moiety of succinyl-CoA is used to form an ester bond with homoserine (the reaction catalyzed by MetA) which is subsequently attacked by the thiol group of cysteine in the MetB reaction with the release of free succinate. Theoretically any homoserine ester will fulfill this role. Indeed *metA* deletion strains are methionine auxotrophs but grow when provided with exogenous acetyl-homoserine in place of methionine (Flavin and Slaughter, 1967, Nagai and Flavin, 1967). Moreover, many plant MetB enzymes prefer acetyl-homoserine over succinyl-homoserine (Hacham et al., 2003). A similar argument can be made for the role of succinyl-CoA in the biosynthetic pathway of lysine and diaminopimelic acid. Ultimately the free succinate generated in the cytoplasm by these pathways has two fates. It is either oxidized to fumarate by SDH or is reattached to CoA by succinyl-CoA ligase (succinyl-CoA synthetase) encoded by the *sucCD* genes (Fig. 3.1). We

hypothesized that if a small amount of succinate was produced in the Δ*lipB* Δ*sdhB* strains then *sucCD* would be essential for scavenging succinate via its ligation to CoA. On the other hand if the role of succinyl-CoA was being fulfilled by another acyl-CoA, such as acetyl-CoA, then *sucCD* could be dispensable. In order to test this hypothesis we constructed a deletion of the *sucCD* genes in the Δ*lipB* Δ*sdhB* strain. The resulting Δ*lipB* Δ*sdhB* Δ*sucCD* strain required lysine and methionine for growth (Table 3.4). This indicated that succinate was indeed produced and must be recycled to succinyl-CoA in the Δ*lipB* Δ*sdhB* strains. The surprising acetate requirement for growth of the Δ*lipB* Δ*sdhB* Δ*sucCD* strain is currently under study.

The pathways of succinate synthesis in Δ*lipB* Δ*sdhB* strains.

The question then became the source(s) of the succinate. Three straightforward sources of succinate in strains lacking functional SDH and OGDH complexes had previously been suggested by Creaghan and Guest (Creaghan and Guest, 1977). These were isocitrate lyase (AceA), fumarate reductase (FrdABCD) and oxidation of succinate semialdehyde (which was proposed to be produced by activity of the E1 subunit of OGDH (Frank et al., 2008)). To test these hypotheses we constructed deletions of the *aceA, frdA* and *sucAB* genes individually and in combination in the Δ*lipB* Δ*sdhB* strain and screened for the ability of these strains to grow without succinate supplementation. The Δ*lipB* Δ*sdhB* strain that contained deletions of *aceA, frdA* and *sucAB* retained succinate-independent growth on glucose minimal (Table 3.4) indicating that yet another source of succinate remained.

Given the elimination of the straightforward sources we considered the activity of aspartate oxidase (NadB), the first enzyme in the *de novo* pathway of nicotinamide adenine dinucleotide (NAD) synthesis. NadB catalyzes the oxidation of aspartate to iminosuccinate and can use either oxygen or fumarate as oxidant (Korshunov and Imlay, 2010). When fumarate is used it is reduced to succinate. The NAD synthetic pathway differs from typical coenzyme biosynthetic pathways in that it has a high flux; the levels of NAD and its derivatives are about

1 mM (Bochner and Ames, 1982, Wimpenny and Firth, 1972). We introduced a Δ*nadB* deletion into the Δ*lipB* Δ*sdhB* Δ*frdA* Δ*aceA* Δ*sucAB* strain. The resulting strain required succinate for growth on glucose minimal medium containing nicotinic acid (Table 3.4). The succinate requirement was alleviated upon introduction of a vector expressing *nadB* (Table 3.4). By construction of various combinations of the four gene deletions in the Δ*lipB* Δ*sdhB* strain, we determined that only three of the four deletions were required to produce the succinate-dependent growth phenotype; Δ*aceA,* Δ*nadB,* and Δ*sucAB* (Fig. 3.4). The succinate requirement for growth of strain FH698 (Δ*lipB* Δ*sdhB* Δ*aceA* Δ*nadB* Δ*sucAB)* was expected to be very low. Indeed, supplementation of glucose minimal medium with only 250 nM succinate gave half maximal growth (Fig. 3.4).

The contribution of OGDH was surprising given that the activity of this enzyme in extracts of the Δ*lipB* Δ*sdhB* strain was consistently below the detection level (~0.005 µmole substrate mg^{-1} protein hr^{-1} (Reed and Cronan, 1993)), and that deletion of *lplA, lipA* or *lpd* in the Δ*lipB* Δ*sdhB* background did not block succinate independent growth (see above). However, it is now clear that the lack of effect of the aforementioned genetic manipulations in the Δ*lipB* Δ*sdhB* background on succinate production is due to the presence of the two other sources of succinate, namely NadB and AceA. In order to further understand the exact role of OGDH, individual deletions of genes encoding its constituent subunits were constructed in the Δ*lipB* Δ*sdhB* Δ*aceA* Δ*nadB* background. An in frame *sucB* gene deletion in the Δ*lipB* Δ*sdhB* Δ*aceA* Δ*nadB* strain resulted in a succinate requirement (Table 3.4) suggesting that full OGDH activity was responsible for the succinate independent growth of the Δ*lipB* Δ*sdhB* Δ*aceA* Δ*nadB* strain, and not just the succinate- or succinate semialdehyde-producing activity of the E1 subunit (*sucA).* Interestingly, introduction of an *lpd* deletion into the Δ*lipB* Δ*sdhB* Δ*aceA* Δ*nadB* strain did not result in a succinate requirement. This result is in line with studies that show that dihydrolipoamide can be oxidized under certain conditions by glutaredoxins with electrons passing to glutathione (Feeney et al., 2011). This also explains why we were unable to detect OGDH activity in the

Δ*lipB* Δ*sdhB* strain since our assay measures reduction of the NAD^+ analogue, 3-acetylpyridine adenine dinucleotide, by Lpd.

Discussion

When Δ*lipB* strains were first isolated "leaky" growth phenotypes on aerobic medium supplemented with nutrients that bypassed the OGDH-catalyzed reaction, i.e. succinate, or lysine plus methionine, were reported (Morris et al., 1995, Reed and Cronan, 1993, Vise and Lascelles, 1967). This acetate-independent growth phenotype was suggestive of the ability of Δ*lipB* strains to produce either lipoate for activation of PDH, or acetate/acetyl-CoA by some other means. The later characterization of LplA (Green et al., 1995) and the detection of a cytoplasmic pool of free octanoate (the precursor of lipoate and a LplA substrate) (Hermes and Cronan, 2009) elucidated the source of lipoate and thus acetyl-CoA in Δ*lipB* strains. In contrast Δ*lipB* Δ*lplA* strains do not exhibit acetate-independent growth. The reported concentration of cytoplasmic octanoate (28.2 μM) is well below the K_m for octanoate of the wild type LplA enzyme (214.3 μM) (Hermes and Cronan, 2009). However, since the origin of cytoplasmic octanoate could be spontaneous hydrolysis of the thioester bond of the acyl-ACP of fatty acid biosynthesis, it is conceivable that strain background and/or growth conditions may affect the availability of free octanoate for lipoate synthesis by the LplA/LipA pathway and thus the potential for acetate-independent growth of Δ*lipB* strains. We observed both acetate plus succinate-dependent and succinate only-dependent growth phenotypes in Δ*lipB* strains constructed in two strain backgrounds; MG1655 and W3110 (data not shown). Despite the presence of cytoplasmic octanoate and LplA, the levels of OGDH are undetectable in Δ*lipB* strains, although PDH is active. A plausible explanation for this phenomenon is that *E. coli* PDH has three LDs whereas OGDH has only a single LD. Only one of the three PDH LDs must be modified for essentially full enzymatic activity (Stepp et al., 1981, Ambrose-Griffin et al., 1980, Angelides and Hammes, 1978, Berman et al., 1981, Danson et al., 1978, Hackert et al., 1983). Thus, PDH

would seem to have a competitive advantage over OGDH when octanoate is limiting.

We have now characterized three Δ*lipB* suppressor strains able to grow on unsupplemented glucose minimal medium. In two of these strains suppression was caused by single point mutations in the *lplA* gene which gave LplA enzymes having significantly reduced K_m values for octanoate (Hermes and Cronan, 2009). These mutant LplA enzymes were thus able to utilize cytoplasmic octanoate to activate PDH and OGDH. In the third Δ*lipB* suppressor strain, described herein, suppression resulted from inactivation of the SDH respiratory enzyme complex (encoded by the *sdhCDAB* operon). SDH catalyzes the oxidation of succinate to fumarate, freeing two protons for participation in ATP synthesis via ATP synthase. Since each molecule of glucose is broken down to two molecules of succinate, and ATP synthase requires 12 protons to produce one molecule of ATP, strains defective in SDH activity are expected to produce one-third less energy than wild type. This is reflected in the growth rate of the Δ*lipB* Δ*sdhB* strain which is only two thirds that of the wild type strain (0.429 hr^{-1} and 0.637 hr^{-1}, respectively; Fig. 3.3A). However, despite its moderate importance in energy production, succinate plays a key biosynthetic role.

Initially it seemed perplexing that blockage of succinate consumption would enable a Δ*lipB* strain to grow on aerobic glucose minimal medium since it had been generally assumed that Δ*lipB* strains lack the ability to produce succinate. However, Creaghan and Guest (Creaghan and Guest, 1977) isolated *sdh* null mutants in their search for suppressors of the succinate requirement of a Δ*lpd* strain and this was taken to indicate that *E. coli* has other modes of succinate synthesis (Cronan and Laporte, 2006). In the Δ*lipB* Δ*sdhB* strain we found that succinate/succinyl-CoA were provided by the activities of isocitrate lyase (AceA), aspartate oxidase (NadB) and OGDH (Fig. 3.5). Any one of the three enzymes provided sufficient succinate for growth. NadB was the unexpected member of this troika. NadB is a very unusual enzyme in that it can use either an organic acid, fumarate, or an inorganic molecule, oxygen, as an electron acceptor. Under

physiological aerobic growth conditions NadB utilizes oxygen as an electron acceptor because the cellular fumarate concentration is low (Korshunov and Imlay, 2010). However, fumarate is the preferred substrate and its presence can block oxygen utilization under aerobic conditions (Korshunov and Imlay, 2010). The intracellular concentration of fumarate in aerobically grown *E. coli* does not exceed 10 μM (Korshunov and Imlay, 2010). However, even such concentrations would be sufficient to provide the low concentrations of succinate required for growth of the Δ*lipB* Δ*sdhB* strains which show half maximal growth at 250 nM succinate (Fig. 3.4). In contrast, Δ*lipB* strains fail to grow under these conditions because these strains require 12 μM succinate for half maximum growth, about a 50-fold greater concentration than that required for the Δ*lipB* Δ*sdhB* strains (Fig. 3.2A).

The contribution to succinate production by OGDH was surprising because OGDH activity in these strains was consistently below the level of detection (Fig. 3.3B) and deletion of genes that are essential to both PDH and OGDH function (*lipA, lplA* or *lpd)* in the Δ*lipB* Δ*sdhB* background affected acetate, but not succinate production (Table 3.4). On the other hand, the remaining, albeit undetectable, OGDH activity may explain why Δ*lipB* strains (and the Δ*lipB* Δ*sdhB* Δ*sucCD* strain of this study) require lysine plus methionine but not diaminopimelic acid for growth. Interestingly, despite reports suggesting low-level expression of fumarate reductase during aerobic growth (Cecchini et al., 1995, Jones and Gunsalus, 1985, Tseng et al., 1996, Tseng et al., 1994), deletion of *frdA* was not required to engender succinate auxotrophy in the Δ*lipB* Δ*sdhB* Δ*aceA* Δ*nadB* Δ*sucAB* strain. Moreover, in this strain background introduction of deletions of either *lplA* or *lpd* in place of the *sucAB* deletion gave strains that did not require succinate but did require acetate (Table 3.4). Therefore 2-oxoglutarate dehydrogenase was able to function without the E3 subunit or the LplA lipoate ligase activity. These results indicate that under conditions where only traces of succinate are required, normally insignificant noncanonical pathways become significant. As mentioned above a glutaredoxin-glutathione couple can replace *lpd* (Feeney et al., 2011) and disruption of *lplA* severely

decreased but did not completely eliminate attachment of lipoate and octanoate to SucB (Morris et al., 1994) indicating the presence of another low activity mechanism to modify SucB. The requirement for SucB modification was demonstrated by the finding that expression of a SucB protein lacking the lipoylated lysine residue (K44) failed to restore succinate independence to the Δ*lipB* Δ*sdhB* Δ*aceA* Δ*nadB* Δ*sucB* strain (Table 3.4). Note that the acetate requirement engendered by replacement of the Δ*sucAB* or Δ*sucB* deletions with Δ*lpd* or Δ*lplA* deletions is readily explained by the fact that, unlike succinyl-CoA, the acetyl-CoA produced by pyruvate dehydrogenase is a major cellular building block.

The structure and regulation of the *sdhCDAB-sucAB-sucCD* operon(s) has been the subject of numerous studies (Conway et al., 2014, Cunningham and Guest, 1998, Masse and Gottesman, 2002, Spencer and Guest, 1985). If *sucB* is cotranscribed with *sdhB,* we would expect reduced SucB expression in Δ*sdhB* strains. If so, the resulting decreased cellular LD concentration could result in a sparing effect on the cytoplasmic octanoate pool, thus freeing it for use in activation of PDH. However, we observed that in the Δ*lipB* background, deletions of the Δ*sdhB* and Δ*sucA* genes give a phenotype that differs from that of the Δ*sucB* deletion, suggesting that these genes may not be strictly cotranscribed. Both the Δ*sucA* and the Δ*sucB* deletions disrupt OGDH function, but only the former spares the succinate pool probably by reducing SDH expression. The Δ*sucB* deletion is not polar on the downstream *sucCD* genes, because the Δ*lipB* Δ*sucB* strain can utilize exogenous succinate, a characteristic that would require functional succinyl-CoA ligase (SucCD). Additionally a plasmid carrying the *sucB* gene alleviates the succinate requirement of the Δ*lipB* Δ*sdhB* Δ*aceA* Δ*nadB* Δ*sucB* strain (Table 3.4).

Finally it should be noted that the ability to decipher the interrelationships of central metabolism is the direct product of advances in *E. coli* genetics that allow facile construction of null mutations in the genes of interest (Datsenko and Wanner, 2000, Court et al., 2002). Although the seminal work of Guest and

coworkers (Creaghan and Guest, 1972, Creaghan and Guest, 1977, Guest and Creaghan, 1973, Langley and Guest, 1978) opened many doors in this area, interpretation of results obtained using the point mutants then available would have been compromised by possible residual activities of the encoded proteins.

Tables

Table 3.1: Description of strains used in this study.

Name	Relevant genotype	Construction or reference
FH6	*lipB::*Tn1000 km^R	(Hermes and Cronan, 2009)
FH34	*lipB::*Tn1000 km^R *sdhB* (amber) lip+	Suppressor of the Δ*lipB* mutation of strain FH6
FH160	Δ*lipB::*FRT::cm^R::FRT	MG1655 X p(ZX221)
FH419	Δ*lipB::*FRT::cm^R::FRT Δ*sdhB::*FRT::km^R::FRT	FH160Xp(JW714)
FH420	Δ*lipB::*FRT Δ*sdhB::*FRT	pCP20 transformation of FH419 (Datsenko and Wanner, 2000)
FH497	Δ*lipB::*FRT Δ*sdhB::*FRT Δ*sucAB::*FRT::cm^R::FRT	Datsenko and Wanner in FH420 using primers sucAKOfor and sucBKOrev
FH663	Δ*lipB::*FRT Δ*sdhB::*FRT Δ*sucCD::*FRT::cm^R::FRT	Datsenko and Wanner in FH420 using primers sucCDKOfor and sucDCKOrev
FH686	Δ*lipB::*FRT Δ*sdhB::*FRT Δ*nadB::*FRT::km^R::FRT	FH420 X p(JW2558)
FH691	Δ*lipB::*FRT Δ*sdhB::*FRT Δ*nadB::*FRT	pCP20 transformation of FH686 (Datsenko and Wanner, 2000)
FH695	Δ*lipB::*FRT Δ*sdhB::*FRT Δ*nadB::*FRT Δ*aceA::*FRT::km^R::FRT	FH691 X p(JW3975)
FH698	Δ*lipB::*FRT Δ*sdhB::*FRT Δ*nadB::*FRT Δ*aceA::*FRT::km^R::FRT Δ*sucAB::*FRT::cm^R::FRT	FH695 X p(FH497)
FH705	Δ*lipB::*FRT Δ*sdhB::*FRT Δ*nadB::*FRT Δ*aceA::*FRT::km^R::FRT Δ*sucB::*FRT::cm^R::FRT	Datsenko and Wanner in FH695 using primers sucBKOfor and sucBKOrev
FH706	Δ*lipB::*FRT Δ*sdhB::*FRT Δ*nadB::*FRT Δ*aceA::*FRT::km^R::FRT Δ*sucA::*FRT::cm^R::FRT	Datsenko and Wanner in FH695 using primers sucAKOfor and sucAKOrev
QC146	Δ*lipB::*FRT Δ*lplA::*FRT	(Christensen and Cronan, 2009)

Table 3.1 Continued.

The following strains were used for mapping and/or as donors to construct deletions of various genes in the above strains.		
Name	**Relevant genotype**	**Construction or reference**
CAG12077	*pagP*::Tn10	(Nichols et al., 1998, Singer et al., 1989)
CAG12147	*nadA*::Tn10	(Nichols et al., 1998, Singer et al., 1989)
CAG12149	*dsbG*::Tn10	(Nichols et al., 1998, Singer et al., 1989)
CAG18433	*asnB*::Tn10	(Nichols et al., 1998, Singer et al., 1989)
CAG18493	Contains a Tn*10* insertion in the intergenic region between genes *ybhL* and *ybhM*	(Nichols et al., 1998, Singer et al., 1989)
FH272	Δ*lipB::*FRT::cm^R::FRT lip+ linked to Tn10 of unknown location	FH160 x P1 (pool of FH34 with random Tn10 insertions), followed by screening for lip+
FH278	Δ*lipB::*FRT::cm^R::FRT lip+ linked to Tn5 (Tn5 located in *ybfC*)	FH160 x P1(pool of FH272 with random Tn5 insertions), followed by screening for lip+
FH302	CAG12077 *lipB::*Tn1000 km^R	CAG12077 Xp(FH6)
FH303	CAG12149 *lipB::*Tn1000 km^R	CAG12149 Xp(FH6)
FH304	CAG18433 *lipB::*Tn1000 km^R	CAG18433 Xp(FH6)
FH305	CAG18493 *lipB::*Tn1000 km^R	CAG18493 Xp(FH6)
FH308	*lipB::*Tn1000 km^R lip+ *nadA*::Tn10	FH34 X p(CAG12147)
FH316	Δ*lipB::*FRT::cm^R::FRT lip+ *nadA*::Tn10	FH160Xp(FH308)
JW112	Δ*lpd::*FRT::km^R::FRT	(Baba et al., 2006)
JW697	Δ*ybgA::*FRT::km^R::FRT	(Baba et al., 2006)
JW709	Δ*ybgD::*FRT::km^R::FRT	(Baba et al., 2006)
JW713	Δ*sdhA::*FRT::km^R::FRT	(Baba et al., 2006)
JW715	Δ*sucA::*FRT::km^R::FRT	(Baba et al., 2006)
JW716	Δ*sucB::*FRT::km^R::FRT	(Baba et al., 2006)
JW717	Δ*sucC::*FRT::km^R::FRT	(Baba et al., 2006)
JW721	Δ*mngA::*FRT::km^R::FRT	(Baba et al., 2006)
JW732	Δ*ybgF::*FRT::km^R::FRT	(Baba et al., 2006)
JW755	Δ*ybhC::*FRT::km^R::FRT	(Baba et al., 2006)
JW855	Δ*poxB::*FRT::km^R::FRT	(Baba et al., 2006)
JW886	Δ*pflB::*FRT::km^R::FRT	(Baba et al., 2006)
JW2558	Δ*nadB::*FRT::km^R::FRT	(Baba et al., 2006)
JW3975	Δ*aceA::*FRT::km^R::FRT	(Baba et al., 2006)
JW4115	Δ*frdA::*FRT::km^R::FRT	(Baba et al., 2006)
JW5098	Δ*ybgO::*FRT::km^R::FRT	(Baba et al., 2006)

Table 3.1 Continued.

Name	Relevant genotype	Construction or reference
JW5100	Δ*tolB::*FRT::km^R::FRT	(Baba et al., 2006)
KER310	W3110 str^R *lipA::*Tn*1000* Km *lipB::*Tn10	(Reed and Cronan, 1993)
TM134	W3110 str^R *lplA::*Tn10	(Morris et al., 1995)
ZX221	W3110 str^R Δ*lipB::*FRT::cm::FRT	(Christensen and Cronan, 2009)

Table 3.2: Sequences of primers used in this study.

Name	Sequence
sucAKOfor	AGTATCCACGGCGAAGTAAGCATAAAAAAGATGCTTAAGG GATCACGATGTGTAGGCTGGAGCTGCTTCG
sucAKOrev	ATCTACGCTACTCATTGTGTATCCTTTATTTATTCGACGTT CAGCGCGTCATATGAATATCCTCCTTAG
sucBKOfor	GATCTGGTTAATGACGCGCTGAACGTCGAATAAATAAAGG ATACACAATGTAGGCTGGAGCTGCTTCG
sucBKOrev	GGTCTACAGTGCAGGTGAAACTTAAACTACTACACGTCCA GCAGCAGACGATATGAATATCCTCCTTAG
sucCDKOfor	GGTCTACGGTTTAAAAGATAACGATTACTGAAGGATGGAC AGAACACATGGTGTAGGCTGGAGCTGCTTC
sucCDrevfor	CGGCGAGGGCTATTTCTTATTACAGATATTTATTTCAGAAC AGTTTTCAGCATATGAATATCCTCCTTAG
NocI-nadB	GCGCCATGGATACTCTCCCTGAACATTC
HindIII-nadB	GCGAAGCTTATCTGTTTATGTAATGATTG
P1	AGTGAACAGCAGAGATACGG
P2	GGCAATCTTCATACCGTCAC
P3	GTGGAGGTAGAGGCGTTCTG
P4	TCGGGAAACCGCGGTGCAGC
P5	GTTATCGTGACCTGGATCAC
P6	AAGCTTCCGCGATTATGGGC
P7	TTGCACTGGTGGTTTACGTG
P8	CGCTTTATCAGCAGAACCTG
P9	CCTGGGTAAAGAAGTTCTCG
P10	GGCTTGAGCAGTTGAAAGTG
P11	AAGGTGTGTGCGGTTCCGAC
P12	TCGGCCATATCAAGTCGATG
P13	TGACACCAATGTGAAGCAGG
P14	ACCGTGGTCGTCTGAACGTG
P15	AACGCGGACGATCCGGAAGC
P16	GTGAGAAACTGTTCGACTGG
P17	ACCACATGCTGCGTCGTCAG
P18	TGGTTAATGACGCGCTGAAC
P19	GAAAGAGTCTGCTCCGGAAG
P20	ATCAAAGATCGTCCGATGGC
P21	GGTTATGCCTGTACTACTCC
P22	ACCTGGCGTTGATCGAAATC
P23	GCGAAGAAACTGGCTGACAG
P24	GTATCCAGCCTGGTCACATT

Table 3.3: Transductional crosses used to map the amber mutation in strain FH34.

Cross	Antibiotic resistance marker location	Linkage	Notes
FH34Xp(CAG12149)	*dsbG*	0%	
FH34Xp(CAG12077)	*pagP*	1%	
FH34Xp(CAG18433)	*asnB*	4.3%	
FH34Xp(CAG18493)	Intergenic region between *ybhL* and *ybhM*	4.3%	
FH34Xp(CAG12147)	*nadA*	69.7%	
FH316Xp(JW755)	*ybhC*	6.9%	This cross indicated that the tet^R marker of FH316 (in *nadA)* was between the lip+ marker and the Km^R marker in *ybhC*
FH316Xp(JW697)	*ybgA*	58%	lip+ to the right of *ybgA*
FH316Xp(JW5098)	*ybgO*	84.9%	lip+ to the right of *ybgO*
FH316Xp(JW709)	*ybgD*	88.9%	
FH316Xp(JW713)	*sdhA*	0%	
FH316Xp(JW715)	*sucA*	0%	
FH316Xp(JW716)	*sucB*	0%	
FH316Xp(JW717)	*sucC*	100%	
FH316Xp(JW721)	*mngA*	83.3%	lip+ to the left of *mngA*
FH316Xp(JW5100)	*tolB*	81.3%	
FH316Xp(JW732)	*ybgF*	72.2%	lip+ to the left of *ybgF*

Table 3.4: Acetate and succinate requirements for aerobic growth on glucose minimal medium of MG1655 strains with various gene deletions.

Genetic Manipulation	Supplement Requirement	
	Acetate	Succinate
Δ*lipB* background (Strains FH6 and FH160)		
None	No	Yes
Δ*lplA*	Yes	Yes
Δ*lipA*	Yes	Yes
Δ*sdhB*	No	No
Δ*sdhA*	No	No
Δ*sucA*	No	No
Δ*sucB*	No	Yes
Δ*sdhB/ p(sdhCDAB)*	No	Yes
Δ*sucA/ p(sdhCDAB)*	No	Yes
Δ*lipB* Δ*sdhB* background (Strains FH34 and FH420)		
Δ*poxB*	No	No
Δ*pflB*	No	No
Δ*poxB pflB*	No	No
Δ*frdA*	No	No
Δ*lplA*	Yes	No
Δ*lipA*	Yes	No
Δ*lpd*	Yes	No
Δ*sucCD*	Yes	Lysine and methionine (but not DAP)
Δ*aceA*	No	No
Δ*sucAB*	No	No
Δ*frdA* Δ*aceA* Δ*sucAB*	No	No
Δ*frdA* Δ*aceA* Δ*sucAB* Δ*nadB*	No	Yes
Δ*frdA* Δ*aceA* Δ*sucAB* Δ*nadB/ p(nadB)*	No	No
Δ*nadB*	No	No
Δ*frdA* Δ*nadB*	No	No
Δ*sucAB* Δ*nadB*	No	No
Δ*frdA* Δ*aceA* Δ*nadB*	No	No
Δ*frdA* Δ*sucAB* Δ*nadB*	No	No
Δ*aceA* Δ*nadB* Δ*sucAB*	No	Yes
Δ*aceA* Δ*nadB* Δ*sucA*	No	Yes
Δ*aceA* Δ*nadB* Δ*sucB*	No	Yes
Δ*aceA* Δ*nadB* Δ*sucAB / p(sucB)*	No	Yes
Δ*aceA* Δ*nadB* Δ*sucB / p(sucB)*	No	No
Δ*aceA* Δ*nadB* Δ*lpd*	Yes	No
Δ*aceA* Δ*nadB* Δ*lplA*	Yes	No
Δ*aceA* Δ*nadB* Δ*sucB / p(sucB^{K44R})*	No	Yes

Figures

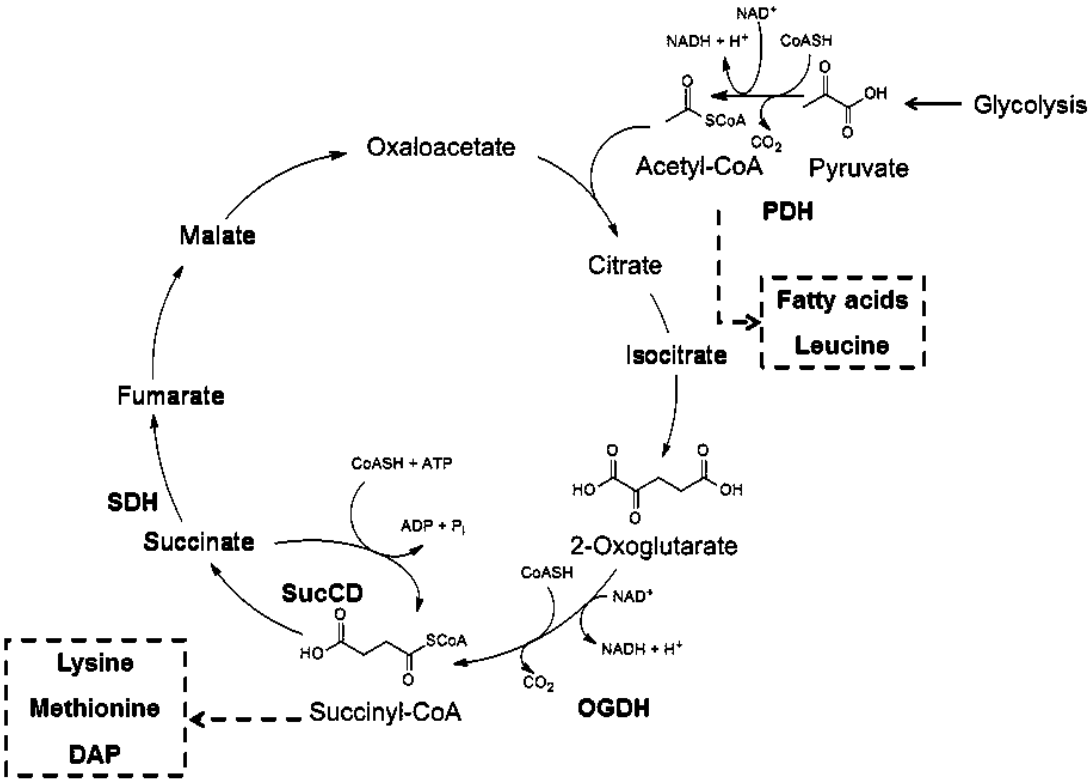


Figure 3.1: Schematic of central metabolism showing the decarboxylation reactions catalyzed by the lipoate-dependent enzyme complexes, pyruvate dehydrogenase (PDH) and 2-oxoglutarate dehydrogenase (OGDH). The dotted arrows point to the biosynthetic pathways that utilize a given intermediate. Also shown is succinate recycling by the succinate:CoA ligase (also called succinyl-CoA synthetase and succinate thiokinase). Abbreviations: PDH, pyruvate dehydrogenase; OGDH, 2-oxoglutarate dehydrogenase; SucCD, succinate:CoA ligase; SDH, succinate dehydrogenase; and DAP, diaminopimelic acid.

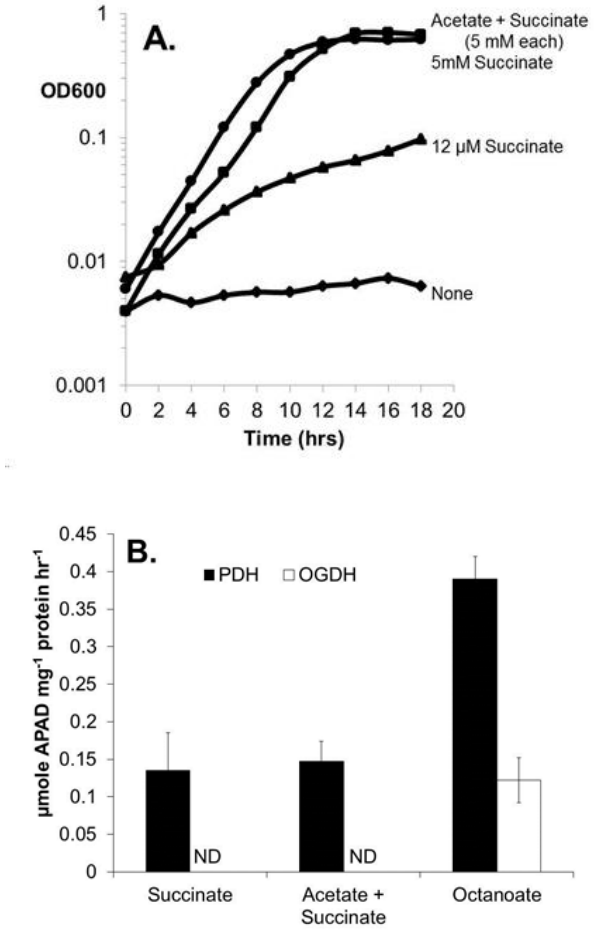


Figure 3.2: The Δ*lipB* strain FH160 contains active PDH consistent with growth on glucose minimal medium without acetate supplementation.
A: Growth characteristics of strain FH160 on glucose minimal medium. Supplementation with a final concentration of 12 µM succinate allowed growth at half the maximal rate.
B: PDH and OGDH activities of extracts of strain FH160**.** Strain FH160 was grown in glucose minimal medium containing the supplements indicated. Although PDH activity was consistently detected, OGDH activity was detected only when octanoate was provided in the growth media to support lipoate synthesis. Error bars indicate the standard deviations of three repeat measurements. ND: not detected (below ~0.005 µmole substrate mg^{-1} protein hr^{-1} (Reed and Cronan, 1993)).

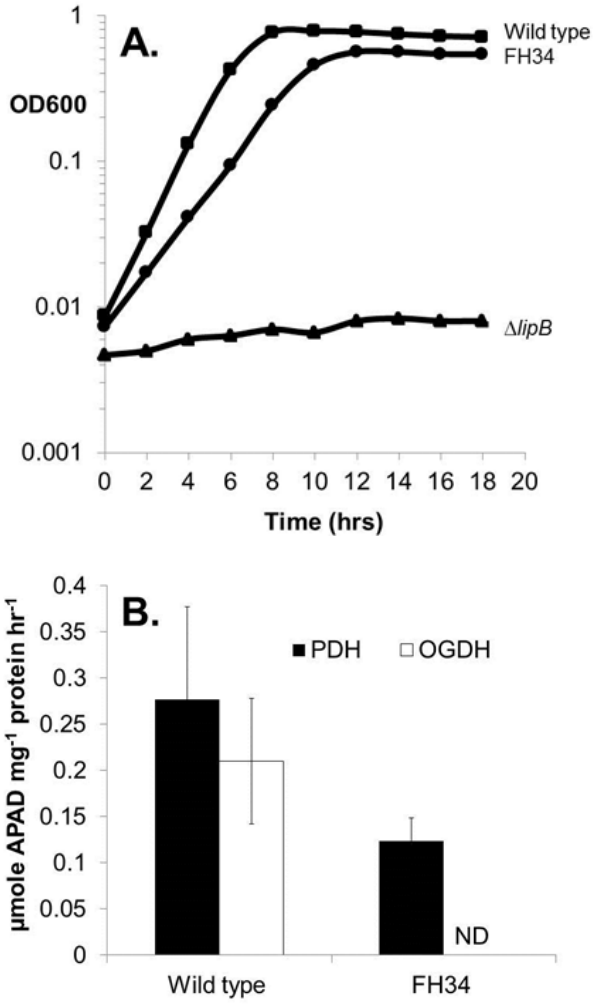


Figure 3.3: Strain FH34 grows in the absence of succinate supplementation despite having no detectable OGDH activity.
A: Growth characteristics on unsupplemented glucose minimal medium of the wild type (MG1655), the Δ*lipB* strain (FH160) and the Δ*lipB* suppressor strain, FH34.
B: PDH and OGDH activities of extracts of strain FH34 and the wild type strain. The strains were grown on glucose minimal medium supplemented with acetate and succinate. Error bars indicate the standard deviations of three repeat measurements. ND: not detected (below ~0.005 µmole substrate mg^{-1} protein hr^{-1} (Reed and Cronan, 1993)).

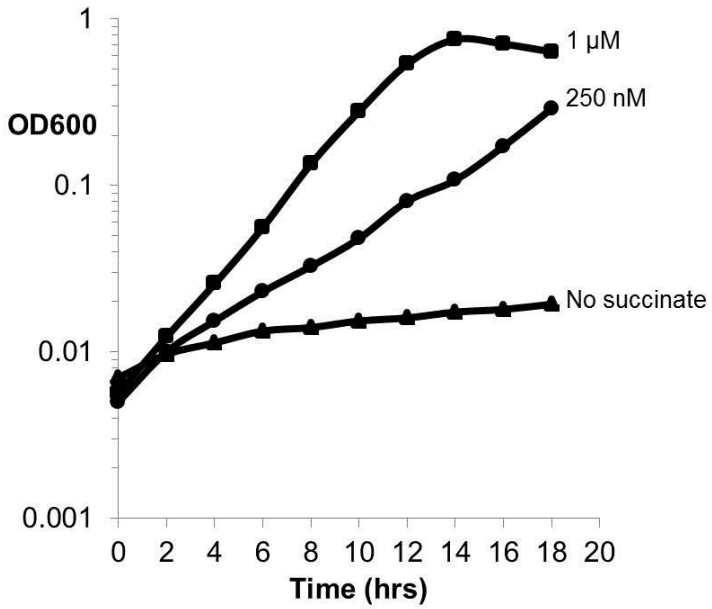


Figure 3.4: The Δ*lipB* Δ*sdhB* Δ*aceA* Δ*nadB* Δ*sucAB* strain (FH698) requires succinate for growth on glucose minimal medium supplemented with nicotinic acid. Nicotinic acid (100 µM) was added to allow synthesis of NAD. The strain was grown in minimal medium containing various concentrations of succinate as shown. Succinate at a final concentration of 250 nM gave half the maximal growth rate.

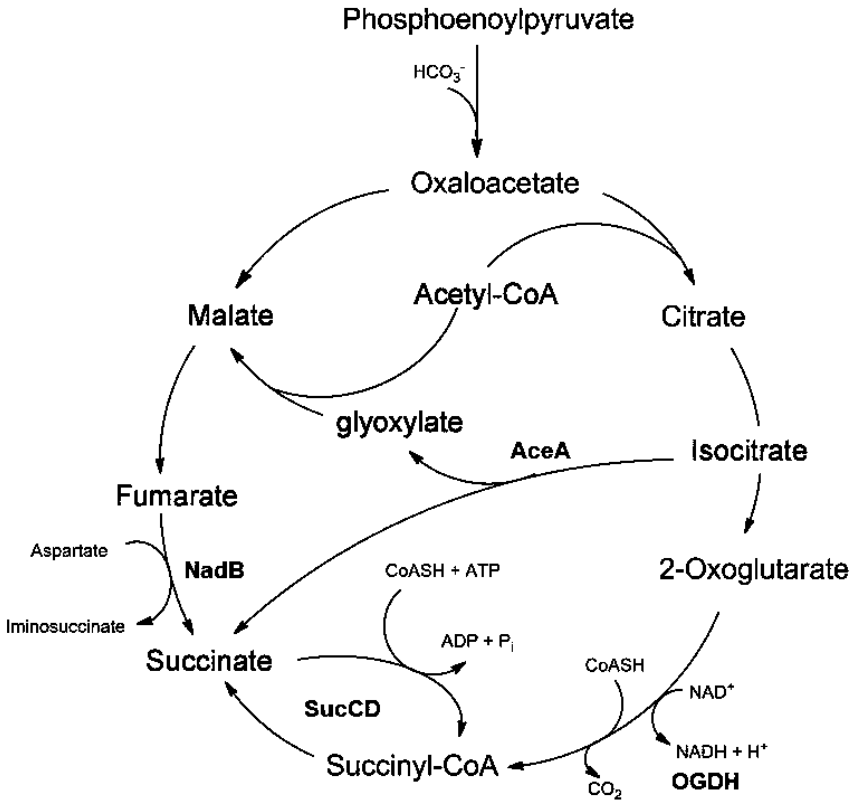


Figure 3.5: Pathways of succinate production in the Δ*lipB* Δ*sdhB* strain. Residual OGDH activity produces succinyl-CoA, whereas the glyoxylate shunt enzyme isocitrate lyase (AceA) cleaves isocitrate to succinate plus glyoxylate which is ultimately converted to malate. Malate is also produced from oxaloacetate in the branched mode of the TCA cycle (shown here). Malate is dehydrated to fumarate which is utilized by aspartate oxidase (NadB) to oxidize aspartate concomitant with reduction of fumarate to succinate. Free succinate from the AceA and NadB reactions is scavenged and converted to its CoA thioester by succinate:CoA ligase (SucCD).

Chapter 4: The Role of the *Saccharomyces cerevisiae* Lipoate Protein Ligase Homologue, Lip3, in Lipoic Acid Synthesis[5]

Introduction

Since the initial demonstration, purification and synthesis of lipoic acid in the early 1940s (Reed, 2001), much has been learned about the synthesis of this essential respiratory cofactor in prokaryotes (Cronan et al., 2005, Cronan, 2008). In *Escherichia coli,* lipoate is assembled on the lipoyl domains (LDs) of the lipoate-dependent enzyme systems from the eight carbon fatty acid, octanoate, in two steps. First, an octanoyltransferase (LipB) transfers an octanoyl moiety from the acyl carrier protein (ACP) of fatty acid synthesis to the LDs (Jordan and Cronan, 2003). A thioester-bound acyl-enzyme intermediate is formed in the process (Zhao et al., 2005). Then lipoyl synthase (LipA) catalyzes replacement of single hydrogen atoms at carbons 6 and 8 with sulfur atoms using radical SAM chemistry (Booker et al., 2007). Alternatively, exogenous lipoate or octanoate can be directly attached to LDs by lipoate protein ligase (LplA) via an acyl-AMP intermediate.

Although the LipA sulfur insertion reaction is conserved in all characterized lipoate synthesis pathways, there are three well-characterized variations to the *E. coli* pathway in the other reactions. In the lipoate auxotrophic archeon *Thermoplasma acidophilum,* the lipoate protein ligase is composed of two separately encoded subunits LplA and LplB that together catalyze formation of the acyl-AMP intermediate (Posner et al., 2009, Christensen and Cronan, 2009). LplB was found to be dispensable for the second half reaction, namely ligation of the acyl moiety to the acceptor lysine on the LDs (Christensen and Cronan, 2009). The Gram-positive bacterium *Bacillus subtilis* lacks a LipB homologue, but encodes three LplA homologues, although only LplJ has the ligase function of *E.*

[5] Results presented in this chapter are published in the journal Yeast (2013) 30(10):415-27.

coli LplA (Martin et al., 2011). One of the two remaining homologues (LipM) is an octanoyltransferase that specifically transfers octanoate from ACP to a small protein, GcvH (Christensen and Cronan, 2010), whereas LipL, the third homologue, is an amidotransferase that transfers the octanoyl moiety from GcvH to the LDs of pyruvate dehydrogenase (PDH), the branched chain oxoacid dehydrogenase, and oxoglutarate dehydrogenase (OGDH) (Christensen et al., 2011b, Martin et al., 2011). LipL is also present in *Listeria monocytogenes* along with two other LplA homologues that are ligases. LplA1 specifically modifies GcvH whereas the LD specificity of LplA2 is undetermined (Christensen et al., 2011a). The lipoate scavenged by *L. monocytogenes* LplAs might come directly from the host environment or might be released from host lipoylated-peptides by the action of the lipoamidase(s) encoded by this bacterium.

The diversity in prokaryotic lipoate attachment mechanisms, summarized in Fig. 1.2, is surprising given the simple structure of the coenzyme. The situation in eukaryotes is not well understood and may contribute further enzymatic diversity. Early studies of the bovine LplA-homologue (LipT; for lipoyltransferase) demonstrated that the enzyme was unable to form the acyl-AMP intermediate (Fujiwara et al., 1994). However LipT was able to transfer the acyl chain from exogenously provided acyl-AMP to LDs *in vitro* giving rise to the proposal that mammalian lipoate attachment requires a “lipoate-activating enzyme”. Accordingly, Fujiwara *et al.* (2001) purified an enzyme from bovine liver that synthesized lipoyl-GMP *in vitro* which in turn could be utilized by LipT to modify LDs (Fujiwara et al., 2001). However this putative lipoate-activating enzyme is one of many poorly characterized medium chain fatty acyl-Coenzyme A (CoA) ligases that have extremely broad substrate specificities (Kasuya et al., 2006, Vessey et al., 2000), and thus the physiological relevance of its *in vitro* lipoyl-GMP forming activity is unclear.

In the yeast, *Saccharomyces cerevisiae,* there are at least three lipoate-dependent enzyme systems; PDH, OGDH and the glycine cleavage system (GCV). PDH catalyzes the oxidative decarboxylation of pyruvate, the endpoint of

glycolysis, to acetyl-CoA and thereby links cytosolic glycolysis and mitochondrial respiration. The acetate moiety of acetyl-CoA is condensed with oxaloacetate to form citrate, the first intermediate of the tricarboxylic acid (TCA) cycle. Mitochondrial acetyl-CoA is also the building block for mitochondrial fatty acid (FA) biosynthesis which produces octanoyl-ACP, the precursor of lipoate (Hiltunen et al., 2010). OGDH catalyzes the oxidative decarboxylation of the TCA cycle intermediate, oxoglutarate, to succinyl-CoA, a precursor of several amino acids, and the source of succinate which is the entry point to the respiratory chain. GCV is involved in cleavage of glycine to ammonia and C1 units and is essential for utilization of glycine as a sole source of nitrogen. All constituents of these enzyme systems, as well as those of mitochondrial fatty acid biosynthesis, are encoded by nuclear genes and have mitochondrial targeting sequences. Their functions are necessary for mitochondrial maintenance and respiratory growth (Contamine and Picard, 2000).

Studies by Schonauer *et al.* (2009) showed that Lip2, Lip5, Lip3 (the LipB, LipA and LplA homologues respectively) as well as Gcv3 (the GcvH homologue) are all required for endogenous lipoate synthesis and attachment (Schonauer et al., 2009). Respiratory growth of a *lip2* strain, which contains a wild type copy of the *lip3* gene, is not supported by exogenous lipoate supplementation (Chen, 1997, Marvin et al., 2001), suggesting that *S. cerevisiae* lacks a lipoate scavenging pathway. On the other hand, early studies by Sulo and Martin (Sulo and Martin, 1993) showed that even though lipoate did not support the respiratory growth of *lip5* strains, the cells contained a substantial level of protein-bound lipoate when grown on lipoate-supplemented media, suggesting the presence of a scavenging mechanism in *S. cerevisiae.*

We sought to further investigate the role of Lip3 in lipoate synthesis in *S. cerevisiae*. We show that Lip3 has octanoyl-CoA: protein transferase activity and present a model for lipoate synthesis in *S. cerevisiae*.

Materials and Methods

General molecular biology techniques:

Genomic DNAs were extracted using the Promega Wizard genomic DNA purification kit. Plasmids were prepared using either the Qiagen minispin or midi kits. PCR was conducted using either Taq (New England Biolabs) or Pfu (Invitrogen) polymerase, and appropriately designed primers from IDT (Table 4.1). Restriction enzymes and ligases were from New England Biolabs. DNA sequencing was conducted by the Core Sequencing Facility of the Carver Biotechnology Center of the University of Illinois, Urbana Champaign.

Yeast suspensions for PCR were prepared by suspending a colony in 30 µl of 0.2% SDS and boiling at 96^{o} for 5 min, then cooling on ice. The suspension was then cleared at 3000xg for 1 min, and 10 µl of supernatant was diluted with 67 µl of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA.

Strains and plasmids:

All *E. coli* strains, yeast strains and plasmids used in this study, as well as their relevant genotypes are listed in Table 4.1.

Plasmid pFH21 was made by digesting PCR-amplified *lip3* with BspHI and HindIII and ligating the product to NcoI and HindIII digested pBAD24. The *lip3* gene used was a synthetic gene composed of codons optimized for expression in *E. coli*. Plasmid pFH23 was constructed by moving the ClaI-HindIII fragment containing *lip3* and the arabinose promoter from pFH21 into pACYC177. Site-directed mutagenesis of the *lip3* gene in plasmid pFH23 was conducted using pfuUltra HF from Invitrogen and appropriately designed primer sets per the manufacturer's recommendations. Vector pFH88 was made by PCR amplification of the *fadK* gene from pRK33 using primers fadK-for and fadK-rev. The PCR product was digested with NcoI and HindIII and ligated to pBAD322C treated with the same restriction enzymes.

To construct plasmid pFH72 the last 360 codons of the *lip3* gene were PCR amplified from a *S. cerevisiae* cell suspension using primers pPICNTlip3-for (which included codons for 6X His tag) and pPIClip3-rev. The PCR product was digested with BamHI and EagI and ligated into pPIC3.5K treated with the same restriction enzymes. Plasmid pFH38 was constructed by ligation independent cloning (Eschenfeldt et al., 2009) of a synthetic *gcv3* gene altered to have *E. coli* optimized codons into pMCSG7.

Vector pFH46 is a pUC-type vector which carries a synthetic 126 bp fragment encoding the first 42 amino acids of the predicted mitochondrial targeting sequence of *S. cerevisiae* Coq3. The sequence was flanked by a BamHI cut site upstream and KpnI and PstI sites immediately downstream. The *lplA* and *lipB* genes were PCR amplified from *E. coli* total genomic DNA using primers mtlplA-for and mtlplA-rev and mtlipB-for and mtlipB-rev, respectively. *B. subtilis lipL* was amplified from plasmid pQC32 using primers mtlipL-for and mtlipL-rev. The amplified genes were ligated into the KpnI and PstI sites of pFH46 and their sequences verified. The result of this step was to introduce a mitochondrial targeting sequence at the N-termini of LplA, LipB and LipL. The resulting plasmids were then digested with BamHI and PstI and the appropriate DNA fragments were ligated to the *E. coli-S. cerevisiae* shuttle vector pCM188 treated with the same restriction enzymes.

pFH18 contains the *lip2* gene synthesized using codons optimized for expression in *E. coli.* The gene was inserted between the NcoI and HindIII sites of pBAD24 under control of an arabinose promoter. pFH87 contains an N-terminal 6X His-tagged *lip2* gene in pQE80K (Qiagen) under control of a *tac* promoter. It was constructed by PCR amplification of *lip2* from pFH18, digestion of the PCR product with BamHI (site included in the primer) and HindIII and ligating the product into pQE80K cut with the same enzymes. pFH87 was transformed into the *E. coli* Δ*lipB* Δ*lplA* strain QC146 to give strain FH590.

E. coli strains were transformed by heat shock treatment (Seidman et al., 1997). To construct a *fadD* gene replacement in strain FH306, a P1 phage stock was

grown on strain RMK75 and the lysate was used to transduce FH306 to chloramphenicol resistance following the method of Miller (1992) (Miller, 1992). The resulting strain, FH307 failed to grow on oleate as sole carbon source.

S. cerevisiae strains were transformed using the lithium acetate/single-stranded carrier DNA/ polyethylene glycol method of Gietz and Woods (2002) (Gietz and Woods, 2002). Transformants were selected on glucose synthetic complete uracil dropout medium (see below). Strain FH553 was made by linearizing pFH72 with SalI and transforming the linear plasmid into *Pichia pastoris* strain GS115 by electroporation as described by the supplier. Transformants having chromosomal integration of the plasmid were selected on glucose synthetic complete histidine dropout medium.

Growth conditions:

Yeast strains were generally grown at 30^{o} on rich media consisting of yeast peptone (YP) containing 1% yeast extract and 2% peptone with either 2% glucose, 3% glycerol or 3% ethanol as carbon sources. Selection for transformants was done on glucose synthetic complete media missing either histidine or urea (Treco, 1993). Methanol (1%) was used to induce protein expression in *P. pastoris* strain FH553.

E. coli strains were grown at 37^{o} on Luria-Bertani (LB) (Miller, 1992) or glucose minimal E media (GMM) (Vogel and Bonner, 1956). Supplements were added to the following concentrations: acetate 5 mM, succinate 5 mM, lipoate 7.5 ng ml^{-1}, octanoate 50 µM, arabinose 0.2%, and glucose 0.4%. Antibiotics were used at the following concentrations (in µg ml^{-1}): sodium ampicillin, 100; spectinomycin sulfate, 50; kanamycin sulfate, 50; and chloramphenicol, 20.

Growth curves:

Strains were inoculated in 5 ml of minimal E supplemented with arabinose, acetate, succinate and appropriate antibiotics and grown two nights at 37^{o}. The cells were collected and washed three times with minimal E and diluted to initial

OD600 of about 0.01 in 300 µl of growth medium. Growth was followed at 37^{o} on a Bioscreen C automated growth curve analysis system set to continuous very strong intensity shaking for aeration. The means and standard deviations of 3 replicates are presented.

Assays of PDH and OGDH activities:

The procedure described in Hermes and Cronan (2009) was followed with a few differences. The cultures were in 50 ml LB supplemented with arabinose, acetate and succinate and either lipoate, octanoate, both, or neither as indicated. Cell pellets were lysed using BugBuster Master Mix from Novagen per the manufacturer's protocol. The means and standard deviations of 3 repeat experiments are presented. p-values were calculated using Student's T-test, using a two-tailed distribution of probability for two independent samples assuming equal variance. Significance was assigned for conditions who's p-values were smaller than 0.05.

Detection of in vivo lipoylation by western blotting:

About 150 µg of total protein was fractionated on 12% SDS gels and then blotted onto a polyvinylidene difluoride membrane using a standard protocol (Gallagher, 2010). Lipoylated domain was detected using rabbit anti-lipoyl protein primary antibody from Calbiochem and goat anti-rabbit antibody from Roche.

Protein expression and purification:

In our hands Lip3 consistently formed inclusion bodies when over expressed in *E. coli* regardless of the strain background and the numerous expression conditions attempted. Fusions to maltose binding protein (MBP) were soluble, but inactive, and Lip3 aggregated upon cleavage of the solubility tag. Purification under denaturing conditions followed by refolding produced inactive protein. We therefore resorted to expressing and purifying Lip3 from the yeast *P. pastoris*. Strain FH553 was cultured in YP glycerol overnight at 30^{o}. The cells were collected and resuspended to OD600 of 0.5 in 400 ml YP methanol in a two-liter

flask and protein production was allowed to proceed overnight. The cells were collected, freeze-thawed and resuspended in breaking buffer (50 mM sodium phosphate (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 5% glycerol). Zymolase was added and the suspension was incubated at 30^{o} for an hour. The extract was cleared by centrifugation. Hexahistidine tagged Lip3 was partially purified using Ni-NTA from Qiagen following the manufacturer's recommendations, and verified using western blotting with anti-his antibody. Protein concentrations were measured at 280 nm using the extension coefficient 55810 $M^{-1}cm^{-1}$ predicted by the program ProtPram (Gasteiger E., 2005). We obtained about 5 µg of protein from 400 ml of culture.

To express hexahistidine-tagged apo Gcv3, strain FH437 was inoculated in LB supplemented with glucose, acetate, succinate, spectinomycin and ampicillin. At OD600 of 0.8, protein production was induced overnight with arabinose. Gcv3 was purified under denaturing conditions with 8 M urea using Ni-NTA chromatography. The protein was refolded by gradually removing the urea by dialysis. Protein concentration was measured at 280 nm using the extension coefficient 26930 $M^{-1}cm^{-1}$ (ProtPram) (Gasteiger E., 2005). Expression and purification of the *E. coli* LD was conducted as described previously (Hermes and Cronan, 2009).

Holo ACP was expressed and purified, and octanoyl-ACP was prepared as described in Hermes and Cronan 2009 (Hermes and Cronan, 2009). Partially purified His-tagged Lip2 was obtained by growing FH590 in 200 ml LB supplemented with glucose, acetate, succinate and kanamycin to OD600 of 0.7. Protein production was induced for 3 h with 1 mM IPTG. The cells were collected and lysed with BugBuster (Novagen). The extract was cleared by centrifugation at 5000xg. Lip2 was found mainly in the insoluble fraction. Soluble Lip2 was obtained from the supernatant using a column of Ni-NTA (Qiagen) following the manufacturer's recommendations. The presence of His-tagged Lip2 in the extracts was verified by western blot using anti-His tag antibodies.

Synthesis of octanoyl-Gcv3:

A 300 µl reaction contained apo-Gcv3, 10 mM sodium phosphate (pH7.0), 0.3mM dithiotheritol, 5 mM $MgCl_2$, 5 mM ATP, 3 mM octanoate (30% radiolabeled and C1) and 5 µM LplA. The reaction was incubated overnight at 37^{o}C. To remove unreacted ATP, 25U of glucokinase (Sigma) and 50 mM glucose were added and the reaction was incubated at 30^{o}C for 2 hours followed by dialysis in a 3.5K cutoff dialysis bag against 50 mM tris (pH 9.0) containing 10 mM EDTA and 10% glycerol overnight at room temperature.

Gel shift activity assays:

To assay Lip3 activity a 20 µl reaction mixture contained 10 mM sodium phosphate buffer (pH 7), 0.3 mM dithiothreitol, about 650 nM Lip3, and either 6 µM apo Gcv3 or 15 µM apo LD. To test for ligase activity 5 mM $MgCl_2$, 5 mM ATP and 1 mM octanoate were added to the reaction. Alternatively 100 µM octanoyl-CoA (from Sigma) was added. The reaction was run for 12 hours at 37^{o}, after which 15 µl was loaded in a 20% native polyacrylamide gel and separated by electrophoresis. To assay Lip2 activity the reactions were set up as described above except Lip2 extract was used instead of Lip3 and octanoyl-ACP or octanoyl-Gcv3 instead of octanoyl-CoA as indicated.

Results

Expression of wild type Lip3 permits growth of an *E. coli* Δ*lipB* Δ*lplA* strain on octanoate, but not lipoate supplemented media.

E. coli strain QC146 is deleted for the *lipB* and *lplA* genes which renders it defective in both synthesis and scavenge of lipoate. Growth of strain QC146 on GMM is strictly dependent on supplementation with acetate and succinate which, respectively, bypass the PDH- and OGDH-catalyzed steps required for TCA cycle function (Herbert and Guest, 1968). We tested the ability of Lip3 to support the growth of strain QC146 either on unsupplemented GMM (i.e. to complement the *lipB* defect), or on GMM supplemented with either lipoate or octanoate (i.e. to complement the *lplA* defect). Strain QC146 containing pFH23, a plasmid that

expresses Lip3, failed to grow on either unsupplemented or lipoate supplemented GMM but grew on octanoate supplemented GMM (Fig. 4.1A). The strain also grew on GMM containing a mixture of lipoate and octanoate (data not shown) which indicated that lipoate neither supported nor inhibited growth of strain QC146 harboring pFH23.

Members of the lipoate protein ligase (Lpl) family have a signature catalytic lysine that is essential for activity. From the sequence alignment of Lip3 with several other Lpl family homologues(Schonauer et al., 2009), K249 was a good candidate for the Lip3 catalytic site lysine residue. Replacing K249 with arginine had deleterious effects on the ability of Lip3 to complement growth of QC146 (Fig. 4.1B), whereas replacing K249 with alanine completely eliminated activity (Fig. 4.1B).

The *E. coli* Δ*lipB* Δ*lplA* strain expressing wild type Lip3 contained active and lipoylated PDH and OGDH complexes.

To confirm that growth of strain QC146 containing the Lip3 expression plasmid pFH23 on octanoate supplemented media was due to activation of PDH and OGDH, the activities of the complexes were assayed in extracts of this strain. The strain was grown in LB medium containing acetate, succinate, arabinose (to induce Lip3 expression) supplemented with either lipoate, octanoate or left unsupplemented. Strain QC146 containing the empty vector grew on these media due to the presence of acetate and succinate (see above). As expected the extracts of the vector containing strain had no detectable activity of either PDH or OGDH even when both lipoate and octanoate had been added to the cultures (Fig. 4.2A). On the other hand, extracts of strain QC146 containing the Lip3 expression plasmid grown without lipoate or octanoate, or with lipoate only had traces of PDH and OGDH activities (Fig. 4.2A). The trace activities were probably due to trace amounts of octanoate in LB medium which can be utilized by Lip3 to activate PDH and OGDH. Addition of octanoate to the cultures of strain QC146 containing pFH23 resulted in significantly higher PDH and OGDH activities (Fig. 4.2A).

To further confirm PDH and OGDH activation in strain QC146 carrying pFH23, cell extracts were assayed for lipoylated PDH and OGDH by western blotting using a commercial anti-lipoyl LD antibody. A faint band corresponding to SucB (the LD-containing subunit of OGDH) but no band for AceF (the LD-containing subunit of PDH) was observed in the extracts from unsupplemented and lipoate supplemented cultures (Fig. 4.2B, lanes 1 and 2). The SucB band was much more prominent in the extract from the octanoate-supplemented culture and an AceF band was also present in this extract (Fig. 4.2B, lane 3). We did not expect to detect lipoyl-GcvH (the LD of GCV) in this experiment because in *E. coli* GCV is expressed only in the presence of glycine.

Complementation of the *E. coli* Δ*lipB* Δ*lplA* strain by expression of Lip3 requires a host-encoded acyl-CoA synthetase.

The finding that Lip3 selectively utilized octanoate, but not lipoate, in vivo was surprising. What was more puzzling was the requirement for octanoate supplementation because we had earlier shown that a pool of intracellular free octanoate is present in *E. coli* (Hermes and Cronan, 2009). This pool is sufficient for activation of PDH and OGDH either when LplA was expressed from a strong promoter or when a mutant enzyme having a lower K_m for octanoate was present. We therefore reasoned that the actual substrate for Lip3 may not be free octanoate *per se* but the much rarer species, octanoyl-CoA. We argued against octanoyl-ACP as the substrate since this is a standard intermediate of fatty acid (and lipoate) synthesis and is thus constitutively present. If octanoyl-CoA was the Lip3 substrate, then a fatty acid-CoA synthetase would be required to convert octanoate to octanoyl-CoA. Hence, growth of the *E. coli* Δ*lipB* Δ*lplA* strain carrying the pFH23 Lip3 expression plasmid on octanoate-supplemented GMM would depend on the presence of the *fadD* fatty acid acyl-CoA synthetase of aerobic β-oxidation. To test this premise, we deleted *fadD* and found that the resulting strain (strain FH307) was unable to grow on octanoate-supplemented GMM (Fig. 4.3A). Growth of the *E. coli* Δ*lipB* Δ*lplA* strain carrying the Lip3 plasmid was slow and it seemed likely that this was due to the fact that octanoate

is a poor substrate for FadD. The acyl-CoA synthetase of the anaerobic β-oxidation pathway FadK has preference for short chain fatty acid substrates (Morgan-Kiss and Cronan, 2004). However *fadK* is only expressed during anaerobiosis (Morgan-Kiss and Cronan, 2004). Hence we placed the *fadK* gene downstream of an arabinose promoter and this plasmid was introduced into strain QC146 carrying the Lip3 expression plasmid pFH23. The resulting strain grew much better on octanoate-supplemented GMM than strain QC146 carrying only pFH23 (Fig. 4.3B), thus providing further support for the hypothesis that the Lip3 substrate is octanoyl-CoA (or derived from it) rather than free octanoate.

Lip3 formed octanoyl-LD and octanoyl-Gcv3 *in vitro* using octanoyl-CoA.

Lip3 activity *in vitro* was tested using an electromobility shift assay. Attachment of octanoate to the lysine residue of the LD or Gcv3 proteins results in loss of a positive charge which gives more rapid migration of the modified protein in native electrophoresis gels relative to the unmodified protein. Lip3 was unable to modify either LD or Gcv3 when provided with free octanoate, ATP and $MgCl_2$ (Fig. 4.4A and data not shown). When octanoyl-CoA was used as acyl donor, Lip3-catalyzed modification of both LD and Gcv3 was seen (Fig. 4.4A, lanes 1 and 5). In contrast to the implications of its *in vivo* activity, Lip3 also catalyzed modification of Gcv3 and LD *in vitro* with octanoyl-ACP as substrate (Fig. 4.4B and 4.5C). No amidotransfer activity was detected for Lip3 *in vitro* (Fig. 4.4C). The extreme insolubility of Lip3 (see MATERIALS AND METHODS) precluded further characterization of Lip3 *in vitro*.

Lip3 functions downstream of Lip2 in *S. cerevisiae*.

As stated earlier lipoate synthesis in *S. cerevisiae* is required for mitochondrial maintenance and respiration. When presented with glucose, *S. cerevisiae* preferentially grows fermentatively even in the presence of oxygen. Fermentative growth does not require mitochondria and therefore to test mitochondrial function (an indicator of lipoate synthesis) *S. cerevisiae* must be grown on a nonfermentable carbon source such as ethanol. We tested growth of *S.*

cerevisae strains carrying deletions of the *lip2, lip3* or *gcv3* genes after transformation with either an empty vector or a plasmid encoding a mitochondrially targeted gene (*E. coli lipB, E. coli lplA*, *or B. subtilis lipL*) on rich ethanol media.

The wild type parent, strain BY4741, grew well on rich medium containing ethanol in place of glucose (Fig. 4.5) whereas the *lip2, lip3* and *gcv3* mutant strains transformed with the empty vector failed to grow, even when the medium was supplemented with octanoate (Fig. 4.5, panel A). Expression of mitochondrially targeted *E. coli* LplA allowed growth of all three mutant strains (Fig. 4.5, panel B) indicating that the mutant strains expressed PDH and OGDH albeit in their inactive (apo) forms. Moreover, these data indicate that Gcv3 *per se* is not required for respiration but is required for downstream lipoylation of PDH and OGDH.

Mitochondrially targeted *B. subtilis* LipL failed to complement the *lip2* mutant strain but complemented the *lip3* mutant strain (Fig. 4.5, panel D). This suggests that Lip2 is the primary source of octanoyl-Gcv3, the LipL substrate. *E. coli* LipB produces octanoyl-Gcv3 as indicated by its ability to complement a *lip2* strain (Fig. 4.5, panel C). However, the inability of LipB to complement the *gcv3* strain indicates that *E. coli* LipB cannot efficiently modify *S. cerevisiae* PDH and OGDH. Finally, the inability of LipB to complement the *lip3* strain indicates that *lip3* acts downstream of the octanoyl-Gcv3 formed by either Lip2 or LipB.

Interestingly, the *lip2* strain expressing LipL exhibited slight growth on ethanol when octanoate was provided (Fig. 4.5, panel D). This suggests that under these conditions, Lip3 (or another as yet unknown enzyme) produces some octanoyl-Gcv3 which is presented to LipL for modification of PDH and OGDH. The *gcv3* strain expressing LipB also grew slightly on octanoate-supplemented ethanol media. This suggests that in the absence of competition with Gcv3 and in the presence of excess octanoate Lip3 can modify PDH and OGDH, albeit poorly.

Discussion

Several studies of transgenic mice and clinical observations of human disease cases have underscored the essential role of lipoate synthesis in health (Mayr et al., 2011, Smith et al., 2012, Yi and Maeda, 2005). Lipoate is an essential cofactor for the respiratory enzyme systems, PDH and OGDH. *S. cerevisiae* mutants defective in lipoate synthesis are thus unable to grow on nonfermentable carbon sources such as ethanol. These strains form characteristically small colonies, called *petites,* when grown on glucose, because the colonies stop growing upon exhaustion of glucose. In contrast colonies of wild type *S. cerevisiae* continue to grow by utilization of the ethanol produced by glucose fermentation (Tzagoloff and Dieckmann, 1990). Petite mutants frequently exhibit complete loss or extensive deletions of mitochondrial DNA (Contamine and Picard, 2000).

There remain several gaps in our understanding of lipoate synthesis in *S. cerevisiae.* Sulo and Martin (Sulo and Martin, 1993) isolated and characterized Lip5, the gene encoding the *S. cerevisiae* LipA homologue. Interestingly, they showed that even though *lip5* strains form petite colonies, the cells contained a substantial level of protein-bound lipoate when grown on lipoate-supplemented media. This observation suggests the presence of a scavenging mechanism in *S. cerevisiae* similar to that of *E. coli* LplA, *B. subtilis* LplJ or the *T. acidophilum* LplA/B complex. Why then are *lip5* strains respiratory deficient? It is possible that the lipoate scavenge pathway modifies only one of the two respiratory enzyme systems, while the endogenous lipoate synthesis pathway targets both systems. The lipoate scavenging enzyme of *S. cerevisiae* has yet to be identified since this study shows that Lip3, the only known Lpl homologue to date in the yeast, is not involved in lipoate scavenge.

A complete set of type II fatty acid biosynthesis genes, including a gene encoding ACP, has been characterized in *S. cerevisiae* mitochondria (Hiltunen et al., 2010). Mitochondrial fatty acid biosynthesis produces octanoyl-ACP, the substrate for the *S. cerevisiae* LipB homologue, Lip2. The *lip2* genes of both *S.*

cerevisiae and *Kluyveromyces lactis* have been cloned and disrupted. Although the Lip2 proteins of the two yeast species have almost identical amino acid sequences, the *S. cerevisiae lip2* mutant strain was respiratory deficient whereas the *K. lactis lip2* strain was not (Chen, 1997, Marvin et al., 2001). Thus the differences in the effects of *lip2* deletion could reflect diversity in lipoate synthesis and/or utilization between the two organisms. We cloned *S. cerevisiae lip2* and showed that it complements an *E. coli* Δ*lipB* Δ*lplA* mutant strain on unsupplemented GMM (Fig. 4.6A). Crude *E. coli* extracts containing Lip2 had octanoyltransferase activity and lacked ligase activity (Fig. 4.6B). It was necessary to confirm that Lip2 is indeed an octanoyltransferase because octanoyltransferases, amidotransferases and lipoate ligases are difficult to distinguish from one another by examination of their amino acid sequences. Members of the three groups share minimal amino acid sequence identity yet have similar structural folds (Ma et al., 2006, Reche, 2000, Christensen et al., 2011b). A case in point is LipM which has the sequence of a lipoate ligase but is an octanoyltransferase rather than a ligase (Christensen and Cronan, 2010). Whereas octanoyltransferases lack an accessory domain, ligases may contain the accessory domain at either the C-terminus of the same protein such as the case with *E. coli* LplA (Fujiwara et al., 2010, Fujiwara et al., 2005), or at the N-terminus, such as the case for *Streptomyces coelicolor* LplA (Christensen and Cronan, 2009), or the accessory domain may be encoded by a completely separate gene, such as the *T. acidophilum* LplB (McManus et al., 2006, Posner et al., 2009, Christensen and Cronan, 2009).

A third and unexpected protein involved in *S. cerevisiae* lipoate synthesis is Gcv3. Gcv3 is a lipoylated protein that is a subunit of the GCV complex of single carbon metabolism together with Gcv1, Gcv2 and Lpd1. Work by Schonauer *et al.* (Schonauer et al., 2009) suggests that lipoylation of Gcv3 is required for lipoylation of PDH and OGDH. They showed that in a *gcv3* strain there is no detectable lipoyl-PDH or OGDH. This was true of *S. cerevisiae* strains containing a complete deletion of *gcv3* and of strains encoding a mutant Gcv3 that lacked the lysine residue modified by lipoate attachment. Deletion of the other GCV

subunits had no effect on lipoylation of Gcv3, PDH or OGDH (Schonauer et al., 2009). The requirement of this apparent “checkpoint” in lipoate synthesis is unclear. Perhaps *S. cerevisiae* uses lipoyl-Gcv3 as a sensor for nutritional standing before proceeding with activation of respiratory enzymes. Furthermore the operation of this putative checkpoint is unclear. Schonauer and coworkers showed that in a *lip2* strain there is no detectable lipoylation of either Gcv3 or the PDH and OGDH, whereas in a *lip3* strain Gcv3 becomes lipoylated (Schonauer et al., 2009). One possible explanation for this is that Lip2 specifically modifies Gcv3, whereas Lip3 modifies PDH and OGDH once Gcv3 lipoylation is complete. In an *in vitro* assay we noticed that when provided with a mixture of Gcv3 and LD, Lip3 modifies only Gcv3 (data not shown). This suggests that Gcv3 has priority for modification in *S. cerevisiae* whether catalyzed by either Lip2 or Lip3. Active GCV might be needed at times when active PDH and OGDH are not. Indeed, when wild type *S. cerevisiae* was grown on glucose the sole lipoylated protein detected was Gcv3 (Fig. 4.7). In contrast the same strain grown under respiratory conditions (on glycerol in place of glucose) contained lipoylated Gcv3, PDH and OGDH (Fig. 4.7).

Note that we were able to bypass the lipoyl-Gcv3 checkpoint to some extent in a *gcv3* strain by expression of mitochondially-tagged versions of either *E. coli* LplA or *E. coli* LipB in the presence of excess free octanoate (Fig. 4.5). The lipoyl-Gcv3 checkpoint could also be bypassed in a *lip2* strain upon expression of *B. subtilis* LipL in the presence of excess free octanoate (Fig. 4.5). The effects of checkpoint bypass on the physiology of *S. cerevisiae* merits further study.

The last known enzyme in the lipoate synthesis pathway of *S. cerevisiae* is Lip3. The substrate for Lip3 had not been identified prior to this work. Lip3 was shown to utilize octanoyl-CoA to activate PDH and OGDH. This substrate could come either directly from peroxisomal fatty acid β-oxidation or from an acyl-ACP:CoA transferase (Fig. 4.1). Free octanoate and octanoyl-Gcv3 were not substrates for Lip3 *in vitro.*

The simplest model which integrates our results with the reported lipoyl-Gcv3 checkpoint is presented in Fig. 4.8. In this model Lip2 specifically modifies Gcv3 using octanoyl-ACP as substrate. When all the Gcv3 has been octanoylated, octanoyl-ACP accumulates. An octanoyl-ACP:CoA transferase then transfers the octanoyl moiety to CoA, providing the substrate used by Lip3 to modify PDH and OGDH. The exact role of octanoyl-Gcv3 in the lipoylation pathway of *S. cereviciae* remains an open question. It is not clear why a *gcv3* mutant doesn't grow on ethanol even though wild type Lip3 is present and octanoyl-ACP is expected to accumulate in this mutant. Perhaps octanoyl-Gcv3 is required for expression, modification or mitochondrial targeting of either Lip3 or the octanoyl-ACP:CoA transferase. It is worth noting that a second distinct role for Gcv3 in RNA processing has been reported in the literature (Schonauer et al., 2008).

Tables

Table 4.1: Primers used in this study. Restriction endonuclease recognition sites are underlined.

Name	Sequence
fadK-for	GGGCCATGGAAGTGACATTAACGTTTAACG
fadK-rev	TCCAAGCTTATTCAATCTCTTCACAGACATCC
pPICNTlip3-for	CGCGGATCCACCATGCATCATCATCATCATCATTTTACA CCTTCAAATGATAACG
pPIClip3-rev	GGGCGGCCGTTATGTGTAACTGTCAATATTCTCC
mtlplA-for	ACGTGGTACCATGTCCACATTACGCCTGCTC
mtlplA-rev	ACGTCTGCAGGCGGGTAACTACCTTACAGC
mtlipB-for	ACGTGGTACCTTGTATCAGGATAAAATTCTTGTCCGCCA GCTCGG
mtlipB-rev	ACGTCTGCAGGGAATTAAGCGGTAATATATTCG
mtlipL-for	ACGTGGTACCATGGCAAACCAACCGATTGATTTACTG
mtlipL-rev	ACGTCTGCAGTTACCCAAATACCTTTGCATTCC

Table 4.2: *E. coli* and yeast strains, and plasmids used in this study.

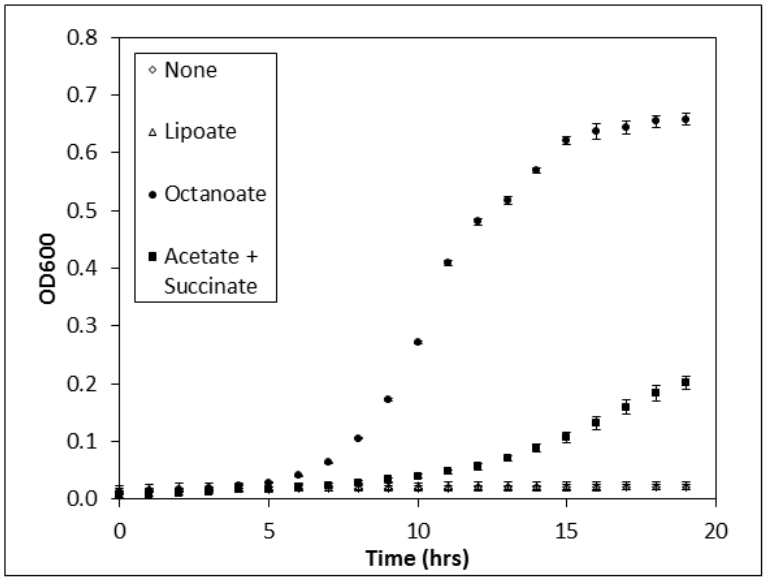
Name	Relevant Genotype	Reference
Yeast strains		
BY4741	*S. cerevisiae* MATa *his3 leu2 met15 ura3*	Open Biosystems
lip2	*S. cerevisiae* MATa *his3 leu2 met15 ura3 lip2*	Open Biosystems
lip3	*S. cerevisiae* MATa *his3 leu2 met15 ura3 lip3*	Open Biosystems
gcv3	*S. cerevisiae* MATa *his3 leu2 met15 ura3 gcv3*	Open Biosystems
GS115	*P. pastoris his4 AOX1 AOX2*	Invitrogen
FH553	GC115 with pFH72 integrated at His4 Expresses recombinant *S. cerevisiae* His-tagged Lip3 under control of the methanol promoter. Lip3 lacks the mitochondrial targeting sequence and is expressed in the cytoplasm.	This study
***E. coli* strains**		
QC146	MG1566 *lipB::FRT lplA::FRT*	(Christensen and Cronan, 2009)
RMK75	MG1655 *fadK::FRT::cm*	(Morgan-Kiss and Cronan, 2004)
FH306	QC146 carrying plasmid pFH23 (expresses Lip3)	This study

Table 4.2 Continued.

Name	Relevant Genotype	Reference
FH307	FH306 *fadD::FRT::cm*	This study
FH437	QC146 carrying pTARA and pFH38. For expression of apo-Gcv3, under control of T7 promoter	This study
FH590	QC146 carrying pFH87. For expression of His tagged Lip2 under tac promoter.	This study
Plasmids		
pACYC177	Cloning vector	(Rose, 1988)
pBAD24	Cloning vector, arabinose promoter	(Guzman et al., 1995)
pBAD322C	Cloning vector, arabinose promoter	(Cronan, 2006)
pCM188	*E. coli- S. cerevisiae* shuttle vector with YC-type yeast replication, Ura3 selection, *cyc1* promoter and two tetO.	(Gari et al., 1997)
pMCSG7	Ligation-independent cloning vector, T7 promoter	(Eschenfeldt et al., 2009)
pPIC3.5K	*P. pastoris* chromosomal integration vector	Invitrogen
pQC32	pBAD322G-*lipL*	(Martin et al., 2011)
pQE80K	Expression vector, tac promoter	Qiagen
pRK33	pET16b-*fadK*	(Morgan-Kiss and Cronan, 2004)
pTARA	pACYC origin, arabinose inducible T7 polymerase	(Wycuff and Matthews, 2000)
pFH18	pBAD-*lip2* in *E. coli* optimized codons	This study
pFH21	pBAD24-*lip3* in *E. coli* optimized codons	This study
pFH23	pACYC177-*lip3* in *E. coli* optimized codons under control of arabinose promoter	This study
pFH38	pMCSG7-*gcv3* for expression of His tagged Gcv3 under T7 promoter	This study
pFH46	pUC vector carrying codons for the first 42 amino acids of of *S. cerevisiae* Coq3 (mitochondrial targeting sequence) between BamHI and KpnI + PstI cut sites	This study
pFH56	*mtlplA* on pCM188	This study
pFH57	*mtlipB* on pCM188	This study
pFH58	*mtlipL* on pCM188	This study
pFH72	pPIC3.5K-*lip3*	This study
pFH87	pQE80K-*lip2*	This study
pFH88	pBAD322C-*fadK*	This study
pFH93	pFH23 derivative expressing K249A Lip3	This study
pFH94	pFH23 derivative expressing K249R Lip3	This study

Figures

A.



B.

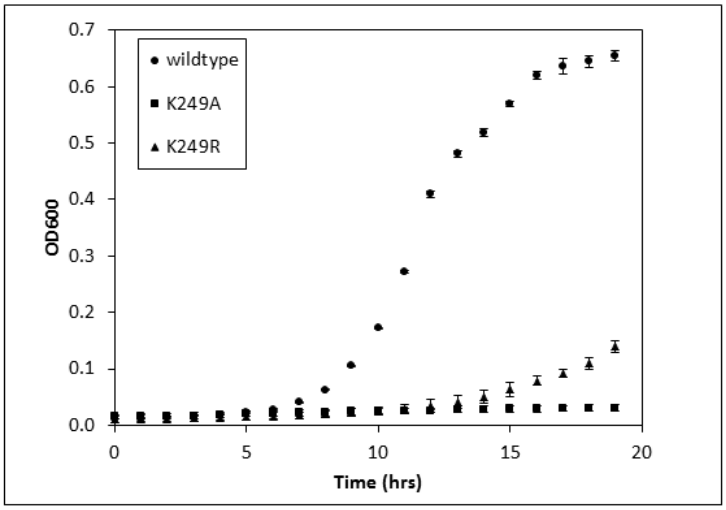
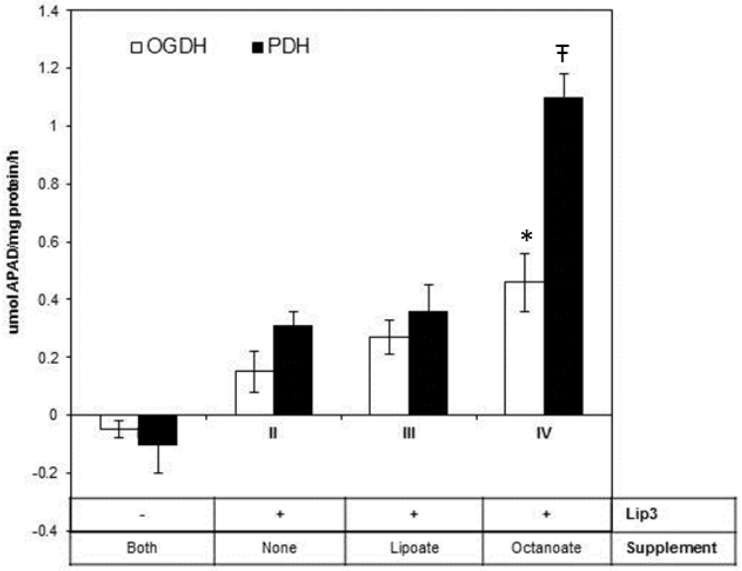


Figure 4.1: Lip3 complementation of the *E. coli* Δ*lipB* Δ*lplA* strain QC146.
A: Growth curves of strain QC146 carrying the Lip3 expression plasmid, pFH23, on either unsupplemented GMM or GMM supplemented with lipoate, octanoate, or a mixture of acetate and succinate as indicated.
B: Replacing the Lip3 active site lysine (K249) with arginine or alanine greatly reduces or entirely eliminates the ability of Lip3 to support growth of QC146 on octanoate-supplemented GMM.

A.



B.

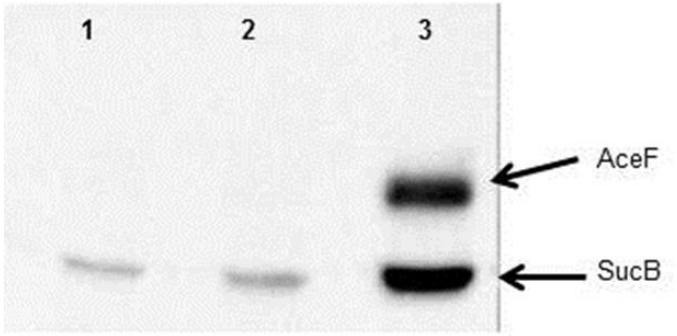
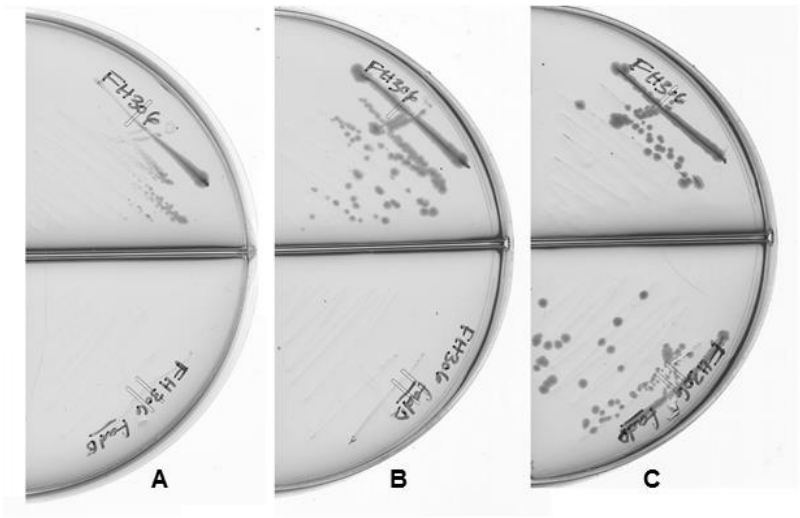


Figure 4.2: Detection of active lipoylated PDH and OGDH complexes in the *E. coli* Δ*lipB* Δ*lplA* strain QC146 expressing Lip3.
A: PDH and OGDH were assayed in extracts of strain QC146 carrying either the empty vector (I) or a plasmid that expressed Lip3 under arabinose control (II – IV). Growth medium was supplemented with lipoate or octanoate, both lipoate and octanoate or left unsupplemented as indicated. The highest levels of PDH and OGDH activities were observed in extracts of strain QC146 carrying the Lip3 expression plasmid pFH23 when octanoate was present in the growth media (IV). * this result is significant relative to that of condition II (*p*-value = 0.003), and condition III (*p*-value = 0.02). Ŧ this result is significant relative to that of condition II (*p*-value = 0.0001), and condition III (*p*-value = 0.0005). Difference in PDH and OGDH activities between conditions II and III are not significant (not shown).
B: Western blot analysis using anti-lipoyl LD antibody of extracts of the strain QC146 carrying pFH23 cultured in LB containing arabinose, acetate and succinate with either lipoate (lane 2), octanoate (lane 3) or neither (lane 1). Whereas lipoylated SucB (the LD of OGDH) was detected in all three extracts, lipoylated AceF (the LD of PDH) was detected only in cells grown the presence of octanoate (3).

A.



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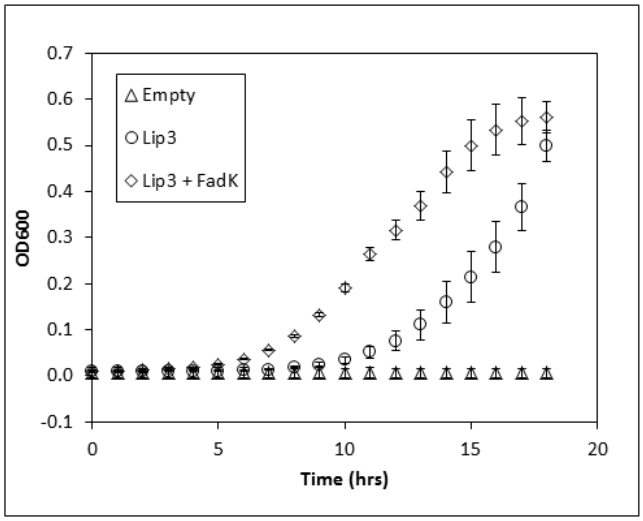
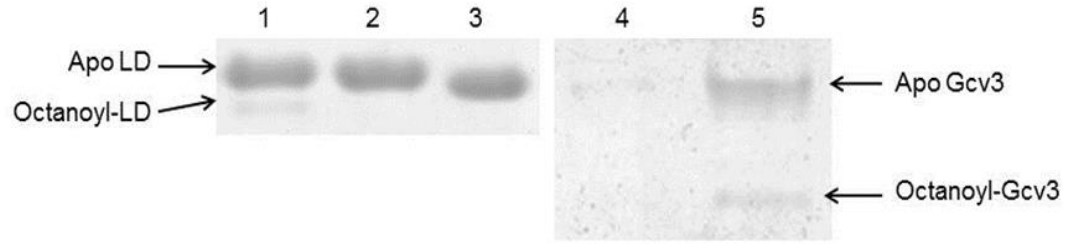


Figure 4.3: Lip3 complementation of the *E. coli* Δ*lipB* Δ*lplA* strain QC146 on octanoate-supplemented GMM requires the presence of the *fadD encoded* acyl-CoA synthetase.

A: Shown are GMM plates unsupplemented (plate A), supplemented with octanoate (plate B), or with succinate and acetate (plate C). The top sector of each plate are strain QC146 carrying a plasmid which expresses Lip3 whereas in the bottom sector of each plate the same strain carried a *fadD* deletion.

B: Over expression of the FadK acyl-CoA synthetase amplifies Lip3 complementation of the *E. coli* Δ*lipB* Δ*lplA* strain QC146. Growth curves on octanoate-supplemented GMM of QC146 carrying either the empty vector, a Lip3 expression plasmid or plasmids expressing both Lip3 and FadK as indicated.

A.



B.

	1	2	3	4
Lip3				+
Apo Gcv3	+			+
Octanoyl-Gcv3		+		
Octanoyl-ACP			+	+

C.

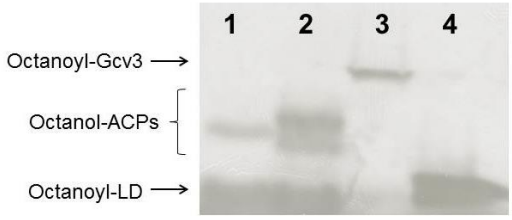


Figure 4.4:
A: *In vitro* detection of Lip3 octanoyl transferase activity by an electrophoresis mobility gel shift assay. Left gel: Lip3 reaction with LD with octanoyl-CoA (lane 1) or with ATP, octanoate and $MgCl_2$ (lane 2). Right gel: Lip3 activity with Gcv3 and octanoyl-CoA (lane 5). Lanes 3 and 4 were loaded with octanoyl-LD and apo-Gcv3 standards, respectively. Lip3 was active with both LD and Gcv3 when octanoyl-CoA was provided (lanes 1 and 5, respectively).
B and C: Octanoyl-ACP is a substrate for Lip3, but not octanoyl-Gcv3. B: Electromobility gel shift assay with Lip3, Gcv3 and octanoyl-ACP from *E. coli* (lane 4). **C:** autoradiograph of Lip3 reaction with LD and various substrates: octanoyl-ACP from *E. coli* (lane 1) octanoyl-ACP from *B. subtilis* (lane 2), and octanoyl-Gcv3 (lane 3). Lane 4 is loaded with octanoyl-LD as standard.

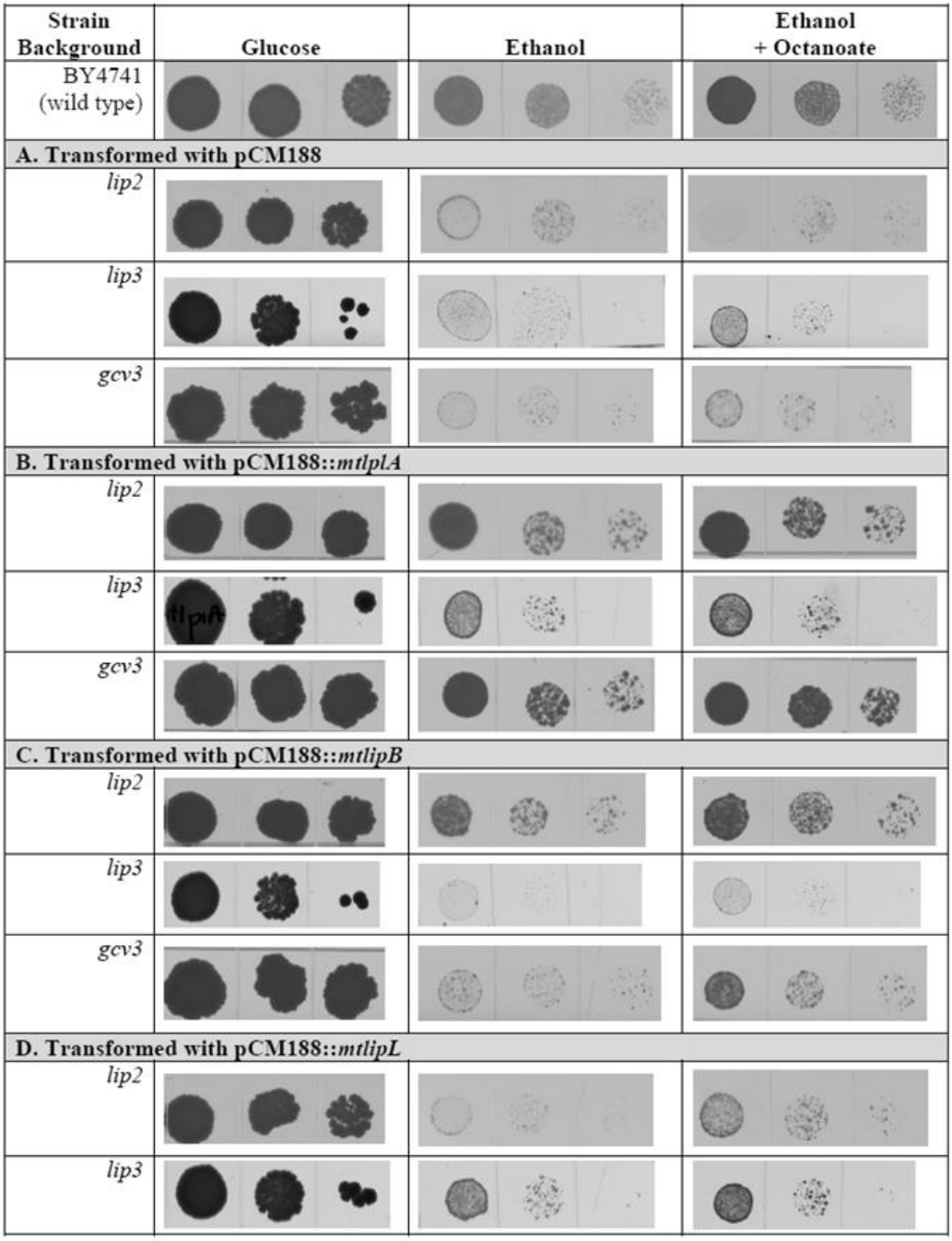
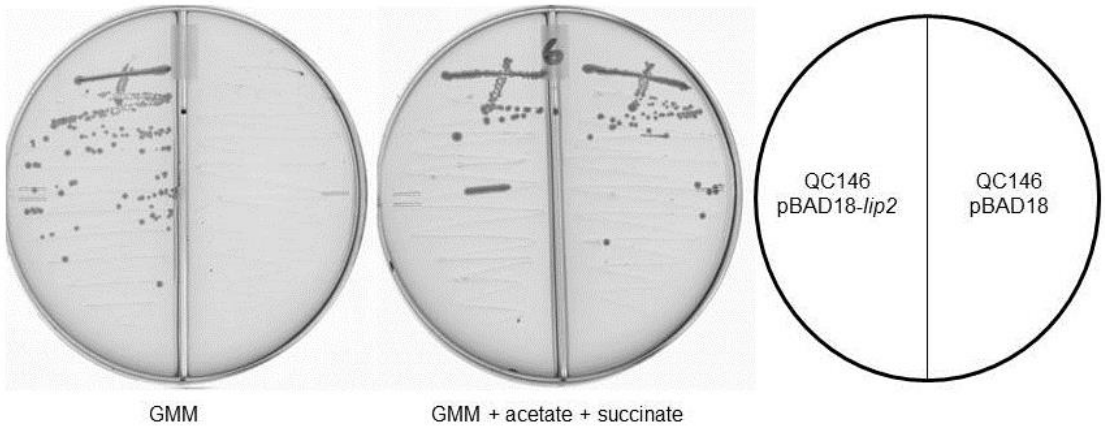


Figure 4.5: Growth phenotypes of *S. cerevisiae lip2, lip3* and *gcv3* strains transformed with different plasmids. All strains were derivatives of BY4741 and were grown on YP glucose, YP ethanol or YP ethanol supplemented with octanoate. Plates were incubated at 30^{o} for up to four days. Panel A: the mutant strains transformed with empty vector. Panel B: the mutant strains transformed with pFH56 expressing mitochondrially targeted *E. coli* LplA. Panel C: the mutant strains transformed with pFH57 expressing mitochondrially targeted *E. coli* LipB. Panel D: the *lip2* and *lip3* strains transformed with pFH58 expressing mitochondrially targeted *B. subtilis* LipL.

A.



B.

	1	2	3	4	5
Octanoate +ATP				+	
Octanoyl-ACP					+
Apo LD	+		+	+	+
Lip2			+	+	+

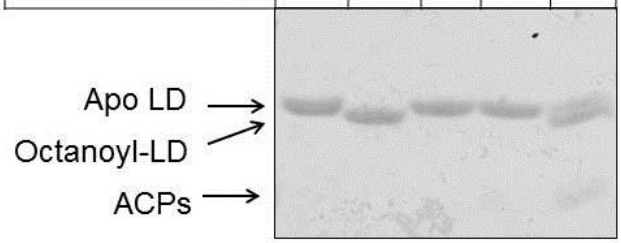


Figure 4.6:
A: Lip2 complements the *E. coli* Δ*lipB* Δ*lplA* strain QC146 on unsupplemented GMM. Left plate: Unsupplemented GMM. Right plate: GMM supplemented with acetate and succinate. The left sector of each plate is strain QC146 carrying plasmid pFH18 which expresses Lip2. The right sector of each plate is strain QC146 carrying the empty vector.
B: Assay of crude extract of a Lip2 expressing strain for octanoyl transferase activity in an electromobility gel shift assay**.** Octanoyltransferase activity is detected when octanoyl-ACP is provided as substrate (lane 5). No ligase activity is detected with octanoate + ATP + $MgCl_2$ (lane 4). Octanoyl-LD is loaded in lane 2 as standard.

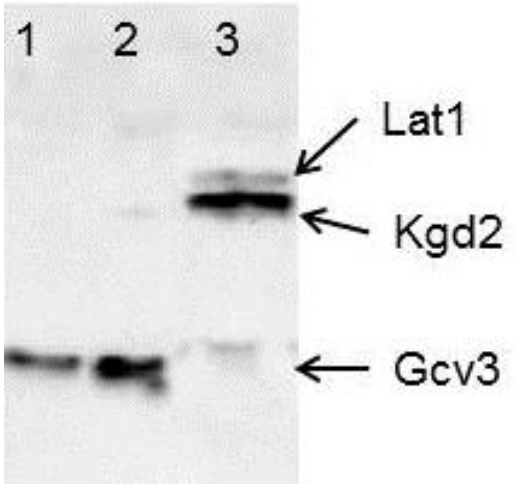


Figure 4.7: Western blot analysis of wild type *S. cerevisiea* extracts using anti lipoyl-LD antibodies. Cells were cultured either in YP glucose (lanes 1 and 2) or YP glycerol (lane 3). Whereas lipoyl-Gcv3 was detected in extracts from both culture conditions, lipoyl-Lat1 (the LD of PDH) and lipoyl-Kgd2 (the LD of OGDH) were detected only under respiratory conditions.

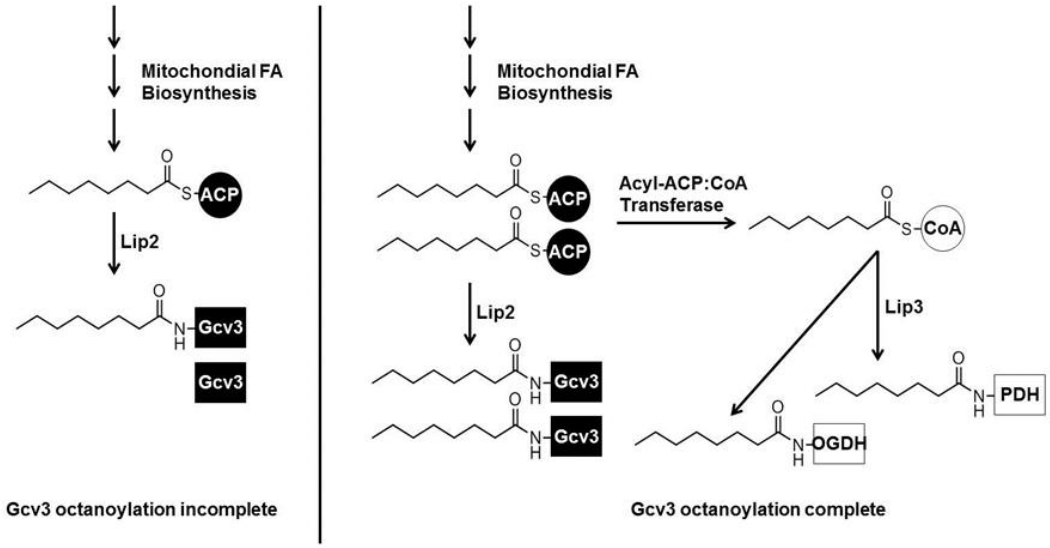


Figure 4.8: Proposed pathway for lipoate synthesis/ attachment in *S. cerevisiae* which accounts for the observed octanoyl-Gcv3 checkpoint. Left: Lip2 specifically modifies Gcv3 using octanoyl-ACP from mitochondrial FA biosynthesis. Right: When all Gcv3 is octanoylated, octanoyl-ACP accumulates. An octanoyl-ACP: CoA transferase transfers the octanoyl moiety to CoA, providing substrate for Lip3 to modify PDH and OGDH. Lip5 catalyzes the sulfur insertions on the octanoyl moiety once it is attached to an LD (not shown).

Chapter 5: Conclusions

Lessons Learned from *Escherichia coli*

The physiological requirement for lipoic acid is extremely low, only a few hundred molecules per cell. Yet under obligate aerobic growth conditions lipoic acid is essential because it is a coenzyme for pyruvate dehydrogenase (PDH) and oxoglutarate dehydrogenase (OGDH) which catalyze key steps in the TCA cycle. In *E. coli,* lipoate is assembled on the lipoyl domains (LDs) of PDH and OGDH from the eight carbon fatty acid, octanoate, in two steps. First, an octanoyltransferase (LipB) transfers an octanoyl moiety from the acyl carrier protein (ACP) of fatty acid synthesis to the LDs. Then lipoyl synthase (LipA) catalyzes replacement of single hydrogen atoms at carbons 6 and 8 with sulfur atoms using radical SAM chemistry. Alternatively, exogenous lipoate or octanoate can be directly attached to LDs by lipoate protein ligase (LplA).

lipB null strains are expected to require acetate and succinate for aerobic growth on glucose minimal media. These supplements, respectively, bypass the PDH- and OGDH-catalyzed steps in the TCA cycle. Often times however, *lipB* strains exhibit a “leaky” growth phenotype, which relies on the presence of the *lipA* and *lplA* genes, and succinate (or lysine and methionine). Findings from this study offer an explanation for this phenomenon.

In this study I characterized two types of *lipB* suppressors. In one type, described in chapter 2, suppression was caused by point mutations in the *lplA* coding region which resulted in enzymes with lowered K_m for octanoate. This was perplexing because at the time no free fatty acids had been detected inside cells. However, with a careful procedure (gentle lysis and conversion of fatty acids to their butyl- instead of methyl- esters), I detected octanoate in the cytoplasm at a concentration of about 28 µM. Whereas this concentration is well below the K_m for octanoate of the wild type LplA enzyme, it is above the K_m of the mutant LplA

proteins. Thus the mutant LplA proteins enable activation of PDH and OGDH and thus growth of the *lipB* strain.

The origin of cytoplasmic octanoate is unknown, but could be spontaneous hydrolysis of the thioester bond of the acyl-ACP of fatty acid biosynthesis. It is thus conceivable that strain background and/or growth conditions may affect the availability of free octanoate for lipoate synthesis by the LplA/LipA pathway and thus the potential for leaky growth of *lipB* strains. During characterization of the second type of suppressor (chapter 3), I observed that the *lipB* strain exhibited growth in the presence of only succinate supplementation and had functional PDH. The mutation causing suppression in this case turned out to be a stop codon in the *sdhB* gene which encodes a subunit of succinate dehydrogenase (SDH). So the question became how does inactivation of succinate catabolism in a strain defective in lipoate synthesis (and thus presumably OGDH activity) alleviate the requirement for exogenous succinate supplementation? There must be other sources of succinate! These other sources were identified as trace levels of OGDH, the isocitrate lyase of the glyoxylate shunt, and aspartate oxidase, the enzyme catalyzing the first step of nicotinamide biosynthesis. With this knowledge I was now able to knockout all the succinate-synthesis pathways and determine that only 250 nM of succinate were required to achieve half-maximal growth in strains defective in succinate catabolism (SDH). This is greatly lower than the amount required for half maximal growth of strain containing functional SDH which is above 12 µM.

Lessons Learned from *Saccharomyces cerevisiae*

Although the LipA catalyzed reaction is conserved in all characterized lipoate synthesis pathways, there are three well-characterized variations to the *E. coli* pathway in the other reactions. In the lipoate auxotrophic archeon *Thermoplasma acidophilum,* the lipoate protein ligase is composed of two separately encoded subunits LplA and LplB that together catalyze formation of the acyl-AMP intermediate. The Gram-positive bacterium *Bacillus subtilis* lacks a LipB homologue, but encodes three LplA homologues, although only LplJ has the

ligase function of *E. coli* LplA. One of the two remaining homologues (LipM) is an octanoyltransferase that specifically transfers octanoate from ACP to a small protein, GcvH. Whereas LipL, the third homologue, is an amidotransferase that transfers the octanoyl moiety from GcvH to the LDs of PDH, the branched chain oxoacid dehydrogenase, and OGDH. LipL is also present in *Listeria monocytogenes* along with two other LplA homologues that are ligases. LplA1 specifically modifies GcvH whereas the LD specificity of LplA2 is undetermined. The diversity in prokaryotic lipoate attachment mechanisms is surprising given the simple structure of the coenzyme. The situation in eukaryotes is not well understood. However with the characterization of the octanoyl-CoA: protein transferase activity of the *S. cerevisiae* lipoate protein ligase homologue (chapter 4), it seems that eukaryotes may contribute further enzymatic diversity to this group of enzymes.

Chapter 6: References

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