An NAD Synthetic Reaction Bypasses the Lipoate Requirement for Aerobic Growth of *Escherichia coli* Strains Blocked in Succinate Catabolism

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SUMMARY

The lipoate coenzyme is essential for function of the pyruvate (PDH) and 2-oxoglutarate (OGDH) dehydrogenases and thus for aerobic growth of *Escherichia coli*. LipB catalyzes the first step in lipoate synthesis, transfer of an octanoyl moiety from the fatty acid synthetic intermediate, octanoyl-ACP, to PDH and OGDH. *E. coli* also encodes LplA, a ligase that in presence of exogenous octanoate (or lipoate) can bypass loss of LipB. LplA imparts *lipB* strains with a “leaky” growth phenotype on aerobic glucose minimal medium supplemented with succinate (which bypasses the OGDH-catalyzed reaction), because it scavenges an endogenous octanoate pool to activate PDH. Here we characterize a *lipB* suppressor strain that did not require succinate supplementation, but did require succinyl-CoA ligase, confirming the presence of alternative source(s) of cytosolic succinate. We report that suppression requires inactivation of succinate dehydrogenase (SDH), which greatly reduces the cellular requirement for succinate. In the suppressor strain succinate 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 an unanticipated source, aspartate oxidase, the enzyme catalyzing the first step of nicotinamide biosynthesis.

Keywords

lipoate; tricarboxylic acid cycle; succinate; aspartate oxidase; pyruvate dehydrogenase; succinate dehydrogenase

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 coli*: pyruvate dehydrogenase (PDH) and 2-oxoglutarate dehydrogenase (OGDH). A third lipoate-dependent enzyme system, the glycine cleavage

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CONFLICT OF INTEREST

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system (GCV), which functions in the metabolism of glycine to C1 units and ammonia, is also present in *E. coli* (Douce *et al.*, 2001, Stauffer, 2004). However, since GCV expression is dependent on the presence of glycine and is not required for aerobic growth, it will not be further discussed.

PDH catalyzes the oxidative decarboxylation of pyruvate, the end product of glycolysis, to acetyl-CoA (Fig. 1A). 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 & 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.

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. 1A). Succinyl-CoA is required for the biosynthesis of the amino acids lysine and methionine and the peptidoglycan component, diaminopimelic acid DAP. Succinyl-CoA is also the source of succinate, the entry point to respiration (Cronan & Laporte, 2006). OGDH activity is dispensable under conditions where the TCA cycle operates as a branched pathway, such as during anaerobic growth (Creaghan & Guest, 1972). In such instances, fumarate is the source of succinate and hence of succinyl-CoA.

The major synthetic pathways that require succinyl-CoA (methionine, lysine and DAP) do not consume the succinate moiety. Rather succinate is released intact into the cytoplasm and can be recycled by succinyl-CoA ligase (Fig. 1A). Hence succinate is said to function “catalytically” in these biosynthetic pathways. In wild type cells however, succinate is consumed by oxidation to fumarate by succinate dehydrogenase (SDH; Fig. 1A) which requires succinyl-CoA to be continuously synthesized by OGDH. Thus mutations in SDH greatly lower the demand for production of new succinyl-CoA by OGDH (Creaghan & Guest, 1977). In SDH mutants very small amounts of succinate suffice due to succinate recycling by succinyl-CoA ligase (Fig. 1A).

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 & 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 distinct genes 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 (Fig. 1C). First, octanoyltransferase (LipB) transfers an octanoyl moiety from the octanoyl-acyl carrier protein (ACP) of fatty acid biosynthesis to the ϵ -amino group of a specific lysine residues in each LD (Jordan & Cronan, 2003, Hassan & 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 (Fig. 1B), 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, Zhao *et al.*, 2003).

Since *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 & Guest, 1968). (However, see the Results section below.) We previously isolated and characterized *lipB* suppressor strains which grew on glucose minimal medium lacking any supplements (Hermes & 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 enzymes' K_m values for free octanoate. This led to a 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 *lipB* defect in these strains was caused by activation of PDH and OGDH by the mutant LplA enzymes using cytosolic octanoate (Hermes & Cronan, 2009). This screen also gave rise to a single *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.

RESULTS

The *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 *lipB* strains would be inactive as a result of an inability to synthesize endogenous lipoate. Thus *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 *lipB* strains grown on bypass medium (Hermes & Cronan, 2009). A somewhat conflicting

observation was previously reported by our laboratory (Reed & Cronan, 1993). The *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 *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 & Cronan, 1993).

We observed that the two *lipB* strains FH6 (*lipB*::Tn1000 km^R) and FH160 (*lipB*::FRT::cm^R::FRT) derived from the wild type strain MG1655 behaved similarly to the *lipB* strain of Reed and Cronan (Reed & Cronan, 1993). Strains FH6 and FH160 required only succinate for growth on glucose minimal medium (Fig. 2A and data not shown). This growth phenotype suggested functional PDH was present and this activity was indeed detected in extracts of strain FH160 (Fig. 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 *lipB* strain FH160 required 12 μ M succinate for half maximal growth in aerobic glucose minimal medium (Fig. 2A). Acetate-independent growth was not observed for *lipB* *lplA* and *lipB* *lipA* strains (Table 1) which indicated that the LplA/LipA pathway was responsible for the residual PDH activity of *lipB* strains (Hermes & 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 *lipB* suppressor strain FH34 is required for PDH activity, but not OGDH activity

Strain FH34 was isolated as a spontaneous suppressor of the *lipB* mutation of strain FH6, which had acquired the ability to grow on unsupplemented glucose minimal medium (Fig. 3A). Like the suppressor strains studied previously (Hermes & Cronan, 2009) FH34 was isolated simply by plating an unmutagenized culture of strain FH6 on glucose minimal medium. 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 acetate 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 strain FH34 derivatives in which these genes were deleted. The resulting strains retained acetate- and succinate-independent growth on glucose minimal medium (Table 1) 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 *lipB* mutation somehow resulted in activation of OGDH. In *lipB* strains the LplA/LipA pathway is the only known 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, *lplA* and *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 *lipB* parental strain (Table 1). The *lplA* and *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 producing pathways.

We had previously reported that *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 & Cronan, 2009). We therefore sequenced the *lplA* gene of strain FH34 and found it to have the wild type sequence. The *lipB* deletion phenotype is known to be suppressed by overexpression of *lplA* (Hermes & Cronan, 2009, 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 & 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 *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 *lipB* parent (Fig. 2B), FH34 extracts contained functional PDH, but no detectable OGDH activity (Fig. 3B). In a further test a *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 & Guest, 1978). However the *lpd* derivative of strain FH34 required acetate, but not succinate for growth (Table 1). 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 *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 *lipB* gene deletion in strain FH34 was localized to the 18 Kbp region between the *ybgO* and *mngA* genes (Table S3). 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 a 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 *lipB* strains.

To confirm that the termination codon caused the growth phenotype of strain FH34, a *lipB 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 1). Moreover, when a low-copy number plasmid encoding the complete *sdhCDAB* operon was introduced into a *lipB sdhB* strain, the resulting derivative was unable to grow without succinate supplementation (Table 1).

Strains containing *lipB sdhA* and *lipB sucA* deletions were also able to grow on unsupplemented glucose minimal medium (Table 1). The *sdhA* gene is located upstream of *sdhB* and encodes another subunit of the SDH complex. Hence, any deletion that inactivates SDH results in suppression of the *lipB* mutation. As seen above in the *lipB sdhB* strain, succinate-independent growth of the *lipB sucA* strain was lost upon introduction of a plasmid encoding the *sdhCDAB* operon (Table 1). This suggested that the *sucA* mutation somehow caused disruption of *sdhB* expression, possibly by destabilizing the polycistronic mRNA (Cunningham & Guest, 1998). However, recent RNA-Seq data argue that, although *sdhCDAB* and *sucAB* are cotranscribed, a somewhat inefficient terminator followed by a secondary promoter are located upstream of *sucA* (Conway *et al.*, 2014). The physiological consequences of this newly recognized complexity are unknown.

Since *sucA* encodes a subunit of OGDH, the phenotype of the *lipB sucA* strain further supports the observation that OGDH function is not essential for succinate production in *lipB* strains that lack SDH activity, such as strain FH34 (*lipB sdhB*). A *lipB sucB* strain however failed to grow on unsupplemented glucose minimal medium (Table 1) indicating that the *sucB* deletion does not exert the effect on the expression of the *sdhCDAB* operon seen for the *sucA* deletion. We note that deletion strains of the Keio collection (Baba *et al.*, 2006) were used to construct the *lipB sucA* and *lipB sucB* strains. These gene deletions are in frame only upon excision of the antibiotic resistance marker. However, the markerless *lipB sucA* and *lipB sucB* versions of our strains retained the phenotypes described above.

Succinate is produced and must be recycled to succinyl-CoA in *lipB 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 & Slaughter, 1967, Nagai & 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. 1A). We hypothesized that if a small amount of succinate was produced in the *lipB 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 *lipB sdhB* strain. The resulting *lipB sdhB sucCD* strain required lysine and methionine for growth (Table 1). This indicated that succinate was indeed produced and must be recycled to succinyl-CoA in the *lipB sdhB* strains. The surprising acetate requirement for growth of the *lipB sdhB sucCD* strain is currently under study.

The pathways of succinate synthesis in *lipB 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 & 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 *lipB sdhB* strain and screened for the ability of these strains to grow without succinate supplementation. The *lipB sdhB* strain that contained deletions of *aceA*, *frdA* and *sucAB* retained succinate-independent growth on glucose minimal (Table 1) indicating that yet another source of succinate remained.

Given the elimination of the straightforward sources of succinate 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 & 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 & Ames, 1982, Wimpenny & Firth, 1972). We introduced a *nadB* deletion into the *lipB sdhB frdA aceA sucAB* strain. The resulting strain required succinate for growth on glucose minimal medium containing nicotinic acid (Table 1). The succinate requirement was alleviated upon introduction of a plasmid expressing *nadB* (Table 1). By construction of various combinations of the four gene deletions in the *lipB sdhB* strain, we determined that only three of the four deletions were required to produce the succinate-dependent growth phenotype; *aceA*, *nadB*, and

sucAB (Fig. 4). The succinate requirement for growth of strain FH698 (*lipB* *sdhB* *aceA* *nadB* *sucAB*) was expected to be very low. Indeed, supplementation of glucose minimal medium with 250 nM succinate gave half maximal growth (Fig. 4).

The contribution of OGDH was surprising given that the activity of this enzyme in extracts of the *lipB* *sdhB* strain was consistently below the detection level (~ 0.005 $\mu\text{mole substrate mg}^{-1}$ protein hr^{-1} (Reed and Cronan, 1993)), and that deletion of *lplA*, *lipA* or *lpd* in the *lipB* *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 *lipB* *sdhB* background on succinate production is due to the presence of the two other sources of succinate, namely NadB and AceA. In order to determine the exact role of OGDH, individual deletions of genes encoding its constituent subunits were constructed in the *lipB* *sdhB* *aceA* *nadB* background. An in frame *sucB* gene deletion in the *lipB* *sdhB* *aceA* *nadB* strain resulted in a succinate requirement (Table 1) suggesting that the overall OGDH reaction was responsible for the succinate independent growth of the *lipB* *sdhB* *aceA* *nadB* strain and that the succinate- or succinate semialdehyde-producing activity of the E1 subunit (*sucA*) did not suffice. Interestingly, introduction of an *lpd* deletion into the *lipB* *sdhB* *aceA* *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 *lipB* *sdhB* strain since our assay measures reduction of the NAD^+ analogue, 3-acetylpyridine adenine dinucleotide, by Lpd.

DISCUSSION

When *lipB* strains were first isolated they were reported to have “leaky” growth phenotypes on aerobic medium supplemented with nutrients that bypassed the OGDH-catalyzed reaction, i.e. succinate, or lysine plus methionine (Morris *et al.*, 1995, Reed & Cronan, 1993). This acetate-independent growth phenotype was suggestive of the ability of *lipB* strains to produce either lipoate for activation of PDH, or acetate/acetyl-CoA by some other means. The characterization of LplA (Morris *et al.*, 1994) and the detection of a cytoplasmic pool of free octanoate (the precursor of lipoate and a LplA substrate) (Hermes & Cronan, 2009) elucidated the source of lipoate and thus acetyl-CoA in *lipB* strains. In contrast *lipB* *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 & 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 *lipB* strains. We observed both acetate plus succinate-dependent and succinate only-dependent growth phenotypes in *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 *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 & 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 *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 & Cronan, 2009). These mutant LplA enzymes were thus able to utilize cytoplasmic octanoate to activate PDH and OGDH. In the third *lipB* suppressor strain, described herein, suppression resulted from inactivation of the SDH respiratory enzyme complex (encoded by the *sdhCDAB* operon).

Initially it seemed perplexing that blockage of succinate consumption would enable a *lipB* strain to grow on aerobic glucose minimal medium since it had been generally assumed that *lipB* strains lack the ability to produce succinate. However, Creaghan and Guest (Creaghan & Guest, 1977) isolated *sdh* null mutants in their search for suppressors of the succinate requirement of a *lplD* strain and this was taken to indicate that *E. coli* has other modes of succinate synthesis (Cronan & Laporte, 2006). In the *lipB sdhB* strain we found that succinate/succinyl-CoA were provided by the activities of isocitrate lyase (AceA), aspartate oxidase (NadB) and OGDH (Fig. 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 utilize either an organic acid, fumarate, or an inorganic molecule, oxygen, as electron acceptor. Under physiological aerobic growth conditions NadB utilizes oxygen as an electron acceptor because the cellular fumarate concentration is low (Korshunov & Imlay, 2010). However, fumarate is the preferred substrate and its presence can block oxygen utilization under aerobic conditions (Korshunov & Imlay, 2010). The intracellular concentration of fumarate in aerobically grown *E. coli* does not exceed 10 μ M (Korshunov & Imlay, 2010). However, even such concentrations would be sufficient to provide the low concentrations of succinate required for growth of the *lipB sdhB* strains which show half maximal growth at 250 nM succinate (Fig. 4). In contrast, *lipB* strains fail to grow under these conditions because these strains require 12 μ M succinate for half maximum growth, about a 50-fold greater concentration than that required for the *lipB sdhB* strains (Fig. 2A).

The contribution to succinate production by OGDH was surprising because OGDH activity in these strains was consistently below the level of detection (Fig. 3B), and deletion of genes that are essential to both PDH and OGDH function (*lipA*, *lplA* or *lplD*) in the *lipB sdhB* background resulted in an acetate requirement but not a succinate requirement (Table 1). On the other hand, the remaining, albeit undetectable, OGDH activity may explain why *lipB* strains (and the *lipB sdhB 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 & Gunsalus, 1985, Tseng *et al.*, 1996, Tseng *et al.*, 1994), deletion of *frdA* was not required to engender succinate auxotrophy in the *lipB sdhB aceA nadB sucAB* strain. Moreover, in this strain background introduction of deletions of either *lplA* or *lplD* in place of the *sucAB*

deletion gave strains that did not require succinate but did require acetate (Table 1). 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 *lipB sdhB aceA nadB sucB* strain (Table 1). Note that the acetate requirement engendered by replacement of the *sucAB* or *sucB* deletions with *lpd* or *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 & Guest, 1998, Masse & Gottesman, 2002, Spencer & Guest, 1985). If *sucB* is cotranscribed with *sdhB*, we would expect reduced SucB expression in *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 *lipB* background, deletions of the *sdhB* and *sucA* genes give a phenotype that differs from that of the *sucB* deletion, suggesting that these genes may not be strictly cotranscribed. Both the *sucA* and the *sucB* deletions disrupt OGDH function, but only the former spares the succinate pool probably by reducing SDH expression. The *sucB* deletion is not polar on the downstream *sucCD* genes, because the *lipB 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 *lipB sdhB aceA nadB sucB* strain (Table 1).

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 & Wanner, 2000, Court *et al.*, 2002). Although the seminal work of Guest and coworkers (Creaghan & Guest, 1972, Creaghan & Guest, 1977, Guest & Creaghan, 1973, Langley & 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.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

The strains used in this study (Table S1) 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 & Wanner, 2000) was applied to the construction of several strains. Primers used are indicated in Table S1 and their sequences in Table S2.

Strain FH34 was isolated in a previous screen by plating strain FH6 on unsupplemented glucose minimal plates (Hermes & 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 & 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 μ M; nicotinic acid, 100 μ M; diaminopimelic acid, 100 μ g ml⁻¹; lysine 10 μ g ml⁻¹; and methionine, 50 μ g ml⁻¹. Antibiotics were used at the following concentration (in μ g ml⁻¹): sodium ampicillin, 100; tetracycline HCl, 15; kanamycin sulfate, 50 and chloramphenicol, 20. The growth temperature was 37°C.

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 μ l minimal media and resuspended in the same medium to a final OD₆₀₀ of 0.15. Each well contained 290 μ l of medium to which 10 μ l of cell suspension was added to give a final starting OD₆₀₀ of 0.005 (about 3×10^8 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 medium lacking succinate to an initial OD₆₀₀ of 0.02 and allowed to grow for 5 to 6 hours.

Four 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 & Cronan, 2011). Site directed mutagenesis of the *sucB* gene of pBH146 was conducted using primers K44Rfor and K44Rrev (Table S2), and *Pfu* turbo DNA polymerase per the manufacturer's recommendations. Plasmid FH112 was constructed by PCR amplification of the *nadB* from genomic DNA using primers NcoI-nadB and HindIII-nadB (Table S2) and cloning the PCR product into NcoI/HindIII digested pKK233-2 (Amann & Brosius, 1985), thus placing *nadB* under *trc* expression.

PDH and OGDH activity assays

The method described in Hermes and Cronan (2009) (Hermes & 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 the mutation into strain FH160 (*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.

This 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^{vir} 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 S2), the region between these two genes was sequenced and compared to the sequence of the same region in GenBank accession U00096.2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Abdel-Hamid AM, Attwood MM, Guest JR. Pyruvate oxidase contributes to the aerobic growth efficiency of *Escherichia coli*. *Microbiology*. 2001; 147:1483–1498. [PubMed: 11390679]
- Amann E, Brosius J. "ATG vectors" for regulated high-level expression of cloned genes in *Escherichia coli*. *Gene*. 1985; 40:183–190. [PubMed: 3007288]
- Ambrose-Griffin MC, Danson MJ, Griffin WG, Hale G, Perham RN. Kinetic analysis of the role of lipoiic acid residues in the pyruvate dehydrogenase multienzyme complex of *Escherichia coli*. *Biochem J*. 1980; 187:393–401. [PubMed: 6772160]
- Angelides KJ, Hammes GG. Mechanism of action of the pyruvate dehydrogenase multienzyme complex from *Escherichia coli*. *Proc Natl Acad Sci U S A*. 1978; 75:4877–4880. [PubMed: 368802]

- Ausubel, F.; Kingston, R.; Moore, D.; Seidman, J.; Smith, J.; Struhl, K. Current Protocols in Molecular Biology. John Wiley and Sons, Inc; 2007.
- Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol. 2006; 2:0008. [PubMed: 16738554]
- Berman JN, Chen GX, Hale G, Perham RN. Lipoic acid residues in a take-over mechanism for the pyruvate dehydrogenase multienzyme complex of *Escherichia coli*. Biochem J. 1981; 199:513–520. [PubMed: 6803766]
- Bochner BR, Ames BN. Complete analysis of cellular nucleotides by two-dimensional thin layer chromatography. J Biol Chem. 1982; 257:9759–9769. [PubMed: 6286632]
- Booker SJ, Cicchillo RM, Grove TL. Self-sacrifice in radical S-adenosylmethionine proteins. Curr Opin Chem Biol. 2007; 11:543–552. [PubMed: 17936058]
- Cecchini G, Sices H, Schroder I, Gunsalus RP. Aerobic inactivation of fumarate reductase from *Escherichia coli* by mutation of the [3Fe-4S]-quinone binding domain. J Bacteriol. 1995; 177:4587–4592. [PubMed: 7642483]
- Chang YY, Wang AY, Cronan JE Jr. Expression of *Escherichia coli* pyruvate oxidase (PoxB) depends on the sigma factor encoded by the *rpoS(katF)* gene. Mol Microbiol. 1994; 11:1019–1028. [PubMed: 8022274]
- Conway T, Creecy JP, Maddox SM, Grissom JE, Conkle TL, Shadid TM, Teramoto J, San Miguel P, Shimada T, Ishihama A, Mori H, Wanner BL. Unprecedented high-resolution view of bacterial operon architecture revealed by RNA sequencing. MBio. 2014;5.
- Court DL, Sawitzke JA, Thomason LC. Genetic engineering using homologous recombination. Annu Rev Genet. 2002; 36:361–388. [PubMed: 12429697]
- Creaghan IT, Guest JR. Amber mutants of the α -ketoglutarate dehydrogenase gene of *Escherichia coli* K12. J Gen Microbiol. 1972; 71:207–220. [PubMed: 4558786]
- Creaghan IT, Guest JR. Suppression of the succinate requirement of lipoamide dehydrogenase mutants of *Escherichia coli* by mutations affecting succinate dehydrogenase activity. J Gen Microbiol. 1977; 102:183–194. [PubMed: 335020]
- Cronan, J.; Laporte, J&D. Tricarboxylic Acid Cycle and Glyoxylate Bypass. In: Böck, RCI.; Kaper, JB.; Karp, PD.; Neidhardt, FC.; Nyström, T.; Slauch, JM.; Squires, CL., editors. EcoSal—*Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press; Washington, D.C.: 2006. <http://www.ecosal.org>
- Cronan, JE. Biotin and Lipoic Acid: Synthesis, Attachment, and Regulation. In: Böck, RCIA.; Kaper, JB.; Karp, PD.; Neidhardt, FC.; Nyström, T.; Slauch, JM.; Squires, CL.; Ussery, D., editors. EcoSal *Escherichia coli* and *Salmonella* : cellular and molecular biology. ASM Press; Washington, D.C.: 2008. p. 3.6.3.5<http://www.ecosal.org>
- Cronan JE, Zhao X, Jiang Y. Function, attachment and synthesis of lipoic acid in *Escherichia coli*. Adv Microb Physiol. 2005; 50:103–146. [PubMed: 16221579]
- Cunningham L, Guest JR. Transcription and transcript processing in the *sdhCDAB-sucABCD* operon of *Escherichia coli*. Microbiology. 1998; 144:2113–2123. Pt 8. [PubMed: 9720032]
- Danson MJ, Fersht AR, Perham RN. Rapid intramolecular coupling of active sites in the pyruvate dehydrogenase complex of *Escherichia coli*: mechanism for rate enhancement in a multimeric structure. Proc Natl Acad Sci U S A. 1978; 75:5386–5390. [PubMed: 214786]
- Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. 2000; 97:6640–6645. [PubMed: 10829079]
- Douce R, Bourguignon J, Neuburger M, Rebeille F. The glycine decarboxylase system: a fascinating complex. Trends Plant Sci. 2001; 6:167–176. [PubMed: 11286922]
- Feeney MA, Veeravalli K, Boyd D, Gon S, Faulkner MJ, Georgiou G, Beckwith J. Repurposing lipoic acid changes electron flow in two important metabolic pathways of *Escherichia coli*. Proc Natl Acad Sci U S A. 2011; 108:7991–7996. [PubMed: 21521794]
- Flavin M, Slaughter C. Enzymatic synthesis of homocysteine or methionine directly from O-succinyl-homoserine. Biochim Biophys Acta. 1967; 132:400–405. [PubMed: 5340123]
- Frank RA, Kay CW, Hirst J, Luisi BF. Off-pathway, oxygen-dependent thiamine radical in the Krebs cycle. J Am Chem Soc. 2008; 130:1662–1668. [PubMed: 18183975]

- Green DE, Morris TW, Green J, Cronan JE Jr, Guest JR. Purification and properties of the lipoate protein ligase of *Escherichia coli*. *Biochem J*. 1995
- Guest JR, Creaghan IT. Gene-protein relationships of the alpha-keto acid dehydrogenase complexes of *Escherichia coli* K12: isolation and characterization of lipoamide dehydrogenase mutants. *J Gen Microbiol*. 1973; 75:197–210. [PubMed: 4578971]
- Hacham Y, Gophna U, Amir R. In vivo analysis of various substrates utilized by cystathionine gamma-synthase and O-acetylhomoserine sulfhydrylase in methionine biosynthesis. *Mol Biol Evol*. 2003; 20:1513–1520. [PubMed: 12832650]
- Hackert ML, Oliver RM, Reed LJ. Evidence for a multiple random coupling mechanism in the alpha-ketoglutarate dehydrogenase multienzyme complex of *Escherichia coli*: a computer model analysis. *Proc Natl Acad Sci U S A*. 1983; 80:2226–2230. [PubMed: 6403946]
- Hassan BH, Cronan JE. Protein-protein interactions in assembly of lipoic acid on the 2-oxoacid dehydrogenases of aerobic metabolism. *J Biol Chem*. 2011; 286:8263–8276. [PubMed: 21209092]
- Herbert AA, Guest JR. Biochemical and genetic studies with lysine+methionine mutants of *Escherichia coli*: lipoic acid and alpha-ketoglutarate dehydrogenase-less mutants. *J Gen Microbiol*. 1968; 53:363–381. [PubMed: 4889470]
- Hermes FA, Cronan JE. Scavenging of cytosolic octanoic acid by mutant LplA lipoate ligases allows growth of *Escherichia coli* strains lacking the LipB octanoyltransferase of lipoic acid synthesis. *J Bacteriol*. 2009; 191:6796–6803. [PubMed: 19684135]
- Jones HM, Gunsalus RP. Transcription of the *Escherichia coli* fumarate reductase genes (*frdABCD*) and their coordinate regulation by oxygen, nitrate, and fumarate. *J Bacteriol*. 1985; 164:1100–1109. [PubMed: 2999070]
- Jordan SW, Cronan JE Jr. Chromosomal amplification of the *Escherichia coli* *lipB* region confers high-level resistance to selenolipoic acid. *J Bacteriol*. 2002; 184:5495–5501. [PubMed: 12218038]
- Jordan SW, Cronan JE Jr. The *Escherichia coli* *lipB* gene encodes lipoyl (octanoyl)-acyl carrier protein:protein transferase. *J Bacteriol*. 2003; 185:1582–1589. [PubMed: 12591875]
- Kita K, Vibat CR, Meinhardt S, Guest JR, Gennis RB. One-step purification from *Escherichia coli* of complex II (succinate: ubiquinone oxidoreductase) associated with succinate-reducible cytochrome b556. *J Biol Chem*. 1989; 264:2672–2677. [PubMed: 2644269]
- Korshunov S, Imlay JA. Two sources of endogenous hydrogen peroxide in *Escherichia coli*. *Mol Microbiol*. 2010; 75:1389–1401. [PubMed: 20149100]
- Langley D, Guest JR. Biochemical genetics of the alpha-keto acid dehydrogenase complexes of *Escherichia coli* K12: genetic characterization and regulatory properties of deletion mutants. *J Gen Microbiol*. 1978; 106:103–117. [PubMed: 349114]
- Larsen RA, Wilson MM, Guss AM, Metcalf WW. Genetic analysis of pigment biosynthesis in *Xanthobacter autotrophicus* Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria. *Arch Microbiol*. 2002; 178:193–201. [PubMed: 12189420]
- Masse E, Gottesman S. A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. *Proc Natl Acad Sci U S A*. 2002; 99:4620–4625. [PubMed: 11917098]
- Miller, JH. A short course in bacterial genetics : a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press; Plainview, N.Y.: 1992.
- Miller JR, Busby RW, Jordan SW, Cheek J, Henshaw TF, Ashley GW, Broderick JB, Cronan JE Jr, Marletta MA. *Escherichia coli* LipA is a lipoyl synthase: in vitro biosynthesis of lipoylated pyruvate dehydrogenase complex from octanoyl-acyl carrier protein. *Biochemistry*. 2000; 39:15166–15178. [PubMed: 11106496]
- Morris TW, Reed KE, Cronan JE Jr. Identification of the gene encoding lipoate-protein ligase A of *Escherichia coli*. Molecular cloning and characterization of the *lplA* gene and gene product. *J Biol Chem*. 1994; 269:16091–16100. [PubMed: 8206909]
- Morris TW, Reed KE, Cronan JE Jr. Lipoic acid metabolism in *Escherichia coli*: the *lplA* and *lipB* genes define redundant pathways for ligation of lipoyl groups to apoprotein. *J Bacteriol*. 1995; 177:1–10. [PubMed: 8002607]
- Nagai S, Flavin M. Acetylhomoserine. An intermediate in the fungal biosynthesis of methionine. *J Biol Chem*. 1967; 242:3884–3895. [PubMed: 6037552]

- Perham RN. Swinging arms and swinging domains in multifunctional enzymes: catalytic machines for multistep reactions. *Annu Rev Biochem.* 2000; 69:961–1004. [PubMed: 10966480]
- Reed KE, Cronan JE Jr. Lipoic acid metabolism in *Escherichia coli*: sequencing and functional characterization of the *lipA* and *lipB* genes. *J Bacteriol.* 1993; 175:1325–1336. [PubMed: 8444795]
- Sawers G, Watson G. A glycyl radical solution: oxygen-dependent interconversion of pyruvate formate-lyase. *Mol Microbiol.* 1998; 29:945–954. [PubMed: 9767563]
- Singer M, Baker TA, Schnitzler G, Deischel SM, Goel M, Dove W, Jaacks KJ, Grossman AD, Erickson JW, Gross CA. A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. *Microbiol Rev.* 1989; 53:1–24. [PubMed: 2540407]
- Spencer ME, Guest JR. Transcription analysis of the *sucAB*, *aceEF* and *lpd* genes of *Escherichia coli*. *Mol Gen Genet.* 1985; 200:145–154. [PubMed: 3897791]
- Stauffer G. Regulation of Serine, Glycine, and One-Carbon Biosynthesis. *EcoSal Plus.* 2004
- Stapp LR, Bleile DM, McRorie DK, Pettit FH, Reed LJ. Use of trypsin and lipoamidase to study the role of lipoic acid moieties in the pyruvate and alpha-ketoglutarate dehydrogenase complexes of *Escherichia coli*. *Biochemistry.* 1981; 20:4555–4560. [PubMed: 6794598]
- Tseng CP, Albrecht J, Gunsalus RP. Effect of microaerophilic cell growth conditions on expression of the aerobic (*cyoABCDE* and *cydAB*) and anaerobic (*narGHJI*, *frdABCD*, and *dmsABC*) respiratory pathway genes in *Escherichia coli*. *J Bacteriol.* 1996; 178:1094–1098. [PubMed: 8576043]
- Tseng CP, Hansen AK, Cotter P, Gunsalus RP. Effect of cell growth rate on expression of the anaerobic respiratory pathway operons *frdABCD*, *dmsABC*, and *narGHJI* of *Escherichia coli*. *J Bacteriol.* 1994; 176:6599–6605. [PubMed: 7961411]
- Vanden Boom TJ, Reed KE, Cronan JE Jr. Lipoic acid metabolism in *Escherichia coli*: isolation of null mutants defective in lipoic acid biosynthesis, molecular cloning and characterization of the *E. coli lip* locus, and identification of the lipoylated protein of the glycine cleavage system. *J Bacteriol.* 1991; 173:6411–6420. [PubMed: 1655709]
- Vogel HJ, Bonner DM. Acetylornithinase of *Escherichia coli*: partial purification and some properties. *J Biol Chem.* 1956; 218:97–106. [PubMed: 13278318]
- Wimpenny JW, Firth A. Levels of nicotinamide adenine dinucleotide and reduced nicotinamide adenine dinucleotide in facultative bacteria and the effect of oxygen. *J Bacteriol.* 1972; 111:24–32. [PubMed: 4360220]
- Zhao X, Miller JR, Cronan JE. The reaction of LipB, the octanoyl-[acyl carrier protein]:protein N-octanoyltransferase of lipoic acid synthesis, proceeds through an acyl-enzyme intermediate. *Biochemistry.* 2005; 44:16737–16746. [PubMed: 16342964]
- Zhao X, Miller JR, Jiang Y, Marletta MA, Cronan JE. Assembly of the covalent linkage between lipoic acid and its cognate enzymes. *Chem Biol.* 2003; 10:1293–1302. [PubMed: 14700636]

Fig. 1. Schematic of central metabolism showing the decarboxylation reactions catalyzed by the lipoate-dependent enzyme complexes, pyruvate dehydrogenase (PDH) and 2-oxoglutarate dehydrogenase (OGDH) and the pathways of protein lipoylation

(A) 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). Enzyme name abbreviations are shown in bold red. Abbreviations: SucCD, succinate:CoA ligase; SDH, succinate dehydrogenase; and DAP, diaminopimelic acid.

Lipoic acid metabolism in *E. coli*. (B) Scavenging of lipoate from the environment. LplA catalyzes the ligase reaction in two steps. First, lipoic acid is activated to lipoyl-AMP with concomitant release of pyrophosphate. In the presence of a lipoyl domain (LD) acceptor protein the lipoyl moiety is transferred to the LD (e.g., the E2 subunit of pyruvate dehydrogenase) by attack of the mixed anhydride by the γ -amino group of a specific lysine residue. (C) Biosynthesis of lipoate. The LipB octanoyl-acyl carrier protein (ACP) transferase, transfers the octanoyl moiety from the fatty acid biosynthetic intermediate, octanoyl-ACP, to the LD domain of a lipoate-accepting protein by attack of the thioester by the lysine residue γ -amino group. The octanoylated domains then become substrates for sulfur insertion by LipA, a radical SAM (S-adenosylmethionine) enzyme that replaces one hydrogen atom in each of octanoate carbons 6 and 8 with sulfur atoms. LplA can also utilize exogenous or endogenous octanoate to bypass the LipB reaction (Hermes & Cronan, 2009, Zhao *et al.*, 2003).

Fig. 2. The *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 μ M 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 μ mole substrate mg^{-1} protein hr^{-1} (Reed & Cronan, 1993).

Fig. 3. Strain FH34 grows in the absence of succinate supplementation despite lacking detectable OGDH activity

(A) Growth characteristics on unsupplemented glucose minimal medium of the wild type (MG1655), the *lipB* strain (FH160) and the *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 & Cronan, 1993).

Fig. 4. The *lipB sdhB aceA nadB sucAB* strain (FH698) requires succinate for growth on glucose minimal medium supplemented with nicotinic acid

Nicotinic acid (100 μM) was added to allow NAD synthesis. 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.

Fig. 5. Pathways of succinate production in the *lipB 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 (as shown). 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).

Table 1

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

Genetic Manipulation	Acetate Requirement	Succinate Requirement
<u><i>lipB</i> background (Strains FH6 and FH160)</u>		
None	No	Yes
<i>lplA</i>	Yes	Yes
<i>lipA</i>	Yes	Yes
<i>sdhB</i>	No	No
<i>sdhA</i>	No	No
<i>sucA</i>	No	No
<i>sucB</i>	No	Yes
<i>sdhB/p(sdhCDAB)</i>	No	Yes
<i>sucA/p(sdhCDAB)</i>	No	Yes
<u><i>lipB sdhB</i> background (Strains FH34 and FH420)</u>		
<i>poxB</i>	No	No
<i>pflB</i>	No	No
<i>poxB pflB</i>	No	No
<i>frdA</i>	No	No
<i>lplA</i>	Yes	No
<i>lipA</i>	Yes	No
<i>lpd</i>	Yes	No
<i>sucCD</i>	Yes	Lysine and methionine (but not DAP)
<i>aceA</i>	No	No
<i>sucAB</i>	No	No
<i>frdA aceA sucAB</i>	No	No
<i>frdA aceA sucAB nadB</i>	No	Yes
<i>frdA aceA sucAB nadB/p(nadB)</i>	No	No
<i>nadB</i>	No	No
<i>frdA nadB</i>	No	No
<i>sucAB nadB</i>	No	No
<i>frdA aceA nadB</i>	No	No
<i>frdA sucAB nadB</i>	No	No
<i>aceA nadB sucAB</i>	No	Yes
<i>aceA nadB sucA</i>	No	Yes
<i>aceA nadB sucB</i>	No	Yes
<i>aceA nadB sucAB/p(sucB)</i>	No	Yes
<i>aceA nadB sucB/p(sucB)</i>	No	No
<i>aceA nadB lpd</i>	Yes	No

Genetic Manipulation	Acetate Requirement	Succinate Requirement
<i>aceA nadB lplA</i>	Yes	No
<i>aceA nadB sucB / p(sucB^{K44R})</i>	No	Yes