

Lipoic Acid Metabolism in Microbial Pathogens

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INTRODUCTION	201
Historical Overview of Lipoic Acid Discovery	201
Structure of Lipoylated Complexes	201
LIPOATE IN CATALYSIS	202
Mechanisms of Catalysis	202
α-Ketoacid dehydrogenase complexes	202
AoDH complex	202
GCV	202
Lipoylated Complexes	203
PDH complex	203
KDH complex	204
BCDH complex	204
AoDH complex	204
GCV	204
MECHANISMS OF LIPOYLATION	205
Lipoate Synthesis	205
Lipoate Scavenging	206
Lipoate Cleavage	206
LIPOATE AS AN ANTIOXIDANT	207
BACTERIAL LIPOATE METABOLISM	207
Gram-Negative Bacteria	207
<i>Alphaproteobacteria</i>	207
<i>Betaproteobacteria</i>	209
<i>Gammaproteobacteria</i>	209
<i>Epsilonproteobacteria</i>	210
<i>Chlamydiae</i>	210
Gram-Positive Bacteria	210
<i>Actinobacteria</i>	211
<i>Firmicutes</i>	213
FUNGAL LIPOATE METABOLISM	215
Mechanisms of Lipoylation	215
Metabolic Regulation	215
Comparative Genomics of Pathogenic Fungi	216
PROTOZOAN LIPOATE METABOLISM	216
Apicomplexans	216
Lipoylated Complexes in Apicomplexans	217
<i>Plasmodium falciparum</i>	217
<i>Toxoplasma gondii</i>	219
<i>Cryptosporidium parvum</i>	220
Mechanisms of Lipoylation in Apicomplexans	220
<i>Plasmodium falciparum</i>	220
<i>Toxoplasma gondii</i>	221
Kinetoplastids	221
Lipoylated Complexes in Kinetoplastids	221
<i>Trypanosoma brucei</i>	221
<i>Leishmania major</i>	222
Metamonada and Amoebozoa	222
<i>Trichomonas vaginalis</i>	222
CONCLUSIONS	222
ACKNOWLEDGMENTS	223
REFERENCES	223

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INTRODUCTION

Lipoate (Fig. 1A) is a highly conserved organosulfur cofactor that is required for the function of several key enzyme complexes in oxidative and one-carbon metabolism. Lipoate was originally discovered as an unknown factor derived from biological extracts that stimulated bacterial growth in the presence of certain carbon sources. These phenomena were ultimately explained by the use of lipoate as a cofactor in multienzyme complexes involved in intermediate metabolism. In addition to its role in catalysis, the redox activity of lipoate also allows it to function as an antioxidant and free-radical scavenger. The acquisition and use of lipoate differ to a surprising degree among microbial pathogens and affect the virulence of these organisms and the pathogenesis of the diseases they cause. This review surveys lipoate metabolism in bacterial, fungal, and protozoan pathogens and explores how it functions in microbial metabolism as well as in non-metabolic processes.

Historical Overview of Lipic Acid Discovery

In the 1930s, Esmond Snell and coworkers observed that the addition of acetate to synthetic media stimulated the growth of lactic acid bacteria (212). Nearly a decade later, Guirard and coworkers observed that some biological preparations were able to replace acetate as a growth factor for lactic acid bacteria (70); the substance that permitted this was termed acetate-replacing factor (ARF). In parallel, O'Kane and Gunsalus showed that *Streptococcus faecalis* (now called *Enterococcus faecalis*) grew equivalently in tryptone-yeast extract and in synthetic medium; however, only the cells grown in tryptone-yeast extract oxidized pyruvate (160). The material in yeast extract that enabled *S. faecalis* to oxidize pyruvate could not be replaced by any known vitamins or cofactors and was called pyruvate oxidation factor (POF) (160). Subsequently, POF was shown to have ARF activity, as was a growth factor (221) described for *Tetrahymena gelii* (211). Pure, crystalline material containing both POF and ARF properties was produced from hydrolyzed liver extracts and was determined to be (*R*)-5-(1,2-dithiolan-3-yl)pentanoic acid (184). This compound was named "lipoic acid" because it was lipophilic, had acidic properties, and was involved in the anabolism of fatty acids. In addition to having ARF and POF activities, pure lipoic acid was soon found to replace another substance, called the "BR factor" (110), which was required for *Butyribacterium rettgeri* growth on lactate as an energy source (111). At the time, lipoate (the deprotonated charge form of lipoic acid which dominates at pHs of above 4.7) was thought to be a new B vitamin (184); however, a disease associated with lipoate deficiency in humans has not been observed. Furthermore, there is increasing evidence that mammals can synthesize lipoate (258). In organisms that generate lipoate endogenously, the cofactor is synthesized from an octanoic acid precursor (168), with stereospecific insertion of the sulfur atom at carbon six to yield the *R* enantiomer, which is the biologically active form (169).

To date, five lipoate-dependent multienzyme complexes have been characterized. Three are α -ketoacid dehydrogenases: pyruvate dehydrogenase (PDH), α -ketoglutarate dehydroge-

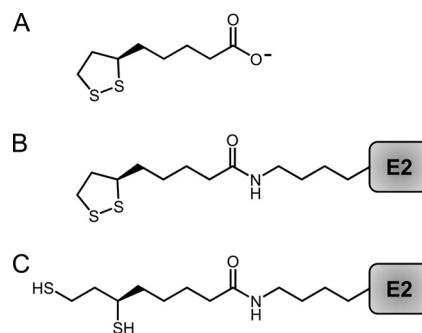


FIG. 1. Lipoyl moieties. (A) The biologically active *R* stereoisomer of lipoate. (B) The oxidized lipoyl cofactor, lipoamide, bound to a conserved lysine residue of the E2 subunit of lipoylated complexes. Lipoamide and dihydrolipoamide are also attached to the H protein of the glycine cleavage complex. (C) The reduced form of the lipoyl cofactor, dihydrolipoamide, shown bound to a conserved lysine residue of the E2 subunit of lipoylated complexes.

nase (KDH), and branched-chain α -ketoacid dehydrogenase (BCDH). These complexes are composed of multiple copies of each of three enzymatic subunits referred to as E1 (often produced as two proteins), E2, and E3 (171). A fourth complex, acetoin dehydrogenase (AoDH), is highly homologous to PDH and shares the three-subunit architecture of the α -ketoacid dehydrogenases (256). The fifth complex, the glycine cleavage complex (GCV), has a different architecture and is composed of four loosely associated proteins called the P, H, T, and L proteins (39). The lipoate cofactor is attached through an amide bond to a conserved lysine residue on the H protein subunit of the GCV and to analogous lysine residues on the E2 subunits of the other complexes. During catalysis, the intramolecular disulfide bond of lipoate cycles between oxidized lipoamide (Fig. 1B) and reduced dihydrolipoamide (Fig. 1C) (171).

Structure of Lipoylated Complexes

The α -ketoacid dehydrogenases and acetoin dehydrogenase are enormous protein complexes containing many copies of the E1, E2, and E3 subunits (171). These complexes are formed around a tightly associated core of E2 trimers which have been observed to form cage-like octahedral complexes of 24 subunits (130) and icosahedral complexes of 60 subunits (97). The amino-terminal region of each E2 subunit contains one or more small (\sim 80-amino-acid) lipoylation domains, and each domain has a single attachment site for lipoate. The E2 core is arranged so that the lipoylation domains are displayed on the outer face of the complex, where they interact with peripheral E1 and E3 subunits. The E2 subunits of the KDH and BCDH contain a single lipoyl domain (12, 170, 187), whereas E2 subunits of the AoDH can contain a second domain (256) and PDH E2 subunits can contain up to three lipoyl domains (171). The E1 subunits of the PDHs of most Gram-negative bacteria and all KDHs are homodimeric (α_2). In contrast, the PDHs of Gram-positive bacteria and all BCDHs and AoDHs are com-

posed of two proteins, E1 α and E1 β , arranged as heterotetramers ($\alpha_2\beta_2$). In both cases, the E1 multimers contain two thiamine pyrophosphate (TPP) cofactors that are thought to communicate through a "proton wire" and act in a reciprocal manner during catalysis (51). Dimeric E1 (or heterotetrameric $\alpha_2\beta_2$ E1) and dimeric E3 subunits are arranged around the E2 cores of α -ketoacid dehydrogenase complexes; although the peripheral subunits bind with a variety of stoichiometries, there are typically more E1s than E3s (171).

The structure of α -ketoacid dehydrogenase complexes and their subunits has been studied by several techniques. X-ray crystallography has been used to determine the structure of the PDH E1 dimer from *Escherichia coli* (9) as well as E3 dimers from many sources, including the earliest structure determined, the E3 from *Azotobacter vinelandii* (201). Crystal structures of complete E2 subunits have not been determined, probably due to the inherent flexibility of these proteins. The N-terminal lipoyl domain (or domains) are connected to a 40-amino-acid peripheral-subunit-binding domain (PSBD) and the C-terminal catalytic domain by flexible linkers. Early nuclear magnetic resonance (NMR) experiments defined the structures of individual lipoyl domains (36) and the PSBDs from E2 subunits (196). Several of the more recently determined E3 subunit structures have been determined as complexes formed between the E3 dimer and a single PSBD derived from the corresponding E2 subunit (126, 154). The structures of E2 catalytic domains have been determined by X-ray crystallography and form either octahedral 24-mers (112, 129) or icosahedral 60-mers (97), depending on the source.

The inherent flexibility of the E2 subunits and the dynamic nature of E1 and E3 binding to the E2 core have so far prevented the crystallization of higher-order complexes. However, reconstituted complexes as well as native complexes have been characterized by cryo-electron microscopy. These structures indicate that the shells of E1 and E3 subunits are separated from the E2 core by an annular gap of 30 to 50 Å in an octahedral complex (239) and of 75 to 90 Å in an icosahedral complex (136, 137). The flexibility of the lipoamide side chain and flexible hinge regions flanking the lipoyl domains in the E2 subunits are thought to facilitate interactions with the E1 and E3 subunits across this gap. The range of motion attributed to the lipoyl domains also allows acyl group transfer (and redox reactions) between lipoyl groups on different E2 proteins throughout the E2 core (170, 187).

In addition to the core E1, E2, and E3 subunits that are characteristic of lipoylated metabolic complexes across taxa, in some species additional proteins that function in complex assembly or regulation are also found in lipoylated complexes. As described in more detail below, such components include regulatory kinases and phosphatases. Additionally, most eukaryotic PDH complexes contain a subunit called the E3-binding protein (E3BP), which is required for recruiting E3 subunits to the complexes. For example, the bovine heart PDH is a 9.5-million-dalton complex composed of 30 copies of heterotetrameric E1, 12 copies of homodimeric E3, and 12 copies of monomeric E3-binding protein arranged around an icosahedral core of 60 E2 subunits (186). In contrast, the protein subunits of the GCV do not form a stable complex but instead behave as independent proteins (39, 156).

LIPOATE IN CATALYSIS

Mechanisms of Catalysis

In the five lipoylated enzyme complexes, lipoate acts both as an electrophile that binds to reaction intermediates (via a thioester or thioether bond) and as a swinging arm that channels the bound substrate between the active sites of different subunits (reviewed in references 171, 185, and 187).

α -Ketoacid dehydrogenase complexes. All three α -ketoacid dehydrogenase complexes catalyze the decarboxylation of α -ketoacids to produce acyl coenzyme A (acyl-CoA), NADH, and CO₂ by similar reaction mechanisms (Fig. 2A). The reaction begins with the thiamine pyrophosphate (TPP)-dependent decarboxylation of the substrate catalyzed by the E1 subunit. The acidic carbon of the TPP thiazole ring attacks the substrate carbonyl carbon (carbon 2), forming a covalent intermediate. Collapse of this intermediate releases CO₂, leaving an activated carbanion species bound to TPP. This species acylates one of the sulfur atoms in lipoamide, leaving the second sulfur atom reduced to a thiol. The E2 active site then catalyzes the transfer of the acyl moiety from dihydrolipoamide to coenzyme A. To regenerate the electrophilic lipoamide form of the cofactor, the E3 subunit, called a dihydrolipoyl dehydrogenase, oxidizes dihydrolipoamide to lipoamide in a NAD-dependent reaction (170). Unlike the E1 and E2 subunits, which are specific to each α -ketoacid dehydrogenase complex, the E3 subunit is often shared between complexes. For example, in *E. coli* the single E3 subunit is encoded in the PDH operon but can also be expressed from an independent transcript, providing E3 subunits for the KDH complex (216). In plants (124, 139) and apicomplexan parasites (135), distinct E3 proteins function in mitochondria and plastids.

AoDH complex. The acetoin dehydrogenase (AoDH) is highly homologous to PDH and shares all of the features described above for the α -ketoacid dehydrogenases (256), but it does not have an α -ketoacid substrate (Fig. 2B). The TPP bound to the E1 subunit attacks the carbonyl carbon of acetoin (3-hydroxy-2-butanone), resulting in a covalent linkage between TPP and 2,3-butanediol. This intermediate collapses, releasing acetaldehyde and leaving TPP with an activated hydroxyethyl group that is poised to acylate the lipoamide cofactor of the E2 subunit. Other than the release of acetaldehyde (rather than CO₂), the reactions catalyzed by AoDH are identical to those catalyzed by PDH and result in the formation of acetyl-CoA.

GCV. While other lipoylated complexes irreversibly decarboxylate α -ketoacids to form acyl-CoA moieties, the glycine cleavage complex (GCV) catalyzes the reversible decarboxylation of glycine to CO₂, NADH, ammonia, and a methylene group that is bound to tetrahydrofolate (THF) to form the one-carbon donor 5,10-CH₂-THF (Fig. 2C). Thus, although the reaction sequence of the GCV is similar to that of the α -ketoacid dehydrogenase complexes, the mechanism varies from that of other lipoylated complexes in subtle but important ways.

The subunits of the GCV are known as the P protein (pyridoxal phosphate-containing protein), H protein (hydrogen carrier protein), T protein (tetrahydrofolate-containing protein), and L protein (lipoamide dehydrogenase), with lipoate covalently bound to the H protein (Fig. 2C) (39). The P protein

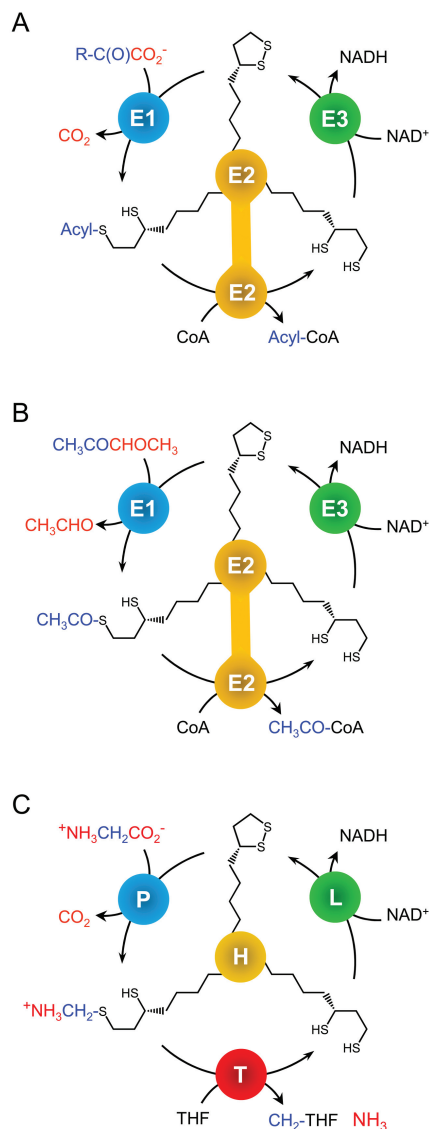


FIG. 2. Reactions of lipoylated complexes. (A) α -Ketoacid dehydrogenase complexes. In the first reaction step of the pyruvate dehydrogenase complex, α -ketoglutarate dehydrogenase complex, and branched-chain α -ketoacid dehydrogenase complex, the E1 subunit decarboxylates the α -ketoacid substrate, and the acyl group is then transferred to the lipoyl cofactor on the E2 subunit. The E2 subunit has catalytic activity in addition to harboring the lipoyl domain, and it transfers the acyl group to coenzyme A (CoA). The lipoate form of the cofactor is regenerated through reduction of NAD⁺ by the E3 subunit. (B) Acetoin dehydrogenase complex. The AoDH is highly homologous to the PDH but uses acetoin (3-hydroxy-2-butanone) as a substrate instead of pyruvate. Reaction of acetoin with the E1 subunit results in release of acetaldehyde and acetylation of lipoamide. The E2 subunit then transfers the acetyl group to CoA, and lipoamide is regenerated by the E3 subunit. (C) Glycine cleavage complex. The GCV catalyzes the reversible oxidative decarboxylation of glycine to generate carbon dioxide, ammonia, and a methylene group that is transferred to the cofactor tetrahydrofolate for use in one-carbon metabolism. The lipoylated H protein acts as a mobile substrate and shuttles between the active sites of the P, T, and L proteins. Note that unlike the E2 subunit of the α -ketoacid dehydrogenase complexes, the H protein does not have catalytic activity. The P protein catalyzes a reaction similar to that of the E1 subunit, and the L protein is analogous to the E3 subunit. (Panel C adapted from reference 39 with permission from Elsevier.)

is analogous to the E1 subunit of the α -ketoacid dehydrogenases and catalyzes the decarboxylation of glycine; however, it depends on a pyridoxal phosphate cofactor instead of TPP. After the oxidative decarboxylation of glycine by the P protein, methyleneamine is covalently attached to dihydrolipoamide on the H protein. Unlike E2 subunits, the H protein does not have catalytic activity but instead acts as a scaffold to protect the unstable intermediate during transfer to the T protein (69). The T protein catalyzes the release of ammonia from methyleneamine and the transfer of the methylene group to THF, forming 5,10-CH₂-THF. The L protein is a dihydrolipoamide dehydrogenase analogous to the E3 subunit of α -ketoacid dehydrogenase complexes, and catalyzes the two-electron oxidation of dihydrolipoamide to regenerate lipoamide and convert NAD⁺ into NADH. Most organisms use the same gene product for the E3 subunit and the L protein (reviewed in references 31 and 39).

Lipoylated Complexes

PDH complex. The pyruvate dehydrogenase (PDH) catalyzes the oxidative decarboxylation of pyruvate to form acetyl coenzyme A (acetyl-CoA). Several key metabolic pathways consume acetyl-CoA, including the tricarboxylic acid (TCA) cycle, fatty acid biosynthesis, and fatty acid elongation pathways and the mevalonate pathway of isoprenoid biosynthesis. *Escherichia coli* contains a single PDH, which is active during aerobic growth. In *E. coli*, the loss of holo-PDH can be bypassed by supplementation with acetate (237). Most eukaryotes contain a mitochondrial PDH, which links glycolysis to the TCA cycle. Plants have an additional PDH in the chloroplast, which generates acetyl-CoA for the *de novo* fatty acid synthase (FAS) in the plastid stroma and also is the primary source of NADH for this pathway (139).

In eukaryotic PDH complexes, an additional protein called the E3-binding protein (previously called "protein X" [37, 100]) is required to tether the E3 subunit to the E2 core (64, 117, 176). The E3-binding protein (E3BP) is homologous to E2 subunits and includes a single lipoyl domain followed by a peripheral-subunit-binding domain (PSBD) and the catalytic domain (77, 155). The lipoyl domain is lipoylated and can be reduced and acetylated by the E3 and E1 subunits of PDH (85, 100, 181). However, E3BPs do not seem to catalyze the transacetylase reaction necessary to generate acetyl-CoA, perhaps due to the absence of a catalytic histidine residue which is present in E2 subunits (77). Truncation of the lipoyl domain of yeast E3BP had little effect on PDH activity or on the formation of the complex (117), demonstrating that this domain is not important for E3BP function. Cleavage of a larger fragment from the N terminus of bovine E3BP resulted in inactive PDH complexes which lacked E3 subunits (64, 176). In these experiments, proteolytic cleavage probably removed the PSBD as well as the lipoyl domain. Thus, the critical role of E3BPs appears to be the binding of the E3 subunit rather than the catalytic activity of the lipoyl domain. Indeed, the genes encoding putative E3BPs from some organisms, such as *Aspergillus fumigatus*, do not seem to contain lipoyl domains.

The PDH is allosterically inhibited by its products, NADH and acetyl-CoA, and by high levels of ATP relative to ADP. In prokaryotes, PDH expression is upregulated by aerobic growth

and excess pyruvate and is suppressed during fermentative growth (31). In eukaryotes, in addition to allosteric regulation of the PDH by accumulation of product, activity is also controlled through phosphorylation of the E1 subunit (122). Under anaerobic conditions, the complex-bound pyruvate dehydrogenase kinase phosphorylates the complex to inactivate it (96, 120, 122), resulting in the conversion of pyruvate to lactate in the cytosol. Repression of PDH activity can subsequently be alleviated by the pyruvate dehydrogenase phosphatase, which is loosely associated with the complex (121, 122).

KDH complex. The α -ketoglutarate dehydrogenase (KDH) converts α -ketoglutarate to succinyl-CoA through a reaction mechanism similar to that of the PDH. Succinyl-CoA can be consumed by the TCA cycle enzyme succinyl-CoA synthetase, or it can be diverted for heme and amino acid biosynthesis (83). In the first step of heme biosynthesis, δ -aminolevulinic acid synthase catalyzes the condensation of glycine and succinyl-CoA to form δ -aminolevulinic acid (δ -ALA) (81). Succinyl-CoA is also used for methionine and lysine biosynthesis in *E. coli* and other organisms that are capable of synthesizing these amino acids. In *E. coli* strains that lack an active KDH, the activity can be bypassed with succinate or, under anaerobic conditions, with lysine and methionine (83). Most eukaryotes contain a single KDH that is located in the mitochondrion. In organisms such as mammals that are auxotrophic for methionine and lysine, the KDH is important for aerobic respiration and for production of heme precursor molecules.

The KDH varies structurally from most PDHs and all known BCDHs in that the E1 subunit is encoded by one gene, which includes regions homologous to both the E1 α and E1 β subunits of other α -ketoacid dehydrogenase complexes. Unlike the eukaryotic PDH, the KDH is not regulated by phosphorylation of the E1 subunit. Instead, it is activated by metabolic intermediates such as a high AMP/ATP ratio (139). In *E. coli*, the expression of the KDH is upregulated during aerobic growth but is highly repressed during fermentative growth (68). This repression results in a branched TCA "cycle" which generates the biosynthetic precursor α -ketoglutarate through an oxidative branch and succinyl-CoA through a reductive branch (215). Several of the pathogens described in later sections of this review contain a branched TCA cycle, and in some cases they lack KDH enzymes.

BCDH complex. The branched-chain α -ketoacid dehydrogenase (BCDH) participates in the degradation of branched-chain amino acids to generate branched-chain CoA (BC-CoA) molecules that can be converted into TCA cycle intermediates or used for branched-chain fatty acid (BCFA) synthesis. During branched-chain amino acid degradation, the amino acids valine, leucine, and isoleucine are deaminated to the corresponding α -ketoacids by the branched-chain amino acid transaminase (BCAT). These α -ketoacids are substrates for the BCDH and are decarboxylated and conjugated to CoA to generate 3-methyl-butanoyl-CoA, isobutyryl-CoA, and 2-methyl-butanoyl-CoA. In many Gram-positive bacteria, the short BC-CoA molecules produced by the BCDH are used chiefly as primers for generating longer branched-chain fatty acids that can have important roles in temperature adaptation by modulating membrane fluidity (223, 260). For example, when the BCDH is disrupted in the bacterial pathogen *Listeria monocytogenes*, the organism becomes deficient in BCFAs and

can no longer adapt to growth in cold conditions (262). The requirement for specific BC-CoA products of the BCDH varies by species. In the bacterium *Bacillus subtilis*, addition of any of the three fatty acid analogs of the BCDH products is sufficient to bypass the mutant enzyme (251). In contrast, *L. monocytogenes* requires 2-methylbutyrate to bypass inactivation of the BCDH (106). Thus, the specific BCFA requirements of an organism dictate which short branched-chain fatty acids can be used to bypass the complex.

In prokaryotes, expression of the BCDH appears to be induced by the accumulation of branched-chain ketoacids (128). In mammalian cells, the BCDH is tightly regulated by phosphorylation and product inhibition in a manner similar to that for the PDH (reviewed in reference 76). Phosphorylation of the E1 α subunit by a complex-bound kinase results in enzyme inactivation (175, 209), which can be reversed by a bound phosphatase (34). The accumulation of branched-chain acyl-CoA products and NADH competitively inhibits the complex (76). In eukaryotes, the BCDH is found in the mitochondrion, where the BC-CoA products can be further metabolized into TCA cycle intermediates such as acetyl-CoA and succinyl-CoA.

AoDH complex. The acetoin dehydrogenase (AoDH) is closely related to the α -ketoacid dehydrogenases and is thought to have evolved from a common PDH ancestor (115). In many bacteria of the *Firmicutes* and *Proteobacteria* phyla, the conversion of pyruvate into acetyl-CoA involves AoDH rather than PDH (reviewed in reference 256). In these bacteria, acetoin (3-hydroxy-2-butanone) is formed from pyruvate in two enzymatic steps (191), providing the substrate for AoDH. Reconstituted AoDH containing the E1, E2, and E3 subunits from the bacterium *Pelobacter carbinolicus* is specific for acetoin and does not use pyruvate or α -ketoglutarate as substrate (162). The E1 α protein contains a region of divergent sequence compared to other α -ketoacid dehydrogenases and appears to be responsible for the substrate specificity of AoDH (115). The E1 β and E2 proteins, and other regions of the E1 α , are highly homologous to those comprising PDH complexes. Like prokaryotic PDH E1 α subunits, the AoDH E1 α does not appear to contain the regulatory phosphorylation site found in eukaryotic PDH. As observed among PDH E2 proteins (187), the E2 proteins of AoDH can have various numbers of lipoyl domains. Two lipoyl domains are found in the AoDH E2 of *P. carbinolicus*, compared to one in *Klebsiella pneumoniae* and in *Cupriavidus necator* (38, 178). The genes encoding the AoDH subunits are organized in a manner similar to that observed for other α -ketoacid dehydrogenases, with the E1 α , E1 β , and E2 subunits encoded in the same gene cluster. The presence of an E3 subunit encoded in this cluster varies by species (256), and in cases where it is absent, a common E3 is presumably shared between the AoDH and the α -ketoacid dehydrogenases. Interestingly, in *P. carbinolicus* an additional gene that encodes lipoate synthase is sandwiched between the genes encoding the AoDH E2 and E3 (163), possibly linking expression of lipoylated metabolic complexes and expression of lipoylating enzymes.

GCV. As discussed above, the glycine cleavage complex (GCV) catalyzes the reversible decarboxylation of glycine. In the direction of glycine catabolism, the GCV generates NADH, CO₂, NH₃, and the one-carbon donor molecule 5,10-CH₂-THF,

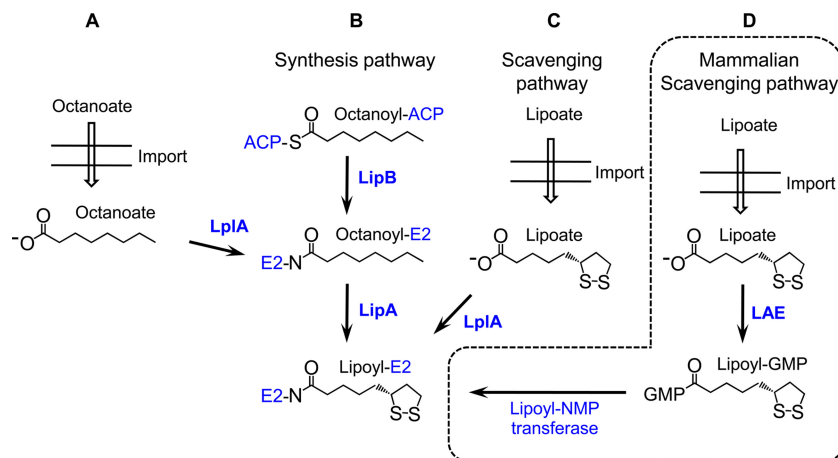


FIG. 3. Comparison of lipoylation strategies. (A and B) In the synthesis pathway, the acyl chain of octanoyl-ACP is transferred by the lipoate (octanoyl) transferase LipB to a conserved lysine residue on the E2 subunit of the α -ketoacid dehydrogenase complexes or the H protein of the GCV. The octanoylated subunit is a substrate for the lipoate synthase, LipA, which catalyzes sulfur insertion. Alternatively, octanoyl-E2 and octanoyl-H protein can be generated by the ATP-dependent ligation of free octanoate by LplA. (C) The primary role of LplA, however, is in lipoate scavenging, where it catalyzes the ATP-dependent ligation of free lipoate to proteins. (D) The mammalian scavenging pathway appears to use a unique approach in which free lipoate is conjugated to GMP in a reaction catalyzed by the lipoate-activating enzyme (LAE). The LplA homolog lipoyl-NMP transferase cannot use free lipoate as substrate and instead ligates GMP-activated lipoate to proteins.

which is required for the biosynthesis of some amino acids and nucleotides (39). The GCV also allows glycine to serve as a carbon and nitrogen source for some organisms. When the GCV favors glycine biosynthesis, glycine can be used for protein translation or as a substrate of δ -aminolevulinic acid synthase in the first step of heme synthesis (81). In eukaryotes, including plants, the GCV has been found to be strictly mitochondrial (39), except in the amitochondriate protozoan *Trichomonas vaginalis*, where components of the GCV are found in organelles related to mitochondria called hydrogenosomes (150).

The direction in which the GCV operates is driven by equilibrium and varies between organisms. In nonphotosynthesizing plant tissues, the GCV operates unidirectionally to catabolize glycine to support the mitochondrial synthesis of serine, which is subsequently trafficked to the cytoplasm and used for the generation of cytoplasmic one-carbon donors (45, 148). In *Saccharomyces cerevisiae*, the GCV functions reversibly, catabolizing glycine or synthesizing it depending on the metabolic state of the cell (173). In *S. cerevisiae* and *E. coli*, the loss of any of the GCV subunits prevents these organisms from using glycine as a sole carbon or nitrogen source but does not otherwise affect growth (173, 174). The expression of GCV proteins in *E. coli* is regulated in a complex manner which includes activation by glycine and repression by downstream purine products (217).

MECHANISMS OF LIPOYLATION

Two mechanisms have been identified for the posttranslational modification of proteins with lipoate: lipoate synthesis and lipoate scavenging (144). Lipoate scavenging refers to the ligation of exogenous free lipoate to target proteins. Conversely, lipoate synthesis refers to the generation of protein-bound lipoate from an octanoylated precursor. These methods of lipoate attachment are best characterized in *E. coli*, which

has independent lipoate synthesis and scavenging pathways (Fig. 3A to C). Despite the highly conserved and almost ubiquitous nature of lipoylated complexes, it is becoming clear that organisms rely on a diverse array of lipoylation strategies to generate the holocomplexes. Here, we use *E. coli* as a model to introduce lipoate synthesis and lipoate scavenging before exploring in subsequent sections how these pathways are employed by microbial pathogens.

Lipoate Synthesis

In *E. coli*, lipoate synthesis proceeds through two reactions catalyzed by an octanoyl transferase, called LipB (144), and a lipoate synthase, called LipA (78, 79, 183) (Fig. 3B). LipB transfers an octanoyl group from octanoyl acyl carrier protein (octanoyl-ACP) to the apoprotein. The transferase does not efficiently use free octanoate as a substrate and consequently is dependent on the type II fatty acid synthase to produce octanoyl-ACP (102). After generation of the octanoylated subunit, two sulfur atoms are inserted by LipA to form the dithiolane ring of lipoate (261). LipA, but not LipB, is essential for lipoate synthesis. LipB can be bypassed by the lipoate ligase, LplA, which can use free octanoate (instead of free lipoate) to octanoylate apoproteins (261) (Fig. 3A). Thus, in *E. coli* the lipoate synthesis pathway relies on LipA and either LipB or LplA to produce lipoylated proteins and does not produce the cofactor as a free acid.

In plants, LipB and LipA paralogs are found in mitochondria and plastids (257). In plastids, where no ligases have been identified, lipoate synthesis is believed to use the octanoyl-ACP product of the plastid type II FAS to lipoylate the plastid PDH. Interestingly, despite the presence of a lipoate ligase in plant mitochondria, a major function of the plant mitochondrial type II FAS appears to be the production of octanoyl-ACP for lipoate synthesis (67). In other eukaryotes, particularly in yeast, there is increasing evidence for mitochondrial

type II FAS and lipoate synthesis (23, 87). In mammals, lipoate is thought to be derived primarily from food and intestinal bacteria and is transported by the bloodstream to target enzymes in the mitochondria of cells (56, 57, 177); however, type II FAS and lipoate synthesis also appear to be functional in these cells (30, 141). Deletion of the lipoate synthase gene in mice results in embryonic lethality which cannot be circumvented with dietary lipoate (258), highlighting the importance of mitochondrial lipoate synthesis in eukaryotic metabolism.

Lipoate Scavenging

Lipoate scavenging involves the attachment of exogenous lipoate to the apo-E2 or H protein subunits by a lipoate ligase (Fig. 3C). *E. coli* contains one lipoate ligase, LplA, which was discovered as a gene product essential for the incorporation of exogenous lipoate into metabolic complexes (143). Studies with pure recombinant LplA showed that it catalyzes a two-step ATP-dependent reaction. In the first step, ATP is used to activate free lipoate to lipoyl-AMP. The conserved lysine residue on the apodomain then reacts at the activated carbonyl of lipoyl-AMP to form the lipoamide bond and release AMP (143). Unlike LipB and LipA, LplA can use octanoate as a substrate (143, 261). Similarly, the lipoate analog 8-bromo-octanoate (BrO) is also a substrate for LplA, resulting in *E. coli* growth inhibition (2, 261).

Despite very low levels of sequence identity, LplA and LipB enzymes were proposed to belong to the same family of cofactor attachment enzymes (171). Consistent with this hypothesis, LplA can catalyze the octanoyl transferase reaction typically catalyzed by LipB, albeit at very low levels (102). X-ray crystal structures of LipB and LplA enzymes confirmed that these enzymes are structurally similar and share the same protein fold observed in the *E. coli* biotin ligase, BirA (253). The structure of the *Mycobacterium tuberculosis* LipB was found to align with the previously determined structures of *Thermoplasma acidophilum* LplA (108) and *E. coli* LplA (58), with a root mean square (RMS) deviation of ~ 2.5 Å for aligned C α atoms (125). Importantly, the fatty acid ligand observed in the *M. tuberculosis* LipB structure could be superimposed with the lipoyl moiety of lipoyl-AMP in the *T. acidophilum* LplA structure, highlighting similarities in the active sites of these enzymes (125). Octanoic acid and its analogs also bind to the analogous active-site pocket in structures of *Thermus thermophilus* LipB (109).

An important distinction between LipB and LplA enzymes is the presence of a C-terminal domain in LplA enzymes that is not found in LipB. Recent structures of *E. coli* LplA show that the C-terminal domain undergoes a significant conformational shift associated with the formation of the lipoyl-AMP conjugate (54). This activation step is not necessary in LipB enzymes since the substrate octanoyl groups are already attached to the acyl carrier protein through a thioester bond. Interestingly, the genomes of many organisms in the domain *Archaea* appear to encode the LplA catalytic domain and the C-terminal domain (dubbed LplB) as separate proteins (27). LplB forms a dimer with the catalytic domain, and both are required to produce lipoyl-AMP conjugate (27, 134). However, LplB is not required to transfer the lipoyl group to a protein containing a lipoyl domain (27). Similarly, mammalian lipoate ligase or-

thologs contain an unrelated C-terminal domain and can catalyze the transfer of lipoyl groups only if supplied with the lipoyl conjugate (53). Thus, the LplB domain, whether expressed as an independent protein or fused to the catalytic domain of a lipoate ligase, appears to be required for the formation of lipoyl-AMP.

While the *E. coli* mechanism of lipoate scavenging is simple, multiple variations of this salvage pathway have been identified. Some organisms, such as *L. monocytogenes* and *Plasmodium falciparum*, contain two ligases (3, 107), which may satisfy different requirements within the cell. In mammalian cells, the two-step ligation reaction catalyzed by LplA has been divided among two enzymes and is GTP dependent (Fig. 3D). This process requires a lipoate-activating enzyme (LAE) that activates free lipoate to lipoyl-GMP (57). A second enzyme, known as lipoyl nucleoside monophosphate (NMP) transferase, then transfers lipoate from lipoyl-GMP to the apodomain (55). The mammalian LAE also functions as a xenobiotic-metabolizing/medium-chain acyl-CoA ligase (57) and can partner with the NMP transferase to aberrantly attach lipoate analogs and xenobiotics to the mammalian PDH E2 (240). Although the mammalian NMP transferases are orthologs of *E. coli* LplA, they are incapable of using free lipoate as substrate and present a lipoylation strategy distinct from that of *E. coli* and other microorganisms (55, 56).

Lipoate Cleavage

Only one enzyme, called lipoamidase (Lpa), is known to specifically cleave the lipoamide bond, and it appears to be unique to the Gram-positive bacterium *Enterococcus faecalis*. In the 1950s, while studying the role of lipoic acid in the activation of the *E. coli* and *E. faecalis* PDHs, Reed and co-workers discovered that a partially purified enzyme activity from *E. faecalis* inactivated the complexes and caused the release of free lipoate (188). The Lpa enzyme has only recently been identified and is an 80-kDa protein with an N-terminal amidase domain featuring a characteristic Ser-Ser-Lys catalytic triad and a C-terminal domain of unknown function (99). It cleaves lipoate from α -ketoacid dehydrogenases and lipoic acid amide and ester small molecules, but it has little to no activity on ϵ -N-biotinyl-L-lysine (biocytin), ϵ -N-acetyl-L-lysine, or ϵ -N-benzoyl-L-lysine (224). *In vivo*, lipoylated proteins seem to be specifically targeted by Lpa, since expression of Lpa is toxic only in *E. coli* strains that rely on lipoate metabolism (214a).

Lipoamidase activity has also been observed in some mammalian sources, including human serum and breast milk (10, 62, 94, 159); however, unlike the *E. faecalis* lipoamidase, these amidase activities do not seem to be specific for lipoate. Instead, lipoamidase activity in human serum appears to derive from an enzyme that also cleaves biotin and is known as biotinidase (62), and lipoamidase activity in human milk has been attributed to cholesterol esterase (94). Free lipoate can also be produced in the mammalian gut by nonenzymatic cleavage through acid hydrolysis. This is thought to be the principal route for generating free lipoate in metazoan animals which obtain their lipoic acid requirement from food and intestinal bacteria (55, 56). Animal pathogens are also able to scavenge the free lipoate generated by host digestion (3, 29, 164).

TABLE 1. Lipoylation enzymes in Gram-negative bacteria

Organism	LplA ^a	LipB and LipA ^b
<i>Proteobacteria</i>		
<i>Alphaproteobacteria</i>		
<i>Rickettsia prowazekii</i> Madrid E		LipB, CAA15299; LipA, CAA15170
<i>Rickettsia rickettsii</i> Iowa		LipB, ABY73303; LipA, ABY73086
<i>Betaproteobacteria</i>		
<i>Bordetella pertussis</i> Tohama I	CAE41593	LipB, CAE40485; LipA, CAE40486
<i>Burkholderia pseudomallei</i> 1710b		LipB, ABA50339; LipA, ABA49561
<i>Neisseria gonorrhoeae</i> FA 1090		LipB, AAW89506; LipA, AAW89507
<i>Neisseria meningitidis</i> Z2491		LipB, CAM08552; LipA, CAM08551
<i>Gammaproteobacteria</i>		
<i>Legionella pneumophila</i> Paris		LipB, CAH12620 ^c ; LipA, CAH11958
<i>Pseudomonas aeruginosa</i> PAO1	AAG07674	LipB, AAG07384; LipA, AAG07383
<i>Salmonella enterica</i> serovar Typhimurium LT2	AAL23391	LipB, AAL19586; LipA, AAL19584
<i>Shigella dysenteriae</i> Sd197	ABB64496	LipB, ABB60757; LipA, ABB60755
<i>Yersinia pestis</i> CO92	CAL21041	LipB, CAL21222; LipA, CAL21221
<i>Vibrio cholerae</i> O395	ABQ20166	LipB, ABQ20610; LipA, ABQ19826
<i>Epsilonproteobacteria</i>		
<i>Helicobacter pylori</i> G27		
<i>Chlamydiae</i>		
<i>Chlamydia trachomatis</i> B/Jali20/OT	CAX10956, CAX10734	LipA, CAX11015 ^d

^a LplA paralogs do not form gene clusters and are listed based on homology to *E. coli* LplA.

^b Genes located in clusters are in bold and listed in the order found in the gene cluster.

^c Annotated as part of secretion system gene cluster "secretion system protein X."

^d The *lipA* gene overlaps by four bases with lipoamide dehydrogenase CAX11014. It is located immediately upstream of a type III secretion system gene cluster.

LIPOATE AS AN ANTIOXIDANT

In addition to their role in the catalysis of metabolic reactions, lipoate and dihydrolipoate also have important functions in redox metabolism (reviewed in references 138 and 165). Lipoate is unique among the antioxidants because it confers antioxidant protection in its reduced (dihydrolipoate) and oxidized (lipoate) forms (165). The functions of lipoate as an antioxidant are wide-ranging. Lipoate and dihydrolipoate form a redox couple that effectively quenches a number of harmful free radicals, including hydroxyl radical, peroxy radical, superoxide radical, and singlet oxygen species. Dihydrolipoate acts synergistically with other antioxidants, indicating that it is able to regenerate the active forms of antioxidants such as vitamin C (104), glutathione (101), coenzyme Q₁₀ (255), and vitamin E (203). As lipoate is soluble in both lipids and aqueous solutions, its ability to interact with other antioxidants provides a bridge between membrane-bound antioxidants, such as tocopherol, and cytoplasmic antioxidants, such as glutathione. Thus, lipoate and dihydrolipoate act as antioxidants directly through radical quenching and indirectly by recycling other antioxidants.

BACTERIAL LIPOATE METABOLISM

Gram-Negative Bacteria

Human bacterial pathogens are predominantly Gram negative and are largely found in the phyla *Chlamydiae* and *Proteobacteria*. These bacteria encompass a morphologically diverse array of species and can be obligate intracellular, facultative intracellular, or extracellular pathogens. Similarly, there is a wide variety in the types of metabolism employed by these bacteria, and they can exist as obligate aerobes, faculta-

tive anaerobes, or microaerophiles. Lipoate metabolism in these organisms is similarly diverse, and in subsequent sections we highlight examples from 13 species of *Proteobacteria* and from the *Chlamydiae* species *Chlamydia trachomatis*. The diversity of lipoate metabolism observed among Gram-negative pathogenic bacteria is illustrated by comparison of the *Proteobacteria Helicobacter pylori* and *Pseudomonas aeruginosa*. Proteins related to lipoate metabolism have not been found in *H. pylori*, while the *P. aeruginosa* genome encodes lipoate synthesis and lipoate-scavenging enzymes as well as the components of five lipoylated protein complexes (Tables 1 and 2). Comparison of lipoate metabolism among Gram-negative pathogens may provide insights into bacterial pathogenesis, as the proteins involved in lipoate synthesis and lipoylated proteins themselves have been implicated in the pathogenesis of some species. For example, in *Burkholderia pseudomallei*, the disruption of lipoate metabolism attenuates virulence (172), while in *Pseudomonas aeruginosa*, a lipoylated complex is required for the proper expression of the toxin secretion system (33). These organisms and others are described in the following sections; however, it is important to note that the proteins encoded in the genomes of these organisms are putative unless experimental evidence is described.

Alphaproteobacteria. Among the *Alphaproteobacteria*, the genus *Rickettsia* contains many obligate intracellular human pathogens. Bacteria from the genus *Rickettsia* are the ancestral bacteria of the endosymbiont that became mitochondria (5), and thus, eukaryotic lipoate metabolism shares common roots with these bacteria. Pathogenic *Rickettsia* species can be divided into two groups: the typhus group and the spotted fever group (182). The etiological agents of Rocky Mountain spotted fever (*Rickettsia rickettsii*) and typhus (*Rickettsia prowazekii*) typify these two phylogenetic groups (241). Although *Rickettsia*

TABLE 2. Lipoylated complexes in Gram-negative bacteria^a

Organism	PDH	KDH	BCDH	GCV	AoDH
<i>Proteobacteria</i>					
<i>Alphaproteobacteria</i>					
<i>Rickettsia prowazekii</i> Madrid E	E1α , CAA14723 ^b ; E1β , CAA14724 ^b ; E2, CAA14979 ^b ; E3, CAA14916	E1 , CAA14647; E2 , CAA14646; E3, CAA15231			
<i>Rickettsia rickettsii</i> Iowa	E1α , ABY72310 ^c ; E1β , ABY72311 ^c ; E2, ABY72664 ^b ; E3, ABY72738	E1 , ABY72191; E2 , ABY72189; E3, ABY73180			
<i>Betaproteobacteria</i>					
<i>Bordetella pertussis</i> Tohama I	E1, CAE41419; E1, CAE41294; E2, CAE41295; E3, CAE41296 ^c ; E3, CAE44944	E1 , CAE41422; E2 , CAE41423; E3, CAE41424; E2 ² , CAE44952 ^d	E1α , CAE44955; E1β , CAE44954	T, CAE40574; H, CAE40575; P, CAE40576	
<i>Burkholderia pseudomallei</i> 1710b	E1, ABA52146; E1, ABA50739; E2, ABA47929; E3, ABA47906 ^e	E1 , ABA48382; E2 , ABA49078; E3, ABA48280	E1α , ABA53431; E1β , ABA52725; E2, ABA51980; E3, ABA51982	T, ABA50545; H, ABA47583; P, ABA48266	
<i>Neisseria gonorrhoeae</i> FA 1090	E1 , AAW89298; E2, AAW89297; E3, AAW89295 ^c	E1 , AAW89613; E2, AAW89612; E3, AAW89611		T, AAW90051; H, AAW90049; P, AAW89976	
<i>Neisseria meningitidis</i> Z2491	E1 , CAM08699; E2, CAM08700; E3, CAM08701 ^c	E1 , CAM08355; E2, CAM08356; E3, CAM08357		T, CAM08008; H, CAM08009; P, CAM09047	
<i>Gammaproteobacteria</i>					
<i>Legionella pneumophila</i> Paris	E1 , CAH12612; E2, CAH12611; E3, CAH12610	E1 , CAH11745; E2, CAH11746	E1α , CAH12666; E1β , CAH12667; E2, CAH12668	T, CAH1280; H, CAH11279; P1, CAH11278; P2, CAH11276	E1α , AAG07537; E1β , AAG07538; E2, AAG07539
<i>Pseudomonas aeruginosa</i> PAO1	E1 , AAG08400; E2, AAG08401; E3, AAG08214	E1 , AAG04974; E2, AAG04975; E3, AAG04976	E1α , AAG05635; E1β , AAG05636; E2, AAG05637; E3, AAG05638; <u>E1α, AAG06805^b</u> ; <u>E1β, AAG06804^b</u> ; <u>E2, AAG06803^b</u>	T, AAG08600; H, AAG08599; P, AAG08598	
<i>Salmonella enterica</i> serovar Typhimurium LT2	E1 , AAL19116; E2, AAL19117; E3, AAL19118	E1 , AAL19680; E2, AAL19681		T, AAL21930; H, AAL21929; P, AL21928	
<i>Shigella dysenteriae</i> Sd197	E1 , ABB60378; E2, ABB60379; E3, ABB60380	E1 , ABB60852; E2, ABB60853		T, ABB63188; H, ABB63189; P, ABB63190	
<i>Yersinia pestis</i> CO92	E1 , CAL22008; E2, CAL22007; E3, CAL22006	E1 , CAL19779; E2, CAL19780		T, CAL19574; H, CAL19573; P, CAL19572	
<i>Vibrio cholerae</i> O395	E1 , ABQ21994; E2, ABQ21180; E3, ABQ20695	E1 , ABQ21464; E2, ABQ21540		T, ABO18764; H, ABQ18676; P, ABQ18474	
<i>Epsilonproteobacteria</i>					
<i>Helicobacter pylori</i> G27					
<i>Chlamydiae</i>					
<i>Chlamydia trachomatis</i> B/Jal120/OT	E1α,β , CAX10792 ^{b,e} ; E2, CAX10853 ^b ; E3, CAX11014 ^{b,f}	E1 , CAX10501; E2, CAX10502		H, CAX10751	

^a Genes located in clusters are in bold or underlined and listed in the order found in the gene cluster.^b Significant similarity to PDH and BCDH complexes.^c E3 proteins contain an N-terminal lipoylation domain.^d Contains a significant deletion and may be a pseudogene.^e Bifunctional protein.^f Gene cluster contains LipA.

species have highly reduced genomes (17), they retain a complete TCA cycle, including a KDH complex (5). The KDH E1 and E2 subunits are encoded in tandem in the *R. rickettsii* and *R. prowazekii* genomes, and two putative E3 subunit genes, either of which could function as part of the KDH complex, are located elsewhere (Table 2). The *Rickettsia* genomes also encode a second lipoylated complex composed of E1 α , E1 β , and E2 subunits located together in a gene cluster. These proteins are similar in sequence to BCDH subunits and to PDH subunits from Gram-positive bacteria, which typically contain E1 α and E1 β PDH proteins. Despite the similarity to BCDH proteins, this complex is likely to function as a PDH. Many enzymes responsible for amino acid metabolism, including several required for the degradation of branched-chain amino acids, are not present in *Rickettsia* species (242). Further evidence that these subunits comprise a putative PDH is derived from evidence that rickettsiae, like mitochondria, may acquire pyruvate directly from the host cell cytoplasm and require the PDH to convert it into acetyl-CoA (5, 190). Thus, *Rickettsia* spp. appear to contain KDH and PDH complexes but lack other lipoylated proteins. Although *Rickettsia* spp. are obligate intracellular bacteria, they do not appear to encode a lipoate ligase that would enable them to scavenge lipoate from the host cell. Instead, both *R. rickettsii* and *R. prowazekii* encode orthologs of *E. coli* LipA and LipB, and these bacteria probably rely on lipoate synthesis to activate the KDH and PDH complexes (Table 1).

Betaproteobacteria. The *Betaproteobacteria* contain several obligate aerobes, including *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Bordetella pertussis*, and *Burkholderia pseudomallei*. These organisms are human pathogens and cause meningitis, gonorrhoea, pertussis (whooping cough), and melioidosis (250), respectively. Consistent with their reliance on respiration, the genomes of these bacteria encode subunits of the PDH and KDH complexes (Table 2), and the genes for each complex are generally found together in an operon. Unlike in *E. coli*, an additional dihydrolipoamide dehydrogenase (E3 subunit) is encoded in the KDH operons of these *Betaproteobacteria*. These pathogens also appear to contain the H, P, and T protein GCV subunits, but they lack an independent dihydrolipoamide dehydrogenase L protein. An additional PDH E1 paralog and subunits of the BCDH complex are encoded in the genomes of *B. pertussis* and *B. pseudomallei* but are not found in the *Neisseria* species.

In *B. pseudomallei*, four putative BCDH genes are arrayed in a complete operon encoding the E1 α , E1 β , E2, and E3 subunits. In contrast, only the genes encoding the E1 α and E1 β BCDH subunits are found in tandem in the *B. pertussis* genome. An additional E2 subunit (CAE44952) and an E3 subunit (CAE44944) are encoded elsewhere in the genome and are not associated with other operons (Table 2). These genes were examined to determine whether they might encode the potential missing BCDH subunits. The E2 homolog is most similar to KDH E2 subunits and contains a single lipoyl domain, but it lacks a central region containing the domain responsible for association with E3 subunits. The E3 homolog appears to be complete, but it is a clear paralog of the PDH E3 and is less similar to the BCDH E3 subunits from other organisms. Thus, the incomplete E2 protein and the apparent

absence of BCDH E2 and E3 orthologs in *B. pertussis* may be a product of gene loss and inactivation over the course of the evolution of *Bordetella* species (167).

The PDH E3 paralog (CAE44944) in *B. pertussis* could have another function distinct from participation in lipoylated complexes. In other microbial species, including *Neisseria meningitidis*, *Listeria monocytogenes*, *Streptococcus pneumoniae*, and the protozoan *Trypanosoma brucei*, there is precedent for E3 subunits adopting other roles (21, 35, 149, 200, 210), possibly involving sugar transport at the plasma membrane (210). In the betaproteobacterium *N. meningitidis*, the PDH E3 is associated with the bacterial envelope (4), a location analogous to that observed in *L. monocytogenes* and *T. brucei* (35, 149, 200). The *N. meningitidis* PDH E3 subunit contains an amino-terminal lipoylation domain in addition to the two lipoylation domains found in the PDH E2 subunit (21). The significance of this additional lipoylation domain is unclear, but it is conserved in the PDH E3 subunit of the related human pathogen *Neisseria gonorrhoeae* and plays a regulatory role in certain Gram-positive bacteria (see “Firmicutes” below).

Most pathogenic *Betaproteobacteria* appear to be capable of synthesizing and scavenging lipoate (Table 1). Orthologs of *E. coli* LipA, LipB, and LplA can be found in the genomes of these bacteria, with one exception. The facultative intracellular pathogen *B. pseudomallei* does not encode a LplA ortholog, suggesting that the bacterium is unable to scavenge lipoate and relies on lipoate synthesis (Table 1). The *B. pseudomallei* *lipB* gene was found to play an important role in growth and survival through a transposon-mediated mutagenesis screen (172). Cells with a disrupted *lipB* gene had a reduced ability to form plaques, indicative of impaired intercellular spreading, and showed reduced resistance to hydrogen peroxide. Since *B. pseudomallei* invades phagocytic as well as nonphagocytic cells, lipoylation may be important for regulating oxidative stress during the intracellular life cycle in addition to its roles in intermediate metabolism. In a murine model, the *lipB* disruption strain showed attenuated virulence, suggesting that lipoate metabolism is important for growth and survival *in vivo* (172). Alternatively, *B. pseudomallei* virulence could be affected if LipB acts as a transcriptional regulator, as observed in the LipB-dependent regulation of *E. coli* Dam methylase (235).

Gammaproteobacteria. Numerous human pathogens are found among the *Gammaproteobacteria*, including the causative agents of Legionnaires’ disease (*Legionella pneumophila*), plague (*Yersinia pestis*), cholera (*Vibrio cholerae*), and dysentery (*Shigella dysenteriae*), the opportunistic pathogen *Pseudomonas aeruginosa*, and the food-borne pathogens *Salmonella enterica* and *E. coli*. Lipoylated complexes in the *Gammaproteobacteria* generally resemble those of *E. coli*; however, two species, *P. aeruginosa* and *L. pneumophila*, have diverged substantially. Unlike *E. coli*, these species both encode subunits of the BCDH, and an acetoin dehydrogenase complex is also present in *P. aeruginosa*. The presence of the BCDH in these species reflects nutritional requirements not present in the other *Gammaproteobacteria*. In *L. pneumophila*, BCFAs are the most abundant fatty acid moieties (147). The BCDH is anticipated to generate the primers for branched-chain fatty acid synthesis in this species, as it does in other bacterial species such as *Listeria monocytogenes* (described in “Gram-Positive Bacteria” below), in which branched-chain fatty acids

predominate. In contrast, BCFAs are found in only trace amounts in *P. aeruginosa* (145, 146), and in this species, the role of the BCDH may be to support the full catabolism of valine, isoleucine, and leucine to TCA cycle intermediates, such as acetyl-CoA and succinyl-CoA. Indeed, the genes encoding branched-chain acyl-CoA dehydrogenases which are required for the further catabolism of branched-chain amino acids can be easily identified in the *P. aeruginosa* genome but not in the *L. monocytogenes* genome.

P. aeruginosa has evolved an unusual mechanism to regulate the activities of its five lipoylated complexes, the PDH, KDH, BCDH, GCV, and acetoin dehydrogenase (Table 2), through the expression of four distinct lipoamide dehydrogenases. Unlike most *Gammaproteobacteria*, which use an E3 subunit common to all lipoylated complexes, pseudomonads express different E3 proteins according to nutrient levels in the cell. Expression of the BCDH E3 subunit LPD-Val is upregulated by valine; likewise, expression of the putative PDH and KDH E3 subunit and GCV L protein, called LPD-Glc, is upregulated by glucose (213, 214). The roles of the two remaining lipoamide dehydrogenases have not been experimentally determined. One of these, called LPD-3, can replace LPD-Glc to generate a functional PDH (25), and, given the similarity between the PDH and acetoin dehydrogenase, it may have a physiological role in the latter complex.

As an opportunistic pathogen, *P. aeruginosa* infects multiple environments within the human host. In immunocompromised individuals, it can cause fatal infections of the lungs, urinary tract, and burn wounds. One of the major virulence determinants of *P. aeruginosa* is a type III secretion system (T3SS), which injects at least four bacterial effector proteins into host cells (44). A transposon-mediated mutagenesis study designed to reveal genes important to the expression of this system identified subunits of the PDH. Mutations in the genes *aceA* and *aceB*, which encode the PDH E1 and E2 subunits, substantially decreased the expression of the T3SS in an *in vitro* culture system (33). These PDH mutant *P. aeruginosa* strains were also avirulent in rats, in contrast with wild-type bacteria that produced lethal pulmonary infections (33).

It was originally proposed that the PDH mediates T3SS expression by acting directly as a transcriptional activator (33), as observed in some members of the genus *Bacillus* (described in "Gram-Positive Bacteria" below) (247). Later studies, however, supported the notion that the metabolic state of the cell has an effect on the expression of the T3SS in *P. aeruginosa* (194). When *aceA* is deleted, induction of the T3SS is abolished; in contrast, when cells are genetically manipulated to accumulate acetyl-CoA through deletion of the citrate synthase gene, induction is enhanced (193). Supplementation with acetate, however, does not restore expression of the T3SS in *aceA* and *aceB* mutant cell lines (33), perhaps due to poor conversion of acetate to acetyl-CoA. Thus, it appears that acetyl-CoA, or a molecule derived from acetyl-CoA, promotes expression of the T3SS (193), linking the activity of PDH to pathogenesis in *P. aeruginosa*.

The link between lipoate metabolism and toxin secretion may also be present in other *Gammaproteobacteria*. In *L. pneumophila*, the lipoate synthesis genes do not occur in the same gene cluster. Instead, the octanoyl transferase is annotated as

secretion system protein X and is part of the secretion system I gene cluster (Table 1).

Epsilonproteobacteria. The *Epsilonproteobacteria* predominantly colonize the digestive tract either as symbionts or pathogens and include species from the genera *Helicobacter* and *Campylobacter*. The microaerophilic epsilonproteobacterium *H. pylori* is one of the few bacterial species that does not encode any lipoylated complexes or enzymes involved in lipoylation. This species does maintain an active TCA cycle (86) but employs anaerobic or microaerophilic alternatives to certain TCA cycle enzymes such as KDH (105). The anaerobic enzyme α -ketoglutarate oxidoreductase (KOR) generates succinyl-CoA in *H. pylori* (93, 232). Similarly, acetyl-CoA is produced by pyruvate:flavodoxin oxidoreductase (POR) instead of PDH (92). The POR enzyme is also found in anaerobic protozoans (152, 234) with minimal or absent lipoate metabolism, including *Trichomonas vaginalis*, *Giardia lamblia*, *Entamoeba histolytica*, and *Cryptosporidium parvum* (see Protozoan Lipoate Metabolism below).

Chlamydiae. *Chlamydia trachomatis*, which causes the eye disease trachoma and the sexually transmitted infection chlamydia, is one of three *Chlamydia* species which commonly cause infection in humans (*C. pneumoniae* and *C. psittaci* also infect humans and can cause pneumonia and influenza-like illnesses) (11). *C. trachomatis* is an obligate intracellular pathogen and is similar in this respect to the *Rickettsia Alphaproteobacteria* described above. Although *C. trachomatis* and *R. prowazekii* are not phylogenetically related, the contents of their genomes are surprisingly similar, perhaps due to the convergent evolution of both obligate intracellular pathogens (263). Both bacteria contain PDH gene clusters encoding the E1 α , E1 β , and E2 subunits, similar to those found in Gram-positive bacteria (Table 2). Both organisms also encode the KDH E1 and E2 subunits in tandem. Unlike *Rickettsia* species, *C. trachomatis* appears to contain a BCDH complex; an unusual feature of this complex is the fusion of the E1 α and E1 β subunits into a single protein (CAX10792). *C. trachomatis* encodes a single E3 subunit, which may function with the PDH, KDH, and BCDH complexes. The E3 gene overlaps with the lipoate synthase gene, perhaps linking lipoate synthesis with the activity of the three lipoylated complexes in *C. trachomatis*.

The genome of *C. trachomatis* appears to have lost all of the GCV components except for the H protein. This may be the result of extensive gene loss in the highly reduced *C. trachomatis* genome (220). Alternatively, the H protein could have another metabolic role in this organism, as observed in the fungus *Saccharomyces cerevisiae* (see Fungal Lipoate Metabolism below). Several pathogens, including the Gram-positive bacteria *Enterococcus faecalis* and *Streptococcus pyogenes* and the protozoans *Plasmodium falciparum*, *Toxoplasma gondii*, and *Trichomonas vaginalis*, appear to have an incomplete GCV, but in these cases, the H protein is always retained (see "Gram-Positive Bacteria" and Protozoan Lipoate Metabolism below).

Gram-Positive Bacteria

Gram-positive bacteria encompass two phyla, *Actinobacteria* and *Firmicutes*. *Firmicutes* generally have genomes with low GC content but are otherwise highly diverse. The *Firmicutes*

TABLE 3. Lipoylation enzymes in Gram-positive bacteria

Organism	LplA ^a	LipB and LipA ^b
<i>Actinobacteria</i>		
<i>Corynebacterium diphtheriae</i> NCTC13129		LipB , CAE50168 ^c ; LipA , CAE50169
<i>Mycobacterium tuberculosis</i> H37Rv		LipB , CAA94273 ^c ; LipA , CAA94258
<i>Mycobacterium leprae</i> TN		LipB , CAC31240 ^c ; LipA , CAC31239
<i>Firmicutes</i>		
<i>Bacilli</i>		
<i>Bacillales</i>		
<i>Bacillus anthracis</i> Ames	AAP25068, AAP28145, AAP29271 ^d	LipA, AAP28874
<i>Listeria monocytogenes</i> EGD-e	CAC99009, CAC98842	
<i>Staphylococcus aureus</i> MSSA476	CAG42736, CAG42075, CAG43266, CAG42323 ^d	LipA, CAG42570
<i>Lactobacillales</i>		
<i>Enterococcus faecalis</i> V583	AAO82441, AAO80474	
<i>Streptococcus pneumoniae</i> R6	AAK99851 ^e	
<i>Streptococcus pyogenes</i> Manfredo	CAM30328 ^e , CAM30198 ^f	
<i>Clostridia</i>		
<i>Clostridium botulinum</i> A strain Hall	ABS36464 ^e , ABS36694	
<i>Clostridium difficile</i> 630	CAJ68519 ^f , CAJ66860 ^e , CAJ67567 ^f	

^a LplA paralogs do not form gene clusters and are listed based on homology to *E. coli* LplA.

^b Genes located in clusters are in bold and listed in the order found in the gene cluster.

^c Located downstream of PDH E2 gene *dlpA*.

^d Highly divergent LplA paralogs that may have LipB activity and function in conjunction with LipA.

^e Located downstream of the AoDH gene cluster.

^f Located near GCV genes.

are further subdivided into the classes *Clostridia*, which contains anaerobic species; *Bacilli*, which is composed of anaerobes and facultative anaerobes; and *Mollicutes*, which contains species that lack cell walls and includes the genus *Mycoplasma*. Five *Firmicutes* genera include species that are pathogenic in humans; they are the *Clostridia* genus *Clostridium* and the *Bacilli* genera *Bacillus*, *Listeria*, *Staphylococcus*, and *Streptococcus*. In contrast to *Firmicutes*, *Actinobacteria* have GC-rich genomes and are predominantly aerobes. Among the *Actinobacteria*, the genera *Mycobacterium* and *Corynebacterium* contain human pathogens.

Lipoate metabolism in the *Actinobacteria* more closely resembles that in some pathogenic Gram-negative bacteria than that in the *Firmicutes*. *Actinobacteria* encode enzymes for lipoate synthesis in a gene cluster, similarly to most Gram-negative bacteria. Also, like many Gram-negative intracellular bacteria, the *Actinobacteria* do not seem to contain a lipoate ligase and thus appear to be unable to salvage lipoate from the host cell. In contrast, *Firmicutes* species encode at least one and in most cases multiple lipoate ligases, but they lack genes for a complete lipoate synthesis pathway (Table 3). The *Firmicutes* *Bacillus anthracis* and *Staphylococcus aureus* do encode lipoate synthase orthologs; however, an accompanying octanoyl transferase is not evident in either species. A second major difference among the Gram-positive phyla is observed in the structures of the PDH E1 subunit and the P protein of the GCV. Like most Gram-negative bacteria, the *Actinobacteria* express the E1 α and - β subunits as a single polypeptide from one structural gene; however, they diverge by encoding the PDH E1, E2, and E3 subunits in widely spaced genes instead of in a gene cluster. In contrast, *Firmicutes* encode the E1 α and - β subunits of the PDH as two genes, similar to the case for eukaryotes (Table 4). *Firmicutes* also express the P protein of the GCV, which is analogous to the E1 subunit of the α -keto-

acid dehydrogenase complexes, as two polypeptides, denoted P1 and P2.

Actinobacteria. *Corynebacterium diphtheriae*, *Mycobacterium tuberculosis*, and *Mycobacterium leprae* are intracellular, aerobic bacteria. They do not appear to encode lipoate ligases and are presumed to depend on lipoate synthesis, similar to the case for the intracellular Gram-negative species *B. pseudomallei*, *L. pneumophila*, *N. gonorrhoeae*, and *N. meningitidis*. The *Actinobacteria* also resemble some of these Gram-negative species by encoding the PDH E1 subunit and the P protein of the GCV as a single polypeptide; however, they diverge through their lack of a KDH (229) (Table 4).

Experimental evidence on the existence and activities of lipoylated complexes in *M. tuberculosis* highlights the difficulty in predicting organismal metabolism from genomic data. *M. tuberculosis* is predicted to encode the E1 α (*pdhA* [CAB08930]), E1 β (*pdhB* [CAB08929]), and E2 (*pdhC* [CAB08928]) subunits of the PDH in an operon, plus an additional PDH E1 subunit (*aceE* [CAA94662]), the KDH E1 and E2 subunits (*sucA* [CAA15904] and *sucB* [CAA94256]), the P, T, and H proteins of a GCV, and three lipoamide dehydrogenase homologs (*lpdA* [CAA17075], *lpdB* [CAE55324], and *lpdC* [CAA17417]). Although from these assignments, *M. tuberculosis* is predicted to contain three lipoylated proteins, only the protein product of *sucB* (CAA94256) has been detected (24). Subsequent studies have shown that this protein forms a functional PDH complex with AceE and LpdC and that the *sucB* gene product has dihydrolipoamide acetyltransferase, not dihydrolipoamide succinyltransferase, activity (229). As such, it has been renamed DlpT. The putative KDH E1 subunit, SucA (CAA15904), has homology to both the KDH E1 and E2 subunits but is an α -ketoglutarate decarboxylase (228). This enzyme partners with a succinic semialdehyde dehydrogenase to form a metabolic route from α -ketoglutarate to succinate.

TABLE 4. Lipoylated complexes in Gram-positive bacteria^a

Organism	PDH	KDH	BCDH	GCV	AoDH
<i>Actinobacteria</i>					
<i>Corynebacterium diphtheriae</i> NCTC13129	E1, CAE50216; E2, CAE50166 ^b ; E3, CAE48873	E1, CAE49520 ^c ; E2, CAE49520 ^c E1, CAA15904 ^c ; E2, CAA15904 ^c		T, CAA94254; H, CAB01475; P, CAB01470	
<i>Mycobacterium tuberculosis</i> H37Rv	E1, CAB08930; E1B, CAB08929; E2, CAB08928; E1, CAA94662; E2, CAA94256 ^{d,e} ; E3, CAA17417 ^d	E1, CAC31476 ^c ; E2, CAC31476 ^c		T, CAC31246; H, CAC31032; P, CAC31027	
<i>Mycobacterium leprae</i> TN	E1, CAC30602; E2, CAC31242 ^b ; E3, CAC31903				
<i>Firmicutes</i>					
<i>Bacilli</i>					
<i>Bacillus anthracis</i> Ames	E1 α , AAP27907; E1B, AAP27906; E2, AAP27905; E3, AAP27904	E1, AAP25228; E2, AAP25227	E3, AAP28101; E1 α , AAP28100; E1B, AAP28099; E2, AAP28098	T, AAP28163; P1, AAP28162; P2, AAP28161; H, AAP28894	E1 α , AAP26611; E1B, AAP26610; E2, AAP26609; E3, AAP26608
<i>Listeria monocytogenes</i> EGD-e	E1 α , CAC99130; E1B, CAC99131; E2, CAC99132; E3, CAC99133		E3, CAC99449; E1 α , CAC99450; E1B, CAC99451; E2, CAC99452	T, CAC99426; P1, CAC99427; P2, CAC99428; H, CAD00503	
<i>Staphylococcus aureus</i> MSSA476	E1 α , CAG42802; E1B, CAG42803; E2, CAG42804; E3, CAG42805	E1, CAG43131; E2, CAG43130	E3, CAG43242; E1 α , CAG43241; E1B, CAG43239; E2, CAG43237	T, CAG43270; P1, CAG43269; P2, CAG43268; H, CAG42548; H, CAG42072	
<i>Lactobacillales</i>					
<i>Enterococcus faecalis</i> V583	E1 α , AAO81144; E1B, AAO81145; E2, AAO81146; E3, AAO81147		E3, AAO81439; E1 α , AAO81438; E1B, AAO81437; E2, AAO81436	H, AAO82216	E1 α , AAK99855; E1B, AAK99854; E2, AAK99853 ^f ; E3, AAK99852 ^{f,g} ; E1 α , CAM30335; E1B, CAM30334; E2, CAM30333; E3, CAM30332 ^{f,g}
<i>Streptococcus pneumoniae</i> R6				H, CAM30195 ^c	
<i>Streptococcus pyogenes</i> Manfredo					
<i>Clostridia</i>					
<i>Clostridium botulinum</i> A strain Hall				T, ABS36011; H, ABS38194; P1, ABS35894; P2, ABS38702; L, ABS36030 ^f	E1 α , ABS38403; E1B, ABS39150; E2, ABS36854; E3, ABS39191 ^c
<i>Clostridium difficile</i> 630				T, CAJ68522 ^{d,e} ; P1, CAJ68522 ^b ; P2, CAJ68523; L, CAJ67557; H, CAJ67563 ^e	E1 α , CAJ66851; E1B, CAJ66850; E2, CAJ66852 ^f ; E3, CAJ66853 ^{f,g}

^a Genes located in clusters are in bold and listed in the order found in the gene cluster.^b LipB and LipA are encoded near this gene cluster.^c A bifunctional protein which lacks a lipoylation domain and has been shown to be α -ketoglutarate decarboxylase (228).^d *M. tuberculosis* PDH E2 (DlaT) and E3 (LpdC) have been shown to function as PDH components (7, 229). Two other potential E3 proteins (LpdA and LpdB) have other roles (8).^e LpdA is encoded near this gene cluster.^f The E2 protein does not contain a lipoylation domain.^g A lipoylation domain is found at the amino terminus of the E3 protein.^h A bifunctional protein.

C. diphtheriae and *M. leprae* each carry a *sucA* gene that is syntenic to the *M. tuberculosis* gene, indicating that the absence of the KDH and presence of an α -ketoglutarate decarboxylase is conserved in *Actinobacteria*.

The function of the putative *M. tuberculosis* PDH operon is mysterious; it does not produce a lipoylated protein detectable in whole-cell lysates or a functional PDH in assays of recombinant proteins (229). Similar gene clusters are notably absent from *C. diphtheriae* and *M. leprae* (Table 4). One hypothesis is that the genes may play a role in bacterial persistence *in vivo*, as *pdhA*, *pdhB*, and *pdhC* are upregulated under a nutrient starvation model of persistence (14).

Genomic predictions of three lipoamide dehydrogenase genes in *M. tuberculosis* are also misleading; among these paralogs, only LpdC, found in the functional PDH complex, is active (7, 8). This contrasts with other bacterial species that have multiple E3 paralogs, such as *P. aeruginosa*, in which each putative E3 gene encodes an active enzyme that functions in specific lipoylated complexes. In these species, the E3 paralogs frequently cluster with other subunits of the lipoylated complex to which they belong (Table 4); in contrast, the *M. tuberculosis* LpdC does not cluster with any PDH subunit genes. This lack of clustering is also common to *C. diphtheriae* and *M. leprae*, which each have a single E3 ortholog, and likely reflects the dispersion of the PDH subunits through the genome in *Actinobacteria*.

Despite having a single lipoylated protein, *M. tuberculosis* has two lipoylated complexes, as DlaT and LpdC are also components of an unusual antioxidant defense complex (24). *M. tuberculosis* persists in host macrophages in the lung alveoli and must employ multiple antioxidant strategies in response to the abundant reactive nitrogen and oxygen species in its environment. DlaT and LpdC function in a NADH-dependent peroxidase and peroxynitrite reductase (NPPR) complex, which also contains the peroxiredoxin alkylhydroperoxide reductase, AhpC, and an adaptor protein with a thioredoxin-like active site, called AhpD (24). In this complex, peroxide and peroxynitrite substrates are reduced by AhpC, which is regenerated by oxidation of AhpD. AhpD is reduced through oxidation of dihydrolipoamide bound to DlaT, and dihydrolipoamide is regenerated in a NADH-dependent reaction by LpdC (24). This oxidation of NADH contrasts with the situation for α -ketoacid dehydrogenase complexes, including the functional *M. tuberculosis* PDH (229), in which NAD^+ is reduced by LpdC to regenerate lipoamide.

Disruption of *dlaT* (Δ *dlaT*) renders both the NPPR and PDH complexes inactive and thus affects both oxidative stress and intermediate metabolism. *In vitro*, *M. tuberculosis* Δ *dlaT* mutants show increased susceptibility to nitrosative stress and macrophage killing and are unable to grow on glucose and glycerol (208). *In vivo*, Δ *dlaT* bacteria persist but do not cause severe pathology. This differential effect, in which disruption of *dlaT* causes less severe effects *in vivo* than *in vitro*, is likely due to a metabolic shift in which fatty acids replace sugars as the major carbon and energy source while the bacteria are growing in the mammalian host (133).

Firmicutes. Lipoate metabolism in the pathogenic *Firmicutes* differs between the *Clostridia* and *Bacilli* classes. Within *Bacilli*, metabolism is further differentiated between the order *Bacillales* (*B. anthracis*, *L. monocytogenes*, and *Staphylococcus aureus*) and lactic acid bacteria belonging to the order *Lactoba-*

cillales (*Streptococcus pneumoniae*, *Streptococcus pyogenes*, and the reference species *Enterococcus faecalis*). *Clostridia* and *Lactobacillales* species share an anaerobic, extracellular lifestyle and a similar strategy for the acquisition of lipoate. According to genomic predictions, they are exclusively lipoate scavengers that encode a single lipoate ligase. In contrast, among *Bacillales*, all species encode multiple lipoate ligase homologs, and *B. anthracis* and *S. aureus* also encode a lipoate synthase. Despite the presence of the lipoate synthase gene in these species, neither appears to contain an octanoyl transferase ortholog (Table 3). It is possible that among the many putative ligases in these species, which include some gene products that are highly divergent from *E. coli* LplA, one may function as an octanoyl transferase. As noted in the introduction, lipoate ligases and octanoyl transferases are structurally related and share the same three-dimensional protein fold. Alternatively, a novel, unidentified transferase may complete the lipoate synthesis pathway in these organisms.

Although *Clostridium* species are similar to the *Lactobacillales* with respect to lipoylating enzymes, they diverge in the conservation of lipoylated complexes. *Clostridium botulinum* and *Clostridium difficile* each encode two lipoylated complexes, an acetoin dehydrogenase and a GCV. Notably, the composition of the GCV varies between these species. *C. difficile* encodes a highly unusual bifunctional protein with homology to the P1 protein subunit and the T protein that occurs in a gene cluster with the P2 protein subunit. The H protein and L protein are encoded elsewhere (Table 4). In contrast, *C. botulinum* features a GCV with a two-subunit P protein, H protein, T protein, and L protein all encoded on separate structural genes; unlike in other bacterial species, the L protein is encoded in the same gene cluster as other GCV subunits. The *Lactobacillales* *S. pneumoniae* and *S. pyogenes* differ from *Clostridia* in that they maintain an acetoin dehydrogenase but appear to lack other lipoylated complexes. In *S. pyogenes* and the model organism *E. faecalis*, the H protein and L protein of the GCV are also present (Table 4). Despite these differences, in both *Streptococcus* and *Clostridium* LplA orthologs are encoded proximally to genes and gene clusters encoding lipoylated complex subunits. Such positioning may reflect the evolution of metabolic controls in which each lipoate ligase lipoylates a particular complex. This specificity may also be present in *P. falciparum* (see "Apicomplexans" below), but it is not always the case in all organisms with multiple lipoate ligases. For example, in *L. monocytogenes*, the two lipoate ligases use different small-molecule substrates (107).

The presence of the H and L proteins and the apparent absence of the P and/or T proteins observed in *E. faecalis* and *S. pyogenes* is the same arrangement observed in the Gram-negative bacterium *C. trachomatis*, as well as in the protozoan parasites *P. falciparum*, *T. gondii*, and *T. vaginalis*. The presence of a lipoate ligase gene near the H protein gene in *S. pyogenes* (46) further suggests that the H protein is lipoylated and has a biological function as a lipoylated protein (Table 4). This repeated, cross-domain pattern, in which the H protein and E3/L protein are retained in the absence of other GCV subunits, suggests that these proteins may have a conserved but yet-undiscovered role distinct from their canonical participation in the GCV.

The single dihydrolipoamide acyltransferase (E2) encoded

in *S. pneumoniae* diverges from the conventional E2 paradigm due to the absence of a lipoyl domain (226). This domain is instead found at the N terminus of the dihydrolipoamide dehydrogenase (DLDH) (210), where it appears to regulate the activity of this enzyme (75). DLDH enzymes with N-terminal lipoyl domains are also found in several other species (Tables 2 and 4), including *Clostridium magnum* (115), *C. difficile*, *C. botulinum*, *S. pyogenes*, *N. gonorrhoeae*, and *N. meningitidis* (210). Notably, in the four latter species, the lipoyl domain on the acyltransferase is also present; however, as observed in *S. pneumoniae*, it is absent from *C. difficile* and *C. magnum*. It thus appears that the lipoyl-E3 domain is conserved in the *Streptococcus*, *Clostridium*, and *Neisseria* genera; however, whether this addition has evolved to replace the lipoyl-E2 varies by species.

In *S. pneumoniae*, no α -ketoacid or acetoin dehydrogenase activity was detected in bacterial lysates (210), indicating that the acetoin dehydrogenase may not be active. Active DLDH, however, is required for infection and pathogenesis *in vivo*. Mutational inactivation of the *S. pneumoniae* DLDH gene did not alter *in vitro* growth but produced bacteria that were avirulent in mouse models of sepsis and lung infection, indicating that the DLDH in *S. pneumoniae* is required for proliferation in the mammalian host (210). In bacteria lacking DLDH, transport of galactose and α -galactoside metabolism were severely compromised, and production of the capsular polysaccharide was reduced by 50% (210). This evidence demonstrates that while a lipoylated complex may be dispensable, the activities of some of its component subunits may not be.

The *Lactobacillales* have a reduced lipoate metabolism compared to *Bacillales* species, which usually contain genes encoding the PDH, KDH, BCDH, and GCV (Table 4). There are some exceptions: the KDH is absent from *L. monocytogenes*, and *Bacillus anthracis* encodes a fifth lipoylated complex, the acetoin dehydrogenase. In both *Bacillales* and *Lactobacillales* there are examples of lipoylation complex proteins executing roles distinct from their participation in metabolic complexes. As described above, the *S. pneumoniae* DLDH appears to be involved in carbohydrate metabolism. In *Bacillus* species, lipoylation complex proteins can play regulatory roles, including possible roles in sporulation.

In the model organism *B. subtilis*, the E2 subunit was first identified as a repressor of DNA replication that bound near the origin (218), and the PDH E2 subunit of the insect pathogen *Bacillus thuringiensis* binds DNA to regulate expression of prototoxin genes during sporulation (247). Efforts to understand genes important for sporulation in *B. subtilis* revealed that subunits of the PDH and KDH complexes are required for this process (47, 52). *Bacillus subtilis* contains the PDH E1 α , E1 β , E2, and E3 subunits (encoded by *pdhA*, *pdhB*, *pdhC*, and *pdhD*, respectively) (157). *pdhA* is refractory to disruption and thus appears to be essential for cell growth, while disruptions of the individual genes *pdhB*, *pdhC*, and *pdhD* all result in sporulation defects (60). Importantly, disruption of *pdhB* and *pdhC* causes sporulation arrest at an earlier stage of sporulation than disruption of *pdhD*, which indicates that the PDH subunits are likely to play a regulatory role in sporulation independent of complex activity (60). Increases in the levels of soluble PDH E1 β and E2 subunits are observed in metabolically exhausted cultures about to sporulate; this suggests that the

complex dissociates when its catalytic substrate is exhausted, leaving the subunits free to bind their regulatory substrates (60). Upregulated expression of *pdhC* and *pdhD* also may occur during sporulation, since two putative binding sites for the sporulation protein Spo0A (required for the expression of some proteins during sporulation) are located in the promoter region of *pdhC* (60). Whether the PDH also plays a regulatory role during the sporulation of *B. anthracis*, which produces spores infective to humans, has not been explored experimentally.

L. monocytogenes is a non-spore-forming member of the order *Bacillales* that is an intracellular, facultative anaerobe. *L. monocytogenes* is a lipoate auxotroph (249) and, like other pathogenic *Bacillales* species, contains multiple lipoate ligase paralogs (63). In *L. monocytogenes*, the two lipoate ligase paralogs, called LplA1 and LplA2, are nonredundant and show adaptation to the bacterium's intracellular lifestyle (164). LplA1 is required for intracellular growth (164) and can use host-derived lipoyl-peptides as substrates (107); in contrast, LplA2 utilizes only free lipoate as a substrate and is dispensable for intracellular growth (107). The ability of LplA1 to use host-derived lipoyl-peptides represents an adaptation to the limited quantities of free lipoate in the host environment. Despite the clear ability of LplA1 to use lipoyl-peptides as substrates, the mechanism by which lipoate is transferred from host-derived lipoyl-peptides to bacterial proteins is unknown (107). This activity involves the cleavage of an amide bond and is therefore expected to proceed through an enzymatic mechanism different from attachment of free lipoate. Thus, LplA1 is anticipated either to have a novel reaction mechanism or to pair with an as-yet-unidentified partner protein to cleave lipoate from host peptides. The *Bacillales* *S. aureus* and *B. anthracis* also have multiple lipoate ligase paralogs; whether these have discrete physiological roles like the *L. monocytogenes* ligases has not been explored.

Disruption of both *L. monocytogenes* lipoate ligases impairs *in vivo* replication but has only a modest effect on bacterial growth in rich medium (106). Growth in minimal medium requires the addition of amino acids and branched-chain fatty acids (BCFAs); however, only BCFAs are needed to restore growth in host fibroblast cells (106). These results suggest that the BCDH has a definitive role in BCFA synthesis during intracellular growth, while the metabolites produced by the PDH and GCV can be scavenged from the host cell (106). Over 95% of the *L. monocytogenes* membrane fatty acids are branched-chain moieties (6), and synthesis of BCFAs is supported by the BCDH, which generates branched-chain fatty acid precursors. The BCDH generates three acyl-CoA moieties; however, in *L. monocytogenes* it is important only for generation of 2-methylbutyryl-CoA (106), which is used in the synthesis of BCFAs with odd-numbered chain lengths. Although *L. monocytogenes* can replicate in rich broth medium in the absence of BCDH activity, in the more resource-constrained intracellular environment, the requirement for the BCDH is acute (106). Taken together, studies of *L. monocytogenes* and *S. pneumoniae* highlight a pattern in which proteins involved in lipoate metabolism are frequently dispensable for *in vitro* culture in nutrient-rich medium but are required for virulent infection *in vivo* in the more resource-constrained host environment.

There is increasing evidence that lipoylated proteins can be secreted from *L. monocytogenes* (15). Since lipoate metabolism

is required for *in vivo* replication, it is unclear what roles these proteins play in bacterial pathogenesis. The GCV H protein, as well as all four subunits of the PDH, have been observed to be associated with the surface of the bacterium (149, 200). This localization is reminiscent of that observed for the E3 proteins of *N. meningitidis*, and *T. brucei* (21, 35). The H protein (231) and the PDH E2 (118) have independently been identified as secreted proteins in *L. monocytogenes*. In the case of the PDH E2, an auxiliary protein secretion system, SecA2, was shown to be responsible for secretion of lipoylated E2 protein (118). Several other Gram-positive pathogenic bacteria have a SecA2 system and secrete bacterial proteins which may have “moonlighting” roles in bacterial pathogenesis that are unrelated to their canonical metabolic roles (195).

FUNGAL LIPOATE METABOLISM

Lipoate metabolism in fungi is linked to cellular metabolism through mechanisms not observed in other organisms. These feedback mechanisms have been best studied in the nonpathogenic yeast *Saccharomyces cerevisiae* (84); however, similarities between this species and pathogenic yeasts, such as *Candida albicans*, suggest that some of these features may be conserved. *Candida albicans* is typical of pathogenic fungi in that it is highly prevalent both in the environment and in the human population but does not normally cause systemic fungemia in humans unless they are immunocompromised. Thus, AIDS sufferers and patients undergoing cancer chemotherapy or organ transplantation are most at risk of developing candidiasis. Similarly, other pathogenic fungi such as those from the genera *Aspergillus*, *Cryptococcus*, *Histoplasma*, and *Pneumocystis* are also environmentally prevalent opportunistic pathogens afflicting those with a weakened immune system. Genetic similarity between these organisms raises the possibility that they could share similar mechanisms of lipoylation and regulation.

Mechanisms of Lipoylation

In yeast, the acquisition and use of lipoate appears to be confined to the mitochondria. Lipoate-dependent enzymes, as well as lipoylation pathway enzymes, are all found in this organelle. Yeasts contain three lipoylated proteins: the E2 subunits of the PDH (158) and KDH (192) and the H protein of the GCV (153). In *S. cerevisiae*, the mitochondrial type II fatty acid biosynthesis pathway produces octanoyl-ACP for the *de novo* synthesis of lipoate (204). As observed in *E. coli*, an octanoyl transferase (LipB) should be necessary for transfer of the octanoyl group to target proteins, followed by generation of lipoate via insertion of sulfur atoms by a lipoate synthase (LipA). Yeasts contain orthologs of LipB (designated Lip2) and LipA (designated Lip5), and both enzymes are required for lipoate synthesis (222) and PDH activity (127) in *S. cerevisiae*. Yeasts also contain an ortholog of the *E. coli* lipoate ligase LplA (designated Lip3); however, this enzyme does not seem to confer the ability to scavenge free lipoate. When lipoate synthesis is blocked by disrupting *lip2* or *lip5*, yeast cannot grow in medium containing lipoate (127, 222). Taken together, these results demonstrate that yeasts acquire lipoate solely through *de novo* synthesis despite the presence of a putative lipoate ligase (Lip3).

Recent studies show that Lip3 plays a role in the lipoate synthesis pathway. Deletion of Lip3 results in lipoylation of only the H protein of the GCV and loss of any detectable PDH and KDH activity (205). Deletion of the H protein disrupts all protein lipoylation in *S. cerevisiae*, indicating that the H protein plays an unusual role in protein lipoylation in addition to its known role in the glycine cleavage complex (the H protein is required when glycine is the sole carbon source [153]). Mutation of the lipoate attachment site on the H protein (K120) also interferes with PDH and KDH lipoylation, but this phenomenon is not observed when other components of the GCV are deleted (205). Thus, Lip2 and Lip5 are sufficient to lipoylate the H protein, but Lip3 and the lipoyl-H protein are required to lipoylate other mitochondrial enzymes. This dependence could be explained by activation of the Lip3 enzyme through binding to the lipoyl-H protein or by the direct transfer of lipoyl moieties from the H protein to other lipoate-dependent proteins. In either case, it is attractive to hypothesize that Lip3, the H protein, and perhaps Lip2 and Lip5 could form a lipoylation complex in yeast (205). Indeed, tandem affinity purification studies identified the H protein as a binding partner for Lip2 in *S. cerevisiae* (114).

Metabolic Regulation

The unusual lipoylation pathway in yeast could provide a mechanism for regulating certain aspects of mitochondrial metabolism. Acetyl-CoA is a key metabolite that is closely linked to the synthesis and use of lipoate. Acetyl-CoA is synthesized by the lipoate-dependent enzyme PDH (179) and serves as the primary carbon source for the synthesis of octanoate by the type II fatty acid synthase (23, 238). Octanoate provides the carbon chain for lipoate synthesis (23, 238) and generation of lipoyl proteins, including the holo-PDH (204). Under conditions of limiting nutrients, low levels of acetyl-CoA could translate into low levels of octanoate synthesis and, consequently, reduced H protein lipoylation. This would result in limited activation of the PDH and KDH. In this scenario, the H protein acts as a metabolic sensor providing a level of control over cellular respiration. Under nutrient-rich conditions, acetyl-CoA derived from a variety of sources, including amino acid catabolism (80), the PDH bypass pathway (19), and the beta-oxidation of fatty acids (16), supports the synthesis of lipoate and the activation of the PDH and KDH (Fig. 4).

Cellular respiration may not be the only pathway affected by lipoate metabolism. Sulo and Martin demonstrated that mitochondrial tRNA processing is perturbed in a *lip5* mutant strain of *S. cerevisiae* (222). Recent studies show that deletion of genes encoding the proteins of type II fatty acid biosynthesis also affects the processing of several mitochondrial RNAs (204). Although the details are not yet clear, a product of fatty acid biosynthesis appears to be necessary for the activation of RNase P (204). Mitochondrial genes are often expressed from multigenic transcripts that are processed by the sequential action of specific endonucleases and exonucleases to produce mature RNAs (42, 142). In yeast, RNase P is an endonuclease composed of a protein called Rpm2, which is encoded in the nuclear genome (140), and an RNA called *RPM1*, which is encoded in the mitochondrial genome (233). The production of mature *RPM1* RNA requires the endonuclease activity of

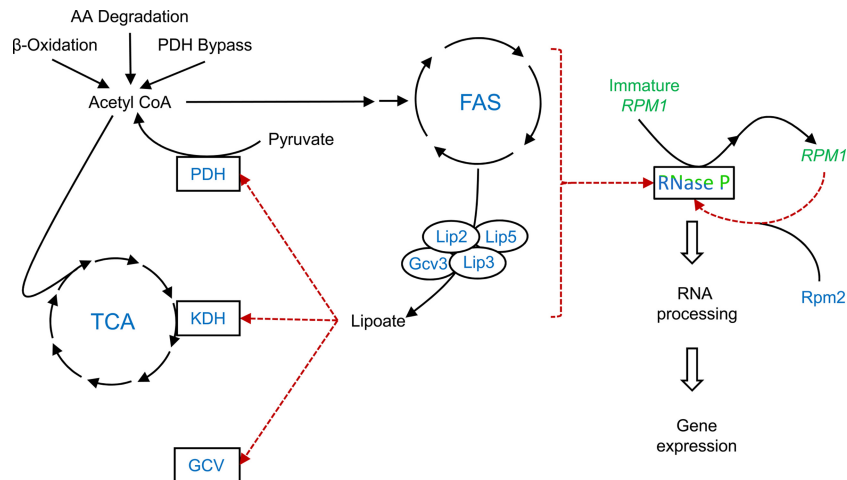


FIG. 4. Metabolic feedback loops affecting gene expression in *Saccharomyces cerevisiae*. Acetyl-CoA is used by the tricarboxylic acid cycle (TCA) and the type II fatty acid synthase (FAS), which provides the substrate for lipoate synthesis. The lipoate synthesis pathway proteins Lip2, Lip3, and Lip5 and the H protein (Gcv3) are required for activation of the lipoate-dependent complexes PDH, KDH, and GCV. An unidentified product of the FAS is required for activation of the ribonucleoprotein RNase P and the processing of RNase P component RPM1 itself. RNase P activation results in efficient processing of RNAs, affecting mitochondrial gene expression. Proteins are colored in blue and RNA in green. Red dotted arrows indicate activation steps.

RNase P, providing a possible feedback mechanism regulating the processing of mitochondrial RNAs (Fig. 4). Deletion of fatty acid biosynthesis genes in *S. cerevisiae* results in defective *RPM1* processing and loss of RNase P activity (204). Defective RNA processing could limit the expression of a broad range of mitochondrial genes, including rRNAs and tRNAs, as well as genes encoding protein components of the respiratory chain complexes and the ATP synthase (50). As discussed above, lipoylation, acetyl-CoA levels, and fatty acid biosynthesis are interdependent and influenced by the metabolic state of the cell. These metabolic pathways may intersect with mitochondrial RNA processing, thus providing a level of regulation affecting mitochondrial gene expression.

Comparative Genomics of Pathogenic Fungi

Saccharomyces cerevisiae and *Candida albicans* are closely related yeast species from the phylogenetic class of *Saccharomycetes*. The *C. albicans* genome contains genes encoding single orthologs of the four proteins (Lip3, Lip5, Lip2, and Gcv3) that are necessary for lipoylation in *S. cerevisiae* (Table 5). Similarly, the lipoate-dependent PDH and KDH proteins, including a PDH E3-binding protein (E3BP), are extremely well-conserved between the two species (Table 5). Disruption of the *PDX1* gene, encoding the E3BP in *S. cerevisiae*, results in the formation of PDH complexes that lack the E3 protein and are no longer able to catalyze the oxidation of pyruvate (117), suggesting an important role for E3BP in fungal metabolism. Thus, the proteins of lipoate metabolism, including E3BP, are highly conserved between *S. cerevisiae* and *C. albicans*, and similar mechanisms of lipoate-linked metabolic regulation may exist in both yeast species. As in *S. cerevisiae*, the mitochondrial genome of *C. albicans* also contains an RNase P *RPM1* RNA. The sequences and lengths of *RPM1* RNAs vary considerably between *Candida* species (95); however, core structural do-

main are conserved (103), making it possible that common mechanisms regulate RNase P activation in pathogenic yeasts.

The proteins of lipoate synthesis are well conserved in other pathogenic fungi. These include the ascomycetes *Aspergillus fumigatus* and *Histoplasma capsulatum* (*Ajellomyces capsulata*) and the basidiomycete *Cryptococcus neoformans*. In all cases, the complete genomes of these organisms encode conserved Lip2, Lip3, Lip5, and Gcv3 proteins with high homology to their orthologs in *S. cerevisiae*. Unlike the lipoate synthesis proteins, the complement of lipoate-dependent proteins seems to vary among these fungi (Table 5). Each genome contains a PDH, KDH, and E3BP; however, in *C. neoformans*, there appear to be two closely related paralogs of E3BP. Unlike *C. albicans*, other pathogenic fungi also contain orthologs of the branched-chain ketoacid dehydrogenase (BCDH). In the case of *A. fumigatus*, there are two BCDH paralogs. In these pathogenic fungi, the lipoate synthesis pathway may be conserved, but there are clearly differences in lipoate metabolism.

PROTOZOAN LIPOATE METABOLISM

Apicomplexans

The phylum Apicomplexa represents a diverse array of protozoans that are exclusively the intracellular parasites of animals. Most apicomplexans contain two endosymbiont organelles, mitochondria and apicoplasts, both of which can harbor lipoate metabolic pathways (29, 73). The apicoplast is a nonphotosynthetic relict plastid and is thought to have arisen from the secondary endosymbiosis of a red alga that had previously incorporated a cyanobacterium as its chloroplast organelle (reviewed in reference 65). Due to this prokaryotic origin, many of the metabolic pathways found in the apicoplast are similar to those found in bacteria (244). Like most endosymbiont organelles, the apicoplast has a reduced organellar

TABLE 5. Lipoate metabolism proteins in fungi

Organism	PDH ^a	KDH	BCDH	GCV	Lipoylation proteins ^b
Ascomycota					
<i>Saccharomyces cerevisiae</i> YJM789	E1 α , EDN63155; E1 β , EDN64829; E2, EDN62741; X, EDN61780; E3, EDN59131	E1, EDN61376; E2, EDN60488		T, EDN60363; H, EDN59717; P, EDN64124	L, EDN63291; B, EDN59455; A, EDN63532; H, EDN59717
<i>Candida albicans</i> WO-1	E1 α , EEQ44823; E1 β , EEQ45094; E2, EEQ46961; X, EEQ41882; E3, EEQ43789	E1, EEQ44163; E2, EEQ43790		T, EEQ46753; H, EEQ41897; P, EEQ42377	L, EEQ46720; B, EEQ42844; A, EEQ45249; H, EEQ41897
<i>Aspergillus fumigatus</i> Af293	E1 α , EAL88407 ^c ; E1 α , EAL87580; E1 β , EAL86696; E2, EAL86900; X, EAL92749 ^d ; E3, EAL87307	E1, EAL89627; E2, EAL93026	E1 α , EAL88734; E1 β , EAL86428; E2, EAL87763; E2, EAL89592	T, EAL90405; H, EAL90537; P, EAL84525	L, EAL88406 ^c ; B, EAL92900; A, EAL92912; H, EAL90537
<i>Ajellomyces capsulatus</i> G186AR	E1 α , EEH03746; E1 β , EEH03442; E2, EEH11714; X, EEH10700 ^d ; E3, EEH03184	E1, EEH08095; E2, EEH03139	E1 α , EEH05633; E1 β , EEH06468; E2, EEH08063	T, EEH09576; H, EEH07882; P, EEH03470	L, EEH08859; B, EEH09657; A, EEH03564; H, EEH07882
Basidiomycota					
<i>Cryptococcus neoformans</i> JEC21	E1 α , AAW44390; E1 β , AAW46579; E2, AAW43137; X, AAW44509 ^c ; X, AAW44510 ^c ; E3, AAW42807	E1, AAW41534; E2, AAW41798	E1 α , AAW43658; E1 β , AAW40904; E2, AAW41028	T, AAW42395; H, AAW47059; P, AAW42121	L, AAW41740; B, AAW44485; A, AAW46468; H, AAW47059

^a E3-binding proteins (X) are shown as part of the PDH complex.

^b Lipoylation proteins with homology to *E. coli* LplA (L), LipB (B), LipA (A), and GcvH (H) are shown.

^c Genes arrayed in tandem.

^d These E3-binding proteins do not seem to contain a lipoylation domain.

genome and contains many proteins which are encoded in the nucleus (91) and trafficked to the organelle (243, 246).

The complete genomes of three apicomplexans that commonly cause severe disease in humans, *Plasmodium falciparum*, *Toxoplasma gondii*, and *Cryptosporidium parvum*, have been sequenced (1, 59, 61). *P. falciparum* is the causative agent of malaria, *T. gondii* is the etiological agent of toxoplasmosis, and *C. parvum* infection manifests as cryptosporidiosis. Here, we focus on lipoylation in *P. falciparum* and *T. gondii*, the two apicomplexans in which lipoate metabolism has been studied experimentally.

Lipoylated Complexes in Apicomplexans

The genomes of *P. falciparum* and *T. gondii* both encode four lipoylated proteins, the E2 subunits of the PDH, KDH, and BCDH and the H protein of the GCV (Table 6). The architecture of the lipoylated protein complexes and their subcellular distribution appear to be very similar in malaria and *T. gondii* parasites. In contrast, the genes encoding lipoylated proteins are absent from the *C. parvum* genome. As discussed below, *C. parvum* appears to have dispensed with its apicoplast organelle and retains a relict mitochondrion with a greatly reduced complement of metabolic pathways.

***Plasmodium falciparum*.** The lipoylated protein complexes found in malaria parasites share many similar features with

those observed in prokaryotes, particularly Gram-positive bacteria. The E1 subunits of the PDH and the BCDH are composed of E1 α and E1 β proteins and presumably form heterotetramers. The *P. falciparum* genome does not appear to encode the regulatory kinases and phosphatases which act on the E1 α subunits of eukaryotic PDH and BCDH complexes, and this type of regulation has not been reported in malaria parasites. The KDH E1 is produced as a single protein and is probably homodimeric, as are its orthologs from other organisms. The *P. falciparum* E2 proteins of the KDH and BCDH have a single lipoyl domain, while the PDH E2 has two lipoyl domains and has been shown to be catalytically active (49). These E2 proteins are expected to form the large multimeric cores of the three α -ketoacid dehydrogenases in malaria parasites. The E3-binding protein associated with the E2 core of eukaryotic PDH complexes does not appear to exist in *P. falciparum* and has not been identified in lipoylation-specific Western blots (3). One of the unusual features of the lipoylated protein complexes in *P. falciparum* is the lack of an identifiable GCV P protein (198). The T protein and H protein components of the GCV are readily identifiable in the genomes of malaria parasites (198), and there may be a divergent P protein in these species that has not yet been identified. Alternatively, the T protein and H protein may participate in some other met-

TABLE 6. Lipopate metabolism proteins in protozoans^a

Organism	PDH	KDH	BCDH	GCV	Lipoylation enzymes ^b
Apicomplexa <i>Plasmodium falciparum</i> 3D7	E1 α , AAN35840; E1 β , AAN37054; E2, AAN35399; E3, CAD51214	E1, CAD51167; E2, CAD52355; E3, AAN36396	E1 α , CAD52260; E1 β , CAD51411; E2, CAB38991	T, CAD52774; H, AAN35923	L, CAD52290; L2, CAD51918 ^c ; B, CAD51137; A, CAD52569 L, EEA98687; L2, EEB03119 ^c ; B, EEA97602; A, EEA99152
<i>Toxoplasma gondii</i> ME49	E1 α , EEA99856; E1 β , EEA98721; E2, EEB00702; E3, EEB03230	E1, EEA99768; E2, EEB03569; E3, EEB00688; ?, EEB02066 ^d	E1 α , EEA99447; E1 α , EEB01412; E1 β , EEA97499; E2, EEB02719	T, EEB01949; H, EEB00438	
<i>Cryptosporidium parvum</i> Iowa II					
Kinetoplastea <i>Trypanosoma brucei</i> TREU927	E1 α , EAN78647; E1 β , AAZ10214; E2, EAN78163; E2, EAN77661 ^c ; E3, EAN80618; E3, AAZ10472; E3, AAZ11094; E3, AAZ13501	E1, EAN79950; E1, EAN79124; E2, EAN80119	E1 α , EAN77496; E1 β , EAN7853; E2, AAZ11544	T, EAN79919; H, EAN76953; P, AAZ12256	L, AAZ12834; B, EAN79891; A, EAN78865
<i>Trypanosoma cruzi</i> CL Brenner	E1 α , EAN92220 E and EAN93231 nE; E1 β , EAN89795 E and EAN97324 nE; E2, EAN98042 E and EAN93711 nE; E2, EAN97566 ^c ; E3, EAN96941 E and EAN90443 nE; E3, EAN82368 E and EAN82673 nE; E3, EAN86891 E and EAN84842 nE; E3, EAN86892 E and EAN84843 nE	E1, EAN83551 E and EAN93176 nE; E1, EAN81565 E and EAN85332 nE; E2, EAN86475 E and EAN81897 nE; E2, EAN83551 E and EAN93176 nE	E1 α , EAN89773 E and EAN86288 nE; E1 β , EAN90241 E and EAN91015 nE; E2, EAN88993 E and EAN90623 nE	T, EAN86200 E and EAN85387 nE; H, EAN96694 E and EAN83079 nE; P, EAN91112 ^c and EAN85656 ^c	L, EAN85875 E and EAN92947 nE; B, EAN91274 E and EAN92615 nE; A, EAN97594 E and EAN90088 nE
<i>Leishmania major</i> Friedlin	E1 α , CAJ04196; E1 β , CAJ05399; E2, CAJ09224; E2, CAJ04084 ^c ; E3, CAJ08862; E3, AAZ09641; E3, CAJ08475; E3, CAJ08476	E1, CAJ09310; E1, AAZ09834; E2, CAJ05698	E1 α , CAJ04806; E1 β , AAZ14234; E2, CAJ01934	T, CAJ09346 ^c ; T, CAJ09347 ^c ; H, AAZ14696; P, CAJ04455	L, CAJ07073; B, CAJ09270; A, CAJ07120
Metamonada and Amoebozoa <i>Entamoeba histolytica</i> HM-1:IMSS					
<i>Trichomonas vaginalis</i> G3					
<i>Giardia lamblia</i> ATCC 50803				H, EAX86583; H, EAY05732; L, EAY01593	L, EAX90813

^a Proteins known or predicted to be located in the apicoplast are in bold. Others are thought to be located in the mitochondria or hydrogenosomes. Esmeralda (E) and nonesmeralda (nE) haplotype genes in *Trypanosoma cruzi* CL Brenner are ~98% identical.

^b Lipoylation enzymes with homology to *E. coli* LipA (L), LipB (B), and LipA (A) are shown.

^c Highly divergent lipopate ligase enzymes referred to as LipL2 in the text. *T. gondii* EEB03119 should probably be annotated similarly to NCLIV_001780 from *Neospora caninum*.

^d This protein is composed primarily of three lipoylation/biotinylation domains, the second of which resembles those found in KDH E2 subunits.

^e Unusually short PDH E2 paralogs conserved in kinetoplastid parasites.

^f Similar genes from unidentified haplotypes.

^g Duplicated genes arrayed in tandem.

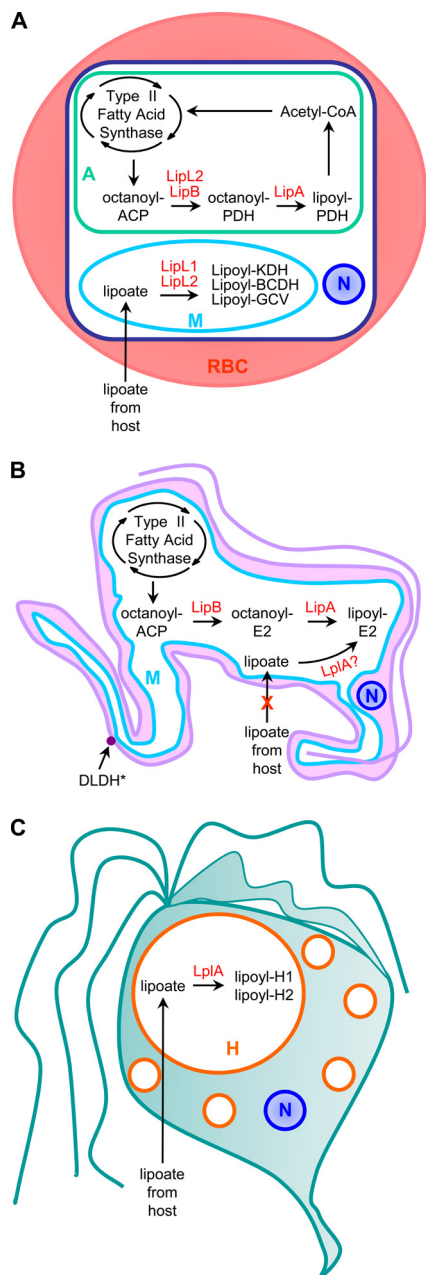


FIG. 5. Comparative lipoylation metabolism in protozoan parasites. (A) *P. falciparum*. In *P. falciparum*, lipoylation synthesis and lipoylation scavenging are segregated by organelle, with lipoylation synthesis occurring in the apicoplast (A) and lipoylation scavenging occurring in the mitochondrion (M). In the apicoplast, the type II fatty acid synthase (FAS) is believed to generate the octanoyl-ACP substrate for lipoylation synthesis. Lipoylation synthesis is then used to lipoylate the sole lipoylated complex in the apicoplast, the PDH. Despite the presence of the lipoylation ligase LipL2 in the apicoplast, the PDH does not appear to be lipoylated through the scavenging pathway. In the mitochondrion, two lipoylation ligases are present, LipL1 and LipL2, and the KDH E2, BCDH E2, and H protein are lipoylated through scavenging. (B) *T. brucei*. In procyclic-form *T. brucei*, lipoylated complexes are found in the mitochondrion (M) and appear to rely on type II FAS for lipoylation. Although a lipoylation ligase is encoded in the genome, supplementation with exogenous lipoylation does not restore lipoylation when lipoylation synthesis is disrupted. In bloodstream-form parasites, the DLDH (*) localizes exclusively to the inner leaflet of the plasma membrane, where it may play a role in sugar transport. (C) *T. vaginalis*. *T. vaginalis* has

abolic pathway. In either case, it is clear from *in vivo* and *in vitro* experiments that the *P. falciparum* H protein is lipoylated (3).

The four lipoylated protein complexes found in *P. falciparum* are partitioned between the mitochondrion and apicoplast organelles (Fig. 5A). All E2 subunits and the H protein are expressed and lipoylated in erythrocytic-stage parasites (3, 74), which is the life cycle stage that causes malaria morbidity and mortality. Unlike plants, which contain independent PDH complexes in the chloroplast and mitochondrion (119), the parasite has a single PDH. The E1 α and E1 β proteins of the *P. falciparum* PDH have been exclusively localized to the apicoplast organelle (49). The apicoplast also contains an E3 dihydrolipoamide dehydrogenase, which is thought to be dedicated to the PDH complex (135). An additional E3 subunit is located in the mitochondrion (135) and must be shared by the lipoylation-dependent complexes in that organelle. So far, the BCDH E1 β protein (72) and the H protein of the GCV (M. D. Spalding and S. T. Prigge, unpublished data) have been localized to the mitochondrion. Presumably, the mitochondrial E3 protein functions in the BCDH and KDH complexes and also serves as the L protein of the GCV. This partitioning of lipoylated complexes indicates that the apicoplast and mitochondrion must each possess functional lipoylation mechanisms (discussed below).

Toxoplasma gondii. The lipoylated protein complexes found in *P. falciparum* are also present in *Toxoplasma*. The genome of *T. gondii* encodes all of the proteins comprising the PDH, BCDH, and KDH complexes. The architecture of these α -ketoacid dehydrogenase complexes is similar to that found in malaria parasites, with the E1 subunits of the PDH and BCDH composed of E1 α and E1 β proteins; however, there are also notable differences. The E2 subunit of the *T. gondii* PDH contains three lipoyl domains (227) rather than the two found in *P. falciparum*. Another difference is that there are two closely related paralogs of the E1 α protein of the BCDH complex in all three strains of *T. gondii* which have been sequenced (strains VEG, GT1, and ME49) (59). The implications of this duplication are not clear; however, it is notable that the *T. gondii* genome encodes the enzymes comprising a complete branched-chain amino acid degradation system (207). Malaria parasites appear to lack this pathway, including the branched-chain amino acid transaminase (BCAT) enzyme, which normally provides deaminated substrates for the BCDH.

T. gondii parasites also encode the GCV H protein and T protein, but they lack an identifiable P protein. The H protein is not recognized in anti-lipoylation Western blot analyses of *T. gondii* tachyzoites, even though the E2 subunits of the three α -ketoacid complexes are detected (29). Failure to detect the H protein could indicate that the H protein is not well expressed or lipoylated in tachyzoites. This would be a departure

two H proteins, and these are the sole lipoylated proteins encoded in its genome. These proteins localize to the hydrogenosomes (H). *T. vaginalis* encodes a lipoylation ligase but no lipoylation synthase or octanoyl transferase, and it is therefore expected to salvage lipoylation from the extracellular milieu.

from lipoate metabolism in *P. falciparum*, where all four lipoylated proteins are expressed and lipoylated during the replicative stages in the mammalian host (3).

As observed for malaria parasites, the PDH resides in the *T. gondii* apicoplast, while the BCDH and KDH localize to the mitochondrion (29). Both the apicoplast and the mitochondrion of *T. gondii* are recognized in immunofluorescence experiments using antibodies specific for lipoylated proteins (29). Thus, as in *P. falciparum*, in *T. gondii* lipoylated complexes and lipoylation mechanisms appear to be present in both organelles.

***Cryptosporidium parvum*.** Unlike *P. falciparum* and *T. gondii*, *C. parvum* does not contain an apicoplast organelle. There is genetic evidence, however, that *Cryptosporidium* evolved from a plastid-containing lineage and subsequently lost this organelle and many of its associated biochemical pathways (91). Although a few genes appear to have been preserved in the nuclear genome after lateral gene transfer from the plastid, these genes do not encode the PDH or proteins of lipoate synthesis typically found in the apicoplast (90). As observed for the microaerophilic bacterium *H. pylori* and amitochondriate protozoans (see "Metamonada and Amoebozoa" below), *C. parvum* contains an anaerobic acetyl-CoA-generating enzyme, pyruvate:NADP oxidoreductase (197), which is located in the cytosol (32).

Similarly, *C. parvum* does not have mitochondria and lacks many of the biochemical pathways typically found in this organelle. The parasite does contain a highly reduced two-membrane compartment called the mitosome that is thought to have descended from a common ancestral apicomplexan mitochondrion (82, 180). This organelle appears to have been retained to carry out a limited number of metabolic functions, such as the synthesis of iron-sulfur clusters (116). The KDH complex found in the *P. falciparum* and *T. gondii* mitochondria is absent from *C. parvum*, as this parasite relies solely on anaerobic metabolism. Genes encoding the BCDH and components of the GCV also appear to be absent from the *C. parvum* genome, as are genes encoding the enzymes involved in lipoylation (Table 6).

Mechanisms of Lipoylation in Apicomplexans

***Plasmodium falciparum*.** Malaria parasites encode the enzymes required for lipoate scavenging and lipoate synthesis, which include single orthologs of the *E. coli* lipoate synthase (LipA) and octanoyl transferase (LipB) and two lipoate ligase orthologs (Table 6). The *P. falciparum* LipA and LipB enzymes (PfLipA and PfLipB) functionally complement their orthologs in *E. coli* gene disruption strains, indicating that the malaria parasite enzymes are capable of synthesizing lipoate (254). Both *P. falciparum* enzymes are encoded in the nucleus and contain amino-terminal targeting peptides with the architecture required for apicoplast import (48, 264). In *P. falciparum*, the targeting peptide of PfLipA directs a reporter protein to a compartment other than the mitochondrion (254), suggesting that the apicoplast is the site of lipoate synthesis in the parasite. The apicoplast also contains a type II fatty acid synthase (123) that is expected to produce the PfLipB substrate octanoyl-ACP, further indicating that a functional lipoate synthesis pathway is housed in the plastid (245). Lipoate synthesized in the apicoplast is not likely to be available for use in the mito-

chondrion, since lipoate is synthesized in a protein-bound form and not as a free acid. Thus, another mechanism of lipoylation is needed in the mitochondrion.

Lipoylation in the mitochondria of malaria parasites occurs through lipoate scavenging. Human serum typically contains 33 to 145 ng/ml lipoate that is noncovalently bound to albumin along with other lipids (165, 225); this is the probable source of scavenged lipoate for malaria parasites. *Plasmodium* species contain two enzymes with lipoate ligase activity (3). This scenario is reminiscent of certain bacteria, such as *L. monocytogenes*, which contain two lipoate ligase paralogs, both of which are homologous to *E. coli* LplA (107). However, in *P. falciparum*, the two ligases are highly divergent (15% pairwise amino acid identity), and only one of them has significant homology to *E. coli* LplA. Because these ligases are not paralogs and seem to represent different classes of lipoate ligases, they are named PfLipL1 and PfLipL2 (3). Both *P. falciparum* lipoate ligases functionally complement LplA in an *E. coli* gene disruption strain, indicating that the malaria parasite enzymes are capable of scavenging free lipoate (3). Analysis of the complementation strains shows that PfLipL1 preferentially lipoylates the *E. coli* KDH, while PfLipL2 almost exclusively lipoylates the *E. coli* PDH (3). As in *L. monocytogenes*, substrate specificity may provide an explanation for why malaria parasites contain two ligases. Another explanation could involve the subcellular location of the two ligases. PfLipL1 contains a mitochondrial transit peptide that targets a reporter protein to the parasite mitochondrion (254). The second ligase, PfLipL2, lacks an identifiable mitochondrial transit peptide; immunofluorescence experiments and targeting of a reporter protein indicate that the enzyme is dually located in the apicoplast and mitochondrion (74).

Thus, malaria parasites contain a functional lipoate synthesis pathway and two functional lipoate ligases. The arrangement of these pathways in *P. falciparum* differs significantly from that observed in plants and photosynthetic microbes such as *Cyanidioschyzon merolae* and *Thalassiosira pseudonana* (29). These organisms contain lipoate synthesis pathways in their mitochondria and plastids; in contrast, malaria parasites have retained only the plastid pathway. Conversely, the plant-like organisms contain a single lipoate ligase found in the mitochondria, whereas malaria parasites seem to contain ligases in both organelles. Despite the presence of functional lipoate ligases in the apicoplast and mitochondrion of *P. falciparum*, exogenously supplied [³⁵S]lipoate was incorporated only into the three mitochondrial complexes, indicating that the PDH is lipoylated solely through the synthetic pathway (3). Thus, it appears that *P. falciparum* has nonredundant lipoylation mechanisms in which the plastid PDH is lipoylated exclusively by lipoate synthesis, while the mitochondrial complexes are lipoylated through scavenging of exogenous lipoate (Fig. 5A).

Biochemical and genetic evidence indicates that lipoate scavenging is required for blood-stage parasite survival. Treatment of parasites with the lipoate analog 8-bromooctanoate (8-BrO) interferes with incorporation of scavenged [³⁵S]lipoate and results in growth inhibition (3). Attempts to disrupt the gene encoding PfLipL1 in *P. falciparum* and in the rodent malaria parasite *Plasmodium berghei* were unsuccessful, which further indicates that lipoate scavenging may be essential in the

blood stages (71). The failure to disrupt PfLipL1 also suggests that the two parasite ligases may not have redundant functions, despite their common localization to the mitochondrion.

Lipoate synthesis also appears to be necessary for the normal growth of blood-stage parasites. Deletion of the gene encoding PfLipB decreases PDH lipoylation by approximately 90% and results in a significantly accelerated life cycle (74). The lipoylated PDH that remains in this disruption strain is likely produced by the octanoyl transferase activity of PfLipL2 in the apicoplast (74). The effects of complete ablation of PDH lipoylation, through deletion of either PfLipA or the PDH E2 subunit, are unknown, although the gene encoding PfLipA is reportedly refractory to deletion (73). Although the results suggest that lipoate synthesis is essential for *P. falciparum* growth, it was recently demonstrated that the type II fatty acid synthase (FAS) enzyme enoyl reductase is dispensable in the blood stages of *P. falciparum* (259). In plants and algae, the critical function of the type II FAS is the production of octanoyl-ACP as the substrate for lipoate biosynthesis (reviewed in reference 131). Thus, disruption of the type II FAS pathway should preclude the biosynthesis of lipoate. It is possible, however, that an undiscovered mechanism for generating lipoyl-PDH may be at work.

***Toxoplasma gondii*.** The organization of lipoylation pathways in *T. gondii* closely mirrors that in *P. falciparum*. Single orthologs of *E. coli* LipB and LipA appear to function in an apicoplast lipoate synthesis pathway. The amino-terminal targeting peptide of *T. gondii* LipA (TgLipA) directs a reporter gene to the apicoplast, and TgLipB is also predicted to be trafficked to the apicoplast (227). TgLipA and TgLipB are expected to function in conjunction with a type II FAS pathway (243) in the apicoplast to lipoylate PDH (227). Like *P. falciparum*, *T. gondii* has a lipoate ligase ortholog of the *E. coli* LplA (TgLipL1) and a second divergent ligase, TgLipL2. Although these ligases have not been studied experimentally, TgLipL1 has a predicted mitochondrial localization (227). Since dual localization to the mitochondrion and apicoplast was observed for PfLipL2, it will be important to experimentally determine the subcellular location of TgLipL2. Overall, it appears to be likely that *T. gondii* parasites have a functional lipoate biosynthesis pathway in the apicoplast and a scavenging pathway in the mitochondrion (227).

T. gondii, like *P. falciparum*, is auxotrophic for lipoate due to a requirement for lipoylated proteins in the mitochondria of these parasites. *T. gondii* parasites grown in lipoate-depleted media show decreased lipoylation of the KDH and BCDH and exhibit slower growth than those cultured in standard media (29). Lipoylation of the PDH is not affected by growth in lipoate-depleted media, presumably due to its reliance on lipoate synthesis in the *T. gondii* apicoplast. Like *P. falciparum*, *T. gondii* is susceptible to lipoate analogs such as 8-BrO that interfere with lipoate scavenging (29). Interestingly, axenic *T. gondii* parasites cultured in the absence of a host cell are unable to scavenge lipoate (29). This raises the possibility that the close interaction between the parasitophorous vacuole and the host mitochondria facilitates the scavenging of lipoate from the host cell (29).

A functional type II FAS appears to be essential in *T. gondii* and is necessary for lipoylation of the PDH. When the acyl carrier protein (ACP) of the type II FAS is disrupted (132) or

T. gondii is treated with the type II FAS inhibitor triclosan, the parasites show growth inhibition and diminished PDH lipoylation (29). Lipoylation of the mitochondrial KDH and BCDH complexes is unaffected under both conditions, supporting the hypothesis that apicoplast and mitochondrial lipoylation are independent. Thus, *T. gondii* parasites require a functional type II FAS for lipoate biosynthesis in the apicoplast as well as exogenous lipoate for lipoylation of the mitochondrial complexes.

Kinetoplastids

Kinetoplastids are a group of flagellated protozoans named for the kinetoplastid, a DNA-containing compartment located near the basal body of the flagellum and within the single mitochondrion. This phylum includes the agents of many neglected tropical diseases, such as *Trypanosoma brucei*, the causative agent of African sleeping sickness; *Trypanosoma cruzi*, the parasite that results in Chagas' disease; and *Leishmania major*, the etiological agent of cutaneous leishmaniasis. Comparison of the *T. brucei*, *T. cruzi*, and *L. major* genomes shows that all three pathogens have substantially similar gene contents, gene orders, and biological processes (43). This similarity extends to lipoate metabolism. Each species encodes a single lipoate ligase as well as a lipoate synthase and an octanoyl transferase (Table 6). These lipoylation pathway enzymes have not been characterized in kinetoplastids; however, the lipoylated protein complexes are well studied. All species encode four lipoylated complexes, a PDH, a BCDH, a KDH, and a GCV. In contrast to the apicomplexans *P. falciparum* and *T. gondii* and the metamonad *Trichomonas vaginalis*, which do not encode all of the subunits of the classical GCV, genes encoding the entire complex are present in kinetoplastids.

Lipoylated Complexes in Kinetoplastids

***Trypanosoma brucei*.** The expression of lipoylated complexes in *T. brucei* differs between the two life cycle stages which have been examined in the laboratory: the procyclic form (PCF) of the parasite, which normally resides in the insect midgut, and the bloodstream form (BSF), which is found in the mammalian host. In both forms, the mitochondrion is believed to be the primary site of lipoate metabolism, although the mitochondrion and its metabolism differ markedly in these two life cycle stages. BSF parasites rely on blood glucose as their primary energy source, excrete pyruvate (20), and lack a functional respiratory chain. In this stage, the mitochondrial metabolic functions are reduced (161), and ATP from glycolysis drives the F_0F_1 -ATPase in reverse in order to maintain the mitochondrial proton gradient (202). Conversely, PCFs feed primarily on proline and threonine in the vector midgut (22) and have a well-developed mitochondrion in which the respiratory chain maintains the mitochondrial proton gradient and ATP is generated by oxidative phosphorylation (13, 40). The activities of TCA cycle enzymes, including the KDH, increase significantly during the transition from BSF to PCF (40), reflecting the changes in metabolism that occur between these life cycle stages. RNA interference (RNAi) depletion of the E1 α subunit of PDH slows the growth of PCF trypanosomes (18), highlighting the importance of pyruvate metabolism at this life cycle stage.

The compositions of the KDH and PDH complexes in PCF-

stage *T. brucei* have been examined using proteomic approaches. The PDH was immunoprecipitated from PCF mitochondria as a five-protein complex containing E1 α , E1 β , E2, E3, and E3BP components with no associated regulatory phosphatases or kinases (166). The KDH was found to contain the same E3 subunit as observed in the PDH complex, as well as an E2 subunit and two independent E1 subunits (166). Both E1 subunits may be functional and may compete with the E3 subunit for binding to the E2 core of the KDH complex. The significance of this arrangement is not clear; however, syntenic orthologs of the two KDH E1 subunits appear to be conserved in the genomes of the other kinetoplastid species (Table 6).

Despite the diminished role of α -ketoacid dehydrogenases in BSF parasites (236), this life cycle stage does possess dihydrolipoamide dehydrogenase (DLDH) (the E3 subunit) activity. In contrast to the procyclic DLDH, which is found in the mitochondrion (28), DLDH associates exclusively with the inner leaflet of the plasma membrane in the bloodstream form of the parasite (35, 98). In *S. pneumoniae*, the import of some sugars is believed to be dependent on DLDH (210) (see "Gram-Positive Bacteria" above). The bloodstream-form DLDH could play a similar role given the prodigious quantity of glucose required to support growth in this stage. Even though the explicit function of the DLDH at the plasma membrane in *T. brucei* is uncertain, it is likely to be important for growth in this stage, as the DLDH, along with trypanothione reductase, is a target of several inhibitors used in the treatment of African trypanosomes (reviewed in reference 113).

Although PDH and KDH activities have not been detected in BSF *T. brucei*, at least one lipoylated protein is expressed during this stage of the life cycle. Antiserum specific for lipoylated proteins recognizes a single 40-kDa protein in this stage as well as in procyclic-form parasites (219). As observed in *T. gondii*, the type II FAS protein ACP is required for proper lipoylation, indicating that lipoylation is responsible for lipoylating the 40-kDa protein. Although a lipoylation ligase is encoded in the *T. brucei* genome (Table 6), supplementation with exogenous octanoate or lipoate does not restore lipoylation of this protein (219) (Fig. 5B). This is reminiscent of results reported for *S. cerevisiae*, which contains a lipoylation ligase (Lip3) but is unable to scavenge lipoate (205). In *S. cerevisiae*, the Lip3 protein, in conjunction with the lipoylation synthase (Lip5), lipoylation transferase (Lip2), and H protein (Gcv3), is required for proper lipoylation of mitochondrial proteins, and a similar situation may exist in *T. brucei*.

Leishmania major. Like *T. brucei* and *T. cruzi*, *L. major* encodes orthologs of bacterial PDH, KDH, BCDH, and GCV proteins (Table 6). Among these, only the role of the GCV in *Leishmania* biology has been characterized, and this complex is necessary for normal replication and pathology *in vivo*. Genetic disruption of the gene encoding the *L. major* GCV P protein reduces parasite virulence and leads to attenuated infections in mice (206). In contrast, the GCV is not required for the *in vitro* growth of parasites in macrophages, although these parasites do show increased sensitivity to elevated glycine or reduced serine levels (206). One reason for a severe *in vivo*, but not *in vitro*, phenotype may be that the GCV allows *L. major* to adapt to metabolic perturbations associated with inflammatory responses *in vivo*. In particular, *L. major* may require the 5,10-CH₂-THF generated by the GCV for serine synthesis by serine

hydroxymethyltransferase (SHMT). Other intracellular pathogens, such as *Francisella tularensis* (248), *Brucella abortus* (88), and *Mycobacterium tuberculosis* (199), are likewise dependent on a functional GCV for virulence.

Metamonada and Amoebozoa

Trichomonas vaginalis. Among the amitochondriate, anaerobic protozoans whose genomes are analyzed here (*Trichomonas vaginalis*, *Giardia lamblia*, and *Entamoeba histolytica*), *T. vaginalis* is the only species that encodes enzymes involved in lipoate metabolism. *T. vaginalis* encodes a single lipoate ligase but does not appear to contain a lipoate synthase or octanoyl transferase. Consistent with its anaerobic lifestyle, *T. vaginalis* does not contain a PDH or KDH. As is the case with *G. lamblia* (230) and *E. histolytica* (189), *T. vaginalis* relies on a pyruvate: ferredoxin oxidoreductase to produce acetyl-CoA (89, 252). *T. vaginalis* diverges from other amitochondriate, anaerobic protozoans, however, in that it contains an L protein and two H protein paralogs, a feature that is so far unique to *T. vaginalis* (Table 6) (150). Both H proteins can be lipoylated, and both can serve as substrates of the L protein, indicating that they can participate in redox reactions involving protein-bound lipoate (150). The L protein is a dimeric dihydrolipoamide dehydrogenase with homology to E3 subunits and L proteins from other species (150). The P and T proteins have not been identified in *T. vaginalis*, suggesting either that they are highly divergent or that the parasite does not contain these proteins. In the latter case, *T. vaginalis* would not have a glycine cleavage system, and the H and L proteins could have alternative functions. This situation is similar to that found in certain *Firmicutes* bacteria, such as *Enterococcus faecalis*, which also appear to lack P and T proteins (Table 6). Interestingly, the L protein found in *T. vaginalis* is most closely related to those of the *Firmicutes* and appears to have been acquired by horizontal gene transfer rather than through a mitochondrial precursor (150).

In *T. vaginalis*, the H proteins and the L protein are located in two-membrane organelles called hydrogenosomes (Fig. 5C) (150), which are related to mitochondria but typically do not contain genetic material (41, 66). Generally, GCV systems work in close conjunction with mitochondrial serine hydroxymethyltransferase (SHMT) enzymes in amino acid metabolism (39). Both enzymes are reversible, so they can generate or consume 5,10-CH₂-THF to break down or synthesize serine and glycine (26). The single SHMT enzyme in *T. vaginalis* has been biochemically characterized and localized to the hydrogenosome organelle (151). Although the presence of SHMT suggests that hydrogenosomes participate in amino acid metabolism, it is not clear whether a functional GCV is involved as well. Thus, the role of the two lipoylated H proteins in *T. vaginalis* remains unknown.

CONCLUSIONS

Lipoate metabolism can be found in most bacterial, fungal, and protozoan pathogens. These organisms acquire lipoate through either *de novo* synthesis or scavenging from the environment, and many pathogens maintain independent lipoate synthesis and scavenging pathways. In apicomplexan parasites,

these pathways are independent but not redundant due to physical partitioning between two organelles. In fungi, it appears that the enzymes typically associated with the synthesis and scavenging pathways are not independent but are all required for lipoate synthesis. In contrast, the intracellular pathogen *L. monocytogenes* is a lipoate auxotroph and contains two lipoate-scavenging enzymes which have nonredundant roles during *in vivo* infection. A similar duplication can be found in the genomes of other Gram-positive bacteria and in certain protozoan parasites. Overall, there is a surprising variety of lipoylation strategies employed by microbial pathogens in response to their adaptation to niche environments.

The composition and function of lipoylated protein complexes also vary dramatically across microbial pathogens. Even among related species, proteins have been duplicated, have been deleted, or have acquired alternative functions. Perhaps the only rule governing the composition and function of lipoylated protein complexes is that there are no rules. Despite the broad variation, several themes emerge.

(i) Many microbial pathogens have multiple dihydrolipoamide dehydrogenase E3 subunits. These can serve to regulate the activity of specific protein complexes, as observed in several species of bacteria, or they can function in different subcellular compartments, as observed in apicomplexan parasites. In both prokaryotes and eukaryotes, E3 proteins have been localized to the plasma membrane, where they may have additional roles in the transport of sugars.

(ii) The genes encoding the lipoylation enzymes LplA, LipB, and LipA are often found adjacent to other lipoate metabolism genes. This is particularly true in Gram-positive bacteria and suggests that there is functional significance to this arrangement, perhaps linking the expression of lipoylation enzymes with the complexes they lipoylate.

(iii) Many microbial pathogens contain an incomplete GCV, but these organisms always retain an H protein. There is likely some other role that the H protein plays in both prokaryotes and eukaryotes. "Orphan" H proteins retain the lysine residue used for lipoate attachment and are always found in conjunction with a dihydrolipoamide dehydrogenase (E3 subunit or L protein) and at least one lipoylation pathway enzyme. This suggests that orphan H proteins are lipoylated (as has been observed in some organisms) and are redox active.

(iv) Duplicated genes, such as the KDH E1 paralog genes conserved in kinetoplastid parasites, can be functional and could serve to modulate the activity of shared protein complexes.

(v) Lipoylated proteins can be critical for the growth and survival of microbial pathogens *in vivo* but dispensable for *in vitro* growth. This is the case in *L. monocytogenes* and *S. pneumoniae*, which require lipoate metabolism to survive in the more resource-constrained environment found *in vivo*. In *B. pseudomallei* and *M. tuberculosis*, lipoylation enzymes and lipoylated proteins have roles in oxidative defense and are required for the virulence of these pathogens. PDH components are required for the proper expression of the type III secretion system in *P. aeruginosa* and for bacterial sporulation in *B. anthracis*. Through different mechanisms, GCV components in several pathogens, such as *F. tularensis*, *B. abortus*, *M. tuberculosis*, and *L. major*, are required for virulence. Overall, lipoate metabolism has been shown to be critical for virulence in many

organisms; however, the mechanisms underlying pathogenicity are as varied as the lipoate metabolic pathways themselves.

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