Lipoic Acid Synthetase Deficiency Causes Neonatal-Onset Epilepsy, Defective Mitochondrial Energy Metabolism, and Glycine Elevation

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Lipoic acid is an essential prosthetic group of four mitochondrial enzymes involved in the oxidative decarboxylation of pyruvate, α-ketoglutarate, and branched chain amino acids and in the glycine cleavage. Lipoic acid is synthesized stepwise within mitochondria through a process that includes lipoic acid synthetase. We identified the homozygous mutation c.746G>A (p.Arg249His) in *LIAS* in an individual with neonatal-onset epilepsy, muscular hypotonia, lactic acidosis, and elevated glycine concentration in plasma and urine. Investigation of the mitochondrial energy metabolism showed reduced oxidation of pyruvate and decreased pyruvate dehydrogenase complex activity. A pronounced reduction of the prosthetic group lipoamide was found in lipoylated proteins.

Lipoic acid, also called thioctic acid, is formed in mitochondria by a series of reactions involving the mitochondrial fatty acid synthase type II (FAS II). Via FAS II, octanoic acid is synthesized in an acyl carrier protein (ACP)-bound fashion. As known from studies in bacteria and yeast, octanoic acid is transferred from octanoyl-ACP to a target protein.[1–3] Further on, two sulfohydryl groups are introduced at positions 6 and 8 on the protein-bound octanoic acid in a stepwise and stereoselective manner. This reaction is catalyzed by the enzyme lipoic acid synthetase (LIAS), which is a highly conserved enzyme found in prokaryotes and eukaryotes (Figure 1). In eukaryotes, lipoic acid functions as a coenzyme of the three mitochondrial dehydrogenase complexes pyruvate dehydrogenase (PDHC), α-ketoglutarate dehydrogenase (α-KGDH), and branched chain keto acid dehydrogenase (BCKDH), which share a similar architecture (three enzymatic subunits: E1, E2, and E3) arranged in an enzyme complex of high molecular weight. Lipoic acid is bound via an amide bond to the ε-amino group of a strictly conserved lysine of the respective E2 subunits. Furthermore, the subunit E3 binding protein of PDHC, which resembles the E2 subunit, is also lipoylated.[4] In a similar fashion, lipoic acid acts as a prosthetic group of the H protein of the glycine cleavage system (GCS).

LIAS-deficient *Escherichia coli* becomes auxotrophic for lipoic acid, which can be taken up and metabolized via the salvage pathway.[5] In contrast, eukaryotes are strictly dependent on the de novo synthesis of the lipoyl group within mitochondria. This has been shown in the *Saccharomyces cerevisiae lip5* mutant (encoding the lipoic acid synthetase), which is unable to utilize lipoic acid supplied in the growth medium.[6] It was also found that early embryonic lethality of LIAS knockout mice cannot be overcome or ameliorated by feeding of lipoic acid to pregnant heterozygous mice.[7]

Here we describe a boy with early-onset lactic acidosis, severe encephalomyopathy, and a pyruvate oxidation defect, in whom a deficiency in lipoic acid synthesis could be delineated as the underlying cause of the disease. The study investigations have been conducted according to the Austrian Gene Technology Act and comply with the Declaration of Helsinki. The investigations are furthermore approved by the head of the Pediatric Department and the head of the Paracelsus Medical University.

The affected individual was the first child of consanguineous Turkish parents. The pregnancy was uneventful, the delivery was spontaneous at term, and the child had Apgar scores of 9/10/10, an umbilical artery pH of 7.35 (normal range: 7.18–7.38), a birth weight of 3,140 g (normal range: 2600–4300 g), a length of 52 cm (normal range: 46–55 cm), and a head circumference of 33 cm (normal range: 32–38 cm). The first two days were uneventful. On the third day of life, the first convulsions were observed. They affected the left arm and leg and also included oral automatisms, which lasted for approximately one minute. The boy was admitted to the hospital on day 4 because of the seizures. He showed muscular hypotonia and poor sucking but did not require infusions. Seizures were controlled by phenobarbitone. On day 8, the clinical condition deteriorated, including recurrent apneas, reduced consciousness, worsened hypotonia, and an increase of convulsions. Lactate was found to be elevated to 4.6 mmol/l (normal < 2.1 mmol/l). Antibiotic treatment with ampicillin and gentamicin was started because of an increase of C-reactive protein. The child became somnolent and showed poor feeding, and lactate in his plasma increased up to 13.0 mmol/l. On day 11, a sudden and dramatic further deterioration occurred, including acute respiratory deficiency and severe lactic acidosis of up to 57.7 mmol/l (base excess −20), necessitating artificial

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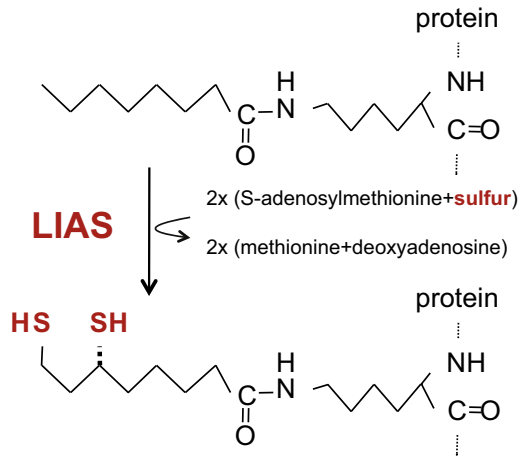


Figure 1. Lipoic Acid Synthetase Catalysis the Formation of the Protein-Bound Prosthetic Group Dihydrolipoamide

ventilation for 17 days. Chest X-ray revealed an infiltration of the lungs. Sonography of the brain, which had been normal initially, now showed severe brain edema. Selective screening for organic and amino acids in urine revealed moderately elevated concentrations of glutaric acid and glycine, respectively. Glycine in plasma was elevated to 906 μmol/l (normal range: 126–384 μmol/l).

After stabilization of this severe neurometabolic crisis, the child developed spastic tetraparesis and presented with symptomatic epilepsy, crying attacks, poor feeding, and lactate elevation between 5 and 7 mmol/l. Brain sonography revealed a multicystic encephalopathy and hydrocephalus ex vacuo. Echocardiography was performed at the age of 11 months and showed a mild hypertrophic cardiomyopathy with mild insufficiency of the right heart. At an age of 2 years, the boy's motor and mental development was severely retarded, and his disease presentation had progressed to include spastic tetraplegia, contractures, microcephaly, and a restless condition including sleep disturbances. Lactate levels were reduced to nearly normal values, with moderate intermittent elevations up to 3.5 mmol/l. At an age of 4 years, the child died at home from a severe respiratory tract infection. The parents had only loose contact with the local hospital and the referring neuropediatrician. An autopsy has not been performed.

A muscle biopsy performed at one month of age showed normal enzyme histochemistry, and electron microscopy revealed abnormally elongated mitochondria with an electron-dense matrix. Respiratory chain enzymes were normal. For reevaluation of the mitochondrial energy metabolism, a needle biopsy of the muscle was performed at 11 months of age. Investigation of oxidation of mitochondrial substrates by intact mitochondria-enriched postnuclear supernatant from a fresh muscle biopsy[8] showed a severely reduced reactivity with pyruvate-containing substrates, whereas acetylcarnitine-containing substrates were normally metabolized (Table 1). This result clearly indicated a defect in the mitochondrial pyruvate oxidation route. The oxidation of acetylcarnitine + malate was reduced in the absence of arsenite but normal in its presence (Table 1). Given that arsenite is an inhibitor of α-KGDH, this result is in line with a defect of the α-KGDH. Measurement of single enzymes of the mitochondrial energy metabolism[9,10] showed a clearly reduced activity of the pyruvate dehydrogenase complex, and other enzymes were only moderately affected (Table 1). Remarkably, immunoblot analysis[11] with an antibody cocktail against PDHC subunits (MSP02, Mitosciences) showed only moderate changes in the protein content: the subunits E1 α and E1 β were found in normal amounts, but E2 and E3 binding proteins were obviously decreased in comparison to a control sample and were related to the ATP synthase subunit OSCP (Figure 2). This result is remarkable because both E2 and E3 binding protein normally carry the prosthetic group lipoic acid.

Mutations in the genes of the PDHC subunits (*PDHA1* [MIM 300502], *PDHB* [MIM 179060], *DLAT* [MIM 608770], *DLD* [MIM 238331], and *PDHX* [MIM 608769]), PDHC phosphatases (*PPAPDC2* [MIM 605993], *PDP2*, and *PDPR*), and thiamine transporters (*SLC19A2* [MIM 603941], *SLC19A3* [MIM 606152], and *SLC25A19* [MIM 606521]) were excluded by Sanger sequencing[8] and expression analysis of these genes. Because the parents were consanguineous, autozygosity mapping (HumanCytoSNP-12v1 BeadChip, Illumina) was performed in this family. Out of seven candidate genes involved in lipoic acid synthesis (*MCAT*, *OXSM* [MIM 610324], *MECR* [MIM 608205], *LIPT1* [MIM 610284], *LIPT2*, *LIAS* [MIM 607031], and *SLC25A26* [MIM 611037]), two localized within the autozygous regions. Additional candidates in this pathway, the PPTase *AASDHPPT* (MIM 607756), the acyl carrier protein *NDUFAB1* (MIM 603836), the possible mtFAS II ketoreductase made up of *CBR4* and *HSD17B8* (MIM 601417), dehydratase *RPP14* (MIM 606112), and malonyl-CoA synthetase *ACSF3* (MIM 614245), were considered later, but all of them lie within heterozygous regions. Furthermore, two enzymes involved in the maturation of the mitochondrial iron sulfur clusters have recently been shown to result in a deficiency of LIAS,[12] because LIAS depends on iron sulfur cluster prosthetic groups.[13] However, *NFU1* (MIM 608100) and *BOLA3* (MIM 613183) were not within autozygous regions. Sequence analysis of *MCAT*, located in a small autozygous region of chromosome 22 and encoding the mitochondrial malonyl CoA:ACP acyltransferase, showed a normal result. In contrast, a potential disease-causing mutation in *LIAS*, encoding the lipoic acid synthetase, was found to map to a large autozygosity region of 19.7 million bases on the short arm of chromosome 4. Sequence analysis of *LIAS* (RefSeq NM_006859.2) revealed a homozygous mutation, c.746G>A (Figure 3), in exon 8, predicted to result in a replacement of arginine 249 by histidine (p.Arg249His). The parents were shown to be heterozygous carriers of this mutation. This position is highly conserved in

Table 1. Investigation of the Mitochondrial Energy Metabolism in Muscle and Fibroblasts

	P-LIAS Muscle	Control Muscle	P-LIAS Fibroblasts	Control Fibroblasts
Substrate Oxidation Rates [nmol/h/mg protein]				
[1 - ^{14}C]pyruvate+malate+ADP	34	263–900		
[1 - ^{14}C]pyruvate+carnitine+ADP	35	302–856		
[1 - ^{14}C]pyruvate+malate (−ADP)	24	32–102		
[U - ^{14}C]malate+pyruvate+malonate+ADP	64	282–874		
[U - ^{14}C]malate+acetylcarn.+malonate+ADP	246	273–678		
[U - ^{14}C]malate+acetylcarn.+arsenite+ADP	182	156–378		
[1,4 - ^{14}C]succinate+acetylcarnitine+ADP	153	167–488		
Enzyme Activities [unit/g protein]				
Citrate synthase	351	150–325	286	242–590
Complex I	26	28–76	30	15–53
Complex I+III	46	64–218	171	102–343
Complex II	23	39–102	88	103–285
Complex II+III	32	65–180	123	167–314
Complex III	179	351–762	262	283–1174
Cytochrome c oxidase	601	306–889	203	392–939
Oligomycin-sensitive ATPase (complex V)	199	70–397	189	36–167
Pyruvate dehydrogenase complex	0.4	6.1–19.8	0.5	6.0–19.7

Functional investigation of postnuclear supernatant prepared from native, unfrozen muscle showed reduced activities in all pyruvate-containing oxidation reactions and was mildly reduced with malate plus pyruvate in the absence of arsenite. Analysis of respiratory chain enzymes, ATPase, and pyruvate dehydrogenase complex revealed strongly reduced activities of the pyruvate dehydrogenase complex in postnuclear supernatant prepared from muscle and isolated mitochondria from fibroblasts in the individual affected with LIAS deficiency (P-LIAS).

eukaryotic and also prokaryotic lipoic acid synthetases (Figure 3). The content of the prosthetic group lipoic acid was investigated by either immunoblot analysis or immunohistochemical staining[14] with a rabbit anti-lipoic acid polyclonal antibody (#437695, Calbiochem) using the lipoic acid auxotrophic *E. coli* strain JRG33.[15] Immunoblot analysis with the lipoic acid antibody showed a severe reduction of the intensities of the E2 subunits of both PDHC and α-KGDH (Figure 4). Because BCKDH and the H protein of the glycine cleavage system are expressed mainly in the liver[16,17] we were not able to show a deficiency of these enzymes because only muscle and fibroblasts were available. Immunohistochemical staining of fibroblasts from the affected individual confirmed a deficiency of lipoic acid by showing loss of staining with the antibody against lipoic acid in comparison to normal staining with an anti-porin antibody (Figure 4). In order to demonstrate the functional relevance of the mutation p.Arg249His, we expressed either the wild-type or the mutated human lipoic acid synthetase in the *E. coli* clone JRG33, which carries the p.Glu195Lys mutation in the endogenous LIAS gene *lipA* and is auxotrophic for lipoic supplied in the growth medium.[18,19] Functional expression of a eukaryotic LIAS in *E. coli* has been previously shown for the enzyme from *Arabidopsis thaliana*.[20] The *E. coli lipA* was cloned into the PstI site of pGEM-3Zf(+) (Promega) after PCR amplification from wild-type genomic DNA with primers lipA-PstI-F 5′-CGAACTGCAGTAAACCCATT GTGATGGAAC-3′ and lipA-PstI-R 5′-CGAACTGCAGGAC GCTCCCTCAATATCT-3′ and digestion with PstI (Fermentas). The start codon of lipA was replaced by the first 21 amino acids of lacZ. The human LIAS was amplified from cDNA of either the affected individual or a control via PCR with the primers LIAS-Hind-F 5′-CCGCAAGCTTGC CAGATAAAAAAAAGGAACTC-3′ and LIAS-Hind-R 5′-CG GCAAGCTTGTGATCTTGAAGGTCTTGTTG-3′, digested with HindIII (New England Biolabs), and cloned into the HindIII site of pGEM-3Zf(+). The first 27 amino acids of human LIAS that are predicted to be a mitochondrial-targeting sequence were replaced by the first 15 amino acids of lacZ. The constructs were transformed into JRG33 carrying the pREP4 plasmid (QIAGEN) and expressed in the presence of 0.2 mmol/l isopropyl β-D-1-thiogalactopyranoside (IPTG). As shown in Figure 5, there was no difference in growth between the wild-type and the mutant human LIAS when grown in the presence of lipoic acid in the medium but there was an obvious growth defect in the mutant cells when grown in the absence of lipoic acid.

Here, we describe an individual with a defect in the synthesis of the prosthetic group lipoic acid. This

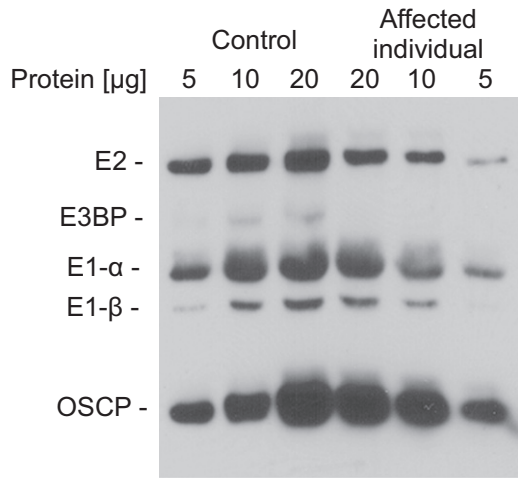
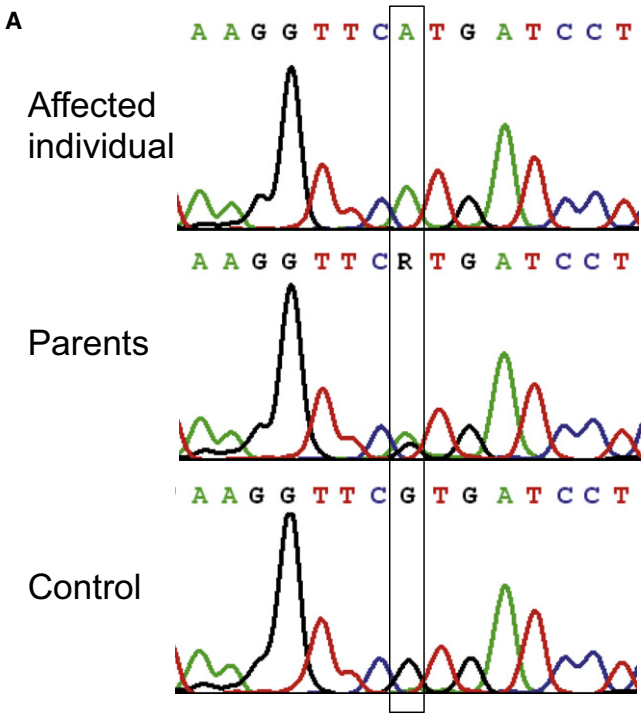


Figure 2. Immunoblot Analysis of PDHC Subunits
Immunoblot analysis with an antibody cocktail against subunits of the pyruvate dehydrogenase complex performed with samples of muscle from the affected individual showed a decrease of E2 and E3 subunit binding protein, whereas the subunits E1α and E1β were found in normal amounts in comparison to the subunit OSCP of the ATP synthase.



B

Affected individual	**H**	
H. sapiens	ETVPELQSKVRDPRANFDQSL	259
M. musculus	ETVPELQRKVRDPRANFDQSL	258
D. melanogaster	ETVEKLTPYVRDRRAHYRQTL	256
S. cerevisiae	ETVESLTPHVRDRRATYRQSL	303
A. thaliana	ETVKRLQRLVRDPRAGYEQSM	280
E. coli	ENVPRIYRQVR-PGADYNWSL	215
St. aureus	ETVRRLTPRVR-ARATYDRTL	206
C. pseudotub.	ETVPRIFKRIR-PAFRYERSL	201
Consensus	*.* : :* : ::	

Figure 3. Mutation Analysis of LIAS
Sequence analysis (A) revealed the mutation c.746G>A (p.Arg249His) in *LIAS*, which affects a phylogenetically conserved amino acid (B).

deficiency was identified during in-depth investigations of a group of so-far-unresolved cases of individuals suspected to have disorders in the mitochondrial energy metabolism and defects in the oxidation of pyruvate. Because genetic investigation of subunit genes of either PDHC or its phosphatases did not reveal any conclusive abnormalities, we considered defects in the metabolism of PDHC cofactors.[21] Elevation of glycine in the diseased individual was a clue for the affection of lipoic acid metabolism because this cofactor is also needed for glycine cleavage. SNP array analysis in this consanguineous family showed, among others, a homozygous region on chromosome 4, which contained *LIAS*, the gene of the lipoic acid synthetase, a candidate gene that turned out to be affected in this individual.

Lipoic acid is needed exclusively in mitochondria and is required for the enzyme reactions of the three keto acid dehydrogenases PDHC, α-KGDH, and BCKDH as well as the glycine cleavage system. The defect of the lipoic acid synthetase resulted in lactic acidosis and glycine elevation, the former reflecting the defects within the mitochondrial energy metabolism and the latter reflecting the defect in the glycine cleavage system. Remarkably, there was no documented elevation of the branched chain amino acids leucine, isoleucine, valine, or even allo-isoleucine in the investigation of amino acids in plasma, in spite of a possible affection of BCKDH. However, the relative concentrations of these amino acids had not been studied systematically in the affected individual, and because the branched chain amino acids are essential amino acids, the concentration of these metabolites depends on the nutritional supply at the time of investigation. Normal results of branched chain amino acids can be found in individuals with maple syrup urine diseases (MIM 248600).[22] It therefore remains open whether a disturbance of branched chain amino acid metabolism contributes to the biochemical and clinical phenotype of disorders in the synthesis of lipoic acid.

The clinical course in the individual with lipoic acid synthetase deficiency was characterized by a severe metabolic crisis with hyperlactatemia and severe convulsions within the first weeks of life. The severe deterioration including massive metabolic acidosis and brain edema was devastating and led to a secondary cystic encephalopathy. Therefore, the clinical course after this crisis was superposed by this severe brain damage.

Given that the deficiency in lipoic acid affects different metabolic pathways, it can be speculated which of them is clinically most relevant. Neonatal seizures are characteristic for severe forms of nonketotic hyperglycinemia

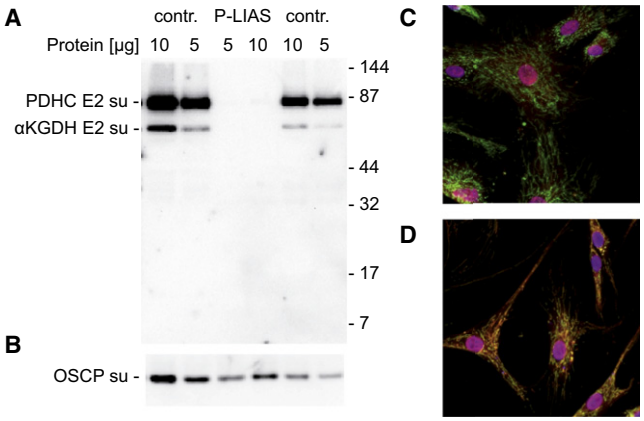


Figure 4. Immunoblot Analysis and Immunoflorescence Staining of Lipoic Acid
Immunoblot analysis with an antibody against lipoic acid (A) showed a severe decrease of the E2 subunits of both PDHC and α-KGDH in the affected individual (P-LIAS) in comparison to control muscle. Subunit OSCP of the ATP synthase (B) was used as a loading control. Immunocytochemical staining of fibroblasts showed a decreased staining with the antibody against lipoic acid (red) compared to porin (green) in cells from the affected individual (C) compared to the control (D).

(NKH [MIM 605899])[23] but can also be found in individuals with mitochondrial encephalopathies, including PDHC deficiency.[24,25] Cardiac involvement, as found in the LIAS-deficient individual, is an unusual finding in NKH[26] as well as in PDHC deficiency. Given that the heart is adapted to use fat rather than glucose as an energy source, it is comprehensible that the heart is not affected in individuals with deficiency of PDHC, an enzyme that is not necessary for fatty acid oxidation. The Krebs cycle, however, is necessary for acetyl-coenzyme A oxidation, and therefore α-KGDH is likely to be important for the cardiac energy metabolism. Isolated metabolic defects of α-KGDH have not been identified so far. However, in three individuals with a mutation in dihydrolipoamide dehydrogenase (*DLD*, E3 subunit) and predominant deficiency of α-KGDH, two of the individuals suffered from hypertrophic cardiomyopathy.[27]

The synthesis of lipoic acid involves several enzymatic steps and depends on the mitochondrial fatty synthesis (FAS II)[1–3] and iron sulfur cluster biosynthesis.[12] We hypothesize that defects in any of these steps might result in a similar biochemical and clinical phenotype. From the experience of the individual with LIAS deficiency, we conclude that in individuals with suspect mitochondrial encephalomyopathy, especially with neonatal-onset lactic acidosis, unclear pyruvate oxidation deficiency, and glycine elevation, a defect in the lipoic acid metabolism should be considered. Determination of the function of the lipoic acid prosthetic group can be performed in fibroblasts and muscle biopsies, either by immunoblotting or by quantification of the hydrolyzed cofactor.

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Web Resources

The URL for data presented herein is as follows:

OMIM (Online Mendelian Inheritance in Man): http://www.omim.org

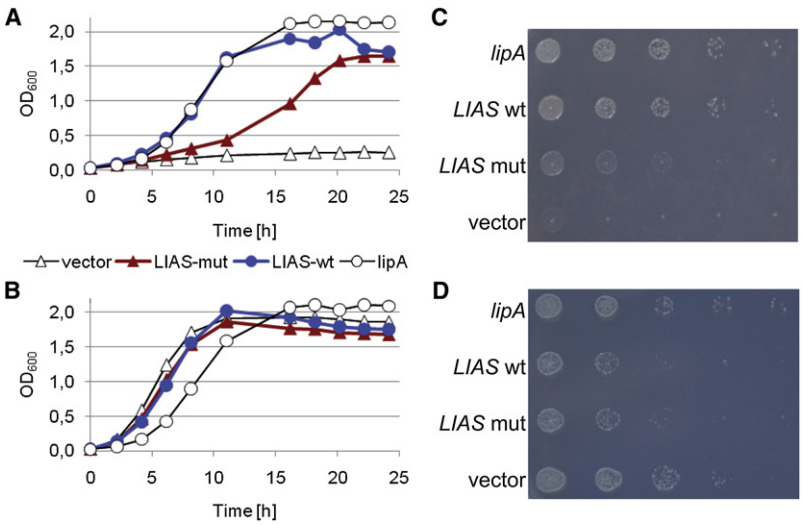

Figure 5. Lipoic Acid Synthetase Complementation Analysis in *Escherichia coli*
Complementation analysis in the lipoic acid synthetase-deficient *E. coli* strain JRG33 revealed a clear growth retardation on lipoic acid-free growth medium[19] (A and C) of cells expressing the mutant (*LIAS*-mut) versus the wild-type (*LIAS*-wt) human LIAS. The strain that expressed the *E. coli* wild-type *lipA* grew at a rate similar to that expressing the wild-type human LIAS, whereas there was no growth with the empty vector (vector). Fast growth was observed with all constructs on the same medium when supplemented with 1 ng/l lipoic acid (B and D).

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