The *Streptomyces coelicolor* Lipoate-protein Ligase Is a Circularly Permuted Version of the *Escherichia coli* Enzyme Composed of Discrete Interacting Domains*

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**Background:** Lipoate-protein ligase salvages lipoic acid from the environment.

**Results:** The domain structure of the *Streptomyces coelicolor* ligase can be restructured into that of the paradigm *Escherichia coli* ligase.

**Conclusion:** The domain architectures of lipoate ligases are plastic.

**Significance:** The domains of bacterial lipoate ligases can act as independent entities.

Lipoate-protein ligases are used to scavenge lipoic acid from the environment and attach the coenzyme to its cognate proteins, which are generally the E2 components of the 2-oxoacid dehydrogenases. The enzymes use ATP to activate lipoate to its dehydrogenases. The enzymes use ATP to activate lipoate to its adenylate, lipoyl-AMP, which remains tightly bound in the active site. This mixed anhydride is attacked by the ε-amino group of a specific lysine present on a highly conserved acceptor protein domain, resulting in the amide-linked coenzyme. The *Streptomyces coelicolor* genome encodes only a single putative lipoate ligase. However, this protein had only low sequence identity (<25%) to the lipoate ligases of demonstrated activity and appears to be a circularly permuted version of the known lipoate ligase proteins in that the canonical C-terminal domain seems to have been transposed to the N terminus. We tested the activity of this protein both by *in vivo* complementation of an *Escherichia coli* ligase-deficient strain and by *in vitro* assays. Moreover, when the domains were rearranged into a protein that mimicked the arrangement found in the canonical lipoate ligases, the enzyme retained complementation activity. Finally, when the two domains were separated into two proteins, both domain-containing proteins were required for complementation and catalysis of the overall ligation reaction *in vitro*. However, only the large domain-containing protein was required for transfer of lipoate from the lipoyl-AMP intermediate to the acceptor proteins, whereas both domain-containing proteins were required to form lipoyl-AMP.

Lipoate acid is an essential sulfur-containing cofactor found in eukaryotes, in most bacteria, and in some archaea. It is required for the function of several key enzymes involved in central carbon metabolism (1). The 2-oxoacid dehydrogenases and the glycine cleavage system contain lipoyl domains (LDs)\(^2\) that are highly conserved and contain a specific lysine residue to which lipoic acid is attached (2, 3) by an amide linkage between the lipoic acid carboxyl group and the lysine residue ε-amino group. The lipoyl moieties plays a unique role in catalysis. The terminal dithiolane ring becomes reduced and acylated with a reaction intermediate, whereas the lipoyllysine arm serves as a tether and a highly mobile carrier of reaction intermediates between the active sites of these multienzyme complexes (1).

In *Escherichia coli*, lipoic acid may be directly scavenged from the environment or synthesized *de novo* (2–6). Studies in our laboratory and others (8, 27) have elucidated two independent pathways in *E. coli* depending on the source of lipoic acid (Fig. 1). If lipoic acid is available in the environment, LpLA catalyzes both the ATP-dependent activation of lipoate to lipoyl-AMP and the subsequent transfer of the activated lipoate moiety to an apo-LD (e.g. the E2 component of a 2-oxoacid dehydrogenase) with concomitant release of AMP (Fig. 1A) (5, 7). When exogenous lipoic acid is absent, LipB, an octanoyl-acyl carrier protein (ACP) transferase, transfers the octanoyl moiety from the fatty acid biosynthetic intermediate octanoyl-ACP to the LD of a lipoate-accepting protein. The octanoylated domains are substrates for sulfur insertion by LipA, a radical S-adenosylmethionine enzyme that replaces single hydrogen atoms on carbons 6 and 8 of octanoate with sulfur atoms (8) to yield dihydrolioproyl-LD, which becomes oxidized to lipoyl-LD (Fig. 1B).

Two different bacterial lipoate synthesis pathways have been described: the *E. coli* LipB-LipA pathway and a more complex pathway in *Bacillus subtilis* that requires two additional proteins (9, 10). Although the function and protein structure of the *E. coli* LpLA lipoate ligase are well established (4, 5, 7, 11), the presence of this activity in other bacteria has been demonstrated only in *B. subtilis*, in which the ligase is called LplJ (10), and in *Listeria monocytogenes*, a bacterium related to *B. subtilis* that is auxotrophic for lipoic acid and that encodes two ligases, both of which function in lipoic acid scavenging (12). Like *B. subtilis*, *Streptomyces coelicolor* is a Gram-positive soil bacterium. This laboratory previously predicted that the putative lipoate-protein ligase of this bacterium might have a circularly permuted architecture relative to the
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well characterized E. coli LplA protein (13). Although the structurally characterized E. coli and Streptococcus pneumoniae LplA proteins have a large N-terminal domain that contains the lipoic acid-binding site plus a small C-terminal domain (Fig. 1C) (11), in the S. coelicolor protein, the small domain appears to have been transposed to the N terminus. Another variation is seen in the mammalian lipoil transferase, which, although sharing 34% sequence identity with E. coli LplA and slightly larger, is unable to synthesize the lipoil-AMP intermediate. The mammalian protein functions only to transfer the lipoil moiety from the adenylate to the lipoate protein (14). A third type of lipoate ligase is found in the thermophile archaeon Thermoplasma acidophilum, in which the ligase is composed of two separate proteins, LplA and LplB, encoded by adjacent genes (13, 15–18). Both LplA and LplB are required for lipoil-AMP formation, but LplA alone is sufficient for lipoil transferase activity (13, 18). A recent crystal structure shows that the two T. acidophilum proteins interact to form a structure with a domain orientation similar to that of the E. coli protein (18). Given the low sequence identity to known ligases plus conservation of the E. coli domain arrangement even in an enzyme in which the arrangement is not dictated by covalent bonding, the question arises as to the whether or not the S. coelicolor protein is a functional LplA protein able to catalyze the overall ligation reaction. Moreover, identification of a protein as encoding a lipoate-protein ligase is not straightforward because members of this biotin protein ligase-LplA-LipB protein family (Pfam Clan CL0040) catalyze three other reactions: octanoyl transfer from octanoyl-ACP, amido transfer in B. subtilis, and ligation of biotin to its cognate proteins. We report that the S. coelicolor protein is indeed a fully functional lipoate ligase and that, upon separation of the two domains into two polypeptide chains, the domains of S. coelicolor LplA interact and carry out both the lipoate activation and lipoil transfer partial reactions.

**EXPERIMENTAL PROCEDURES**

**Chemicals, Bacterial Strains, and Growth Medium**—The antibiotics and most chemicals used in this study were purchased from Sigma and Fisher unless noted otherwise. American Radiolabeled Chemicals provided [α-32P]ATP. Oligonucleotide primers were synthesized by Integrated DNA Technologies and are shown in Table 1. PCR amplification was performed using Pfu (Stratagene) or Taq (New England Biolabs) polymerase. Restriction enzymes and T4 DNA ligase were supplied by New England Biolabs. DNA sequencing was performed by AGCT, Inc. Invitrogen provided the Ni2+-agarose column. S. coelicolor A3 genomic DNA was from a laboratory stock. Antibiotics were used at the following concentrations: ampicillin sodium, 100 μg/ml; kanamycin sulfate, 50 μg/ml; and chloramphenicol, 40 μg/ml. L-Arabinose was used at a final concentration of 0.2%. The bacterial strains used were derivatives of E. coli K12 (Table 1). The rich medium used for E. coli growth was LB broth.

**Plasmid Constructions**—All plasmids used and constructed in this study are shown in Table 1. The S. coelicolor lplA gene (Kyoto Encyclopedia of Genes and Genomes (KEGG) entry SCO6423) was amplified by PCR from genomic DNA of strain A3 using Pfu DNA polymerase with primers Xc001 and Xc002, which added BspHI and HindIII restriction sites. The product was digested with BspHI and HindIII restriction enzymes and ligated into Ncol- and HindIII-cut pBAD332C downstream of an arabinose-inducible promoter to give plasmid pXC001. The putative lplA gene was amplified in a similar manner using the primers listed in Table 2 and inserted into the BamHI and Xhol sites of pET28b to express an N-terminally hexahistidine-tagged protein.

Plasmid pGS331, which expresses an E. coli E2 LD under the control of the tac promoter (19, 20), was the source of the LD71_EC domain. The LDs of S. coelicolor branched-chain 2-oxoacid dehydrogenase E2 (KEGG entry SCO3815), S. coelicolor gcvH (KEGG entry SCO5471), and E. coli gcvH (KEGG entry SCO5471) were amplified by PCR from genomic DNA of strain A3 using Pfu DNA polymerase with primers Xc001 and Xc002, which added BspHI and HindIII restriction sites. The product was digested with BspHI and HindIII restriction enzymes and ligated into Ncol- and HindIII-cut pBAD332C downstream of an arabinose-inducible promoter to give plasmid pXC001. The putative lplA gene was amplified in a similar manner using the primers listed in Table 2 and inserted into the BamHI and Xhol sites of pET28b to express an N-terminally hexahistidine-tagged protein.

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**FIGURE 1.** Lipoic acid metabolism in E. coli. A, when lipoic acid is provided in the environment, LplA catalyzes the ligation reaction in two steps. First, lipoic acid is activated to lipoil-AMP with concomitant release of pyrophosphate. In the presence of a LD acceptor protein, the lipoil moiety is transferred to the LD (e.g. the E2 component of pyruvate dehydrogenase). B, LipB, which is an octanoyl-ACP transferase, transfers the octanoyl moiety from the fatty acid biosynthetic intermediate octanoyl-ACP to the LD of a lipoate-accepting protein. The octanoylated domains then become substrates for sulfur insertion by LipA, a radical S-adenosylmethionine enzyme that replaces one hydrogen atom in each of octanoate carbons 6 and 8 with sulfur atoms. C, different arrangements of coding sequences and domains found in E. coli LplA, the putative S. coelicolor enzyme, and the T. acidophilum bipartite ligase. For review, see Ref. 2.
entry b2904) were amplified by PCR from the corresponding genomic DNAs with primers XC005–XC010. All primers added BspHI and HindIII sites. The products were digested with these restriction enzymes and ligated into Ncol- and HindIII-digested pET28b to give plasmids pXC003–pXC005 (Table 1). Using the same approach, the large and small domains of \( S. \) coelicolor were inserted into plasmids pBAD322C and pKK232, respectively, using primers XC015/XC016 and XC017/XC018 to give pXC006 and pXC007. Using primers XC019/XC020 and XC021/XC022, the two domains were also individually inserted into the pET28b vector to obtain pXC008 and pXC009.

To obtain the circularly permuted \( S. \) coelicolor \( lplA \) gene, overlap extension PCR was performed. The large and small domains of \( S. \) coelicolor \( lplA \) were first amplified by PCR using primers XC024-XC025 and XC016–XC023 (Table 2), respectively. The \( E. \) coli \( lplA \) linker sequence was added to primers XC023 and XC024 to provide a spacer between the large and small domains of the circularly permuted \( lplA \) gene from \( S. \) coelicolor. PCR products from the first round of amplification were purified using a QIAquick PCR purification kit (Qiagen). In the second round of PCR amplification, the purified large and small domain PCR products were used as templates, and XC025 and XC016 were used as primers to insert Ncol and HindIII sites. The final PCR product was digested with the same enzymes and inserted into pBAD322C digested with the same enzymes to give pXC010. All plasmids constructed were verified by DNA sequencing.

### Protein Expression and Purification

The coding sequences of the wild-type \( S. \) coelicolor \( lplA \) gene and its small and large domains were inserted into vector pET28b to generate pXC002, pXC008, and pXC009, respectively, to express N-terminally hexahistidine-tagged proteins. Each plasmid was expressed in the \( E. \) coli Tuner (BL21) strain. One-liter cell cultures expressing wild-type \( LplA \) and the \( LplA \) small domain and 4-liter cultures of the \( LplA \) large domain were grown at 37 °C in LB broth until \( A_{600} = 0.8 \), induced with 0.5 mM isopropyl 1-thio-\( \beta \)-d-galactopyranoside, and allowed to grow for another 16 h at 18 °C before harvesting. The cells were collected by centrifugation and resuspended in lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, and 1 mM dithiothreitol, pH 7.4) and lysed by French pressure cell treatment. Cell debris was removed by centrifugation at 48,000 × g for 40 min. The supernatant was loaded onto a nickel chelate resin (Qiagen) and allowed to bind to the resin for >5 h. The column was washed with 10 column volumes of wash buffer (50 mM sodium phosphate, 300 mM NaCl, 50 mM imidazole, and 1 mM dithiothreitol, pH 7.4) to remove contaminating proteins, and the His-tagged protein was eluted with wash buffer containing 200 mM imidazole. The protein was concentrated by ultrafiltration (Amicon Ultra 10- or 3-kDa cutoff) and dialyzed overnight. Protein concentrations were determined at 280 nm using extinction coefficients calculated from the ProtParam program on the ExPASy tool website. Protein purity was monitored by SDS-PAGE.

### Purification of LD Substrates

The E2 LD and GcvH (glycine cleavage \( H \)) proteins from \( E. \) coli and \( S. \) coelicolor were purified by precipitation and ion exchange chromatography according to the methods described previously (6). Plasmids pXC003–pXC005 encoded the native proteins (Table 2). The anion exchange chromatography protocol allowed resolution of the apo and holo forms of the domain as shown on a 20% native polyacrylamide gel. Pure apo-domain was dialyzed, flash-frozen in dry ice, and stored in –80 °C. The purified LDs were submitted to the University of Illinois at Urbana-Champaign Mass Spectrometry Laboratory for electrospray mass spectral analysis.

### Complementation Analyses

The \( E. \) coli \( \Delta lplB \) \( \Delta lplA \) strain QC146 was used for complementation. To prevent carryover of lipoic acid, all plasmid-carrying strains were grown for 1 day on the same medium containing 0.4% glycerol, appropriate antibiotics, 5 mM acetate, and 5 mM succinate to bypass the lipoic acid-requiring aerobic pathways. Strains were then grown overnight at 37 °C on M9 minimal plates (41) with and without supplementation with lipoic acid (1 mM). Glycerol was used as the carbon source in the absence of arabinose.

### Western Blot Analysis

Strains MG1655, QC146, XC001, and XC002 were grown to \( A_{600} = 0.8 \) in 20 ml of LB broth with

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**Table 1**

| Bacterial strains and plasmids | Relevant genotype or description | Ref. or derivation |
|-------------------------------|---------------------------------|--------------------|
| **E. coli strain** | | |
| MG1655 | Wild-type \( E. \) coli K12 | Lab stock |
| DH5α | Δ(argF lacZΔM15 recA1 endA1) | Lab stock |
| QC146 | ΔlipB ΔlplA of MG1655 | Novagen |
| XC001 | QC146 carrying pXC001 | This study |
| XC002 | QC146 carrying pXC010 | This study |
| **Plasmid** | | |
| pET28b | T7 promoter expression vector, Kan\( ^{\beta} \) | Novagen |
| pBAD322C | Low copy expression vector, Cm\( ^{\beta} \) | This study |
| pXC001 | pBAD322C encoding \( S. \) coelicolor \( lplA \) | This study |
| pXC002 | pET28b encoding N-terminally hexahistidine-tagged \( S. \) coelicolor \( lplA \) | This study |
| pG533 | \( E. \) coli E2 LD | Refs. 19 and 20 |
| pXC003 | pET28b encoding native \( S. \) coelicolor \( E2 LD \) | This study |
| pXC004 | pET28b encoding native \( S. \) coelicolor GcvH | This study |
| pXC005 | pET28b encoding native \( E. \) coli GcvH | This study |
| pXC006 | pBAD322C encoding \( S. \) coelicolor \( lplA \) small domain | This study |
| pXC007 | pKK223 encoding \( S. \) coelicolor \( lplA \) large domain | This study |
| pXC008 | pET28 encoding N-terminally hexahistidine-tagged small domain of \( S. \) coelicolor \( lplA \) | This study |
| pXC009 | pET28 encoding N-terminally hexahistidine-tagged large domain of \( S. \) coelicolor \( lplA \) | This study |
| pXC010 | pBAD322C encoding circularly permuted \( S. \) coelicolor \( lplA \) | This study |
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**Table 2**

| Oligonucleotide | Restriction site | Sequence (5′-3′) |
|-----------------|-----------------|------------------|
| XC001           | LplA forward, BspHI | ATATGTGCCCTGGCCACGGAGCGG |
| XC002           | LplA reverse, HindIII | ATCGAAGTCTGGCCACGGAGCGG |
| XC003           | LplA forward, BamHI | AGCTGTTATGCTGGCCACGGAGCGG |
| XC004           | LplA reverse, XhoI | GCCCTGAGTCGACGGACCGGGGCCAAG |
| XC005           | S. coelicolor E2 LD forward, BspHI | ATGCTTATGCTGGCCACGGAGCGG |
| XC006           | S. coelicolor E2 LD reverse, HindIII | ATGCTTATGCTGGCCACGGAGCGG |
| XC007           | S. coelicolor GcvH forward, BamHI | AGCTGTTATGCTGGCCACGGAGCGG |
| XC008           | S. coelicolor GcvH reverse, HindIII | ATGCTTATGCTGGCCACGGAGCGG |
| XC009           | E. coli GcvH forward, BspHI | ATGCTTATGCTGGCCACGGAGCGG |
| XC010           | E. coli GcvH reverse, HindIII | ATGCTTATGCTGGCCACGGAGCGG |
| XC011           | Small domain forward, BspHI | ATGCTTATGCTGGCCACGGAGCGG |
| XC012           | Small domain reverse, HindIII | ATGCTTATGCTGGCCACGGAGCGG |
| XC013           | Small domain forward, EcoRI | ATGCTTATGCTGGCCACGGAGCGG |
| XC014           | Large domain reverse, Psf | ATGCTTATGCTGGCCACGGAGCGG |
| XC015           | Large domain forward, NdeI | ATGCTTATGCTGGCCACGGAGCGG |
| XC016           | Large domain reverse, XhoI | ATGCTTATGCTGGCCACGGAGCGG |
| XC017           | Large domain forward, XhoI | ATGCTTATGCTGGCCACGGAGCGG |
| XC018           | Large domain reverse, XhoI | ATGCTTATGCTGGCCACGGAGCGG |
| XC019           | Small domain forward, NdeI | ATGCTTATGCTGGCCACGGAGCGG |
| XC020           | Small domain reverse, XhoI | ATGCTTATGCTGGCCACGGAGCGG |
| XC021           | Large domain forward, NdeI | ATGCTTATGCTGGCCACGGAGCGG |
| XC022           | Large domain reverse, XhoI | ATGCTTATGCTGGCCACGGAGCGG |
| XC023           | Small domain forward, with E. coli linker | ATGCTTATGCTGGCCACGGAGCGG |
| XC024           | Large domain reverse, with E. coli linker | ATGCTTATGCTGGCCACGGAGCGG |
| XC025           | Large domain forward, Ncol | ATGCTTATGCTGGCCACGGAGCGG |

additional lipoic acid (5 μg/ml) and arabinose (0.2%). Soluble fractions of whole cell extracts were analyzed by SDS-PAGE. Equal amounts of protein were loaded and separated on a 12% SDS-polyacrylamide gel and transferred by electrophoresis to Immobilon-P membranes (Millipore) for 15 min at 15 V. The membranes were preblocked with TBS buffer (100 mM Tris base and 0.9% NaCl, pH 7.5) containing 0.1% Tween 20 and 5% nonfat milk powder. The membranes were probed for 1 h with an anti-lipoil protein primary antibody (Calbiochem) diluted 1:10,000 in the above buffer. Following incubation with a goat anti-rabbit secondary antibody (diluted 1:5000; Roche Applied Science), the labeled proteins (pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase) were detected using Quantity One software.

**Structural Modeling and Sequence Alignment**—A model of the small domain of S. coelicolor LplA was determined by threading it with the T. acidophilum LplB crystal structure (Protein Data Bank ID 3R07, chain C) using the automated mode of SWISS-MODEL (21–23). The S. coelicolor LplA large domain was likewise threaded using T. acidophilum LplA (Protein Data Bank ID 3R07, chain A) as the template. The final image was generated using the UCSF Chimera package (24). Sequence alignment was conducted using ClustalW2 (25), and the final output shown in Figs. 3 and 5 was created by ESPript 3.0 (26).

**Gel Shift Assay for LD Modification Analysis**—The reaction (20 μl) contained 50 mM sodium phosphate, pH 7.0, 1 mM sodium lipoate, 5 mM disodium ATP, 5 mM dithiothreitol, 1 mM MgCl₂, and 20 μM apo-LD (or apo-GcvH). The S. coelicolor small (2 μM) and large (4 μM) domains were added as indicated in Fig. 8. The reactions (20 μl) were incubated at 37 °C for 4 h, loaded on a 15% native polyacrylamide gel containing 2.5 M urea, and separated by electrophoresis.

Lipoil-AMP was chemically synthesized according to the method of Reed et al. (27). The product was confirmed by mass spectral analysis performed by the University of Illinois at Urbana-Champaign Mass Spectrometry Laboratory. Lipoil-AMP was dissolved in 100 mM sodium phosphate, pH 7.0. The reaction (20 μl) contained the following components: 50 mM sodium phosphate, pH 7.0, 1 mM tris(2-carboxyethyl)phosphine, 5 mM dithiothreitol, 0.1 mM MgCl₂, 20 μM S. coelicolor apo-LD, 1 mM synthetic lipoyl-AMP, and one of the following: 2 μM small domain, 4 μM large domain, or 2 μM wild-type S. coelicolor LplA. A gel shift assay to analyze S. coelicolor LD modification was performed as described above after incubation of the reaction at 37 °C for 4 h.

**Mass Spectrometry of LDs**—The reaction mixtures (100 μl) contained 50 mM sodium phosphate, pH 7.0, 1 mM sodium lipoate, 5 mM disodium ATP, 5 mM dithiothreitol, 1 mM MgCl₂, and 20 μM apo-LD with or without 2 μM wild-type S. coelicolor LplA and were incubated at 37 °C for 4 h as indicated. The reactions were dialyzed overnight against 200 mM ammonium acetate buffer and dried the next day under a nitrogen stream. Samples were submitted to the University of Illinois at Urbana-Champaign Mass Spectrometry Laboratory for electrospray mass spectrometric analysis.

**Assay of Enzymatic Lipoil-AMP Intermediate Formation**—The reactions contained 50 mM sodium phosphate, pH 7.0, 1 mM tris(2-carboxyethyl)phosphine, 10 mM [α-32P]ATP, 10 μM MgCl₂, 0.1 mM sodium lipoate, 20 or 10 μM apo-LD as the acceptor protein, 20 or 10 μM small domain, 8 or 4 μM large domain, and 2 μM wild-type S. coelicolor LplA as indicated in Fig. 8. The reaction was incubated for 1 h at 37 °C. One μl of each reaction mixture was spotted on cellulose TLC plates and developed in isobutyrlic acid/NH₄OH/water (66:1:33). The TLC plates were dried overnight, exposed to a phosphorimaging plate, and visualized using a Fujifilm FLA-3000 system.

**Bioinformatics and Phylogenetic Analysis**—The amino acid sequences of different species were retrieved from the BLAST page of the ExPaSy server using the Swiss-Prot+TrEMBL+TrEMBL_NEW method (28, 29). Both the number of best scoring sequences and the number of best alignments were set to 1000. The e-values of the sequences selected were between 10⁻¹⁰ and 10⁻⁵. Multiple sequence alignments were done using ClustalW. Gap-rich columns were ignored (30). The poorly conserved N and C termini were removed. The phylogenetic tree was constructed using the maximum likelihood method with the PhyML program (31, 32). PHYLIP
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FIGURE 2. Expression of the S. coelicolor protein complements growth of the E. coli ΔlipB ΔlipA strain QC146. Strain QC146 was transformed with a pBAD322C-derived plasmid that expresses S. coelicolor LplA from an arabinose-inducible promoter. The control strains were the wild-type strain and strain QC146 containing the empty vector (pBAD322C). Complementation proceeded both in the presence and absence of arabinose induction. The plates above lacked arabinose.

Interleaved was used for alignment. Bootstrap analysis was set to 1000 replicates.

RESULTS

The Putative S. coelicolor LplA Protein Is a Bona Fide Lipoate-protein Ligase—The amino acid sequence of the putative S. coelicolor LplA protein shares only marginal sequence identity (~25%) with E. coli LplA and the other lipoate ligases of known activity, and the putative small domain shows no significant similarity. Indeed, even when the domains were rearranged in silico to allow alignment over the complete E. coli LplA sequence the identity is only 24%. To determine whether the S. coelicolor protein has LplA activity, we first tested its ability to restore growth of the strain QC146 with lipoic acid supplementation. To determine whether the putative L. lactis LplA gene would complement growth of the E. coli ΔlipB ΔlipA strain QC146 (13). The ΔlipB and ΔlipA deletions of this strain result in an inability to synthesize lipoate or to scavenge lipoic acid from the medium (5, 13). The putative S. coelicolor lplA gene was inserted into the arabinoinducible vector pBAD322C and transformed into strain QC146. Complementation was tested on M9 minimal agar plates with glycerol as the sole carbon source to avoid bypass of succinate- and acetate-dependent growth by fermentative metabolism (33). Due to the E. coli QC146 ΔlipA mutation, the strain is unable to grow with lipoic acid supplementation; however, growth proceeds robustly when a plasmid encoding lipoate ligase activity is present (5, 10, 13). Upon expression of the putative S. coelicolor lplA-expressing gene, the E. coli strain grew well, but only when the medium contained lipoic acid (Fig. 2). Growth proceeded in either the presence or absence of arabinose. Growth in the absence of arabinose suggests that only low levels of the S. coelicolor protein are required to catalyze attachment of exogenous lipoic acid to the 2-oxoacid dehydrogenases of E. coli.

To characterize the function of S. coelicolor LplA in vitro, we purified the protein to homogeneity (see “Experimental Procedures”) (Fig. 3C). The ligation activity of the protein was tested in vitro with the E2 LD and GcvH proteins from both E. coli and S. coelicolor as acceptor proteins (Figs. 3 and 5). Note that the lysine residue modified by lipoate attachment in the S. coelicolor dehydrogenase domain is within the AKA sequence rather than the typical DKA sequence found in the E. coli 2-oxoacid dehydrogenase domains (Fig. 3). However, the aspartate residue does not play an important role in lipoylation. The AKA sequence of an Azotobacter vinelandii LD is fully lipoylated when expressed in E. coli (34), and proteins with valine (E. coli GcvH), histidine (pea GcvH) (35), or methionine (36) in place of the aspartate are excellent lipoylation substrates. The residue following the modified lysine is also not strictly conserved. In S. coelicolor GcvH, the lysine residue is within an AKS sequence (see Fig. 5). However, methionine and valine are also functional as the residues following the lysine residue (36). The use of E2 LDs allows detection of modification by gel shift assays and electrospray mass spectrometry. We found that, in the presence of lipoic acid and ATP, the E2 LD was modified as shown by the more rapid migration of the protein on native gel electrophoresis due to loss of the positive lysine charge upon modification (Fig. 4A). Electrospray mass spectrometry further confirmed that the molecular mass of the modified E2 LD was the same that as of the LD (Fig. 4B). The GcvH proteins are slightly larger than the E2 LDs, and their modification could not be distinguished on native gels. Thus, [1-14C]octanoic acid, a substrate of E. coli LplA (in vivo and in vitro (6, 7)), was used in place of lipoic acid. S. coelicolor LplA modified both E. coli and S. coelicolor GcvH proteins, although the native protein seemed to be a better substrate (Fig. 5C). These data demonstrate that, despite its atypical domain arrangement and its low sequence similarity to the documented lipoate ligases, S. coelicolor LplA is a fully functional lipoate ligase.

S. coelicolor LplA Retains Function When Its Domain Architecture Is Altered to That of E. coli LplA—The finding that the separate proteins of the T. acidophilum ligase interact to form a structure with the same domain orientation as that of the E. coli ligase suggests that restructing the domain orientation of S. coelicolor LplA into that of E. coli might result in an inactive protein. To test this, we constructed a circularly permuted version of S. coelicolor LplA with the domain orientation of E. coli LplA. Construction of circularly permuted proteins requires cleavage of the native sequence without perturbation of the domain structures and covalent linking of the original N and C termini without untoward disturbance of the structure (Fig. 6A). Because the structure of S. coelicolor LplA was unknown, the first challenge was to find an appropriate junction between the small and large domains. SWISS-MODEL Workspace was used to construct a model of S. coelicolor LplA using the T. acidophilum LplA-LplB structure (18) as the template. Several candidate cleavage positions were chosen and tested for the ability to complement growth of strain QC146. The next challenge was to find a proper linker to fuse the two domains in their new orientation. For this purpose, we utilized the interdomain linker of E. coli LplA, an 8-residue segment with sequence FGQAPAFS that orients the two domains to form a substrate binding-pocket in the lipoate activation step and that plays a role in rotation of the small domain in the lipoyl moiety transfer step (11).

We inserted the genes encoding the circularly permuted proteins into the pBAD322C vector and tested complementation of E. coli strain QC146 as described above. Only one of the three constructs allowed growth: the least truncated construct with the cleavage position between His-126 and Ala-127. The rearranged S. coelicolor LplA protein shared ~24% identity with E. coli LplA. To test the enzyme activity of this protein in vitro, we purified the N-terminally hexahistidine-tagged protein by nickel chelate chromatography. Unfortunately, the protein
showed no detectable activity in vitro in either the gel shift or radioactive assays. Several additional constructs encoding proteins with cleavable and C-terminal hexahistidine tags were made, but all failed to show detectable in vitro activity. The difference in the in vivo and in vitro results is most likely explained by the greater sensitivity of the in vivo assay. Only trace amounts of lipoic acid are required for growth of E. coli (33). Indeed, Western blot analysis of cell extracts with anti-lipoic acid antibody showed low levels of lipoylation in strain QC146 expressing the circularly permuted LplA protein (Fig. 6C). A protein band of lipoylated 2-oxoglutarate dehydrogenase E2 was readily seen, whereas the lipoylated pyruvate dehydrogenase E2 band was faint (Fig. 6C). The faint pyruvate dehydrogenase band is due to the presence of three LDs on the E2 protein, which causes the protein to run in abnormally diffuse bands on SDS gels (only one of the three domains is required for enzyme activity) (7, 37). Therefore, the complementation data were confirmed by the Western blot results.

Both the Large and Small Domains Are Required to Activate Lipoate to Lipoyl-AMP, whereas Only the Large Domain Is Required for Lipoyl Transfer Activity—The success of the circular permutation arrangement suggested that the interactions between the two domains of S. coelicolor LplA are sufficiently strong that the enzyme might function when the two domains are separate protein molecules, as is the case in the enzyme from the archaeon T. acidophilum. With the same cleavage site used to make the permuted construct, we separated the domains in silico and threaded the structure of the large and small domains using SWISS-MODEL Workspace on the structures of LplA and LplB of T. acidophilum, respectively (Fig. 6B). Despite the low sequence identities between the two ligases, threading was successful. The subgenes encoding the S. coelicolor LplA small and large domains were inserted into vectors pBAD322C and pKK223, respectively. Complementation of the E. coli lipoate auxotrophic strain QC146 showed that the strain grew well when both plasmids were present, whereas strain QC146 expressing either plasmid alone failed to grow (Fig. 7). This indicates that both domains are required for the overall lipoylation reaction. To test which domain(s) are required for each partial reaction, we purified the two domains using N-terminal hexahistidine tags. Purification of the large domain was problematical due to precipitation of the protein. After several protocols were tried, we found that nickel ion affinity chromatography followed by size exclusion chromatography gave modest amounts of soluble protein (Fig. 8A). The overall lipoylation activity was tested by gel shift assay with the S. coelicolor LD as the acceptor protein (data not shown) and by mass spectral analysis (Fig. 8B). LD modification was seen only when both
domains were present. To test the first step of the enzyme reaction (activation of lipoic acid with ATP to form lipoyl-AMP), [α-32P]ATP was used as the substrate. The reaction products were analyzed by cellulose TLC and visualized by phosphorimaging. In this system, lipoyl-AMP is the most rapidly migrating product (Fig. 8C). When wild-type S. coelicolor LplA was assayed in the absence of the LD, lipoyl-AMP was formed. On the basis of prior results with various lipoate and biotin ligases, we expected that most of the lipoyl-AMP would remain stably bound within the active site, thus limiting the amount of product formed. Indeed, this seems to be the case because upon addition of the LD acceptor protein, the accumulation of the other product, AMP, markedly increased due to transfer of the lipoyl group from lipoyl-AMP to the LD. Most important, lipoyl-AMP was formed only when both domain-containing proteins were present, and thus, two domains are required for lipoate activation. Finally, we tested the role of each domain in the second step (transfer of the lipoyl moiety to the LD) using chemically synthesized lipoyl-AMP as the substrate. Gel shift assays (Fig. 8D) indicated that LD modification required only the large domain of S. coelicolor LplA.

The Conserved GDFF Sequence Does Not Play a Significant Role in S. coelicolor LplA Domain Interactions—Previous studies in our laboratory demonstrated that, in the lipoate ligase family, the small domain contains a highly conserved GDFF motif (13), in which the aspartate residue is best conserved. In E. coli, the Asp residue faces the catalytic domain and is in close proximity to the loop formed by residues 69–76 (11), which are highly conserved and form the active site that binds lipoate in the catalytic domain. We substituted several different residues (Ala, Lys, and Arg) for Asp-66 in the S. coelicolor LplA small domain. Growth curves showed that the mutation reduced the rate of exponential growth of the E. coli lipoate auxotroph strain QC146, but mutant strains continued to grow and reached the same final cell density (data not shown). Thus, the conserved Asp residue plays only a modest role in enzyme activity. This is consistent with the large number of interactions between the large and small subunits seen in the T. acidophilum ligase structure (18).

Bioinformatics Analysis of S. coelicolor LplA—A phylogenetic tree was constructed using the maximum likelihood method. The phylogeny of S. coelicolor LplA with the circularly permuted architecture was determined with other LplA homologs (Fig. 9). E. coli LplA with the C-terminal small domain and the mammalian (Bos taurus) lipoyl transferase that has a small domain of unknown function were included as an outgroup. This analysis revealed that the S. coelicolor LplA homologs form a close clade with other Actinomycetes. Interestingly, some strains of Rhizobiales and Burkholderiales have proteins that are highly similar to strains of S. coelicolor, although they have a remote evolutionary ancestry.

DISCUSSION

The Pfam-A biotin/lipoate A/B protein liga family includes both classical ligases and other enzymes catalyzing acyl transfer. The classical ligases (those that produce AMP) seem to have a consistent overall architecture: a large N-terminal domain and a small C-terminal domain. This architecture persists even when the domains reside in separate proteins, as in the T. acidophilum lipoate ligase. However this "rule" has now been broken by the demonstration that a ligase with the opposite architecture, S. coelicolor LplA, is fully functional. Moreover, the S. coelicolor enzyme retains some activity when it is manipu-
lated into the classical ligase architecture and also functions when the two domains are divided into separate proteins. *S. coelicolor* LplA stands out among the classical ligases of this family. The bacterial biotin ligases have a large N-terminal domain and a small C-terminal domain. The catalytic region of the eukaryotic biotin ligases follows the same architecture, although these enzymes have very large and variable N-terminal extensions that double the size of the proteins and that function in binding the acceptor substrate. Thus far, no example of a bipartite biotin ligase has been reported; thus, the interesting notion that, in analogy to LplA, the C-terminal domain of the biotin enzymes is required for biotin-adenylate synthesis but not for biotin transfer has not been tested.

The most curious of the lipoate ligase family proteins are the human and bovine lipoyl transferases, which are unable to perform the first partial reaction: synthesis of the adenylate. The large domains of the mammalian lipoyl transferases and *E. coli* LplA share ~30% identity, whereas the small domains show few...
identical residues. In the lipoate activation partial reaction, the small domain of *E. coli* LplA moves toward the large domain to form the lipoic acid-binding pocket, whereas in the second partial reaction, the small domain rotates away from the large domain by $180^\circ$ (11). In contrast, the small domain of the bovine lipoyl transferase is always extended, and the region dynamically moves to the ligand side and forms an adenylate-binding loop (38). However, in the *E. coli* LplA structure, the loop equivalent to the adenylate-binding loop is disordered because of steric hindrance caused by the altered conformation of the small domain (11). Despite these differences, we investigated whether the presence of the *S. coelicolor* small domain would permit lipoate activation by the human lipoyl transferase large domain. This was tested both as separate domains and by fusing the two domains using the *E. coli* LplA interdomain linker (as in the reverse *S. coelicolor* construct). We also fused the *E. coli* small domain to the human lipoyl transferase large domain at a triplet sequence (WDW) that is conserved in both proteins and that is located within a highly conserved region close to the ends of both large domain sequences. None of these constructs allowed growth of the *E. coli* lipoate auxotroph strain QC146. Note that adenylate

**FIGURE 7. Lipoylation activity of the individual separated domains of *S. coelicolor* LplA.** The *E. coli* ΔlipB ΔlplA strain QC146 was transformed with a plasmid encoding the small domain (DOM) and/or a second plasmid encoding the large domain. Wild-type MG1655 was used as the control strain.

**FIGURE 8. Purification of the small and large domains of *S. coelicolor* LplA and the enzymatic role of each domain.** A, SDS-PAGE profile of the purified small and large domains of *S. coelicolor* LplA. Lane 1, small domain (13 kDa); lane 2, large domain (29 kDa). M, molecular mass. B, electrospray mass spectrometric analysis of lipoylated LD73_SC (calculated mass of 7896.5 Da) produced by a mixture of the separately expressed and purified *S. coelicolor* small and large domain-containing proteins. intensity; a.u., arbitrary units. C, TLC analysis of products formed from $[^{32}P]ATP$. Ligase activity was assayed using different forms of *S. coelicolor* (Sc.) LplA. Both synthesis of lipoyl-5'-AMP and transfer of the lipoyl moiety to the LD73_SC acceptor protein are shown. ++ indicates that the concentration of enzymes added was twice than that indicated by +. D, transfer of the lipoyl moiety from synthetic lipoyl-AMP assayed by the gel shift assay. Lane 1, apo-LD LD73_SC; lane 2, holo-LD LD_73SC; lanes 3 and 4, only the small (S) domain; lanes 5 and 6, only the large (L) domain; lanes 7 and 8, both domains. Note that transfer to the acceptor protein resulted in an increase in AMP production.
A New Form of Lipoate-protein Ligase

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Phylogenetic tree of lipoate-protein ligases of the Actinomycetes. The tree is drawn to scale. The branch lengths are in the same units as the evolutionary distances. E. coli LplA and B. taurus lipoyl transferase were used as the related outgroup.

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