Bacterial lyso-form lipoproteins are synthesized via an intramolecular acyl chain migration

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All bacterial lipoproteins share a variably acylated N-terminal cysteine residue. Gram-negative bacterial lipoproteins are triacylated with a thioether-linked diacylglycerol moiety and an N-acyl chain. The latter is transferred from a membrane phospholipid donor to the α-amino terminus by the enzyme lipoprotein N-acyltransferase (Lnt), using an active-site cysteine thioester covalent intermediate. Many Gram-positive Firmicutes also have N-acylated lipoproteins, but the enzymes catalyzing N-acylation remain uncharacterized. The integral membrane protein Lit (lipoprotein intramolecular transacylase) from the opportunistic nosocomial pathogen Enterococcus faecalis synthesizes a specific lysoform lipoprotein (N-acyl S-monoacylglycerol) chemotype by an unknown mechanism that helps this bacterium evade immune recognition by the Toll-like receptor 2 family complex. Here, we used a deuterium-labeled lipoprotein substrate with reconstituted Lit to investigate intramolecular acyl chain transfer. We observed that Lit transfers the sn-2 ester-linked lipid from the diacylglycerol moiety to the α-amino terminus without forming a covalent thioester intermediate. Utilizing Mut-Seq to analyze an alanine scan library of Lit alleles, we identified two stretches of functionally important amino acid residues containing two conserved histidines. Topology maps based on reporter fusion assays and cysteine accessibility placed both histidines in the extracellular half of the cytoplasmic membrane. We propose a general acid–base catalytic mechanism, invoking direct nucleophilic attack by the substrate α-amino group on the sn-2 ester to form a cyclic tetrahedral intermediate that then collapses to produce lyso-lipoprotein. Lit is a unique example of an intramolecular transacylase differentiated from that catalyzed by Lnt, and provides insight into the heterogeneity of bacterial lipoprotein biosynthetic systems.

Lipoproteins are ubiquitous membrane-anchored proteins located on the extra-cytoplasmic surface of bacterial cell membranes (1–6). Lipoproteins perform a wide variety of functions at the membrane surface, including in the capture of nutrients, in adhesion, and as structural components of the cell envelope. Preprolipoproteins are exported from the cytoplasm and post-translationally modified by lipoprotein diacylglycerol transferase (Lgt) with a diacylglycerol moiety from a neighboring glycerophospholipid (Fig. 1) (7). Lipoprotein signal peptidase II (Lsp) then recognizes the lipobox and cleaves the leader peptide at the Cys + 1 position, liberating the cysteine α-amino group and forming a diacylated lipoprotein (DA-LP) (8). Although Lgt and Lsp are highly conserved in the lipoprotein biosynthetic pathways from diverse prokaryotes, further acyl tailoring modifications can occur. In the majority of Gram-negative bacteria and some high-GC Gram-positive species, lipoprotein N-acyltransferase (Lnt) attaches a single amide-linked acyl chain to the α-amino group of DA-LP using a neighboring phospholipid as the acyl donor to complete triacylated lipoprotein (TA-LP) (9). Despite lacking an Lnt sequence ortholog, several low-GC Gram-positive Firmicutes also produce lipoproteins with N-terminal modifications (10).

We previously identified an integral membrane protein in Enterococcus faecalis that forms lipoproteins with N-acyl-S-monoacyl (sn-1)-modified cysteine residues (11). This novel lipoprotein chemotype (lyso-LP) is directed by an enzyme (WMC_RS08810) that is narrowly distributed among enterococci, being confined to a subset of intestinal–associated strains that includes the opportunistic nosocomial pathogens Enterococcus faecalis and Enterococcus faecium (12). Lyso-LP formation may thus play a role in host adaptation, as lipoproteins are key ligands for Toll-like receptor 2 (TLR2) complexes of the innate immune system (13). E. faecalis expressing lyso-LP is recognized with over 100-fold less sensitivity by TLR2 compared with DA-LP expressing isogenic strains (14). Lyso-LP forming paralogs that are specifically induced by copper have been characterized on horizontally transmissible plasmid in Listeria monocytogenes (normally a DA-LP producer) (14). Copper, a widely used agricultural microbiocide and a natural defense mechanism in phagocytes (15, 16), was proposed to complex less tightly with N-acylated lyso-LP compared with the free α-amino nitrogen atom in DA-LP (14).

For the lyso-LP forming enzymes, there are no functionally characterized sequence orthologs, no conserved domains with annotation, and the catalytic mechanism is unknown. Two possible mechanisms for lyso-LP formation have been proposed: (i) N-acylation by an Lnt-like mechanism coupled with a O-deacylation sn-2 lipase activity; or (ii) transacylation through direct transfer of the sn-2 acyl chain from the diacylglycerol moiety to the N terminus (5). Because WMC_RS08810 is only ~200 amino acids, we initially favored an intramolecular
Lyso-LP formation by lit

Figure 1. Lipoprotein biosynthetic pathways with Lnt and Lit N-terminal tailoring. The first two steps of the lipoprotein biosynthetic pathway are highly conserved in all prokaryotes. Lgt catalyzes thioether bond formation using a glycerophospholipid donor, followed by proteolytic cleavage of the signal peptide by Lsp to make DA-LP. In E. coli and other Gram-negative bacteria, Lit then transfers the sn-1 lipid from a glycerophospholipid to the ω-amino position of a lipoprotein (top). Lit from E. faecalis and other Firmicutes is proposed to function as an O-to-N intramolecular transacylase (bottom), in which case [d5]-DA-LP substrate with ω-labeled acyl chains (labels colored red) should be retained in the lyso-0-LP product. IM, inner membrane.

Results

Isolation of deuterium-labeled LppK58A-strep tag DA-LP substrate

To test whether Lit functions as an inter- or intramolecular transacylase (Fig. 1), an E. coli strain was first engineered to produce substrate for in vitro reconstitution reactions with Lit (Fig. 2). DA-LP substrate with deuterium specifically incorporated into the diacylglycerol fatty acid ω termini could be used to ascertain N-acyl chain origin. Most Gram-negative bacteria, however, including E. coli, require N-acylation of lipoproteins for efficient trafficking from the cytoplasmic membrane to the outer membrane (23). Without N-acylation, outer membrane bound lipoproteins accumulate in the cytoplasmic membrane and some, notably Braun’s lipoprotein (Lpp), form aberrant cross-links to peptidoglycan (24). The chromosomal lpp was therefore deleted and replaced with a streptomycin-tagged LppK58A allele to prevent covalent lysine-peptidoglycan cross-linking (23–26). LolCDE, the essential ABC (ABC) transporter that traffics lipoproteins from the inner to outer membrane in E. coli (27), was overexpressed to enhance transport of the non-optimal DA-LP substrate. In this genetic background, int could be deleted without loss of cell viability as has been reported (23).

Next, we rewired the fatty acid β-oxidation pathway of E. coli to uniformly label lipoproteins in the five terminal ω deuterium atoms in both acyl chains of DA-LppK58A-strep tags when fed octanoic-7,7,8,8,8-[d5] acid (Fig. 2). E. coli will not readily take up exogenous medium and short chain fatty acids (28–30), however, because the cognate acyl-CoAs are poor ligands for the fatty acid metabolism regulatory protein FadR (31). Therefore we deleted FadR to de-repress expression of exogenous fatty acid catabolism genes, including the outer membrane transporter FadL (31, 32). The long chain fatty acid:acyl-CoA ligase FadD of E. coli has low intrinsic activity with octanoate substrate, so the FadD(V451A) allele was introduced to enhance conversion of octanoate to acetyl-CoA (33, 34). To prevent further entry into the β-oxidation cycle, which would catabolize labeled octyl-CoA to acetyl-CoA and randomly distribute the deuterium label, the first enzyme in the β-oxidation cycle acyl-CoA dehydrogenase (FadE) was deleted as well (35). We have previously shown that the Pseudomonas aeruginosa enzyme PA3286 when expressed in E. coli specifically condenses octyl-CoA with malonyl-ACP (acyl carrier protein) to form the fatty acid synthesis (FAS) intermediate β-keto decanoyl-ACP (36). By expressing PA3286, terminally labeled β-keto decanoyl-ACP could now be made from fed octanoic-7,7,8,8,8-[d5] acid (Fig. 2). Elongation to long chain acyl-ACP by FAS and incorporation into membrane glycerophospholipids by the acyltransferases PslB and PslC (37) thus provides labeled substrate for Lgt to form pro-DA-LP (7). Additionally, because the de novo fatty acid initiation enzyme FabH can be deleted in E. coli K-12 (38, 39), fabH was removed to favor incorporation of exogenously fed octanoate by shrinking the endogenous cellular fatty acid pool. Although the growth rate of the final DA-LP labeling strain (TXM1111) was significantly decreased in standard lysogeny broth (LB) when compared with that of the parental WT and intermediate strains, octanoate supplementation of both solid (not shown) and liquid media improved growth...
Long chain fatty acids such as palmitate, which are not shunted by PA3286 (36), did not improve growth and is consistent with a functional medium acyl-CoA substrate chain shunt rescuing defective de novo fatty acid initiation.

\[d_5\]-DA-LppK58A-strep from strain TXM1111 is dually labeled and retains acyl chain asymmetry

Growth enhancement by octanoate, but not palmitate, suggested efficient incorporation of octanoate by the engineered PA3286 shunt (Fig. 2A). MALDI-TOF MS analysis of trypsinized [\(d_5\)]-DA-LppK58A-strep purified from TXM1111 grown in media supplemented with octanoic-7,7,8,8,8-[\(d_5\)] acid confirmed a new series of peaks shifted by 10 u compared with the negative control \([\text{fabH}}\) strain KA775 (Fig. 3, A versus D, Table 1). The most abundant peak (1167.8 u) can be assigned to the N-terminal lipopeptide of LppK58A with 10 deuterium atoms, consistent with two [\(d_5\)]-labeled acyl chains (Fig. 3C). A low abundance peak corresponding to singly labeled lipopeptide can be observed at 1162.6 u. Notably absent is the unlabeled lipopeptide peak (1157.6 u), which is the most abundant ion in the spectrum of the control strain KA775. To further characterize both lipoprotein structure and extent of labeling, the 1167.8 u peak was fragmented by tandem MS/MS (Fig. 3B). The y series ions corresponding to the SSNAK peptide of Lpp were observed as expected, whereas fragment ions 575.6 u, 607.7 u, and 647.7 u confirm a free \(\alpha\)-amino terminus (Fig. 3C). Ions 575.6 u and 607.7 u, respectively, represent the dehydroalanyl peptide and the thiolated congener, generated by the neutral loss of the diacylthioglycerol and diacylglycerol moieties. The 647.7 u ion is consistent with the loss of both labeled fatty acids, a characteristic DA-LP fragment peak (11, 40, 41). The MS/MS spectrum of the 1157.8 u parent ion of unlabeled Lpp from KA775 shared these nonacylated fragment ions, confirming peptide identity, free N-terminal structure, and supports specific isotope incorporation within the acyl chains in TXM1111 (Fig. 3, E and F). Distinct between the two spectra, additional ions at 909.1 u and 927.2 u of [\(d_5\)]-DA-Lpp from TXM1111 correspond to the parent ion having lost a labeled [\(d_5\)]-C_{16:1} fatty acid.
acid (C_{15}D_{9}H_{24}COOH) or [d_5]-C_{16:1} ketene (C_{14}D_{5}H_{22}CH=CO), respectively (Fig. 3B). The corresponding ions in the unla-
beled sample are shifted 5 u lower (904.0 and 921.6 u) (Fig. 3, E and F). The acyl chain composition for the most abundant DA-
LP species can thus be inferred as one monounsaturated (C_{16:1}) and one saturated (C_{16:0}) palmitate fatty acids.

Figure 3. MALDI-TOF MS and MS/MS analysis of [d_5]-DA-LppK58A-strep tag purified from strain TXM1111 and KA775. Trypsinized Lpp lipopeptides purified from strains grown with [d_5]-octanoate were extracted from TXM1111 (A) or the control strain KA775 (D) and analyzed by MALDI-TOF MS. B, MS/MS spectra of the 1167.8 u parent ion was used to elucidate the N-terminal structure of Lpp as [d_5]-DA-LppK58A with two labeled acyl chains (C). MS/MS spectra of the 1157.8 u parent ion (E) confirmed a free α-amino N terminus in DA-LppK58A (F). Calculated peak assignments are detailed in Table 1.

The sn-1 or sn-2 assignments were not determined by MS/MS unless indicated.
1Peaks not observed (Fig. 3).
2Peaks not observed (Fig. 3).
3Potassium adduct of 1157.6 u (Fig. 3D).

| Total acyl chain length | Number of [d_5]-labeled acyl chains |
|------------------------|-----------------------------------|
| 32:1                   | 2                                 |
| 34:1                   | 1                                 |
| 34:1                   | 2                                 |
| 34:1                   | 1                                 |

Table 1
Calculated masses of [d_5]-DA-LppK58A-strep

| [M + H] (u) | [M + Na] (u) | Total acyl chain length | Number of [d_5]-labeled acyl chains |
|-------------|--------------|-------------------------|-------------------------------------|
| 1139.6      | 1161.6       | 30:1                    | 2                                   |
| 1157.6      | 1184.6       | 32:1                   | 1                                   |
| 1162.6      | 1189.6       | 32:1                   | 2                                   |
| 1185.6      | 1190.6       | 34:1                   | 0                                   |
| 1190.6      | 1212.6       | 34:1                   | 1                                   |
| 1195.7      | 1217.7       | 34:1                   | 2                                   |
We next determined the position of the C_{16:1} fatty acid, either sn-1 or sn-2. Although WT *E. coli* preferentially incorporates unsaturated fatty acids at sn-2 (37, 42), the TXM1111 labeling strain has highly altered fatty acid metabolism and much slower growth kinetics (Fig. 2). Deletion of the fatty acid metabolic regulator FadR not only de-represses β-oxidation, but also decreases transcription of the unsaturated fatty acid biosynthetic enzymes *fabAB* (43). We utilized the sn-1 regiospecificity of lipoprotein lipase (LPL) from *Pseudomonas* spp., as reported by Asanuma et al. (44), to de-O-acylate [d_{5}]-DA-LppK58A-strep. The MS spectra of digested [d_{5}]-DA-LppK58A-strep contains a prominent peak at 924.6 u consistent in mass with the loss of the labeled saturated [d_{5}]-C_{16:0} acyl chain, allowing regiospecific assignment of C_{16:1} to the sn-2 position in the [d_{5}]-DA-LppK58A-strep substrate (Fig. S1). Together, these data indicate that the majority of [d_{5}]-DA-LppK58A-strep population purified from strain TXM1111 is dually labeled with two C_{16} [d_{5}]-acyl chains and has preferentially incorporated C_{16:1} in the sn-2 position.

**Reconstitution of lit:lipoprotein N-acyltransferase activity in vitro**

With structurally defined [d_{5}]-DA-LppK58A-strep substrate in hand, we next reconstituted Lit enzyme activity. We have previously shown that replacement of Lnt with Lit produces lyso-form LppK58A (11), indicating Lit is expressed and active in *E. coli*. For *in vitro* reaction conditions, we used conditions established by Hillmann et al. (45) with Lnt so activity could be directly compared in a substrate competition assay. Inner membranes were isolated from *E. coli* strains expressing Lnt, Lit, or both, and used as the enzyme source. Membranes were mixed with purified recombinant DA-LppK58A-strep lipoprotein substrate in the presence of detergent, and product formation was assayed by α-strep tag immunoblotting (Fig. 4A). When DA-LppK58A is incubated with *E. coli* Lnt, a higher molecular weight band is observed over time, consistent with conversion of DA-LP into TA-LP. DA-LP and lyso-LP cannot be separated by SDS-PAGE because they are the same mass (11). The persistence of a lower molecular weight band in reactions after 24 h with both Lnt and Lit (Lnt/Lit) is indirect evidence of Lit activity, however, as any DA-LppK58A converted to the lyso-LP by Lit cannot be converted back into the TA-LP chumotyped by Lnt. This also supports an *intra*-molecular transfer, as a bifunctional N-acyltransferase (DA-LP to TA-LP) and de-O-acylation lipase (TA-LP to lyso-LP) Lit activity would be expected to convert the entire substrate population into lyso-LP (Fig. 1, *bottom panel*).

To confirm Lit-catalyzed lyso-LppK58A formation under our reconstitution conditions, Lit reactions were separated by SDS-PAGE and the product was analyzed by MS. The parent spectra obtained from membranes containing neither Lit/Lnt, nor only Lit, shared a prominent peak at 1157.7 u, consistent with diacylated Lpp lipopeptide (Fig. S2). The lyso-LP diagnostic N-acyl(C_{16:1}) dehydroalanyl fragment ion at 811.2 u was only observed for reactions that included Lit (sodium adduct, 1179.7 u) (Fig. 4B). The preference for C_{16:1} incorporation at the N terminus is in full agreement with the *in vivo* acyltransferase activity of Lit (11).

**Lit catalyzes an intramolecular transfer of the sn-2 acyl chain to the N terminus**

To determine the origin of the amide-linked N terminus acyl chain in lyso-LP, Lit activity was reconstituted as above using deuterium-labeled [d_{5}]-DA-LppK58A-strep substrate. If Lit functions intermoleurally, using a phospholipid acyl chain donor similar to that of Lnt (45), an unlabeled acyl chain from the phospholipid pool will be incorporated into the N-terminal lipopeptide. If Lit functions intramoleurally, the [d_{5}]-C_{16:1}-labeled acyl chain should migrate to the N terminus. Membranes were prepared from *ltr*^{-} *E. coli* cells and incubated with the [d_{5}]-DA-LppK58A-strep tag substrate. Lipoprotein products were affinity purified with StrepTactin-coated magnetic beads, and analyzed by MS. Membranes with Lnt yielded products with masses consistent with TA*-LppK58A-strep (1406.3 u) (Fig. 5A). This mass is 10 u higher than the previously characterized unlabeled variant (11), consistent with two [d_{5}]-labeled acyl chains and a single unlabeled acyl chain. Fragmentation produced the 814 u dehydroalanyl ion, indicating an unlabeled C_{16:0} N-acyl chain at the α-amino terminus (Fig. 5B). Additional peaks at 885.9 u, 1146.9 u, and 1164.9 u confirmed the retention of both [d_{5}]-acyl chains on the diacylglycerol moiety and assignment of all acyl chains positions (Fig. 5C). The bulk phospholipid pool in the reconstituted system is thus the acyl donor used by Lnt in TA-LP synthesis, as previously reported (46).

The reaction was repeated with membranes containing only Lit. Parent spectra of isolated lipoprotein products revealed a predominant peak at 1168.0 u, consistent in mass with LppK58A-strep containing two [d_{5}]-acyl chains (Fig. 5D). As this ion could possibly be unreacted [d_{5}]-DA-LppK58A-strep substrate, the corresponding sodium adduct 1189.9 u was subject to MS/MS (Fig. 5E). This ion preferentially fragmented to give the expected mass for the [N-[d_{5}]-acyl-dehydroalanyl ion (816.2 u) (Fig. 5F). These data confirms that Lit is an intramolecular sn-2 ester to N-terminal amide lipoprotein transacylase (Fig. 1).

**Mut-seq analysis of important amino acids in lit**

There are few examples of enzymes catalyzing analogous intramolecular ester to amide acyl chain migration reactions. We initially presumed one of the three cysteine residues (Cys-16, Cys-100, and Cys-187) in Lit formed an acyl thioester type intermediate, as has been characterized for Cys-387 in the active site of Lnt (19, 26, 45, 47). However, none of the cysteine residues in the chromosomal copy of Lit (11) appear to be conserved in the plasmid borne, copper-inducible Lit2 paralog (14). To better understand how Lit catalyzes transfer of the sn-2 acyl chain, we established a selection system to monitor growth rescue in *E. coli* by an alanine (Ala) scan library of *E. faecalis* Lit alleles (Fig. 6A). We have previously shown *lit* can functionally replace *ltr* in an *E. coli* Δlpp background through lyso-LP formation, with the N-acylation restoring lipoprotein trafficking to the outer membrane (11). A plasmid-based Ala
scan allele library of the lit gene was systematically constructed to replace every coding amino acid with an Ala triplet (GCG) and introduced into an E. coli strain that conditionally expresses lnt (PBAD-lnt) provided arabinose inducer is present (Fig. 6A). Plasmids were isolated from the input library as well as from a culture serially passaged in the absence of arabinose inducer. Residues important for Lit function and phenotypic rescue were determined using Mut-seq analysis of next generation sequencing data to measure depletion ratios (17, 18). Every coding triplet except two nonsynonymous triplets of the 212 amino acids were detected above background in the input library (Fig. S3A). Deletion of any three Cys residues did not attenuate function (Fig. 6B), indicating the active site does not utilize a thioester intermediate and further differentiates Lit from Lnt. Except for L118A that modestly increased fitness (Fig. S3B), conversion to Ala at a single position either had no effect or led to a decrease in fitness when Lnt was depleted. Two prominent stretches of amino acids (S1 and S2) where Ala replacement led to marked depletion were identified (Fig. 6B), suggesting local regions of defined structure and the potential location of catalytic residues. Interestingly, many functionally important aromatic residues are located within both of these stretches (His-89, Phe-90 from S1 and Phe-149, Phe-153, Phe-156, His-157, Phe-161, Trp-166, Phe-168, Phe-184 from S2), in addition to a single N-terminal tyrosine residue (Tyr-36). We tested whether these residues were important due to changes in protein expression levels or protein stability by immunoblotting (Fig. 6C). Although the level of Lit varied between each Ala mutant, all constructs expressed protein at levels at least equal to that observed in WT Lit. All mutants displaying significant depletion ratios in the Mut-seq analysis when reconstructed and introduced into E. coli PBAD-lnt recapitulated the phenotype in a spot titer assay (Fig. 6D). A role for these aromatic residues in establishing tertiary structure is thus more likely. Aromatic residues, through $\pi-\pi$ and other interactions, are critical determinants in the self-assembly of multipass $\alpha$-helical transmembrane (TM) segments in integral membrane proteins (48–50). Phe in particular is one of the most common partners in interhelical contact pairs (51). Lit is predicted to encode multiple TM domains (see below), suggesting these key residues may be part of dynamic interhelical interactions involved in lipoprotein substrate gating and/or binding.

Residues deemed to be important by Mut-seq analysis would be expected to be highly conserved across Lit sequences from different organisms. Sequence alignment confirms that most depleted Ala substitution positions are indeed highly conserved residues across Lit from different bacterial genera (chromosomal Lit) as well as in plasmid-borne Lit2 paralogs located in a copper-resistance operon (Fig. 7). However, not all conserved residues are required for function. Both Ser-83 and Glu-181 are highly conserved, polar, and located within important stretches (S1 and S2), but are not necessary for catalysis (Fig. 6D). Overall, Mut-seq analysis implicated few polar residues as critical to function, which is surprising given the catalytic residues are likely polar. Presumably, the $sn$-2 position O-acyl chain shown to be transferred intramolecularly (Figs. 1, bottom panel, and 5D) is subject to nucleophilic attack by either: (i) an amino acid side chain to form an acylated covalent enzyme intermediate or (ii) through direct attack by the $\alpha$-amino terminus of the of DA-LP substrate itself. Two prime candidates for general acid-
base chemistry to activate the carbonyl are the functionally required (Fig. 6, B and D) and invariant (Fig. 7) His-89 and His-157 residues. We randomized both histidine positions with all 20 amino acids to determine whether any substitutions besides Ala could retain function and performed a second round of Mut-seq analysis. All amino acid replacements were rapidly depleted from the population (Fig. S4), confirming a key role of these His residues in Lit function.

Membrane topology of lit

As Lit is the sole member of a novel class of enzymes with no sequence similarity to characterized acyltransferases, we sought to map the membrane topology of Lit. According to several membrane topology mapping algorithms (Table S2), Lit is predicted to have four TM passes along with one intracellular and two extracellular loops, with both the N and C termini located in the cytoplasm (Fig. 8A). There is, however, poor consensus in boundaries for the second predicted extracellular loop (loop 3), which overlaps with S2 identified in Mut-seq analysis (Fig. 8B). To gain further insight into the membrane topology, a series of C-terminal reporter fusions using either β-gal (LacZ) or alkaline phosphatase (PhoA) were constructed within each putative loop. Localization of the reporter proteins to the cytoplasm (LacZ) and the periplasm (PhoA) was assessed using the respective colorimetric substrates (52). LacZ fusions within loop 2 (LitR129-LacZ) and at the C terminus (LitK212-LacZ) confirmed cytoplasmic locations, whereas loop 1 PhoA fusion (LitD52-PhoA) indicated an extracellular periplasmic location (Fig. 8, B and C). Loop 3 reporters for both intra- and extracellular (LitD173-LacZ and LitD173-PhoA)
Lyso-LP formation by lit

Figure 6. Mut-seq analysis of *E. faecalis* Lit. A, a library of plasmids expressing *lit* from *E. faecalis* was systematically constructed to replace every amino acid (212 total) with an alanine residue. The plasmid library was pooled, transformed into *E. coli* IPPpBAD-int, and maintained in media with 0.2% arabinose (w/v) inducer so that *int* remained expressed. The library was then passed in the absence of arabinose to make growth dependent on the activity of the respective *lit* X-to-Ala mutant allele. Plasmid DNA was isolated from the library before and after withdrawal of arabinose, the *lit* coding region was amplified with flanking primers, and the amplicon pools were subject to next generation sequencing. B, representation of each Ala codon (GCG) triplet substitution in the initial library was calculated as a percent of total reads containing Ala substitution, and compared with the composition after passing to determine depletion ratios (Fig. S3). Residues rechecked by spot titer are shaded black, negative controls are indicated (*), and the putative catalytic His residues subjected to randomization (Fig. S4) are boxed. The EF Lit coding region targeted for Ala scan mutagenesis is indicated along with the two functional stretches (S1 and S2). Flanking regions (10 in-frame triplets upstream of ATG start codon and eight in-frame triplets downstream) are shown for competition to spontaneous mutations/sequencing error rate. C, the level of Lit expression for each Lit Ala point mutant was determined by immunoblotting with anti-strep tag. Sta, molecular weight standard; Vector, empty vector. D, strains of *E. coli* expressing each Lit variant were 10-fold serially diluted and spotted on LB agar supplemented with either glucose (+ Glc) or arabinose (+ Ara) to repress or induce expression of IPPpBAD-int.

respectively) orientations were negative. Because loop 3 contains the functionally important S2 amino acid stretch, we tested additional fusion sites within loop 3 (after residues Leu-159, Ala-165, Leu-179, and Met-185) as well as an intervening linker to separate the reporter enzyme domain from Lit (18-residue linker VPDSYTQVASWTEPPFC) (53)). However, no reporter enzyme function was observed (data not shown).

The failure to reconstitute activity for any fusion reporter construct in loop 3 suggests a highly structured local region closely associated with or even possibly embedded within the membrane. This would explain the difficulty in predicting a clear boundary consensus for TM3 (Table S2). As LacZ/PhoA fusion experimental analysis was likewise inconclusive, the substituted cysteine accessibility method (SCAM) (54) was used to interrogate topology at multiple positions within loop 3 (Fig. 8D). Cysteine exchanges were made within each loop and in both termini using a strep-tagged Lit construct lacking all endogenous cysteine residues (C16A, C100A, C187A). Methoxypolyethylene glycol maleimide (mal-PEG, average molecular mass 5,000 Da) was used to detect free cysteines after pretreatment with either membrane-permeable (N-ethylmaleimide (NEM)) or impermeable (2-sulfonatoethyl methanethiosulfonate (MTSES) or 4-acetamido-4’-maleimidylstilbene-2,2’-disulfonic acid (AMS)) thiol reactive probes (55, 56). Lit without any cysteine residues was unreactive with mal-PEG. Intracellular cysteine residues (N terminus L6C, intracellular loop 2 L124C, and C terminus S209C) were only protected by pretreatment with the membrane-permeable probe NEM, indicating a cytoplasmic location and suggested by the Lit-LacZ reporter construct activity assay (Fig. 8B). The loop 2 construct (V54C) was protected by all probes, consistent with the extracellular topology suggested by the Lit-PhoA reporter fusion data (Fig. 8C). Three positions were chosen within the putative loop 3 (A165C, L171C, and A178C), and the membrane-impermeable probes (MTSES and AMS) protected Lit from mal-PEG labeling in all three cases. The AMS (536.4 g/mol) membrane-impermeable probe, which is larger than MTSES (242.2 g/mol), induced an upward molecular mass shift in AMS-labeled samples in conjunction with decreased amounts of Lit-mal-PEG adduct. These positions within loop 3 of Lit thus appear to be at least somewhat exposed to small molecule polar probes on the extracellular membrane surface, although it remains unknown whether loop 3 is truly an extended disordered loop as shown in Fig. 8A or in a state that is more intimately associated with the membrane.

Discussion

Bacteria have evolved large and diverse enzyme families to catalyze acyl transfers (57). Lit, however, has no apparent sequence similarity to any of these enzyme families, reflecting the unique nature of an enzyme-catalyzed intramolecular acyl chain migration. From a thermodynamic perspective, the ester-to-amide acyl chain exchange would be expected to favor lysol-PL over DA-PL at equilibrium. Indeed, residual DA-PL is at the limit of detection by MALDI-TOF MS in WT *E. faecalis* (11).
Unlike the unimolecular Lit reaction mechanism, Lnt in Gram-negative bacteria catalyzes a bimolecular acyl transfer reaction. The high effective phospholipid donor concentration, in concert with the \(N\)-acyl substrate selectivity of the Lol transport system (58), favors chemotype conversion of the lipoprotein population to TA-LP. Lol acts as a quality control mechanism to ensure only mature Lnt-processed lipoproteins are removed from the inner membrane, a particularly relevant factor in controlling the DA-LP/TA-LP ratio given that out of an estimated 3-4 million total proteins in a typical \(E. coli\) cell (59), up to a million are outer membrane-bound lipoproteins (3, 60, 61).

Because there is no Lol system to ensure \(N\)-acylation of the bulk of lipoproteins in Gram-positive bacteria, Lit must act rapidly as many lipoproteins are part of multicomponent protein complexes that will become inaccessible once assembled. For instance, if only \(\sim 1\%\) of the lipoprotein population does not get converted to lyso-LP, the TLR2 detection sensitivity will be doubled because DA-LP is detected with 100-fold more sensitivity (14). An intramolecular transfer mechanism may also help explain why both \(\text{lit}\) genes occur in tandem on a horizontally transmissible chromosomal copper-resistance operon (14). The Lit enzymes have a narrow phylogenetic distribution and thus limited amino acid sequence divergence (Fig. 7). None the less, residues identified by Mut-seq analysis as important for function are highly conserved (Fig. 6D) and predicted TM segments (by Protter (26); see Fig. 8) are boxed in gray with uncertainty in the boundaries of loop 3 (L3) denoted by brackets and asterisks (Table S2).

**Figure 7. Primary amino acid sequence alignment of chromosomal Lit and accessory Lit2 proteins.** Sequences of the chromosomal Lit (\(\text{Lit}\)) and the copper-resistance associated transposon/plasmid located (\(\text{Lit2}\)) paralogs from the indicated strains were generated by STRAP (36). Highly conserved amino acids are colored (purple for 100% and yellow for retention in all but one sequence). Residues deemed essential (green) or nonessential (blue) by Mut-seq analysis and confirmed for function (Fig. 6D) are indicated. Predicted TM segments (by Protter (26); see Fig. 8) are boxed in gray with uncertainty in the boundaries of loop 3 (L3) denoted by brackets and asterisks (Table S2).
residues and possible active site candidates were deemed essential by Mut-seq. Given the absence of any obvious candidates for an acyl thioester (Cys) or analogous ester (Ser/Thr) or mixed anhydride (Asp/Glu) type intermediate, there are no mechanistic parallels with Lnt. This underscores the independent origin of lipoprotein N-terminal tailoring reactions in what is otherwise a highly conserved pathway.

Two polar residues with high depletion ratios, located within stretches of functional importance, and absolutely required for activity were His-89 and His-157 (Fig. 6). In addition to being conserved (Fig. 7), both His residues were irreplaceable by any other amino acid (Fig. S4). Topology mapping places both residues in an extracellular or membrane interface having access to DA-LP substrate (Fig. 8). In the case of His-157, precise mapping of TM3 and the putative loop 3 boundary encompassing S2 was complicated. Computational (Table S2) and experimental colorimetric fusion reporter assays could not verify topology (Fig. 8, B and C). SCAM labeling with membrane-impermeable thiol-reactive probes did support a degree of extracellular exposure of the putative loop 3 (Fig. 8D). It is possible that this TM3-loop3 boundary region is partially embedded or parallel with the membrane, defining a cavity that directly interacts...
with DA-LP. Histidine residues are common catalytic residue in many acyltransferases (57), and in the case of Lit, presents two possible catalytic mechanisms. In a general acid-base mechanism (Fig. 9), Lit positions the free α-amino terminus of DA-LP substrate in proximity to the N-terminal histidine residue. A second plausible mechanism involves direct nucleophilic attack by His on the α-carbonyl to form a covalent acyl-Lit enzyme intermediate. Lit represents a novel class of acyltransferases catalyzing lipoprotein N-acylation in select Firmicutes. Lit shares no primary sequence or mechanistic similarity with the only other characterized lipoprotein N-acylating enzyme Lnt from Gram-negative bacteria. There are other options for lipoprotein N-acylating systems besides Lit and Lnt as well, including the recently identified two-component LnsAB system in Staphylococcus aureus, which bears no resemblance to either Lit or Lnt.3 In the context of the lyso-LP formation by Lit, what is the advantage of intramolecular transacylation over other known mechanisms of N-acylation? Extracellular proteins in Firmicutes have evolved to exclude cysteine as many lack machinery to reduce aberrant disulfide bonds formed under oxidative stress (38). Lit does not utilize an active site cysteine (Fig. 6), a relevant detail.

Table 2

Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant genotype/phenotype | Reference |
|-------------------|----------------------------|-----------|
| **Escherichia coli** | | |
| BL21(DE3) strains | | |
| KA729 | pET22b (+)-Eflit (from E. faecalis ATCC 19433) | This study |
| KA736 | lpp::Cm<sup>+</sup> + pET22b (+)-Eflit | This study |
| KA801 | lpp::Cm<sup>+</sup> Int::Spec<sup>+</sup> chiQ::Apr<sup>+</sup> + pET22b (+)-Eflit | This study |
| KA818 | lpp::Cm<sup>+</sup> Int::Spec<sup>+</sup> chiQ::Apr<sup>+</sup> + pET22b (+)-Eflit + pKA810 | This study |
| KA893 | lpp::Cm<sup>+</sup> flanked by FRT sites | This study |
| BW25113 strains | | |
| TXM327 | lpp::Cm<sup>+</sup> | 8 |
| KA349 | lpp::Cm<sup>+</sup> ybeX-(Kan<sup>+</sup> -rrnB TT-araC-P<sub>BAD</sub>-inr) | 11 |
| TXM541 | got::Kan<sup>+</sup> -rrnB TT-araC-P<sub>BAD</sub>-inr, Int::Spec<sup>+</sup>, chiQ::Apr<sup>+</sup> | 8 |
| KA775 | TXM327 Int::Spec<sup>+</sup> + pCT763 (spontaneous suppressor mutant) | Laboratory stock |
| KA827 | phoA::Kan<sup>+</sup> | |
| KA845 | E. coli BW25113 fadR-Trm<sup>+</sup> | This study |
| SS851 | KA827 + pET22b (+)-Eflit; Carb<sup>+</sup> | This study |
| SS904 | KA827 + pET22b (+)-lacZ; Carb<sup>+</sup> | This study |
| SS915 | KA827 + pET22b (+)-EflitK1219-(-9)lacZ fusion; Carb<sup>+</sup> | This study |
| SS916 | KA827 + pET22b (+)-EflitK1219-(-13)phoA fusion; Carb<sup>+</sup> | This study |
| SS932 | KA827 + pET22b (+)-EflitD173-(-9)lacZ fusion; Carb<sup>+</sup> | This study |
| SS935 | KA827 + pET22b (+)-EflitR129-(-9)lacZ fusion; Carb<sup>+</sup> | This study |
| SS936 | KA827 + pET22b (+)-EflitR129-(-13)phoA fusion; Carb<sup>+</sup> | This study |
| SS940 | KA827 + pET22b (+)-EflitD173-(-13)phoA fusion; Carb<sup>+</sup> | This study |
| SS950 | KA827 + pET22b (+)-EflitD52-(-13)phoA fusion; Carb<sup>+</sup> | This study |
| SS951 | KA827 + pET22b (+)-EflitD52-(-9)lacZ fusion; Carb<sup>+</sup> | This study |
| SS964 | KA827 + pET22b (+)-phoA; Carb<sup>+</sup> | This study |
| TXM1014 | KA845 lpp::Kan<sup>+</sup> flanked by FRT sites | This study |
| TXM1015 | TXM1014 lpp::FRT | This study |
| TXM1018 | TXM1015 fadE::Tet<sup>+</sup> | This study |
| TXM1019 | TXM1018 + pTXM1019<sup>+</sup> | This study |
| TXM1036 | TXM1036 Int::Sp<sup>+</sup> | This study |
| TXM1058 | TXM1036 fabH::Cm<sup>+</sup> flanked by FRT sites | This study |
| TXM1067 | TXM1038 fabH::FRT | This study |
| TXM1111 | TXM1067 + pTM1100 | This study |
| **Plasmids** | | |
| pCL25 | Cloning vector with RepA origin | 39 |
| pSEVA434 | General cloning vector with pBBR1 origin | 71 |
| pKAS22 | pCL25(ori) lpp(K58A)-Strep-tag K<sup>+</sup>n<sup>+</sup> | 8 |
| pET22b<sup>+</sup> | Expression vector with T7 promoter lacI; Carb<sup>+</sup> | Novagen |
| pET22b<sup>+</sup>-Eflit | pET22b<sup>+</sup> (+) Eflit (from E. faecalis ATCC 19433), 210 amino acid ORF | This study |
| pET22b<sup>+</sup>-Eflit strep step | pET22b<sup>+</sup> (+) Eflit strep tag (from E. faecalis ATCC 19433) lacking all cysteine residues | This study |
| pET22b<sup>+</sup>-Eflit strep | pET22b<sup>+</sup> (+) Eflit strep tag (from E. faecalis ATCC 19433) lacking all cysteine residues | This study |
| C16A C100A C187A | | |
| pLI<sup>+</sup>-Ppen Gfpmut2 | Mic-copy plasmid number with P<sub>pen</sub> promoter controlling insert expression; Carb<sup>+</sup> | 72 |
| pLI<sup>+</sup>-Ppen | pLI<sup>+</sup>-Ppen lit lacking all endogenous cysteine residues | This study |
| **pLI<sup>+</sup>-Eflit strep** | | |
| C16A C100A C187A | | |
| pCT763 | pKA522 PA3286 (from Pseudomonas aeruginosa) | This study |
| pKA810 | pCL25(ori) Tmp<sup>+</sup>-lpp(K58A)-Strep-tag | This study |
| pTXM1016 | pBRRI (ori) P<sub>ara</sub>-IolCDE-PA3286 Kan<sup>+</sup> | This study |
| pTM1100 | pCL25(ori) lpp(K58A)-Strep-tag-fadD<sup>+</sup>(151A) Cm<sup>+</sup> | This study |

1Resistance phenotypes: Apr<sup>+</sup>, apramycin; Carb<sup>+</sup>, carbencillin; Cm<sup>+</sup>, chloramphenicol; Kan<sup>+</sup>, kanamycin; Sp<sup>+</sup>, spectinomycin; Tet<sup>+</sup>, tetracycline; and Tmp<sup>+</sup>, trimethoprim.

3 J. Gardiner, 4th, G. Komazin, M. Matsuo, K. Cole, F. Gotz, and T. C. Meredith, unpublished data.
Lyso-LP formation by lit

![Diagram of Lyso-LP formation by lit]

**Figure 9. Putative catalytic mechanism of Lit.** The direct attack mechanism using general acid-base catalysis to activate the sn-2 carbonyl of DA-LP substrate. Nucleophilic attack by the α-amino group forms a 8-member cyclic intermediate that collapses to yield N-acyl containing lyso-LP product. His-89 and His-157 are candidate residues for directing protonation and deprotonation events in the catalytic cycle.

given Lit2 is specifically induced by copper, which readily oxidizes thiols. Formation of TA-LP by Lnt type activity also generates a lysophospholipid byproduct from the glycerophospholipid acyl donor (46). Physically similar to detergents, lysophospholipids are cytolytic and perturb membrane integrity (66). In E. coli, lysophospholipids are reacylated by the phospholipid repair system donor (46). As more is learned regarding the various lipoprotein systems in different bacteria, insights into why each system has been acquired in a particular host will become more clear.

**Experimental procedures**

**Bacterial strains and growth conditions**

_E. coli_ strains used in this study, listed in Table 2, are derivatives of either K-12 (BW25113) or BL21(DE3). All strains were grown in lysogeny broth-Miller medium (LB) at 37 °C with agitation. Antibiotic markers were selected with carbenicillin (100 µg/ml), apramycin (100 µg/ml), spectinomycin (50 µg/ml), tetracycline (5 µg/ml), kanamycin (15 or 30 µg/ml), and trimethoprim (50 µg/ml). Where appropriate, fatty acids were supplemented to 100 µg/ml.

**Construction of deletion strains and plasmids**

Gene deletions in _E. coli_ were constructed using the Red recombinase method and transduced into recipient strains by P1vir (73). Plasmids were assembled using the In-Fusion HD cloning kit (Takara Bio). Plasmids with point mutations were constructed by inverse PCR. Primers used in this study are listed in Table S1.

**Deuterium labeling and affinity purification of Lpp(K58A) strep tag**

Strain TXM1111 was constructed and used to produce deuterium labeled [d₅]-labeled *DA-LppK58A-strep tag. For labeling, octanolic-7,7,8,8,8-[d₅]-acid (CDN Isotopes) was added to LB solid media at 100 µg/ml. After overnight incubation of strain TXM1111 at 37 °C, colonies were collected from the agar surface by scraping and washed with PBS (PBS, pH 7.4, 5.6 mM Na₂HPO₄, 1.06 mM KH₂PO₄, 154 mM NaCl). Labeled DA-LppK58A-strep tag was purified from the resulting biomass using affinity chromatography as described previously (11), except additional purification was performed to isolate the DA-LppK58A-strep tag from any phospholipid contaminants. After purification using Strep-Tactin®XT column (Iba Life Scien-

ces), protein was further purified using a 1-ml HiTrap Q HP anion exchange column (GE Healthcare) in buffer containing 100 mM Tris-HCl, pH 7.4, 50 mM NaCl, and 0.5 mM n-dodecyl-β-d-maltoside (DDM). The Q column flow through containing DA-LppK58A-strep tag was then loaded onto Superdex 10/300 sizing column (GE Healthcare) equilibrated with 10 mM Heps, pH 7.4, and 0.5 mM DDM buffer. Fractions containing purified protein were combined and flash frozen. Labeled DA-LppK58A-strep tag was characterized by MALDI-TOF MS and lipase treatment (described below). Purified protein was determined to contain two [d₅]-labeled acyl chains on the α-amino terminus (referred to as [d₅]-DA-LppK58A-strep tag). Unlabeled DA-LppK58A-strep tag preparations were likewise isolated from _E. coli_ TXM1111 but grown in LB agar containing unlabeled sodium octanoate (Sigma).

**Growth assay with fatty acids**

Single colonies of strains WT BW25113, TXM1018, TXM1029, and TXM1111 were pregrown in LB media. For TXM1111, pregrowth media contained sodium octanoate. Once cultures had reached exponential growth, cells were washed once with LB, inoculated into fresh LB to an OD₆₀⁰ of 0.0001 (≈5 × 10⁵ colony forming units/ml), and supplemented with either 100 µg/ml of sodium palmitate (Sigma) or sodium octanoate. Cultures were grown at 37 °C for 19 h before measuring the final OD₆₀⁰.

**Isolation of inner membranes for enzyme reconstitution assays**

As a source of Lnt, Lit, and Lnt/Lit, inner membranes of KA893, KA801, and KA736, respectively, were harvested as previously described, with some modifications (11). One liter of cells were grown to an OD₆₀⁰ of 1.0, harvested by centrifugation, washed once with PBS, and stored frozen until use. Cell pellets were resuspended in ~35 ml of 50 mM Tris, pH 7.8 (adjusted with HCl), containing 1 mM EDTA and disrupted with 3 passes through a French pressure cell at 14,000 p.s.i. Phenylmethylsulfonyl fluoride (PMSF) was then added to 1 mM and the lysates centrifuged at 3,200 × g for 10 min at 4 °C to remove unbroken cells. Total membranes were collected by ultracentrifugation at 110,000 × g for 90 min at 4 °C, then homogenized in 3 ml of 10 mM Heps, pH 7.4 (adjusted with NaOH), with a 26-gauge needle. The membrane suspension was layered on top of a discontinuous sucrose gradient as described previously and centrifuged at 80,000 × g for 19 h at 4 °C. The top volume of sucrose was discarded and the inner membrane layer (~4-6 ml) transferred to a new ultracentrifuge
indicated time intervals, 10-
Purified DA-LppK58A was then added to 0.004 mg/ml and total protein and sonicated for 1 min in a water sonicator bath.

Small scale reaction with unlabeled DA-LppK58A-strep tag

The reaction buffer, composed of 50 mM Tris, pH 7.2, 150 mM NaCl, and 0.1% Triton X-100, was optimized previously by Hillmann et al. (45) in activity assays with purified Lnt enzyme. To the reaction, inner membrane fractions containing Lnt, Lit, or Lnt/Lit were added to a final concentration of 0.75 mg/ml of total protein and sonicated for 1 min in a water sonicator bath. Purified DA-LppK58A was then added to 0.004 mg/ml and mixed by further sonication before incubation at 37°C. At the indicated time intervals, 10-μl samples were removed and the reaction halted with SDS-PAGE loading buffer and flash frozen.

Large scale lip acylation reaction and product purification

To 500 μl in the aforementioned reaction buffer supplemented with 1 mM PMSF and 0.75 mg/ml of total inner membrane protein fractions containing Lnt or Lit, 3 μg total of purified deuterium-labeled [d5]-DA-LppK58A-strep tag substrate was added. Reactions were incubated at 37°C overnight with constant shaking and intermittent sonication (~30 s in sonicator bath). MagStrep “type 3” XT beads (IBA Life Sciences) were used to purify LppK58A from the reaction following the manufacturer’s recommendations with minor modifications. The reaction was first clarified by centrifugation at 4,000 × g for 2 min, then combined with an excess of 3 μl of magnetic beads. The reaction was inverted for 1.5 h at 4°C and the beads washed as recommended. LppK58A-strep tag was eluted from the beads under denaturing conditions by boiling in 25 μl of SDS loading buffer for 10 min. Samples were taken from the start and end points of the initial reaction, the pelleted fraction, the unbound fraction, and the final elution fraction and visualized by Western blotting to assess for recovery, with an estimated ~40% recovery of total strep-tagged protein in the eluent. The entire elution fraction was loaded into a single lane on an SDS-PAGE gel for immunoblotting and further analysis by MS (see below).

MALDI-TOF MS analysis

For MS analysis of lipoprotein substrates, preparations were transferred from a 16.5% Tris-Tricine SDS-PAGE gel to a nitrocellulose membrane. The band corresponding to Lpp was excised, digested in a trypsin solution overnight, then sequentially washed with 0.1% TFA, 10% acetonitrile, and finally 20% acetonitrile before eluting the N-terminal lipopeptides in 10 mg/ml of α-cyano-4-hydroxycinnamic acid in 2:1 chloroform-methanol, as described previously (11, 40). MS and MS/MS spectra were acquired on an Ultraflextreme (Bruker Daltonics) MALDI-TOF mass spectrometer in positive anion mode.

Alanine scanning mutagenesis and Mut-seq analysis of E. faecalis lit

The lit gene was amplified from E. faecalis genomic DNA (primers TM1990 and SS1314) and cloned into pET22b(+) using the Ndel/Xhol restriction sites. This resulting plasmid (pET22b(+)-Eflit) was then used as template for inverse PCR using primer pairs that introduced an alanine codon (GCC) at each position. Primers were 25 bp long, with 6 bp on the 5’-side and 16 bp on the 3’-side retaining homology to DNA flanking the targeted codon. The forward and reverse primers shared 15 bp of complimentary homology on the 5’ ends to facilitate assembly by re-circularization. Separate PCR (212 total for each Lit amino acid encoding triplet) were set up in 96-well microplates with pET22b(+)-Eflit as template (1 ng/20-μl reaction). After PCR, 5-μl aliquots were resolved on 1% Tris-Tri-cine gel and immunobotted for LppK58A-strep tag using Precision Protein StrepTactin-horseradish peroxidase (strep-HRP) conjugate (Bio-Rad) as described previously (11).

Lipoprotein lipase treatment [d5]-DA-LppK58A-strep tag

Lyophilized LPL from Pseudomonas sp. (Sigma) was reconstituted in water to 1 mg/ml, then added to a final concentration of 80 ng/μl in a 15-μl reaction with 1.8 μg total of [d5]-DA-LppK58A-strep tag. The reaction was incubated at 37°C for 19 h and halted by the addition of SDS loading buffer and boiling for 10 min. The reaction volume was loaded into a single lane on an SDS-PAGE gel for immunoblotting and further analysis by MS (see below).
Lyso-LP formation by lit

calculated by summing the number of G, C, and G reads at positions one, two, and three of the in-frame triplet, respectively. This sum was then divided by the average coverage at the corresponding three base positions. To correct for synonymous changes involving bases held in common with GCG, this raw value was adjusted by dividing by 3 for HDH, by 2 for GDH/HCH/HDG, and 1 for HCG/GDG/GCH targeted codons to generate GCG enrichment values for each in-frame triplet in the library. Depletion ratios were then calculated by dividing the enrichment values for each Lit codon in the input library by the int-depleted library after three passages.

Phenotypic confirmation of select lit Ala mutants

Select mutants were re-made by inverse PCR and transformed into KA349 using the method described above for the Ala library. Transformants were grown to mid-logarithmic growth in LB supplemented with 0.2% arabinose, washed, and serially diluted in PBS before spot tittering on LB agar plates containing either 0.2% glucose or 0.2% arabinose to compare growth in LB supplemented with 0.2% arabinose, washed, and then incubated overnight at 37 °C before imaging. To measure Lit protein expression levels and complementation efficiency, plates were incubated overnight after spotting total protein extracts obtained from exponentially growing cells (StrepMAB-Classic HRP conjugate, IBA Life Sciences) using PAGE Tris glycine gel, transferred to nitrocellulose membranes, and immunoblotted with StreptMAB-Classic HRP conjugate (IBA Life Sciences) using total protein extracts obtained from exponentially growing cells at identical optical densities.

His-89 and His-157 randomization and Mut-seq analysis

Primers targeting His-89 (TM2182 and TM2183) and His-157 (TM2184 and TM2185) were designed to introduce the degenerate NNK/MNN triplet and create a library of 32 unique triplicates, encompassing 31 amino acids and a stop codon. All 20 amino acids are represented by at least one codon. Plasmids construction, selection post int-depletion, and next generation sequencing was performed as described above for the alanine scan library. Reads from each passage were aligned to the reference WT lit sequence (10,000- to 25,000-fold coverage depth at His-89 and His-157 to generate at minimal 100 reads per randomized codon). Representation of each NNK codon was calculated as a percentage of the entire library in the input and passaged libraries.

LacZ and PhoA lit fusion activity assays

A series of pET22b(+)−based constructs were made by fusing lit to either β-gal (lacZ) or alkaline phosphatase (phoA) reporters lacking their endogenous N-terminal signal peptides (from amino acid +13 for PhoA and +9 for LacZ) (26). Fusions were made after each predicted TM pass in every loop of Lit. Constructs encoding native lacZ and phoA genes were used as positive controls. For colorimetric activity assays on solid media, the indicated strains were grown on LB agar containing 100 μg/ml of carbenicillin and 1 mM isopropyl β-D-1-thiogalactopyranoside. To assay for LacZ activity, media also contained 40 μg/ml of 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal), and for PhoA activity, 40 μg/ml of 5-bromo-4-chloro-3-indoly phosphate (BCIP).

SCAM for lit topology mapping

Native cysteine residues were replaced by three rounds of inverse PCR using the corresponding alanine scan library primers to construct pET22b(+)−Eflit strep C16A, C100A, C187A plasmid. The cysteine-less lit gene was amplified by PCR (primers TM2149 and TM2150) and cloned into the XbaI/AsgI sites of pLI50-Pras for low level constitutive expression. Inverse PCR using the corresponding primers (Table S1) was used to reintroduce Cys in targeted positions. SCAM experiments were conducted as described (55, 56), except probe labeling was conducted with 2.5 mm thiol probe NEM (Acros Organics), MTSES, (Biotium), or AMS (Invitrogen) for 20 min before quenching with addition of 100 mM cysteine. Cells were then washed three times with PBS before proceeding to lysis and labeling with 5 mm mal-PEG (average molecular mass 5,000 Da, Sigma). Lysed protein extracts were resolved on a 12% SDS-PAGE Tris glycine gel, transferred to nitrocellulose membranes, and immunoblotted with StreptMAB-Classic HRP conjugate (IBA Life Sciences).

Data availability

All data described herein are contained within this manuscript.

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Conflict of interest—The authors declare that they have no conflicts of interest.

Abbreviations—The abbreviations used are: Lgt, lipoprotein diacylglycerol transferase; Lsp, lipoprotein signal peptidase II; Lit, lipoprotein intramolecular transferase; ABC, ATP-binding cassette; ACP, acyl carrier protein; AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CoA, coenzyme A; DA-1P, diacylated lipoprotein; DDM, n-dodecyl-β-d-maltoside; HRP, horseradish peroxidase; LacZ, β-galactosidase; LB, lysogeny broth-Miller medium; LPL, lipoprotein lipase; Lyso-LP, lysophosphatidylcholine; mal-PEG, methoxypolyethylene glycol maleimide;
MTSES, 2-sulfonatoethyl methanethiosulfonate; NEM, N-ethylmaleimide; PhoA, alkaline phosphatase; PMSEF, phenylmethylsulfonyl fluoride; SCAM, substituted cysteine accessibility method; TAE, Tris acetate-EDTA; TA-LP, triacylated lipoprotein; TM, transmembrane; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; mACP, malonyl-ACP; FAS, fatty acid synthesis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Llp, lipoprotein.

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