Chapter 13

Measurement of Lysophospholipid Transport Across the Membrane Using *Escherichia coli* Spheroplasts

Yibin Lin, Lei Zheng, and Mikhail Bogdanov

Abstract

In the inner membrane of Gram-negative bacteria lysophospholipid transporter (LplT) and the bifunctional acyl-acyl carrier protein (ACP) synthetase/2-acylglycerolphosphoethanolamine acyltransferase (Aas) form a glycerophospholipid remodeling system, which is capable of facilitating rapid retrograde translocation of lyso forms of phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin across the cytoplasmic membrane. This coupled remodeling enzyme tandem provides an effective method for the measurement of substrate specificity of the lipid regeneration and lysophospholipid transport per se across the membrane. This chapter describes two distinct but complementary methods for the measurement of lysophospholipid transport across membrane using *Escherichia coli* spheroplasts.

Key words Lysophospholipid flipping, Lysophospholipid transporter, Aas, Glycerophospholipid remodeling system, Thin-layer chromatography, Spheroplasts

1 Introduction

Lysophospholipids (LPLs) are small bioactive lipid molecules characterized by a single carbon chain and a polar head group. Two subgroups can be distinguished: molecules containing the sphingoid base backbone (lysosphingolipids) and molecules containing the glycerol backbone (lysoglycerophospholipids). The LPL structure renders these lipids more hydrophilic and versatile than their corresponding phospholipids [1]. Distinct from their diacyl counterparts, these inverted cone-shaped molecules share physic characteristics of detergents, enabling modification of local membrane properties such as curvature and micellization [2]. In eukaryotic cells, it is now widely accepted that LPLs function as mediators through G-protein-coupled receptors (GPCRs) [3]. Intracellular or extracellular LPL transport across the membrane is required to activate corresponding GPCR, such as sphingosine-1-phosphate (S1P) [4], or for the uptake of bioactive substances, such as docosahexaenoic acid (DHA) [5]. The role of LPLs remains poorly

Guillaume Drin (ed.), *Intracellular Lipid Transport: Methods and Protocols*, Methods in Molecular Biology, vol. 1949, https://doi.org/10.1007/978-1-4939-9136-5_13,

characterized in bacteria. As a protective barrier, bacterial membranes are often stressed by harsh environments and challenged by external perturbations, caused by exposure to bile acids, different temperatures, or diverse phospholipases (PLAs). Bile acids and heat shock cause a dramatic increase in the lysophosphatidylethanolamine (LPE) level in the *E. coli*, *Vibrio cholerae*, and *Yersinia pseudotuberculosis* membranes, respectively [6–8]. Disrupting bacterial membranes by PLAs is one of the major host cell defense mechanisms used to kill an invading bacterium [9]. LPE can be taken by bacteria from exogenous sources [10] or a "silent" outer membrane phospholipase A_1 (PldA) and lipid A palmitoyltransferase PagP with broad diacylated lipid specificity can be activated in stressed cells to form LPLs as products and by-products, respectively [11, 12]. LPL transport across the membrane and the phospholipid remodeling were reported to be important for maintaining bacterial membrane stability and bacterial survival [9], cell division and other functions [2]. Although the LPL turnover membrane is functionally important [2, 9, 13], the mechanism of LPL turnover and its physiological significance remain obscure and require further investigation. Development of a high-efficiency method for measurement of LPL transport across the membrane is required for addressing some of these outstanding questions.

In the inner membrane of Gram-negative bacteria, LplT was identified as lysophospholipid transporter, which is capable of facilitating rapid retrograde translocation of LPE, lysophosphatidylglycerol (LPG), monoacyl-cardiolipin (MCL), diacyl-cardiolipin (DCL) across the cytoplasmic membrane [13]. Aas is a bifunctional enzyme catalyzing acyl transfer to LPL, generating diacyl form of the phospholipid [14, 15]. The LplT-Aas system has been reported to translocate and facilitate remodeling of all three bacterial major phospholipids including PE, PG, and CL with comparable translocation and remodeling efficiencies [13]. The LplT-Aas system in Gram-negative bacteria provides an efficient method for measuring LPL transport across the membrane (Fig. 1). Two methods have been developed and used to monitor LPL transport across the membrane based on the mechanism of functioning of LplT-Aas system in Gram-negative bacteria. The general design of the experiments to measure the LPL transport across the membrane is outlined in Fig. 2. ^{32}P-labeled diacyl phospholipids are prepared by growing various recombinant *E. coli* strains (Table 1) in LB broth containing 5 μCi/mL ^{32}P-orthophosphate. ^{32}P-labeled PE is synthesized in *E. coli* strain UE54 (MG1655 *lpp-2Δara714 rcsF::mini-Tn10 cam pgsA::FRT-kan-FRT*) containing up to 95% of PE [16]. ^{32}P-labeled PG and CL are made in the *E. coli* AL95 strain (*pss 93::kan lacY::Tn9*), lacking the ability to synthesize PE. This strain contains only acidic phospholipids, such as PG and CL (45–50%, respectively) and PA which altogether build a negatively charged membrane consisting of 100% of negatively charged phospholipids

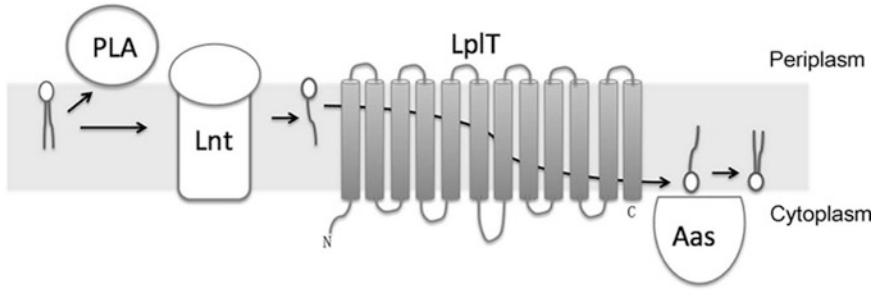

Fig. 1 A topological diagram of the LPL biogenesis and remodeling in the Gram-negative bacterial inner membrane. Only lysophospholipids generated from diacylphospholipids by PLA-mediated deacylation or lipoprotein acyltransferase (Lnt)-catalyzed transacylation reactions within inner membrane of *Escherichia coli* are shown. LplT flips the resultant lysophospholipids across the inner membrane for reacylation by Aas to the cytoplasmic side. Figure and legend are freely reproduced with modification from reference [13]

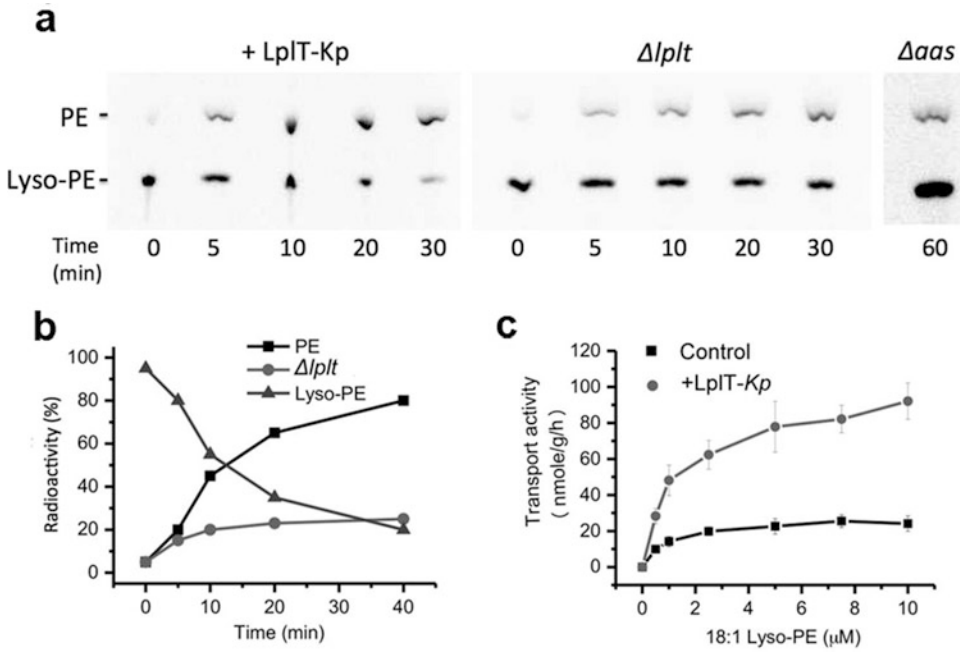

Fig. 2 Two methods for the detection of LPL transport across membrane using *E. coli* spheroplasts. (**a**) TLC-based translocation assay. LPL is mixed with spheroplasts prepared from *E. coli* Δ*lplt* cells expressing plasmid-borne LplT protein. *E. coli* Δ*lplt* cells harboring an empty vector or Δ*aas* cells are used as a negative control. The reactions are stopped by 0.5 M NaCl in 0.5 N HCl at the indicated times, and total lipids are extracted and then separated by TLC. The substrates and acylated products shown in TLC plate can be quantified using a phosphorimaging system. (**b**) Silicone oil-spin method. LPL is mixed with spheroplasts prepared from *E. coli* Δ*lplt*/*aas* cells expressing LplT or Δ*lplt*/*aas* cells harboring an empty vector (control). After incubation, samples are centrifuged through a layer of silicone oil to stop the reaction and separate spheroplasts from non-transported LPLs. After centrifugation, aliquots of the perchloric acid phase are removed and the radioactivity is counted

Table 1
Bacterial strains

Stain	Genotype	Grow condition	Application	References
UE54	*MG1655 lpp-2Δara714 rcsF::mini-Tn10 cam pgsA::FRT-kan-FRT*	LB	95% PE	[16]
AL95	*pss 93::kan lacY::Tn9*	LB + 50 mM $MgCl_2$	40% PG, 45% CL	[17]
AL95	*pss 93::kan lacY::Tn9; pAC-PCSlp-Sp-Gm*	LB + 50 mM $MgCl_2$	PC	[18]
SM2-1	*plsC(Ts)*	LB	20% PLA	[19]

[17]. Strain AL95 carrying plasmid pAC-PCSlp-Sp-Gm is used to prepare ^{32}P-labeled phosphatidylcholine (PC) from cells synthesizing this foreign lipid in an amount equivalent to that of PE (75%) [18]. Radiolabeled LPE, LPC, LPG, MCL, and DCL are prepared by digestion of the corresponding purified ^{32}P-labeled phospholipids with phospholipase A_2 (PLA_2). Lysophosphatidic acid (LPA) is made in the temperature-sensitive *E. coli* strain SM2-1 (*plsC*(Ts)) accumulating this LPL (up to 20%) after a shift to growth at nonpermissive temperature [19]. Lysophospholipid transporters are expressed in the appropriate *E. coli* strains, and the spheroplasts are made using the basic lysozyme-EDTA method [20]. For thin-layer chromatography (TLC)-based LPL translocation assay, radioactive LPLs are mixed with cold counterparts to fit an apparent μM binding affinity [13] and resuspended in ethanol. Transport assays are initiated by adding substrates into spheroplast solutions. At the indicated time, reactions are terminated by adding a chloroform/methanol solvent system. The total lipids are extracted and separated by TLC. The Aas-dependent formation of diacyl form of lipids from corresponding lyso form can be quantified from TLC plate, which reflects those LPLs that has been translocated across the membrane (Fig. 3a, b). This is an indirect approach to measure LPL transport across the membrane. However, this technique, which requires coupled functioning of lysophospholipid transporter as well as acyltransferase/acyl-ACP synthetase has its own limits due to two facts [13]: (1) Substrates flipped into the spheroplasts are indistinguishable from non-transported substrates monitored by TLC because to maintain stability the spheroplasts were not collected by centrifugation before lipid extraction; (2) Whether LplT transport activity is facilitated by downstream Aas-mediated continuous reacylation of transported LPLs driving their downhill uptake is unknown. Although this assay can be used to test substrate specificity of LplT/Aas transport/acylation regeneration system in order to characterize LplT transport activity per se, the transport reaction should be separated from assay mixture by

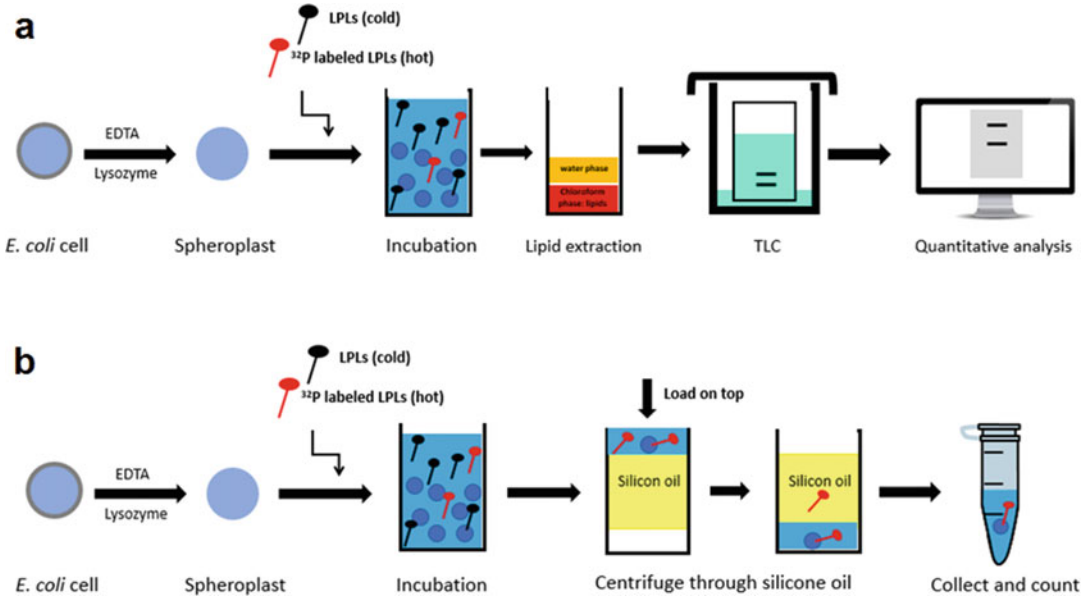

Fig. 3 LPE transport across the *E. coli* inner membrane measured by TLC-based translocation assay and silicone oil-spin method. (**a**) TLC for separating substrates and acylated products. (**b**) Quantification of the substrates and acylated products shown in (**a**). (**c**) Transport assay of LPE by LplT protein using silicone oil-spin method. For detailed experimental procedure *see* Fig. 2 and the main text. Figure and legend are freely reproduced with modification from [13]

centrifugation through a layer of silicon oil. Due to their different sedimentation rates and miscibility with oil, non-transported lipids are retained in silicon oil and the upper layer, whereas the pelleted spheroplasts containing transported substrates are assessed by scintillation counting to measure LplT transport kinetics (Fig. 3c). To eliminate any coupling effect of Aas, the transport assay should be performed in Δ*aas* mutant expressing LplT from the plasmid. In this transport assay, LPLs (premixed "cold" and "hot") were added to the spheroplasts to a desired final concentration as indicated and incubated at 37 °C for 30 min. After incubation, samples were centrifuged through a layer of silicone oil [21] to stop the reaction and separate spheroplasts from non-transported LPLs. After centrifugation, samples of the perchloric acid phase were removed and the radioactivity was measured by a liquid scintillation counter. The silicon oil-spin method does not require functional acyltransferase/acyl-ACP synthetase. These two methods each have strengths and limitations, yet combining them make it possible to accurately measure LPL transport across membrane. In this chapter, we describe these two methods. Although this protocol was developed based on a Gram-negative bacteria glycerophospholipid remodeling system, it can be extended to any other lysophospholipid transporters that can be expressed in Gram-negative bacteria inner

membrane. TLC-less silicone oil-spin method can be adapted to any membrane protein and expression system to study kinetics of LPL translocation.

2 Materials

2.1 Lipid Extraction

1. 0.5 M NaCl in 0.5 N HCl solution.
2. Methanol/chloroform solvent system (2:1, v/v).
3. Vortex equipped with microtube foam rack for multiple polyallomer tubes.
4. Tabletop centrifuge.
5. Savant Speed Vacuum Concentrator.

2.2 TLC

1. EMD Millipore TLC Silica Gel 60 F_{254}.
2. TLC developing tanks with lids for use with TLC plates up to 20 × 20 cm.
3. Plain Whatman 3 M filter paper sheets.
4. Oven for activation of TLC.
5. Tank with regenerable indicating desiccant.
6. 1.2% (w/v) boric acid in ethanol/water (1:1, v/v).
7. Developing solvent system: Chloroform/methanol/water/ammonia (39% w/v, 8.56 N), (60:37.5:3:1, v/v/v/v).
8. X-Ray developing cassettes.
9. Kodak HR GP plate.
10. Imaging system such as a Fluor-S Max MultiImager (Bio-Rad Laboratories) or similar.

2.3 Isolation of Lipids from TLC

1. X-Ray Film such as Thermo Scientific CL-XPosure Film.
2. X-ray Film Auto Processor.

2.4 Cell Culture

1. Luria–Bertani (LB) liquid medium: 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl.
2. Luria–Bertani (LB) agar plate: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 16 g/L agar.
3. 2 M $MgCl_2$ stock solution sterilized by passing through a 0.22 μm syringe filter.
4. 10% arabinose stock solution sterilized by passing through a 0.22 μm syringe filter and stocked fresh.
5. 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) stock solution sterilized by passing through a 0.22 μm syringe filter.

6. 100 mM choline chloride stock solution sterilized by passing through a 0.22 μm syringe filter.

2.5 Preparation of ^{32}P-Labeling LPLs

1. *E. coli* strain UE54 (*MG1655 lpp-2Δara714 rcsF::mini-Tn10 cam pgsA::FRT-kan-FRT*) is used to make ^{32}P-labeled PE. *E. coli* AL95 strain (*pss 93::kan lacY::Tn9*), lacking the ability to synthesize PE, is used to make ^{32}P-labeled PG and CL. Strain AL95 carrying plasmid pAC-PCSlp-Sp-Gm is used to prepare ^{32}P-labeled phosphatidylcholine (PC). The temperature-sensitive *E. coli* strain SM2-1 (*plsC(Ts)*) is used to make lysophosphatidic acid (LPA) (*see* Table 1).
2. Phosphorus-32 radionuclide orthophosphoric acid.
3. Phospholipase A_2 (PLA_2) from porcine pancreas or venom.
4. Digestion solution: 0.1 M HEPES-NaOH, pH 7.5, 0.1 M KCl, 10 mM $CaCl_2$ and 1% DDM (*n*-Dodecyl-β-D-maltoside).

2.6 Preparation of E. coli Spheroplasts

1. Spheroplast solution A: Add 125 μL of 0.1 M Tris–HCl, pH 8, 114 μL of 2 M sucrose, 21 μL of 1% EDTA, pH 7, and 21 μL of 0.5 mg/mL lysozyme into a 1.5 mL Eppendorf tube, and complete with ddH_2O to 0.5 mL.
2. Spheroplast solution B: Add 125 μL of 0.1 M Tris–HCl, pH 8 and 114 μL of 2 M sucrose into a 1.5 mL Eppendorf tube, and complete with ddH_2O to 0.5 mL.
3. 10 mM $MgCl_2$, 0.75 M sucrose solution.

2.7 TLC-based LPL Translocation Assay

1. Lipids: 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (18:1 Lyso PE), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (18:1 Lyso PG), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (18:1 Lyso PC), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate (sodium salt) (18:1 Lyso PA), monolyso heart CA, dilysocardiolipin (heart, bovine) (sodium salt).
2. Imaging system such as a Fluor-S Max MultiImager (Bio-Rad Laboratories) or similar.
3. Quantity One 1-D Analysis Software (Bio-Rad Laboratories).
4. Water bath.

2.8 Silicone Oil-Spin Method

1. Silicone oil ($d = 1.05$).
2. 22% perchloric acid.
3. LKB Wallac Liquid Scintillation Counter (Model 2109) or similar.
4. *E. coli* dry weight: 2.8×10^{-13} g per cell (adopted from Bio-Rad Laboratories).

3 Methods

3.1 Lipid Extraction

1. Resuspend 1 g of cell pellet, 0.5 mL of reaction solution, or 1 g of powder in 1.5 mL of 0.5 M NaCl in 0.5 N HCl solution and transfer it to a 15 mL falcon tube (*see* **Note 1**).
2. Add 4.5 mL of methanol/chloroform (2:1, v/v) mixture, and vortex for 20 min.
3. After vortexing, add another 1.5 mL of 0.5 M NaCl in 0.5 N HCl solution and continue to vortex for 5 min.
4. Centrifuge at 2000 × *g* for 5 min.
5. Discard upper water-methanol phase, carefully transfer lower chloroform lipid phase to a new Eppendorf tube.
6. Dry the lipid extract in a Savant Speed Vacuum Concentrator.

3.2 TLC

1. Line the inner walls of a developing tank with Whatman 3 M paper, which absorbs the solvent and helps to saturate a tank by providing more surface area for evaporation of vapor rising from both the sides of tank in an even manner. Use a bench but not a hood to saturate a tank (*see* **Note 2**).
2. Prepare developing solvent system immediately before use. Mix it in a tightly closed 150–200 mL bottle and then transfer it into the tank. Seal the tank and wait about 2 h, which allows the tank atmosphere to be thoroughly saturated with solvent vapor before using.
3. Impregnate Silica Gel G thin-layer plates for 1 min in 1.2% (w/v) boric acid in ethanol/water (1:1, v/v) and let the plates standing on the absorbent paper towels for 5 min. Dry the plates 5 min on air, and then put the plates into the oven (90 °C) for 60 min (*see* **Note 2**). Remove the plates from the oven and desiccate them for 15 min (*see* **Note 2**).
4. Subject 50 μL of lipid samples onto Silica Gel G thin-layer plates in their predetermined positions (pencil marks) along with a line drawn 2 cm above the bottom of the plate with TLC spotting capillary tubes. Leave at least 1 cm between sample positions and the edge of the silica plate.
5. Once fully loaded, position the plate in the solvent in the tank, and run the TLC until the solvent front reaches 5 mm below the top of the TLC plate.
6. Quickly remove the plate and allow them to dry in the air (under a fume hood) before exposing to an X-ray or Bio-Rad developing screens for several hours to several days.

7. The phospholipid bands were visualized by developing the film, and bands corresponding to ^{32}P-labeled phospholipids are quantified using the Quantity One software (Bio-Rad Laboratories).

3.3 Purification of Lipids from TLC

1. Perform TLC for ^{32}P-labeled lipids mixture following the method as shown in Subheading 3.2.
2. Expose air-dried TLC plate to an X-ray film for 2 h.
3. Develop the film, and use this film to localize/map the bands on the TLC plate that correspond to the desired lyso- or diacyl phospholipid.
4. Wet the phospholipid spot with water, and then scrape it gently and carefully transfer the powder containing the wanted lipid into a 15 mL falcon tube.
5. Perform lipid extraction following the method as shown in Subheading 3.1 (*see* **Note 1**).

3.4 Preparation of ^{32}P-Labeling LPLs

3.4.1 Preparation of ^{32}P-Labeled LPE

1. To recover *E. coli* strain UE54 from glycerol stock, open the tube and use a sterile loop, toothpick, or pipette tip to scrape some of the frozen bacteria off of the top. Do not let the glycerol stock unthaw! Streak the bacteria onto an LB agar plate.
2. Grow bacteria overnight at 37 °C.
3. Next day, pick a single colony and grow bacteria overnight in 20 mL of LB broth containing 5 μCi/mL ^{32}P-orthophosphate (final) at 37 °C in a 50 mL falcon tube (*see* **Note 3**).
4. Overnight growth cells were harvested by centrifugation at 4000 × *g* for 10 min.
5. Perform lipid extractions following the method described in Subheading 3.1.
6. Dry the lipid extract in a Savant Speed Vacuum Concentrator.
7. Generate LPE from PE: Redissolve the dried lipid extract with 0.5 mL of digestion solution. Sonicate in ice by 59-s bursts (amplitude 16%). Add 10 U of PLA_2 from porcine pancreas and incubate at 37 °C for overnight with shaking.
8. Extract lipids following the same method as in **step 5**.
9. Dry the lipid extract in a Savant Speed Vacuum Concentrator. Redissolve the dried lipid extract with 50 μL of chloroform.
10. Perform TLC to analyze results of PLA_2 digest reactions (*see* **Note 4**) following the method as shown in Subheading 3.2.
11. Purify LPE from TLC following the method as shown in Subheading 3.3.

3.4.2 Generation of LPG, D-CL, and M-CL

1. Revive an *E. coli* strain AL95 from glycerol stock on LB-agar supplemented with 50 mM $MgCl_2$ (from 2.5 M sterile stock) since these cells strictly require a millimolar amount of magnesium for growth (*see* **Note 3**).
2. Grow bacteria overnight at 37 °C.
3. Next day, pick a single colony and grow bacteria overnight in 20 mL of LB broth containing 5 μCi/mL ^{32}P-orthophosphate (final concentration) and 50 mM $MgCl_2$ at 37 °C in a 50 mL falcon tube.
4. Overnight growth cells were harvested by centrifugation at 4000 × *g* for 10 min.
5. Perform lipid extractions following the method as shown in Subheading 3.1.
6. Dry the lipid extract in a Savant Speed Vacuum Concentrator. Redissolve the dried lipid extract with 50 μL of chloroform.
7. Purify PG and CL from the TLC plate following the method as shown in Subheading 3.3.
8. Generation of LPG from PG: Redissolve the dried PG with 0.5 mL of digestion solution. Sonicate in ice by 59-s bursts (amplitude 16%). Add 10 U of PLA_2 from porcine pancreas and incubate at 37 °C overnight under shaking.
9. Generate D-CL and M-CL from CL: Redissolve the dried CL with 0.5 mL of digestion solution. Sonicate in ice by 59-s bursts (amplitude 16%). Add 10 U of PLA_2 from porcine pancreas. To make D-CL, incubate the reaction mixture at 37 °C for 2 h under shaking. To make M-CL, keep the reaction mixture at 37 °C overnight under shaking.
10. Extract lipids following the same method as shown in **step 5**.
11. Dry the lipid extract in a Savant Speed Vacuum Concentrator. Redissolve the dried lipid extract with 50 μL of chloroform.
12. Perform TLC to analyze results of PLA_2 digest reactions following the method as shown in Subheading 3.2 (*see* **Note 4**).
13. Purify LPLs from TLC following the method as shown in Subheading 3.3.

3.4.3 Generation of LPC

1. Revive an *E. coli* strain AL95 carrying plasmid pAC-PCSlp-Sp-Gm from glycerol stock on LB-agar plates supplemented with 50 mM $MgCl_2$ as described in **step 1** of Subheading 3.4.2 (*see* **Note 3**).
2. Grow bacteria overnight.
3. Next day, pick a single colony and grow bacteria overnight in 4 mL of LB broth supplemented with 50 mM $MgCl_2$ at 37 °C in a 10 mL plastic culture tube.

4. Next day, dilute overnight culture 1/100 into 20 mL of LB broth supplemented with 0.2% arabinose, 2 mM choline, and 5 μCi/mL ^{32}P-orthophosphate and culture cells at 37 °C in a 50 mL falcon tube (*see* **Note 3**).
5. When cell culture reaches an OD_{600} of 0.6–0.8, harvest the cells by centrifugation at 4000 × *g* for 10 min.
6. Perform lipid extractions following the method as shown in Subheading 3.1.
7. Dry the lipid extract in a Savant Speed Vacuum Concentrator.
8. Generate LPC from PC: Redissolve the dried lipid extract with 0.5 mL of digestion solution. Sonicate in ice by 59-s bursts (amplitude 16%). Add 10 U of PLA_2 from porcine pancreas and incubate at 37 °C overnight under shaking.
9. Extract lipids following the same method as in **step 6**.
10. Dry the lipid extract in a Savant Speed Vacuum Concentrator. Redissolve the dried lipid extract with 50 μL of chloroform.
11. Perform TLC to analyze the result of PLA_2 digest reactions following the method as shown in Subheading 3.2 (*see* **Note 4**).
12. Purify LPC from TLC following the method as shown in Subheading 3.3.

3.4.4 Generation of LPA

1. Recover temperature-sensitive *E. coli* strain SM2-1 from glycerol stock.
2. Grow bacteria overnight at 30 °C.
3. Next day, pick a single colony and grow bacteria overnight in 4 mL of LB broth at 30 °C in a 10 mL plastic culture tube.
4. Next day, dilute overnight culture to 1/100 into 100 mL of LB broth and culture cells at 30 °C in a 250 mL glass Erlenmeyer Flask.
5. When cell culture reaches an OD_{600} of 0.6–0.8, add 5 μCi/mL ^{32}P-orthophosphate to the culture, and culture cells at 42 °C for 2 h (*see* **Note 5**).
6. Harvest cells by centrifugation at 4000 × *g*.
7. Perform lipid extractions following the method as described in Subheading 3.1.
8. Dry the lipid extract in a Savant Speed Vacuum Concentrator. Redissolve the dried lipid extract with 50 μL of chloroform.
9. Perform TLC to analyze the yield of LPA following the method as described in Subheading 3.2.
10. Purify LPA from TLC following the method as described in Subheading 3.3.

3.5 Preparation of E. coli Spheroplasts

1. Revive *E. coli* WT or Δ*lplT*, Δ*aas*, Δ*lplT/aas* mutant strains from glycerol stock as described above.
2. Grow bacteria overnight at 37 °C.
3. Next day, pick a single colony and grow bacteria overnight in 4 mL of LB broth supplemented with antibiotic if needed at 37 °C in a 10 mL plastic culture tube.
4. Next day, dilute overnight culture to 1/100 into 20 mL of LB broth supplemented with 0.1 mM IPTG and antibiotic if needed, and culture cells at 37 °C in a 50 mL falcon tube for 2 h (*see* **Note 6**).
5. Harvest the cells by centrifugation at 4000 × *g*.
6. For 3×10^9 cells, resuspend the pellet in a total of 500 μL of spheroplast solution A and incubate it at 25 °C.
7. Monitor the formation of spheroplasts: every 5 min withdraw and inject 100 μL of reaction mixture (**step 6**) into 2 mL of water or a solution of 10 mM $MgCl_2$, 0.75 M sucrose, and compare the OD_{600} of each other. The spheroplasts are stable in the solution of 10 mM $MgCl_2$, 0.75 M sucrose (OD_{600} does not change), but will rupture (break) in plain water (OD_{600} drops immediately) (*see* **Note 7**).
8. Pellet osmotically unstable spheroplasts by centrifugation at 6000 × *g*.
9. Wash spheroplasts three times with spheroplast solution B by resuspending the pellet in the same solution and re-pelleting the sample.
10. Resuspend spheroplasts in solution B at 10 mg/mL of total protein.

3.6 TLC-based LPL Translocation Assay

1. Weight "cold" LPLs and dissolve them in chloroform.
2. Dissolve radioactive LPLs with chloroform and mix them with their cold counterparts.
3. Dry the lipid mixture in a Savant Speed Vacuum Concentrator and resuspend it in ethanol to a final concentration of 200 μM by sonication (59-s bursts, amplitude 16%).
4. Add 10 μM (final concentration) of substrates into spheroplast solutions and incubate in a 37 °C water bath (*see* **Note 8**).
5. At the indicated time of 0, 5 min, 10 min, 30 min, and 60 min, transfer 0.5 mL of reaction solution into a 15 mL falcon tube containing 0.5 M NaCl in 0.5 N HCl to stop the reaction. Extract lipids following the method as described in Subheading 3.1 (*see* **Note 8**).
6. Perform TLC as shown as described in Subheading 3.2 for LPL translocation analysis.

7. Expose dried TLC to the imaging screen for appropriate time and store the image using a Molecular Imager FX (Bio-Rad Laboratories) (Fig. 3a).
8. Process and quantify the stored images using Quantity One software (Bio-Rad Laboratories) (Fig. 3b).

3.7 Silicone Oil-Spin Method

1. Prepare "cold" and "hot" lysophospholipid mixture following the method as shown in Subheading 3.5.
2. Add different concentration of substrates into spheroplast solutions and incubate in a 37 °C water bath for 30 min.
3. After incubation, transfer 0.5 mL samples from the reaction mixtures and layer them onto 0.15 mL of 22% perchloric acid solution and 0.50 mL of silicone oil ($d = 1.05$) in microcentrifuge tubes. Centrifuge through the silicone oil in an Eppendorf microcentrifuge at 14,000 × *g* for 5 min at room temperature (*see* **Note 9**).
4. After centrifugation, discard the upper phase, carefully transfer the lower phase into a new Eppendorf tube. Use 1 mL and 200 μL Gilson pipetmans to remove upper and withdraw lower phases, respectively.
5. Measure the radioactivity of the lower phase using a liquid scintillation counter.
6. Normalize the data to nmol/g total protein/h based on the specific radioactivity of the lipids and considering a *E. coli* dry weight of 2.8×10^{-13} g per cell (Fig. 3c).

4 Notes

1. To extract lipids from 1 g of cell pellet, 0.5 mL of reaction solution, or 1 g of powder, 1.5 mL of 0.5 N NaCl in 0.1 N HCl, 4.5 mL of methanol/chloroform (2:1, v/v), and 1.5 mL of 0.5 N NaCl in 0.1 N HCl (1:3:1, v/v/v) solution should be added sequentially. Dry material, such as a silica gel powder scraped from TLC plate, should be first saturated with 0.5 N NaCl in 0.1 N HCl alone by vigorous vortexing. Indeed, if silica flakes/powder are dry and chloroform/methanol is added first, the efficiency of extraction is weaker. Usually, all volumes can be scaled up or down depending on demand (number of cells or silica gel powder). Lower phase should be collected carefully without contamination from the upper phase. To extract lipids from wet or liquid solution (cell pellet or suspension), vortexing for 20 min should be enough.
2. The tank should be prepared 2 h before use to allow the chamber air to be thoroughly impregnated with solvent vapor. TLC plate should be heated for at least 1 h, but should

not be heated for too long, such as overnight. Activated TLC adsorbs water vapor from the air and becomes hydrated. Therefore, after heating in an oven, a TLC plate should be desiccated for 10 min in the tank with regenerable indicating desiccant. An activated TLC can be kept in a cabinet with continuous air flow dehumidified by passing through a polycarbonate drying column with activated regenerable indicating desiccant. The column should be connected in-line between a drying cabinet and compressed air line or gas cylinder.

Developing solvent for TLC should be freshly prepared. Only 28–30% ammonia hydroxide (e.g., highest concentration possible) should be used to prepare a solvent system. The concentrations of ammonia and boric acid solution can change after a long-time use, which may affect the mobility and separation of individual phospholipid TLC. The solutions should to be properly renewed. Before exposing to an X-ray screen, TLC plate should be dried completely; otherwise, chloroform may damage the Bio-Rad imaging screen.

3. *E. coli* UE54 should be always grown overnight in order to receive the cells with the maximal amount of PE (95–97%). PE-deficient cells should be always grown on LB-agar supplemented with 50 mM $MgCl_2$. Although in AL95 strain carrying plasmid pAC-PCSlp-Sp-Gm zwitterionic PE is substituted by the same amount of net neutral PC, these cells still require millimolar Mg for growth. These two strains should be checked out routinely by growth on LB-agar plates with and without 50 mM $MgCl_2$ (no growth without Mg should be observed). To fully express *pcsA* gene and get the cells with 75% PC, the cells should be outgrown first overnight in the presence of 50 mM $MgCl_2$ and then diluted in the morning to 1/100 or more (to start growth approximately from an OD_{600} of 0.025).
4. PLA_2 from porcine pancreas has a higher activity against phospholipids. Under the experimental condition described in this protocol, PLA_2 can completely hydrolyze PE, PG, and PC substrate into the corresponding LPLs. As PLA_2 can use CL, triacyl-CL, diacyl-CL, and monoacyl-CL as substrates, TLC, as shown in Subheading 3.2, was used to monitor the duration of reaction. Triacyl-CL, diacyl-CL, and monoacyl-CL can be purified from TLC plates as shown in Subheading 3.3.
5. The temperature-sensitive *E. coli* strain SM2-1 can be used to generate LPA, but the yield is relatively low (about 10% of total lipids). To get more LPA, the number of cells should be scaled up, and the volumes of solvent for extracting lipids from cell pellet and TLC spot should be adjusted accordingly.
6. The expression of plasmid-borne membrane proteins (LplT, Aas) for functional assays should be used with caution because

the overexpression of integral membrane proteins often causes cell death and physiological interpretations problematic by overloading the cytoplasmic membranes, by disrupting the membrane integrity, or "jamming" a translocon and triggering the accumulation of membrane protein into newly made intracytoplasmic membranes [22]. To overcome these limitations, the use of lower-copy number plasmids, the induction of protein expression for short time, and the omission of inducer are recommended for the expression of target membrane proteins for functional assays. In this protocol, 0.1 mM IPTG was used to produce the desired membrane proteins in the cytoplasmic membrane rather than in intracytoplasmic membranes and inclusion bodies in a misfolded state.

7. To make spheroplasts, cells should be collected from middle log phase. Usually, the volumes of spheroplast solution A and B can be scaled up or down on demand. As spheroplasts are unstable, they should be always freshly prepared. All operations must be gentle to avoid spheroplasts rupture. Spheroplast formation and stability should be thoroughly monitored nephelometrically by comparing the OD_{600} of a 100 μL spheroplast solution with 2 mL of either plain water or a solution of 10 mM $MgCl_2$, 0.75 M sucrose, respectively.
8. For TLC-based translocation assay, the final concentration of ethanol should not be higher than 5%. As spheroplasts are fragile structures, we do not recommend incubating the spheroplasts at 37 °C for more than 1 h.
9. For silicone oil-spin method, the density of silicone oil is a critical factor for ensuring the success and reproducibility. It must be greater than that of the incubation medium used, but lower than that of the perchloric acid or the cells themselves. Silicone oil ($d = 1.05$) and 22% perchloric acid are well suited for experiments with Gram-negative bacteria cells. Different cell types with unknown densities can be determined empirically by appropriate centrifugation conditions [23].

Acknowledgments

This work was supported by NIH grants R01GM097290 and R01GM098572 (to L. Z.) and European Union Marie Skłodowska-Curie Grant H2020-MSCA-RISE-2015-690853 and NATO Science for Peace and Security Programme-SPS 985291 and Program of Competitive Growth of Kazan Federal University (to M. B.).

References

1. Offermanns S, Rosenthal W (eds) (2008) Encyclopedia of molecular pharmacology, 2nd edn. Springer, Berlin; New York
2. Zheng L, Lin Y, Lu S et al (2017) Biogenesis, transport and remodeling of lysophospholipids in Gram-negative bacteria. Biochim Biophys Acta 1862:1404–1413
3. Makide K, Kitamura H, Sato Y et al (2009) Emerging lysophospholipid mediators, lysophosphatidylserine, lysophosphatidylthreonine, lysophosphatidylethanolamine and lysophosphatidylglycerol. Prostaglandins Other Lipid Mediat 89:135–139
4. Kawahara A, Nishi T, Hisano Y et al (2009) The sphingolipid transporter spns2 functions in migration of zebrafish myocardial precursors. Science 323:524–527
5. Nguyen LN, Ma D, Shui G et al (2014) Mfsd2a is a transporter for the essential omega-3 fatty acid docosahexaenoic acid. Nature 509:503–506
6. Kern R, Joseleau-Petit D, Chattopadhyay MK et al (2001) Chaperone-like properties of lysophospholipids. Biochem Biophys Res Commun 289:1268–1274
7. Giles DK, Hankins JV, Guan Z et al (2011) Remodelling of the Vibrio cholerae membrane by incorporation of exogenous fatty acids from host and aquatic environments. Mol Microbiol 79:716–728
8. Davydova L, Bakholdina S, Barkina M et al (2016) Effects of elevated growth temperature and heat shock on the lipid composition of the inner and outer membranes of Yersinia pseudotuberculosis. Biochimie 123:103–109
9. Lin Y, Bogdanov M, Lu S et al (2018) The phospholipid-repair system LplT/Aas in gram-negative bacteria protects the bacterial membrane envelope from host phospholipase A2 attack. J Biol Chem 293:3386–3398
10. Kol MA, Kuster DW, Boumann HA et al (2004) Uptake and remodeling of exogenous phosphatidylethanolamine in E. coli. Biochim Biophys Acta 1636:205–212
11. Malinverni JC, Silhavy TJ (2009) An ABC transport system that maintains lipid asymmetry in the gram-negative outer membrane. Proc Natl Acad Sci U S A 106:8009–8014
12. Bishop RE (2008) Structural biology of membrane-intrinsic beta-barrel enzymes: sentinels of the bacterial outer membrane. Biochim Biophys Acta 1778:1881–1896
13. Lin Y, Bogdanov M, Tong S et al (2016) Substrate selectivity of lysophospholipid transporter LplT involved in membrane phospholipid remodeling in Escherichia coli. J Biol Chem 291:2136–2149
14. Hsu L, Jackowski S, Rock CO (1991) Isolation and characterization of Escherichia coli K-12 mutants lacking both 2-acyl-glycerophosphoethanolamine acyltransferase and acyl-acyl carrier protein synthetase activity. J Biol Chem 266:13783–13788
15. Harvat EM, Zhang YM, Tran CV et al (2005) Lysophospholipid flipping across the Escherichia coli inner membrane catalyzed by a transporter (LplT) belonging to the major facilitator superfamily. J Biol Chem 280:12028–12034
16. Shiba Y, Yokoyama Y, Aono Y et al (2004) Activation of the Rcs signal transduction system is responsible for the thermosensitive growth defect of an Escherichia coli mutant lacking phosphatidylglycerol and cardiolipin. J Bacteriol 186:6526–6535
17. Bogdanov M, Heacock PN, Dowhan W (2002) A polytopic membrane protein displays a reversible topology dependent on membrane lipid composition. EMBO J 21:2107–2116
18. Bogdanov M, Heacock P, Guan Z et al (2010) Plasticity of lipid-protein interactions in the function and topogenesis of the membrane protein lactose permease from Escherichia coli. Proc Natl Acad Sci U S A 107:15057–15062
19. Coleman J (1990) Characterization of Escherichia coli cells deficient in 1-acyl-sn-glycerol-3-phosphate acyltransferase activity. J Biol Chem 265:17215–17221
20. HR K (1971) Preparation and characterization of bacterial membranes. Methods Enzymol XXII:99–120
21. Bogdanov M, Dowhan W (1995) Phosphatidylethanolamine is required for in vivo function of the membrane-associated lactose permease of Escherichia coli. J Biol Chem 270:732–739
22. Bogdanov M (2017) Mapping of membrane protein topology by substituted cysteine accessibility method (SCAM). Methods Mol Biol 1615:105–128
23. Tyson CA, Frazier JM (eds) (1994) In vitro toxicity indicators. Methods in toxicology. Academic, San Diego