Appl Microbiol Biotechnol (2016) 100:4255–4267
DOI 10.1007/s00253-016-7468-x

MINI-REVIEW

Organization and function of anionic phospholipids in bacteria

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Received: 4 February 2016 / Revised: 4 March 2016 / Accepted: 8 March 2016 / Published online: 30 March 2016

Abstract In addition to playing a central role as a permeability barrier for controlling the diffusion of molecules and ions in and out of bacterial cells, phospholipid (PL) membranes regulate the spatial and temporal position and function of membrane proteins that play an essential role in a variety of cellular functions. Based on the very large number of membrane-associated proteins encoded in genomes, an understanding of the role of PLs may be central to understanding bacterial cell biology. This area of microbiology has received considerable attention over the past two decades, and the local enrichment of anionic PLs has emerged as a candidate mechanism for biomolecular organization in bacterial cells. In this review, we summarize the current understanding of anionic PLs in bacteria, including their biosynthesis, subcellular localization, and physiological relevance, discuss evidence and mechanisms for enriching anionic PLs in membranes, and conclude with an assessment of future directions for this area of bacterial biochemistry, biophysics, and cell biology.

Keywords Anionic phospholipids · Cardiolipin · Subcellular localization · Bacteria · Cell · Membrane curvature

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Introduction

Bacterial membranes primarily consist of phospholipids (PLs) that contain a hydrophilic phosphate head group and two hydrophobic acyl chains (tails). The amphipathic characteristic of PLs is responsible for their formation of bilayer structures in aqueous environments that create a physical barrier and localize and concentrate molecules and materials within cells (i.e., in the cytoplasm) from the extracellular environment. Bacteria synthesize a diverse collection of PLs that differ in the number and length of acyl chains, the number, position, and geometry of unsaturated bonds, and the structure, polarity, and charge of head groups. Figure 1 depicts the chemical structures of the three major families of PLs in bacterial membranes: phosphatidylethanolamine (PE) is zwitterionic, and phosphatidylglycerol (PG) and cardiolipin (CL) are anionic. In addition to the major PLs listed above, bacteria produce additional PLs that are less prevalent, including phosphatidylcholine (PC) and phosphatidylinositol (PI), and a spectrum of lipids that lack phosphorus, such as ornithine (OL) and sulfoquinovosyl diacylglycerol (SQDG) (Sohlenkamp and Geiger 2016).

The structure of the cell wall separates the majority of bacteria into two families. Gram-positive bacteria contain a cytoplasmic membrane surrounded by a thick (~30–100-nm thick) layer of peptidoglycan; in contrast, Gram-negative bacteria contain two distinct bilayer membranes—the cytoplasmic and outer membrane—surrounding a thin layer of peptidoglycan (~3–5-nm thick) (Fig. 2). The cytoplasmic membrane of Gram-positive bacteria generally contains lipoteichoic acids, PG, and CL. In contrast, the cytoplasmic membrane of Gram-negative bacteria is primarily composed of PE, PG, and CL. The outer membrane and cytoplasmic membranes of

Gram-negative bacteria have a similar phospholipid profile; the primary difference between the two membranes is the presence of lipopolysaccharides in the outer membrane (Silhavy et al. 2010; Sohlenkamp and Geiger 2016).

Several lines of experimental evidence support PL heterogeneity in bacterial membranes and a connection between lipid composition and biomolecular function (Barak and Muchova 2013; Matsumoto et al. 2006). Concentrated regions of anionic PLs in membranes have been hypothesized to sort proteins into different regions in bacterial cells and regulate a variety of processes, including ATP synthesis, chromosomal replication, cell division, protein translocation across membranes, DNA repair, and cell shape determination (Arias-Cartin et al. 2012; de Vrije et al. 1988; Gold et al. 2010; Jyothikumar et al. 2012; Lin et al. 2015; Mileykovskaya and Dowhan 2005; Rajendram et al. 2015; Saxena et al. 2013). There are conceptual parallels between this phenomena and 'lipid rafts' in eukaryotic membranes, in which local differences in lipid concentration are hypothesized to arise from the formation of phase-ordered regions that sort proteins and localize cellular processes (Lingwood and Simons 2010). The characterization of mechanisms underlying the formation of localized regions of anionic PLs and their physiological relevance in bacteria is an active area of research. In this review, we provide a current outlook of the biosynthesis, localization, and function of anionic PLs in bacterial cells, discuss what remains unknown, and provide suggestions for the next steps in this field.

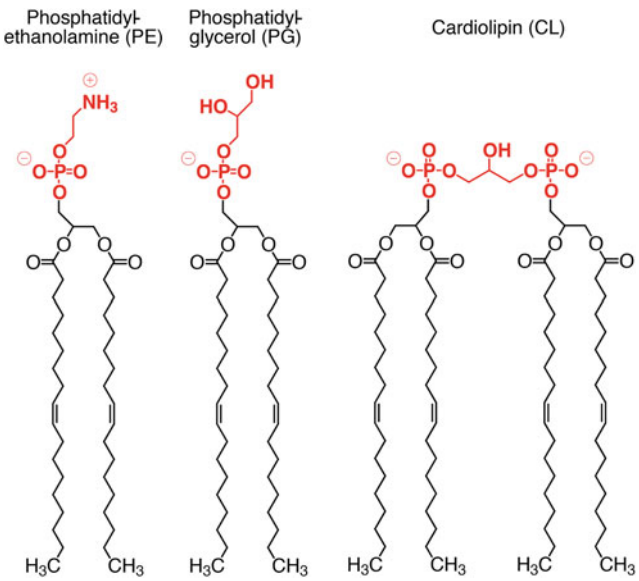


Fig. 1 Chemical structures of the major PLs in bacteria. For simplicity, PLs are shown with unsaturated 18-carbon tails; however, these molecules can have various acyl chains that differ in the length, number, position, and geometry of unsaturated bonds. PL head groups are *bold* and highlighted in *red*. CL contains two phosphate groups; it is reported to have a net charge of −1 at physiological pH as one phosphate is protonated and forms an intramolecular hydrogen bond with the secondary hydroxyl group on the glycerol head group. This figure was reproduced with permission from the following reference (Oliver et al. 2014). Copyright © American Society for Microbiology

Biosynthesis of bacterial anionic PLs

Figure 3 highlights the common biosynthetic pathways for the major anionic PLs in bacteria (i.e., PG and CL). In most bacteria, cytidine diphosphate-diacylglycerol (CDP-DAG) is the precursor of anionic PLs and is formed by incorporating cytidine triphosphate (CTP) into phosphatidic acid (PA) through CDP-DAG synthase (CdsA). CDP-DAG can be converted to either PE or PG and CL through two distinct pathways. In the pathway to PE, phosphatidylserine (PS) synthase (PssA) converts CDP-DAG to PS using L-serine as the phosphatidyl acceptor and PS decarboxylase (PsdA) decarboxylates PS to form PE. In the pathway to PG and CL, PG synthase (PgsA) catalyzes the transfer of the phosphatidyl group from CDP-DAG to glycerol-3-phosphate (G3P) to form PG phosphate (PGP), which is subsequently dephosphorylated by phosphatidylglycerophosphate phosphatase (Pgp) to produce PG. CL synthase (Cls) catalyzes the condensation of two PG molecules to form CL. A family of Cls enzymes has been classified into prokaryotic and eukaryotic types. In contrast to the mechanisms for CL synthesis in most bacteria, eukaryotic cells synthesize CL through a CDP-DAG-dependent Cls

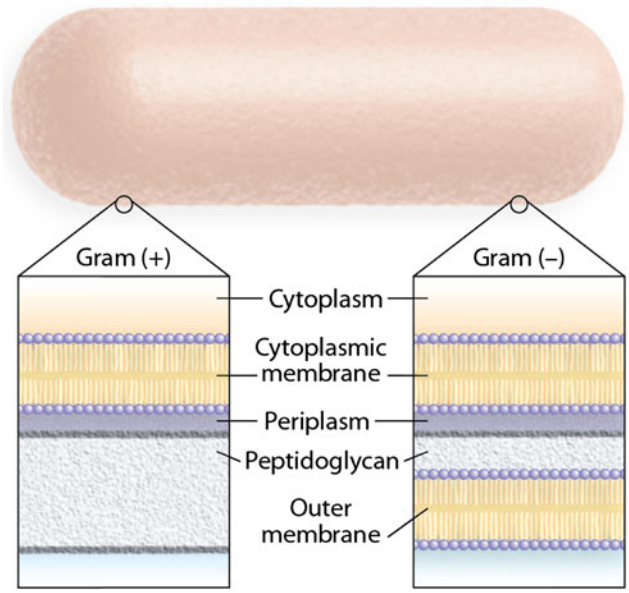
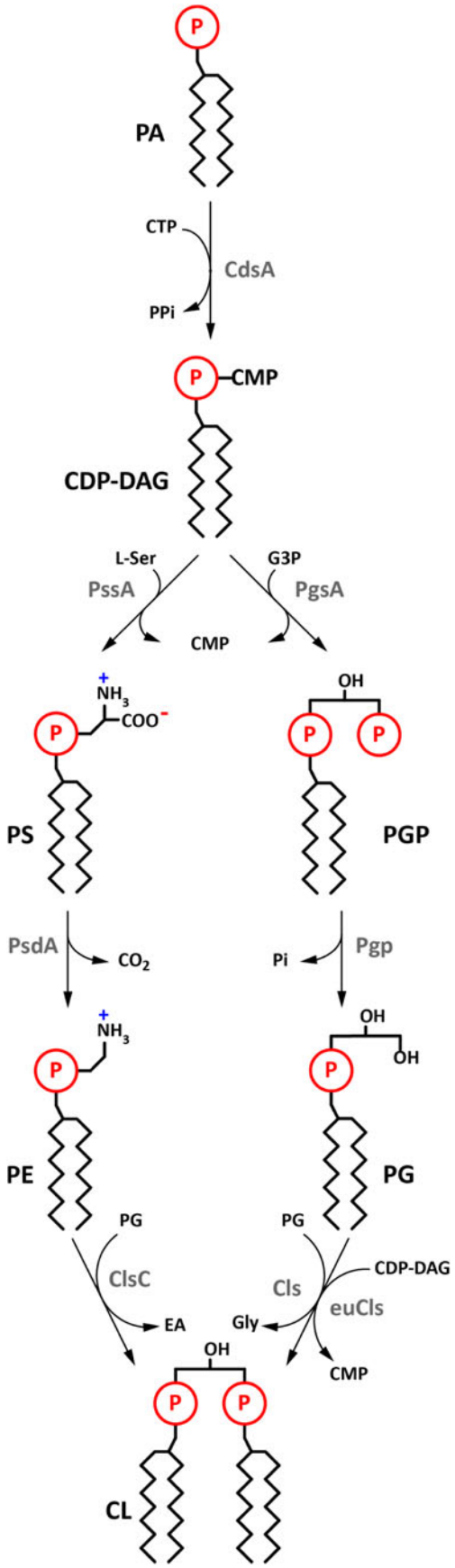


Fig. 2 A cartoon depicting the structure of the cell envelope of Gram-positive and Gram-negative bacteria. The cell envelope of Gram-positive bacteria contains a cytoplasmic membrane surrounded by a thick layer of peptidoglycan. In contrast, the cell envelope of Gram-negative bacteria consists of a cytoplasmic membrane and outer membrane, with a thin layer of peptidoglycan positioned between the membranes. This figure was reproduced with permission from the following reference (Foss et al. 2011). Copyright © 2011 American Chemical Society



◀ **Fig. 3** Biosynthesis of bacterial PLs. This figure depicts the most common pathways and enzymes for the biosynthesis of PLs in bacteria. See the text for an explanation of the pathways. *CMP* cytidine monophosphate, *CTP* cytidine triphosphate, *EA* ethanolamine, *euCls* eukaryotic-type Cls, *G3P* glycerol-3-phosphate, *Gly* glycerol, *L-ser* L-serine, *PPi* pyrophosphate, *Pi* inorganic phosphate. For simplicity, a *red P surrounded by a circle* represents the glycerol phosphate head group of the PLs, *black zigzag lines* represent acyl portions of PLs, and the unique chemistry of each head groups is shown

that uses CDP-DAG as the phosphatidyl donor and PG as the acceptor. This "eukaryotic-like" Cls has recently been identified in many actinobacteria, including *Streptomyces coelicolor* (Sandoval-Calderon et al. 2009).

Many bacteria possess a single version of PgsA. Knocking out *cls* and detecting that CL was still present indicated that some bacteria contain multiple isoforms of Cls. For example, three Cls have been identified in *Escherichia coli*; ClsA, ClsB, and ClsC show sequence homology and contain two phospholipase D domains, which represent a characteristic biochemical feature of bacterial Cls. ClsA produces CL in the log phase and stationary phase, while ClsB and ClsC only synthesize CL in the stationary phase. Similar to the Cls enzymes in other bacteria, ClsA and ClsB synthesize CL from two molecules of PG. In contrast, ClsC catalyzes the formation of CL by transferring a phosphatidyl moiety from PE to PG (Tan et al. 2012). *E. coli* PssA catalyzes the formation of PS, contains a phospholipase D domain, and is hypothesized to have Cls activity; however this hypothesis is untested, and an *E. coli* Δ*clsABC* mutant does not produce any detectable CL, thereby making this concept unlikely (Nishijima et al. 1988; Tan et al. 2012). *Staphylococcus aureus* contains two Cls isoforms—referred to as Cls1 and Cls2—that synthesize CL from two molecules of PG. Cls2 contributes the majority of CL produced by *S. aureus* during growth in both log and stationary phases as measured by thin-layer chromatography (TLC). No CL synthesis is detected when both *cls1* and *cls2* are deleted (Koprivnjak et al. 2011; Kuhn et al. 2015).

Bacteria maintain the compositional balance between zwitterionic and anionic PLs in membranes by biochemically regulating the two families of PL synthases. Table 1 summarizes the major PL compositions in the membranes of widely studied model bacteria. In *E. coli*, the membrane composition is balanced by a feedback mechanism between the PssA–PsdA and PgsA–Pgp pathways: PssA is a peripheral membrane protein that enzymatically synthesizes PS and is activated by interacting with PG and CL in the membrane. PsdA performs the enzymatic step for conversion of PS to PE. As the concentration of PE in the membrane increases due to the PssA–PsdA pathway, the membrane association and activity of PssA decreases, which slows the rate of PE production, thereby accelerating the rate of PG/CL formation through the PgsA–Pgp pathway. This feedback mechanism enables the cell to control

Table 1 Major PL composition in membranes of different model bacteria

Bacterial strain	Percentage of total PLs in membranes (%)[a]			Reference
	PE	PG	CL	
Gram-negative bacteria				
Escherichia coli	80	15	5	Romantsov et al. 2007
Caulobacter crescentus	ND[b]	78	9	Contreras et al. 1978
Pseudomonas aeruginosa	60	21	11	Conrad and Gilleland 1981
Proteus mirabilis	76	13	6	Gmeiner and Martin 1976
Gram-positive bacteria				
Bacillus subtilis	49	25	8	Lopez et al. 2006
Staphylococcus aureus	ND[b]	50	32	Tsai et al. 2011
Streptococcus pneumonia	ND[b]	60	40	Trombe et al. 1979

[a] Percentage calculated according to the phosphate contents of PLs extracted from cells in log phase. PLs were separated and quantified on TLC plates (see references for details)

[b] Not detected

the PL composition in membranes (Linde et al. 2004; Salamon et al. 2000; Zhang and Rock 2008).

The concentrations of anionic PLs in bacterial membranes vary in response to growth phase, salinity, pH, osmolality, and organic solvents (Dowhan 1997; Hiraoka et al. 1993; Koprivnjak et al. 2011; Lopez et al. 2006; Ohniwa et al. 2013; Romantsov et al. 2007; Shibuya et al. 1985; Tan et al. 2012; Tsai et al. 2011). The overall CL concentration in bacterial membranes can vary by a factor of ~2; for example, the concentration of CL can increase by ~200 % as cells enter stationary phase compared to cells in the log phase of cell growth (Tan et al. 2012). *S. aureus* cells generally have an increase in their CL content after they are engulfed by neutrophils (Koprivnjak et al. 2011). Changes in Cls enzyme activity or its expression level alter the amount of CL in the membrane and these mechanisms are hypothesized to be important for bacterial adaptation to environmental stress. In vitro enzyme assays using purified *E. coli* Cls suggest that its activity is product inhibited and thereby regulated by CL concentration (Ragolia and Tropp 1994). Product inhibition of Cls may play an important role in regulating CL synthesis under normal growth conditions (i.e., in the absence of extracellular stress).

Subcellular distribution of anionic PLs in bacterial membranes

The fluid mosaic model of the membrane was originally formulated upon the model of PLs distributed homogeneously (Singer and Nicolson 1972) and has been modified to account for observations of PL domains in cell membranes. Several studies have demonstrated the presence of lateral PL heterogeneity or PL domains in bacterial membranes (Matsumoto et al. 2006). For example, the segregation of PE and PG into different domains in *E. coli* membranes was demonstrated utilizing the biophysical properties of pyrene–lipid probes (Vanounou et al. 2003). Several fluorescent lipophilic probes display a heterogeneous distribution in mycobacterial cells, reflecting lateral PL heterogeneity in membranes (Christensen et al. 1999).

The anionic PL-specific fluorescent dye 10-*N*-nonyl acridine orange (NAO) has been used to visualize anionic PL-enriched membrane domains. NAO is hypothesized to bind to anionic PLs through (i) an electrostatic interaction between the positive charge on the acridine amino moiety and the negative phosphate groups of anionic PLs and (ii) hydrophobic interactions between the hydrophobic region of the acridine ring and the aliphatic region of the bilayer (Petit et al. 1992). CL was proposed to orient NAO such that excitation produces an excimer that red shifts the emission wavelength of the fluorophore (for NAO bound to CL, $\lambda ex,max = 474$ nm, and $\lambda em,max = 640$ nm; for NAO bound to other anionic PLs, $\lambda ex,max = 495$ nm and $\lambda em,max = 525$ nm) (Petit et al. 1994; Petit et al. 1992). This spectroscopic signature was widely applied to characterizing CL in bacteria and mitochondria. For example, the accumulation of the red-shifted fluorescence signal at the cell poles and division septum in NAO-treated rod-shaped bacteria led to the hypothesis that CL is concentrated at these regions of the membrane (Kawai et al. 2004; Mileykovskaya and Dowhan 2000). In *Bacillus subtilis* cells, regions of similar fluorescence were also observed in engulfment membranes and forespore membranes during sporulation and attributed to CL localization (Kawai et al. 2004). The polar localization of CL was verified in a dye-independent manner using *E. coli* strains with point mutations in the MinC, MinD, or MinE proteins that produced minicells with membranes that largely represented the polar regions of the cell due to misplacement of the division site. Quantifying the PL compositions in the membranes of minicells (poles)

and vegetative cells (whole cells) using TLC and mass spectrometry demonstrated that CL is concentrated at the cell poles (Koppelman et al. 2001; Oliver et al. 2014).

A recent study demonstrated that NAO binds promiscuously to all anionic PLs in vitro, including PG, CL, PS, and PA, and displays spectroscopic changes (i.e., the "diagnostic" red shift) previously considered to be unique for the interaction between NAO and CL (Oliver et al. 2014). Cells of the triple *cls* knockout *E. coli* strain BKT12—containing no measurable CL by mass spectrometry—treated with NAO retained red-shifted fluorescence localized at the cell poles and septa. Presumably, another member(s) of the anionic PL family is enriched at these regions of the cells. Mass spectrometric analysis of *E. coli* minicells demonstrated that the polar membranes are enriched in PG and CL and contain small amounts of different anionic PLs. Removing CL (e.g., in *E. coli* strain BKT12) increased the concentration of polarly localized PG (Fig. 4).

PG has been hypothesized to form spiral structures in membranes that extend along the long axis of *B. subtilis* cells based on the pattern of the fluorescent lipophilic dye FM4-64, which is a cationic styryl compound that has been suggested to preferentially associate with anionic PLs (Barak et al. 2008). The spiral lipid structures are absent in cells lacking PG. A recent study suggests that this phenomenon is due to depolarization of the membrane potential and that the FM dye is not a useful fluorophore for observing PG (Strahl et al. 2014). The absence of spiral patterns of FM4-64 localization in *B. subtilis* protoplasts and cells depleted of MurG suggests that they may be connected to peptidoglycan assembly and structure (Muchova et al. 2011). Spiral PL domains have not been observed in *E. coli*; however, FM dyes display a heterogeneous pattern of membrane labeling (Fishov and Woldringh 1999). The interaction between FM4-64 and specific PLs has yet to be determined at a level of detail that enables the conclusively determination of the observations of cells labeled with this fluorophore. Experimental evidence both supports the existence of these structures as biologically relevant and suggests that they are artifacts of fluorescent labeling techniques. Experiments designed to determine the biological significance

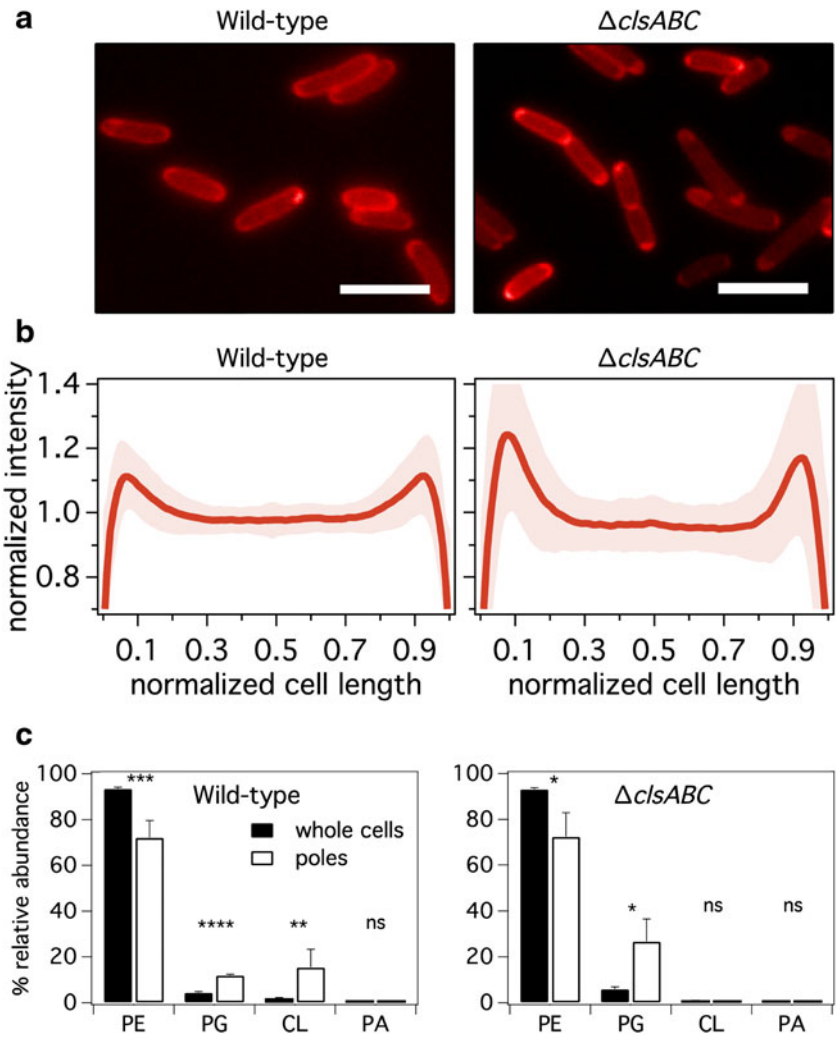


Fig. 4 **a** Microscopy images of *E. coli* wild-type and *ΔclsABC* (BKT12) cells labeled with NAO. NAO red fluorescence concentrates at the cell poles and septa. Scale bar, 5 μm. **b** Red fluorescence intensity profiles of cells labeled with NAO versus cell length. The *shaded space surrounding the NAO red fluorescence intensity profiles* indicates the standard error of the intensity at each point. Dividing cells were intentionally excluded from the analysis. **c** Percent abundances of PE, PG, CL, and PA determined by liquid chromatography-mass spectrometry (LC-MS) of minicell-producing *E. coli* wild-type and ΔclsABC strains (*ns* nonsignificant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$). PG and CL localize at the cell poles of wild-type cells. The polar membranes of *ΔclsABC* cells are enriched in PG. This figure was reproduced with permission from the following reference (Oliver et al. 2014).

of the spirals lipid structures would help form a picture of their biological and structural relevance.

Mechanisms of localizing anionic PLs

Clustering of CL

CL was first isolated from bovine heart and its structure was determined in a complex with the photoreaction center in *Rhodobacter sphaeroides* using X-ray crystallography (McAuley et al. 1999). The volume of the CL head group is small relative to the volume occupied by its four large acyl tails, creating a large intrinsic negative curvature ($-1.3\ nm^{-1}$) and influencing the structure, physical properties, and dynamics of the membrane. A recent study of planar supported lipid bilayers (SLBs) suggests that CL induces double bilayers or nonlamellar structures having a large local mean curvature (Unsay et al. 2013). The same study also found that CL increases the fluidity and decreases the mechanical stability of planar SLBs probably through decreasing the packing of PLs in membranes. CL can also cause local curvature changes in the membrane of giant unilamellar vesicles (Tomsie et al. 2005). In the presence of divalent cations, CL forms a hexagonal phase with a curvature of $\sim -1.3\ nm^{-1}$ due to the interaction of cations with the phosphate groups on CL (Powell and Hui 1996). Divalent cations also bind to the head group of PE and produce a curvature of $\sim -0.48\ nm^{-1}$ (Hamai et al. 2006). Atomic force microscopy imaging of planar SLBs indicates that CL and PE can self-associate into domains that differ in height from the rest of the membrane, thereby illustrating the role of the intrinsic curvature of PLs in the formation of membrane domains (Domenech et al. 2006, 2007; Sennato et al. 2005).

Computational models have been used to explain the preferential localization of CL at the cell poles in rod-shaped bacteria by considering the large osmotic pressure across the cell wall arising due to the mismatch in the concentration of solutes inside and outside of bacterial cells. One model suggests that the large osmotic pressure (~3–5 kPa) (Koch and Pinette 1987) pins the cytoplasmic membrane (bilayer stiffness of ~20 k_BT) (Phillips et al. 2009) against the stiff layer of peptidoglycan (stiffness of ~25–45 MPa) (Yao et al. 1999) that surrounds the cytoplasmic cell membrane, thereby creating elastic strain that is stored in the membrane (Huang et al. 2006; Mukhopadhyay et al. 2008). In this model, short-range interactions between molecules of CL create small domains that localize at regions of the membrane with largest negative mean curvature (e.g., the poles), dissipate the elastic strain on the membrane, and reduce the surface energy potential imposed by bending the bilayer. Because of its large negative curvature, CL is hypothesized to preferentially localize in the inner leaflet of the cytoplasmic membrane and concentrate at the polar membranes due to the enhanced curvature of these cellular regions relative to the cylindrical mid-cell. A repulsion arising from the osmotic force pinning the membrane to the cell wall prevents CL from forming large aggregates. Instead, CL forms finite-sized domains that are large enough to reduce membrane elastic stress and localize at the cell poles. In agreement with the curvature model, several studies have demonstrated the relationship between curvature and CL localization (Renner et al. 2013; Renner and Weibel 2011). This model also predicts a critical concentration for the microphase separation of CL below which the entropy of lipid mixing prevents the formation of CL domains, which is consistent with an observation that CL is not concentrated at the cell poles of a *clsA* deletion strain of *E. coli* with reduced CL concentration (Romantsov et al. 2007). One caveat to these studies is that CL localization was indirectly measured using NAO, and recent studies indicate that this fluorophore may not distinguish between different anionic PLs (Oliver et al. 2014); consequently, membrane localization of CL domains may partially or entirely consist of other anionic PLs. An aggregation-induced emission-active fluorophore with high selectivity to CL versus other mitochondrial membrane PLs has been reported; however, this probe has yet to be tested in bacteria (Leung et al. 2014).

Interactions of PLs with proteins

Labeling *B. subtilis* cells with the PE-specific fluorescent cyclic peptide Ro09-0198 (Ro) leads to the accumulation of fluorescence at regions of the cell with the largest mean curvature (i.e., poles, septa, and forespores) (Nishibori et al. 2005). Although PE favors membranes with a negative curvature (Hamai et al. 2006), the curvature-mediated mechanism proposed above is not sufficient to explain the cellular localization of PE due to its small intrinsic curvature. Similarly, the model is insufficient to explain the polar/septal localization of PG that occurs in cells of *E. coli* strain BKT12 (i.e., lacking CL), as PG has a smaller curvature than PE ($\sim -0.1\ nm^{-1}$ for PG) (Alley et al. 2008). The curvature-based model provides a model for explaining the preferential localization of CL in rod-shaped bacteria; however, a caveat to this model is that one osmotic-mechanical model suggests that the cytoplasm and periplasm are isoosmotic and that the relevant osmotic pressure in bacteria primarily occurs between the periplasm and the extracellular space (Cayley et al. 2000). In this model, the difference in solute concentration in the cytoplasm and periplasm is insufficient to create a large force to pin the cytoplasmic membrane against the peptidoglycan layer. In addition, the curvature model is not applicable to cocci-shaped bacteria in which membrane curvature is the same throughout the cell. Membrane domains enriched in anionic PLs have been observed in the spherical bacterium *Streptococcus pyogenes* using NAO (Rosch et al. 2007),

suggesting that other mechanisms and strategies are involved in localizing anionic PLs to specific regions of cells.

The interaction of proteins with specific PLs provides another mechanism for concentrating lipids in membranes. One possible hypothesis is that membrane domain formation in bacteria is triggered by the coupled transcription-translation-insertion (transertion) of proteins into membranes (Norris 1995). This hypothesis is based on the transertion of membrane proteins occurring at a high frequency (Kennell and Riezman 1977) and many integral membrane proteins (e.g., ATP synthase, NADH dehydrogenase) that have been characterized to bind to specific PLs (e.g., PG, CL) (Dancey and Shapiro 1977; Laage et al. 2015). As a result, compact membrane regions (domains) enriched in specific proteins and PLs are formed. As predicted by the model, drugs (e.g., rifampicin, chloramphenicol, puromycin) that interfere the transertion process of membrane proteins cause dissipation of membrane domains (Binenbaum et al. 1999).

In principal, peripheral membrane proteins can also trigger the formation of PL domains through the interaction of charged residues in the proteins and PLs. An example in support of this hypothesis is the phase separation of CL in model membranes induced by mitochondrial creatine kinase (Epand et al. 2007). Creatine kinase contains clusters of cationic residues that interact with CL, neutralize membrane charge, and reduce the electrostatic repulsion between negatively charged head groups on anionic PLs, thereby promoting the concentration of CL into domains. Another example is the cationic antimicrobial peptide Ltc1 isolated from the Latarcin family, which induces PG domain formation in model bacterial membranes (Polyansky et al. 2010). A recent study provides evidence that the peripheral membrane protein MreB induces PL domains along the cylindrical walls of rod-shaped bacteria based on the protein binding to the membrane (Strahl et al. 2014). This study suggests that the length of acyl chains is responsible for the formation of PL domains—likely arising from the energetics of chain packing—however, the mechanism underlying this protein-lipid interaction remains unsolved. Several groups have demonstrated that the positioning of MreB in rod-shaped bacterial cells is curvature dependent (Renner et al. 2013; Ursell et al. 2014). Bacteria may use both geometry and protein interactions to localize PLs; for example, in principle, the localization of a protein at curved membranes may recruit specific anionic PLs. Despite uncertainty regarding the mechanisms involved in PL localization, the existence of PL domains in bacterial membranes has gained strong traction through biophysical and optical measurements. These regions of the membrane provide specialized environments for the function of membrane proteins. Although the historic view of bacteria is that they do not contain subcellular compartments for organizing biomolecules, membrane heterogeneity and other structural features of cells may instead provide this function. We summarize bacterial processes that regulated by specific anionic PLs and their interacting proteins in Table 2.

Functional roles of anionic PLs

ATP synthesis

CL is found in archaea, bacteria, and eukarya, and its head group contains two phosphate groups with different pKa values (pKa_1 = 2.8 and pKa_2 = 7.5–9.5). CL is reported to have a net charge of −1 at physiological pH because the second phosphate gets protonated and forms an intra-molecular hydrogen bond with the hydroxyl group of the central glycerol moiety (Kates et al. 1993). CL is an essential component of energy-transducing membranes as it can serve as a proton trap for energy-transducing complexes that create and operate off of the cellular ΔpH. In addition, CL interacts tightly with energy-transducing complexes in membranes, fills clefts at the interface between proteins and membranes, and stabilizes protein complexes and regulates their function (Arias-Cartin et al. 2012). CL has been observed to bind to several bacterial proteins in X-ray structures, including the *R. sphaeroides* bacterial reaction center (McAuley et al. 1999), and *R. sphaeroides* cytochrome *c* oxidase (C*c*O) (Zhang et al. 2011), *E. coli* formate dehydrogenase (Jormakka et al. 2002), and *E. coli* succinate dehydrogenase (Yankovskaya et al. 2003). Compared to other PLs, CL more effectively restores the activity of several purified respiratory complexes, such as lactate dehydrogenase (Tanaka et al. 1976), NADH dehydrogenase (Dancey and Shapiro 1977), succinate dehydrogenase (Esfahani et al. 1977), and nitrate reductase (NarGHI) (Arias-Cartin et al. 2011). Although the interaction of CL and these energy-transducing complexes is

Table 2 Summary of bacterial processes regulated by specific anionic PLs and their interacting proteins

Bacterial process	Anionic PL	Protein
ATP synthesis	CL	Energy-transducing proteins[a]
DNA replication	CL	DnaA
Protein translocation	PG[b]	SecA
	CL[c]	EpsE/EpsL, SecYEG
Cell division	PG[b]	MinD, FtsA/FtsZ
	CL[c]	FtsA, MinD, MinE
Osmo-adaptation	CL	ProP
DNA repair	PG, CL	RecA

[a] CL promotes the activity of several energy-transducing proteins. See text for details

[b] PG regulates protein translocation and cell division in Gram-positive bacteria. See text for references

[c] CL regulates protein translocation and cell division in Gram-negative bacteria. See text for references

considered to be essential for their assembly and function, this dependency has not yet been established in vivo. A previous study of the NarGHI complex demonstrated that a PE-deficient mutant of *E. coli* that contains a high level of anionic PLs has enhanced NarGHI activity (Arias-Cartin et al. 2011). The dependence of NarGHI binding to CL on the protein structure and function may also be applicable to other respiratory complexes. However, a previous study demonstrated that a CL deficiency in *R. sphaeroides* does not impair the structure and function of C*c*O or cause significant growth defects (Zhang et al. 2011). Hence, it remains unclear whether CL is essential for the structure and function of these complexes of bacterial respiratory proteins.

Chromosomal replication

Anionic PLs play a role in chromosomal replication by regulating DnaA (Saxena et al. 2013). An *E. coli* strain deficient in anionic PLs has impaired growth and inhibited chromosomal replication with a concomitant reduction in the amount of cellular DNA (Fingland et al. 2012). Anionic PLs bind DnaA through electrostatic interactions and are proposed to promote the conversion of ADP-DnaA to ATP-DnaA, which binds to *oriC* and initiates the replication of DNA at the mid-cell. After transcriptional initiation, the origin moves towards the cell poles and anionic PLs inhibit the interaction of DnaA and *oriC*, thus preventing the re-initiation of chromosomal replication. CL is the most effective of the anionic PLs at promoting the conversion of ADP-DnaA to ATP-DnaA and inhibiting the DnaA-*oriC* interaction (Castuma et al. 1993; Crooke et al. 1992; Kitchen et al. 1999; Sekimizu and Kornberg 1988; Yung and Kornberg 1988). This crosstalk between DnaA and anionic PLs assures initiation occurs only once per cell cycle. DnaA forms helical structures along the longitudinal axis of *E. coli* cells (Boeneman et al. 2009). It remains unclear how anionic PLs that accumulate at polar/septal regions assist in defining the subcellular localization of DnaA and the DNA replication site. To the best of our understanding, there are no conclusive measurements of the temporal localization of anionic PLs in bacterial membranes.

Protein translocation

The subcellular distribution of anionic PLs provides a mechanism for positioning the protein translocon. The Sec machinery is reportedly organized into spiral-like structures in *B. subtilis* that disappear in a strain depleted of PG. PG increases the ATPase activity of SecA and forms spiral structures in *B. subtilis*, suggesting that PG may determine the subcellular sites for exporting proteins through the SecA-YEG pathway in this bacterium (Campo et al. 2004; Lill et al. 1990). In contrast, the Sec translocon in *S. pyogenes* forms a single ExPortal membrane domain for protein secretion that is enriched in PG (Rosch et al. 2007). In Gram-negative bacteria, the subcellular localization of the Sec machinery appears to be CL-dependent. The *Vibrio cholerae* Eps system exports cholera toxin across the outer membrane and localizes at the cell poles. CL interacts with the EpsE/EpsL complex and stimulates its ATPase activity through stabilizing the oligomerization state of EpsE (Camberg et al. 2007). In *E. coli*, CL binds tightly to the SecYEG protein complex, stabilizes its dimeric form, and stimulates the ATPase activity of SecA. The SecYEG complex is arranged in spiral-like structures in *E. coli* cells. SecYEG spirals are observed less frequently in a CL-deficient strain, suggesting that the spatial distribution of the *E. coli* protein translocon relies on CL (Gold et al. 2010). The connection between CL, its organization at the cell poles, and protein export remains a puzzle.

Cell division

Anionic PLs have been hypothesized to play an important role in determining the cell division site by regulating the subcellular distribution of FtsA and MinD. Both of these proteins contain an amphipathic helix enriched in positively charged amino acids and preferentially interact with anionic PLs, in particular CL (Mileykovskaya and Dowhan 2005; Mileykovskaya et al. 2003). FtsA is a bacterial homolog of eukaryotic actin that recruits the bacterial tubulin homolog FtsZ to the membrane, where it polymerizes into a ring structure (Z-ring) and creates a constriction force at the division site. MinD is a component of the Min system that prevents the placement of the Z-ring at the cell poles. After its association with the *E. coli* membrane, MinD polymerizes into a dynamic helical structure that attaches to MinC and oscillates between the cell poles. MinC is a FtsZ inhibitor that prevents Z-ring formation. MinE is another component of the *E. coli* Min system that interacts with MinD and promotes its ATP hydrolysis activity, resulting in detachment of MinD from the membrane. MinE interacts with the membrane through an N-terminal helix that has a preference for binding to anionic PLs, especially for CL (Hsieh et al. 2010; Shih et al. 2011). MinD and MinE have differential affinities for binding to anionic PLs that may affect their retention times on the membrane (Renner and Weibel 2012; Vecchiarelli et al. 2014). In a model of the Min system, MinE forms a ring structure (E-ring) near the mid-cell and confines the MinCD complex to the polar regions of the cell, enabling Z-ring formation at the mid-cell (Drew et al. 2005). Anionic PL domains may play a role in MinD nucleation at the cell poles and stabilize the ring structure of MinE and FtsA/FtsZ at the septal region of the cell (Shih et al. 2003). In support of this model, a recent study demonstrated that the dynamic oscillation of MinCDE proteins can be reconstituted in an artificial cell-shaped compartment in which a negatively charged membrane containing PG

or CL was required to create protein gradients that position FtsZ at the middle of the cell-like compartment (Zieske and Schwille 2014).

MinD does not oscillate between the *B. subtilis* cell poles; instead, it co-localizes with PG spirals positioned along the long axis of the cell (Barak et al. 2008). Similar to MinD, FtsA/FtsZ is also reported to form helical structures that are positioned along the length of cells (Ben-Yehuda and Losick 2002). During cell division, the DivIVA/MinJ complex—that is functionally synonymous to MinE in *E. coli*—recruits MinCD to the cell poles, enabling Z-ring formation at the mid-cell (Bramkamp et al. 2008; Edwards and Errington 1997). Polar localization of DivIVA depends on negative membrane curvature of the cell poles instead of CL domains (Lenarcic et al. 2009; Ramamurthi and Losick 2009).

Adaptation to environmental stress

A growing body of evidence suggests that anionic PLs play a fundamental role in the adaptation of bacteria to environmental stress. For example, CL accumulates in *E. coli* cells in response to osmotic stress and promotes the polar localization of ProP, an osmosensory transporter that senses a high osmolality and regulates the concentrations of organic osmolytes in the cytoplasm. In a CL-deficient strain, the localization of ProP to the cell poles is reduced, causing impaired growth of this mutant under osmotic stress (Romantsov et al. 2007, 2008). The requirement of CL for osmoadaptation has been found in other bacteria, including *R. sphaeroides* (Catucci et al. 2004), *S. aureus* (Tsai et al. 2011) and *B. subtilis* (Lopez et al. 2006). The concentration of CL also increases when bacterial cells enter the stationary phase of growth or low-pH environments. Cells deficient in CL have reduced survival under stress conditions. It has been suggested that bacteria require additional energy to grow in the presence of environmental stress and an increase in the amount of CL may enhance the activity of respiratory complexes and increase ATP production. Recent studies have found that the PhoPQ system transports CL from the cytoplasmic membrane to the outer membrane in the gastrointestinal pathogen *Salmonella typhimurium*, during its response to a decrease in pH and the presence of cationic antimicrobial peptides in the hosts. An increase in CL in the outer membrane may contribute to constructing the barrier necessary for bacterial survival within host tissues (Dalebroux et al. 2014, 2015). Recent studies demonstrate that anionic PLs facilitate DNA repair by stabilizing RecA filament bundles in *E. coli* (Rajendram et al. 2015) and that the concentration of CL in *R. sphaeroides* cells correlates with their shape and plays an important role in forming biofilms (Lin et al. 2015). A CL-deficient mutant of *Pseudomonas putida* is susceptible to several antibiotics (Bernal et al. 2007). These data suggest that anionic PLs enable bacteria to adapt to changes in their environments and survive.

Future directions

Several decades of studies have demonstrated that anionic PLs play roles in bacterial functions. These lipids appear to provide mechanisms of positioning and regulating biochemical machinery in cells, which can be viewed as having some similarities to the function of organelles in eukaryotic cells. Among the anionic PLs, CL has a unique molecular shape and high binding affinity for many proteins that plays an important role in regulating the position and function of proteins in cells. Bacterial strains lacking CL are stable and do not display any growth or obvious physiological abnormalities, suggesting that this PL is not essential (Matsumoto 2001). Several studies have demonstrated that PG can override the absence of CL in *E. coli* and restore the interaction of proteins with the membrane (Oliver et al. 2014; Romantsov et al. 2009; Shibuya et al. 1985). It is possible that different families of anionic PLs provide a mechanism of redundancy; a *pgsA*-null *E. coli* strain lacking PG and CL (UE54) remains viable, yet only if the outer membrane lipoprotein is mutated or removed. NAO-labeled cells of strain UE54 display fluorescence at the polar/septal regions of cells, indicative of concentrated regions of anionic PLs. Analysis of PL composition of UE54 minicells demonstrated that the anionic PLs *N*-acyl-PE and PA are enriched at the cell poles (Mileykovskaya et al. 2009). Polar localization of PG, PA, and *N*-acyl-PE in bacteria suggests that they maintain the anionic character of polar membranes in the absence of CL. It remains unclear whether these anionic PLs have interchangeable functions in regulating bacterial biochemistry. In vitro studies using liposomes or planar SLBs containing different anionic PLs may be useful in understanding their effects on the structure and function of bacterial proteins.

CL is a major component of the inner membrane of mitochondria and plays an important role in the function of a range of proteins in mitochondrial energy-transducing membranes. CL has been suggested to cluster into domains and interacts with ATP synthase at the apex of mitochondrial cristae (Mileykovskaya and Dowhan 2009). In addition, CL binds the mitofilin/MINOS protein complex at the mitochondrial cristae junctions (Weber et al. 2013). The localization of CL at these highly curved membrane regions may provide a mechanism for controlling mitochondrial cristae morphology. In support of this hypothesis, a *Saccharomyces cerevisiae* mutant lacking CL shows an aberrant morphology of mitochondrial cristae (Mileykovskaya and Dowhan 2009). Large changes in membrane shape and the formation of membrane invaginations are also observed in bacteria. Photosynthetic bacteria such as *R. sphaeroides* form intracytoplasmic

membranes (ICMs)—by delamination of the cytoplasmic membrane from the peptidoglycan layer of the cell wall—and accommodate the photosynthetic apparatus (Chory et al. 1984). Membrane invaginations can also be induced by overexpression of several membrane proteins in *E. coli*, including the ATP synthase. Interestingly, *E. coli* intracellular membranes are enriched in CL (Arechaga 2013). An understanding of the physiological, biochemical, and biophysical mechanisms underlying membrane internalization in bacteria and its connection to the organization of anionic PLs may provide insight into the endosymbiotic theory of mitochondria evolving from α-proteobacteria.

Acknowledgments T.-Y. Lin acknowledges a Dr. James Chieh-Hsia Mao Wisconsin Distinguished Graduate Fellowship from the Department of Biochemistry, University of Wisconsin-Madison. Research in this area of our lab is supported by the National Science Foundation (under award DMR-1121288), the National Institutes of Health (1DP2OD008735), and the United States Department of Agriculture (WIS01594).

Compliance with ethical standards

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest Ti-Yu Lin declares that he has no conflict of interest. Douglas B. Weibel declares that he has no conflict of interest.

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