Biochimica et Biophysica Acta 1788 (2009) 2092–2100

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Review

Cardiolipin and the osmotic stress responses of bacteria

Tatyana Romantsov [a], Ziqiang Guan [b], Janet M. Wood [a,*]

[a] Department of Molecular and Cellular Biology, University of Guelph, Science Complex, 488 Gordon St., Guelph, ON, Canada N1G 2W1
[b] Department of Biochemistry, Duke University Medical Center, Durham, NC 27710, USA

ARTICLE INFO

Article history:
Received 7 October 2008
Received in revised form 7 June 2009
Accepted 10 June 2009
Available online 17 June 2009

Keywords:
Osmotic stress
Bacteria
Cardiolipin
ProP
MscL

ABSTRACT

Cells control their own hydration by accumulating solutes when they are exposed to high osmolality media and releasing solutes in response to osmotic down-shocks. Osmosensory transporters mediate solute accumulation and mechanosensitive channels mediate solute release. *Escherichia coli* serves as a paradigm for studies of cellular osmoregulation. Growth in media of high salinity alters the phospholipid headgroup and fatty acid compositions of bacterial cytoplasmic membranes, in many cases increasing the ratio of anionic to zwitterionic lipid. In *E. coli*, the proportion of cardiolipin (CL) increases as the proportion of phosphatidylethanolamine (PE) decreases when osmotic stress is imposed with an electrolyte or a non-electrolyte. Osmotic induction of the gene encoding CL synthase (*cls*) contributes to these changes. The proportion of phosphatidylglycerol (PG) increases at the expense of PE in *cls*$^-$ bacteria and, in *Bacillus subtilis*, the genes encoding CL and PG synthases (*clsA* and *pgsA*) are both osmotically regulated. CL is concentrated at the poles of diverse bacterial cells. A FlAsH-tagged variant of osmosensory transporter ProP is also concentrated at *E. coli* cell poles. Polar concentration of ProP is CL-dependent whereas polar concentration of its paralogue LacY, a H^+-lactose symporter, is not. The proportion of anionic lipids (CL and PG) modulates the function of ProP *in vivo* and *in vitro*. These effects suggest that the osmotic induction of CL synthesis and co-localization of ProP with CL at the cell poles adjust the osmolality range over which ProP activity is controlled by placing it in a CL-rich membrane environment. In contrast, a GFP-tagged variant of mechanosensitive channel MscL is not concentrated at the cell poles but anionic lipids bind to a specific site on each subunit of MscL and influence its function *in vitro*. The sub-cellular locations and lipid dependencies of other osmosensory systems are not known. Varying CL content is a key element of osmotic adaptation by bacteria but much remains to be learned about its roles in the localization and function of osmoregulatory proteins.

Contents

1. Introduction

Changes in extracellular osmotic pressure can elicit rapid water fluxes that concentrate or dilute the cytoplasm of living cells, disrupting their structure and function. Cells respond to osmotic stress by actively adjusting the distributions of selected osmoregulatory solutes across the cytoplasmic membrane [5,8,11,37,42,57,90]. They accumulate solutes to forestall dehydration of the cytoplasm as external osmotic pressure increases and release solutes to prevent cell lysis as external osmotic pressure decreases. Bacteria use K^+, organic anions like glutamate and uncharged or zwitterionic organic

* Corresponding author. Tel.: +1 519 824 4120x53866; fax: +1 519 837 1802.
E-mail address: jwood@uoguelph.ca (J.M. Wood).

0005-2736/$ – see front matter © 2009 Published by Elsevier B.V.
doi:10.1016/j.bbamem.2009.06.010

compounds as osmoregulatory solutes. Organic solutes that accumulate in cells under osmotic stress without impairing cell functions are often called osmolytes [4] or compatible solutes [1]. Potassium glutamate accumulation is an effective first response to osmotic upshifts and it can activate other osmoregulatory responses, but organic osmolyte accumulation is preferred [1]. The osmolytes elicit more complete cytoplasmic rehydration while favoring native macromolecular structures and interactions [4,71,72].

Arrays of osmoregulatory systems mediate solute accumulation and release by each organism. Fig. 1 illustrates the osmoregulatory systems of *Escherichia coli*, a paradigm for studies of cellular osmoregulation. In response to osmotic upshifts, transporters mediate the uptake of K^+ or osmolytes (e.g. choline, ectoine, glycine betaine, proline) and cytoplasmic enzymes catalyze trehalose or glycine betaine synthesis. Mechanosensitive channels mediate solute release when osmotic pressure falls. Current research focuses on the mechanisms that regulate the level and activity of each system, integrating their functions in response to osmotic shifts that may differ in rate, extent and duration [1].

Bacterial phospholipid composition also varies when bacteria are grown in media that differ in salinity and/or osmolality. Here we review emerging evidence that increasing cardiolipin content is a key player in bacterial adaptation to osmotic stress.

2. Osmoregulation of bacterial phospholipid composition

The cell surfaces of Gram negative bacteria include the cytoplasmic membrane, a thin peptidoglycan layer and an outer membrane with a phospholipid inner leaflet and a lipopolysaccharide outer leaflet. Gram positive bacteria have a thicker peptidoglycan layer and no outer membrane. The compositions of total lipid extracts are usually reported. For Gram negative bacteria, such data represent the average composition of the cytoplasmic membrane plus the inner leaflet of the outer membrane.

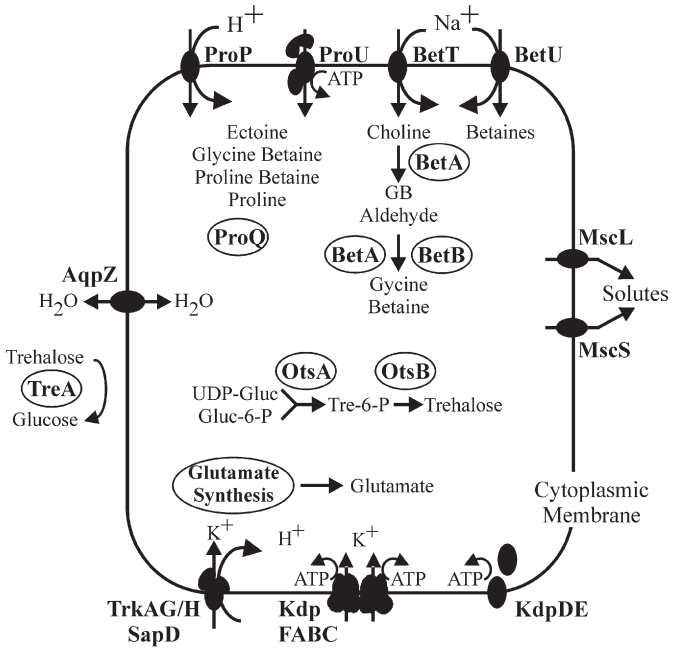

Fig. 1. Osmoregulatory systems of *Escherichia coli*. Aquaporin AqpZ mediates passive transmembrane water flux. K^+ transporter TrkA(G/H)/SapD mediates K^+ accumulation in response to high osmotic pressure; K^+ transporter KdpFABC contributes to a lesser extent. KdpDE is a two component regulatory system that controls *kdpFABC* transcription in response to K^+ supply and osmotic stress. Suppression of glutamate catabolism leads to its accumulation as K^+ counterion. At high osmotic pressure trehalase (TreA) hydrolyzes extracellular trehalose while OtsA and OtsB mediate cytoplasmic trehalose synthesis and genes encoding a trehalose catabolic system are repressed. Transporters ProP, ProU, BetT and BetU mediate organic osmolyte accumulation at high osmotic pressure. ProQ regulates ProP by an unknown mechanism. Enzymes BetA and BetB mediate glycine betaine synthesis from choline. Mechanosensitive channels MscL and MscS mediate solute efflux in response to decreasing osmotic pressure.

The membranes of many (but not all) bacteria are comprised primarily of a zwitterionic phospholipid plus anionic phospholipids. For example the predominant lipids of *E. coli* are phosphatidylethanolamine (PE) plus phosphatidylglycerol (PG) and cardiolipin (diphosphatidylglycerol, CL). As discussed elsewhere in this volume, the zwitterionic and anionic lipids arise from CDP-diacylglycerol via separate pathways and CL is derived from PG. The proportion of anionic phospholipid increases as the proportion of zwitterionic phospholipid decreases when bacteria are grown in media of increasing salinity [6,7,21,45,47,48,73,74,78,85,86,88]. This behaviour is shared by Gram positive and Gram negative, halophilic and halotolerant bacteria (see Table 1 (representative data) and reviews [73,78,80]). Russell et al. reported that glycine betaine reversed the effects of salinity on the phospholipid composition of *Lactobacillus plantarum* [79]. The uptake and cytoplasmic accumulation of osmolytes like glycine betaine protects cells from osmotic stress, so this observation reinforced the link between lipid composition and the osmotic stress response (Fig. 1). It will be interesting to learn whether other bacteria behave similarly.

At least for *E. coli*, changes in phospholipid composition with salinity are superimposed on changes in phospholipid composition due to growth phase (Table 1). The proportion of CL may also increase if energy metabolism is impaired with colicin K, dinitrophenol, penicillin or cyanide or by infection with bacteriophage T4 [14,15]. Experiments must therefore be carefully designed to differentiate phospholipid changes resulting from high salinity-induced entry to stationary phase from phospholipid changes resulting from high salinity *per se*. CL dominates the increase in the proportion of anionic lipid for *E. coli* and some other bacteria whereas PG rises most dramatically in others (Table 1, compare *Bacillus subtilis* and *Oceanomonas baumannii*). The phospholipid compositions of *E. coli* cells from late exponential phase cultures changed in the same ways when the osmotic pressure was elevated to the same extent with NaCl or sucrose [86]. Thus these changes constitute responses to the osmotic pressure, not the ionic strength, salinity or ion composition.

Bacterial fatty acid composition is also affected by the salinity of the growth medium and the growth phase (Table 1; [73,77,78,80]). The proportion of cyclopropane fatty acids increases when Gram negative bacteria are cultivated in high salinity media or enter stationary phase. Total fatty acid compositions of lipid hydrolysates are usually reported, obscuring any reciprocal changes in fatty acid composition among phospholipid species or correlations between headgroup and acyl chain changes. McGarrity and Armstrong reported that the acyl chain composition of CL from exponential or stationary phase *E. coli* differed from that of PE or PG, and salinity altered the acyl chain composition of CL from stationary phase, but not exponential phase bacteria [51]. In addition, isosmolar NaCl and sucrose had different effects on the fatty acid compositions of cells from exponential and stationary phase cultures [52]. Comparisons of salinity and osmolality effects will continue to be important since the ionic strength will influence the structures of membranes comprised of lipids with ionic headgroups.

Much remains to be learned as high resolution techniques are applied to more precisely delineate stress-induced changes in phospholipid composition (e.g. [62]). For example, we used liquid chromatography coupled with tandem mass spectrometry to analyze phospholipid extracts from *cls*$^+$ and *cls*$^-$ *E. coli* strains cultivated to exponential phase in low and high salinity media. The arrays of PE, PG and CL species were similar for bacteria cultivated at low and high salinity, but those containing fatty acids with odd-numbers of carbon atoms (likely cyclopropane fatty acids), increased relative to those containing fatty acids with only even numbers of carbon atoms. This

Table 1
Impact of increasing medium osmolality on the cardiolipin content of bacterial cells.

Organism	Growth medium[a]		Growth phase	Phospholipid headgroups (mol%)[b]			Acyl chains[c]	Ref.
	Base	NaCl (M)		Lipid	LOM	HOM		
Bacillus subtilis	LB	1.5	NR[d]	PE	20	17	↑ Straight (versus branched) saturated chains	[46,47]
				PG	42	32	↓ Saturation	
				LPG	14	5		
				CL	24	46		
Escherichia coli	MOPS	0.3[e]	Exponential	PE	79	74	NR[d]	[74]
				PG	18	18		
				CL	4	8		
	MOPS	0.3	Stationary	PE	91	86	NR[d]	[74]
				PG	3	5		
				CL	6	9		
Lactococcus lactis	CDM	0.3	Exponential	NR	NR	NR	↑ Cyclopropane ↑ Saturation	[31]
Oceanomonas baumannii	TSB	2.1	Mid-exponential	PE	71	44	↑ Saturation	[6]
				PG	23	48	↓ Length	
				CL	6	8		
	MSM	1.2	Mid-exponential	PE	63	51	↓ Saturation	[7]
				PG	20	40	↑ C16, ↓ C12, C18	
				CL	17	5		
Vibrio sp. DSM14379	PYE	1.7	Late exponential	PE + LPE	78	68	↑ Saturation	[21]
				PG + CL	22	32		

[a] The growth media were: CDM, Chemically Defined Medium [31]; LB, Luria–Bertani medium [47]; MOPS, MOPS-based Minimal Medium [86]; MSM, Minimal Salts Medium [7]; PYE, Peptone Yeast Extract [21]; TSB, Tryptone Soya Broth [6].
[b] Bacteria were cultivated in the indicated Base medium (Low Osmolality Medium, LOM) and in the same medium supplemented with NaCl at the indicated concentration (High Osmolality Medium, HOM) to the indicated growth phase. The phospholipids were PE, phosphatidylethanolamine; LPE, lysophosphatylethanolamine; PG, phosphatidylglycerol, LPG, lysophosphatidylglycerol; CL, cardiolipin or diphosphatidylglycerol.
[c] ↑ is increased; ↓ is decreased.
[d] NR is not reported.
[e] The phospholipid compositions of bacteria cultivated in media adjusted to equivalent osmolalities with NaCl and sucrose were the same [86].

effect was most pronounced for PE (e.g. 16:0/17:1 versus 16:016:1) (Fig. 2 and Table 2).

3. Osmoregulation of CL synthesis

Bacterial CL synthases catalyze the condensation of two PG molecules to yield CL and glycerol [81]. The *cls* locus of *E. coli* and the *clsA* locus of *B. subtilis* (previously denoted *ywnE*) encode CL synthases with similar sequences (27% sequence identity). The designation *cls* is used below to denote both loci. The *cls* locus of *E. coli* encodes a well characterized CL synthase [84], and *E. coli* and *B. subtilis* with *cls* defects are CL-deficient [14,45,74]. Analyses performed with *lacZ* fusions revealed 2- to 3-fold induction of *cls* transcription by osmotic stress in both organisms [45,74]. Expression of *cls* also increased 2.5-fold as *E. coli* entered stationary phase in a low osmolality medium [33]. The impact of osmotic stress on CL synthase activity has not been reported.

Other enzymes appear to contribute to CL synthesis by *E. coli* since *cls*$^-$ bacteria retain traces of CL [14]. One report indicates that *cls*$^-$ *B. subtilis* is devoid of CL [45] but another indicates that residual CL is present in such bacteria [40]. The CL synthase activity of *E. coli* rises in stationary phase [36] and the proportion of CL rises in stationary phase for both *cls*$^+$ and *cls*$^-$ bacteria [14,74]. Phosphatidylserine synthase may catalyze the residual CL synthesis [59]. The genomes of *E. coli* and *B. subtilis* encode additional Cls sequence homologues (YbhO and YmdC in *E. coli*; YwiE and YwjE in *B. subtilis*) [32,45]). The YbhO protein of *E. coli* has CL synthase activity *in vitro* [32]. However the physiological roles of these Cls homologues remain unclear as disruption of the encoding loci did not perturb bacterial phospholipid composition, at least under the conditions tested [32,45].

Defects at *cls* elevated the proportion of PG in *E. coli* and *B. subtilis*, and that proportion was further elevated by osmotic stress [45,74]. Indeed, López et al. reported osmotic induction of *pgsA*, encoding PG synthase, in *B. subtilis* [45]. Such regulation could be designed to control the relative proportions of zwitterionic and anionic lipids in the bacterial membranes [45,74]. Nishijima et al. reported a strain-dependent increase in the proportions of CL and PE at the expense of PG as *E. coli* cultures entered stationary phase [59]. However the effects of osmotic stress and the transition to stationary phase on the proportion of CL were not additive. For at least one *E. coli* strain, the proportion of CL rose almost two-fold on entry to stationary phase in low osmolality medium but it was independent of growth phase for bacteria grown at high osmolality [74]. Feedback inhibition of CL synthase may impose a limit on CL accumulation at high osmolality [70]. The net effect of these changes was to minimize the difference in the proportion of headgroup phosphate in anionic phospholipid between *cls*$^+$ and *cls*$^-$ bacteria, perhaps thereby minimizing the difference in membrane surface charge density [74].

4. Cardiolipin is concentrated at the poles of bacterial cells

Lipophilic fluorescent dyes, including FM 4-64 ([N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide]) and NAO (10N-nonyl acridine orange), have helped researchers to discover the distributions of phospholipids in bacterial membranes. FM 4–64 binds to phospholipid membranes non-specifically [89,92]. NAO was found more than thirty years ago to be a specific marker of the CL-rich inner mitochondrial membrane in living eukaryotic cells [82]. Only green fluorescence was observed in pioneer studies with NAO. However, when NAO was applied to liposomes with increasing concentrations of CL the NAO fluorescence changed from green (525 nm) to red (640 nm) [24]. Petit et al. showed that NAO binds with higher affinity to negatively charged phospholipids (CL, phosphatidylinositol and phosphatidylserine) than to zwitterionic phospholipids (e.g. PE) [64]. In addition, they demonstrated that NAO forms a dimer when it associates with CL, which contains two phosphate groups per molecule. At low NAO concentration the red excimer fluorescence replaces the green monomer fluorescence. Thus the red fluorescence indicates association of NAO with CL rather than other anionic lipids, including phosphatidylinositol

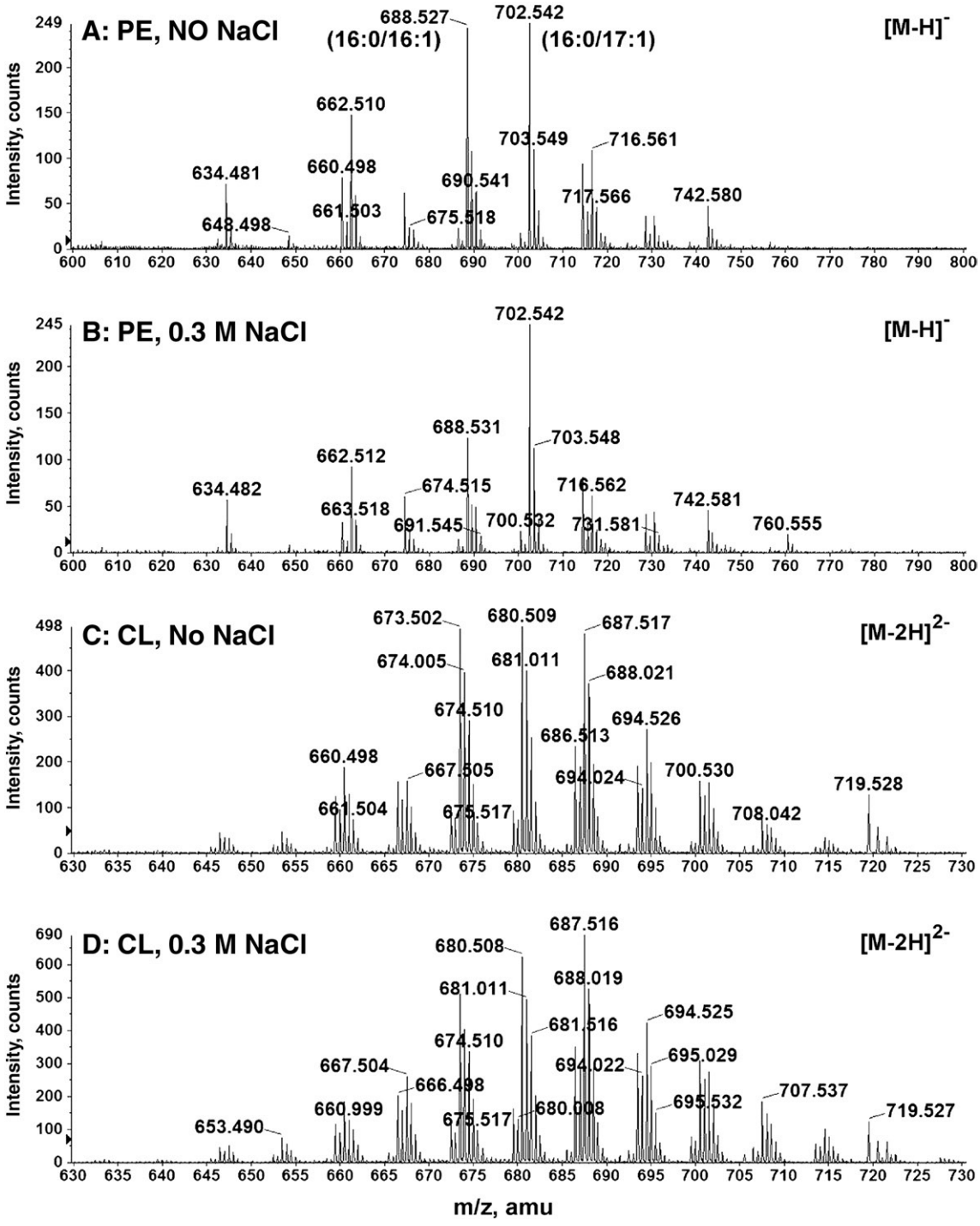

Fig. 2. Impact of salinity on the fatty acid compositions of *E. coli* phospholipids. Negative ion ESI mass spectra were obtained for phospholipid molecular species extracted from *E. coli* strain MG1655 (wild-type) that had been cultivated to exponential phase in: (A and C) low or (B and D) high salinity media. Spectra of PE (A, B) and CL (C, D) species are shown and mass assignments for major species of PE, PG (not shown) and CL are listed in Table 2. The PE species containing fatty acids with odd-numbers of carbon atoms (e.g. m/z 702.5 ion signals for PE (16:0/17:1)) increased relative to those containing fatty acids with only even numbers of carbon atoms. The fatty acids with odd-numbers of carbon atoms are presumably cyclopropane fatty acids. The bacteria were cultivated in NaCl-free MOPS medium [58] (low salinity) or MOPS medium containing 0.3 M NaCl (high salinity medium) as described by Tsatskis et al. [86]. Phospholipid extracts were prepared, species were fractionated and mass spectra were acquired using a procedure based on that of Guan et al. [30]. Normal phase LC was coupled to a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (Applied Biosystems, Foster City, CA), equipped with an electrospray source. Normal phase LC using an Ascentis® Si HPLC Column (5 μm, 25 cm×2.1 mm) was performed on an Agilent 1200 Quaternary LC system. The fatty acid compositions of the phospholipid species were identified by collision-induced dissociation tandem mass spectrometry (MS/MS).

or phosphatidylserine. This CL-specificity decreases at higher NAO concentrations as would be expected since NAO has a much lower affinity for these monoacidic phospholipids ($K_a = 7\times10^4$ M^{-1}) than for CL ($K_a = 2\times10^6$ M^{-1}) [64].

The mechanism of the specific fluorescence shift in the presence of CL is still unclear. Mileykovskaya et al. proposed that the nonyl group of NAO inserts between the phosphate groups at the hydrophobic surface generated by the two outer acyl chains of CL. Thus the green fluorescence of NAO monomers may be replaced by the red fluorescence of NAO excimers as NAO forms an array of parallel twisted stacks on the surface of CL-rich membranes [55]. In contrast to other CL probes, such as adriamycin which only works *in vitro*, NAO

Table 2
Phospholipids from *E. coli* cells grown at low and high salinity.

Phosphatidylethanolamine[a] (PE)		Phosphatidylglycerol[a] (PG)		Cardiolipin[b] (CL)	
m/*z*	Fatty acids	*m*/*z*	Fatty acids	*m*/*z*	Fatty acids
634.48	14:0/14:0	693.51	16:0/14:0	660	68:1
662.51	14:0/16:0	705.51	15:0/16:1	667	69:1
688.53	16:0/16:1	719.51	16:0/16:1	673	70:2
702.54	16:0/17:1	733.52	16:0/17:1	680.5	71:2
716.56	16:0/18:1	747.55	16:0/18:1	687.5	72:2
742.58	18:1/18:1	761.57	16:0/19:1	694.5	73:2

The table lists major phospholipid species found in extracts from *E. coli* cells grown at low and high salinity (see Fig. 2).
[a] The PE and PG species were detected as $[M–H]^-$ ions by ESI/MS in the negative ion mode. Thus the masses of PE or PG species are the observed *m*/*z* values plus the mass of a proton. For example, the mass of the *m*/*z* 702.5 PE is 703.5 (see also [25]).
[b] The CL species were detected as $[M–2H]^{2-}$ ions by ESI/MS in the negative ion mode, so the masses of CL species are two (2) times the *m*/*z* values of the ion peaks, and then plus the mass of two protons. For example, the mass of the *m*/*z* 680.5 CL species is 1363.0 (see also [26]). In this case, total numbers of carbons and total numbers of double bonds in four (4) fatty acids are listed.

spontaneously diffuses through membranes [50]. NAO is therefore a useful indicator of CL concentration in the membranes of intact cells.

Visualization of membrane lipids via fluorescence microcopy has helped to reveal lateral lipid phase separations in bacterial membranes. The selective staining of cell poles and septal regions by fluorescent lipophilic dyes has been observed in mycobacteria [12] and *E. coli* [23]. Moreover, this kind of membrane heterogeneity correlated with the position of the nucleoid in *E. coli* [23]. These observations were interpreted as showing that membrane domains with different phospholipid compositions exist in bacteria and they may play an important role in the bacterial cell cycle and cell division.

Obvious CL domains were discovered at the poles and septa in *E. coli* [54], *B. subtilis* [40], *Streptococcus pyogenes* [76], and *Pseudomonas putida* [3] by using NAO staining. The red fluorescence indicative of CL domains was not detected in *cls*$^-$ bacteria but green fluorescence was uniformly distributed over their surfaces. Mileykovskaya et al. suggested that the residual green fluorescence indicated association of NAO with PG [29], based on the results of Petit et al. for monoacidic phospholipids (see above) [34]. Analyses of the phospholipid compositions of *E. coli* minicells (DNA-free small cells that are products of abnormal cell division on the cell poles) corroborated the results obtained with fluorescence microscopy. The proportion of CL in minicells was higher than that in normal cells from the same culture, consistent with the polar localization of CL in bacterial cells or spores [41,75]. However the proportion of PG in the minicells was lower than that in normal cells from exponential phase cultures of low and high salinity, so that the two also differed almost 2-fold in PG:PE ratio [75]. It is possible that the low PG content of minicells arose in part from continuing conversion of PG to CL after polar septation. These results suggest that *E. coli* cells maintain a significant lateral lipid phase separation between their side walls and poles. CL-rich domains were also found in the polar septa created during sporulation by *B. subtilis* [40]. Concentration of CL at the cell poles and polar septation were delayed in bacteria with defects at *cls* or at all three putative CL synthase loci (*clsA ywjE ywiE*) [40]. This was the first evidence for a functional role of CL *in vivo*.

We used NAO as a tool to further explore the link between osmoregulation of CL synthesis and the sub-cellular localization of CL in *E. coli*. We observed that the CL content increased whereas PE content decreased, and the PG content remained unchanged, as *cls*$^+$ cells were grown at increasing osmolalities (0.1–0.7 mol kg^{-1}) [52, 39]. The proportion of CL was low and did not vary with osmolality in *cls*$^-$ bacteria. Moreover, the proportions of CL and PE increased from exponential to stationary phase in both *cls*$^+$ and *cls*$^-$ bacteria, while the proportion of PG decreased [32, 39]. The intensity of red fluorescence at the poles of *E. coli* cells varied with their CL content as it varied with the bacterial genotype (*cls*$^-$ or *cls*$^+$), growth medium osmolality and culture growth phase [75]. Osborn et al. reported differences in phospholipid headgroup composition between the cytoplasmic and outer membranes of *Salmonella enterica* pathovar *Typhiumurium*, which is closely related to *E. coli* [61]. They found the proportion of anionic lipid in the cytoplasmic membrane to be more than 2-fold higher than that in the outer membrane (40% versus 19%) and the CL/PG ratio of the cytoplasmic membrane to be more than 2-fold higher than that of the outer membrane (0.20 versus 0.09). It would be interesting to know whether the distribution of phospholipids between the cytoplasmic and outer membranes of *E. coli* differs and/or changes with growth medium osmolality.

There is a lot of evidence that CL concentrates at bacterial cell poles whereas PG does not [2,3,40,41,54,74–76], but the mechanism by which CL concentrates at cell poles is unclear. For example, polar CL concentration could result from its biophysical properties or from preferential interaction of CL with one or more proteins directed to the cell poles during growth. Wingreen and his colleagues have proposed a mathematical model of CL-domain formation in *E. coli* based entirely on biophysical principles [39,56]. They predict that CL domains may spontaneously concentrate in the inner leaflet of the cytoplasmic membrane at the poles of rod-shaped cells because of an interaction between the conical shape of CL molecules and the membrane curvature [39]. In the membrane plane, the surface area of CL headgroups is small relative to the surface area of the linked acyl chains. Thus the CL molecules would be best accommodated in the membrane leaflet closest to the cytoplasm, with their headgroups facing the cytoplasm and their acyl chains extending towards the convex monolayer surface in the membrane core. Huang et al. indicate that this physical mechanism of CL aggregation at the cell poles is consistent with the CL concentration, size of aggregates or lipid domains, and cell curvature found in *cls*$^+$ *E. coli*. It is also consistent with the failure of CL to concentrate at the poles of *cls*$^-$ bacteria from exponential phase cultures [39,56]. However this model is predicated on the unproven assumption that CL is confined to the cytoplasmic leaflet of the cytoplasmic membrane.

This model was extended with the proposal that turgor pressure contributes to the polar concentration of CL by pressing the cytoplasmic membrane against the cell wall [56]. In bacteria, the cytoplasmic membrane is surrounded by a peptidoglycan mesh with holes on the order of 2–5 nm in diameter [22]. This mesh is much thicker in Gram positive than in Gram negative bacteria and it is bounded by the outer membrane in Gram negative bacteria. Thus the model proposed by Wingreen and his colleagues could relate polar CL localization to the maintenance of turgor pressure via osmoregulation. However, the assumption that turgor pressure physically "pins" the cytoplasmic membrane to the cell wall is also unproven. Indeed there is evidence that, under at least some conditions, the cytoplasm and periplasm are isosmotic and turgor pressure is exerted on the peptidoglycan/outer membrane complex [9,83]. Nevertheless, this model represents an important step towards understanding the mechanism of membrane domain formation, its relationship to

osmoregulation and the role of membrane heterogeneity in physiological processes of bacterial cells, such as protein–lipid interactions and cell division.

5. Cardiolipin and the osmoregulatory responses of *E. coli*

A *cls* defect was initially reported to have only small effects on the growth and survival of *E. coli* [59]. It was interesting to find that, though small, the reductions in colony size on solid medium and growth rate in minimal medium due to a *cls* mutation became more marked as growth medium osmolality increased [74]. Growth of *B. subtilis* in a hypersaline medium was also impaired by a *clsA* defect [45]. The reasons why CL deficiency impairs growth are not fully understood. However, recent reports show that CL can influence the functions of the osmoregulatory proteins MscL (a mechanosensitive channel that releases cytoplasmic solutes when cells are subjected to osmotic down-shocks) and ProP (an osmosensory transporter that senses increasing osmotic pressure and responds by mediating osmolyte uptake into the cytoplasm) (see Figs. 1 and 3). That work is discussed below.

To understand how CL might influence osmoregulatory systems, it is helpful to understand how lipids influence the structures and functions of membrane proteins. Changes to membrane phospholipid composition, in particular CL content, could influence the activities of membrane proteins in many ways [43]. Membrane lipids may reside in the bulk lipid phase (not interacting directly with a particular membrane protein but influencing membrane properties) or among the annular lipids that directly contact an integral membrane protein. As discussed above, lateral phase separation occurs in bacterial cytoplasmic membranes. Thus within each cell, different cytoplasmic membrane proteins may reside in membrane domains of different lipid composition. In addition, co-factor lipids associate tightly at specific sites on some membrane proteins and may be required for their functions. In principle, changing lipid structure could influence membrane protein function by altering chemical and/or physical properties of the membrane (e.g. via chemical phenomena like hydrophobic interactions, hydrogen bonding and electrostatic interactions, or by affecting physical properties like membrane fluidity, membrane tension or interfacial curvature). For example, osmotically induced changes of the phospholipid composition of *B. subtilis* have been shown to alter physical properties of its cytoplasmic membrane [45,46]. Some effects of lipids on protein function require direct lipid–protein interactions and others do not [43,90].

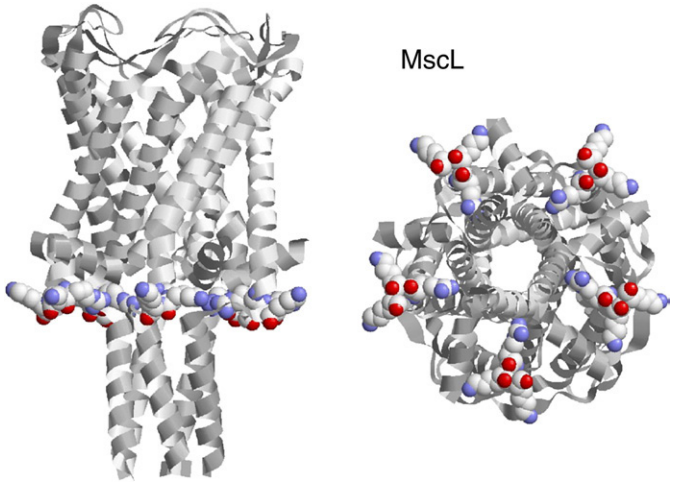

Fig. 3. Structure of mechanosensitive channel MscL. The figure shows the crystal structure of mechanosensitive channel MscL from *M. tuberculosis* (top, PDB ID 2OAR [10]), visualized from the membrane plane (left) and from the cytoplasm (right). Clusters of basic residues that contribute to the association of anionic lipids with MscL (Arg-98, Lys-99 and Lys-100) are highlighted [67].

Efforts by Lee and his colleagues to characterize the interactions of lipids with mechanosensitive channel MscL offer a paradigm for the elucidation of lipid–protein interactions that is directly relevant to bacterial osmotic stress responses [43]. MscL is a channel of large conductance that opens to release cytoplasmic solutes when membrane tension increases *in vivo* or after reconstitution of the purified protein in proteoliposomes, *in vitro* [13]. Thus lipid–protein interactions are critical for MscL function. MscL is a homopentameric protein in which each subunit is comprised of two transmembrane α-helices, a single periplasmic loop and cytoplasmic termini. The crystal structure of MscL from *Mycobacterium tuberculosis* [10] is being exploited as researchers make and test predictions regarding MscL structure and function (Fig. 3). Recently Powl et al. identified a "hot spot" at which anionic lipids (PG, CL and phosphatidylserine) bind to MscL [66,67]. Their data suggest that a cluster of basic residues in the C-terminal domain of MscL promotes binding of anionic lipid to a site that faces the cytoplasmic leaflet of the cytoplasmic membrane (Fig. 3). Furthermore Powl et al. measured efflux of calcein, a self-quenching fluorescent dye, from MscL-containing proteoliposomes of variable phospholipid composition. Anionic lipids (CL, PG or phosphatidic acid) influenced the rate and extent of calcein flux through *E. coli* MscL *in vitro* [68]. Norman et al. reported broad distribution of a MscL-GFP fusion protein within the cytoplasmic membrane of *E. coli* [60], but MscL may find itself in a CL- or PG-enriched membrane when bacteria adapt to high osmolality media. It will be interesting to learn whether osmotically induced changes in membrane phospholipid composition bring about physiologically significant changes to the function of MscL during subsequent osmotic down-shocks.

There is direct evidence that CL has physiologically relevant effects on the function of osmosensory transporter ProP. The activity of ProP, an osmolyte-H^+ symporter (Fig. 1), increases with osmolality in intact *E. coli* [16], right side out cytoplasmic membrane vesicles [19], and proteoliposomes reconstituted with the purified protein [69]. This behaviour contrasts with the osmotic inhibition of respiration and other transporters [38]. For example, ProP approached half its maximal activity as the activity of lactose transporter LacY fell 2-fold when the osmolality of the medium suspending *E. coli* membrane vesicles rose from 0.25 mol/kg to 0.48 mol/kg [19]. LacY and ProP are structurally related members of the Major Facilitator Superfamily (MFS) and both are H^+-solute symporters [18,91]. ProP addresses the deleterious effects of increasing osmolality on cell structures and functions, including lactose uptake via LacY, by mediating the cytoplasmic accumulation of compounds such as proline, glycine betaine and ectoine [49]. ProP acts, in concert with membrane lipid, as an osmosensor and an osmoregulatory transporter.

ProP activity is a sigmoid function of assay medium osmolality, rising from a negligible to a maximum value over an osmolality range of approximately 0.2 mol/kg [16] (Fig. 4A). ProP activity, and hence the amplitude of its osmotic activation, is proportional to the proton motive force [19,86]. The relationship between transport activity and osmolality (the osmotic activation profile) depends on the structure of ProP [17,20,34,44,75,87,91] and its cellular environment [16,19,86]. We recently found that the CL content of *E. coli* and the osmolality at which ProP activity is half maximal vary in parallel with bacterial growth medium osmolality [86,87] (Fig. 4B). Thus CL was implicated in a mechanism that tunes ProP activity to vary with osmolality in the ambient range (that of bacterial growth).

Noting evidence that CL concentrates at the poles of *E. coli* cells [54], we visualized the sub-cellular location of ProP. FlAsH-EDT_2 is a membrane-permeant biarsenical fluorescein derivative that becomes fluorescent when it binds tightly and specifically to proteins that include the tetracysteine tag CCPGCC [28,29]. FlAsH-labeling is a powerful cell biological tool because it permits protein visualization by fluorescence microscopy with minimal perturbation of target protein structure. The tetracysteine tag was inserted at the cytoplasmic N-

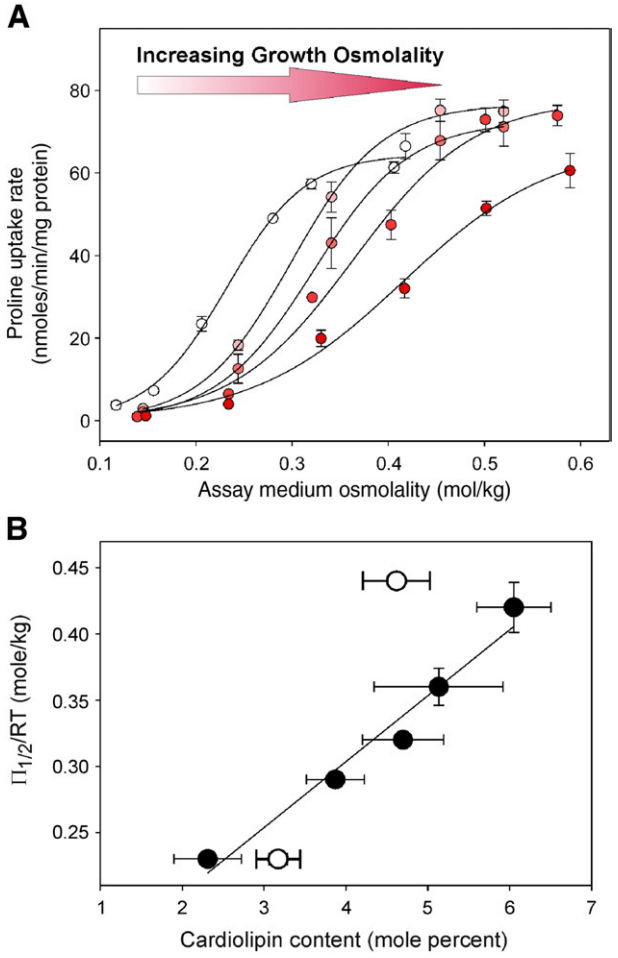

Fig. 4. Osmotic activation and CL-mediated osmotic adaptation of osmosensory transporter ProP in *E. coli*. Panel A, ProP activity is a sigmoid function of osmolality (osmotic activation) and the osmolality required to activate ProP increases with growth medium osmolality (osmotic adaptation). Bacteria were cultivated in minimal medium with an osmolality of 0.14 mol/kg (white circles) or in the same medium supplemented with NaCl to attain osmolalities of 0.43, 0.52, 0.60 or 0.70 mol/kg (increasingly dark red circles). Initial rates of proline uptake via ProP (a_0) were measured using assay media adjusted to the indicated osmolalities (Π/RT) and lines were created by fitting the data to the equation $a_0 = A_{max}\,(1+\exp\,[-(\Pi-\Pi_{1/2})/(RTB)])^{-1}$ by non-linear regression as described by Tsatskis et al. [86]. R is the gas constant, T is the temperature, A_{max} is the activity that would be attained at infinite osmolality, $\Pi_{1/2}/RT$ is the osmolality at which $a_0 = 1/2A_{max}$, and parameter B is inversely proportional to the slope of the response curve. Panel B, The osmolality required to activate ProP is directly proportional to the CL content in bacteria cultivated in media of increasing osmolality. The osmolality at which ProP activity was half maximal ($\Pi_{1/2}$/RT, mol/kg) was estimated by regression analysis (see panel A) and the proportion of CL in bacterial membrane extracts was estimated by thin layer chromatography as described by Tsatskis et al. [86]. The bacteria were cultivated in media of increasing salinity (solid symbols) or in media without or with sucrose (open symbols). Panels A and B were reproduced from Figs. 2 and 5C of Tsatskis et al. [86].

terminus of ProP and the resulting, fully functional variant was labeled with FlAsH *in vivo*. Like CL, FlAsH-ProP was concentrated at the cell poles [74]. Furthermore, the polar concentration of ProP was CL-dependent [74]. The fraction of cells with ProP at the cell poles rose with the proportion of CL, as it varied with the bacterial genotype (*cls*$^-$ or *cls*$^+$), growth medium osmolality and culture growth phase (Fig. 5). Using minicells, the CL content at the cell poles was estimated to be at least 7.4 mol% when that of normal cells from the same culture was 3.6 mol%. The polar concentration of CL was not contingent on the presence of ProP (Romantsov and Wood, unpublished data). When FlAsH-labeling was used to visualize ProP homologue LacY, polar fluorescent patches appeared in approximately one half of the cells, regardless of CL content [75]. Thus CL-dependent polar concentration is characteristic of osmoregulatory protein ProP [53]. Efforts to determine whether CL influences the sub-cellular locations of other osmoregulatory proteins are in progress.

Concentration of ProP in the CL-rich environment at the cell poles has important functional consequences [75]. Proteoliposomes were prepared with histidine-tagged ProP (ProP-His_6), a polar lipid extract from *E. coli* and varying quantities of synthetic PG or CL. The osmolality at which ProP attained half its maximal activity was a direct function of their anionic lipid content. However the properties of ProP were much more sensitive to CL than to PG and they were more sensitive to lipid composition in cells than in proteoliposomes. ProP function was influenced by the PG content of *cls*$^-$ bacteria, even though ProP was not concentrated at the poles of these cells, so ProP function depended on its membrane environment, not its sub-cellular location *per se*. These data suggest that, in wild-type bacteria, osmotic induction of CL synthesis and co-localization of ProP with CL at the cell poles adjust the osmolality range over which ProP activity is controlled so that it will match ambient conditions.

Although fluorescence microscopy tells us that ProP and CL co-localize at the poles of *E. coli* cells it does not tell us whether CL interacts with ProP as an annular or co-factor lipid. The mechanism by which CL alters the osmotic activation profile of ProP is not known, but hints regarding ProP–lipid interactions are emerging from our structure–function analyses. ProP orthologues are known or predicted to occur in many bacteria. Each is predicted to consist of 12 transmembrane α-helices. This prediction was verified for *E. coli* ProP, which has a fold similar to those of MFS members LacY, GlpT and OxlT, and cytoplasmic N- and C-termini [91]. *E. coli* ProP has a dimer interface that includes transmembrane helix XII and an intermolecular, antiparallel α-helical coiled-coil formed by the extended, cytoplasmic C-termini of adjacent monomers [20,34,44]. The NMR structure of the coiled-coil is known [35,93] and its sequence-structure relationships have been extensively studied [20,86,87]. However, some ProP orthologues lack the C-terminal coiled-coil [65]. Orthologues with and without the coiled-coil are osmosensory transporters, but a higher osmolality is required to activate those without a coiled-coil, and those in which coiled-coil formation is impaired by mutation, than those with this structure [20,27,63,86,87].

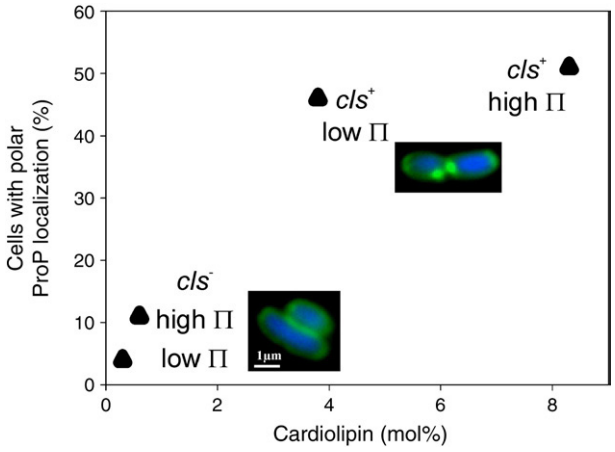

Fig. 5. Concentration of osmosensory transporter ProP at *E. coli* cell poles is cardiolipin-dependent. The percentage of *E. coli* cells with ProP concentrated at the cell poles is plotted versus the cardiolipin content of the cell membrane. Tetracysteine-tagged ProP was expressed in *cls*$^+$ and *cls*$^-$ cells that were cultured to exponential phase in media supplemented with NaCl to attain osmolalities of 0.15 or 0.7 mol/kg. The sub-cellular localization of ProP was visualized by FlAsH-labeling and fluorescence microscopy [75]. The insets show images of representative bacteria, cultivated in low osmolality medium, in which the FlAsH-labeled ProP variant is not (*cls*$^-$ bacteria) and is (*cls*$^+$ bacteria) concentrated at the cell poles (green fluorescence, FlAsH-labeled ProP; blue fluorescence, nucleoid stained by DAPI (4′,6′-diamidino-2-phenylindole)). The CL content of lipid extracts from bacteria prepared in the same way was determined by thin layer chromatography [74,86].

Stabilization of the coiled-coil via covalent cross-linking of introduced cysteines reversed the impact of increasing CL content on the osmotic activation of ProP *in vivo* [75]. Thus the C-terminal coiled-coil appears to function in opposition to CL, lowering the osmolality at which ProP can activate.

Strikingly, mutations R488I and I474P disrupt the antiparallel coiled-coil, raise the osmolality at which ProP activates and prevent concentration of ProP at the cell poles [75]. These results led us to propose that the ProP C-termini may associate with the CL-rich membrane at the cell poles, thereby raising the osmolality required to activate ProP by blocking coiled-coil formation. Both the surface of the ProP coiled-coil and the CL-rich membrane are anionic. However coiled-coil formation may compete with binding of amphipathic, monomeric ProP C-termini to the surface of a CL-rich membrane. Alternatively, as for MscL, CL headgroups could bind to clusters of positively charged side-chains associated with the cytoplasmic loops of ProP [91] or an unidentified protein, concentrated with CL at the bacterial cell poles, could associate with the ProP C-terminus to alter ProP function [87].

6. Conclusion

Growth in media of high salinity alters the phospholipid headgroup and fatty acid compositions of bacterial cytoplasmic membranes. Although CL is synthesized from PG, the proportion of CL in *E. coli* increases as the proportion of PE decreases and PG content remains constant when osmotic stress is imposed with an electrolyte or a non-electrolyte. These changes result, at least in part, from osmotic induction of the gene encoding CL synthase (Cls). The *pgsA* gene, encoding PG synthase, may also be osmotically induced. CL is concentrated at the poles of diverse bacterial cells. Concentration of osmosensory transporter ProP at the poles of *E. coli* cells increases with CL content and depends on the structure of the transporter's cytoplasmic, carboxyl terminal domain. The proportions of anionic lipids (CL or PG) modulate the function of ProP *in vivo* and *in vitro*. These effects suggest that the osmotic induction of CL synthesis and co-localization of ProP with CL at the cell poles adjust the osmolality range over which ProP activity is controlled to match ambient conditions. Anionic lipids bind to a specific site on each subunit of mechanosensitive channel MscL and influence its function *in vitro*.

Much remains to be learned about the role of CL in osmotic adaptation. How do the proportions of diverse phospholipid species (headgroups and acyl chains) vary with osmotic stress, and how do these changes alter physical and chemical properties of the membrane? What is the alternative pathway for CL synthesis, and how is synthesis of PG and CL controlled in response to osmolality and culture growth phase? Why and by what mechanism do CL and some proteins concentrate at the poles of bacterial cells, and why is polar concentration of ProP in *E. coli* CL-dependent? By what mechanism do anionic lipids influence the functions of MscL, ProP and, possibly, other osmoregulatory proteins? What physiological advantages are conferred by regulating phospholipid composition and adjusting the locations of lipids and proteins on the cell surface?

Acknowledgements

We are grateful to Dyanne Brewer for discussions of the data and to Doreen Culham, Charles Deutch, Michelle Smith, Geordie Wright and Adam Zuiani for their comments on the manuscript. The Wood Lab is supported by the Natural Sciences and Engineering Research Council of Canada and the Canadian Institutes of Health Research. The mass spectrometry facility in the Department of Biochemistry at the Duke University Medical Center and Z.G. were supported by the LIPID MAPS Large Scale Collaborative Grant number GM069338 from the National Institutes of Health, USA.

References

[1] K. Altendorf, I.R. Booth, J.D. Gralla, J.-C. Greie, A.Z. Rosenthal, J.M. Wood, Module 5.4.5 Osmotic Stress, in: A. Böck, R. Curtiss III, J.B. Kaper, P.D. Karp, F.C. Neidhardt, T. Nyström, J.M. Slauch, C.L. Squires (Eds.), American Society for Microbiology, 2009.
[2] I. Barák, K. Muchová, A.J. Wilkinson, P.J. O'Toole, N. Pavlendová, Lipid spirals in *Bacillus subtilis* and their role in cell division, Mol. Microbiol. 68 (2008) 1315–1327.
[3] P. Bernal, J. Muñoz-Rojas, A. Hurtado, J.L. Ramos, A. Segura, A *Pseudomonas putida* cardiolipin synthesis mutant exhibits increased sensitivity to drugs related to transport functionality, Environ. Microbiol. 9 (2007) 1135–1145.
[4] D.W. Bolen, Protein stabilization by naturally occurring osmolytes, Meth. Mol. Biol. 168 (2001) 17–36.
[5] C.W. Bourque, Central mechanisms of osmosensation and systemic osmoregulation, Nat. Rev. Neurosci. 9 (2008) 519–531.
[6] G.R. Brown, I.C. Sutcliffe, D. Bendell, S.P. Cummings, The modification of the membrane of *Oceanomonas baumannii* when subjected to both osmotic and organic solvent stress, FEMS Microbiol. Lett. 189 (2000) 149–154.
[7] G.R. Brown, I.C. Sutcliffe, S.P. Cummings, Combined solvent and water activity stresses on turgor regulation and membrane adaptation in *Oceanimonas baumannii* ATCC 700832, Antonie Van Leeuwenhoek 83 (2003) 275–283.
[8] M.B. Burg, J.D. Ferraris, Intracellular organic osmolytes: function and regulation, J. Biol. Chem. 283 (2008) 7309–7313.
[9] D.S. Cayley, H.J. Guttman, M.T. Record Jr., Biophysical characterization of changes in amounts and activity of *Escherichia coli* cell and compartment water and turgor pressure in response to osmotic stress, Biophys. J. 78 (2000) 1748–1764.
[10] G. Chang, R.H. Spencer, A.T. Lee, M.T. Barclay, D.C. Rees, Structure of the MscL homolog from *Mycobacterium tuberculosis*: a gated mechanosensitive ion channel, Science 282 (1998) 2220–2226.
[11] K.P. Choe, K. Strange, Molecular and genetic characterization of osmosensing and signal transduction in the nematode *Caenorhabditis elegans*, FEBS J. 274 (2007) 5782–5789.
[12] H. Christensen, N.J. Garton, R.W. Horobin, D.E. Minnikin, M.R. Barer, Lipid domains of mycobacteria studied with fluorescent molecular probes, Mol. Microbiol. 31 (1999) 1561–1572.
[13] B. Corry, B. Martinac, Bacterial mechanosensitive channels: experiment and theory, Biochim. Biophys. Acta 1778 (2008) 1859–1870.
[14] J.E. Cronan, Bacterial membrane lipids: where do we stand? Annu. Rev. Microbiol. 57 (2003) 203–224.
[15] J.E. Cronan, P.R. Vagelos, Metabolism and function of the membrane phospholipids of *Escherichia coli*, Biochim. Biophys. Acta 265 (1972) 25–60.
[16] D.E. Culham, J. Henderson, R.A. Crane, J.M. Wood, Osmosensor ProP of *Escherichia coli* responds to the concentration, chemistry and molecular size of osmolytes in the proteoliposome lumen, Biochem. 42 (2003) 410–420.
[17] D.E. Culham, A. Hillar, J. Henderson, A. Ly, Y.I. Vernikovska, K.I. Racher, J.M. Boggs, J. M. Wood, Creation of a fully functional, cysteine-less variant of osmosensor and proton-osmoprotectant symporter ProP from *Escherichia coli* and its application to assess the transporter's membrane orientation, Biochemistry 42 (2003) 11815–11823.
[18] D.E. Culham, B. Lasby, A.G. Marangoni, J.L. Milner, B.A. Steer, R.W. van Nues, J.M. Wood, Isolation and sequencing of *Escherichia coli* gene *proP* reveals unusual structural features of the osmoregulatory proline/betaine transporter, ProP, J. Mol. Biol. 229 (1993) 268–276.
[19] D.E. Culham, T. Romantsov, J.M. Wood, Roles of K^+, H^+, H_2O and $\Delta\Psi$ in solute transport mediated by Major Facilitator Superfamily members ProP and LacY, Biochemistry 47 (2008) 8176–8185.
[20] D.E. Culham, B. Tripet, K.I. Racher, R.T. Voegele, R.S. Hodges, J.M. Wood, The role of the carboxyl terminal α-helical coiled-coil domain in osmosensing by transporter ProP of *Escherichia coli*, J. Mol. Recog. 13 (2000) 1–14.
[21] T. Danevcic, L. Rilfors, J. Strancar, G. Lindblom, D. Stopar, Effects of lipid composition on the membrane activity and lipid phase behaviour of *Vibrio* sp. DSM14379 cells grown at various NaCl concentrations, Biochim. Biophys. Acta 1712 (2005) 1–8.
[22] A.J. Dijkstra, W. Keck, Peptidoglycan as a barrier to transenvelope transport, J. Bacteriol. 178 (1996) 5555–5562.
[23] I. Fishov, C. Woldringh, Visualization of membrane domains in *Escherichia coli*, Mol. Microbiol. 32 (1999) 1166–1172.
[24] P.F. Gallet, A. Maftah, J.M. Petit, M. Denis-Gay, R. Julien, Direct cardiolipin assay in yeast using the red fluorescence emission of 10-N-nonyl acridine orange, Eur. J. Biochem. 228 (1995) 113–119.
[25] T.A. Garrett, Z. Guan, C.R. Raetz, Analysis of ubiquinones, dolichols, and dolichol diphosphate-oligosaccharides by liquid chromatography-electrospray ionization-mass spectrometry, Meth. Enzymol. 432 (2007) 117–143.
[26] T.A. Garrett, R. Kordestani, C.R. Raetz, Quantification of cardiolipin by liquid chromatography-electrospray ionization mass spectrometry, Meth. Enzymol. 433 (2007) 213–230.
[27] G. Gouesbet, A. Trautwetter, S. Bonnassie, L.F. Wu, C. Blanco, Characterization of the *Erwinia chrysanthemi* osmoprotectant transporter gene *ousA*, J. Bacteriol. 178 (1996) 447–455.
[28] B.A. Griffin, S.R. Adams, J. Jones, R.Y. Tsien, Fluorescent labeling of recombinant proteins in living cells with FlAsH, Meth. Enzymol. 327 (2000) 565–578.
[29] B.A. Griffin, S.R. Adams, R.Y. Tsien, Specific covalent labeling of recombinant protein molecules inside live cells, Science 281 (1998) 269–272.
[30] Z. Guan, S. Li, D.C. Smith, W.A. Shaw, C.R. Raetz, Identification of N-acylphosphatidylserine molecules in eukaryotic cells, Biochem. 46 (2007) 14500–14513.
[31] A. Guillot, D. Obis, M.-Y. Mistou, Fatty acid membrane composition and activation of glycine-betaine transport in *Lactococcus lactis* subjected to osmotic stress, Int. J. Food Microbiol. 55 (2000) 47–51.

[32] D. Guo, B.E. Tropp, A second *Escherichia coli* protein with CL synthase activity, Biochim. Biophys. Acta 1483 (2000) 263–274.
[33] S. Heber, B.E. Tropp, Genetic regulation of cardiolipin synthase in *Escherichia coli*, Biochim. Biophys. Acta 1129 (1991) 1–12.
[34] A. Hillar, D.E. Culham, Ya.I. Vernikovska, J.M. Wood, J.M. Boggs, Formation of an antiparallel, intermolecular coiled-coil is associated with *in vivo* dimerization of osmosensor and osmoprotectant transporter ProP in *Escherichia coli*, Biochemistry 44 (2005) 10170–10180.
[35] A. Hillar, B. Tripet, D. Zoetewey, J.M. Wood, R.S. Hodges, J.M. Boggs, Detection of α-helical coiled-coil dimer formation by spin-labeled synthetic peptides: a model parallel coiled-coil peptide and the antiparallel coiled-coil formed by a replica of the ProP C-terminus, Biochemistry 42 (2003) 15170–15178.
[36] S. Hiraoka, H. Matsuzaki, I. Shibuya, Active increase in cardiolipin synthesis in the stationary growth phase and its physiological significance in *Escherichia coli*, FEBS Lett. 336 (1993) 221–224.
[37] S. Hohmann, Osmotic stress signaling and osmoadaptation in yeasts, Microbiol. Mol. Biol. Rev. 66 (2002) 300–372.
[38] C. Houssin, N. Eynard, E. Shechter, A. Ghazi, Effect of osmotic pressure on membrane energy-linked functions in *Escherichia coli*, Biochim. Biophys. Acta 1056 (1991) 76–84.
[39] K.C. Huang, R. Mukhopadhyay, N.S. Wingreen, A curvature-mediated mechanism for localization of lipids to bacterial poles, PLoS Comput. Biol. 2 (2006) e151.
[40] F. Kawai, M. Shoda, R. Harashima, Y. Sadaie, H. Hara, K. Matsumoto, Cardiolipin domains in *Bacillus subtilis* Marburg membranes, J. Bacteriol. 186 (2004) 1475–1483.
[41] C.-M. Koppelman, T. den Blaauwen, M.C. Duursma, R.M.A. Heeren, N. Nanninga, *Escherichia coli* minicell membranes are enriched in cardiolipin, J. Bacteriol. 183 (2001) 6144–6147.
[42] F. Lang, Mechanisms and significance of cell volume regulation, J. Am. Coll. Nutr. 26 (2007) 613S–623S.
[43] A.G. Lee, How lipids affect the activities of integral membrane proteins, Biochim. Biophys. Acta 1666 (2004) 62–87.
[44] F. Liu, D.E. Culham, Y.I. Vernikovska, R.A.B. Keates, J.M. Boggs, J.M. Wood, Structure and function of the XIIth transmembrane segment in osmosensor and osmoprotectant transporter ProP of *Escherichia coli*, Biochemistry 46 (2007) 5647–5655.
[45] C.S. López, A.F. Alice, H. Heras, E.A. Rivas, C. Sánchez-Rivas, Role of anionic phospholipids in the adaptation of *Bacillus subtilis* to high salinity, Microbiology 152 (2006) 605–616.
[46] C.S. López, H. Heras, H. Garda, S. Ruzal, C. Sánchez-Rivas, E. Rivas, Biochemical and biophysical studies of *Bacillus subtilis* envelopes under hyperosmotic stress, Int. J. Food Microbiol. 55 (2000) 137–142.
[47] C.S. López, H. Heras, S.M. Ruzal, C. Sánchez-Rivas, E.A. Rivas, Variations of the envelope composition of *Bacillus subtilis* during growth in hyperosmotic medium, Curr. Microbiol. 36 (1998) 55–61.
[48] M.C. Machado, C.S. López, H. Heras, E.A. Rivas, Osmotic response in *Lactobacillus casei* ATCC 393: biochemical and biophysical characteristics of membrane, Arch. Biochem. Biophys. 422 (2004) 61–70.
[49] S.V. MacMillan, D.A. Alexander, D.E. Culham, H.J. Kunte, E.V. Marshall, D. Rochon, J.M. Wood, The ion coupling and organic substrate specificities of osmoregulatory transporter ProP in *Escherichia coli*, Biochim. Biophys. Acta 1420 (1999) 30–44.
[50] A. Maftah, J.M. Petit, M.H. Ratinaud, R. Julien, 10-N nonyl-acridine orange: a fluorescent probe which stains mitochondria independently of their energetic state, Biochem. Biophys. Res. Commun. 164 (1989) 185–190.
[51] J.T. McGarrity, J.B. Armstrong, The effect of salt on phospholipid fatty acid composition in *Escherichia coli* K-12, Biochim. Biophys. Acta 398 (1975) 258–264.
[52] J.T. McGarrity, J.B. Armstrong, The effect of salt on phospholipid fatty acid composition in *Escherichia coli*, Can. J. Microbiol. 27 (1981) 835–840.
[53] E. Mileykovskaya, Subcellular localization of *Escherichia coli* osmosensory transporter ProP: focus on cardiolipin membrane domains, Mol. Microbiol. 64 (2007) 1419–1422.
[54] E. Mileykovskaya, W. Dowhan, Visualization of phospholipid domains in *Escherichia coli* by using the cardiolipin-specific fluorescent dye 10-N-nonyl acridine orange, J. Bacteriol. 182 (2000) 1172–1175.
[55] E. Mileykovskaya, W. Dowhan, R.L. Birke, D. Zheng, L. Lutterodt, T.H. Haines, Cardiolipin binds nonyl acridine orange by aggregating the dye at exposed hydrophobic domains on bilayer surfaces, FEBS Lett. 507 (2001) 187–190.
[56] R. Mukhopadhyay, K.C. Huang, N.S. Wingreen, Lipid localization in bacterial cells through curvature-mediated microphase separation, Biophys. J. 95 (2008) 1034–1049.
[57] R. Munns, M. Tester, Mechanisms of salinity tolerance, Annu. Rev. Plant Biol. 59 (2008) 651–681.
[58] F.C. Neidhardt, P.L. Bloch, D.F. Smith, Culture medium for enterobacteria, J. Bacteriol. 119 (1974) 736–747.
[59] S. Nishijima, Y. Asami, N. Uetake, S. Yamagoe, A. Ohta, I. Shibuya, Disruption of the *Escherichia coli cls* gene responsible for cardiolipin synthesis, J. Bacteriol. 170 (1988) 775–780.
[60] C. Norman, Z.W. Liu, P. Rigby, A. Raso, Y. Petrov, B. Martinac, Visualisation of the mechanosensitive channel of large conductance in bacteria using confocal microscopy, Europ. Biophys. J. 34 (2005) 396–402.
[61] M.J. Osborn, J.E. Gander, E. Parisi, J. Carson, Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of cytoplasmic and outer membrane, J, Biol. Chem. 247 (1972) 3962–3972.
[62] N. Ozcan, C.S. Ejsing, A. Shevchenko, A. Lipski, S. Morbach, R. Krämer, Osmolality, temperature, and membrane lipid composition modulate the activity of betaine transporter BetP in *Corynebacterium glutamicum*, J. Bacteriol. 189 (2007) 7485–7496.
[63] H. Peter, B. Weil, A. Burkovski, R. Krämer, S. Morbach, *Corynebacterium glutamicum* is equipped with four secondary carriers for compatible solutes: identification, sequencing, and characterisation of the proline/ectoine uptake system ProP and the ectoine/proline/glycine betaine carrier EctP, J. Bacteriol. 180 (1998) 6005–6012.
[64] J.M. Petit, A. Maftah, M.H. Ratinaud, R. Julien, 10N-nonyl acridine orange interacts with cardiolipin and allows the quantification of this phospholipid in isolated mitochondria, Eur. J. Biochem. 209 (1992) 267–273.
[65] B. Poolman, J.J. Spitzer, J.M. Wood, Bacterial osmosensing: roles of membrane structure and electrostatics in lipid–protein and protein–protein interactions, Biochim. Biophys. Acta 1666 (2004) 88–104.
[66] A.M. Powl, J.M. East, A.G. Lee, Lipid–protein interactions studied by introduction of a tryptophan residue: the mechanosensitive channel MscL, Biochemistry 42 (2003) 14306–14317.
[67] A.M. Powl, J.M. East, A.G. Lee, Heterogeneity in the binding of lipid molecules to the surface of a membrane protein: hot spots for anionic lipids on the mechanosensitive channel of large conductance MscL and effects on conformation, Biochemistry 44 (2005) 5873–5883.
[68] A.M. Powl, J.M. East, A.G. Lee, Anionic phospholipids affect the rate and extent of flux through the mechanosensitive channel of large conductance MscL, Biochemistry 47 (2008) 4317–4328.
[69] K.I. Racher, R.T. Voegele, E.V. Marshall, D.E. Culham, J.M. Wood, H. Jung, M. Bacon, M.T. Cairns, S.M. Ferguson, W.-J. Liang, P.J.F. Henderson, G. White, F.R. Hallett, Purification and reconstitution of an osmosensor: transporter ProP of *Escherichia coli* senses and responds to osmotic shifts, Biochemistry 38 (1999) 1676–1684.
[70] L. Ragolia, B.E. Tropp, The effects of phosphoglycerides on *Escherichia coli* cardiolipin synthase, Biochim. Biophys. Acta 1214 (1994) 323–332.
[71] M.T. Record Jr., E.S. Courtenay, D.S. Cayley, H.J. Guttman, Responses of *E. coli* to osmotic stress: large changes in amounts of cytoplasmic solutes and water, Trends Biochem. Sci. 23 (1998) 143–148.
[72] M.T. Record Jr., E.S. Courtenay, S. Cayley, H.J. Guttman, Biophysical compensation mechanisms buffering *E. coli* protein-nucleic acid interactions against changing environments, Trends Biochem. Sci. 23 (1998) 190–194.
[73] L. Rilfors, G. Lindblom, Regulation of lipid composition in biological membranes—biophysical studies of lipids and lipid synthesizing enzymes, Colloids Surf. B Biointerfaces 26 (2002) 112–124.
[74] T. Romantsov, S. Helbig, D.E. Culham, C. Gill, L. Stalker, J.M. Wood, Cardiolipin promotes polar localization of osmosensory transporter ProP in *Escherichia coli*, Mol. Microbiol. 64 (2007) 1455–1465.
[75] T. Romantsov, L. Stalker, D.E. Culham, J.M. Wood, Cardiolipin controls the osmotic stress response and the subcellular location of transporter ProP in *Escherichia coli*, J. Biol. Chem. 283 (2008) 12314–12323.
[76] J.W. Rosch, F.F. Hsu, M.G. Caparon, Anionic lipids enriched at the ExPortal of *Streptococcus pyogenes*, J. Bacteriol. 189 (2007) 801–806.
[77] N.J. Russell, Adaptive modifications in membranes of halotolerant and halophilic microorganisms, J. Bioenerg. Biomembrane. 21 (1989) 93–113.
[78] N.J. Russell, Lipids of halophilic and halotolerant microorganisms, in: R.H. Vreeland, L.I. Hochstein (Eds.), The Biology of Halophilic Bacteria, CRC Press, Boca Raton, 1993, pp. 163–210.
[79] N.J. Russell, R.I. Evans, P.F. ter Steeg, J. Hellemons, A. Verheul, T. Abee, Membranes as a target for stress adaptation, Int. J. Food Microbiol. 28 (1995) 255–261.
[80] J. Sajbidor, Effect of some environmental factors on the content and composition of microbial membrane lipids, CRC Crit. Rev. Biotechnol. 17 (1997) 87–103.
[81] M. Schlame, Cardiolipin synthesis for the assembly of bacterial and mitochondrial membranes, J. Lipid Res. 49 (2008) 1607–1620.
[82] M. Septinus, W. Seiffert, H.W. Zimmerman, Hydrophohic acridine dyes for fluorescence staining of mitochondria in living cells 1. Thermodynamic and spectroscopic properties of 10-n-alkyl-acridinium-orange-chlorides, Histochemistry 79 (1983) 443–456.
[83] J.B. Stock, B. Rauch, S. Roseman, Periplasmic space in *Salmonella typhimurium* and *Escherichia coli*, J. Biol. Chem. 252 (1977) 7850–7861.
[84] B.E. Tropp, Cardiolipin synthase from *Escherichia coli*, Biochim. Biophys. Acta 1348 (1997) 192–200.
[85] Y.A. Trotsenko, V.N. Khmelenina, Biology of extremophilic and extremotolerant methanotrophs, Arch. Microbiol. 177 (2002) 123–131.
[86] Y. Tsatskis, J. Khambati, M. Dobson, M. Bogdanov, W. Dowhan, J.M. Wood, The osmotic activation of transporter ProP is tuned by both its C-terminal coiled-coil and osmotically induced changes in phospholipid composition, J. Biol. Chem. 280 (2005) 41387–41394.
[87] Y. Tsatskis, S.C. Kwok, E. Becker, C. Gill, M.N. Smith, L. Stalker, R.S. Hodges, J.M. Wood, Coiled-coil orientation switching places osmosensory transporter ProP in an osmolality-refractory state, Biochemistry 47 (2007) 60–72.
[88] C. Vargas, A. Kallimanis, A.I. Koukkou, M.I. Calderon, D. Canovas, F. Iglesias-Guerra, C. Drainas, A. Ventosa, J.J. Nieto, Contribution of chemical changes in membrane lipids to the osmoadaptation of the halophilic bacterium *Chromohalobacter salexigens*, Syst. Appl. Microbiol. 28 (2005) 571–581.
[89] T.A. Vida, S.D. Emr, A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast, J. Cell Biol. 128 (1995) 779–792.
[90] J.M. Wood, Osmosensing by bacteria: signals and membrane-based sensors, Microbiol. Mol. Biol. Rev. 63 (1999) 230–262.
[91] J.M. Wood, D.E. Culham, A. Hillar, Y.I. Vernikovska, F. Liu, J.M. Boggs, R.A.B. Keates, Structural model for the osmosensor, transporter, and osmoregulator ProP of *Escherichia coli*, Biochemistry 44 (2005) 5634–5646.
[92] A. Zaritsky, C. Woldringh, I. Fishov, N.O. Vischer, M. Einav, Varying division planes of secondary constrictions in spheroidal *Escherichia coli* cells, Microbiology 145 (1999) 1015–1022.
[93] D.L. Zoetewey, B.P. Tripet, T.G. Kutateladze, M.J. Overduin, J.M. Wood, R.S. Hodges, Solution structure of the C-terminal antiparallel coiled-coil domain from *Escherichia coli* osmosensor ProP, J. Mol. Biol. 334 (2003) 1063–1076.