Fluorescence microscopy has revealed that the phospholipid cardiolipin (CL) and FlAsH-labeled transporters ProP and LacY are concentrated at the poles of *Escherichia coli* cells. The proportion of CL among *E. coli* phospholipids can be varied *in vivo* as it is decreased by *cls* mutations and it increases with the osmolality of the growth medium. In this report we compare the localization of CL, ProP, and LacY with that of other cytoplasmic membrane proteins. The proportion of cells in which FlAsH-labeled membrane proteins were concentrated at the cell poles was determined as a function of protein expression level and CL content. Each tagged protein was expressed from a pBAD24-derived plasmid; tagged ProP was also expressed from the chromosome. The osmosensory transporter ProP and the mechanosensitive channel MscS concentrated at the poles at frequencies correlated with the cellular CL content. The lactose transporter LacY was found at the poles at a high and CL-independent frequency. ProW (a component of the osmoregulatory transporter ProU), AqpZ (an aquaporin), and MscL (a mechanosensitive channel) were concentrated at the poles in a minority of cells, and this polar localization was CL independent. The frequency of polar localization was independent of induction (at arabinose concentrations up to 1 mM) for proteins encoded by pBAD24-derived plasmids. Complementation studies showed that ProW, AqpZ, MscS, and MscL remained functional after introduction of the FlAsH tag (CCPGCC). These data suggest that CL-dependent polar localization in *E. coli* cells is not a general characteristic of transporters, channels, or osmoregulatory proteins. Polar localization can be frequent and CL independent (as observed for LacY), frequent and CL dependent (as observed for ProP and MscS), or infrequent (as observed for AqpZ, ProW, and MscL).

Modern developments in fluorescence microscopy have led to a new understanding of the organization of bacterial cells, particularly protein and lipid localization (21, 56). Analysis of the subcellular localization of diverse proteins and lipids has shown that they are not uniformly distributed. The phospholipid cardiolipin (CL) localizes at the poles and septal regions (36), and there is evidence for segregation of phosphatidyl-ethanolamine (PE) from phosphatidylglycerol (PG) in the membranes of living *Escherichia coli* cells (69). Localization of many proteins that are integral or peripheral to the cytoplasmic membrane has been studied by fusing them to green fluorescent protein (GFP) (or its derivatives), and it is possible to classify the fusion proteins according to their subcellular localization. The first group, comprised of proteins that are concentrated at the cell poles, includes chemoreceptors (31, 62), the lactose permease LacY (43), and the metabolic sensor kinases DcuS and CitA (55). Members of the second group form helices that extend from pole to pole and include MreB (25), MinD (57), the Sec protein export system (58), and RNase E, which is the main component of the RNA degradosome in *E. coli* (67). Other proteins may appear to be similarly distributed due to their association with the Sec system (58). Members of the third group are uniformly distributed and include the mechanosensitive channel MscL (45) and the sensor kinase KdpD (32).

The polar localization of proteins appears to be a critical feature of the complicated internal localization of bacteria. For example, it is important for temporally and spatially accurate placement of the septum during cell division (15). However, the mechanism of protein organization at bacterial cell poles is still unclear, and in many cases its functional role has not been determined. Do the poles merely serve as a receptacle for proteins, superstructures, or membrane domains with no functional effects, or is this location functionally important for membrane proteins and lipids?

Recent evidence indicates that the subcellular localization of the transporter ProP in *E. coli* is related to membrane phospholipid composition, cardiolipin localization, and ProP function (51, 52). *E. coli* cells from cultures grown to exponential phase contain mostly the zwitterionic phospholipid PE (approximately 75 mol%) and the anionic phospholipids PG (approximately 20 mol%) and CL (approximately 5 mol%) (8). (Note that cardiolipin is diphosphatidylglycerol.) However, the phospholipid composition depends on the bacterial growth conditions. We found that the proportion of CL among *E. coli* lipids varies directly with growth medium osmolality (68), and...
increased CL synthesis was at least partially attributed to regulation of the \( c l s \) locus encoding cardiolipin synthase (52). There is residual CL in \( c l s \) bacteria, indicating that there is an alternative pathway for CL synthesis (51). The CL-specific fluorescent dye 10-N-onyl-acridine orange (NAO) was used to show that CL clusters at the poles and septa in growing \( E. coli \) cells (36, 52). This result was corroborated by analyzing the phospholipid composition of \( E. coli \) minicells (DNA-free cells resulting from asymmetric cell division) (24, 51).

ProP is an osmosensory transporter that senses increasing osmolality and responds by mediating the cytoplasmic accumulation of organic osmolytes (e.g., proline, glycine betaine, and ectoine). Biochemical regulation of the ProP protein ensures that ProP activity increases with increasing assay medium osmolality (49). We showed that ProP and CL colocalize at the poles and near the septa of dividing \( E. coli \) cells and that the polar concentration of ProP correlates with the polar concentration of CL (52). Moreover, we showed that the osmolality required to activate ProP increased in parallel to the CL content when \( E. coli \) was cultivated in media with increasing osmolality (51, 52, 68). The osmolality required to activate ProP was also a direct function of CL content in proteoliposomes reconstituted with purified ProP (51). We concluded that concentration at the cell poles controlled the osmoregulatory function of ProP by placing the transporter in a cardiolipin-rich environment.

To determine whether CL-dependent membrane protein localization is a general phenomenon in \( E. coli \), we compared the subcellular localization of ProP with that of its paralogue LacY, a well-characterized lactose transporter (16). LacY and ProP are both members of the major facilitator superfamily. LacY, a well-characterized lactose transporter (16). LacY and subcellular localization of ProP with that of its paralogue labeled protein concentrated at the poles as a function of concentration at the cell poles controlled the osmoregulatory function of ProP (51). We concluded that concentration at the cell poles controlled the osmoregulatory function of ProP by placing the transporter in a cardiolipin-rich environment.

In this work we further explored the relationship between CL and protein localization in \( E. coli \). We compared ProP with other proteins related to cellular osmoregulation. Bacteria use arrays of osmoregulatory mechanisms to survive and function when the osmotic pressure of their environment changes. In \( E. coli \), the aquaporin AgpZ mediates transmembrane water flux, the transporters ProP, ProU, BetT, and BetU mediate organic osmolyte accumulation at high osmotic pressure, and the mechanosensitive (MS) channels Mscl and Msce mediate solute efflux in response to osmotic downshift (71). Localization of these proteins might be expected since AgpZ might influence cell morphology changes by accelerating water flux at particular positions on the cell surface and the pressure sensitivities of Mscl and Msce are known to depend on membrane curvature in vitro (18).

For ProP and LacY, we labeled the inserted peptide tag CCPGCC with the biarsenical fluorescent reagent FlAsH-EDT \(_2\) (fluorescein arsenical helix binder, bis-EDT adduct) (1, 2) to examine the subcellular localization of AgpZ, the integral membrane component ProW of the osmoregulatory ATP-binding cassette (ABC) transporter ProU, and the MS channel proteins Mscl and Msce in \( c l s \) and \( c l s \) bacteria. Fluorescence microscopy was used to determine the proportion of cells with labeled protein concentrated at the poles as a function of bacterial CL content and protein expression level. For ProP, the frequency with which Mscl was concentrated at cell poles was proportional to the level and polar concentration of CL. LacY concentrated at the cell poles at a high and CL-indepedent frequency. The frequencies with which AgpZ, Mscl, and ProW concentrated at the cell poles and septa were low (at 12%) and CL independent.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** Strains and plasmids used in this work are listed in Table 1. Strain NCM3308 (MG1655 uppZ::Cam [60]) was obtained from Sydney Kustu (Berkeley, CA); strains MM294-Strept \( e \)(spontaneous streptomycin-resistant mutant of MM294 [\( E. coli \) Genetic Stock Center no. 6315; \( F^-\) glnA44 lac \( \lambda^-\) recBC1 endA1 spoT1 thi-1 rildR1 creS1]) and MM1211 (MM294-Strept \( a\) uppZ::lacZamK) (7) were obtained from Giuseppe Calamita (University of Bari, Bari, Italy).

Unless otherwise stated, the bacteria were cultivated in Luria-Bertani (LB) medium (37) or in NaCl-free morpholinepropanesulfonic acid (MOPS) medium (44) with glycerol (0.4%, vol/vol) as the carbon source, NH\(_4\)Cl (9.5 mM) as the nitrogen source, tryptophan (245 \( \mu \)M), and thiamine (1 mg/ml) to meet auxotrophic requirements. Ampicillin (100 \( \mu \)g/ml) was added to maintain plasmids, and arabinose was added as indicated below to adjust gene expression from the \( pBA\) promoter of vector pBAD24 (17). NaCl was added to adjust the osmolality, and osmolalities were measured with a Wescor vapor pressure osmometer (Wesco Scientific Inc., UT). To monitor growth in liquid medium, cultures (5 ml) were prepared in 125-ml sidearm flasks and incubated at 37°C with rotary shaking at 200 rpm. Optical densities were monitored with a Bausch and Lomb Spectronic 8 spectrophotometer.

**Plasmids and molecular biological manipulations.** The basic molecular biological techniques used were performed as described by Sambrook and Russell (54). PCR were carried out as described by Brown and Wood (6). Oligonucleotides were purchased from Cortec DNA Services (Kingston, ON, Canada). Construction of plasmid pDC224 encoding MCPPGCC-ProP and plasmid pDC245 encoding MCPPGCC-LacY was described previously (51, 52). The genes encoding ProW, Mscl, Msce, and AgpZ were each amplified using PCR primers that introduced restriction endonuclease cleavage sites appropriate for insertion downstream from the AraC-controlled \( pBA\) promoter in the vector pBAD24 (enzymes NcoI and HindIII) (17). Each amplicon was cloned into this vector, and the desired recombinant plasmids were recovered from the ligation mixture by standard chemical transformation of \( E. coli \) DH5\( \alpha \) (19). Site-directed mutagenesis was performed as described by Culham et al. (12) to introduce the oligonucleotide sequence encoding the desired FlAsH tag (Table 1). The entire sequence of the gene encoding each protein was confirmed (GenAlEx/TIC, Guelph, ON, Canada). Plasmids pTR3 and pTR4 (encoding ProW and its FlAsH-tagged derivative, respectively) were introduced into \( E. coli \) strains WG1224 and WG1225 (encoding MscL, MscS, and AqpZ and their FlAsH-tagged derivatives [Table 1]) were introduced into strains WG1230 (encoding Mscl and Msce, and AqpZ) and their FlAsH-tagged derivatives (Table 1) were introduced into strains WG1230 (encoding Mscl and Msce, and AqpZ) and their FlAsH-tagged derivatives (Table 1) were introduced into strains WG1230 and WG1250 (encoding Mscl and Msce, and AqpZ) and their FlAsH-tagged derivatives (Table 1) were introduced into strains WG1230 and WG1250 (encoding Mscl and Msce, and AqpZ) and their FlAsH-tagged derivatives (Table 1) were introduced into strains WG1230 and WG1250 (encoding Mscl and Msce, and AqpZ) and their FlAsH-tagged derivatives (Table 1).
plasmid pDC288. The proP678::Lumio strain was then introduced into the chromosome of E. coli strain W3110 by allelic exchange as described by Link et al. (29). Chloramphenicol- and kanamycin-sensitive isolates that survived the Sac selection were screened on MOPS medium supplemented with 0.6 M NaCl and 1 mM proline to identify the isolates that were ProP+ (11). Chromosomal insertion of proP678::Lumio into one such isolate, WG1259, was confirmed by PCR amplification and cleavage of the resulting amplicon to verify introduction of an NcI site during creation of proP678::Lumio. The allelic change in the background strain was confirmed by PCR of the BamHI site using primers 1 and 2 (11).

**Plasmids**

- **pTR10**: E. coli ORF mscS inserted into expression vector pBAD24
- **pTR11**: pTR10 derivative that encodes MscS-CCPGCC
- **pTR12**: E. coli ORF mscL inserted into expression vector pBAD24
- **pTR13**: pTR12 derivative that encodes MscL-CCPGCC
- **pTR14**: E. coli ORF agrZ inserted into expression vector pBAD24
- **pTR15**: pTR14 derivative that encodes MvCCPGCC-AgrZ
- **pTR17**: pDC10 derivative that encodes MLCCPGCC-ProP
- **pDC288**: pKO3 derivative with proP678::Lumio inserted at the BamHI site

- **MG1465**: Wild type
- **MJF465**: ΔnscL::Cm ΔaggR ΔproP::kan
- **MM294-Strnp**: Streptomycin-resistant derivative of MM294 (F- glv44 λ− rfbC? endA1 spoTI?)
- **MM1211**: ΔntrC MOPS proP::Tn10 lacZ::kan
- **NCM3306**: MG1655 ΔaggR::ram
- **WG170**: F− lacZ trp rpsL thi (ΔproP)A101 proP219
- **WG350**: F− lacZ trp rpsL thi (ΔproP)A101 (ΔproU)600 (ΔproP-melAB)212
- **WG269**: F− lacZ trp rpsL thi (ΔproP)A101 proP219 (ΔproU)1 zdd-351::Tn10
- **WG980**: WG350 clp::Tn10Del3
- **WG1127**: WG350 aprlW859::FRT
- **WG1128**: WG980 aprlW859::FRT
- **WG1224**: WG350 aprlR837::FRT
- **WG1259**: WG350 proP678::Lumio
- **WG1260**: WG1259 clp::Tn10Del3

**Strains**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
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<th>Source or reference</th>
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<td>pDC288</td>
<td>pKO3 derivative with proP678::Lumio inserted at the BamHI site</td>
<td>This study</td>
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\[ A = A_0 + A_{\text{max}} \cdot (1 - e^{-kt}) \]  

where \( A_0 \) and \( A_{\text{max}} \) are the absorbance at zero time and the absorbance at infinite time, respectively.

**Lysis of E. coli upon osmotic downshock.** Cultures were grown, cells were treated, and lysis of the cells was measured exactly as described by Levina et al. (27).

**Preparation of giant spheroplasts.** Spheroplasts were prepared from E. coli strain MJF465 lacking MscL, MscS, and MscK essentially as described previously (34, 53). Plasmids pTR10 to pTR13 encoding MscL and MscW with and without the FlAsH tag were introduced into this strain. A culture was grown in LB medium until the OD\(_{600}\) was 0.4 to 0.5 and then diluted 1:10 into Luria-Bertani medium, and cephalixin was added to a concentration of 60 \( \mu \)g/ml. The culture was then incubated at 42°C for 2 to 2.5 h until single-cell filaments reached a length (50 to 150 \( \mu \)m) sufficient for formation of giant spheroplasts (diameter, 5 to 10 \( \mu \)m). After this, arabinose (final concentration, 0.0015%) and glycerol (0.4%) were added, and the cells were incubated at 25°C and 180 rpm for about 1 h. Then filaments were harvested by centrifugation, and the pellet was rinsed without resuspension by gentle addition of 1 ml of 0.8 M sucrose (0.4% glycerol). After a second centrifugation, the supernatant was removed, and the pellet was resuspended in 2.5 ml of 0.8 M sucrose (0.4% glycerol). Then reagents were added in the following order: 150 \( \mu \)l of 1 M Tris (pH 7.2), 120 \( \mu \)l of lysoyzyme (5 mg/ml), 50 \( \mu \)l of DNase I (5 mg/ml), and 150 \( \mu \)l of 0.125 M EDTA. The mixture was incubated at room temperature for 3 to 5 min to hydrolyze the peptidoglycan layer, and the progress of spheroplast formation was followed under a microscope. At the end of this incubation, 1 ml of a solution containing 20 mM MgCl\(_2\) (to remove the EDTA and activate the DNase), 0.7 M sucrose, 0.4% glycerol, and 10 mM Tris (pH 7.2) was added. Aliquots were prepared and stored in a freezer at −20°C.

**Electrophysiological recordings.** Single-channel analysis was performed with giant spheroplasts (64). Borosilicate glass pipettes (Drummond Scientific Co., Broomall, PA) were pulled using a Flaming/Brown pipette puller (P-87; Sutter Instrument Co., Novato, CA) to obtain a diameter which corresponded to a pipette resistance between 3.0 and 6.0 M\( \Omega \) (to remove the EDTA and activate the DNase), 0.7 M sucrose, 0.4% glycerol, and 10 mM Tris (pH 7.2). Aliquots were prepared and stored in a freezer at −20°C.

**Protein assays.** The protein concentrations of cell suspensions were determined by the bicinchoninic acid assay (59), using a BCA kit from Pierce (Rockford, IL) with bovine serum albumin as the standard.

**RESULTS**

**Analysis of lipid and protein localization: CL and ProP as prototypes.** We previously reported that the level and polar concentration of CL depend on the bacterial genotype (CL bacteria retain only low levels of CL) and growth medium osmolality (the cellular CL content increases with increasing growth medium osmolality) (51, 52, 68). The subcellular localization of bacterial components like CL can be illustrated by reproducing representative fluorescence micrographs (Fig. 1), but selected images do not reveal how frequently a feature is observed in a bacterial population. Such images can be supplemented by reporting the proportion of cells in which membrane components are concentrated at cell poles and septa. For example, we observed concentration of CL-specific, red NAO fluorescence at the poles in very different proportions of CL+ and CL− E. coli cells (Fig. 2G). CL-specific red fluorescence was evident at the poles in 73% of CL+ bacteria and in less than 3% of CL bacteria from liquid cultures with a variety of osmolalities. Within each population (CL− or CL+), the frequency of polar localization did not vary significantly with growth osmolality (Fig. 2G) or CL content (Fig. 2H). Systematic variation might have occurred for CL contents in the range from 0.6 to 4 mol% that is not accessible to us in vivo. As reported previously, the intensities of the polar CL “patches” varied (51, 52). Concentration of CL at the cell poles in some bacteria (particularly CL bacteria) may have eluded detection by fluorescence microscopy, or CL may concentrate at the cell poles less frequently when it is present as a smaller mole fraction of the membrane phospholipid (22, 41).

We previously exploited the well-controlled, low-level protein expression provided by the pBAD24 vector (17) and the propensity of FlAsH-EDT2 to specifically label small hexapeptide tags in vivo (2) to show that the transporters ProP and LacY concentrate at the poles of E. coli cells (51, 52). Here we extended these results by comparing the behavior of plasmid- and chromosome-encoded ProP and by further quantifying the relationship between CL content and the proportion of cells with ProP or LacY concentrated at the poles.

We previously reported that insertion of the oligonucleotide encoding the FlAsH tag at the 5′ end of proP impaired the expression of this gene from the pBAD24 vector (as indicated by Western blotting) and that the activities of FlAsH-tagged ProP and untagged ProP are similar (52). Here we show that the cellular levels of FlAsH-tagged ProP expressed from a pBAD24-derived plasmid (P\(_{\text{BAD}}\) promoter, no arabinose induction) or from the chromosome (native proP promoters, no osmotic induction) are lower than the level of native ProP expressed from the chromosome (native proP promoters, no osmotic induction) (Fig. 3A). This conclusion is based on measurements of both protein levels (Western blotting) and transport activity. Tagged ProP was expressed from the plasmid...
FIG. 2. Frequencies with which CL, ProP, LacY, ProW, MscS, MscL, and AqpZ were concentrated at cell poles. Bacteria expressing the proteins were cultivated and imaged as described in the legend to Fig. 1, employing MOPS media adjusted with NaCl to the indicated range of osmolalities and lacking arabinose. CL was detected by staining with NAO as described in Materials and Methods. The frequency with which CL or each protein was concentrated at a cell pole (one or both poles) was determined by visual inspection for three groups of 100 randomly selected cells. Adjustment of the depth of focus was required to fully examine each cell. Each protein was found to concentrate at the septa of some dividing cells. This was always accompanied by concentration of the protein at the other pole of the same cell and was therefore counted as polar.
The proportion of cells in which FlAsH fluorescence was concentrated at the poles did not vary when expression of plasmid-encoded, tagged ProP was induced with up to 100 μM arabinose (see Table S1 in the supplemental material). The cellular level of tagged ProP varies from well below to approximately 2-fold above that of the native, chromosome-encoded transporter with arabinose induction in this range (Fig. 3A) (52). SDS-PAGE analysis and fluorescence imaging of the proteins in FlAsH-labeled bacteria revealed good labeling specificity and no significant degradation of FlAsH-labeled ProP (Fig. 3B). We therefore concluded that neither the polar localization of ProP nor its correlation with CL localization is an artifact of ProP expression from a heterologous promoter, of its under- or overexpression, or of its degradation. For analogous studies performed with this and other tags, we cannot rule out effects of the FlAsH tag or FlAsH labeling on ProP localization.

Analysis of protein localization: other membrane proteins. We extended the approach described above to compare the subcellular localization of ProP with that of the transporter LacY, the aquaporin AqpZ, and three osmoregulatory proteins (ProW, MscL, and MscS). Each protein was expressed from a pBAD24-derived plasmid, and the optimal FlAsH tag was introduced at the N or C terminus of a protein subunit. Each tagged protein complemented the corresponding genetic defect (see reference 51 for LacY and data below for the other proteins). For each protein, FlAsH-EDT₂ labeling of cells cultivated without arabinose and fluorescence microscopy revealed some cells in which fluorescence was concentrated at the poles and/or septa (Fig. 1). As discussed further below, both the proportion of cells with fluorescence concentrated at the poles and the correlation of this behavior with CL content were protein specific. For each protein, the frequency of polar localization was independent of the protein expression level after induction with up to 1 mM arabinose (see Table S1 in the supplemental material). The ranges of protein expression obtained here with the pBAD system may be higher or lower than those obtained when each native protein is expressed from the chromosome via its native promoter.

To assess the integrity of the tagged and FlAsH-labeled proteins, we imaged SDS-PAGE gels displaying the arrays of proteins present in each preparation after FlAsH labeling (Fig. 3B). Induction (1 mM arabinose) was used to obtain protein levels detectable with this technique. The background labeling was weak (Fig. 3B, control lane). Each protein migrated with concentration. The data are expressed means ± standard deviations for the three determinations. The frequency data are plotted versus the osmolality of the bacterial growth medium (left panels) and the proportions of CL among the phospholipids of bacteria cultivated under the conditions described above (right panels). The CL contents were determined previously and reported by Tsatskis et al. (68) and Romantsov et al. (51). ProP₀ indicates plasmid-encoded ProP, and ProPₑ indicates chromosome-encoded ProP. All other proteins were plasmid encoded (P).
an electrophoretic mobility close to that reported previously (35, 42, 49, 50, 65, 66). More slowly migrating species may represent dimers of the target proteins, and more rapidly migrating species, perhaps representing degraded protein, were observed at extremely low levels.

Data reported below indicate the subcellular localization of proteins in which FlAsH-EDT2 labeled the introduced 6-aminooacid tag CCPGCC, not the localization of the native protein. This shortcoming is shared with many other studies of protein localization.

Transporters LacY and ProW. We previously reported that FlAsH fluorescence was concentrated at the poles in approximately one-half the cells expressing MCCPGCC-LacY from a pBAD24 plasmid (34). Three other expression plasmids, pTr3, pTr4, and pTr5, showed that this was also true for the FlAsH-tagged variants of ProW (Table 2).

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>ProW variant</th>
<th>Glycine betaine uptake rate (nmol min⁻¹ mg protein⁻¹)</th>
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<td>proU⁺ (proVWX⁺)</td>
<td>ProW</td>
<td>28.2 ± 0.5</td>
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<tr>
<td>ΔproU</td>
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<td>0.08 ± 0.08</td>
</tr>
<tr>
<td>ΔproW pBAD24</td>
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<tr>
<td>ΔproW pTr3</td>
<td>ProW</td>
<td>10.8 ± 0.15</td>
</tr>
<tr>
<td>ΔproW pTr4</td>
<td>MCCPGCC-ProW</td>
<td>9.1 ± 0.8</td>
</tr>
</tbody>
</table>

* The host bacteria either were proU⁺ (WG170) or harbored an in-frame proW deletion (WG350 ΔproWS95:FRT).

b Bacteria were cultivated and transport assays were performed as described in Materials and Methods. Cells were grown under osmotic stress conditions (0.3 M NaCl) to induce proU transcription.

c Cells were induced with 1 mM arabinose for one generation (~2.5 h).

Betaine uptake activities were conferred by plasmids encoding ProW (pTr3) and MCCPGCC-ProW (pTr4). This similarity suggested that the FlAsH tag had not disrupted ProW function. However, even after arabinose induction, each activity was three times lower than the activity of ProW encoded by the intact, chromosomal proU operon (proVWX) (Table 2). The difference may have arisen from assembly of plasmid-encoded ProW with chromosome-encoded ProV and ProX.

Mechanosensitive channels. MscL and MscS (also known as YggB) are mechanosensitive channels with large and small conductance, respectively, that open to release cytoplasmic solutes after an osmotic downshock. Each of these proteins is an oligomer consisting of identical subunits (five subunits for MscL and seven subunits for MscS). We introduced the FlAsH tag at the C terminus of each channel protein because the pressure sensitivities of the proteins were not affected when other C-terminal tags were attached and overall the channels behaved like the wild-type channels (4, 34, 64). Staining with FlAsH-EDT2 and fluorescence microscopy revealed that polar localization of MscS was evident in a larger proportion of cells than polar localization of MscL (Fig. 2C) and that polar localization of MscS varied with the cellular CL content, whereas the polar localization of MscL did not vary with the cellular CL content (Fig. 2D).

The osmotic lysis method of Levina et al. (27) was used to determine whether introduction of the tag affected channel function in vivo. Strain MJF465 is a mutant that lacks MscS, MscL, and MscK. Plasmids encoding wild-type and FlAsH-tagged MscS or MscL were introduced into this strain to test the abilities of these channels to protect cells during sudden downshock. Cell lysis, indicated by release of material with absorbance at 260 nm, occurred after downshock of strain MJF465 with or without the pBAD24 vector (Fig. 4A) but not after downshock of this strain expressing the wild-type or FlAsH-tagged MscS or MscL protein (Fig. 4B). Thus, the MscS channel protein behaved like the transporter ProP, whereas the MscL channel protein behaved like the transport protein ProW in terms of subcellular localization.

Patch-clamp analysis was also used to check channel activity. Giant spheroplasts were made by using standard methods (Materials and Methods) and were analyzed by cell-attached recording. The slope conductances were not significantly affected by tagging the channels (Table 3) and were close to those recorded previously (34, 64). The channel opening threshold was not affected by the tag; however, subtle differences were observed when negative voltage was applied to the tagged and wild-type MscL-containing spheroplasts. The tagged MscL protein showed an unusual propensity to open at subconducting levels (Fig. 5). This prevented us from obtaining a slope conductance for this tagged protein, especially at applied voltages less than ~40 mV. In contrast, no significant
differences were seen at negative voltages in the channel gating behavior of tagged and wild-type MscS protein. Indeed, the FlAsH-MscS protein exhibited normal rectification (Fig. 6A) between the positive and negative voltages (Table 3), as well as desensitization or inactivation (Fig. 6B), which is typical of the behavior of wild-type MscS in giant spheroplasts.

Aquaporin AqpZ. We introduced the FlAsH tag at the N terminus of AqpZ (a homotetramer) because an AqpZ derivative with a 23-residue N-terminal extension containing 10 consecutive His residues was functional in proteoliposome studies (5). Staining with FlAsH-EDT2 and fluorescence microscopy revealed polar localization at a low frequency (Fig. 2E) that did not vary with the cellular CL content (Fig. 2F). The frequencies with which AqpZ was concentrated at the cell poles were 12% in cls bacteria and 10% in cls bacteria. Thus, the aquaporin AqpZ behaved like the transport protein ProW and the channel protein MscL in terms of subcellular localization.

Multiple methods were used to assess the function of AqpZ and its FlAsH-tagged derivative. Calamita et al. reported differences in colony phenotype and growth in batch culture between E. coli strains MM294-StreptR (aqpZ::cam) and MM1211 (aqpZ::cam) (7), but Soupene et al. found no such differences between a wild-type E. coli strain (MG1655) and strain NCM3306 (MG1655 aqpZ::cam) (60). Examining all four strains, we observed no difference in growth characteristic of the aqpZ defect (data not shown). Spectroscopy (33) and electron microscopy (14) have revealed differences between wild-type and AqpZ-deficient bacteria. Mallo and Ashby used stopped-flow light-scattering spectroscopy to show that the response of E. coli MG1655 to an

![FIG. 4. Analysis of MscS and MscL function using osmotic lysis of E. coli. Bacteria were grown in the presence of 0.5 M NaCl, filtered, and resuspended in the presence (filled symbols) or absence (open symbols) of 0.5 M NaCl as described by Levina et al. (27). At intervals, samples were centrifuged, and the OD260 of the supernatant was recorded. (A) Circles, MJF465 (mscL::Cm ΔyggB ΔkefA::kan); triangles, MJF465/pBAD24. (B) Circles, MJF465/pTR10 (MscS'); triangles, MJF465/pTR11 (MscS-CCPGCC); squares, MJF465/pTR12 (MscL'); diamonds, MJF465/pTR13 (MscL-CCPGCC).](http://jb.asm.org/)

![FIG. 5. Patch-clamp recordings of FlAsH-labeled MscL. The recordings are of inside-out excised patches from giant spheroplasts of E. coli strain MJF465 (mscL mscS mscK) expressing FlAsH-labeled MscL. (A) Recording at 30 mV; (B) recording at −30 mV. Flickery channel behavior is seen at positive voltages (expansion), and subconducting states are apparent at negative voltages. C and O1 to O9 indicate closed and open channel levels, respectively.](http://jb.asm.org/)
osmotic upshift imposed with proline was much faster than that of E. coli NCM3306, particularly for bacteria from stationary-phase cultures (33).

Stopped-flow spectroscopy was used to compare the functions of wild-type and FlAsH-tagged AqpZ. Strains MG1655 and NCM3306 and their plasmid-bearing derivatives were used for these experiments as phase-contrast microscopy showed that strains MM294-StreptR and MM1211 are filamentous (data not shown). The absorbance increased when osmotic upshocks were imposed with glycerol but not when upshocks were imposed with NaCl or proline (data not shown). The changes in scattering were fitted to a single exponential function to obtain the rate constants ($k_w$) shown in Table 4. The $k_w$ for $aqpZ^+$ bacteria (MG1655) was much larger than that for $aqpZ$ bacteria (NCM3306) from stationary-phase cultures, and the differences in $k_w$ between $aqpZ^+$ and $aqpZ$ bacteria from exponential-phase cultures were much smaller. Our $k_w$ values are lower than those reported by Mallo and Ashby, but we observed qualitatively similar effects of culture growth phase and the $aqpZ$ defect. The osmotic shock response of AqpZ-deficient bacteria became slower in stationary phase as the response of the wild-type bacteria accelerated.

The abilities of AqpZ and its FlAsH-tagged derivative to complement the $aqpZ$ deficiency during expression from plasmid pBAD24 were compared. As before, $k_w$ did not vary widely for bacteria from exponential-phase cultures (Table 4). In contrast, plasmid-based expression of either AqpZ or MV CCPGCC-AqpZ clearly complemented the AqpZ deficiency in cells from stationary-phase cultures (Table 4 and Fig. 7). This suggests that the particularly large effects of AqpZ on the osmotic stress response in stationary-phase bacteria result from factors other than stationary-phase (RpoS-dependent) induction of $aqpZ$ expression. Comparable responses were observed for bacteria expressing AqpZ without arabinose induction and for bacteria expressing MV CCPGCC-AqpZ after induction with 1 mM arabinose. Insertion of the oligonucleotide encoding the N-terminal FlAsH tag may have impaired expression of the tagged variant (as seen for ProP and LacY) and/or the FlAsH tag may have impaired channel function.

**DISCUSSION**

We have examined the localization of the proteins ProP, LacY, ProW, MscS, MscL, and AqpZ in E. coli by using reagent FlAsH-EDT$_2$ to specifically label an introduced

TABLE 4. Osmotic upshock responses of bacteria expressing AqpZ and its FlAsH-tagged variant

<table>
<thead>
<tr>
<th>Strain</th>
<th>AqpZ variant</th>
<th>$k_w$ (s$^{-1}$)$^a$</th>
<th>Upshock imposed with NaCl on culture from:</th>
<th>Upshock imposed with proline on culture from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Exponential phase</td>
<td>Stationary phase</td>
<td>Exponential phase</td>
</tr>
<tr>
<td>MG1655</td>
<td>AqpZ</td>
<td>6.82 ± 0.05</td>
<td>19.24 ± 0.37</td>
<td>5.56 ± 0.04</td>
</tr>
<tr>
<td>NCM3306</td>
<td>None</td>
<td>6.02 ± 0.04</td>
<td>0.480 ± 0.004</td>
<td>5.19 ± 0.03</td>
</tr>
<tr>
<td>NCM3306/pBAD24</td>
<td>None</td>
<td>6.00 ± 0.07</td>
<td>0.450 ± 0.004</td>
<td>5.09 ± 0.07</td>
</tr>
<tr>
<td>NCM3306/pTR14</td>
<td>AqpZ</td>
<td>7.31 ± 0.10</td>
<td>27.64 ± 0.40</td>
<td>7.18 ± 0.06</td>
</tr>
<tr>
<td>NCM3306/pTR15</td>
<td>MV CCPGCC-AqpZ</td>
<td>6.94 ± 0.09</td>
<td>0.984 ± 0.005</td>
<td>5.37 ± 0.06</td>
</tr>
<tr>
<td>NCM3306/pTR14$^b$</td>
<td>AqpZ</td>
<td>7.08 ± 0.06</td>
<td>29.19 ± 0.36</td>
<td>7.76 ± 0.04</td>
</tr>
<tr>
<td>NCM3306/pTR15$^b$</td>
<td>MV CCPGCC-AqpZ</td>
<td>7.07 ± 0.06</td>
<td>29.38 ± 0.15</td>
<td>7.95 ± 0.03</td>
</tr>
</tbody>
</table>

$^a$ Rate constants ($k_w$) for the optical density change following osmotic upshocks (0.5 mol/kg) imposed on bacteria at 5°C were determined by stopped-flow light-scattering spectroscopy as described in Materials and Methods.

$^b$ Expression of $aqpZ$ was induced with 1 mM arabinose for the last 2 h of growth.
hexapeptide tag (51, 52; this study). This study addressed a number of technical issues important for lipid and protein localization studies and provided evidence that ProP and MscS are unusual in showing CL-dependent localization at the cell poles.

Care is required to maximize the probability that protein localization determined with an introduced tag reflects localization of the native protein expressed at levels in the physiological range. We employed FlAsH labeling in this study because we anticipated that the small “CCPGCC” tag would perturb protein structure, function, and localization less than an added domain comprised of an entire fluorescent protein, like GFP. Our CCPGCC-tagged proteins were functional (Tables 2 to 4; Fig. 4 to 7). As observed in previous work employing GFP, we found that LacY was concentrated at the cell poles in a large proportion of bacteria, whereas MscL was not (Fig. 1 and 2; see Table S1 in the supplemental material). This lends credence to both visualization techniques, but it is still possible that the introduced tag and FlAsH labeling influenced the protein localization observed during this study.

Artifacts due to anomalous protein expression levels are a particular concern for protein localization studies. Overexpressed proteins can accumulate at the cell poles as inclusion bodies (28). The appearance of the regions of membrane protein fluorescence concentrated at the cell poles that we report here is clearly different from the appearance of inclusion bodies that can be visualized by phase-contrast microscopy. However, it is difficult to rule out artifacts due to the polar accumulation of protein aggregates visible by fluorescence microscopy but not by phase-contrast microscopy. Conversely, an apparently uniform distribution of a tagged protein on the cell surface could mask more specific localization of a native protein that is normally present at lower levels.

Our studies included two approaches to deal with this problem. Since anti-ProP antibodies are available, we compared the localization of tagged ProP when it was expressed from the native proP promoters in the chromosome to its localization when it was expressed at lower, comparable, or higher levels from the arabinose-inducible P_BAD promoter of a pBAD24-derived plasmid (Fig. 2A and B; see Table S1 in the supplemental material). (Note that the highest levels of ProP expression employed during this study were only approximately 2-fold higher than the levels of the native protein present after osmotic induction.) Polar concentration of ProP was seen at comparable frequencies, and the frequencies were similarly correlated with bacterial CL content at each expression level. We concluded that neither concentration of ProP at the cell poles nor its correlation with cellular CL content is an artifact of the ProP expression level.

Antibodies were not available for the other proteins included in our study. We therefore determined the conditions under which each tagged protein, expressed from a pBAD24-derived plasmid, restored function to a physiological level. We then determined protein localization at the minimum level attainable with plasmid pBAD24 (i.e., without arabinose induction) (Fig. 1 and 2) and, if it was higher, the expression level required to restore physiological function (see Table S1 in the supplemental material). For each protein we found the same frequency of polar localization and the same correlation (or lack of correlation) with CL levels at various protein expression levels. However, we cannot rule out the possibility that the native expression levels of these proteins are outside the range employed in our study.

Taking the approach described above, we observed polar localization of FlAsH-labeled ProW, MscL, and AqpZ at low frequencies (less than 20%) in cls or cls” bacteria cultivated at

FIG. 7. Analysis of AqpZ function by stopped-flow spectroscopy. E. coli strains NCM3306/pBAD24 (AqpZ”), NCM3306/pTR14 (AqpZ”), and NCM3306/pTR15 (MVCCPGCC-AqpZ) were grown in LB medium to an OD_600 of 0.5 (stationary phase) and then upshocked with 0.5 M NaCl (A) or 0.5 M proline (B) in PBS as described in Materials and Methods. Strain NCM3306/pTR15 was induced with 1 mM arabinose. The time course of the change in absorbance after osmotic upshock was measured at 600 nm. The curves represent averages of five replicates, and data from 0.1 to 5 s were fitted to a single exponential equation to obtain k_w (see Materials and Methods and Table 4). a.u., absorbance units.
a variety of osmolalities (Fig. 2; see Table S1 in the supplemental material). Cellular processes that are not protein specific may cause these proteins to be concentrated at the poles in a small proportion of cells. In contrast, LacY, ProP, and MscS were concentrated more frequently at the cell poles, and for ProP and MscS this behavior correlated with cellular CL content (Fig. 2 and 3; see Table S1).

At least two questions arise from observations of lipid and protein localization in bacterial cells. How are proteins targeted to particular subcellular locations, and what is the functional significance of lipid and protein localization? The mechanisms targeting proteins to cell poles remain unclear, but our data suggest a role for CL in the polar localization of ProP and MscS (51, 52) (Fig. 1 to 3; see Table S1 in the supplemental material). We previously reported that concentration of ProP at the cell poles is CL dependent and suggested that CL controls the polar localization of ProP (51, 52). Other workers proposed that, when it reaches a critical mole fraction, CL may concentrate spontaneously at the highly curved cell poles (22, 41). Here we compared the proportions of cells with CL, ProP, or MscS concentrated at the poles of bacteria with various CL contents. Concentration of CL at the poles was detected 24-fold more frequently in \(\text{cls}^+\) bacteria than in \(\text{cls}^-\) bacteria, but the frequencies did not vary significantly as the CL content was further modulated by varying the growth osmolality (Fig. 2G and H). We do not know whether the frequency of polar localization would be modulated in the critical but experimentally inaccessible range of CL contents between that of \(\text{cls}^-\) bacteria cultivated at high osmolality (0.8 mol%) and that of \(\text{cls}^+\) bacteria cultivated at low osmolality (4 mol%).

In contrast to the results for CL, the frequencies with which ProP and MscS were concentrated at the poles varied with both bacterial genotype (\(\text{cls}^+\) or \(\text{cls}^-\)) and growth osmolality (Fig. 2A to D). Concentration of ProP at the poles was detected 16-fold more frequently in \(\text{cls}^+\) bacteria cultivated at high osmolality than in \(\text{cls}^-\) bacteria cultivated at low osmolality, whereas the corresponding ratio for MscS was only 2-fold. The different correlations of CL localization, ProP localization, and MscS localization with CL content may indicate that they do not reflect direct interaction of ProP or MscS with CL or even the same underlying phenomenon. Other changes are known to accompany the \(\text{cls}\) mutation (e.g., changes in PG content) and growth in media with variable osmolality (e.g., widespread induction or repression of gene expression [70]). ProP and/or MscS may interact with unknown proteins targeted to the cell poles in a CL-dependent manner rather than with CL itself. The molecular basis for colocalization of membrane proteins with CL at the cell poles will be understood better when the structures of the polar membrane domains are better characterized.

The functional importance of protein localization is not always evident, and phospholipid-dependent protein function is not always simply correlated with protein-lipid colocalization. Polar localization of some proteins has been correlated with functional changes. For example, the localization of ProP in the CL-rich environment at the cell poles tunes its sensitivity to osmotic pressure (51), the chemoreceptor arrays located at cell poles function in chemotactic signal integration (23, 61), and concentration at cell poles increased when the sensor kinases DcuS and CitA were provided with their effectors (fumarate and citrate, respectively) (55). The osmolality at which ProP attains one-half its maximal activity correlates with the anionic phospholipid content (CL plus PG) in both cells and proteoliposomes, although ProP function is more sensitive to CL than to PG (51). However, the proportion of PG increased with growth medium osmolality in \(\text{cls}^-\) bacteria, and ProP function was influenced by the varying PG content even though ProP was not concentrated at the poles of these cells. Thus, ProP function depends on its phospholipid environment and not on its polar localization per se.

This study provides the first evidence that MscS concentrates at the poles of \(E.\ coli\) cells in a CL-dependent manner. Such localization would be expected to influence channel function because the pressure sensitivity of MscS is known to depend on membrane curvature in vitro (18). Aggregation of MscS at the cell poles could influence both its channel function and the impact of its structural transitions on membrane strain (20, 40).

Future work will determine whether other proteins included in this study function in a lipid-dependent manner, but this study shows that their function is not simply correlated with their subcellular localization. For example CL, PG, and phosphatidylserine can bind to a specific site on MscL (47, 48), and CL, PG, and phosphatidic acid influenced the rate and extent of calcein flux through \(E.\ coli\) MscL in vitro (46). The effects of phospholipid headgroup composition on purified and reconstituted MscL were also examined by patch clamping in vitro (39). The data suggested that the lipid headgroup composition altered MscL activity by changing the biophysical properties of the membrane rather than by interacting specifically with MscL. Thus, further investigation is required to define the impact of lipids on MscL function in vivo, but MscL does not appear to colocalize with CL at the cell poles. Stopped-flow spectroscopy suggests that \(\text{aqpZ}\) \(E.\ coli\) cells from stationary-phase cultures shrink more slowly in response to osmotic upshifts than bacteria from log-phase cultures and that the impact of AqpZ on this response is much greater in bacteria from stationary-phase cultures than in bacteria from log-phase cultures, even when \(\text{aqpZ}\) is expressed from an RpoS-independent promoter (Table 4). Elevated CL levels are characteristic of \(E.\ coli\) cells from stationary-phase cultures (52), so AqpZ function may be CL dependent, even though AqpZ does not colocalize with CL at the cell poles. Overall, lipid-dependent function and lipid-dependent subcellular localization are not always linked.

This study revealed that CL-dependent localization at cell poles is not a general characteristic of transporters, channels, or osmoregulatory proteins in \(E.\ coli\). Polar localization can be frequent and CL independent (as observed for LacY), frequent and CL dependent (as observed for ProP and MscS), or infrequent (as observed for AqpZ, ProW, and MscL).

**ACKNOWLEDGMENTS**

We are grateful to Sydney Kustu and Giuseppe Calamita for provision of \(E.\ coli\) strains, to Todd Gillis for use of the stopped-flow spectrometer, to Gordon Kirby and Allison MacKay for use of the Typhoon 9410 variable-mode imager, to France-Isabelle Auzanneau, Karsten Brandt, Doreen Culham, Michelle Smith, and Danielle Visschedyk for help with the experiments, and to Michael Ashby for discussions of the data.

This work was supported by discovery grants awarded to J.M.W. and France-Isabelle Auzanneau by the Natural Sciences and Engineering
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AUTHOR’S CORRECTION

Protein Localization in *Escherichia coli* Cells: Comparison of the Cytoplasmic Membrane Proteins ProP, LacY, ProW, AqpZ, MscS, and MscL

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In particular, we are grateful to Paul R. Rohde for optimizing the preparation of giant *E. coli* spheroplasts used for the patch-clamp recording from FIAsh-labeled MscL and MscS Channels.