Monoglucosyldiacylglycerol, a Foreign Lipid, Can Substitute for Phosphatidylethanolamine in Essential Membrane-associated Functions in Escherichia coli*

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The mechanisms by which lipid bilayer properties govern or influence membrane protein functions are little understood, but a liquid-crystalline state and the presence of anionic and nonbilayer (NB)-prone lipids seem important. An Escherichia coli mutant lacking the major membrane lipid phosphatidylethanolamine (NB-prone) requires divalent cations for viability and cell integrity and is impaired in several membrane functions that are corrected by introduction of the "foreign" NB-prone neutral glycolipid α-monoglucosyldiacylglycerol (MGlcDAG) synthesized by the MGlcDAG synthase from Acholeplasma laidlawii. Dependence on Mg\(^{2+}\) was reduced, and cellular yields and division malfunction were greatly improved. The increased passive membrane permeability of the mutant was not abolished, but protein-mediated osmotic stress adaptation to salts and sucrose was recovered by the presence of MGlcDAG. MGlcDAG also restored tryptophan prototrophy and active transport function of lactose permease, both critically dependent on phosphatidylethanolamine. Three mechanisms can explain the observed effects: NB-prone MGlcDAG improves the quenched lateral pressure profile across the bilayer; neutral MGlcDAG dilutes the high anionic lipid surface charge; MGlcDAG provides a neutral lipid that can hydrogen bond and/or partially ionize. The reduced dependence on Mg\(^{2+}\) and lack of correction by high monovalent salts strongly support the essential nature of the NB properties of MGlcDAG.

The lipid bilayer of biological membranes acts as a permeability barrier permitting maintenance of essential ion gradients and is also the local environment for integral and peripheral membrane proteins. Important bilayer structural features are a liquid-crystalline state, an optimal length of the lipid chains, and critical fractions of anionic and nonbilayer- (NB)1

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The abbreviations used are: NB, nonbilayer; alMGS, A. laidlawii 1,2-diacylglycerol 3-glucosyltransferase; CL, cardiolipin; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; GFP, green fluorescent protein; LacY, lactose permease; MGlcDAG, α-monoglucosyldiacylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PIPES, 1,4-piperazineethanesulfonic acid; TMG, methyl-β-D-thio-d-galactopyranoside.
cations at millimolar concentrations. Only those divalent cations that supported growth of the mutant also restored the temperature-dependent bilayer/NG phase transition profile of the extracted lipids to that of lipids from wild type cells in the absence of divalent cations (18). Restoration of the normal phase behavior was caused specifically by the interaction of cations with CL, which is a major lipid in the mutant (19). Cells lacking both PE and CL are not viable (17). However, this compensatory mechanism is less likely to work for processes dependent on the chemical rather than the physical properties of PE. Cell division is strongly defective in the mutant lacking PE, resulting in very long cell filaments even with divalent cations present, probably partly because of misassembly or mislocalization of the FtsZ-dependent division machinery (20) and components of the Min system (11). Furthermore, an alteration in the mutant cell envelope structure or physical properties also results in a direct activation of the Cpx stress response pathway (21). Another striking feature of the mutant is a misfolding of the integral membrane transport proteins lactose permease (LacY) and phenylalanine permease; the first six or first two transmembrane helices are inserted in an inverted orientation in the membrane in the former (22) or latter protein (23), respectively. These and other studies of the mutant indicate a strong dependence on PE for a number of membrane-associated functions.

In addition to PE, monoglycosyldiacylglycerols are major NB-prone membrane lipids present in most photosynthetic membranes and many Gram-positive bacteria. To determine whether the affected functions in the E. coli lipid mutant are dependent on the chemical structure of PE or on its NB character we have expressed the gene for the α-monoglycosyldiacylglycerol (MGlcDAG) synthase (a glucosyltransferase) from the mycoplasma Acholeplasma laidlawii (24) in E. coli cells lacking PE. MGlcDAG is an uncharged and major NB-prone lipid that is crucial for bilayer packing properties in the A. laidlawii (25) membrane, varying in amounts from 5 to 50 mol % depending on growth conditions (26). This new E. coli lipid mutant contains up to 55% of the “foreign lipid” MGlcDAG. The MGlcDAG-containing PE-lacking strain grows faster, is less dependent on divalent cations, and exhibits partially restored cell division and active sugar transport by LacY. The MGlcDAG strain also compensates for osmotic stress better, and membrane permeability properties are changed but not restored.

**EXPERIMENTAL PROCEDURES**

**Construction of E. coli Lipid Mutants—**Plasmid pET-MGlcDAG (24) carries a DNA fragment encoding the A. laidlawii membrane-associated 1,2-diacylglycerol 3-glucosyltransferase (aMGlS) (EC 2.4.1.157). The N-terminal methionine of aMGlS was fused to a fragment encoding a new initiation codon followed by information encoding 6 histidines, a threonin pro tease site, a factor Xa pro tease site, and 3 additional amino acids derived from the vector. This chimeric gene was excised using Ncol and BamHI (5' to 3') and ligated into plasmid pTEF1/Zeo (Invitrogen) using the same restriction sites. The aMGlS gene completely replaced the vector zeocin resistance gene and was positioned following sequence precedes the N-terminal Met of aMGlS: MGSSHH-HHHHSSGLVPRGSHMEEELRGR. This plasmid, pTFED1/Neo (Invitrogen) using the same restriction sites. The aMGlS gene completely replaced the vector zeocin resistance gene and was positioned downstream of the artificial prokaryotic constitutive EM7 promoter. The following sequence precedes the N-terminal Met of aMGlS: MGSSHH-HHHHSSGLVPRGSHMEEELRGR. This plasmid, pTFED1/Neo, also carries the β-lactamase gene. Strain AD93/pD72 (pSOS8-ban*psps*can* rPS101 temperature-sensitive replicon) (17) was transformed with pTMG3 selecting for ApR to generate AD93/pDD72/pTMG3 (MGlcDAG-containing PE-lacking). Strain AD93/pTMG3 (MGlcDAG-containing PE-lacking) was generated by curing pD72 by growth at 42 °C in Luria-Bertani (LB) medium, supplemented with 50 μg/mL MglS, for 4 h prior to plating to LB agar plates (with 50 μg/mL MglS), and incubating for 2 days at 42 °C (17). Colonies unable to synthesize PE were identified by screening for the lack of growth in the absence of 50 μg/mL MglS followed by lipid analysis.

**Growth Conditions—**Unless otherwise stated all E. coli strains were grown under the same conditions before as during experiments, and all cells compared in a given experiment were grown under the same conditions. Unless otherwise stated bacteria were grown in LB broth containing 5 μg/mL NaCl with 10 μM MgCl2 at 30 °C. Plasmids expressing aMGlS were maintained by supplementing growth medium with 50 μg/mL ampicillin.

**Osmotic Stress and Solvent Exposure Experiments—**After growing cells overnight in LB containing 5 μg/mL NaCl and 10 μM MgCl2 glass tubes were prepared with different concentrations of salts, sugars, and organic solvents in LB containing 5 μg/mL NaCl with 10 μM MgCl2 in a total volume of 5 ml. The cells were grown for 24 h at 30 °C with 150 strokes/min in a water bath shaker. Absorbance at 600 nm was measured at the start and after growth with the additives. All osmotic response experiments were done at least in duplicate.

**Growth with Sterols—**Stock solutions in ethanol (10 mg/ml) were made for cholesterol, 5-cholen-3-one and 4-cholene-3-one. These were diluted to the indicated concentration in sterile LB containing 4 μg/mL bovine serum albumin as sterol carrier and stirred for 2 h. Cells were grown with 0, 30, and 100 μM sterols under the same conditions as in the osmotic stress and solvent experiments.

**Lipid Analysis—**To incorporate radiolabel metabolically into the fatty acyl chains of the lipids, overnight cell cultures were inoculated into LB medium with different additives (e.g. salts or solvents) containing 0.2 μCi/ml [14C]acetic acid. After 24 h the cells were harvested by centrifugation, and lipids were extracted from cell pellets by extensive stirring in chloroform/methanol (2:1, v/v), followed by centrifugation and a second extraction in methanol. The lipids were concentrated by evaporation, separated on Silica Gel 60 TLC plates (Merck) in chloroform/methanol/acetic acid (65:25:10, v/v/v), and visualized and quantified by electronic autoradiography (Packard Instant Imager).

**Separation of Outer and Inner Membranes—Spheroplasts and membranes were prepared from cells harvested in mid-exponential phase. These were suspended in 10 mM potassium PIPES, pH 7.0, 0.75 μm sucrose, 10 μM MgSO4, 2.5% (w/v) LiCl, and 50 μg/ml chloramphenicol. After the addition of 2 mg/ml lysozyme, cells were gently shaken at 37 °C for 55 min for AD93 or 90 min for AD93/pTMG3 and AD93/pD72. The formation of spheroplasts was followed by light microscopy. Intact spheroplasts were collected by centrifugation at 3,000 × g for 10 min at room temperature and resuspended at 10 mg of total protein/ml in the above buffer without LiCl (modified from Ref. 28). Spheroplasts were broken by ultrasonication for 3 × 15 s on ice. Removal of whole spheroplasts was accomplished by centrifugation at 3,000 rpm for 5 min, and the total membrane fraction was collected by centrifugation at 35,000 rpm for 60 min. Membranes were resuspended in 1 ml of 25% (w/v) sucrose and layered on top of a sucrose gradient made from 0.5 ml of 55% (bottom) and 2.1 ml each of 50, 45, 40, 35, and 30% sucrose in 0.1 M Tris-HCl, pH 8.0, and 0.1 M EDTA, and centrifuged at Beckman SW-50.1 for 45 min at 35,000 rpm. Membrane fractions were captured with a CCD camera (Sony), and Image Access 3.0 software (Imagis Bildverarbeitung AG). Editing of images was made in Adobe Photoshop 6.0.

**Localization of aMGlS-GFP—**Full size (398 amino acids) aMGlS was cloned upstream and in-frame with the gene encoding green fluorescent protein (GFP) using a PCR primer approach based on the sequence of the aMGlS DNA (24) and the vector pGFPuv from (Clontech) (30). Potential potential fusion signal for spheroplasts membranes by aMGlS in AD93/pTMG3 was compared with aMGlS expressed from a T7 promoter in vector pET15b carried in E. coli BL21(DE3) (cf. (31)). On the microscope the cells were mixed 1:1 (v/v) with 0.7% low melting agarose. Cells were viewed with a Zeiss Axioplan 2 (cholamine filter) fluorescence microscope. Images were made from a CCD camera (Sony), and Image Access 3.0 software (Imagis Bildverarbeitung AG). Editing of images was made in Adobe Photoshop 6.0.

**Light Microscopy—**E. coli cell layer distribution was viewed with an Olympus BX60 microscope. Images were captured with an Optronix DEI-750 video camera and edited by Adobe Photoshop 4.0. Membranes were stained by adding 1 μM FM4-64 (Molecular Probes) to overnight cultures and stirred for 3 h at 30 °C before washing three times with phosphate-buffered saline buffer. Potential potential fusion signal for spheroplasts membranes by aMGlS in AD93/pTMG3 was compared with aMGlS expressed from a T7 promoter in vector pET15b carried in E. coli BL21(DE3) (cf. (31)). On the microscope the cells were mixed 1:1 (v/v) with 0.7% low melting agarose. Cells were viewed with a Zeiss Axioplan 2 (cholamine filter) fluorescence microscope. Images were made from a CCD camera (Sony), and Image Access 3.0 software (Imagis Bildverarbeitung AG). Editing of images was made in Adobe Photoshop 6.0.

The construction was verified by DNA sequencing. Enzymatic activity of the aMGlS-GFP was analyzed after induction with isopropyl-β-D-thiogalacto-
topyranoside by labeling of E. coli lipids in vivo with radioactive acetate (24) and by assay for MGlcDAG synthase activity after detergent solubilization (24). The plasmid encoding aMGS-GFP was transformed into AD93/pDD72 by standard techniques. Localization of aMGS-GFP in E. coli strains was analyzed by fluorescence microscopy using a Zeiss Axiosoplan 2 microscope with fluorescein isothiocyanate filters. Deconvoluted images of cells were obtained with a Delta Vision wild field optical sectioning microscope (Applied Precision, Issaquah, WA) equipped with a ×100 oil-immersion objective and visualized with a cooled charge-coupled device camera and fluorescein isothiocyanate filter set; z axis optical sections were taken at 0.1-μm intervals. Deconvolution of raw data was performed with five rounds of integration.

Membrane Permeability—Leakiness and permeability of cell membranes were assayed by antibiotic sensitivity and by RNase release from the periplasmic space. For antibiotic sensitivity experiments PDM antibiotic sensitivity discs (AB Biodisc, Sweden) were used. All discs contained 30 μg of antibiotic except for rifampicin, which was at 50 μg. Cells were spread evenly on LB plates before the discs were positioned. Plates were incubated for 24 h before measurements of the size of growth inhibition around the discs. Experiments were made in triplicate. Leakage of the outer membrane for larger molecules was estimated by release of periplasmic RNase. Colonies of AD93/pDD72, AD93, and AD93/pTMG3 were replica plated onto LB agar plates with 50 μM MgCl₂. The plates were overlaid with soft agar containing 1.5% carboxylic cyanine. Leakiness of the outer membrane for larger molecules was estimated on the plasmid-borne lipid-synthesizing genes present (Fig. 4).

RESULTS

Levels of a Foreign Lipid in E. coli—Strain AD93 lacks the major phospholipid PE because of inactivation of the gene (psaA) encoding phosphatidylether synthase (17) and requires divalent cations such as Mg²⁺ in the growth medium for optimal growth and viability. The lack of PE is compensated by an increase in PG and CL to maintain a normal protein:phospholipid ratio. E. coli contains only trace amounts of neutral uncharged lipids such as diacylglycerol (34), which is derived from PG and PE during synthesis of membrane-derived oligosaccharides localized to the periplasmic space (35). This rapidly metabolized pool of diacylglycerol (plus UDP-glucose) is sufficient to make significant amounts of MGlcDAG after introduction of plasmids encoding aMGS (24). The presence of plasmid-borne copies of the psaA gene in AD93 results in wild type PE levels, phospholipid composition, and phenotype (17). Membrane lipids were labeled with [¹⁴C]acetate during growth, and analyses revealed substantial differences dependent on the plasmid-borne lipid-synthesizing genes present (Fig. 1, A and B). AD93 (denoted as PE⁻) and AD93/pDD72 (denoted as PE⁺) showed the expected mutant and wild type lipid compositions, respectively (17); all cells were analyzed after growth into stationary phase (36). AD93/pTMG3 (denoted as PE⁻/MGlcDAG⁺) contained up to 55 mol % MGlcDAG depending on growth conditions (see below). AD93/pDD72/pTMG3 (PE⁺/MGlcDAG⁺) contained only a maximum of 10 mol % MGlcDAG and a corresponding reduction of the PE amount (data not shown), analogous to previous data (24). Separation and isolation of inner and outer membranes of AD93, AD93/pDD72, and AD93/pTMG3 showed fractions of the lipids in both membranes corresponding to those in Fig. 1B, indicating that MGlcDAG could be transported to the outer membrane (data not shown). MGlcDAG was also substantially more resistant than the phospholipids to endogenous lipolytic degradation during prolonged incubation of the membrane fractions.

MGlcDAG-containing Strain Is Less Dependent on Mg²⁺—The presence of divalent cations such as Mg²⁺ is essential for the growth and viability of AD93 (17) and the wild type phase transition (bilayer/nonbilayer) properties of lipids extracted from AD93 (18). Growth of the strains with different concentrations of MgCl₂ showed that the PE·MGlcDAG⁻ strain is less dependent on MgCl₂ compared with the PE⁻ strain (Fig. 2). Both strains grew faster with increasing Mg²⁺, but in general PE·MGlcDAG⁻ cells grew faster and reached higher culture densities at stationary phase than PE⁻ cells. Neither strain grew as well as PE⁺ cells, which have about a 20-min doubling time and reach an A₅₆₀ of 2–3 in the stationary phase (not shown). When grown with 5 mM MgCl₂ (Fig. 2 inset, note linear scale for A₅₆₀), PE⁻ cells lysed as they approached late log or stationary phase, whereas PE·MGlcDAG⁻ cells were stable. MGlcDAG amounts were at 50–55 mol % at 10 mM MgCl₂ and decreased to 20–30 mol % at 50 mM MgCl₂ with little variability at different stages of growth (data not shown). The purified MGlcDAG synthase is stimulated by Mg²⁺, but at the same time increasing ion concentrations may screen the anionic lipid charge also essential for enzyme activity (37). For the following experiments 10 mM MgCl₂ supplementation was chosen, yield-
ing maximum amounts of MGlcDAG.

Cell Division and Cell Morphology—Despite the presence of Mg\(^{2+}\) and the associated compensation of lipid packing properties (18), strain AD93 cannot divide properly. Long, multinucleoid filaments are formed, probably because of misassembly of cell division proteins in the absence of PE resulting in failure of cells to constrict (20). However, the presence of MGlcDAG partially suppressed this morphological phenotype of PE-lacking cells. Comparison of the cell length showed progressively longer and more filamentous cells in the following order: PE\(^{-} < \) PE/MGlcDAG\(^{-} < \) PE containing E. coli (Fig. 3, A, B, and E) with average cell lengths of 1, 3, and 7 \(\mu\)m, respectively.

Overexpression of some membrane proteins, including ones that synthesize lipids (38, 39) and cell wall precursors (40) or depletion of some membrane assembly proteins (41), may result in the formation of new membrane structures in the cytoplasm. Given the NB character of MGlcDAG, it was also important to establish whether the large amount of this lipid was properly integrated into the membranes. The lipophilic stain FM4-64 has visualized membrane domains in Bacillus subtilis (43). Staining of the lipid variants with FM4-64 showed normal membranes (Fig. 3, C and D) with more brightly stained areas at the poles of PE/ MGlcDAG\(^{-} \) cells and along the length of PE\(^{-} \) cells that appear very similar to 10-N-nonyl acridine orange-staining of lipid- or CL-enriched domains in wild type and PE\(^{-} \) cells, respectively (44). A few constriction rings were visible in both the PE\(^{-} \) and PE/MGlcDAG\(^{-} \) cells but more frequently in the latter. However, extensive formation of intracellular membranes in a banded pattern was observed (data not shown) when allMGS was strongly overexpressed from the T7 promoter (as in (30)), analogous to findings for the overproduced structurally similar E. coli glycosyltransferase MurG (40). Because extensive overproduction of allMGS does not increase MGlcDAG levels further, the distortions in membrane structure are most likely because of protein overproduction from the T7 promoter. The similarity in the staining pattern with that observed with 10-N-nonyl acridine orange for wild type cells suggests little or no intracellular compartments in MGlcDAG-containing cells.

Changes in Osmotic Stress Tolerance—E. coli has several systems to deal with osmostress adaptation (45). Osmotic stress tolerance was studied for the three E. coli lipid variants by exposing the cells to large variations in the concentration of NaCl, KCl (both 0–0.75 M), and sucrose (0–1.5 M) during growth in LB medium containing 10 mM Mg\(^{2+}\). For both K\(^{+}\) and Na\(^{+}\) an almost linear relationship between the added external and resulting internal ion concentration have been recorded in E. coli, reaching 85 and 45% intracellularly, respectively, of the extracellular values (46–48). Growth was compared with standard conditions without salt stress, where PE\(^{-} \) cells grew better than the two lipid variants (cf. Fig. 2), as described under “Experimental Procedures.” AD93 (PE\(^{+}\)) was significantly more sensitive to high concentrations of NaCl and KCl than the PE\(^{-} \) and PE/MGlcDAG\(^{-} \) strains (Fig. 4). Similar sensitivity of PE\(^{-} \) cells and insensitivity of PE\(^{+} \) and PE/ MGlcDAG\(^{-} \) cells were observed with high sucrose (uncharged) concentrations (data not shown). Betaine (1–5 mM), which is normally concentrated by specific transporters in E. coli to counteract high external osmotic pressures, did not improve the tolerance of AD93. For all three osmotic inducers the PE/ MGlcDAG\(^{-} \) strain was least sensitive to osmotic changes. However, for the two strains lacking PE moderate growth medium additions of the major intracellular ion K\(^{+}\) stimulated growth (Fig. 4), indicating that they may have a malfunctioning permeability barrier. Na\(^{+}\) is normally much lower inside E. coli and substantially less affected by external additions, cf. above and Refs. 46 and 47. Osmotically comparable additions of sucrose were also less inhibitory for AD93 than were salt ions, and low amounts stimulated growth of the PE/MGlcDAG\(^{-} \) strain (data not shown). Sucrose, at the concentrations used here, has a substantial ability to promote nonbilayer tendencies of membrane lipids (49). Overall, osmotic stress responses were regained or improved in the MGlcDAG\(^{-} \) cells compared with the PE\(^{-} \) cells.

Analysis of membrane lipid composition revealed that the amounts of the polar lipid species as a function of osmolarity of the growth medium were fairly similar except for the PE/ MGlcDAG\(^{-} \) strain (shown in Fig. 4). The amount of MGlcDAG was lowered from more than 50 mol % to 30 mol % by increasing concentrations of either K\(^{+}\) or Na\(^{+}\) without affecting stress tolerance. However, increasing sucrose did not decrease MGlcDAG amounts (data not shown); therefore, it is unlikely that the reduced level of MGlcDAG is caused by reduced avail-

Fig. 2. Dependence on Mg\(^{2+}\) for PE-lacking strains. Growth curves for AD93 (PE\(^{-}\), open symbols) and AD93/ pTMG3 (PE/MGlcDAG\(^{-}\), closed symbols) grown with different concentrations of Mg\(^{2+}\) are shown. Overnight cultures were grown in LB medium (10 g/liter NaCl) with 50 mM MgCl\(_2\), washed once with plain LB medium, and then diluted into LB medium containing MgCl\(_2\) as indicated (numbers are mM) to A\(_{600}\) 0.05. After 8 h of growth, cells were diluted into the same medium for the second time to A\(_{600}\) 0.02, and growth was followed as a function of A\(_{600}\).
ability of diacylglycerol as a precursor resulting from increased osmolarity because adding either salt or sucrose to the growth medium results in reduced synthesis of membrane-derived oligosaccharide (50), the source of diacylglycerol. It is more likely that the membrane binding of alMGS is prevented by increasing salt, as revealed in vitro (30), where the reduced fraction bound is not easily released by salt treatment typical for additional hydrophobic interactions (30). This hypothesis was supported by studies of the intracellular localization of enzymatically active alMGS-GFP (Fig. 5). The alMGS-GFP was more localized to the cytosol than attached to the membranes when high salt (A and C versus B and D, respectively) was present in the growth medium. The same effect was seen in both wild type E. coli (A and B) and AD93 (C and D). There was also a tendency of the membrane-bound alMGS-GFP to be more concentrated at the cell poles in both PE⁺ and PE⁻ cells. In AD93 alMGS-GFP was also enriched in what appeared to be domains in the membrane similar to the localization pattern of CL (44) and MinD (11) in AD93. Membrane localization as opposed to inclusion bodies containing alMGS-GFP in AD93 cells was confirmed by deconvoluted images of an individual optical section of a cell (E). Hence, increased intracellular ions partially abolish MGlcDAG synthesis, but the lower amounts of the lipid remaining were still sufficient for response to stress.

PE Is Important for Maintenance of Membrane Permeability—The passive permeability of the outer membrane was assayed indirectly by analyzing the sensitivity of strains to antibiotics as described under “Experimental Procedures.” Doxycycline, tetracycline, neomycin, rifampicin, and streptomycin block protein synthesis in the cytoplasm, and therefore these antibiotics must pass through both the outer and inner membrane to inhibit growth. Cephalothin and vancomycin, on the other hand, block cell wall synthesis in the periplasmic space, and these antibiotics only have to pass the outer membrane to be active. Polymyxin incorporates into both inner and outer membranes and therefore is a reporter of

Fig. 3. Cell length and morphology of lipid variants. Comparison of cell morphology by light microscopy for AD93 (PE⁺) (A) and AD93/pTMG3 (PE⁻/MGlcDAG⁺) (B) cells grown on LB agar plates containing 50 mM MgCl₂ and by fluorescence microscopy for AD93 (C) and AD93/pTMG3 (D) grown in LB with 10 mM MgCl₂ and stained with FM4-64. A and B were viewed with an Olympus BX60 microscope; images were captured with an Optronics DEI-750 video camera and edited by Adobe Photoshop 4.0. C and D were viewed with a Zeiss Axioplan 2 fluorescence microscope (rhodamine filter). Images were captured at 63 times enlargement (ocular ×1.25) with a CCD camera (Sony) and Image Access 3.0 software (Imagic Bildverarbeitung AG). Editing of images was done with Paint Shop Pro 4.12. E, analysis of cell length for the lipid variants grown in LB medium with 50 mM MgCl₂: AD93 (PE⁺) (red triangles), AD93/pDD72 (PE⁻) (green triangles), and AD93/pTMG3 (PE⁻/MGlcDAG⁺) (blue circles). Cells were viewed with the Olympus microscope and images captured with the Optronics camera. Pictures of 10 fields for every culture were taken, and the length of the individual cells was measured manually.
membrane stability. The size of zones of no growth around the antibiotic discs is reflective of the permeability spectra of the three lipid variants studied. The maximum molecular mass of molecules allowed to pass through the outer membrane porins is ~600 Da (51), which, except of vancomycin (1,449 Da), is on the order or greater than the antibiotics tested. Generally, the PE cells were less permeable (more resistant) than the PE or PE/MGlcDAG+ cells (Fig. 6A), indicating the importance of lipid surface charge (cf. lipid composition in Fig. 1). Hydrophobicity may also be a parameter involved because the presence of MGlcDAG increased the sensitivity to the more hydrophobic tetracycline over the more hydrophilic doxycycline. The most selective differences were observed for the large and hydrophilic vancomycin that cannot pass the outer membrane porins. A partial reduction of the PE amounts in a temperature-sensitive psaA mutant strain (52) yielded substantially smaller effects by vancomycin than in Fig. 6A. Hence, the zwitterionic character or charge of PE is important for a lower passive permeability barrier of the membranes, and the NB-prone but uncharged MGlcDAG cannot fully replace PE as a permeability barrier except in the case of cephalothin.

The barrier properties of the outer membranes were also tested with a larger probe, i.e. leakiness through the outer membrane of the periplasmic enzyme RNase (53) as indicated by extracellular digestion of RNA embedded in agar plates. PE and PE/MGlcDAG+ cells displayed a “periplasmic leakiness” phenotype, whereas PE− cells did not show this characteristic (Fig. 6B). These experiments were performed with 50 mM MgCl2, which should stabilize the outer membrane lipopolysaccharide. Still, the barrier properties to such large molecules as proteins as well as vancomycin were substantially weakened in the absence of PE, and MGlcDAG failed to correct this defect.

Solvents and Sterols Cannot Substitute for NB-prone Lipid—
Organic solvents and sterols can modify the packing preferences of membrane lipids by intercalating in the interface or deep among the acyl chains and even induce various NB aggregates (54, 55). 0.15–3.5 mM ethanol, 20–150 mM benzylalcohol, 2–100 mM dodecane, 0.5–3 mM octanol, and 30–100 μM cholesterol, 5-cholestene-3-one or 4-cholestene-3-one, respectively, were selected for their potencies with A. laidlawii (56) and analyzed here for their ability to modify growth properties, cell morphology, and lipid composition of the E. coli lipid variants. Ethanol promotes normal (type I) nonbilayer structures in lipid model systems, in addition to lowering lipid chain order, whereas the other additives promote reversed (type II) nonbilayer structures albeit with varying efficiencies (see Ref. 56 and references therein). Usable solvent concentrations were first screened, and then potential effects were investigated. Cholesterol can be incorporated to high concentrations into E. coli inner membranes and other bacteria, in sufficient amounts to affect lipid chain order substantially (57). However, the sterols used here were without growth-promoting effects for the lipid mutants. The higher concentrations listed above of all solvents inhibited growth, but for none of these additives except dodecane was there any significant changes or improvements revealed at lower concentrations (data not shown). Dodecane at 5 mM caused a small stimulation in growth for the PE strain and an increase in MGlcDAG amounts of ~10 mol % in the MGlcDAG+ strain. This is attributed to the established NB-promoting abilities of dodecane; the bilayer binding and activity of the aIMGS enzyme is moderately stimulated in vitro by such NB properties (30). We conclude that the lack of PE in AD93 and its concurrent packing properties cannot be replaced or substituted for by incorporation of NB-promoting solvents or sterols in vivo.

MGlcDAG Improves Lactose Transport—LacY, the E. coli membrane transporter for lactose, when assembled in PE cells, cannot accumulate substrate against a concentration gradient even though membrane potential required for substrate uptake is unaffected (33). However, facilitated transport of substrate is unaffected by the lack of PE. In minimal defined medium significantly higher levels of lactose were required as a carbon source for PE cells than PE/MGlcDAG+ cells (Table I). These levels of lactose are consistent with the Km values for facilitated and active transport of lactose (58), respectively. Likewise, PE cells were also dependent on tryptophan for growth in this minimal medium in contrast to the PE+ or PE/MGlcDAG+ cells. This latter requirement for tryptophan is cause by a defect in the active transport of aromatic amino acids.

The function of LacY was assayed by measurement of the uptake of the nonhydrolyzable substrate analog TMG in cells lacking a chromosomal copy of lacY and overproducing LacY from plasmid-borne copies of the lacY gene as described under “Experimental Procedures.” There was no active transport by PE cells (no change in the presence of FCCP) compared with the PE− cells, whereas in PE/MGlcDAG+ cells a significant level of active transport activity was observed (Fig. 7A); the level of accumulation in PE+ and PE/MGlcDAG+ cells in the presence of the uncoupler FCCP was the same as the level in PE cells with or without FCCP (data not shown). Western blot analyses using a polyclonal antibody indicated that LacY

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amounts in PE/H6126/MGlcDAG/H11001 cells (Fig. 7B) were 80% of the level in PE/H11001 cells (Fig. 7C); specific transport activity in the former cells was normalized to the latter cells to account for this difference. Hence, active transport function in PE/H6126 cells is partially restored (about 30% of normal) by the presence of MGlcDAG.

DISCUSSION

Synthesis of large amounts of a “foreign” NB-prone, uncharged membrane lipid in the E. coli PE’ mutant AD93 caused several profound changes in the cell. Compared with the paired AD93, the MGlcDAG-containing strain displayed shorter cells, faster growth, less dependence on Mg\(^{2+}\) ions, better osmotic tolerance, and a functional LacY but was still compromised in its outer membrane permeability barrier. Large variations in amounts of E. coli endogenous lipids were observed previously for a number of lipid enzyme mutants, where a decrease in especially PE was associated with abnormal cell division, decreased osmotic tolerance, outer membrane leakiness, dysfunction of several solute transport systems, and antibiotic hypersensitivity (16). MGlcDAG could substitute for PE in correcting the above phenotypes in at least three ways: (i) provide an increase in NB-forming lipids to increase the membrane curvature elastic stress; (ii) dilute the high negative charge density of lipid monolayers composed of only anionic lipids; (iii) provide specific chemical support similar to that of PE for cellular processes.

PE and MGlcDAG have several physico-chemical properties in common (for review, see Ref. 60). Both lipids form bilayer (L\(_b\)) and NB (reversed hexagonal and/or cubic phases) liquid-crystalline phases under similar conditions. They also have similar radii of spontaneous curvature and cause a slight increase in the average chain ordering of lipid bilayers. However, MGlcDAG seems to be more prone to form NB phases, and PE is less sensitive to temperature and acyl chain modifications (60). The \textit{in vivo} lipid mixtures of wild type E. coli, where PE is the major species, are closer on the temperature scale to a NB phase transition than are \textit{A. laidlawii} lipid mixtures (61). Both PE and MGlcDAG may also partly phase separate from their lipid environment under certain conditions (62, 63), and PE is potentially enriched around membrane proteins in \textit{E. coli} (64). Likewise, they can both become ionized and engage in hydrogen bonding (65, 66), in contrast to phosphatidylethanolamine, which does not substitute for PE in supporting LacY function (67, 68). Finally, the chemical differences between the zwitterionic PE headgroup, and the uncharged, stiff, ring-shaped glucose of MGlcDAG are substantial, although both would dampen the
negative charge density of a bilayer composed of only anionic lipids. However, the charge properties of PE are somewhat dampened by the formation of an internal charge paired ring, which cannot be attained by phosphatidylethanolamine (69, 70). Hence, the properties and improvements recorded here for AD93 by the introduction of MGlcDAG must be interpreted in terms of these physico-chemical similarities and differences. Furthermore, the inherent regulatory properties of the alMGS enzyme are important. The maximum amounts of MGlcDAG maintained in AD93, i.e. up to 50 mol%, are very similar to the maximum amounts recorded for A. laidlawii in vivo (25, 26) and coincidentally also to the maximum amounts of the major NB-prone, “packing analog” β-monogalactosyl-DAG in chloroplast thylakoids (71). A larger fraction of these lipids may be detrimental to membranes.

The reduced dependence on Mg\(^{2+}\) necessary to support growth and viability of AD93 containing MGlcDAG is consistent with increasing the NB character of the membrane. The effectiveness of divalent cations in supporting growth of AD93 (Ca\(^{2+}\) > Mg\(^{2+}\) > Sr\(^{2+}\) with Ba\(^{2+}\) being ineffective) parallels the potency of these cations in inducing NB structures in the lipids (primarily CL) extracted from AD93 (18, 19). In *E. coli* intracellular levels of Mg\(^{2+}\) are maintained in the high mM range by active transport systems (72), whereas Ca\(^{2+}\) and Sr\(^{2+}\) are in the μM range and actively excluded from the cytoplasm (73), suggesting that divalent cation supplementation of the growth medium is required for the outer monolayer of the inner membrane and/or the outer membrane; MGlcDAG was also found in the outer membrane. The requirement for divalent cations cannot be substituted by high concentrations of monovalent cations or polyamines, indicating that a simple charge damping or high ionic strength is not the molecular basis for the requirement. Therefore, the NB-forming potential of MGlcDAG is the most likely property responsible for reducing the dependence on divalent cations.

Supplementation of AD93 with MGlcDAG resulted in cell morphology substantially more like wild type cells than observed for filamentous PE\(^{-}\) cells (Fig. 3). Septum localization and constriction demand several integral membrane or amphitropic proteins of the Min and Fts systems that (74) associate with the membrane through other division proteins or with phospholipids (75). In AD93 localization of FtsZ and associated proteins is correct, but aberrant structural organization is observed, and subsequent ring constriction is only rarely seen (20). Because cell division in the PE/MGlcDAG\(^{-}\) strain appears more precise than a PE\(^{-}\) strain, MGlcDAG can partially replace a demand for PE in the assembly of the division machinery, further demonstrating the importance of membrane lipids in the cell division process. The amphitropic *E. coli* and *B. subtilis* cell division protein MinD, like alMGS (Fig. 5A), localizes to the membrane (76). MinD *in vitro* shows a preference for anionic lipids, and in PE\(^{-}\) cells GFP-MinD assembles into dynamic focal clusters that often appear to follow a helical “zigzag” path instead of the broad zones typical of cells with normal phospholipid content (11). The aberrant behavior of MinD in PE\(^{-}\) cells may be caused by an increase in its affinity for the anionic lipid-enriched membrane surface and a change in the orientation of the MinD membrane binding domain with the membrane surface (11). The partial correction in cell division properties brought about by MGlcDAG may be the result of the reduction of anionic surface charge of the membrane in combination with stimulation of MinD bilayer binding by the MGlcDAG NB properties (cf. (12)), which parallel the role of PE in wild type cells.

alMGS membrane association and activity also depend on anionic and NB-prone lipids (30), so it is not surprising that alMGS-GFP also shows an aberrant membrane localization pattern in PE\(^{-}\) cells highly enriched in anionic lipids (Fig. 5, A and C) similar to that observed for GFP-MinD. Like MinD, alMGS has a similar (but longer) anionic-lipid binding motif that is influenced positively by NB-prone lipids. In AD93 alMGS also exhibits a focal point rather than diffuse membrane association similar to the distribution of CL or anionic lipid domains along the surface of PE\(^{-}\) cells or at the poles of PE\(^{-}\) cells (44). In PE\(^{-}\) cells the higher proportion of anionic lipid relative to wild type cells may stimulate alMGS activity and explain the higher MGlcDAG content in the former cells. The alMGS enzyme is evolutionarily designed to work in a cytoplasm containing substantially lower ionic strength and divalent cation content than *E. coli*. Therefore, addition of salt to the growth medium is consistent with a lower MGlcDAG content through reducing alMGS membrane association and in concordance with the behavior of the enzyme in high salt *in vitro* (30).

Given the permeability “handicap” of the outer membrane of

| Cells                              | AD93/pDD72 (PE\(^{-}\)) | AD93/pTMG3 (PE\(^{-}\)/MGlcDAG\(^{-}\)) | AD93 (PE\(^{-}\)) |
|-------------------------------------|--------------------------|----------------------------------------|------------------|
| Minimal requirement for lactose    | 1 μM                     | 1 μM                                   | 250 μM           |
| Time for colony formation          | 16 h at 30 °C            | 16 h at 37 °C                          | 36 h at 37 °C    |
| Growth requirement for tryptophan   | Prototroph               | Prototroph                             | Auxotroph        |

Fig. 7. *LacY* transport function in *E. coli* lipid variants. A. uptake of TMG, normalized to total cell protein, by PE\(^{+}\) (AL95/pDD72GM/pT7-5LacY), PE\(^{-}\) (AL95/pT7-5LacY), and PE/MGlcDAG\(^{-}\) (AL95/pTMG-LacY) cells as described under “Experimental Procedures.” Uptake of TMG by (AL95/pTMG-LacY) was normalized for the reduced level of LacY relative to the level of LacY in AL95/pDD72GM/pT7-5LacY as shown by Western blot analysis (protein loads of 5 μg (lane 1), 10 μg (lane 2), and 20 μg (lane 3)) in B and C.
the MGlCDAG/PE strain, its fully restored ability to tolerate osmotic up shifts is quite remarkable (Fig. 4). Bacteria, including E. coli, have effective protein-mediated systems to cope with different types of osmotic stress (osmoadaptation). Membrane elastic stress and lipid packing properties seem to be key regulators of osmoadaptation processes as observed in vivo and for several membrane-reconstituted, pure proteins (45, 77).

Even when the MGlCDAG fraction was reduced from 55 to 30 mol % by quenching the aMGs activity at higher intracellular mol % by quenching the aMGs activity at higher intracellular

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Bacteriol. 146, 1151–1153
58. Viitanen, P., Newman, M. J., Foster, D. L., Wilson, T. H., and Kaback, H. R. (1986) Methods Enzymol. 125, 429–452
59. Nesmeyanova, M. A., Tsfasman, I. M., Karamyshev, A. L., and Suzina, N. E. (1991) World J. Microbiol. Biotechnol. 7, 394–406
60. Mannock, D. A., Lewis, R. N., McElhaney, R. N., Harper, P. E., Turner, D. C., and Gruner, S. M. (2001) Eur. Biophys. J. 30, 537–554
61. Niemi, A. E., Andersson, A. S., Rilfors, L., Lindblom, G., and Arvidson, G. (1997) Eur. Biophys. J. 26, 485–493
62. Yang, L., Ding, L., and Huang, H. W. (2003) Biochemistry 42, 6631–6635
63. Storm, P., Li, L., Kinnunen, P., and Wieslander, A. (2003) Eur. J. Biochem. 270, 1699–1709
64. Vanouzov, S., Parola, A. H., and Fishov, I. (2003) Mol. Microbiol. 49, 1067–1079
65. Urban, F., and Shaffer, P. A. (1932) J. Biol. Chem. 94, 697–715
66. Lewis, B. E., and Schramm, V. L. (2003) J. Am. Chem. Soc. 125, 7872–7877
67. Bogdanov, M., Sun, J., Kaback, H. R., and Dowhan, W. (1996) J. Biol. Chem. 271, 11018–11018
68. Wang, X., Bogdanov, M., and Dowhan, W. (2002) EMBO J. 21, 5673–5681
69. Seimiya, T., Ashida, M., Muramatsu, T., Hara, I., and Hayashi, M. (1978) Chem. Phys. Lipids 22, 221–226
70. Zhukov, A. V., Kusnetsova, E. I., and Vereshchagin, A. G. (1996) Chem. Phys. Lipids 82, 1–6
71. Dermann, P., and Benning, C. (2002) Trends Plant Sci. 7, 112–118
72. Lusk, J. E., and Kennedy, E. P. (1969) J. Biol. Chem. 244, 1653–1655
73. Gangola, P., and Rosen, B. P. (1987) J. Biol. Chem. 262, 12570–12574
74. Errington, J., Daniel, R. A., and Scheffers, D. J. (2003) Microbiol. Mol. Biol. Rev. 67, 52–85
75. Lutkenhaus, J. (2002) Curr. Opin. Microbiol. 5, 548–552
76. Sze, T. H., Rowland, S. L., Rothfield, L. I., and King, G. F. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 15693–15698
77. Poolman, B., Blount, P., Folgering, J. H., Friesen, R. H., Moe, P. C., and van der Heide, T. (2002) Mol. Microbiol. 44, 889–902
78. Bogdanov, M., Umeda, M., and Dowhan, W. (1999) J. Biol. Chem. 274, 12339–12345
79. Brahetz, W., Liebl, W., and Schleifer, K. H. (1993) J. Bacteriol. 175, 7488–7491
80. Driessen, A. J., Zheng, T., Int Veld, G., Op den Kamp, J. A., and Konings, W. N. (1988) Biochemistry 27, 865–872
81. van der Heide, T., Stuart, M. C. A., and Poolman, B. (2001) EMBO J. 20, 7022–7032
Monoglucoylglycerol, a Foreign Lipid, Can Substitute for Phosphatidylethanolamine in Essential Membrane-associated Functions in *Escherichia coli*

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