Published in final edited form as:

Appl Microbiol Biotechnol. 2015 January ; 99(1): 241–254. doi:10.1007/s00253-014-6082-z.

Biosynthetic preparation of selectively deuterated phosphatidylcholine in genetically modified *Escherichia coli*

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Abstract

Phosphatidylcholine (PC) is a major component of eukaryotic cell membranes and one of the most commonly used phospholipids for reconstitution of membrane proteins into carrier systems such as lipid vesicles, micelles and nanodiscs. Selectively deuterated versions of this lipid have many applications, especially in structural studies using techniques such as NMR, neutron reflectivity and small-angle neutron scattering. Here we present a comprehensive study of selective deuteration of phosphatidylcholine through biosynthesis in a genetically modified strain of *Escherichia coli*. By carefully tuning the deuteration level in *E. coli* growth media and varying the deuteration of supplemented carbon sources, we show that it is possible to achieve a controlled deuteration for three distinct parts of the PC lipid molecule, namely the (a) lipid head group, (b) glycerol backbone and (c) fatty acyl tail. This biosynthetic approach paves the way for the synthesis of specifically deuterated, physiologically relevant phospholipid species which remain difficult to obtain through standard chemical synthesis.

Keywords

Glycerophospholipids; Phosphatidylcholine; Selective deuteration; Biosynthesis; *E. coli*; Neutron scattering; NMR; Mass spectrometry; Evolution in the test tube

Introduction

Deuterium-labelled phospholipids were originally developed for the analysis of lipid pathways and for the purposes of mass spectrometry as internal standards (Rohwedder 1985). They have also found use in solid state NMR and neutron diffraction techniques for the study of structural organization and dynamics of biological membranes (Akesson et al. 2012; Hagn et al. 2013; Jacques et al. 2012). Additionally, deuterated phospholipids are used in membrane-mimicking carriers to study membrane proteins (Hagn et al. 2013; Jacques et al. 2012). In ^1H -NMR studies of complex membrane protein systems, full lipid deuteration leads to the complete suppression of their ^1H signal (Hagn et al. 2013; Varga et al. 2007).

This results in lipid ‘transparency’ and significantly simplifies data analysis enabling both secondary and tertiary structural analyses of embedded membrane proteins (Hagn et al. 2013). In neutron reflectometry, the advantages lie in obtaining different contrasts for different parts of the lipid bilayer (Akesson et al. 2012). Controlling the deuteration levels allows specific parts of the lipid molecules to be highlighted (Majewski et al. 2000). This can then be exploited not only in studies of the effects of the lipid environment on membrane protein systems but allows for the study of different lipid systems and their interactions with various ligands and potential drugs (Akesson et al. 2012; Molloy 2010). In small-angle neutron scattering (SANS) studies, specific deuteration of lipids allows solvent contrast variation to render membrane protein carrier systems such as lipid vesicles (Hunt et al. 1997) or other membrane-mimicking carriers (Jacques et al. 2012) fully or nearly invisible. This potentially allows for analysing the SANS data with well-established data analysis methods originally developed for proteins in solution (Konarev et al. 2006).

The available labelling approaches for production of biomolecules such as lipids are chemical and microbial synthesis, or a combination of the two, when different levels of deuteration within the molecules are required (Bragina and Chupin 1997). For recombinant proteins, microbial expression is the preferred deuteration method—often with *Escherichia coli* as the expression host (Leiting et al. 1998). This organism can be adapted to grow in minimal media in high levels of D₂O (Leiting et al. 1998), and the use of D₂O can be combined with different deuterated carbon sources such as sodium acetate, glycerol or glucose. This type of approach is now widely used in neutron protein crystallography (Cuypers et al. 2013a; Howard et al. 2011), SANS (Cuypers et al. 2013b; Laux et al. 2008; Rochel et al. 2011; Vijayakrishnan et al. 2010; Zhu et al. 2010) and neutron reflectivity (Grage et al. 2011; Hellstrand et al. 2013) and has previously also been used in neutron fibre diffraction studies of natural and synthetic polymers (Gardner et al. 2004; Shotton et al. 1997). Deuterated phospholipids, on the other hand, are generally produced by chemical synthesis, which allows for the combination of various fatty acyl residues to obtain the desired mixtures of partially labelled or perdeuterated, saturated phospholipid species as well as selective deuteration of specific groups (Bragina and Chupin 1997). However, the synthesis of selectively deuterated versions of physiologically relevant unsaturated phosphatidylcholines (PCs), which are the major membrane-forming phospholipids in eukaryotes (van Meer et al. 2008), still remains difficult. For example, obtaining PC with non-identical fatty acyl residues at positions *sn*-1 and -2 of glycerol requires rather complex organic synthesis. In addition, adjustments of the head-group deuteration level at the final stages of lipid synthesis can lead to modifications of the double bonds in unsaturated acyl tails (de Kruijff et al. 1978). These monounsaturated PC species with a fatty acyl length between 16 and 18 carbon atoms are, however, the physiologically most relevant for biophysical and biochemical studies of membranes and membrane proteins due to their ability to form stable bilayers at a broad range of pH, temperature and ionic strength. Thus, they are the lipids of choice for the reconstitution of membrane proteins into various carrier-systems such as lipid vesicles, micelles and nanodiscs.

We recently reported a small-angle neutron scattering study of a so-called stealth nanodisc system, i.e. a version of the ApoA1-derived nanodisc carrier (Bayburt et al. 2002), which is

contrast optimized for SANS-based structural studies of membrane proteins in solution (Maric et al. 2014). This approach for SANS contrast optimization is a crucial step as a part of a general quest towards developing nanodisc carriers for low resolution structural studies of membrane proteins in solution or at interfaces (Rambo and Tainer 2013; Skar-Gislinge et al. 2010; Wadsater et al. 2012; Wadsater et al. 2011). Here we describe in more detail the novel methods that were developed for the production of selectively deuterated, physiologically relevant PCs. This was done using *E. coli* strain AL95/pAC-PCS_{lp}-Sp-Gm genetically engineered to produce PC instead of its wild-type lipid phosphatidylethanolamine (PE) (Bogdanov et al. 2010). These methods allow controlled, site-specific deuteration of three distinct parts of the PC lipid molecule (lipid head group, glycerol backbone, fatty acyl tail) by varying the D₂O concentration of the growth media as well as deuteration of the supplementing carbon sources. This choice of the host enabled the possibility of reaching very high degrees of deuteration (Paliy et al. 2003) while the very simple phospholipid composition of *E. coli* (Raetz 1978) (as compared to yeast for example (Kaneko et al. 1976)) facilitated both biosynthesis and subsequent lipid extraction and purification. This has established a new platform for the preparation of physiologically relevant deuterium-labelled PCs and should be applicable also for the deuteration of other phospholipids and membrane components.

Materials and methods

Materials

All chemicals and solvents were obtained (in the highest commercially available purity) from Sigma-Aldrich (Denmark) and used as supplied unless stated otherwise. Deuterated glycerol (d8, 99 %) and deuterated choline chloride (trimethyl-d9, 98 %) were from Eurisotop (St-Aubin Cedex, France) and Silantes[®] rich deuterated *E. coli* OD2 medium was purchased from Silantes GmbH (München, Germany). Minimal medium was composed of 6.86 g/l (NH₄)₂SO₄, 1.56 g/l KH₂PO₄, 6.48 g/l Na₂HPO₄·2H₂O, 0.49 g/l diammoniumhydrogen-citrate, 0.25 g/l MgSO₄·7H₂O, 1.0 ml/l of a salt mix (0.5 g/l CaCl₂·2H₂O, 16.7 g/l FeCl₃·6H₂O, 0.18 g/l, ZnSO₄·7H₂O, 0.16 g/l CuSO₄·5H₂O, 0.15 g/l MnSO₄·4H₂O, 0.18 g/l CoCl₂·6H₂O, 20.1 g/l EDTA). Phospholipid standards and 1,2-dioleoyl-*sn*-glycerol-3-cytidine diphosphate (CDP-DAG) were purchased from Avanti Polar Lipids Inc. (Birmingham, AL, USA) and used as supplied. L-[3-³H] Serine (20 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Triton X-100 (electrophoresis grade) was supplied by Thermo Fisher Scientific (New Hampshire, USA). Matrix-assisted laser desorption and ionization (MALDI) matrices used were acidic 2,5-dihydroxybenzoic (DHB) acid as 0.5 M solution in methanol (Schiller et al. 1999) and basic 9-aminoacridine (9-AA) hemihydrate (Acros Organics, Geel, Belgium) applied as 10 mg/ml solution in 60/40 (v/v) isopropanol/ acetonitrile (Sun et al. 2008). Thin-layer chromatography (TLC) silica gel 60 plates were from Merck (Darmstadt, Germany). Ultrapure water (Ultra Clear Basic, SG, resistivity 18.2 M Ω×cm) was exclusively used throughout this study.

Bacterial strain and cultivation conditions

E. coli strain AL95 (*pssA*: *kan^R lacY*: *Tn9 cam^R*) carrying the plasmid pAC-PCS_{IP}-Sp-Gm (*P_{araB}-pcs Sp^R Gm^R*), which allows for the expression of PC synthase encoded by the *Legionella pneumophila pcsA* gene under control of an arabinose inducible promoter (Bogdanov et al. 2010), was adapted to deuterated minimal medium after a multistage adaptation process according to a modified method by Artero et al. (Artero et al. 2005). Briefly, in phase I, a fresh overnight culture of AL95 strain carrying the plasmid and grown in Luria-Bertani (LB) broth supplemented with 50 mM MgCl₂ was diluted to an initial cell density of 0.95 (*A*₆₀₀) into flasks containing minimal medium supplemented with 5 g/l glycerol and 10 mg/l gentamicin. To ensure initial growth, divalent metal ions in the form of 50 mM MgSO₄ solution were added to the flask cultures in addition to 10 % Silantes[®] rich medium. After overnight incubation at 37 °C, this process was repeated daily over a period of 3 weeks. During phase II, cells were amplified (1 in 10) in minimal medium based on D₂O supplemented with 50 mM MgSO₄ solution and 10 % deuterated Silantes[®] rich medium. This process was then repeated for 1 week (for adaptation scheme, see Fig. 1a). The adapted cultures were amplified in minimal medium containing different levels of D₂O as well as different variations of the used deuterated carbon sources; glycerol (C₃D₈O₃) and choline chloride (C₅H₅D₉ClNO) (Table 1). The cells were induced with 0.2 % arabinose (unlabelled) and 2 mM choline chloride as previously described (Bogdanov et al. 2010). After incubation at 37 °C for 24 h, cells were harvested by centrifugation (10,000×g, 20 min, 4 °C), and washed with MiliQ water.

To prepare the cell-free total cell extracts for phosphatidylserine synthase assays, strain AL95 lacking PE was grown in LB supplemented with 50 mM MgCl₂ as previously described (Bogdanov et al. 2010; DeChavigny et al. 1991). Strain AL95 carrying plasmid pAC-PCS_{IP}-Sp-Gm capable of synthesizing PC instead PE was grown in LB supplemented with 50 mM MgCl₂, 0.2 % arabinose and 2 mM choline as previously described (Bogdanov et al. 2010).

Phospholipid extraction and purification

Total lipids were extracted by a modified method by Bligh and Dyer (Bligh and Dyer 1959). In short, cell pellets were resuspended in H₂O (5 ml H₂O/g of cell paste) and sonicated (10×2 s pulses at about 20 % power using a Branson Sonifier[®] 450 Sonicator). Methanol (2.2 ml) and chloroform (1 ml) were added per 1-ml cell suspension aliquot. After 30 min at 25 °C, phase separation was induced by addition of chloroform (1 ml) and H₂O (1 ml) followed by centrifugation (800×g, 10 min, 4 °C). The chloroform phase was collected and the process repeated two more times. The chloroform phase was stored at –20 °C and the residual aqueous phase subsequently removed. The final organic phase was dried using a nitrogen stream, and lipid extracts were stored at –20 °C. For further purification, 50 mg of total lipid extracts were applied to a silica gel column (1×15 cm, silica gel 60, 230/400 mesh, 6 g), and lipids were separated according to phospholipid head group by eluting with varying ratios of chloroform/methanol [9:1 (200 ml), 4:1 (200 ml), 1:1 (200 ml), 1:4 (200 ml), 100 % methanol (300 ml)] mixtures.

Phospholipid analysis

Phospholipids were separated by thin-layer chromatography (TLC) using chloroform/ethanol/water/triethylamine (30/35/ 7/35, v/v/v/v) and identified through comparison with known standards chromatographed on the same plate after staining with primuline (5 mg primuline in 100 ml acetone/water, 80/ 20, v/v) (White et al. 1998). For improved separation of phosphatidylglycerol (PG) and cardiolipin (CL), plates were developed in chloroform/acetone/methanol/acetic acid/water (30/40/10/10/5, v/v/v/v/v). For further analysis, lipid spots from primuline-stained TLC were scraped off and extracted three times with 100 µl chloroform/methanol/0.9 % aqueous NaCl (1/1/1, v/v/v). Total phospholipid content was determined by digesting lipids in deionized water and perchloric acid for 1 h at 180 °C followed by addition of ammonium molybdate and ascorbic acid. After further heating to 80 °C for 10 min, the sample was cooled and the absorbance was read at 812 nm to quantify total lipid phosphorus as previously described (Rouser et al. 1966).

MALDI-TOF mass spectrometry

For MS analysis, lipid spots from primuline stained TLC were scraped off and extracted as described above. The obtained lipid fractions (concentrated to a volume of 10 µl) as well as total extracts (10 µl) were independently pre-mixed 1/1 (v/v) with the different matrix compounds (DHB and 9-AA) prior to deposition onto the MALDI target and investigated by positive and negative ion-mode MALDI-TOF MS, respectively. MALDI-TOF mass spectra were acquired on a Bruker Autoflex mass spectrometer (Bruker Daltonics, Bremen, Germany) which utilizes a pulsed nitrogen laser (337-nm wavelength). The extraction voltage was 20 kV and gated matrix suppression was applied to prevent the saturation of the detector by matrix ions (Petkovic et al. 2001). For each mass spectrum, 100 single laser shots were averaged. In order to enhance the resolution, all spectra were acquired in the reflector mode using delayed extraction conditions. See Fuchs et al. 2007 and Schiller et al. 2004 for a more detailed methodological description of MALDI-TOF MS with the focus on lipid analysis. All data were processed with the software 'Flex Analysis' version 2.2 (Bruker Daltonics). Selected lipids were also analysed subsequent to phospholipase A₂ (PLA₂) digestion in order to determine the fatty acyl composition as previously described (Fuchs et al. 2007).

Deuteration level determination

The level of deuteration, X_D , for the lipid species obtained from adapted AL95 preparations with varying levels of D₂O was determined by mass spectrometry. This involved a comparison of the measured lipid species mass with the theoretical value calculated for its fully deuterated analogue. Hence, X_D was calculated as below:

$$X_D = \frac{m_{DPC} - m_{HPC}}{m_{100\%DPC} - m_{HPC}} \quad (1)$$

Where m_{DPC} is the average mass obtained for the deuterated species, m_{HPC} is the mass of unlabelled PC and $m_{100\%DPC}$ is the theoretical mass of fully deuterated phospholipid.

NMR spectroscopy

The localization of the incorporated deuterium on the lipid PC molecules was determined by ^1H -NMR spectroscopy through comparison of ca. 5 % (v/v) solutions of deuterated phosphatidylcholine with an equivalent solution of unlabelled phosphatidylcholine also synthesized in *E. coli*. All lipids were dissolved in chloroform- d (99.96, 0.03 % (v/v) tetramethylsilane (TMS)) and all spectra were recorded on a Bruker Avance 300 MHz instrument.

Assay of phosphatidylserine synthase

E. coli cell pellets were suspended in 0.1 M Tris-HCl buffer, pH 7.4 and subjected to 3 cycles of sonication (1 min on with 1 min off intervals at amplitude 40 % using a Branson Digital Sonifier) on ice after which unbroken cells were removed by centrifugation (5000×g, 5 min). Phosphatidylserine synthase activity was measured as previously described by Larson and Dowhan (Larson and Dowhan 1976). Aliquots of cell free extract were added to the assay mixture (0.06 ml) containing 0.67 mM CDP-DAG, 0.5 mM L -[3- ^3H] serine (2000 cpm/nmol, 50,000 cpm per assay), 0.1 % Triton X-100 and 0.1 M Tris-HCl, pH 7.4. CDP-DAG was first evaporated from a chloroform solution under vacuum using a Savant Speed Vac Concentrator. A 10× stock solution of CDP-DAG at a final concentration of 6.7 mM was prepared by sonication (30 s) on ice at amplitude 20 % (Branson Digital Sonifier) in 100 mM Tris-HCl buffer, pH 7.4, supplemented with 0.1 % Triton X-100. The reaction was stopped by adding 0.5 ml of acidic methanol (0.1 N HCl in methanol) followed by chloroform (1.5 ml) and 3 ml of a 1 M solution of MgCl_2 forming a two-phase system. The chloroform phase was evaporated and the level of radiolabel incorporated into phospholipid determined by scintillation counting. One unit of enzymatic activity is defined as the amount of enzyme that incorporates 1 nmol of serine into chloroform soluble material in 1 min. Specific activity is expressed as units per mg protein determined by Pierce BCA Protein Assay (Thermo Fisher Scientific New Hampshire, USA).

Results

Growing *E. coli* AL95/pAC-PCS_{1p}-Sp-Gm bacteria in a deuterated medium required a process of adaptation (Katz and Crespi 1966). Initial amplification of the strain in minimal medium according to the method by Artero et al. (Artero et al. 2005) resulted in no significant cell growth neither in unlabelled nor deuterated conditions (data not shown). This result was expected as the auxotrophic strain requires the presence of amino acids and high amounts of divalent metal ions for growth (Bogdanov et al. 2010; DeChavigny et al. 1991). Supplementation with 10 % rich medium (Silantes[®]) was necessary to reach a cell density (OD_{600}) of 2 following overnight growth (Fig. 1b, black squares). However, the required high amount of magnesium salts proved to be insoluble in rich Silantes[®] media as well as in minimal media due to their high phosphate content. After culturing for 3 weeks in the Silantes[®] supplemented medium (phase I) and 1 week in Silantes[®] supplemented medium based on D_2O (phase II), a significant increase in bacterial cell growth was observed, resulting in cell densities (OD_{600}) of 9 for the adapted AL95 strain (Fig. 1b, grey circles). After the adaptation process, the strain was able to grow on minimal medium without supplementation (Phase III).

The adaptation process led to substantial changes in the lipid composition of the strain. While the original AL95/pAC-PCS_{lp}-Sp-Gm produced predominantly PC, PG and CL, with no detectable PE, a clear reversion to high amounts of PE was detected in the D₂O adapted AL95 strain (Fig. 1c). It has previously been shown that magnesium is a requirement for growth of the *E. coli* cells lacking PE (Bogdanov et al. 2010; DeChavigny et al. 1991). Therefore, it is most likely that the presence of PE in adapted AL95 resulted from a gain of function of the *pssA* gene due to the lower (and finally zero) concentration of divalent metal ions used in the media preparations during the adaptation process (see below).

Figure 1d shows the proportion of the four individual phospholipid classes in wild-type *E. coli* (WT), AL95/pAC-PCS_{lp}-Sp-Gm before adaptation (AL95) and AL95/pAC-PCS_{lp}-Sp-Gm after adaptation (Adapted AL95). Before adaptation, the lipid composition of the PC-producing strain consisted of up to 60 % PC together with 40 % CL and a small contribution (less than 1.5 %) of PG. The well-adapted strain, however, showed more than 50 % PE together with almost equal amounts of PC and PG (22 and 23 %, respectively) and a small contribution (less than 1 %) of CL component. Even under conditions of reversion to a more wild type-like lipid composition for the well-adapted strain, the ability to synthesize PC was obvious, leading to (for *E. coli*) a new lipid distribution for the four most abundant phospholipids. This may explain the improved bacterial growth in minimal conditions as compared to AL95/pAC-PCS_{lp}-Sp-Gm bacteria before adaptation and wild-type *E. coli*.

Phenotypic characterization of the adapted AL95

Numerous attempts have been made to suppress the divalent metal ion and amino acid supplemented medium requirement of *E. coli* strains lacking PE, and all have resulted in regain of wild-type phosphatidylserine synthase activity. The kanamycin-resistance (*kan*^R) cassette inserted into the chromosomal *pssA* gene did not result in any deletion of genetic information due to the presence of a complete coding sequence interrupted by a single copy of *kan*^R (Bogdanov et al. 2010).

Therefore, its exact excision would result in a functional *pssA* gene. The adapted AL95 strain was no longer resistant to kanamycin but retained resistant to the chloramphenicol used to interrupt the *lacY* gene in this strain. As noted above, the strain grew robustly in the absence of amino acid and MgCl₂ supplementation. Assays for phosphatidylserine synthase activity showed no detectable activity prior to adaption for AL95 with or without plasmid pAC-PCS_{lp}-Sp-Gm. However, the specific activity for the adapted AL95 carrying plasmid pAC-PCS_{lp}-Sp-Gm was 17 units/mg. This level is comparable to that of the AL95 wild-type parent strain W3899 (18 units/mg) assayed at the same time.

AL95/pAC-PCS_{lp}-Sp-Gm was grown in minimal medium supplemented with 10 % Silantes[®] rich medium during the adaptation process without kanamycin selection for the insertion cassette in the *pssA* gene. This resulted in precipitation of an unknown amount of the magnesium supplement and selected for revertants expressing phosphatidylserine synthase. Final culture in D₂O was carried out in minimal medium without amino acids, magnesium or kanamycin. Interestingly, a significant increase in mutation rates in general and reverse mutations from antibiotic resistance to sensitivity in particular has been noted in

E. coli cultured in concentrations of D₂O greater than 50 % (De Giovanni and Zamenhof 1963).

Fatty acyl distribution of deuterated phospholipids from adapted *E. coli* AL95

After the adaptation process, the adapted *E. coli* AL95 was cultured in deuterated medium and the phospholipids were extracted, purified using silica gel column chromatography and characterized by mass spectrometry. For a preparation of adapted AL95 where 100 % D₂O was used together with deuterated glycerol as carbon source, this gave a narrow distribution of highly deuterated phospholipid species enabling the determination of their fatty acyl composition (Fig. 2). Lipids extracted from the strain harvested in the stationary phase showed predominantly 1-palmitoyl-2-palmitoleoyl (16:0/16:1) PC and its cyclopropane modified analogue (16:0/17:0cyclo) (see Fig. 2 *insert*).

The species containing the cyclopropane residue was the most abundant, as is usually the case with *E. coli* lipids, and was further analysed (Fig. 2). The peak maximum at m/z 810 (H⁺ adduct) corresponded to the PC species where 63 hydrogen atoms are replaced by deuterium. This assignment could be made because it is known that 9-AA results primarily in H⁺ adduct formation while the generation of Na⁺ adducts (m/z 832) plays only a minor role (Sun et al. 2008). In contrast to PC which is preferentially detected as positive ion, all other phospholipids of interest were more easily detectable in the negative ion mode. PE 16:0/17:0 cyclo and PG 16:0/17:0 cycles were detected at m/z 769 and 802, respectively, which indicated the incorporation of either 66 or 68 deuterium atoms and, thus, gave data comparable to PC. Remarkably, there was in all cases a signal with a marked isotope pattern at m/z 769 (PC), 728 (PE) and 761 (PG). These signals have not been assigned so far. However, the mass difference in comparison to the most intense signals was always 41 mass units. This made an assignment even more difficult because the head groups of all discussed phospholipids are different and thus the generation of fragment ions during the MS process was very unlikely. Although we were also able to detect some deuterated cardiolipin (CL) species, these particular species are not discussed here due to poor signal-to-noise ratio in accordance with the previous study on CL detection (Eibisch et al. 2011) in particular since deuteration leads to an additional decrease of sensitivity.

Deuteration levels of phosphatidylcholine in adapted *E. coli* AL95

Adapted AL95 was cultivated in an increasing level of D₂O in order to monitor the effects of D₂O on PC molecules with deuterated minimal medium ranging from 50 to 100 % D₂O. This was done in the presence of both deuterated and unlabelled glycerol, while the choline was kept unlabelled. The deuteration level for the resulting PC species was determined through comparison of the actual average mass with that of the theoretical mass of fully deuterated species according to Eq. 1. This calculation assumes that no major changes in the fatty acyl composition are present as a result of deuteration, as it was shown by comparing the highly deuterated species produced in 100 % D₂O with the analogous lipids produced in H₂O (Fig. 2). As expected, the increased level of D₂O in the growth medium resulted in increased deuteration of PC for preparations with unlabelled as well as deuterated glycerol (Fig. 3a black and red, respectively). It was observed that the PC lipids biosynthesized in both 50 and 75 % D₂O displayed a very broad m/z distribution when analysed by mass

spectrometry. This was likely to be a result of random deuterium incorporation in the lipid molecules leading to different species of partially deuterated PC making the fatty acyl analysis challenging. Assuming 80 possible deuterium atoms for fully deuterated 16:0/17:0 cyclo PC, it can be seen that the use of unlabelled glycerol as carbon source resulted in an increase in the deuteration of the most abundant PC species from 21 to 83 % (Fig. 3a black) while for the preparations using deuterated glycerol, the deuteration of PC naturally increased from 43 to 90 % (Fig. 3a red). Full deuteration was not observed for any of the species indicating that the unlabelled choline chloride was also incorporated into the lipid molecules, lowering the overall level of deuteration.

NMR analysis of the PC extracted and purified from preparations grown in an increasing level of D₂O showed a gradual decrease of the ¹H resonance (Fig. 3b). This was most obvious for the large signal at 1.2 ppm corresponding to the deuteration of the majority of the hydrogens of the fatty acyl chains as the level of D₂O in the growth medium was increased. The signals at 3.9 and 4.4 ppm belonging to the ethylene hydrogens and the signal at 3.4 ppm corresponding to the methyl groups (all belonging to the choline head group) were observed in all preparations where the supplemented choline was unlabelled. The glycerol signals, on the other hand, at 4.0, 4.2 and 5.2 ppm were not observed in any of the preparations where deuterated glycerol was used as a supplement. These data indicated that the carbon source had an effect on the PC head-group deuteration while the increasing fatty acyl residue deuteration depended particularly on the total amount of D₂O. This is consistent with previous studies where fatty acyl residues obtained from *E. coli* cells grown in D₂O showed replacement of almost every hydrogen by deuterium without supplementation of deuterated carbon sources (Saito et al. 1980).

Head-group deuteration of PC synthesized in adapted AL95

To determine the effect of deuteration for both the glycerol and the choline moiety of the PC head group, the adapted AL95 was cultivated in growth medium supplemented with deuterated glycerol and deuterated choline and their unlabelled analogues according to Fig. 4.

The positive ion MALDI-TOF mass spectra of PC lipids synthesized in deuterated media at ~100 % D₂O while varying the deuteration of both the glycerol and the choline moiety is shown in Fig. 4 as a function of the deuteration of both the glycerol and the choline moiety. The PC species were compared with commercially available hydrogenated POPC and D₃₁-POPC (Avanti Polar Lipids Inc.) after digestion with phospholipase PLA₂ (which cleaves exclusively the fatty acyl chain in *sn*-2 position) to yield LPC 16:0 aiding a more accurate differentiation of both fatty acyl residues. This method was chosen because the MALDI MS system used did not possess a dedicated collision cell. The peaks at *m/z* 526.5 and 549.5 correspond to the respective proton and sodium adducts of 16:0 LPC with fully deuterated fatty acyl residues and a hydrogenated head. LPC was obtained from D₃₁-POPC after digestion with PLA₂ and showed a rather narrow mass distribution due to the lacking possibility of exchange reactions with the solvent. A similar mass distribution was also observed for highly deuterated preparations of PC obtained through *E. coli* biosynthesis. PC extracted from cells cultivated with unlabelled carbon sources (H_{gly}H_{cho}-PC) showed an

almost identical profile as that of D31-POPC with major peaks at m/z 526.5 and 548.5 corresponding to the proton and sodium adduct of LPC 16:0, respectively. Deuterated PC extracted from cells cultivated with deuterated glycerol ($D_{gly}H_{cho}$ -PC) showed an increase of exactly 5 amu as compared with $H_{gly}H_{cho}$ -PC resulting in peaks at m/z 531.5 and 553.5. This is in good agreement with the mass of five deuterium atoms of the glycerol moiety in the lipid molecule. In contrast to this, the preparation of PC where deuterated choline was used together with unlabelled glycerol ($H_{gly}D_{cho}$ -PC) resulted in a mass difference of exactly 9 amu (m/z 535.6 and 557.6), corresponding to the nine available deuterium atoms from the supplemented choline. The $D_{gly}D_{cho}$ -PC preparation, where both carbon sources were deuterated, showed peaks at m/z 540.6 and 562.6, i.e. an increase of 14 amu when compared to both $H_{gly}H_{cho}$ -PC and D31-POPC. This mass difference agrees well with the difference between the protonated and deuterated carbon sources added to the media with choline possessing nine and glycerol five hydrogen atoms.

The extent of deuteration of the remaining cellular lipids from adapted AL95 preparations with varying deuterated carbon source (Table 1) was also determined by MALDI-TOF MS, and the masses of the most abundant phospholipid species are summarized in Table 2. While deuterium-enriched glycerol and choline had an effect on the deuteration of the obtained PC, the remaining lipids were affected solely by glycerol. These data showed that choline was not metabolized into the WT *E. coli* lipids and confirmed the lipid synthesis pathways as suggested by Dowhan (Dowhan 2013).

Deuterium localization in PC synthesized in adapted AL95

For PC species obtained from preparations of adapted AL95 cultivated in deuterated media and varying deuterated carbon sources (according to Table 1), the location of the deuterium atoms within the head group was investigated by 1H -NMR (Fig. 5). The preparation of PC in the presence of unlabelled carbon sources ($H_{gly}H_{cho}$ -PC) showed a head group identical to that of unlabelled PC. The two signals seen at 4.04 and 4.50 ppm in the $D_{gly}D_{cho}$ -PC preparation (Fig. 5, $D_{gly}D_{cho}$ -PC) were in agreement with the ethylene hydrogens of the choline chloride head group ($C_5H_5D_9ClNO$). Although the deuterated species gave a resonance slightly downfield-shifted in comparison to the hydrogenated sample, these signals were present in all preparations and this could also be confirmed by ^{13}C -NMR (data not shown). The signal at 3.4 ppm assigned to the methyl groups of the choline moiety was present in both preparations of PC where unlabelled choline was used ($H_{gly}H_{cho}$ -PC and $D_{gly}H_{cho}$ -PC). However, this signal was completely lacking in preparations where deuterated choline was used ($H_{gly}D_{cho}$ -PC and $D_{gly}D_{cho}$ -PC), confirming the direct choline incorporation into the PC head group. Similarly, the 1H signals at 4.0 and 4.2 as well as 5.2 ppm (corresponding to the glycerol backbone of the PC) were absent in preparations where deuterated glycerol was used ($D_{gly}H_{cho}$ -PC and $D_{gly}D_{cho}$ -PC) but were still present in PC obtained from preparations where the growth medium was supplemented with unlabelled glycerol. The many weak signals seen in NMR for the deuterated analogues (Fig. 3b and our previous study) were associated with the acyl residues and indicated that the remaining hydrogens were randomly distributed throughout the fatty acyl chains.

The combined ^1H -NMR and MALDI-TOF data showed that both glycerol and choline were taken up by adapted AL95 and incorporated directly into the PC head groups without modifications. The data presented clearly indicate that head-group deuteration of PC can be controlled by carbon source supplementation while the degree of deuteration for the fatty acyl chains is dependent on the total amount of D_2O in the media. Therefore, it should also be possible to prepare PC species with selective head-group deuteration while the fatty acyl chains are kept unlabelled. Examples of the selectively deuterated PC species obtained at ~100 % D_2O are illustrated in Fig. 6. While in this study, we never used fully deuterated carbon source in combination with 100 % D_2O ; these data also indicate that 100 % D_2O in combination with 100 % deuterated carbon source should yield 100 % deuterated lipids.

Discussion

Adaptation to deuterated conditions is harsh for any organism and in the case of higher organisms (e.g. insect and mammalian cells) has so far only been accomplished at low levels of deuteration. Microorganisms appear to have considerable difficulty adapting to growth in D_2O as compared to adjusting to a single deuterated carbon source only (Katz and Crespi 1966). For bacteria such as *E. coli*, the deuterium adaptation process, when successful, has been shown to lead to many unusual features (Katz and Crespi 1966). In this work, we have shown that the *E. coli* strain AL95/pAC-PCS_{lp}-Sp-Gm, designed so that PC replaces the native phospholipid PE, could be adapted to grow under fully deuterated minimal media conditions. This was, however, accompanied by severe metabolic modifications of the strain as a function of the growth medium. The austerity of the adaptation process and the limiting choice of deuterated supplements led to changes in both auxotrophy and lipid composition of the strain. A clear reversion towards the production of high amounts of PE was observed after the adaptation process, as had previously been observed for preparations with insufficient Mg^{2+} concentration in the growth media (Bogdanov et al. 2010; DeChavigny et al. 1991). While the adapted strain continued to produce PC, the adaptation process resulted in a novel lipid composition, which was associated with an enhanced biomass production as compared to both non-adapted AL95 strain and wild-type *E. coli*.

Although the *pssA* null strain used in these experiments reverted to a *pssA*⁺, the utility of such strains for production of deuterated lipids ‘foreign’ to *E. coli* was demonstrated. Other foreign lipids such as phosphatidylinositol (Xia and Dowhan 1995), several mono-, di- and tri-saccharide derivatives of diacylglycerols (Wikstrom et al. 2009) (Xie et al. 2006), and *O*-lysyl phosphatidylglycerol (P. Heacock and W. Dowhan, unpublished data) have been synthesized in wild-type and PE-lacking *E. coli* strains. Although the levels of these lipids are higher in strains lacking PE, their level still ranges from 10 to 40 % of total lipid in the presence of PE. Clearly, a complete deletion of the *pssA* gene, e.g. by an antibiotic resistance cassette using the lambda phage recombination system (Thomason et al. 2014), would facilitate adaptation to minimal medium without the potential for regain of PE synthesis. However, it is unlikely that a PE-lacking strain can be propagated in the absence of divalent metal ion supplementation since extensive attempts have been made to eliminate this requirement (P. Heacock and W. Dowhan, unpublished data). Further improvements would be to delete the *cfa* gene encoding cyclopropane fatty acid synthase (Jordi et al. 1992); and *gpsA* gene encoding glycerol-3-phosphate dehydrogenase (Cronan and Bell

1974) in a *pssA* null background. In the former case, cyclopropane modification of fatty acids would be prevented. In latter case, the conversion of dihydroxyacetone phosphate to *sn*-glycerol-3-phosphate would be prevented allowing specific labelling of the glycerol backbone of lipids by medium supplemented with deuterated glycerol. The yields of deuterated PC that could be obtained through the biosynthetic approach are in the range of 50 mg/l for flask cultures—clearly sufficient for use in techniques such as NMR, SANS and neutron reflectivity. Based on the amount of cell paste obtained in a pilot fermentation study, it is also possible to scale the production up to over 200 mg/l; however, it remains to be analysed whether under those conditions the deuteration levels and fatty acyl compositions are maintained. The PC lipids can easily be extracted and purified in high yields using established methods. However, enabling purification procedures by means of preparative high-performance liquid chromatography (HPLC) may be preferable for large-scale preparations.

Mass spectrometry showed no major difference in fatty acyl length or degree of saturation for the biosynthetically deuterated PC species when compared to their hydrogenated analogues with possibilities for regulation of the fatty acyl composition during biosynthesis. The cyclopropanated residues are typical of *E. coli* lipids from cells harvested in the stationary growth phase (Magnuson et al. 1993) and have been shown to improve stability while maintaining the overall membrane fluidity (Dufourc et al. 1983; Law 1971). The fatty acyl distribution can be regulated during the bacterial growth cycle (Magnuson et al. 1993). It should be noted that harvesting cells in the stationary growth phase is recommended in terms of the efficiency and effective cost of production of deuterated phospholipid.

The biologically produced PC lipids can be deuterated to a very high extent, and the level of deuteration can be easily controlled by adjusting the total level of D₂O in the growth medium. Direct carbon source incorporation into the PC head groups provides additional control to selectively deuterate this part of the molecule through supplementation of selectively deuterated carbon sources to the growth media. While the *E. coli* strain AL95/pAC-PCS_{IP}-Sp-Gm was originally engineered to test the effects of the eukaryotic model lipids on bacterial membrane proteins, this project showed that it can also be used as a production platform for physiologically relevant deuterium-labelled phosphatidylcholines and that this approach is also applicable for the deuteration of other physiologically relevant phospholipids and membrane components. The lipids obtained in this way were, in combination with deuterated versions of the ApoA1-derived membrane scaffold proteins (Bayburt et al. 2002), recently used in a small-angle neutron scattering study of a so-called stealth nanodisc system contrast optimized for SANS-based structural studies of membrane proteins in solution (Maric et al. 2014). Utilizing the novel methods for the production of selectively deuterated, physiologically relevant PCs allowed controlled, site-specific deuteration of three distinct parts of the PC lipid molecule (lipid head group, glycerol backbone, fatty acyl tail) which were successfully used to assemble both neutron-invisible nanodiscs as well as vesicles for use in low resolution structural studies of membrane proteins in solution.

Acknowledgments

The authors gratefully acknowledge the access to the Deuteration Laboratory (D-Lab) PSB platform within ILL's Life Sciences Group, at the Institut Laue-Langevin in Grenoble. We thank the European Spallation Source, the UNIK research initiative of the Danish Ministry of Science, Technology and Innovation through the 'Center for Synthetic Biology' at University of Copenhagen, by the Danish National, and the Research Foundation through the PUMPKIN Center of Excellence for co-funding of the project Dr Schiller thanks the German Research Council (SFB 1052/B6) for financial support Professor Forsyth acknowledges support from the UK Engineering and Physical Sciences Research Council (EPSRC) for the original creation of ILL's Deuteration Laboratory Deuteration Laboratory under grants GR/R99393/01 and EP/ C015452/1, from the EU NMI3 programme under FP7, and also through the EU CRISP programme (grant number 283745). Professor Dowhan acknowledges support from the United States National Institutes of Health under grant GM R37-20748 and the John Dunn Research Foundation

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Fig. 1.

Adaptation of *E. coli* AL95/pAC-PCS_{I_p}-Sp-Gm to growth in D₂O. **a** Scheme illustrating AL95 adaptation to minimal deuterated medium. Supplementation consists of 10 % Silantes® rich medium. **b** AL95 carrying the plasmid pAC-PCS_{I_p}-Sp-Gm growth curves in minimal deuterated medium with 10 % Silantes® supplementation (*black squares*) and adapted AL95 in minimal medium without any supplementation (*grey circles*). Results are the means from two independent experiments; the SD is less than 5 % of the mean for all data shown. **c** Total lipid extracts separated by thin-layer chromatography from wild-type *E. coli* (WT), AL95 before adaptation (AL95) and after adaptation (Adapted AL95). The solvent system was chloroform/ethanol/water/triethylamine (30/35/7/35, v/v/v/v). Lipids were visualized by primuline staining. **d** Phospholipid composition of wild-type *E. coli* (WT), AL95 before adaptation (AL95) and after adaptation (Adapted AL95).

Phosphatidylethanolamine, PE, (*black*), phosphatidylcholine, PC, (*white*), phosphatidylglycerol, PG, (*grey*) and cardiolipin, CL, (*striped*). Results are the means from four independent experiments; the SD is less than 3 % of the mean for all data shown. For quantification, lipids were separated by thin-layer chromatography using chloroform/acetone/methanol/acetic acid/water (30/40/10/10/5, v/v/v/v/v).

Fig. 2.

Positive phosphatidylcholine (PC) and negative ion (all other phospholipids) MALDI-TOF mass spectra of phospholipid species extracted from adapted AL95 grown in ~100 % D₂O supplemented with deuterated glycerol and unlabelled choline. Spectra were recorded in the presence of 9-aminoacridine as the matrix and only the most abundant species (16:0/17:0 cyclo) were investigated in more detail and compositional details are given exclusively for this species. *Insert:* the two most abundant PC species from adapted AL95 cultivated in unlabelled medium and harvested in the stationary phase. Abbreviations:

phosphatidylcholine (*PC*), phosphatidylethanolamine (*PE*), phosphatidylglycerol (*PG*), cardiolipin (*CL*). Although *CL* species were detectable, they were not investigated in more detail due to the poor signal-to-noise ratio

Fig. 3.

Increased levels of D₂O lead to increasingly deuterated phosphatidylcholine (PC). **a** Level of deuteration in the PC molecule as a function of the D₂O level in the growth medium containing unlabelled glycerol (*black*) and deuterated glycerol (*red*). Deuteration levels were calculated from mass spectrometry data through comparison of the average mass obtained with the calculated theoretical mass of fully deuterated species. **b** ¹H-NMR spectra of increasingly deuterated PC normalized to the intensity of choline ethylene resonance. From top to bottom: PC synthesized in AL95 under unlabelled conditions (*Control*), PC produced

in adapted AL95 grown in 50 % D₂O (50% D₂O), PC produced in adapted AL95 grown in 75 % D₂O (75% D₂O) and PC produced in adapted AL95 grown in 100 % D₂O (100% D₂O) with deuterated glycerol and unlabelled choline in the three preparations. *Insert:* hydrogen positions and the corresponding peaks are indicated with red letters for aliphatic methylene groups from increasingly deuterated medium and for choline methyl and ethylene groups from unlabelled supplementation. The *asterisks* indicate signals of minor contaminants present in two of the samples

Fig. 4.

Positive ion MALDI-TOF spectra of the different species of purified PC extracted from adapted AL95 cultivated in deuterated media and varying carbon source deuteration subsequent to digestion with phospholipase PLA₂ shows selective deuteration. From top to bottom: synthetic hydrogenated LPC (obtained by digestion of *POPC*), synthetic LPC with hydrogenated head group and deuterated fatty acyl chain (from *D₃₁-POPC*), PC synthesized in 100 % D₂O supplemented with H-glycerol (C₃H₈O₃) and H-choline (C₅H₁₄ClNO) (*H_{gly}H_{cho}-PC*), PC synthesized in 100 % D₂O supplemented with D-glycerol (C₃D₈O₃) and H-choline (C₅H₁₄ClNO) (*D_{gly}H_{cho}-PC*), PC synthesized in 100 % D₂O supplemented with H-glycerol (C₃H₈O₃) and D-choline (C₅H₅D₉ClNO) (*H_{gly}D_{cho}-PC*), PC synthesized in 100 % D₂O supplemented with D-glycerol (C₃D₈O₃) and D-choline (C₅H₅D₉ClNO) (*D_{gly}D_{cho}-PC*). Spectra were recorded in the presence of DHB as matrix. Peaks are marked with their *m/z* values and assignments are indicated

Fig. 5.

Selective head-group deuteration of phosphatidylcholine (PC) can be monitored through ^1H -NMR. From top to bottom: PC synthesized in AL95 under unlabelled conditions (*Control*), PC synthesized in deuterated media containing hydrogenated glycerol and hydrogenated choline ($H_{\text{gly}}H_{\text{cho}}\text{-PC}$), PC synthesized in deuterated media containing deuterated glycerol and unlabelled choline ($D_{\text{gly}}H_{\text{cho}}\text{-PC}$), PC synthesized in deuterated media supplemented with unlabelled glycerol and deuterated choline ($H_{\text{gly}}D_{\text{cho}}\text{-PC}$) and PC synthesized in deuterated media with deuterated glycerol and deuterated choline ($D_{\text{gly}}D_{\text{cho}}\text{-PC}$). Spectrum intensities are normalized to the choline ethylene signals. Signals at 3.55 ppm originate from alkene protons. *Insert*: PC head group with hydrogen positions and their corresponding protons indicated by red letters

Fig. 6.

Examples of the selectively deuterated PC species synthesized by *E. coli* in dependence on the extent of deuteration. **a** PC synthesized in deuterated media containing both unlabelled glycerol and unlabelled choline (H_{Gly}H_{Cho}-PC). **b** PC synthesized in deuterated media supplemented with D₈-glycerol and unlabelled choline (D_{Gly}H_{Cho}-PC). **c** PC synthesized in deuterated media containing unlabelled glycerol and D₉-choline (H_{Gly}D_{Cho}-PC). **d** PC synthesized in adapted AL95 grown in deuterated media supplemented with D₈-glycerol and D₉-choline (D_{Gly}D_{Cho}-PC). NMR analysis shows the presence of fatty acids having approximately four randomly distributed hydrogens left

Table 1

Deuterium variation in carbon source supplemented to growth medium

Growth media	Carbon supplement	
	Glycerol	Choline
D _{gly} D _{cho}	C ₃ D ₈ O ₃	C ₅ H ₅ D ₉ CINO
D _{gly} H _{cho}	C ₃ D ₈ O ₃	C ₅ H ₁₄ CINO
H _{gly} D _{cho}	C ₃ H ₈ O ₃	C ₅ H ₅ D ₉ CINO
H _{gly} H _{cho}	C ₃ H ₈ O ₃	C ₅ H ₁₄ CINO

Table 2

m/z values (determined by MALDI-TOF MS) of major lipid species from AL95 after deuterium variation in bacterial growth media

Lipid species	Mass					
	D _{gly} D _{cho}	D _{gly} H _{cho}	H _{gly} D _{cho}	H _{gly} H _{cho}		
PC 16:0/17 cyclo	818	810	813		804	
PE 16:0/17 cyclo	774	769	763		763	
PG 16:0/17 cyclo	806	802	792		792	