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Cardiolipin synthases of *Escherichia coli* have phospholipid class specific phospholipase D activity dependent on endogenous and foreign phospholipids

Aike Jeucken, J. Bernd Helms, Jos F. Brouwers*

Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 2, 3584CM Utrecht, the Netherlands

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ABSTRACT

E. coli has three Cls-isoenzymes for cardiolipin (CL) synthesis but the differences between these three enzymes remain unresolved. All three Cls enzymes contain the phospholipase D (PLD) characteristic HKD motive and synthesize CL using PLD activity. Here, using LC-MS we show the effect of overexpressing or deletion of the three individual Cls enzymes on the lipidome, which included changes in lipid class distribution and CL species profiles. We demonstrate, for the first time, that overexpression of only ClsB resulted in the appreciable synthesis of a variety of phosphatidylalcohols, thereby establishing a 'classic' PLD activity for this enzyme: phospholipid headgroup exchange. Endogenous *E. coli* lipids and primary alcohols were substrates for this trans-phosphatidylation reaction. Furthermore, we show that endogenous levels of ClsA mediated a similar trans-phosphatidylation reaction to form phosphatidylalcohols, however this reaction was dependent on the presence of the foreign phospholipid class phosphatidylcholine (PC). This allows us to clarify the different specificities of the cardiolipin synthases.

1. Introduction

The *Escherichia coli* phospholipidome consists of approximately 70% phosphatidylethanolamine (PE), 20% phosphatidylglycerol (PG) and 5–10% cardiolipin (CL), with variations depending on different growth phases and conditions [1]. Together they form the bilayer of the inner membrane of *E. coli*, thus maintaining cell integrity and allowing membrane related processes [2]. The different lipid classes have different functions. CL for example, plays a critical role in dynamic organization of membranes and in cell division [3].

The initial steps in the *E. coli* lipid bio-synthesis pathway comprise the synthesis of phosphatidic acid (PA), followed by condensation of cytidine triphosphate with PA to form cytidine diphosphate-diacylglycerol (CDP-DAG). From here the pathway diverts into the direction of PE or PG *via* the intermediates phosphatidylserine (PS), or phosphatidylglycerophosphate (PGP) respectively [4, 5]. PG is the main substrate for CL synthesis [6], see Fig. 1A for an overview of the pathways. The dianionic CL is a unique phospholipid class because it has two PA moieties connected by a glycerol. In eukaryotes, one molecule CDP-DAG and one molecule PG are the substrates for the production of one molecule CL [7]. However, like most other prokaryotes, *E. coli* synthesizes CL by a condensation reaction of a phospholipid and a molecule of PG.

E. coli has three genes encoding for CL synthases namely; *clsA* (*cls*), *clsB* (*ybhO*), and *clsC* (*ymdC*) [8]. The reason for the presence of these three genes encoding CL enzymes remains unclear. The three synthases do have their own distinct features; ClsA, previously named Cls, is the first discovered CL synthase and is the major contributor to CL synthesis. It synthesizes CL during exponential growth and it uses two PG molecules as substrates, resulting in CL and a free glycerol [6]. ClsB, previously named YbhO, produces CL *in vitro*, but *in vivo* activity remains unclear [9]. This enzyme can also produce PG *in vitro* by using a PE molecule and glycerol [10]. ClsC, previously named YmdC, is the most recently discovered CL synthase. It uses one PE molecule as the phosphatidyl donor and one PG molecule to produce CL. It requires co-expression of the YmdB protein encoded by the upstream gene *ymdB* to be fully active [8]. All three enzymes have two HKD motifs that are characteristic for the phospholipase D (PLD) superfamily to which they belong [8, 9, 11].

PLD enzymes hydrolyze several phospholipid classes generating PA and the corresponding alcohol from the headgroup. A PLD reaction to form PA has so far not been described in *E. coli*. However, depending on the conditions, PLD enzymes can also catalyze a transphosphatidylation reaction, using primary alcohols, rather than a water molecule, and a

* Corresponding author.
E-mail address: j.brouwers@uu.nl (J.F. Brouwers).

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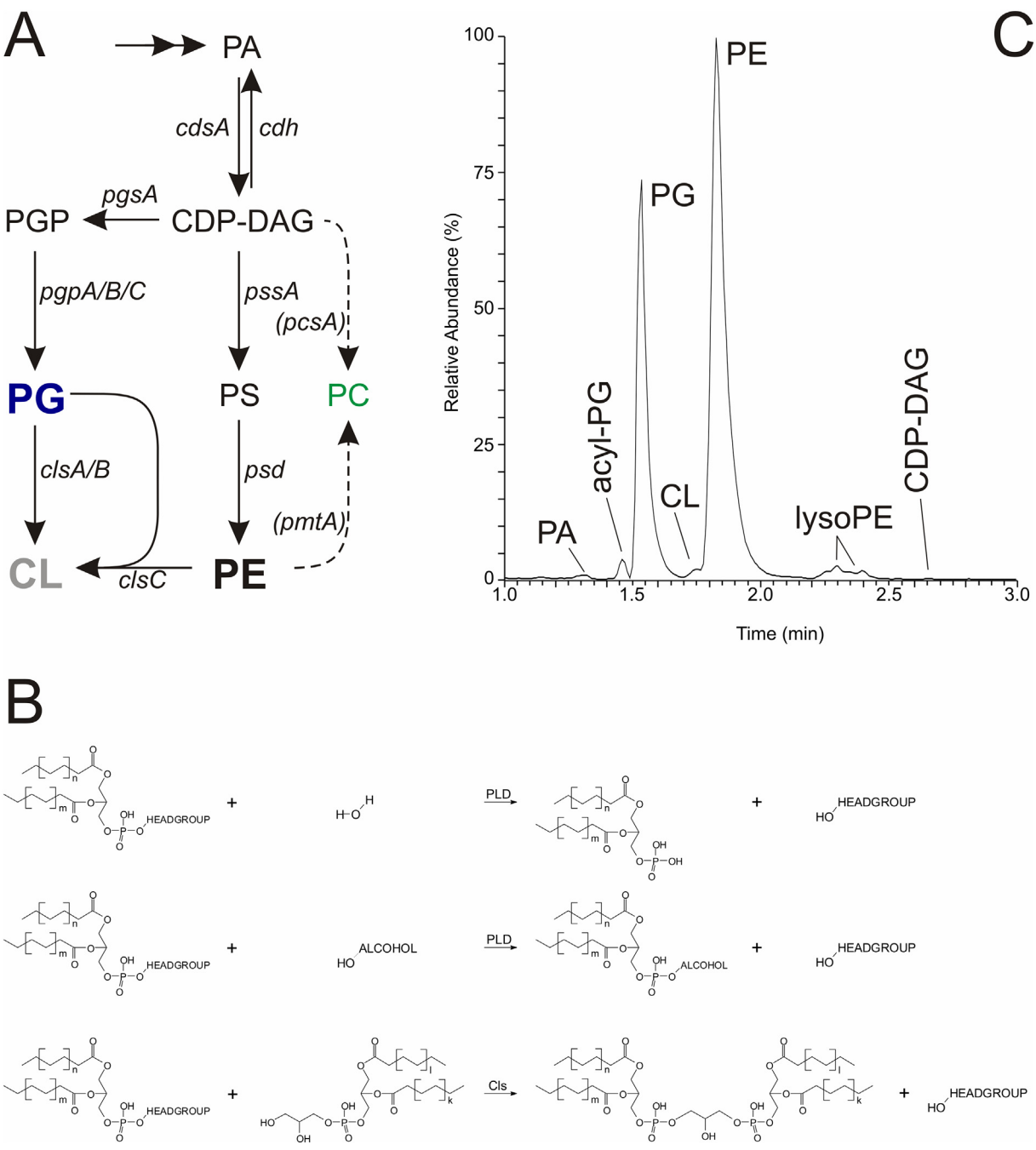

Fig. 1. Phospholipid synthesis in *E. coli* (BW25113). A: Overview of pathways and involved enzymes for phospholipid synthesis in *E. coli* (continuous arrows). Introduced enzymes are given in parenthesis and their reactions by dashed arrows. B: Phospholipase D (PLD) catalyzes the hydrolysis of the phosphodiester linkage in a phospholipid, liberating the headgroup (top). In the presence of primary alcohols, the alcohol is a preferred substrate, leading to a phosphatidylalcohol (middle). Cardiolipin synthase has a PLD activity using a phospholipid and a molecule of PG to synthesize CL. C: Lipid analysis by LC-MS of steady-state *E. coli* (BW25113). The phospholipidome consists mainly of PE (67%), PG (30%), and CL (2%). The remaining lipids consist of trace amounts of PA (< 0.1%), CDP-DAG (< 0.1%), lysoPE (< 0.1%), lysoPG (< 0.1%), PS (< 0.1%), and acyl-PG (< 0.1%).

phospholipid to generate phosphatidylalcohols. PLD enzymes have a much higher preference for primary alcohols as compared to water. Therefore, activity of PLD is often identified by using n-butanol or other primary alcohols followed by detection of phosphatidylbutanol (PBut) or other phoshatidylalcohols [12–15]. See Fig. 1B for the reactions performed by PLD enzymes and the formation of CL.

In this study we investigated PLD activity of the three Cls enzymes in *E. coli* by using individual overexpressing transformants and single knockout mutants. We tested primary alcohols and a variety of both native and a foreign phospholipid class as substrates. We show the Cls enzymes have distinct substrate specificity for their PLD activity in the presence of phosphatidylcholine (PC), a foreign lipid class that is not present in wild type *E. coli*. Our combined results demonstrate the plasticity of the *E. coli* lipidome as the bacterium remains viable in the presence of substantial amounts of phosphatidylalcohols and its capacity to metabolize foreign lipids, suggesting highly flexible adaptations towards different environmental conditions.

2. Materials and methods

2.1. Materials

Isopropyl β-D-1-thiogalactopyranoside (IPTG) was obtained from Melford (Suffolk, UK). NaCl, $MgSO_4$, $CaCl_2$, Na_2HPO_4, KH_2PO_4, Fe(II) $SO_4{\cdot}7H_2O$, formic acid and ethanol were purchased from Merck Chemicals (Darmstad, Germany). Chloramphenicol was obtained from Boehringer Mannheim (Mannheim, Germany). Trypton, agar, choline chloride, gentamycin, kanamycin, arabinose were purchased from Sigma (St. Louis, MO). Yeast extract and glycerol were obtained from MP Biomedicals (Strasbourg, France). NH_4Cl, n-octanol, n-propanol and n-butanol were purchased from J.T. Baker (Deventer, The Netherlands). CAS amino acids were obtained from ICN Biomedicals (Aurora, Ohio). Methanol, acetonitril, acetone and ammonium formate were purchased from BioSolve (Valkenswaard, The Netherlands), chloroform was obtained from Roth (Karlsruhe, Germany) and were all HPLC/MS grade.

2.2. Bacterial strains and plasmids

Plasmids for overexpressing the cls genes were isolated from AG1 (ME5305) host cells, part of the ASKA(−) collection [16], obtained from NBRP (NIG, Japan): *E. coli*. These pCA24N plasmids were used to transform BW25113 cells. The used knockout mutants are part of the Keio collection [17], obtained from NBRP (NIG, Japan): *E. coli*. The plasmid PAC-PCSlp-Sp-Gm (kind gift from Mikhail Bogdanov, Ph.D) [18] was used for transforming BW25113 cells and expression the pcsA gene. The plasmid pET_PmtA (kind gift from Meriyem Aktas, Ph.D) [19] was used for expression of the pmtA in BL21 cells.

2.3. Growth conditions

Liquid LB medium (10 g/l Trypton, 10 g/l NaCl, 5 g/l yeast extract) was used in the pre-cultures of *E. coli*. For solid medium 15 g/l agar was added. The main-cultures were grown using M9 minimal medium (6 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 0.5 g/l NaCl, 1 g/l NH_4Cl, 2 g/l CAS amino acids, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 3 mg/l Fe(II)$SO_4{\cdot}7H_2O$) and 0.5% glycerol (v/v) as carbon source. All growth was performed at 37 °C.

For strain selection purposes medium was supplemented with 34 μg/ml chloramphenicol or 50 μg/ml kanamycin or 10 μg/ml gentamycin, or a combination of these three. Final concentration of 40 μM IPTG was used for induction of the cls proteins expression since the ORF on the pCA24N plasmid is under control of IPTG-inducible promoter, P T5-*lac*. Plasmid borne pmtA expression was achieved by induction of host chromosome born T7 RNA polymerase by a final concentration of 40 μM IPTG. Final concentration of 0.2% arabinose was used for induction of the PC synthase since the pcsA gene is under induction control by arabinose of the promoter ParaB. 2 mM choline was provided for PC synthesis, unless otherwise noted. 150 μl cultures were grown in 96 wells plates, in an orbital shaker. Alcohols were added in noted concentrations, from the start of the main culture. The plate was covered using a clear film.

2.4. Lipid extraction

Bacterial cultures were transferred to glass coated 96 wells plates with conical bottom, and centrifuged (1800*g*, 20 min, 4 °C). The obtained pellets were resuspended in 150 μl chloroform/methanol (1:1 v/v), extracted for 1 h at 4 °C, followed by centrifugation (1800*g*, 20 min, 4 °C). The plate was covered and placed in the autosampler.

2.5. Liquid chromatography mass spectrometry of lipids

Chromatography of 10 μl of the supernatant was performed on a hydrophilic interaction liquid chromatography (HILIC) column (2.6 μm HILIC 100 Å, 50 × 4.6 mm, Phenomenex, Torrance, CA), by elution with a gradient from ACN/Acetone (9:1, v/v) to ACN/H2O (7:3, v/v) with 10 mM ammonium formate, and both with 0.1% formic acid. Flow rate was 1 ml/min. The column outlet of the LC was either connected to a heated electrospray ionization (HESI) source of a LTQ-XL mass spectrometer or a Fusion mass spectrometer (both from ThermoFisher Scientific, Waltham, MA). Full scan spectra were collected from m/z 350–1750 at a scan speed of 3 scans/s (LTQ-XL). On the Fusion full spectra were collected from m/z 400 to 1600 at a resolution of 120.000. Parallel data dependent MS2 was done in the linear ion trap at 30% HCD collision energy.

2.6. Data analysis

Data were converted to mzXML or mzML format and analyzed using XCMS version 1.52.0 running under R version 3.4.3 (R Development Core Team: A language and environment for statistical computing, 2016. URL http://www.R-project.org). Principle Component Analysis provided by the R package pcaMethods [20] was used to visualize the multidimensional LC-MS data.

3. Results

3.1. Wild type E. coli displays no phospholipid headgroup exchange activity

Under normal growth conditions, the phospholipidome of *E. coli* consists of approximately 70% PE, 30% PG and 2% CL, as determined by LC-MS (Fig. 1C, in agreement with previous reports [1]). Small amounts of CDP-DAG, PA, and lyso-PE were also detected (Fig. 1C), but as these lipids contributed only in trace amounts to any of the experiments, they were excluded from further analyses.

To identify PLD activity other than CL synthesis in *E. coli* BW25113, bacterial cells were grown in the presence or absence of a variety of primary alcohols and subsequently analyzed for the appearance of the corresponding phosphatidylalcohols by LC-MS. In the presence of 0.5% n-butanol (v/v, corresponding to 55 mM), a known substrate for PLD mediated headgroup exchange [13], *E. coli* growth was inhibited by approximately 50% but no detectable formation of PBut was observed (not shown). Similar results of moderate inhibition of bacterial growth without the presence of phosphatidylalcohols were also obtained with other primary alcohols (data not shown). Small adaptations of the *E. coli* phospholipidome to the presence of these primary alcohols were found as revealed by principal component analysis (PCA) of the phospholipidome (Fig. 2). In PCA, the *n*-dimensional complexity of the lipidome, resulting from the presence of *n* molecular species, is reduced by the construction of a set of principal components (PC-1, PC-2, …) that are linear combinations of (correlating) lipid species. The resulting PCA score plot (Fig. 2, left panel) shows the location of the lipidome of the analyzed samples, mapped to the two principle components that best represent the original variance present in the sample's lipidomes. Hence, similar lipidomes map close together, as seen for the each alcohol's triplicate cultures in Fig. 2. The corresponding loading plot (Fig. 2, right panel) visualizes how individual lipid species contribute to the differences of the lipidomes: species at the origin do not contribute to this variance, species furthest away may be considered marker lipids. The changes in lipidomes after growth in the presence of various alcohols, did not involve the formation of respective phosphatidylalcohols, but consisted of a limited change in the level of many pre-existing and common lipid species (Fig. 2, right panel).

3.2. Alteration in Cls enzymes expression affects the phospholipidome of E. coli

The *E. coli* genome contains three CL synthases and we investigated the contribution of the individual Cls enzymes to the lipidome. Therefore, we analyzed the lipid profiles of *E. coli cls* knockout mutants and transformants overexpressing one of the individual Cls isoforms

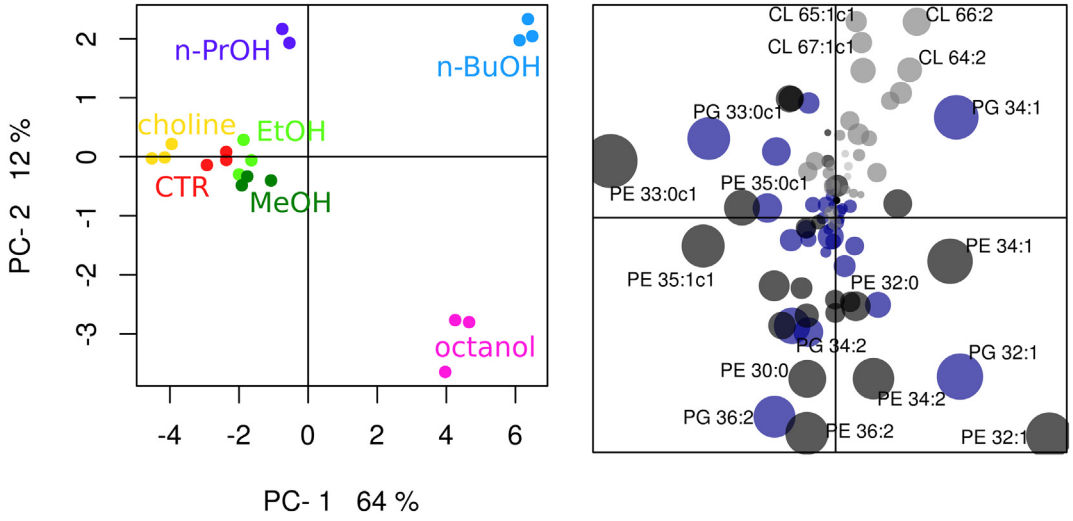

Fig. 2. Minor adaptations of the phospholipidome of *E. coli* when grown in the presence of different alcohols. Multiple primary alcohols (methanol (MeOH, dark green), ethanol (EtOH, light green), n-propanol (n-PrOH, blue), n-butanol (n-BuOH, light blue), each at 55 mM, choline (yellow) at 2 mM and octanol (pink) at 5.5 mM) were supplied to the medium of *E. coli* BW25113, and bacteria were grown to stationary phase. After extraction, lipids were analyzed using LC-MS and Principle Component Analysis (PCA). "CTR" indicates that no alcohol was added to the growth medium (red). No phosphatidylalcohols were detected. The left panel shows the score plot, the right panel shows the loading plot of the PCA in which lipid species are colored based on their phospholipid class (PE, PG, CL, dark grey, dark blue, grey, respectively).

ClsA, ClsB, or ClsC. In accordance with literature, all three knockout mutants still produce CL, presumably due to the presence of two other Cls enzymes (Fig. 3A). A modest increase in total CL content was observed in the strains overexpressing either ClsB or ClsC (from 2.0% to 3.5%, and 2.9%, respectively, $p = 0.003$ and 0.0006). An unexpected but minor decrease of CL content was observed in the ClsA overexpression (from 2.0% to 1.0%, $p = 0.006$). Overexpression of ClsB also led to an increase of PE (from 67% to 79%, $p < 0.005$,) and a decrease of PG content (from 31% to 14%, $p < 0.005$). PE and PG levels remained unaltered upon overexpression of ClsA or ClsC.

PCA of the corresponding CL species revealed enzyme specific changes in the CL species composition, demonstrating that the three Cls enzymes produce distinct CL species profiles (Fig. 3B and C). The transformant with the empty vector, used for overexpression of the Cls enzymes, also lead to a small change in CL species composition, but not to a change in lipid class distribution (Fig. 3B and C).

3.3. Overexpression of ClsB, but not ClsA or ClsC, leads to formation of phosphatidylalcohols

To investigative if overexpressing any of the Cls enzymes leads to formation of phoshatidylalcohols in WT *E. coli*, the individual Cls enzymes were overexpressed in the presence of n-butanol. Overexpression of ClsA and ClsC did not lead to the formation of PBut or any other foreign phospholipid, but overexpression of ClsB led to a significant production of PBut (Fig. 4). The presence of phosphatidylethanol (PEth) resulted in all likelihood from the fact that the antibiotics used in these experiments were dissolved in ethanol before addition to the bacterial culture, resulting in approximately 0.1% ethanol in the growth medium. Remarkably, incorporation of these large amounts (> 10%) of non-physiological phospholipid classes into the membranes did not hamper growth rate or biomass production ($p = 0.46$ and 0.36 respectively).

As shown in Fig. 5, formation of phosphatidylalcohol was dependent on the induction of ClsB expression by IPTG and the presence of an alcohol in the culture medium (ethanol and n-butanol in Fig. 5G and J). Other primary alcohols, ranging from methanol to octanol and choline, were also shown to be substrates for ClsB, albeit with varying degree of efficiency of the respective phosphatidylalcohol synthesis (Supplementary Fig. 1). Although the species composition of the non-physiological phosphatidylalcohol classes were slightly distinct from one another, the main molecular species of PE and PG resembled the main species of the phosphatidylalcohols. Since all tested primary alcohols were substrates for the ClsB mediated PLD reaction, we expanded the tested substrates to more complex alcohols. The secondary alcohols 2-propanol and inositol as well as the tertiary alcohol *tert*-butanol did not lead to synthesis of their corresponding phosphatidylalcohols, as expected for PLD reactions (data not shown).

3.4. The foreign phospholipid PC as a substrate for PLD activity

We have shown that ClsB can use primary alcohols and a bacterial phospholipid for PLD activity and subsequent synthesis of significant amounts of non-physiological phospholipids. This raises the question whether non-physiological phospholipid classes can also be used as a PLD substrate by any of the Cls enzymes. This may be particularly relevant in the metabolism of foreign phospholipids that *E. coli* encounters. We therefore introduced the foreign phospholipid PC in *E. coli* as a potential substrate for PLD activity. This phospholipid is abundantly present in mammalian cells but absent in WT *E. coli* (Fig. 1C). To this end, WT *E. coli* was transformed with a plasmid encoding PC synthase (pcsA) from *Legionella pneumophilia* [18]. This PC synthase uses CDP-DAG and choline to produce PC. PC became one of the most abundant lipids in the transformed *E. coli* strain at the expense of a reduced biomass in the stationary phase (Supplementary Fig. 2).

As shown in Fig. 6 and Supplementary Fig. 2, synthesis of PC is dependent on the addition of choline to the medium. Surprisingly, the additional presence of n-propanol as a primary alcohol to this PC-containing *E. coli* leads to the formation of phosphatidylpropanol (PPro) as a lipid class without overexpression of any of the Cls enzymes (Fig. 6). No synthesis of PPro was observed when PC formation was prevented by abolishing choline from the medium. This indicates that the presence of PC is essential for the observed PLD activity. Additional evidence for the PC requirement of phosphatidylalcohol synthesis was obtained by varying the amount of PC and determining the effect on phosphatidylalcohol levels in *E. coli*. To this end, *E. coli* was grown with various concentrations of choline and at a constant concentration of n-butanol. Under these conditions a choline-concentration dependent increase was observed in both PC and PBut (Fig. 6B), at the expense of PE and PG concentrations (data not shown). Thus, in the presence of PC

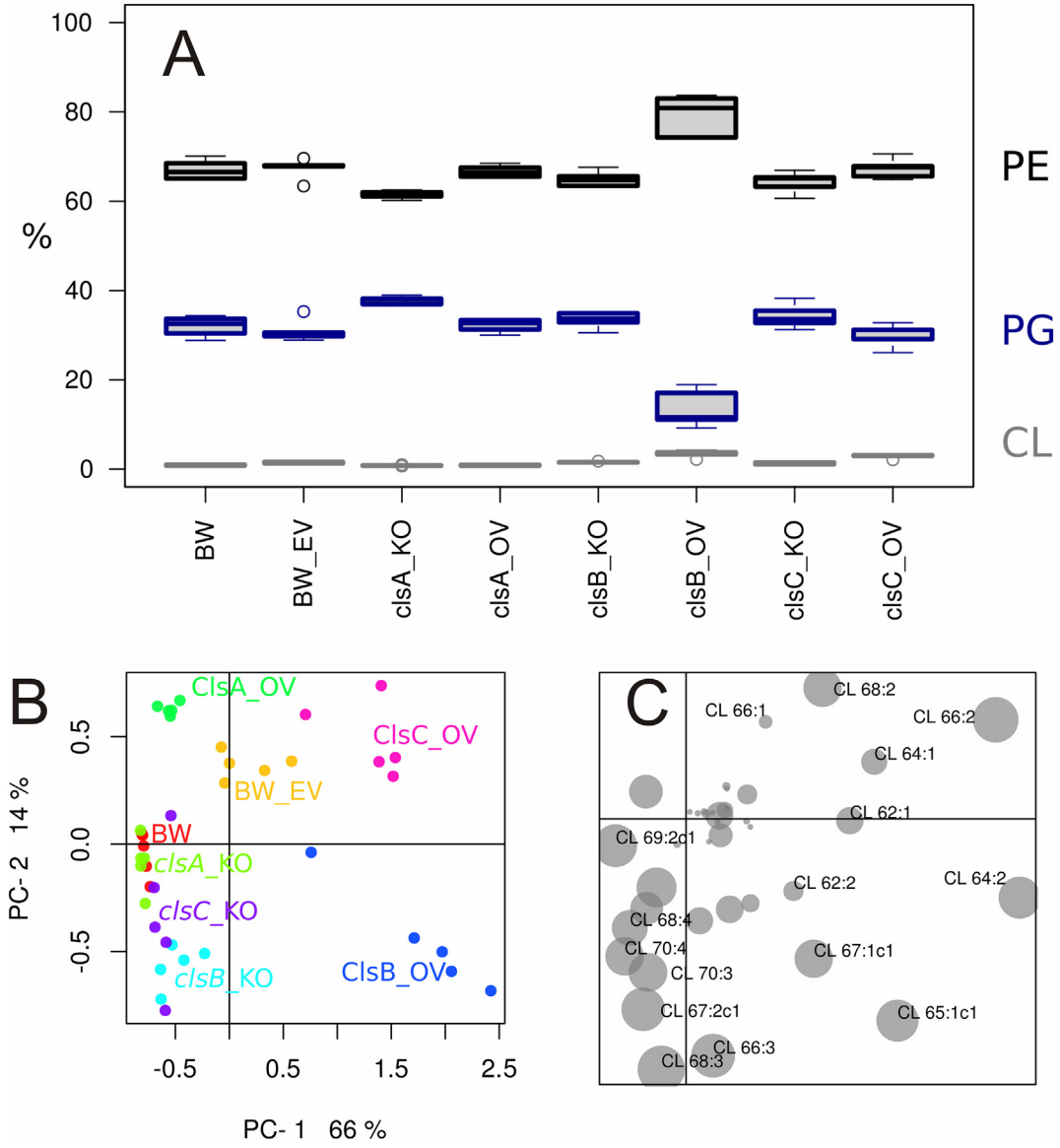

Fig. 3. Effect of knocking out cls genes or overexpressing the Cls enzymes on lipid class distribution and CL species in *E. coli*. cls knockout mutants ("KO") and strains overexpressing Cls enzymes ("OV") (40 μM IPTG final concentration) were grown to stationary phase and subsequently their lipids analyzed by LC-MS. For comparison lipid analysis of WT ("BW") and WT plus the empty vector ("BW_EV") were included (panel A). All three individual cls deletion strains still synthesize CL. PCA is depicted in panel B and C, showing the score plot and the corresponding loading plot, respectively. ClsC overexpression led to an increase in those CL species that are found in the top right corner of the loading plot, for ClsB overexpression the CL species in the bottom right corner of the loading plot were preferred products.

and primary alcohols, *E. coli* can synthesize phosphatidylalcohols without the need for overexpressing any of the Cls enzymes. To exclude the formal possibility that pcsA is able to directly synthesize a variety of phosphatidylalcohols itself, either *via* an unexpected PLD activity of the PcsA enzyme or a substitution of n-propanol for choline in the biosynthetic pathway of PC, we applied an alternative route for PC synthesis by using the PE methylation pathway. Phosphatidylethanolamine *N*-methyltransferase (PmtA) from *Agrobacterium tumefaciens*. PmtA, shown to be functional in *E. coli* upon recombinant expression, is a PE methylating enzyme capable of methylating PE three times to form PC [19]. Indeed expression of pmtA led to the formation of PC, albeit in lower quantities than with pcsA (Supplementary Fig. 3). Addition of n-propanol as a primary alcohol to the pmtA expressing strain again led to synthesis of the corresponding phosphatidylalcohol (Supplementary Fig. 3). This confirms that the presence of PC allows for the synthesis of phosphatidylalcohols without overexpressing any of the Cls enzymes.

3.5. clsA deletion leads to abolishment of PC dependent phosphatidylalcohol formation

We then investigated which of the three Cls isoenzymes was responsible for this PC dependent PLD activity in *E. coli*. Therefore, each of the three *cls* knockout mutants was transformed with the pcsA plasmid. All three were capable of synthesizing PC in abundant quantities (Fig. 7). However, addition of a primary alcohol no longer led to phosphatidylalcohol formation in the *clsA* knockout mutant whereas levels of phosphatidylalcohols were unaltered in the *clsB* and *clsC* knockout mutants.

Taken together our results show that depending on substrate availability (both phospholipids and primary alcohols) different Cls enzymes can execute their PLD activity.

4. Discussion

Wild type *E. coli* has three different Cls enzymes, all capable of

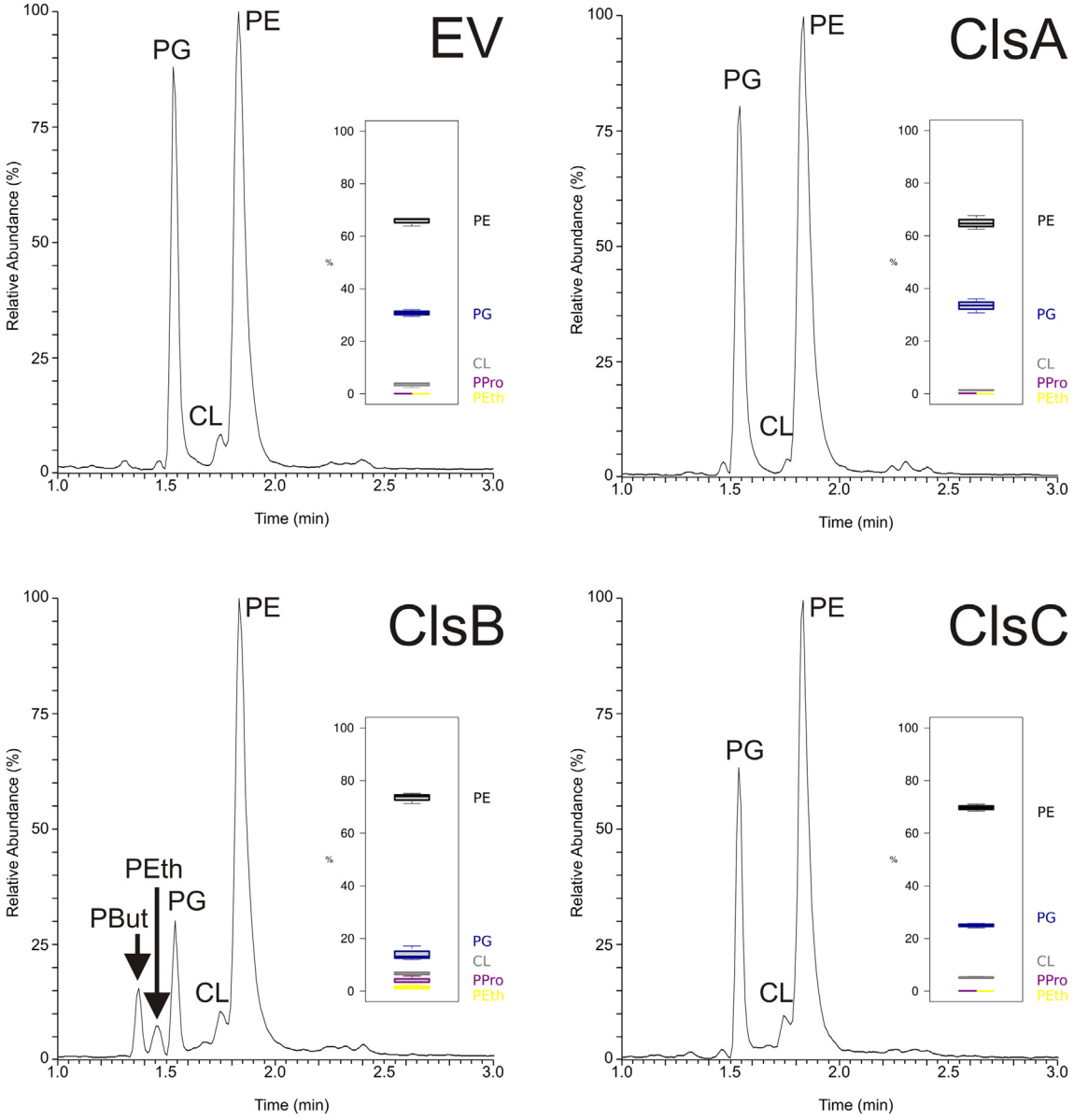

Fig. 4. Overexpression of ClsB results in the formation of phosphatidylalcohol. Base peak chromatograms of lipids from *E. coli*, grown to stationary phase in the presence of 40 μM IPTG 55 and mM n-butanol, harbouring the empty vector ("EV") or overexpressing one of the individual Cls enzymes ("ClsA", "ClsB", "ClsC"). Eluting directly before PG is acyl-PG, the abundance of which did not change upon overexpression of any of the Cls enzymes. Only the ClsB overexpressing strain resulted in the synthesis of phosphatidylalcohols.

synthesizing CL [8] and thereby exhibiting PLD activity. Under normal wild type conditions, *i.e.* with endogenous expression levels of the different Cls enzymes and native lipids in combination with primary alcohols, formation of phoshatidylalcohols could not be demonstrated. Here we presented two different conditions that lead to synthesis of phosphatidylalcohols as a measure of PLD activity: i) overexpression of ClsB demonstrates PLD activity using endogenous phospholipids and primary alcohols, which is not observed with endogenous expression levels of ClsB; and ii) in the presence of PC as a foreign lipid, PLD activity was demonstrated at endogenous expression levels of the ClsA enzyme.

E. coli has a marked redundancy in CL synthesis as none of the Cls enzymes is essential [8]. We indeed found that CL levels remained mostly unaltered when the individual *cls* genes were deleted (Fig. 3). The observed increase in CL levels upon overexpression of ClsB coincided with a decrease in PG levels ($p < 0.005$) and an increase in PE ($p < 0.005$). For ClsC overexpression there was surprisingly also an increase in CL levels but no statistically significant change in PG nor PE ($p = 0.43$ and 0.98 respectively). The synthesis of one molecule of CL goes at the expense of two molecules of PG and/or PE. It is surprising that ClsC overexpression did not lead to a lower PE content, as ClsC is believed to synthesize CL form PE and PG in a 1:1 ratio. On the other hand, the reduction in PG (compared to the *clsC* KO) is more than twice the increase in CL, so CL synthesis was not the only process contributing to PG reduction. Which other processes lead to this reduction and how big this effect is compared to the increased CL synthesis, remains unclear. These data, therefore, do not necessarily contradict the concept of ClsC using PE and PG in a 1:1 ratio. Overexpression of ClsA did not lead to an increase of CL levels as was observed after ClsB or ClsC overexpression. When we investigated the lipidomic phenotypes of overexpressing Cls enzymes or the knockout mutants, we observed only modest changes to the CL species fingerprint. This was corroborated by the fact that even overexpression of the empty vector alone already led to a phenotypic shift in the CL-specific lipidome (from

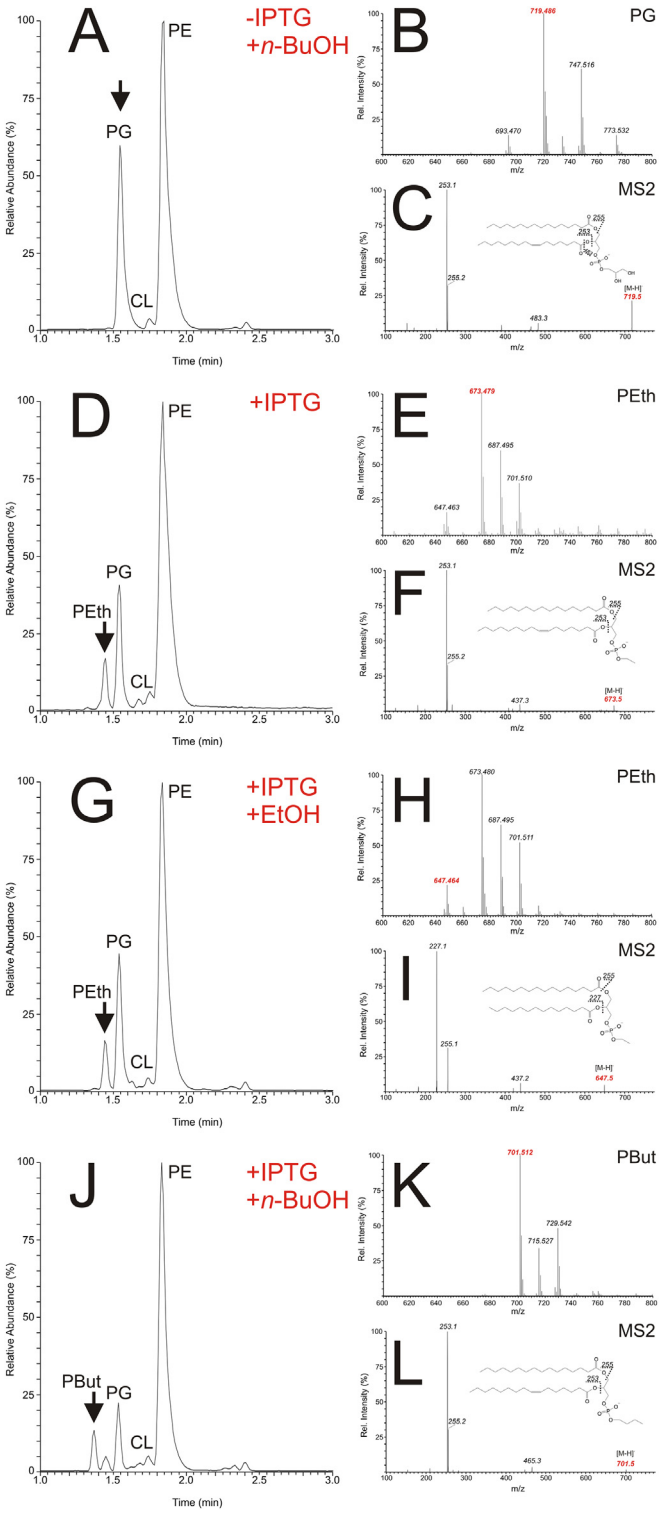

Fig. 5. Synthesis of phosphatidylalcohols upon overexpression of ClsB in *E. coli*. The ClsB overexpressing strain was grown to stationary phase in the absence (panels A–C) or presence (panels D–L) of IPTG, with 55 mM n-butanol (panels A–C, J–L) or with 55 mM ethanol (panels G–I) followed by lipid extraction and LC-MS/MS analysis. No phosphatidylalcohols were observed in the absence of IPTG (panel A). Under these conditions a full scan- and a fragmentation spectra of PG/PG 32:1 are shown (panels B, C). Induction of ClsB expression led to the formation of PEth (Panel D), and full scan- and a fragmentation spectra of a PEth 32:1 are shown (panels E, F). Addition of both ethanol and IPTG lead to a higher PEth content (panel G), and full scan- and a fragmentation spectra of PEth 30:0 are shown (panels H, I). Addition of both n-butanol and IPTG led to PEth and PBut formation (panel J), and full scan- and a fragmentation spectra of PBut 32:1 are shown (panels K, L).

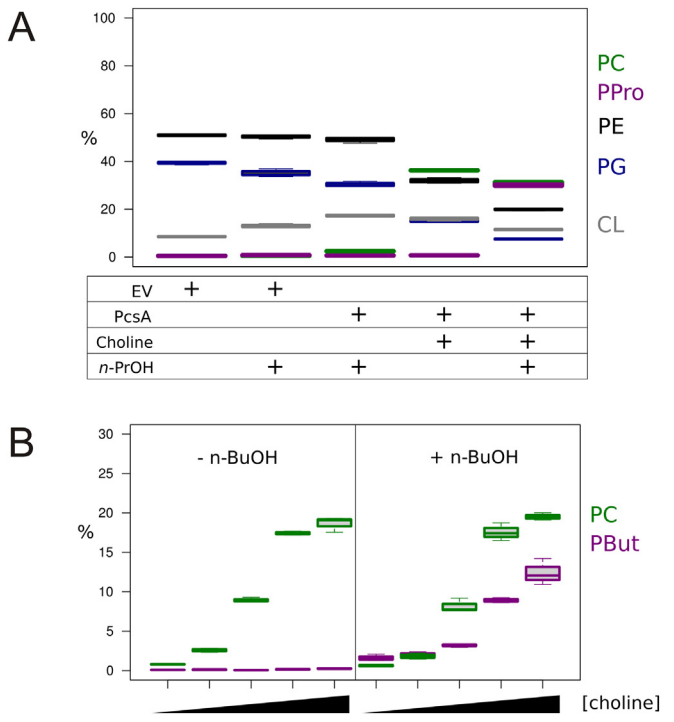

Fig. 6. The foreign lipid classes PC and PPro become the most abundant lipid classes upon PC synthase expression in the presence of propanol and are dependent on choline. *E. coli* transformed with empty vector ("BWEV", lane 1 and 2) or with the PC synthase containing plasmid ("PC synthase" lane 3–5) were grown to stationary phase (panel A). Expression of the PC synthase was induced using 0.2% arabinose, choline was supplied (2 mM) for PC synthesis (lane 4 and 5), and 55 mM n-propanol was added (lane 2, 3, and 5). *E. coli* with only the control plasmid did not produce PC nor PPro (lane 1 and 2). Induction of the PC synthase, without available choline did not result in production of PC nor PPro (lane 3). Addition of choline resulted in abundant PC production (lane 4). Only addition of both n-propanol and choline resulted in production of both PC and PPro (lane 5). PC and PBut distribution of *E. coli* is shown in panel B. *E. coli* transformed with the PC synthase was grown using different concentrations of choline (0.0 0.02, 0.2, 2.0, 10 mM) (lane 1–5, and lane 6–10), without n-butanol (lane 1–5) and plus 0.75% n-butanol (lane 6–10). PC production is dependent on the concentration of choline (lane 1–5). When supplied with n-butanol, PBut was synthesized in a choline concentration dependent manner (lane 6–10).

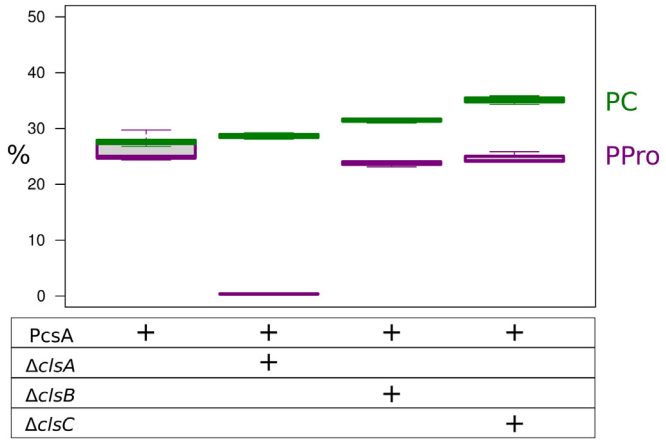

Fig. 7. The PC headgroup exchange activity is abolished in the clsA knockout mutant. Foreign phospholipid class distribution in *E. coli* expressing the PC synthase (lane 1), and the cls knockout mutants expressing the PC synthase (lane 2–4), were grown under pcsA inducing conditions, supplied with choline and 55 mM n-propanol. All strains produced PC (lane 1–4), only the knockout mutant of clsA did no longer produce PPro.

−0.782 ± 0.032 to 0.158 ± 0.284 on principal component 1, Fig. 3B) that was at least comparable to the any of the *cls* deletions (Fig. 3B).

The fact that we observed a change in the lipidome of cells exposed to primary alcohols (Fig. 2) demonstrates that these molecules have an effect on normal physiological processes in *E. coli*. Alcohols can change the fluidity of the lipid bilayer and that *E. coli* can adapt to this by changing the lipidome have been observed before by others [21–23]. The alcohols we tested had different effects on the lipid composition as can be concluded from the PCA score plots (Fig. 2, left panel) where the various alcohols do not overlap. The nature of this variable effect, however, is not a result of these alcohols being substrates for PLD reactions, as none of these alcohols were introduced as a foreign headgroup in the phospholipidome.

ClsB-mediated enzymatic trans-phosphatidylation was demonstrated recently both *in vivo* and *in vitro* [10]. Here, we demonstrated, in addition to endogenous phospholipids, that foreign phospholipid headgroups can be introduced upon overexpression of ClsB, but not ClsA or ClsC, in the presence of a primary alcohol (Fig. 4). From this it can be concluded that i) only ClsB can use endogenous phospholipids and short chain primary alcohols as substrates leading to phospholipid headgroup exchange and ii) ClsB activity in WT *E. coli* is very low, as PBut is hardly visible in WT *E. coli* expressing only the empty vector or one of the other Cls enzymes. Indeed, ClsB levels have been reported before to be very low in WT *E. coli* [9]. Overexpression of ClsB and the concomitant synthesis of PBut did not result in a different growth rate ($p = 0.46$) nor in a difference in the maximum OD600 in the stationary phase ($p = 0.36$), demonstrating a remarkable tolerance of *E. coli* for foreign phospholipids in its membranes. The presence of PEth is a first indication that PLD activity of ClsB has a broad substrate specificity. Indeed, both shorter (methanol, ethanol, n-propanol and n-butanol) as well as longer alcohols (octanol) led to synthesis of new, foreign, lipid classes in *E. coli* when ClsB was overexpressed (Fig. 5 and Supplementary Fig. 1).

Tolerance for foreign lipid classes was exemplified when *Legionella pcsA* was expressed in *E. coli* in the presence of n-propanol (Fig. 6). The two most abundant phospholipid classes under these conditions, PC and PPro, are both foreign lipid classes to *E. coli* and together comprised two third of the total phospholipidome, without interfering with a steady cell growth. The tolerance to incorporate foreign lipids of *E. coli* has been shown before by others by introducing phosphatidylinositol, glycosyl-diacylglycerol, diglycosyl-diacylglycerol, and o-lysyl-phoshatidylglycerol, [24–27] however, the formation of phosphatidylalcohols by endogenous enzymes in *E. coli* has so far not been reported.

In the presence of PC, the synthesis of PPro had become independent of ClsB overexpression. It thus seems likely that WT *E. coli* has, next to ClsB, a second PLD activity that uses PC, but not endogenous PE or PG as a substrate for headgroup exchange. To confirm this we first ruled out the possibility that PPro was a direct product of the expressed *Legionella pcsA*, by substituting *pcsA* for *pmtA* as an alternative for PC synthesis. Indeed PPro synthesis was preserved, albeit now at much lower levels for both foreign phospholipid classes. The correlation between PC levels and phosphatidylalcohol levels was firmly established when PC levels were controlled by regulating choline availability (Fig. 6B). ClsA was the most likely candidate for this PC dependent PLD activity since the CL synthases are the only enzymes in *E. coli* with the PLD specific double HKD motif, and ClsA is by far the most active Cls under these conditions [8]. Deletion of *clsA* indeed abolished the PC dependent PPro synthesis, whereas deletion of one of the other *cls* genes did not (Fig. 7). Hence, we conclude that under these conditions ClsA is capable to perform a headgroup exchange, leading to phosphatidylalcohols.

The mechanism of action in the presence of PC remains unclear. It is attractive to speculate that PC may serve as a direct substrate for ClsA, thus resulting in a direct conversion of PC to phosphatidylalcohol. The fact that we did not observe a decrease in PC levels suggests that either PC is not a substrate or that PC synthesis is upregulated under these conditions. The abundant presence of the foreign lipid PC may induce conformational changes in the ClsA enzyme which has two predicted transmembrane domains [8]. A resulting loss of PG specificity in CL synthesis, could then result in a gain of headgroup exchange activity for ClsA. Further research is required to elucidate the mode of action of ClsA in the presence of PC.

Taken together, we have demonstrated that ClsA is able to produce phosphatidylalcohols in the presence of a foreign lipid class, but not when only endogenous phospholipids are present. ClsB, on the other hand, clearly is able to perform headgroup exchange on endogenous phospholipids, as we demonstrated for a variety of primary alcohols. For ClsC, we could not demonstrate such headgroup exchange for either an endogenous or foreign phospholipid class. However, the distinct species composition of CL after ClsC overexpression suggests that the Cls enzymes prefer distinct substrates. Thus, the various Cls enzymes each utilize their own distinct lipid pool.

Apart from a first demonstration of PLD activity resulting in foreign lipid classes in *E. coli*, our findings open up the possibility for this micro-organism as a production platform for a large variety of phospholipid classes using only endogenous enzymes and primary alcohols.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Transparency document

The Transparency document associated with this article can be found in the online version.

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