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Uptake and remodeling of exogenous phosphatidylethanolamine in *E. coli*

Matthijs A. Kol[a], Diederik W.D. Kuster[a], Henry A. Boumann[a], Hans de Cock[b], Albert J.R. Heck[c], Ben de Kruijff[a], Anton I.P.M. de Kroon[a,*]

[a] *Department of Biochemistry of Membranes, Institute of Biomembranes, Ctr. Biomem. and Lipid Enz. (CBLE), Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands*
[b] *Department of Molecular Microbiology, Institute of Biomembranes, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands*
[c] *Department of Biomolecular Mass Spectrometry, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands*

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Abstract

The fate of exogenous short-chain analogues of phosphatidylethanolamine and phosphatidylserine was studied in a deep-rough derivative of *E. coli* mutant strain AD93 that cannot synthesize phosphatidylethanolamine de novo. Using mass spectrometry, it was shown that dicaproyl(di 6:0)-phosphatidylethanolamine is extensively remodeled, eventually adopting the phosphatidylethanolamine species profile of the parental wild-type strain of AD93. Dicaproyl-phosphatidylserine was decarboxylated to form phosphatidylethanolamine, and yielded a species profile, which strongly resembled that of the introduced phosphatidylethanolamine. This demonstrates transport of phosphatidylserine to the cytosolic leaflet of the inner membrane. The changes of the species profile of phosphatidylethanolamine indicate that the short-chain phospholipids are most likely remodeled via two consecutive acyl chain substitutions, and at least part of this remodeling involves transport to the inner membrane.

Keywords: Phosphatidylethanolamine; Phosphatidylserine; Cytosolic leaflet

1. Introduction

Newly synthesized membrane phospholipids (PL) are often remodeled in their acyl chains. The combined action of phospholipases A (PLAs) and acylating enzymes create new molecular species which, in eukaryotes, have been shown to play important roles in signaling processes (see, e.g. Ref. [1] for a recent review). Remodeling requires an acyl chain donor, and an accepting (lyso)phospholipid. The donor can either be an existing phospholipid, or an activated acyl chain on a carrier, and various transacylation activities with distinct substrate specificities result in a plethora of PL classes and species [2]. Creating a lysophospholipid requires PLA activity, which has been demonstrated in various cellular fractions of eukaryotes (reviewed in Ref. [3]) and also in prokaryotes (see, e.g. Ref. [4]). Henk van den Bosch and his collaborators have studied in-depth the PLA activity in mitochondria (see, e.g. Ref. [5]). The ubiquitous presence of phospholipases suggests that lipid remodeling is evolutionary conserved.

Indeed, lipid remodeling has also been shown to occur in the prokaryotes. Hellion et al. [6] showed that lysophosphatidylethanolamine is converted to its diacyl analogue by both the inner and outer membrane fractions of *E. coli*. Transacylation [7] and reacylation of lysophospholipids via acyl-acyl carrier protein (ACP) [8] and acyl-CoA [9] have been described. Apart from these early studies, very little is known about lipid remodeling reactions in bacteria, despite the growing interest in and importance of remodeling reactions in eukaryotes.

Using mass spectrometry, in this study we analyzed remodeling of phosphatidylethanolamine (PE), the most abundant phospholipid in wild-type *E. coli*. This technique nowadays provides a powerful tool for analyzing lipid species compositions with high sensitivity [10,11]. The approach we took was to follow the fate of exogenous

Abbreviations: ACP, acyl carrier protein; CFA, cyclopropane fatty acid; CoA, coenzyme A; DCPE, dicaproylphosphatidylethanolamine; DCPS, dicaproylphosphatidylserine; LPS, lipopolysaccharide; PE, phosphatidylethanolamine; Pi, inorganic phosphate; PL, phospholipid(s); PLA, phospholipase A; PS, phosphatidylserine

* Corresponding author. Tel.: +31-30-2533-424; fax: +31-30-2533-969.
E-mail address: a.i.p.m.dekroon@chem.uu.nl (A.I.P.M. de Kroon).

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short-chain PE and PS in growing cells of *E. coli* strain AD93 [12]. This strain has a disrupted PS synthase (*pss*) gene, and therefore cannot synthesize PE. AD93 requires divalent cations for growth, which together with the increased levels of cardiolipin can provide the membrane with the essential intrinsic curvature stress normally provided by PE in wild-type *E. coli* [13]. The absence of endogenous PE facilitates the detection of PE taken up by the cells. Being the predominant phospholipid in *E. coli* under wild-type conditions, it serves as an acyl chain donor for the outer membrane lipoprotein LPP [14], though it is not the exclusive donor as was shown in a PE-deficient strain [15]. PE and other phospholipids also act as acyl chain donors for the lipopolysaccharide (LPS) precursor lipid X [16,17]. Additionally, PE has been shown to be important for membrane protein insertion [18,19]. Therefore, knowledge of the fate of exogenously added PE might eventually provide a tool to restore functions that are blocked or reduced in a PE-deficient background.

To increase the passive uptake of lipids from the medium, we constructed a deep-rough mutant of AD93 with a LPS moiety of reduced length, which results in reduced barrier property of the outer membrane and thus increased uptake, as has been shown for *E. coli* [20] and *Salmonella* [21]. It is demonstrated that exogenously added short-chain PE is remodeled, most likely via two sequential deacylation/reacylation steps, to the wild-type PE profile of the parent strain of AD93. Short-chain PS is rapidly decarboxylated to form PE, which is also remodeled towards a lipid species profile resembling that of the wild-type, demonstrating that remodeling occurred subsequent to a localization of PS in the inner membrane. Uptake of PE results in a partial loss of the auxotrophy for divalent cations, indicative for functional integration of PE in the membrane fraction.

2. Materials and methods

2.1. Chemicals

$MgCl_2$ (Baker, 1 M stock solution) and kanamycin A (Sigma, a 10 mg/ml stock solution) were dissolved in water, filter sterilized (0.2 μm filters, Schleicher&Schuell) before use, and stored at RT and −20 °C, respectively. Dicaproylphosphatidylethanolamine (DCPE, di-6:0 PE) and dicaproyl-phosphatidylserine (DCPS, di-6:0 PS) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), dissolved as a 250 mM stock solution in ethanol and stored at −20 °C. All other chemicals used were analytical grade or better.

2.2. Growth of bacteria and isolation of membranes

Wild-type parent strain *E. coli* W3899 (F^+ *nadB7 supE*) and CE 1229 [22] were grown in LB medium (10 g of Bacto-tryptone (Difco), 5 g of Bacto-yeast extract (Difco) and 10 g of NaCl/liter supplemented with 0, 25 or 50 mM $MgCl_2$ as indicated. AD93 (*pss93::kan recA, srl::*Tn10 $nadB^+$, derivative of W3899) [12] and deep-rough AD93 (drAD93, see forthcoming) were grown in LB medium supplemented with 50 μg/ml kanamycin and 25 or 50 mM $MgCl_2$ (final concentration) as indicated. In short-time incubations, cells were grown to an OD_{600} of 1.2 at 50 mM Mg^{2+}, diluted 1:1 with LB medium without Mg^{2+}, and incubated for 0–12 h. To study remodeling of DCPE by growing drAD93, a pre-culture of drAD93 was grown at 50 mM Mg^{2+} to an OD_{600} value of 0.5, and subsequently diluted to OD_{600} 0.05 and grown for 24–72 h at 25 mM Mg^{2+}. Growth media were supplemented with 250 μM DCPE or DCPS unless indicated otherwise. Cells were grown at 37 °C under continuous shaking, and harvested at different time points by centrifugation for 10 min at 10,000 rpm in a Sorvall RC-5 with SS-34 rotor. The pellet was washed with 10 ml 0.9% (w/v) NaCl, pelleted and resuspended in the same solution.

2.3. Isolation and characterization of drAD93

AD93 was grown overnight to the stationary phase. An aliquot of 100 μl cell suspension was added to 4 ml 0.8% LB-agar solution at 45 °C, containing 100 μl P1-phage stock (10^9 phages/ml), 50 mM $MgCl_2$ and 50 μg/ml kanamycin. The molten agar cell suspension was poured onto a LB-agar/kan/Mg plate, and individual phage-resistant colonies were selected and plated on LB-agar/kan/Mg. LPS-analysis was performed as described [23]. In short, colonies were inoculated in liquid medium grown to an OD_{600} ~ 1 and harvested. The pellet was resuspended in 50 mM Tris, 2 mM EDTA pH 8.5, frozen and thawed, and then subjected to three cycles of sonication on ice (each cycle 15 s, Branson tip sonicator with microtip operated at maximum allowed output). Subsequently, the membrane fraction was collected by centrifugation (15 min, 14,000 rpm) resuspended in an aliquot of non-reducing sample buffer (50 mM Tris (pH 6.8), 2% SDS (w/v), 0.1 bromophenol blue (w/v) and 10% glycerol (v/v)), and incubated with proteinase K (100 μg/ml final concentration) for 1 h at 60 °C. The LPS length was determined with SDS-PAGE (15% gel) and silver-staining [24].

2.4. Phenotype suppression assays

LB-agar plates were used containing LB, agar (1.5%), kanamycin (50 μg/ml) and various concentrations of $MgCl_2$. AD93 and drAD93 were grown overnight. An aliquot of ~150 μl of cell suspension was pipetted on LB-agar plates supplemented with kanamycin. The plates were grown at 37 °C for 5 days. To check for suppression of the auxotrophy for divalent cations by the presence of DCPE in the plates, plates were prepared with and without 250 μM DCPE.

2.5. ESI-MS/MS experiments

Lipid extracts were dissolved at 2 mM total phospholipid in chloroform/methanol/water/formic acid 10:75:15 (v/v/v). After addition of 1.5 volumes of formic acid, samples were subjected to electro spray ionization-tandem mass spectrometry (ESI-MS/MS) using a Quattro Ultima™ triple quadrupole instrument (Micromass) and introduced via a nano-electrospray at a flow rate of 40–60 nl/min. A capillary voltage of 1.5 kV and a cone voltage of 30 V were applied in the positive ion mode. Argon was used to induce collision-activated dissociation at 30 eV. Both $[M+H]^+$ and $[M+Na]^+$ adducts of PE molecular species were detected by neutral loss scanning at *m/z* 141 for the characteristic PE head group [10,11]. Peak assignments, listed in Table 1, are based on the *m/z* values combined with available data in literature [25,26]. In addition, product ion scans were performed to confirm the identity of individual PE molecular species. Spectra were obtained by averaging 20–40 repetitive scans of 10 s and are representative for at least two independent experiments with similar results.

2.6. General procedures

Phospholipid extracts of washed cells prepared as described above were begotten by the method of Bligh and Dyer [27], which quantitatively extracts long chain phospholipids. In control experiments, the recovery of DCPS and DCPE was found to be ~ 50% and ~ 60%, respectively, due to their higher water solubility. Phospholipids were separated on precoated TLC-plates (Merck), impregnated with 2.4% boric acid in ethanol/water 1:1 (v/v), essentially as described previously [28]. 1D-TLCs were developed in chloroform/methanol/water/ammoniumhydroxide 120:75:6:2 (v/v/v/v). 2D-TLC was performed with chloroform/methanol/ammoniumhydroxide 71:25:4 (v/v/v) in the first dimension and chloroform/methanol/acetic acid 65:25:10 (v/v/v) in the second dimension. Phospholipids were detected by staining with iodine vapor, and subsequently scraped off for quantification. Phospholipid amounts and concentrations are always presented based on inorganic phosphate (P_i-PL), determined after destruction as described [29]. OD_{600} measurements were performed with a Unicam Helios epsilon spectrophotometer.

Table 1
E. coli PE molecular species identified in this study

$[M+H]^+$ *m/z*	$[M+Na]^+$ *m/z*	PE molecular species[a]
412	434	12:0 (6:0–6:0)
524	546	20:0 (14:0–6:0)
552	574	22:0 (16:0–6:0)
564	586	23:0 (17_{cyclo}:0–6:0)
636	658	28:0 (14:0–14:0)
662	684	30:1 (14:0–16:1)
664	686	30:0 (14:0–16:0)
676	698	31:0 (17_{cyclo}–14:0)
690	712	32:1 (16:0–16:1)
692	714	32:0 (16:0–16:0)
704	726	33:0 (17_{cyclo}:0–16:0)
716	738	34:2 (16:1–18:1/17_{cyclo}–17_{cyclo})
718	740	34:1 (16:0–18:1/16:1–18:0)
732	754	35:0 (17_{cyclo}–18:0)
744	766	36:2 (18:1–18:1)
746	768	36:1 (18:0–18:1)

[a] The fatty acyl chains at *sn*1 and *sn*2 positions are indicated in arbitrary order.

3. Results and discussion

3.1. Deep-rough AD93 remodels DCPE

We attempted to get uptake of PE from the medium by the AD93 cells, and selected the water-soluble short-chain DCPE for that purpose to be added to the medium. However, after 24 h of growth in the presence of 25 μM DCPE, hardly any uptake of PE was observed (0.3 ± 0.2%, (n = 3), of total membrane P_i-PL, not shown). This is probably due to the tightness of the outer membrane of *E. coli* [30], which blocks entry of the short-chain lipids.

For wild-type *E. coli*, it has been demonstrated that uptake of lipidic components from the medium can be facilitated by decreasing the polysaccharide length of LPS, as in deep-rough mutants [20,31]. Therefore, a deep-rough mutant of AD93 was isolated. For this purpose, a series of P1-resistant AD93 mutants was selected and screened for reduced LPS length. Fig. 1A shows that one of the P1-resistant mutants (arrow) has an LPS length comparable to that of a known deep-rough mutant CE1229 [22]. The drAD93 still has the characteristic lipid composition of AD93, lacking PE, and with an increased CL content (Fig. 1B: compare the TLCs of lipid extracts with that of wild-type *E. coli* W3899, which has a low cardiolipin content and a high level of PE). Growing drAD93 with 25 μM DCPE for 24 h resulted in significant uptake of PE (not shown). We optimized uptake in the deep-rough mutant, by increasing the time of incubation to 54 h, and including 250 μM DCPE in the medium. This resulted in the appearance of PE in the lipid extract of these cells, which was not observed when grown without PE (compare Fig. 2A and B). The extent of uptake gradually increased to ± 4% PE of total cellular P_i-PL after 72 h (Fig. 2C). Using TLC analysis, at early time points uptake was not detectable, yet occurred as will be shown shortly. Thus, the deep-rough mutation in AD93 allows for uptake of short-chain phospholipids from the medium. The drAD93 mutant had a generation time of 4.7 ± 0.9 h, slightly longer than that of AD93 (3.7 ± 0.1 h, n = 3, data not shown) and grew to a stationary OD_{600} of ~ 1. The lag phase of growth was variable.

The fatty acid profile of the membrane incorporated PE was followed in time using mass spectrometry, allowing detection of PE in the low picomole region [10]. To this end, late log cell cultures were incubated with DCPE and

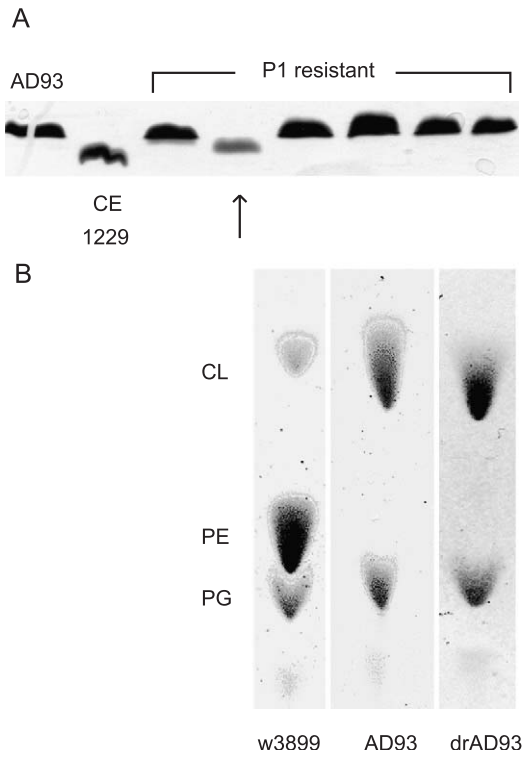

Fig. 1. (A) Part of a 15% SDS-PAGE gel, showing the LPS bands of AD93, a known deep-rough *E. coli* mutant (CE 1229) and a series of individual P1-resistant colonies from an overnight culture of AD93. LPS was visualized by silver staining, and the arrow indicates the mutant drAD93 with reduced LPS length, which was used in the subsequent lipid uptake experiments. (B) Thin-layer chromatograms of lipid extracts of the wild-type parent strain W3899, its derivative AD93, and the deep-rough derivative of AD93, visualized by iodine staining.

harvested at different time points. Subsequently, the lipid extracts from the cells were analyzed for the different PE species which are summarized in Table 1. At $t=30$ min, the mass spectrum of the PE species shows peaks at (m/z) 412 and 434, corresponding to the protonated and sodiated forms of DCPE, respectively (Fig. 3A). Interestingly, also a substantial amount of the DCPE has already been partially remodeled, as evidenced by, e.g. the appearance of a major peak at m/z 552, which originates from 22:0 PE. It is most likely derived from DCPE, with one of the two caproyl (6:0) acyl chains replaced by a palmitoyl (16:0) acyl chain. The occurrence of this PE species argues strongly against a mechanism which involves complete deacylation of DCPE after which the glycero-PE moiety is reacylated with naturally occurring acyl chains. It also indicates that PL head group transfer does not occur under these circumstances, since head group transfer of the PE head group from DCPE to diacylglycerol (with a wild-type lipid acyl chain profile), would lead directly to PE with a wild-type acyl chain profile.

After 3 h of incubation (Fig. 3B) the cells are entering the stationary phase. A new intermediate species is coming up in the spectrum at m/z 564, representing 17_{cyclo}-6:0 PE. The formation of cyclopropane fatty acids (CFAs) coincides with *E. coli* entering the stationary phase [26]. The presence of this new intermediate demonstrates that the 17_{cyclo} acyl chain is also a substrate for remodeling of short-chain PE. The lack of PE peaks between m/z 434 and m/z 524 in all the spectra indicates that no smaller intermediates than 14:0–6:0 are formed. This argues against a putative mechanism of elongation of the acyl chains, while they are attached to the glycero-PE moiety. From these observations, we conclude that the first step in remodeling of PE most likely involves a deacylation/reacylation reaction.

Between m/z 650 and m/z 750 (Fig. 3B), now PE species are emerging that have two long acyl chains as, e.g. the peak at m/z 704, representing 17_{cyclo}-16:0 PE. This molecule is most likely derived from an early intermediate species, e.g. 16:0–6:0, of which the second 6:0 acyl chain has now been

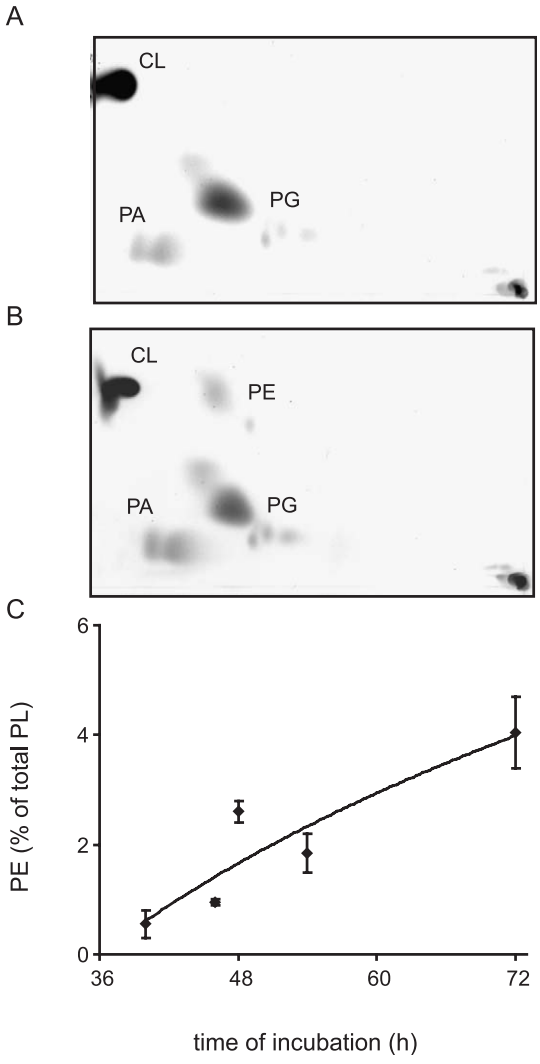

Fig. 2. Iodine-stained 2D-TLC (vertical, first dimension; horizontal, second dimension; origin, lower right corner) of lipid extracts of deep-rough AD93, grown for 54 h in liquid culture in the absence (A) or presence (B) of 250 μM DCPE. The major phospholipid species PE, PG and CL, as well as phosphatidic acid (PA) are indicated. Quantification of a time series of these experiments revealed the time-dependent appearance of PE in *E. coli* cellular lipid extracts (C).

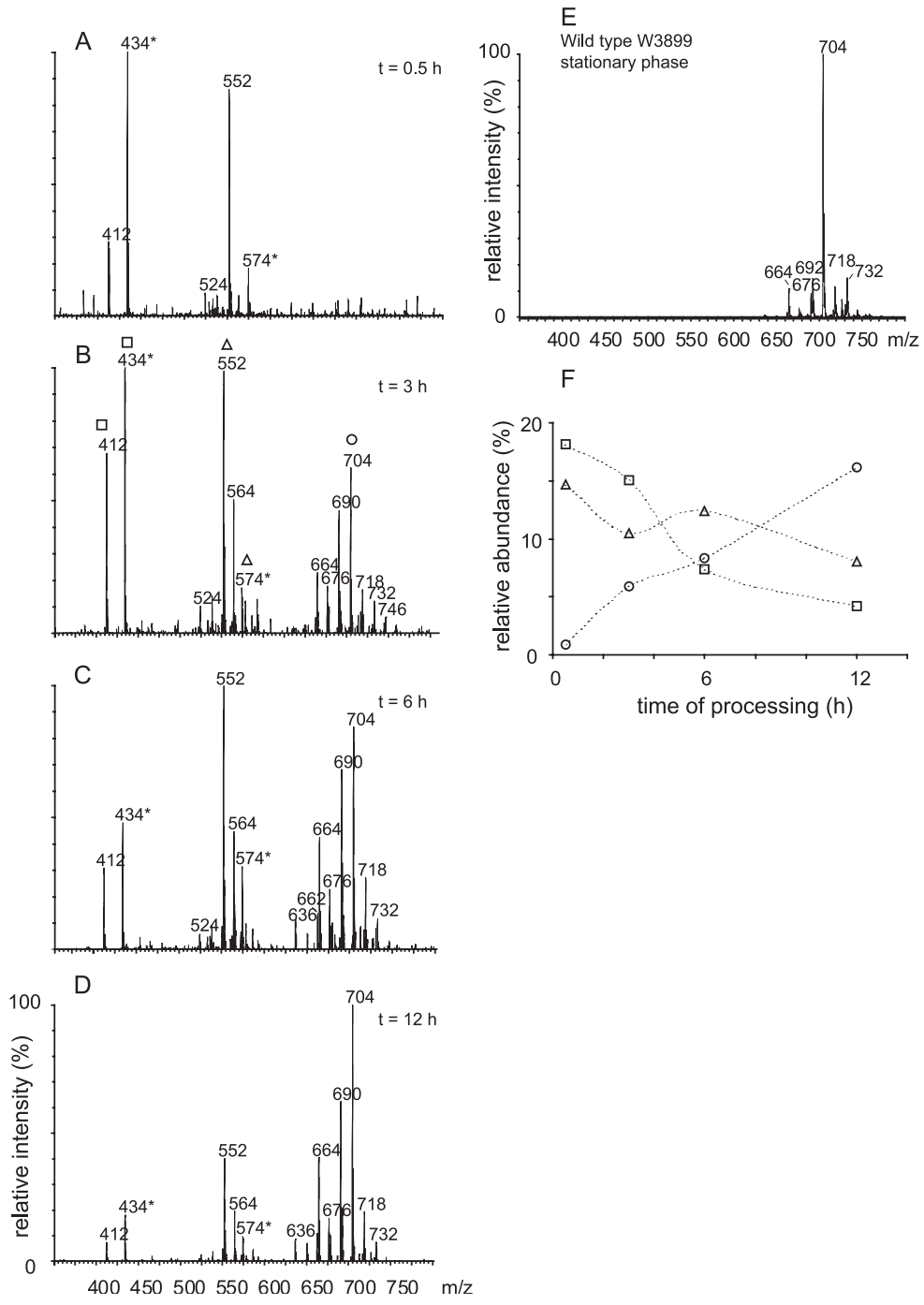

Fig. 3. Mass spectrometric analysis of the PE species profile of late log/stationary drAD93 cultures. In panels A through D, drAD93 was incubated with 250 μM DCPE and 25 mM Mg^{2+}, and harvested at the time points indicated (see Materials and methods for details). For comparison, panel E shows a typical lipid species profile of wild-type *E. coli* W3899 grown to stationary phase. Peaks marked with *asterisks* indicate sodiated species. Panel F shows the extent of remodeling, expressed as the change in relative abundance of the three marker PE species marked with the corresponding symbols in B. The duplicate experiment indicated an error of typically ~ 3% (not shown). For quantification, the peak intensities as a percentage of total intensity of each spectrum was determined.

replaced by a 17_{cyclo} acyl chain. Interestingly, 18-carbon acyl chains are now also found, e.g. at *m*/*z* 732, whereas these seem to be absent as intermediate species (e.g. 18:0–6:0 is not present in the spectrum). This is indicative of specificity for the first acylation reaction, followed by a second deacylation/reacylation in which more variability with respect to donor acyl chains is tolerated. Upon further incubation for 6 and 12 h (Fig. 3C and D), a gradual shift is observed towards a complex PE species profile resembling the wild-type PE species profile of the stationary phase (Fig. 3E). The 6:0–6:0-PE, the 16:0–6:0-PE intermediate, and the 16:0–17_{cyclo}-PE peaks, marked with the symbols in Fig. 3B, are the main

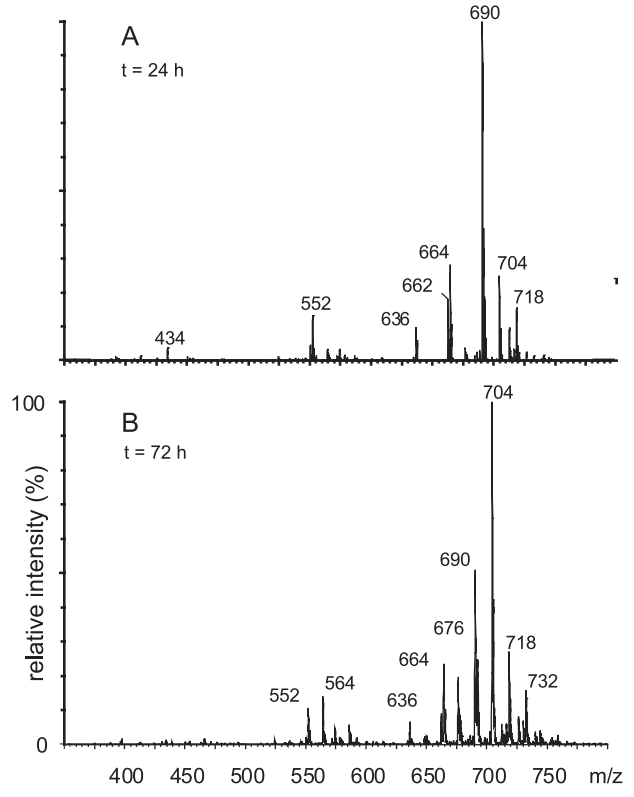

Fig. 4. Mass spectrometric analysis of the PE species profile of growing drAD93. Cell culture was diluted to OD_{600} 0.05 and grown in the presence of 250 μM DCPE for 24 h (A), or to stationary phase after 72 h (B) at 25 mM Mg^{2+}.

species present in panels A through D. Quantification of these peaks, as depicted in Fig. 3F, reveals the extent of remodeling. The relative presence of DCPE decreases (squares), whereas the relative amount of 16:0–17_{cyclo}-PE increases (circles) during time. The relative amount of 16:0–6:0-PE is initially more or less constant and decreases moderately after longer incubation (triangles). This pattern is strongly reminiscent of a typical precursor–product relation between these main species.

Having established remodeling in the late log and stationary phase, we were also interested to see whether DCPE is remodeled during growth. Fig. 4A shows a spectrum of PE species after 24 h of growth from a diluted cell suspension (see Materials and methods), demonstrating extensive lipid remodeling during growth, as evidenced by the low relative amounts of DCPE and intermediate species. Whereas in Fig. 3B–E, the major PE species contains a CFA characteristic for the stationary phase (*m*/*z* 704), here the 32:1 (16:0–16:1) peak at *m*/*z* 690 is predominant. These fatty acids are known to account for ~ 50% of phospholipid acyl chains during normal growth [25]. After prolonged incubation, the typical stationary lipid species profile is again obtained (Fig. 4B).

In conclusion, DCPE is taken up and remodeled by *E. coli* cells irrespective of the growth phase. Remodeling occurs via two consecutive acylation/deacylation reactions, of which the second probably has a broader specificity.

3.2. Remodeling involves passage to the inner leaflet of the plasma membrane

To get insight into the possible role of the inner membrane in lipid remodeling, we performed a similar series of experiments where DCPS was added to the medium in which drAD93 was grown. Since the decarboxylation of PS in AD93 is functional, the last step in synthesis of PE from PS proceeds normally. Phosphatidylserine decarboxylase (*psd*, [32]) is active at the cytosolic side of the inner membrane, and thus represents a marker for phospholipid transport to the inner leaflet of the inner membrane [33]. After the shortest time of incubation, no PE molecular species could be detected above the background (not shown). However, we were able to detect PE in cells grown on DCPS for 3 h and longer (Fig. 5), showing that DCPS was also taken up from the medium albeit slower, possibly due to interactions with the divalent cations present in the growth medium.

In contrast to PE, lipid species of phosphatidylserine were not detected in the mass spectrometry experiments, i.e. the PS content of the mixture was below the detection limit under the conditions used of 1% of total phospholipid (not shown). This indicates that PS was quickly transported to the inner leaflet of the inner membrane and subsequently decarboxylated, as has been shown previously for endogenously synthesized PS [33]. The change in PE species

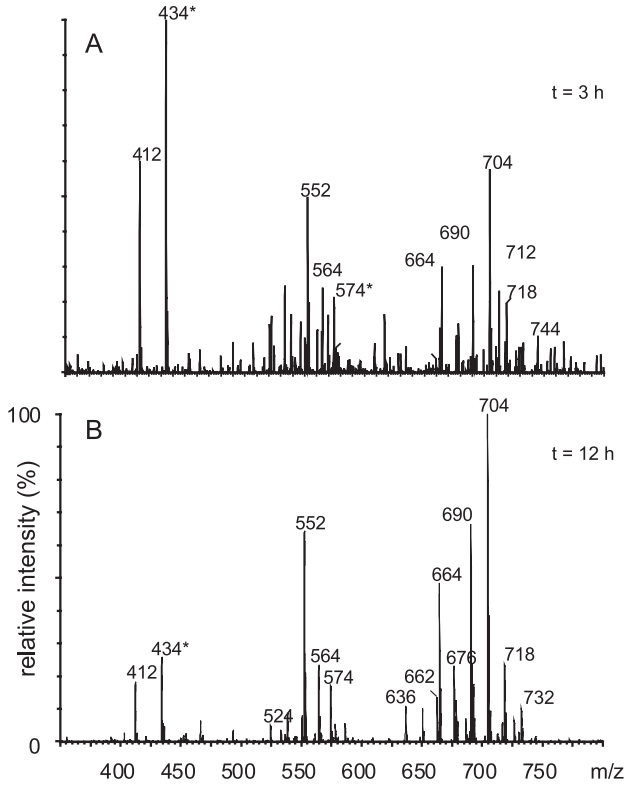

Fig. 5. Mass spectrometric analysis of the PE species profile of drAD93, grown in the presence of 250 μM DCPS, harvested after 3 h (A) and 12 h (B) of growth. Experimental details as in Fig. 3.

profile with time of growth is remarkably similar to that of cells grown on DCPE, as is shown for growth for 3 and 12 h in Fig. 5A and B, respectively (compare to Fig. 3B and D, respectively). Also similarly as for incubation with DCPE, prolonged incubation of growing cells with DCPS results in a wild-type like profile of PE species (not shown).

These data demonstrate that DCPS is translocated to the inner membrane, where it is transformed into PE, which subsequently is remodeled. This makes it unlikely that the deacylation and reacylation takes place in the outer membrane exclusively, since then the occurrence of remodeled PS species might be expected. Furthermore, involvement of the outer membrane phospholipase OMPLA in deacylation of DCPE is unlikely, since a long acyl chain is one of the minimum requirements for OMPLA substrates [34].

There are two mechanisms that could lead to lipid remodeling via intermediate species, i.e. transacylation and reacylation. In transacylation, another PL is acyl chain donor and a lysophospholipid the acceptor. The reaction results in formation of a new lysophospholipid (the former donor) and a diacylphospholipid. In reacylation, acyl-ACP or acyl-CoA is the donor, and a diacylphospholipid is formed. Since CFAs are synthesized while attached to the diacylphospholipid [26], it is most likely that transacylation is the predominant mechanism, although the involvement of reacylation (to form 16:0–6:0 PE, but not 17_{cyclo}–6:0 PE) cannot be excluded.

Both mechanisms require a lysophospholipid as acceptor. As OMPLA is most likely not involved, the question remains how the lyso-caproyl PE is formed. It could be that DCPE is an acyl chain donor for, e.g. LPP, which uses lipid modification to anchor itself in the outer membrane. However, such a short acyl chain would probably be a poor lipid anchor. Alternatively, DCPE could serve as substrate for PagP as lipid donor for lipid X. However, PagP was reported to be specific for palmitate (16:0) [17]. Possibly, DCPE is a substrate for the protein associated with the rather enigmatic detergent-sensitive phospholipase activity described in *E. coli* [35]. Given the evolutionary relationship between bacteria and mitochondria, it is tempting to speculate that this phospholipase activity, and the activity that Henk van den Bosch et al. studied, are related.

3.3. Remodeled PE is functional

Since PE has been shown to be important for *E. coli*, we next tested whether the PE taken up from the medium was functionally integrated in the membrane fraction in an in vivo assay. As a measure for functional integration, we tested whether the incorporated PE could overcome the auxotrophy for divalent cations. Fig. 6 shows that drAD93 at 10 mM $MgCl_2$ grows with and without DCPE present, whereas it cannot grow at 5 mM $MgCl_2$. However, with DCPE present in the plates, some colonies survive at this Mg^{2+} concentration, indicating that the remodeled PE can functionally complement the growth phenotype. Possibly, this system can be used as a genetic screen to identify the putative proteins involved in lipid trafficking in *E. coli*, e.g.

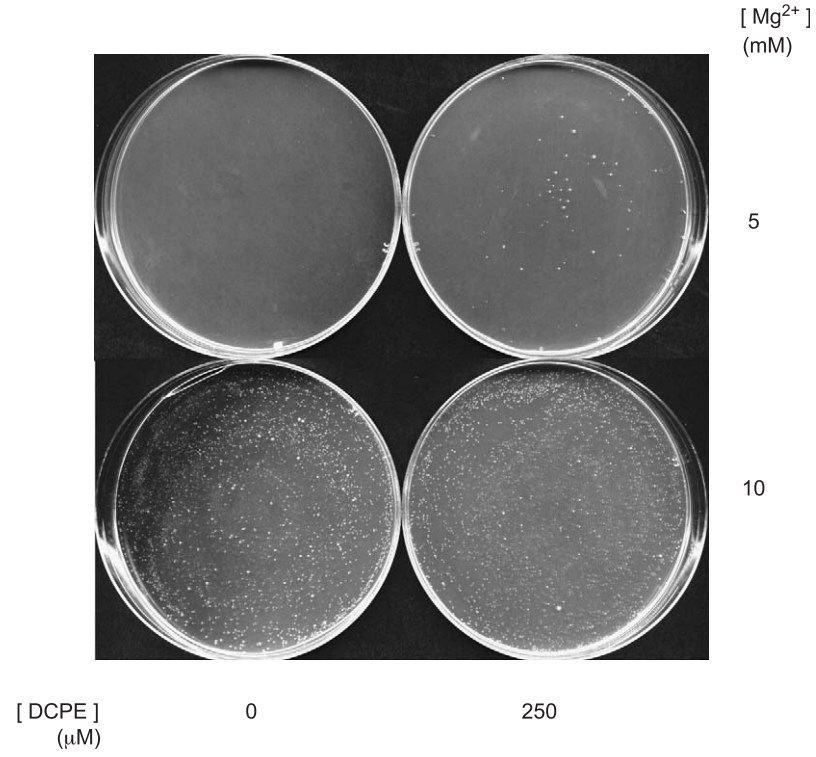

Fig. 6. Growth of drAD93 on LB-agar plates, supplemented with 5 mM and 10 mM $MgCl_2$, and in the presence and absence of 250 μM DCPE.

by selecting mutants with increased capacity for uptake and transport of PE.

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