Studying a Synthetic Phospholipid Biosynthesis Pathway Using a Cell Lysate Expression System

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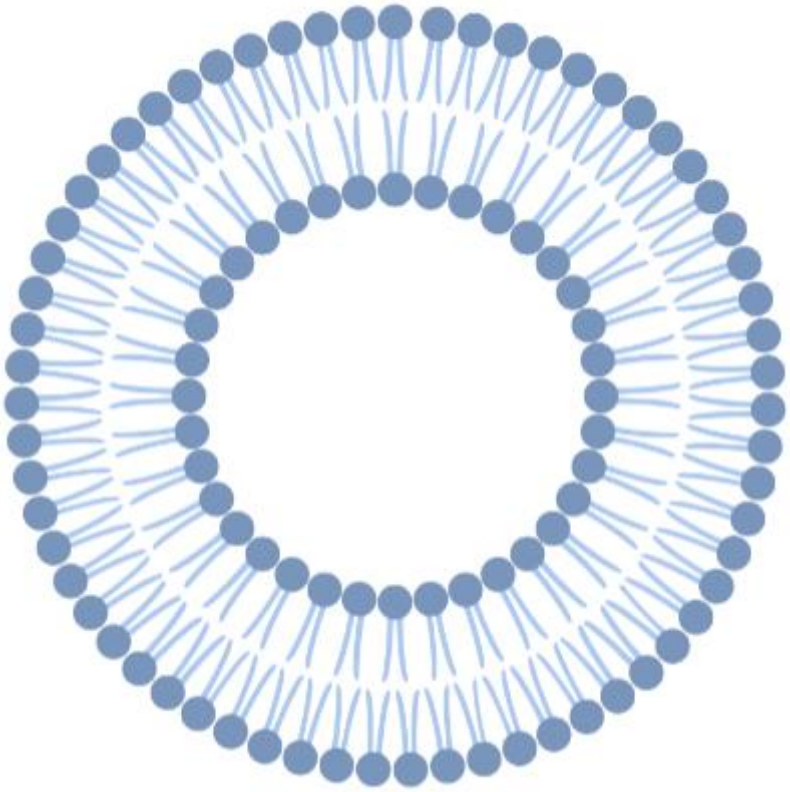

Abstract

An important module in the construction of a bottom-up synthetic cell will be the generation of a cellular membrane, which will likely be composed of phospholipids. Previous research has indicated the possibility of generating a functional cellular membrane using a synthetic phospholipid biosynthesis pathway. This biosynthesis pathway was already shown to be functional in an expression system using PURE. The aim of this research project was to study the phospholipid biosynthesis pathway in a cell lysate expression system. The influence of insertases SecYEG and YidC on co-translational insertion of this biosynthesis pathway into liposomal membranes was studied using mass spectrometry. Furthermore, the possibility of bulk lipid production in such a lysate expression system was elucidated using thin layer chromatography as an analysis method. It was found that while such a lysate expression system does allow for robust production of lipids, it is also prone to interference by background processes. However, analysis using thin layer chromatography coupled with fluorescent imaging also seemed to indicate the potential for bulk lipid production using such a system.

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Introduction

General

In the field of synthetic biology, the construction of a bottom-up synthetic cell would constitute a major breakthrough. This branch of biology is concerned with the construction of novel biological systems which are not found in nature[1]. Synthetic biology can be divided into two approaches, top-down and bottom-up[2]. The top-down approach alters already existing biological systems, such that they are better able to perform a certain task[3]. Top-down synthetic biology often makes use of a host-cell, called a chassis[3]. Using such a chassis has the benefit of already having the necessary biological components at hand. However, the use of a chassis also brings the risk of interference between endogenous processes and the synthetic system. The bottom-up approach takes a more *de novo* approach, creating truly artificial systems. One of the challenges of this approach is the lack of a full understanding of how cellular activity emerges from individual cellular components[4]. Furthermore, in a living cell there are a multitude of cellular activities which all influence each other. Therefore, an abstraction is required from this complex network of activities towards more confined modules. Such modules would constitute a minimal system which produces the same functionality as the more complex cellular activity[4]. Precisely what modules would be required for the realization of a fully functional synthetic cell is as of yet unknown. Furthermore, no fully autonomous synthetic cell has been constructed yet. However, one of these modules will undoubtedly be the ability to self-organize into a compartment[5].

Self-assembly into a compartment would require the presence of a boundary layer, which is most often encountered in the form of a phospholipid bilayer in nature. The lipid bilayer constitutes two major functions in biological cells. Firstly, it provides a barrier between the internal cytosol and the external environment. Secondly, it acts as a selective filter which controls the flow of compounds between the cell and its environment[6]. Through these two major functions, the cellular membrane creates a compartment which allows for crowding of molecules. This crowding of molecules in turn allows for the catalysis of chemical reactions[7].

An essential feature of the lipid membrane which is required for life, is the ability to expand and divide. In order to achieve this, the biosynthesis of lipids is necessary. Biological cell membranes are comprised of a wide variety of molecules, amphiphilic lipids as well as the proteins embedded in them[8]. Membrane-bound proteins perform a wide range of functions, from transport to catalysis of metabolic reactions. For their proper functioning they often rely on a matrix in which they can be properly folded. This matrix is provided by the lipids of the cellular membrane[9]. The complexity of the membranes of cells encountered in nature grants them the ability to carry out highly specialized tasks. However, as the construction of a synthetic cell will be achieved with the use of minimal modules, its lipid membrane will not contain such a wide variety of different compounds.

The major component of biological membranes are phospholipids, which are encountered in all forms of life[10]. Phospholipids are amphiphilic molecules, comprised of a hydrophilic headgroup coupled to a hydrophobic backbone. Phospholipids are capable of assembling into a bilayer, which allows for the formation of a compartment. Furthermore, they can also be involved in other cellular activities such as signal transduction and protein targeting[11]. These characteristics make phospholipids ideal candidates for synthetic cell membranes. The inner membrane of model organism *Escherichia coli* is comprised of two major components: phosphatidylethanolamine (PE) and phosphatidylglycerol (PG)[11]. While this membrane also contains additional lipid types, it was found that only PE and PG are required for proper functionality[11]. If the membrane of a potential synthetic cell would be based on this natural lipid bilayer, then it would only require the synthesis of these two phospholipids.

Phospholipid biosynthesis pathway

Production of the two phospholipids PE and PG can be achieved with a synthetic biosynthesis pathway comprised of 8 enzymes (Figure 1)[11]. This biosynthetic pathway catalyzes the chain reaction from fatty acids into the two desired phospholipids. The pathway starts with acyl-chain formation. This can be achieved in two ways, using the fatty acid synthesis complex or the enzyme FadD. The fatty acid synthesis complex is comprised of multiple proteins, and can be coupled to glycolytic enzymes to facilitate biosynthesis of acyl chains from glucose[12]. However, a simpler alternative is provided by the fatty acyl-CoA synthetase FadD, which facilitates the coupling of fatty acid to CoA moieties. Depending on the available fatty acids, this enzyme is capable of activating a range of varying acyl chains[13]. Acyl-

CoA produced by FadD is used by the enzymes PlsB and PlsC. PlsB is a glycerol-3-phosphate acyltransferase which catalyzes the acylation of a glycerol-3-phosphate (G3P) moiety to yield lysophosphatidic acid (LPA). LPA is then again acylated to create PA by the lysophosphatidic acyltransferase PlsC. From this phospholipid, the intermediate lipid cytidine diphosphate-diacylglycerol (CDP-DAG) can be produced. This conversion is catalyzed by the CDP-DAG synthase CdsA, using cytidine triphosphate (CTP) as a reagent. This intermediate lipid is then used in into the two phospholipids PE and PG through two distinct pathways. Phosphatidylserine (PS) synthase PssA can convert CDP-DAG into PS using Serine as a reagent. PS decarboxylase Psd subsequently converts PS into PE. Phosphatidylglycerol-3-phosphate (PGP) synthase PgsA can convert CDP-DAG into PGP. This PGP can then be used to produce PG through removal of 3-phosphate by the PGP phosphatase PgpA. In this manner fatty acids can be converted into the two phospholipids PE and PG, using only eight essential enzymes.

Exterkate *et al.* already showed the feasibility of this biosynthesis pathway, through reconstitution of these eight enzymes into liposomes[11]. However, if this pathway is to be implemented as a module for membrane synthesis in synthetic cells it cannot be reliant on reconstitution into existing membranes. A potential synthetic cell will be DNA-driven, as such this biosynthesis pathway should be coupled to an expression system. Where the enzymes are encoded on a genome and are inserted into the membrane following expression. Scott *et al.* generated a system wherein these eight enzymes were brought to expression and showed activity within liposomes[14]. In their experiments the enzymes were brought to expression in the PURE system, an *in vitro* translation and transcription system. However, while they did witness expression and activity of these eight enzymes, production was very low[14]. This low activity might have been due to the lack of an insertion mechanism which could facilitate co-translational insertion in their expression system. In order to elucidate the effect of such an insertion system on the activity of this biosynthesis pathway, an expression system using cell lysate was generated. Furthermore, experiments were also conducted to test whether such an expression system in cell lysate would be capable of bulk lipid production.

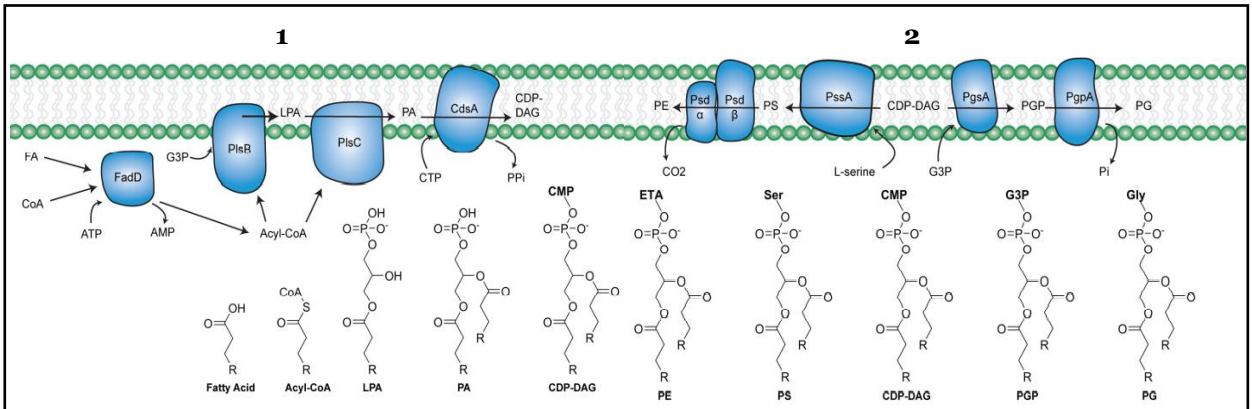

Figure 1. Schematic overview of the phospholipid biosynthesis pathway, which facilitates production of PE and PG. 1) Production of intermediate lipid CDP-DAG. 2) Conversion of CDP-DAG into phospholipids PE and PG. (Adapted from Exterkate *et al.*, September 2018.)

Research project

The aim of this research project was to study the phospholipid biosynthesis pathway in a lysate expression system. This research aim was divided into two subprojects. The aim of the first subproject was studying the co-translational insertion of this biosynthesis pathway into liposomal membranes. Many of the enzymes of the phospholipid biosynthesis pathway were predicted to insert into the lipid membrane in a co-translational manner. Membrane proteins often rely on co-translational insertion into the lipid membrane, due to the fact they are prone to aggregate following exposure to an aqueous environment[15]. In bacterial cells, co-translational insertion of membrane proteins is often facilitated by the Sec-translocon[16]. Targeting to this translocon can be facilitated by the signal recognition particle (SRP), which is recruited to the translating ribosome by hydrophobic regions on the nascent chain of the protein that is being produced[15]. The SRP guides the ribosome to the lipid membrane, where its receptor FtsY guides the SRP-ribosome complex to the Sec-translocon. The Sec-translocon is then

responsible for co-translational insertion of the nascent chain into the membrane. Insertion into the lipid membrane is facilitated by the heterotrimeric SecYEG complex[17]. Another important insertase present in bacterial cells is the protein YidC, which is also targeted by the SRP pathway[18]. This insertase can either function on its own, or in cooperation with the SecYEG complex. On its own, YidC is only capable of inserting relatively simple small transmembrane segments (TMs)[18]. However, the cooperation between SecYEG and YidC can facilitate the insertion of membrane proteins containing multiple complex TMs. In this interaction SecYEG facilitates insertion of TMs into the lipid membrane, where YidC functions as a chaperone that assists them in their correct folding[19]. To elucidate the effect of these insertases on the proper functioning of the enzymes of the phospholipid biosynthesis pathway, experiments were carried out in cell lysate. First, liposomes were constructed for each of the enzymes-to-be-tested. In these liposomes the two insertases were reconstituted, in such a way that liposomes with all possible combinations of SecYEG and YidC would be generated. These liposomes had a dual function, they functioned as a lipid membrane in which the enzyme could be inserted and also contained its substrate lipid. The two aforementioned insertases were reconstituted into these liposomes. If co-translational insertion into the liposomal membrane would be successful, the substrate would be converted into product. This conversion could then be analyzed using mass spectrometry (MS).

The second subproject was aimed at looking into the potential of bulk lipid production in a cell lysate expression system. While Scott *et al.* were able to successfully link expression and functionality of the phospholipid biosynthesis pathway using a PURE system, their lipid yield was very low [14]. An assumption was made that utilizing a cell lysate expression system, instead of this PURE system, would significantly increase this lipid yield. This assumption was partly based on the distinction between the top-down and bottom-up approach in synthetic biology. In this comparison, the PURE system could be seen as the true bottom-up approach. Because this artificially created system only contains the minimal components required for transcription and translation of protein. A cell lysate expression system could be seen as a more top-down approach to expression of the biosynthesis pathway. As this system is generated by reduction of already existing biological systems, in the form of removal of the total membrane fraction of lysed *E. coli* cells. While the PURE system would offer better control over the activity of the expression system, it would also be less robust. In contrast, a cell lysate system would already contain all required components for bulk production of product, at the risk of interference with background reactions. In order to test the potential of such a cell lysate expression system, the production of PA from fatty acids was studied. Fluorescently tagged fatty acids were supplied to a lysate expression system containing the enzyme-to-be-studied, and their incorporation into product could be analyzed using thin layer chromatography (TLC) combined with fluorescent imaging.

Materials and Methods

Transformation

Transformations were carried out with plasmids containing genes encoding for *E. coli* enzymes SecYEG, YidC, FadD and PgsA (Table 1). 100 µl of $CaCl_2$ competent *Escherichia coli* BL21 cells were mixed with 1 µl of plasmid (100 ng/µl). Cells were incubated on ice for 30 minutes and subsequently heat shocked at 42 °C for 1 minute. 1 ml of preheated LB medium (37 °C) (LB broth, Lennox) was added, and the cells were incubated at 37 °C for 60 minutes. Cells were plated on selective medium containing antibiotic, either ampicillin (100 µg/ml) (ampicillin sodium salt SIGMA) or kanamycin (50 µg/ml) (kanamycin sulfate from *S. kanamyceticus* SIGMA). 100 µl of transformed cells were also added to selective liquid medium, containing 100 µg/ml antibiotic, and grown overnight at 37 °C. All cloning was performed in *E. coli*, all genes except for *PssA* were isolated from *E. coli*. The PssA gene was isolated from *B. subtilis*.

Overexpression

Overnight cultures were used to inoculate LB medium to an OD_{600} of 0.05, and incubated (37 °C, 200 RPM) until an OD_{600} of 0.6 was measured (Excella E25, New Brunswick Scientific). Following induction with 0.5 mM IPTG, cultures were incubated for an additional 2 hours (37 °C, 200 RPM). Culture was then transferred to centrifuge flasks and spun down (4 °C, 6000 rcf, 15 minutes) (J-26S XP, Avanti). Cell pellet was resuspended in buffer A (50 mM Tris-HCl pH8, 100 mM KCl, 15% glycerol) and spun down (4 °C, 6000 rcf, 15 minutes). Cell pellet was again resuspended in buffer A. 1 spatula tip of DNase (Deoxyribonuclease I from bovine pancreas DN25) and a 1 pellet of protease-inhibitor (EDTA-free EASY pack, Roche) were added to this suspension (per 4 liter of overexpression culture). Cell suspension was lysed by French press (Constant Cell Disruption Systems, LA biosystems) at 13 kPSI. Membrane proteins SecYEG, YidC, and PgsA were isolated in the total membrane fraction. Lysed cells were spun down (4 °C, 6000 rcf, 15 minutes). Cell lysate was further centrifuged (4 °C, 40000 rpm, 90 minutes) (Optima XE-90 Ultracentrifuge, Beckman Coulter, rotor: JA25.50) to isolate total membrane fraction. Isolated membranes were resuspended in buffer A and stored at -80 °C. Samples of total membrane fractions were loaded onto a 12% SDS gel to check for the presence of overexpressed protein. Cytosolic FadD was isolated in the cytosolic fraction following lysis. Lysed cells were centrifuged (4 °C, 20000 rcf, 15 minutes) (J-26S XP, Avanti), after which the supernatant was collected. Cytosolic fraction was isolated through centrifugation (4 °C, 235000 rcf, 60 minutes) (Optima XE-90 Ultracentrifuge, Beckman Coulter).

Table 1. All plasmids used in this research project

Plasmid	Description	Source
pME001	*fadD* gene with C-terminal His-tag from *E.coli* MG1655. Expression factor (KanR), T7 promoter	(Exterkate *et al., 2018*)
pAC015	*pgsA* gene with C-terminus His-tag from E. coli MG1655. Expression vector (KanR), T7 promoter	(Exterkate *et al.*, 2018)
pET610	N-terminal SecY-his-EG with ΔHincII in secE under *trc* promotor	(Kaufmann et al., 1999)
pEHhisYidC	C-terminus his-YidC under *lac* promotor	(Van Der Laan et al., 2001)
pRSF-PlsC	*pslC* gene from *E. coli* BL21 cloned into pRSF-Duet1 under *T7* promotor	In house plasmid
pRSF-CdsA	cdsA gene from *E. coli* BL21 cloned into pRSF-Duet1 under *T7* promotor	In house plasmid
pRSF-Pss	*pss* gene from *B. subtilis* cloned into pRSF-Duet1 under *T7* promotor	In house plasmid
pRSF-PsD	*psd* gene from *E. coli* BL21 cloned into pRSF-Duet1 under *T7* promotor	In house plasmid
pRSF-PgsA	*pgsA* gene from *E. coli* BL21 cloned into pRSF-Duet1 under *T7* promotor	In house plasmid
pRSF-PgpA	*pgpA* gene from *E. coli* BL21 cloned into pRSF-Duet1 under *T7* promotor	In house plasmid

His-tag Purification

All buffers used for the purification of SecYEG, YidC, PgsA, and FadD can be found in Table 2. Total membrane fraction and solubilization buffer were mixed in a 1:1 ratio and incubated (4 °C, roller bank, 30 minutes). 0.4 times total membrane fraction volume of NTA (ni-NTA Agarose, QIAGEN) was washed (3 times: 5x volume MQ water added, spun down at 12000 rcf, soluble fraction disposed) and added to the incubated sample. In the case of SecYEG purification, 10 mM of imidazole was also added. Samples were incubated (4 °C, roller bank, 15 minutes). Sample with NTA was run through a column (Bio-spin Disposable Chromatography Columns, BIORAD) and washed 3 times with 5 times column volume of washing buffer. After washing, 1 column volume of elution buffer was added to the NTA fraction which was incubated for 15 (4 °C , roller bank, 15 minutes). After flowthrough of elution step was collected, 2 times 1 column volume of elution buffer was pipetted onto the NTA fraction and also collected. Samples of elution flowthrough were loaded onto a 12% SDS gel to check for the presence of the correct purified protein. Samples of elution flowthrough were also analyzed with absorbance at 280 nm (UV-3100PC, VWR). The following molar extinction coefficients were used to calculate protein concentrations: (SecYEG – 71000 $M^{-1}cm^{-1}$, YidC – 96260 $M^{-1}cm^{-1}$, FadD – 53290 $M^{-1}cm^{-1}$, PgsA – 66460 $M^{-1}cm^{-1}$).

Table 2. All buffers used for His-tag purification of the proteins SecYEG, YidC, FadD, and PgsA

Component	SecYEG Solubilization	SecYEG Wash	SecYEG Elution	YidC Solubilization	YidC Wash	YidC Elution
DDM	4%	0.05%	0.05%	4%	0.1%	0.1%
Tris-HCl pH8	50 mM	50 mM	50 mM	50 mM	50 mM	50 mM
KCl	100 mM	100 mM	100 mM	100 mM	100 mM	100 mM
Imidazole pH8	N.A.	40 mM	300 mM	N.A.	30 mM	400 mM (pH)7

Component	FadD Wash	FadD Elution	PgsA Solubilizat	PgsA Wash	PgsA Elution
DDM	0.05%	0.05%	4%	0.05%	0.05%
Tris-HCl pH8	50 mM	50 mM	50 mM	50 mM	50 mM
KCl	100 mM	100 mM	100 mM	100 mM	100 mM
Imidazole pH8	20 mM	300 mM	N.A.	40 mM	300 mM

LUV formation

Lipid mixtures were prepared from chloroform stocks (Avanti Polar Lipids, Inc.) to an end-concentration of lipids of 5 mM, the different lipid compositions that were prepared for the various experiments are denoted in Table 3. In almost all of the various constructed LUV types PC, PG, and PE were present in the same molarities. PC made up 35% of all lipids, PG and PE both made up 30% of all lipids. Which left 5% of total lipids for the substrate lipid of the reaction which was catalyzed by the enzyme-to-be-tested. This varied for the enzymes PssA, PgsA, and PgpA. In their case 37% of total lipids was made up of PC ,and 3% of total lipids was made up by substrate lipid CDP-DAG. If not specified otherwise, dioleoyl variants (DO) of phospholipids were used. In the case of Psd, which produces DOPE from DOPS, POPE was used for the construction of LUVs because it allows for differentiation between produced PE and background PE during MS analysis. The PssA, PgsA, and PgpA LUVs contain POPG instead of DOPG, because the enzyme PgpA produces DOPG from DO CDP-DAG. Furthermore, the LUVs for PgpA do not contain its substrate PGP but CDP-DAG. This was done to facilitate production of PGP by the enzyme PgsA, which was to be reconstituted into the liposomes belonging to the enzyme PgpA. This was due to a lack of commercially available PGP, therefore reconstituted PgsA would act as a means of PGP production in the LUVs for PgpA. Additionally, the substrate lipid for the LUVs, LPA, was added to the liposomes after reconstitution of insertases. Which was done to avoid washing out of this lipid during washing steps with BioBeads. Furthermore, LPA is able to self-insert into liposomes, without the need of extrusion.

After the chloroform was evaporated from the samples, they were dissolved in liposome buffer (50 mM Tris-HCl pH8, 100 mM KCl, 2 mM DDT). LUVs were formed by extrusion, using a filter with pore diameters of 400 nm (polycarbonate membrane, AVESTIN). Absorbance kinetics at 540 nm were measured with a spectrophotometer. Triton (Triton X-100, SIGMA) was added to the sample until a value of 40% of the absorbance peak was reached, indicating successful destabilization of the liposomes.

Table 3. Lipid compositions of LUVs constructed for the various enzymes of the phospholipid biosynthesis pathway, unless specified otherwise DO lipids were used

Enzyme	PC	PG	PE	PX
PlsC	1.75 mM	1.5 mM	1.5 mM	0.25 mM LPA
CdsA	1.75 mM	1.5 mM	1.5 mM	0.25 mM PA
PssA	1.85 mM	1.5 mM **PO**	1.5 mM	0.15 mM CDP-DAG
Psd	1.75 mM	1.5 mM	1.5 mM **PO**	0.25 mM PS
PgsA	1.85 mM	1.5 mM **PO**	1.5 mM	0.15 mM CDP-DAG
PgpA	1.85 mM	1.5 mM **PO**	1.5 mM	0.15 mM CDP-DAG

For reconstitution of protein, a ratio of 500:1 lipid to protein was used. In the case of a reconstitution of two types of proteins, a ratio of 500:1:1 lipid to protein was used. The LUV-protein mixtures were incubated shortly (4 °C, roller bank, 15 minutes). 20 mg of dried Bio-Beads (Bio-Beads SM2 Adsorbent medium, BIORAD) were added to 200 µl of sample, and incubated (4 °C, roller bank, 90 minutes). Sample was transferred to fresh BioBeads 4 times (40 mg for 45 minutes, 60 mg for 45 minutes, 60 mg overnight, 60 mg for 30 minutes), every time the sample was transferred 200 µl of liposome buffer was added until a final volume of 1 ml was reached. The sample was then transferred to centrifuge tubes and spun down (4 °C, 254000 rcf, 30 minutes) (Optima MAX-XP Ultracentrifuge, Beckman Coulter). Pellet was resuspended in 100 µl liposome buffer. Samples of LUVs were loaded onto a 12% SDS gel to check for successful reconstitution of SecYEG and YidC. Following formation of LUVs, MS analysis was used to check for the correct lipid composition (data not shown).

FadD activity assay

An activity assay was carried out on purified FadD in assay buffer (50 mM Tris-HCl pH8, 10 mM $MgCl_2$, 100 mM KCl, 15% glycerol, 2 mM DTT). FadD was added to this buffer in a 0.5 µM concentration, together with additional reagents: 550 µM oleic acid, 250 µM CoA, 1 mM ATP. Reactions were carried out in an end-volume of 100 µl of assay buffer and incubated overnight at 37 °C.

Lysate Reactions

Expression- and- activity of the various enzymes were tested in a reaction mixture containing *E. coli* cell lysate, from which the total membrane fraction had been removed through centrifugation at 135000 rcf. Plasmids containing genes encoding for the various enzymes were added to reaction mixtures with an end-volume of 20 µl. A master mix (cell lysate, energy mix, T7 polymerase, tRNAs, RNase inhibitor, 2 mM 20 of the essential amino acids, 2mM Methionine+Cysteine, 0.01 µM Creatine Kinase) was used, to which additional components were added (Table 4). Reactions were incubated overnight at 30 °C. All reactions were set-up under RNase free conditions. An in-house energy mix was used (75 mM Tris/KOH pH7.5, 35 µg/ml Folinic acid, 28 mM NH_4OAc-Tetra, 11 mM $Mg(OAc)_2$, 0.05% NaN3, 1.7 mM DTT, 1.2 mM NTPs, 0.65 mM cAMP, 80 mM creatine phosphate, 200 mM K-glutamate, 0.175 g/l tRNA, 2% PEG8000, 1 mM spermidine).

Table 4.
Additional components for lysate reactions

Compound	Concentration
Additional Reagents	
LUVs	0.25 mM
Oleic Acid	0.025 mM
CTP	0.05 mM
LPA	0.05 mM
CoA	0.05 mM
FadD	~50 µM
Thin Layer Chromatography	
BODIPY	0.0125 mM
Oleic Acid	0.0125 mM

Butanol Extractions

In order to prepare reaction samples for analysis with MS, butanol extractions were carried out. To each sample, 100 µl of MQ water and 150 µl of water-saturated butanol were added. 5 µl of a 25 mg/ml stock of 10:0 PG (Avanti Polar Lipids, Inc.) was also added to each sample, to act as an internal standard for MS analysis. Samples were thoroughly vortexed and centrifuged (RT, 16000 rcf, 2 minutes) to achieve phase separation. 100 µl of the butanol layer was pipetted into a glass vial (1.5 ml screw neck vial, VWR) and 100 µl of water-saturated butanol was again added to the sample. This sample was again thoroughly

mixed and centrifuged (RT, 16000 rcf, 2 minutes). An additional 100 µl of the butanol layer was pipetted into the same glass vial, which was placed under a nitrogen gas stream (REACTI-THERM III, Thermo Scientific). Lipid film was resuspended in 50 µl of methanol, which was pipetted into a (0.1 ml micro insert, VWR).

Mass Spectrometry

As described in[20] (A Versatile Method to Separate Complex Lipid Mixtures Using 1-Butanol as Eluent in a Reverse-Phase UHPLC-ESI-MS System, N.A.W. de Kok). In short, 5 µl of sample was run over COSMOSIL 5C4-AR-300 Packed Column, 4.6 mm I.D. × 150 mm (Nacalai USA, Inc.) operating at 40 °C with a flow rate of 500 µL/min. Separation of the compounds was achieved by a gradient of Eluent A (MQ:MeCN (40:60), containing 5 mM of ammonium formate) and eluent B (MQ:MeCN:1-BuOH (0.5:10:90), containing 5 mM ammonium formate). Analysis of data was performed using Thermo Scientific XCalibur, by use of the Genesis algorithm based automated peak area detection and integration. The internal standard of 10:0 PG was used to normalize total ion count between samples.

Lysate Reactions with Fluorescent Probe

The lysate reactions which were to be analyzed using thin layer chromatography contained fluorescently tagged oleic acid (BODIPY™ FL C16 (4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Hexadecanoic Acid), Avanti). The reactions mixtures were similar to the ones described in table 4, with the exception of added fluorescent compound. This BODIPY moiety was added in a 1:1 ratio with regular oleic acid, to the same end-concentration of fatty acids as in the regular lysate reactions. Reactions were carried out in an end-volume of 40 µl, containing the same concentrations of compounds. Reactions were incubated overnight at 30 °C. Butanol extractions were carried out on these samples, without the addition of internal standard 10:0 PG. Furthermore, lipid film was resuspended in 30 µl methanol.

Thin Layer Chromatography

For the TLC, 25 ml of a liquid phase composed of 50:10:1 chloroform:methanol:water was used. TLC sheets (silica gel 60, VWR) were loaded with 2 µl of sample and inserted into the container containing liquid phase. Chromatography was performed for 8 minutes, after which the sheet was taken from the container and air dried. Fluorescent imaging was performed (emission filter: 515Y (SYBR) band-pass). Molybdenum blue (molybdenum blue spray reagent, SIGMA) was used as a phosphate stain, after which high resolution images were made of the TLC sheet (ImageScanner, Amersham Biosciences). Fluorescent images were analyzed through comparison of band-heights with molybdenum blue stained lipid stock controls. Furthermore, ImageJ was used for quantitative analysis of fluorescent signal through intensity measurements.

Results

Transmembrane domain predictions

Whether or not an enzyme relies on insertases for its proper insertion into- or localization to the lipid membrane is partially dependent on the presence of TMs in its sequence, among other factors such as the presence of a signal sequence. An algorithm was used (DeepTMHMM) to make a prediction as to the presence of such segments in the eight enzymes of the phospholipid biosynthesis pathway (Table 5)[21]. The first enzyme of this pathway, FadD, is known to be cytosolic[22]. As such it is not surprising that it is predicted to not contain any TMs in its sequence. However, there are two other proteins that are also predicted to not contain any TMs: PlsB and Psd. In the case of these two enzymes, which are known to localize to the membrane, this points to a more peripheral association. Meaning that they do not fully insert into the membrane, but likely contain amphiphilic helices which allow for association to the lipid membrane.

Table 5. Predictions of the transmembrane segments present in the enzymes of the phospholipid biosynthesis pathway.

Enzyme	FadD	PlsB	PlsC	CdsA	PssA	Psd	PgsA	PgpA
TMs	Cytosolic	0	1	9	6	0	6	5

Purification of insertases

In order to test the effects of SecYEG and YidC on the functioning of the various enzymes of the phospholipid biosynthesis pathway, these insertases were first purified. Both proteins were overexpressed in *E. coli* BL21 cells. His-tag purification was used to acquire pure samples of both SecYEG and YidC, small amounts of these samples were loaded onto SDS-gels to analyze their purity (Figure 2). These gels show bands at slightly lower positions than expected from the molecular mass of the various proteins. However, this can be explained by the fact that both these insertases are membrane-bound themselves and as such contain a high degree of non-polar residues. These non-polar residues are more readily bound by SDS moieties than their polar counterparts, and proteins that contain a high degree of non-polar amino acids will thus receive a relatively more negative charge. This more negative charge allows them to migrate further down the gel than their molecular size would normally allow for. Furthermore, only two bands are visible for the SecYEG-overexpressed sample, while this complex is made up of three proteins. This is due to the fact that SecG has been shown to be bound weakly by SDS, causing only a very faint band to appear. However, since SecYEG is a stable complex, the presence of bands corresponding to SecY and SecE was taken as confirmation of the presence of the whole complex. Absorbance at 280 nm was used to determine the protein-concentrations of the purified samples. Molar extinction coefficients of the proteins were calculated with the use of a web calculator[23]. SecYEG purifications yielded elutions with concentrations between 20 µM and 40 µM, which proved to be sufficient for proper reconstitution into LUVs. YidC purifications yielded higher concentrations, between 60 µM and 80 µM.

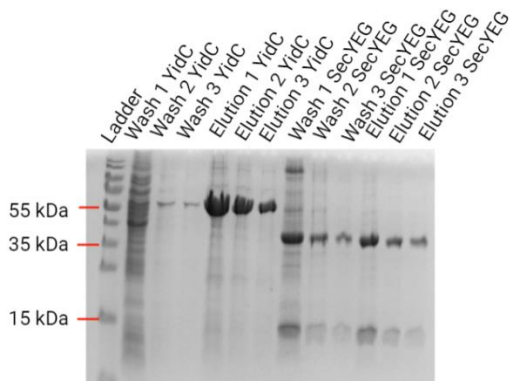

Figure 2. SDS gel showing washing- and elution samples from YidC- and SecYEG purification. YidC has a molecular mass of 61.8 kDa, but shows a band at ~55 kDa. SecY has a molecular mass of 48.5 kDa, but shows a band at ~35 kDa. SecE has a molecular mass of 13.6 kDa, and shows a band slightly below 15 kDa. SecG has a molecular mass of 11.3 kDa but does not show a band on a 12% SDS gel.

Formation of large unilamellar vesicles

As explained before, the synthetic phospholipid biosynthesis pathway consists of eight proteins which facilitate the production of PE and PG. LUVs were constructed for use in lysate experiments concerning these enzymes. However, LUVs were not constructed for all eight enzymes. The first enzyme of the biosynthesis pathway, FadD, was already known to be cytosolic. As such it is not expected to require any insertase function for its activity, thus no liposomes were constructed for this enzyme. No LUVs were constructed for the second enzyme of the biosynthesis pathway as well. As PlsB was predicted to not contain any TMs, furthermore it was assumed that it did not rely on insertases for its proper localization. Thus, the decision was made to only test this enzyme in lysate reactions if time in the research project allowed for it. Since this was not the case, no LUVs were constructed for this enzyme as well. LUVs were constructed for the six other enzymes, and insertases were reconstituted into them. The liposomes for the three enzymes PssA, PgsA, and PgpA were identical. However, purified PgsA would be reconstituted into liposomes for PgpA experiments, to act as a PGP producer. Reconstituted LUVs were loaded onto SDS gels to check for successful reconstitution of insertases SecYEG and YidC (Figure 3).

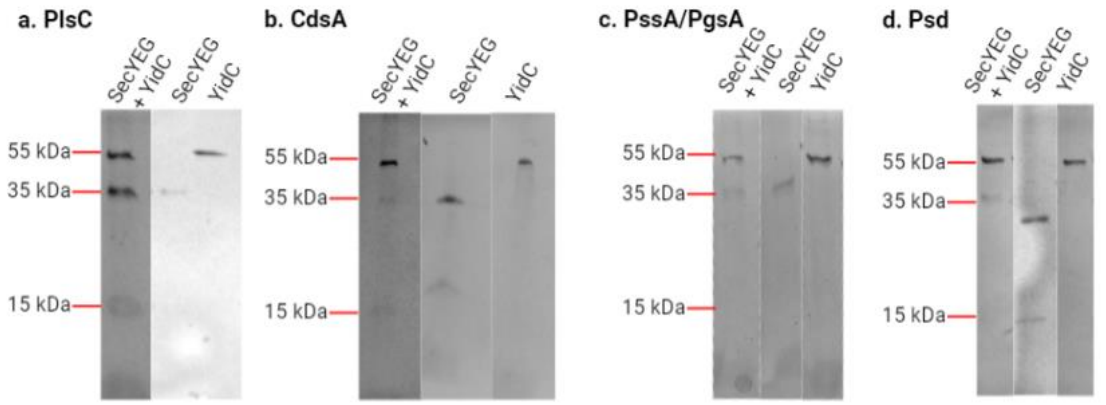

Figure 3. a) SDS gel showing successful reconstitution of insertases into PlsC LUVs b) successful reconstitution of insertases into CdsA LUVs, YidC shows a thicker band than SecYEG because it is a larger protein c) SDS gel pictures showing successful reconstitution of insertases in liposomes belonging to PssA and PgsA d) SDS pictures showing successful reconstitution of insertases in liposomes belonging to Psd, the difference in positioning between the SecYEG bands on the SecYEG and SecYEG+YidC samples is due to the fact that these pictures are taken from different gels

Purification of FadD and PgsA

Two enzymes in the phospholipid biosynthesis pathway use acyl-CoA as a reagent in their catalyzed reactions, where it acts as a fatty acid carrier. PlsB makes use of acyl-CoA to couple an acyl-chain to a G3P moiety, to yield LPA. Subsequently, PlsC requires acyl-CoA to yield an acyl-chain for conversion of LPA into PA. However, because of the high costs of commercially available purified acyl-CoA, a means of acyl-CoA production in the lysate reactions had to be realized. The cytosolic enzyme FadD provided a means of such acyl-CoA production. Following purification, this enzyme could be added to reaction mixtures together with the necessary reagents and produce acyl-CoA for the enzymatic reactions of PlsB and PlsC. FadD was overexpressed in *E. coli* BL21 cells, and His-tag purification was performed on the cytosolic fraction to acquire purified FadD. SDS page showed a strong band at the expected height, indicating successful purification of FadD (Figure 4a). Absorbance at 280 nm was used to determine the protein concentrations of the purified samples, which yielded concentrations between 60-70 µM. In order to test the activity of the purified FadD, activity assays were set up and analyzed with mass spectrometry (MS) (Figure 4b). The reaction containing both purified FadD and ATP shows production of acyl-CoA. Both the reaction containing no FadD and the reaction containing no ATP show no, or very low, production of acyl-CoA. A second reaction containing FadD and ATP was set up, but terminated with butanol after addition of reagents, this sample shows some production of acyl-CoA. This is probably due to the short time between addition of reagents and butanol. These results were taken as

proof of activity of purified FadD, and as confirmation that this enzyme could be used in later experimental setups.

Additionally, the enzyme PgsA was purified to be reconstituted into liposomes for PgpA experiments. The protein was overexpressed in *E. coli* BL21 cells, and purified using His-tag purification (Figure 4c). Because of technical problems with the mass spectrometer, it was not possible to carry out an activity assay for this enzyme during this research project. Therefore no conclusions can be drawn as to the activity of this purified protein.

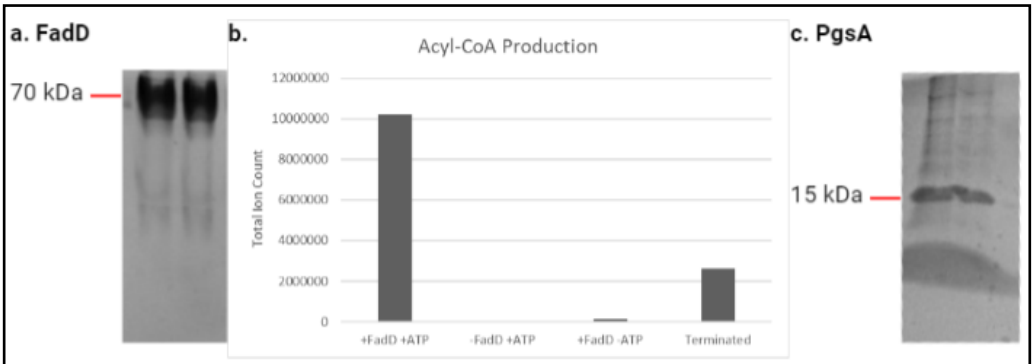

Figure 4. a) SDS gel showing purified FadD. FadD has a molecular mass of 62.3 kDa b) Table showing results of FadD activity assay, analyzed with MS. In the presence of ATP, purified FadD shows production of acyl-CoA. Terminated sample contains both FadD and ATP but was terminated shortly after addition of reagents, with the use of butanol c) SDS gel showing purified PgsA. PgsA has a molecular mass of 20.7 kDa, on a 12% SDS gel it runs at ~15 kDa. However, this can be explained by the fact that PgsA is a membrane protein.

Lysate experiments

The primary aim of this research project was to study the effect of insertases on the insertion and functioning of the phospholipid biosynthesis pathway. In order to study this relationship, an expression system in cell lysate was used. Plasmids containing the various enzymes would be brought to expression in a reaction mixture containing cell lysate, and would be co-translationally inserted into liposomal membranes under the right conditions. If correct association to- or insertion into the membrane was obtained, then the substrate lipids present in the liposomal membrane would be converted into product. This conversion of one lipid type into another could then be analyzed using MS. The lysate which was used in this experimental setup was harvested from *E. coli* cells, and centrifugation was used to extract the total membrane fraction from the cell lysate. This was necessary for two reasons, firstly to avoid background lipid signals during MS analysis of samples. Secondly, most of the enzymes used in this synthetic biosynthesis pathway were originally from *E. coli* cells. As the inner membrane of *E. coli* cells would contain these enzymes, the total membrane fraction had to be removed to avoid enzymatic background activity.

PssA

Due to technical problems with the mass spectrometer, only two enzymatic reactions could be analyzed using this expression-insertion setup. First PssA was tested in cell lysate reaction mixtures. This enzyme catalyzes the following reaction: CDP-DAG + L-Serine → PS. CDP-DAG was already present as the substrate lipid in the liposomes constructed for PssA, and L-Serine was present in the amino acid mix used in the reaction. Reactions with added plasmid were performed in duplicates. Two control samples were also used, a negative control containing no plasmid and a background control comprised of only liposomes in MQ water. Reaction mixtures were incubated at 30 °C overnight and lipids were extracted using a butanol extraction, where 10:0 PG was used as an internal standard. MS analysis was carried out on the PssA lysate reactions (Figure 5). Conversion of CDP-DAG into DOPS was detected in all duplicates, in liposomes with reconstituted insertases as well as in empty liposomes. This was not as expected, PssA likely depends on some insertase activity for its proper insertion into the membrane as it is predicted to contain multiple TMs. Furthermore, conversion of substrate into product was also

detected in all negative controls, which did not contain any enzyme. This signal might be due to contaminations with either lipids or enzymes from the lysate. However, it could also be due to contamination of MS samples with samples that were analyzed previously, due to insufficient washing cycles with methanol. This might also explain the slight PS signal in the background controls. However, the strong signal in the background control of the liposomes containing YidC is still strikingly high. Furthermore, the negative control of this liposome type also indicates high conversion of CDP-DAG into PS. Additionally, in all LUVs containing reconstituted insertases, a drop in substrate lipid was detected compared to the background control. However, this did not coincide with an increase in product lipid, or only a very slight one. This seems to indicate the presence of another enzyme which catalyzes the conversion of the substrate lipid into something other than the product lipid. Because this does not seem to be the case in the empty liposomes, it might be explained by co-purification of unwanted enzyme during purification of SecYEG and YidC. All these unexpected results seem to indicate that these samples are very likely not reliable. Due to technical issues with the mass spectrometer, it was not possible to repeat this experiment with differing conditions to elucidate the cause for these unexpected results.

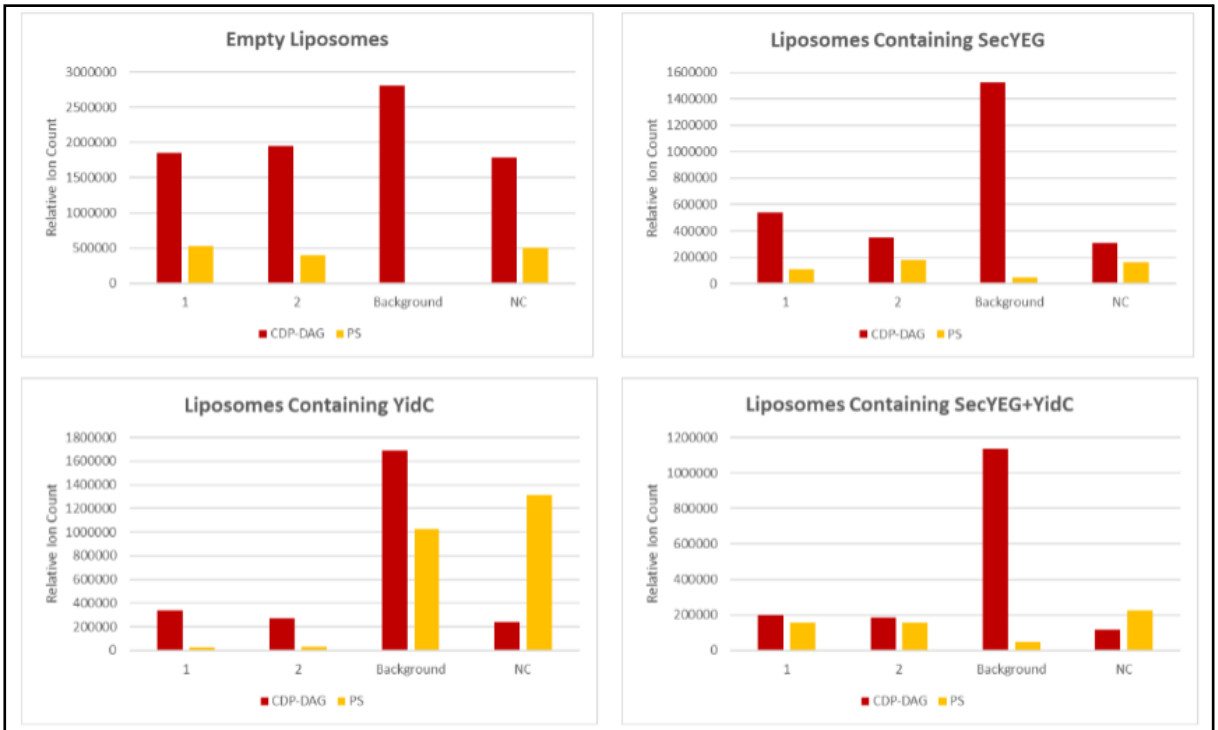

Figure 5. Results from MS analysis of PssA reactions in cell lysate in the presence of LUVs. Reactions containing plasmid with PssA were performed in duplicates (1, 2), a sample containing only liposomes was used as a background control (background), and a negative control (NC) containing no plasmid was used. All samples were normalized using 10:0 PG. Conversion of CDP-DAG into PS can be seen in all positive samples, however it is also detected in the negative controls. Furthermore, in the samples belonging to liposomes containing YidC, conversion is detected in the background sample but not (or very slightly) in the duplicates.

Psd

The second enzyme that was tested with the lysate expression system was Psd, which converts DOPS into DOPE. It catalyzes the following reaction: PS → PE + CO_2. PS was already present as the substrate lipid in the liposomes constructed for this enzyme. The experimental setup for Psd was similar to that of PssA. MS analysis was also carried out on the lysate reactions for this enzyme (Figure 6). Conversion of PS into PE was detected in all positive duplicates, as was expected based on preliminary in house results from M. J. den Uijl (data not shown). Since Psd was predicted to not contain any transmembrane domains, it likely does not depend on insertase activity for its proper localization in the liposomes. However, conversion of substrate into product also seems to be detected in the negative controls of all liposome types. In some cases even to a higher degree than the positive samples. This could indicate background activity of enzyme contamination or background signal from lipid contamination. Additionally, it could also be due to insufficient washing steps in the MS protocol, and therefore contaminations with previous samples. The background samples, composed of just the liposomes used in these reactions, also show some signal for PE. This solidifies the hypothesis that these unexpected results might be due to contaminations of some sort. Additionally, the background control for the SecYEG LUVs shows an unexpectedly low amount of substrate lipid PS. If compared to the duplicates and negative control, the background control should have contained more PS. These unexpected findings seem to point to unreliability of results, just as in the analysis of the PssA lysate reactions. Because of technical problems with the MS, it was also not possible to repeat this experiment and test this hypothesis.

Figure 6. Results from MS analysis of Psd lysate reactions in the presence of LUVs. Samples were normalized with 10:0 PG. All positive duplicates (1, 2) show conversion of PS into PE. Furthermore, all negative controls (NC) also show high conversion of substrate into product. Conversion is also detected in background samples (background), containing just liposomes. These findings seem to indicate unreliability of results.

Thin layer chromatography

Because of the inability to continue with MS analysis of experiments, the primary research aim for this research project had to be abandoned. Therefore, a secondary research aim was devised. This secondary research aim was to study the possibility of bulk lipid production in a lysate expression system, using thin layer chromatography as a means of analysis. Due to time constraints and technical difficulties, it was not possible to study the entire phospholipid biosynthesis pathway. The chain reaction from fatty acid to PA was therefore chosen, involving the enzymes FadD, PlsB, and PlsC. TLC allows for the identification of compounds based on their hydrophobicity, which infers different running speeds. By using known lipid stocks as controls, product formation in lysate reactions could be analyzed. However, due to the small volumes of these lysate reactions only very small amounts of product could be formed. Due to these small amounts of compound, detection using a normal molybdenum blue staining was insufficient for analysis. Therefore, fluorescently labeled fatty acids were used as a probe in these enzymatic reactions. If these fatty acid probes could be incorporated in the production of phospholipids, then these phospholipids could be visualized using fluorescent imaging. First the correct concentration of fluorescent probe in the lysate reactions had to be determined. In order to do this, different dilutions were spotted directly onto a TLC sheet and subsequently imaged to determine the minimal required concentration (data not shown). It was found that a ratio of 10:1 of fatty acid:fluorescent probe yielded a sufficiently clear fluorescent signal. However, since incorporation of fluorescently tagged fatty acids into enzymatic reactions seemed rather inefficient a 1:1 ratio was used for lysate reactions later on.

FadD

In order to determine whether the purified FadD was still active, and able to incorporate the fluorescent probe into acyl-CoA, an activity assay was performed. The activity assay itself was similar to the assay which was performed directly following purification, however this time fluorescently tagged fatty acids were supplied as 10% of the total fatty acid concentration. Reactions were performed in duplicates. The reactions were incubated overnight at 30 °C, and a butanol extraction was performed to extract all lipids. Samples were spotted onto a TLC sheet and fluorescently imaged (figure 7a). Both positive FadD samples showed secondary signals, apart from the fatty acids, belonging to acyl-CoA. This indicated that the purified enzyme was still active, and able to incorporate fluorescently tagged fatty acids into acyl-CoA production. Quantitative analysis was carried out through intensity measurements of fluorescent signal (Figure 7b). There seems to be a difference in conversion efficiency between the duplicates, but conversion of fluorescently tagged fatty acids into Acyl-CoA lies between 30%-40%. The cause for the lack of 100% conversion of fatty acids into acyl-CoA is the limited amount of added CoA.

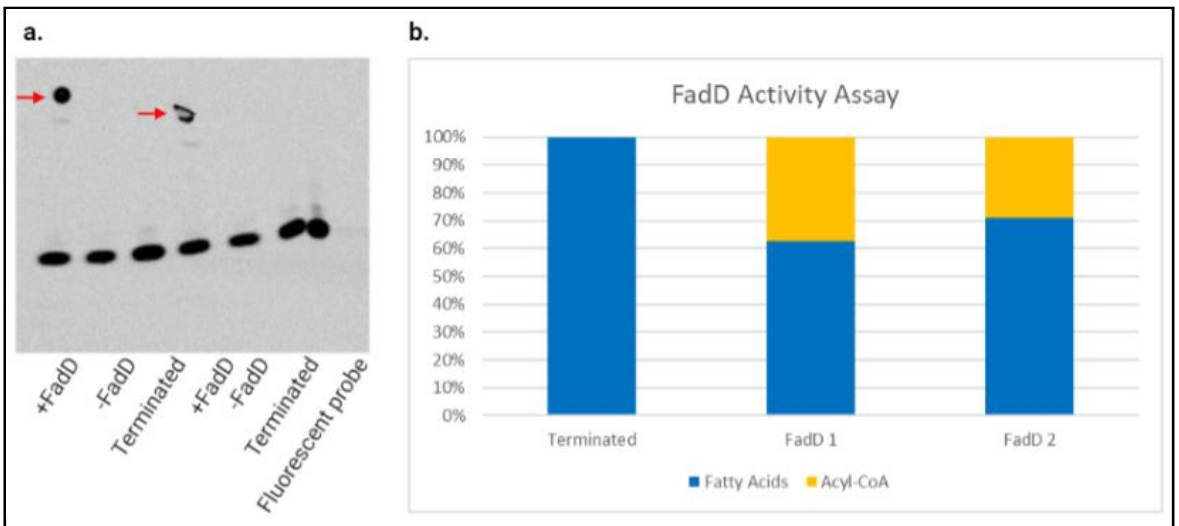

Figure 7. a) Fluorescent image of TLC analysis of FadD activity assay. The TLC sheet was run slightly tilted due to errors in the submerging of the sheet. On the far right the control of fluorescent probe is visible. The duplicates of positive FadD samples show production of acyl-CoA, and incorporation of the fluorescent probe into acyl-CoA (red arrows). Both negative controls and both terminated reactions show no presence of acyl-CoA b) Quantitative analysis of acyl-CoA production in duplicates compared to the terminated reactions, there is a slight difference in conversion between the two duplicates. Conversion of fluorescently tagged fatty acids into acyl-CoA seems to lie between 30%-40%

PlsB

PlsB is the second enzyme of the PA production pathway, and catalyzes the conversion of acyl-CoA into LPA. In order to study the production of LPA from a mixed fatty acid feed, containing fluorescent probes, reaction mixtures containing cell lysate were set up. In this setup 50% of total fatty acids were comprised of fluorescent probes. Furthermore, SUVs were constructed with a composition of 40% PC, 30% PG, and 30% PE. Samples were loaded onto TLC and a fluorescent image was made (figure 8a). Acyl-Coa production seems to be present in all three samples, even the control without FadD. However, in this sample it does seem to be significantly lower than in the samples with added FadD. The terminated reaction shows no presence of acyl-CoA. Furthermore, LPA production seems to have occurred in two samples, +PlsB/+FadD and -PlsB/+FadD. The terminated reaction shows no presence of LPA. However, the production of both acyl-CoA and LPA in the absence of added enzyme does seem to indicate contamination of lysate with enzymes. This could be possible considering the lysate was spun down to remove the total membrane fraction, however FadD is a cytosolic protein and PlsB is only associated to the membrane, not inserted into it. It might be possible that some protein was not spun out during preparation of cell lysate. Additionally, quantitative analysis was carried out by measuring intensity of fluorescent signal (Figure 8b). Strikingly, a higher conversion of fatty acids into acyl-CoA was observed in this lysate reaction than in the FadD activity assay (50% compared to 30-40%). Both the sample containing added PlsB and the sample without added PlsB show production of LPA. The +PlsB sample shows a conversion of fatty acids into LPA of 2,91%, whereas the -PlsB sample shows a conversion of 2,10%. Again, these numbers seem to point at contaminations of the cell lysate. Furthermore, these results indicate that PlsB does not convert fluorescently tagged Acyl-CoA into LPA with a high efficiency.

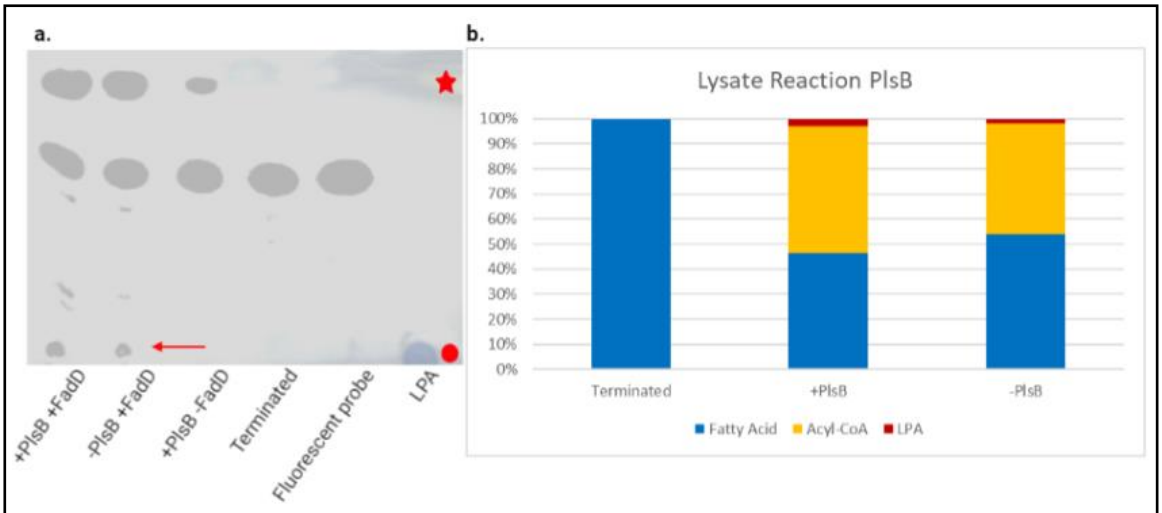

Figure 8. a) Combined fluorescent/stained TLC sheet of PlsB lysate reaction. On the far right the LPA control has been stained with molybdenum blue (red circle). Production of acyl-CoA is visible in all three samples on the left (red star), also in the sample which contained no added FadD. Furthermore, two sample seem to show production of LPA (red arrow). The sample containing both PlsB and FadD, and the sample containing only added FadD. Terminated reaction shows no presence of either acyl-CoA or LPA. These findings again seem to point to contamination of lysate with enzymes b) Quantitative analysis of LPA production in PlsB lysate reaction in the presence of FadD. Both the sample containing added PlsB and the sample without added PlsB seem to produce LPA, however production is slightly higher in the +PlsB sample

PlsC

The last enzyme involved in PA production is PlsC, which converts LPA into PA. A reaction was performed to study the efficacy of PA production in cell lysate using this enzyme. Again a ratio of 50% of total lipid concentration was made up by fluorescently labeled fatty acid. For this lysate reaction, SUVs were constructed with a composition of 35% PC, 30% PG, 30% PE, and 5% LPA. Figure 9a shows the result of these reactions. Production of acyl-CoA seems to be present in all three reaction samples, except for the terminated sample. The sample with no added FadD also seems to show production of acyl-CoA, although to a lesser extent, again indicating possible contamination of the cell lysate. Furthermore, one sample, +PlsC/+FadD, also seems to indicate presence of LPA. However, no PlsB was added to this sample, again indicating contamination of some sort. This sample also shows an additional band between LPA and fatty acid, which likely belongs to production of PA by PlsC. However, the molybdenum staining of the PA control on this TLC sheet was unsuccessful. Correlating this band to PA controls on other sheets does seem to strengthen this assumption, however it is still inconclusive. This is due to the fact that the positioning of compounds on a TLC greatly depend on the exact composition of the liquid phase and runtime. Between different TLC experiments these variables might differ. Quantitative analysis into the production of this assumed PA was also performed by intensity measurements (Figure 9b). Both samples that contained added FadD show conversion of fatty acids into acyl-CoA. However, the sample containing added PlsC seems to have much more conversion of fatty acids into acyl-CoA (~40% compared to ~15%). Furthermore, the +PlsC sample shows a conversion of fatty acids into PA of 3,03%. Additionally, there seems to be no background production of PA in the -PlsC sample.

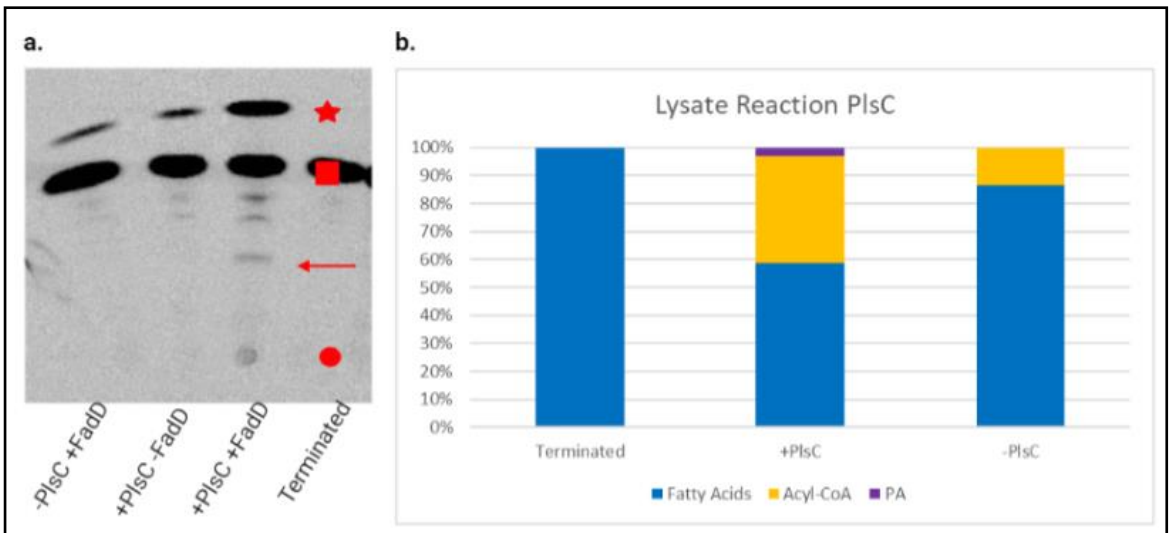

Figure 9. a) Fluorescent image of TLC of PlsC lysate reaction. On the right, the terminated sample shows a clear band of fatty acids (red square). All three other samples show the presence of acyl-CoA (red star), even the sample with no added FadD. One sample shows the presence of fluorescently tagged LPA (Red circle), +PlsC/+FadD, while this sample does not contain added PlsB. An additional band is visible between Fatty acid and LPA, in the +PlsC/+FadD sample, which might correspond to PA (red arrow). However, the molybdenum blue staining of the PA control on this TLC sheet was unsuccessful. Results do seem to indicate production of PA, however they are inconclusive and also seem to indicate contamination of lysate with enzymes. b) Quantitative analysis of PA production in PlsC lysate reaction in the presence of FadD. Both the sample with added PlsC and without added PlsC show conversion of fatty acids into acyl-CoA, however this seems much lower in the -PlsC sample. The +PlsC sample also shows production of PA, indicating functionality of PlsC

Discussion

The aim of this research project was to study the synthetic phospholipid biosynthesis pathway in a lysate expression system, which was done in two subprojects. The first subproject was aimed at studying the effect of insertases on the correct insertion of the enzymes of this synthetic pathway into the membrane of liposomes. This localization would be linked to enzymatic activity, which could be analyzed using MS. The second subproject was to study the possibility of bulk lipid production in a lysate expression system. Only a part of the biosynthesis pathway, towards the production of PA, was studied in this manner. Analysis of bulk lipid production experiments was performed using TLC, and fluorescently tagged fatty acids. While both subprojects were quite distinct, they were both aimed at the primary goal of studying the synthetic phospholipid biosynthesis pathway in a lysate expression system.

It was shown that the enzymes involved in production of PA were able to incorporate fluorescently tagged fatty acids into their products. The enzyme PlsC was brought to expression in a cell lysate mixture containing SUVs with its substrate LPA present in their membranes. Fluorescent imaging, combined with TLC, indicated that this enzyme was able to acylate LPA with fluorescently labeled fatty acids to produce PA. Quantitative analysis showed that PlsC was capable of incorporating ~3% of labeled fatty acids into PA. However, this number might be skewed due to two reasons. Firstly, the purified FadD that was also added to the reactions as a means of acyl-CoA production was only capable of converting ~40% of fluorescently labeled fatty acids into acyl-CoA. Furthermore, the presence of this fluorescent label might very well have an effect on catalytic efficiency of the enzyme. The fluorescent label on the fatty acid tail makes this moiety much more bulky than normal fatty acids, therefore it might be less readily used in enzymatic reactions involved in PA production. Taking these two factors into account, it might be assumed that the actual efficiency of PA production by PlsC in a cell lysate expression system would be higher than this analysis indicates. The same consideration holds true for the quantitative analysis of LPA production by PlsB. However, in this experiment background activity was also detected. The reaction containing added PlsB showed a conversion of fluorescently labeled fatty acid into LPA of ~3%, whereas the reaction without added PlsB showed a conversion of ~2%. This background activity was likely due to contamination of the cell lysate with enzyme, as no fluorescently labeled LPA would be present in *E. coli* cells. This contamination makes analysis of catalytic efficiency of PlsB in a cell lysate expression system difficult. Purified FadD was shown to be capable of conversion of fatty acids into acyl-CoA using both MS- and TLC analysis. MS analysis was carried out on an activity assay in an artificial reaction buffer, where FadD was shown to be capable of acyl-CoA production. Additionally, TLC analysis showed that FadD was capable of incorporating fluorescently tagged fatty acids into its enzymatic reaction in cell lysate. Therefore this enzyme could be used as a means of acyl-CoA production in subsequent experiments.

MS analysis of lysate reaction concerning the enzymes PssA and Psd yielded unexpected results. The lysate reactions for the enzyme PssA showed conversion of CDP-DAG into PS across all liposome types, including liposomes with no reconstituted insertases present. This was unexpected because TM predictions showed that PssA likely contains multiple TMs, as such it was expected to rely on insertases for its insertion into the liposomal membrane and subsequent enzymatic activity. Furthermore, negative controls also showed conversion of substrate into product across all liposome types. This indicated contaminations of some sort. The background controls showed that the detected PS was not present in the liposomes that were used. Therefore, the contaminations were likely from the cell lysate that was used. However, the background control for the liposomes containing reconstituted YidC does show a high amount of PS to already be present. This might be due to human error in the setting up of the mass spectrometer. Additionally, all samples containing liposomes with reconstituted insertases showed a drop in substrate lipid compared with their background controls, without the subsequent increase in product lipid. This might be explained by co-purification of unwanted enzymes during insertase purification, where these enzymes convert the substrate lipids into something other than the desired product. The analysis of the lysate reactions belonging to Psd shows similar unexpected results. However, conversion of substrate into product in all liposome types was expected for this enzyme based on TM predictions and preliminary in house results. MS analysis of these two lysate reactions did not yield conclusive results, as there is a high probability of contaminations. Due to technical issues there was no opportunity to repeat or alter these experiments during this research project.

Concluding remarks

In conclusion, the use of a cell lysate expression system does seem suited to bulk lipid production. However, the use of a more top-down system compared to the bottom-up PURE system does seem to have its drawbacks. While the cell lysate expression system does offer a more robust way of phospholipid production, it also brings about a high degree of interference by background processes. This was noticeable in both MS and TLC analyses. This problem of background activities might be overcome by the use of differently treated cell lysate. Furthermore, the use fluorescently labeled fatty acids does seem compatible with the PA production pathway. However, there is a high chance that the presence of such a large fluorescent moiety on the fatty acids does influence incorporation into the phospholipid biosynthesis. Therefore, MS remains the preferred method of analysis. Additionally, further analysis of lysate reactions are required to make draw any conclusions about the influence of insertases on the functioning of the biosynthesis pathway.

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